Modulation of cognitive behaviour in mice via targeted molecular and systemic pharmacological interference of glycine transporter 1 function

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Modulation of Cognitive Behaviour in Mice via Targeted Molecular and Systemic Pharmacological Interference of Glycine Transporter 1 Function

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Doctor of Sciences

presented by

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General Discussion

Overview

Functional dissociation between complete and neuronal GlyT1 deletion in forebrain

GlyT1 disruption restricted to forebrain neurons enhances the formation of associative links without compromising selectivity is associative learning

GlyT1 disruption in forebrain neurons is associated with enhanced cognitive flexibility/adaptability

Deletion/inhibition of GlyT1 in forebrain neurons: A potential approach to achieve cognitive enhancement?
Summary

The N-methyl-D-aspartate receptor (NMDAR) assumes a pivotal role in synaptic plasticity, learning and memory (Tang et al., 1999), and impaired NMDAR function has been implicated in a number of cognitive disorders and neuropsychiatric diseases including schizophrenia (c.f. Javitt 2007). This has generated growing interest in agents that enhance NMDAR activation in order to alleviate cognitive deficiency or to achieve cognitive enhancement in otherwise healthy individuals. NMDAR activation requires the simultaneous binding of glutamate and the co-agonist glycine (McBain and Mayer 1994). Local glycine levels at NMDAR-containing glutamatergic synapses are tightly regulated by the glycine transporter 1 (GlyT1) which mediates the re-uptake of synaptic glycine into the cytosol (Cubelos et al., et al., 2005; Gomez et al., 2003). Inhibition of GlyT1 and its resulting increase in extra-cellular glycine therefore provides a promising approach to augment NMDAR activation via enhanced occupancy of the glycine binding site.

The overall basic question the present thesis sought to address was to evaluate the impact of reduced GlyT1 function on NMDAR mediated transmission and the expression of higher cognitive behaviour including learning and memory, and to evaluate whether such interventions may prove useful to enhance cognitive functions in non-clinical situations. To achieve this, we made extensive use of genetically engineered mice with a specific functional defect of GlyT1 due to molecular deletion of the GlyT1 gene. To this end, two conditional GlyT1 knockout mouse lines were generated with either neuron or neuron and glia specific loss of GlyT1 in the forebrain, thus allowing us to compare the in vivo regulatory functions of neuronal and global (neuron and glia) GlyT1. This was complemented by a pharmacological approach using the selective GlyT1 inhibitor SSR504734 by which the acute short-term effect of GlyT1 inhibition in wild-type mice can be examined.

The first series of experiments revealed that GlyT1 disruption restricted to forebrain neurons increased NMDAR currents and enhanced cognitive functions across a variety of learning situations. The mutation led to enhanced associative learning, and it improved object recognition memory when the demand on retention was high (Appendix I, Chapter 2). This was accompanied by increased latent inhibition (LI) effect pointing towards enhanced flexibility in associative learning in the mutant mice (Appendix I). Furthermore, the mutation facilitated new learning under conditions of high levels proactive interference (Chapter 3). These findings indicate that forebrain neuronal GlyT1 deletion does not indiscriminately strengthen the expression of learned behaviour at the expense of selectivity and flexibility in learning. In contrast, flexibility was actually enhanced. Hence, the constellation of behavioural phenotypes identified by the first series of experiments is suggestive of a general enhancement in behavioural adaptability. In addition, neuron-specific GlyT1 disruption in forebrain reduced the responsiveness to the motor stimulating effect of psychomimetic drugs (Appendix I and II), which is suggestive of an antipsychotic profile of mutation.

The next series of experiments evaluated the effect of complete forebrain specific GlyT1 disruption on NMDAR function and memory processes, providing a comparison to the more specific forebrain
neuronal GlyT1 deletion (Chapters 4-6). A functional impact of the mutation on NMDAR function was evidenced by a near-complete absence of a response to NMDAR blockade by PCP presumably due to increased synaptic glycine, which was supported by a drastic reduction in GlyT1 specific glycine re-uptake implying an increase in extra-cellular glycine. A pro-cognitive effect of the mutation was revealed on working memory and object recognition memory. Associative learning, on the other hand, was not enhanced in the mutant mice. Furthermore, the altered NMDAR properties did not translate into enhanced hippocampal NMDAR currents as previously seen in mice lacking GlyT1 specifically in forebrain neurons. This constitutes a notable physiological difference between the two GlyT1 knockout mouse lines which may account for their phenotypic differences and the wider range of effects associated with forebrain neuronal loss of GlyT1. These findings demonstrate that inhibition of GlyT1 represents a feasible approach to modulate learning and memory, and specifically illustrate the differential impacts on cognitive processes via forebrain selective (neuron vs. neuron plus glia) GlyT1-targeted manipulations. This possibly points towards a functional dissociation between neuronal and non-neuronal GlyT1 in the regulation of higher cognitive behaviour which is highly instructive for the design of novel GlyT1 inhibitors aiming at enhancing cognitive functions.

In order to further dissect the cognitive effects of complete and neuronal GlyT1 deletion in forebrain additional cognitive tests were conducted which revealed other phenotypic differences between the two GlyT1 knockout lines. First, the expression of prepulse inhibition (PPI), a measure of sensorimotor gating (e.g. Braff and Geyer 1990), was attenuated when GlyT1 deletion was restricted to forebrain neurons, but was unaffected when GlyT1 was disrupted in a cell-type non-specific manner throughout the forebrain (Chapter 5). Second, defect of neuronal but not complete GlyT1 deletion in forebrain exerted multiple effects on associative learning in a series of Pavlovian conditioned freezing procedures (Chapter 6). The results of these experiments showed that forebrain neuronal GlyT1 disruption increased both the magnitude and the persistence of the conditioned freezing response to a discrete conditioned tone stimulus (CS) previously having been paired with a foot shock unconditioned stimulus (US) and enhanced contextual fear when the animals were explicitly conditioned to a novel context in the absence of any discrete CS. Again, such excessive conditioned responding was not realized at the cost of selectivity in associative learning because the sensitivity to the trace conditioning effect, in which a protracted interval between the offset of the conditioned stimulus (CS) and the onset of the unconditioned stimulus (US) attenuates the subsequent conditioned responding to the CS, was more pronounced in the mutant mice. This pattern of results lends further support to the suggestion that specific GlyT1 deletion in forebrain neurons can facilitate selectivity in associative learning, and may be indicative of enhanced cognitive flexibility, rather than a simple strengthening of any potential associative links between stimuli.

The last series of experiments was designed to evaluate the behavioural effects of systemic GlyT1 blockade achieved by SSR504734, a selective GlyT1 inhibitor which has been shown to enhance NMDAR function (Depoortère et al., 2005). SSR504734’s promnesic potential was assessed on
working memory function using an automated operant continuous delayed alternation task (Chapter 7). The results showed that SSR504734 enhanced working memory performance in a dose- and delay-dependent manner. This is in agreement with the working memory enhancement previously seen in complete forebrain specific GlyT1 knockout mice indicating that GlyT1 inhibition/deletion in a cell-type non-specific manner constitutes a feasible approach to improve working memory. The effect of SSR504734 on the responsiveness to psychomimetic drug challenge was examined in Appendix IV and V. It was found that SSR504734 attenuated the motor-stimulant effect of PCP (Appendix IV), but exacerbated the behavioural effects induced by dopaminergic agonists (Appendix IV and V, also see Depoortère et al., 2005). This may indicate that the more direct impact of GlyT1 blockade on NMDAR-mediated glutamatergic neurotransmission can subsequently lead to changes in dopaminergic activity which may contribute to the behavioural effects of SSR504734.

Together, the experimental investigations presented in this thesis provide clear evidence that inhibition of GlyT1 can lead to some forms of cognitive enhancement, but the emergence of such effects is largely dependent on its selective inhibition/deletion in forebrain neurons as opposed to complete forebrain GlyT1 deletion. The effects of forebrain neuronal GlyT1 deletion are beyond merely strengthening memory of things past (e.g. enhanced object recognition memory), but are realized in tune with on-going purposeful behaviour resulting in enhanced adaptability to changes that lead to a current goal, as evidenced by improved reversal or new-learning in the water maze and the T-maze (Chapter 3). These findings lend support to the suggestion that inhibition of GlyT1 may represent a promising strategy to modulate cognitive behaviour with potential applications in the treatment of cognitive impairments in a number of diseases and for non-clinical purposes to boost mental functions in otherwise healthy people or to halt cognitive decline in normal ageing.

On the other hand, the attenuation of PPI by GlyT1 deletion in forebrain neurons and the potentiation of dopamine agonists induced behaviours by pharmacological GlyT1 blockade may be interpreted as signs linked to schizophrenia, which is contrary to the antipsychotic profile of GlyT1 inhibition revealed in other tests (Appendix I and II, Chapter 4). This illustrates that any change in cognitive behaviour may lead to both beneficial and harmful effects, including possible tradeoffs when enhancement of one function may lead to a malfunction of another. Thus, these costs and benefits are situation-dependent, and their balance needs to be subjected to empirical testing.

Finally, the precise mechanisms in which these changes in cognitive functions are realized in terms of brain-circuitry remain poorly understood. Selectivity of the GlyT1 molecular deletion therefore requires further refinement in its expression to advance in this direction.
Zusammenfassung


Der Hauptfokus der hier vorliegenden Doktorarbeit war darauf gerichtet zu untersuchen, inwieweit eine reduzierte Funktion des GlyT1 die durch den NMDAR vermittelte Neurotransmission sowie höheres kognitives Verhalten wie Lernen und Gedächtnis beeinflusst. Zudem sollte untersucht werden, ob solche Manipulationen zur Behandlung von kognitiven Störungen verwendet werden können oder aber ob sie zur Steigerung der kognitiven Leistungsfähigkeit in nicht-pathologischen Situationen führen. Zu diesem Zweck wurden genetisch modifizierte Mäuse untersucht, die aufgrund einer Deletion im entsprechenden Gen einen spezifischen funktionellen Defekt im GlyT1 haben. Um genauer überprüfen zu können, inwiefern sich die Funktionen von Glycin in einem Neuronen-spezifischen und einem generellen Sinn (Neuronen und Glia) unterscheiden, wurden zwei spezifische knockout-Mauslinien generiert; wobei eine Linie einen generellen Ausfall der GlyT1 Funktion im gesamten Vorderhirn hatte, bei der anderen dieser Ausfall jedoch auf Neuronen des Vorderhirns beschränkt blieb. Der selektive GlyT1 Inhibitor SSR504734 wurde als ein pharmakologischer Ansatz benutzt, um die kurzfristigen Effekte einer GlyT1-Blockierung eruieren zu können.

Flexibilität stärkt; die Flexibilität war im Gegenteil sogar verbessert. Die Konstellation der beobachteten Verhaltensphänotypen in der ersten Reihe von Experimenten weist darauf hin, dass eine erhöhte NMDAR Funktion zu einer generellen Verbesserung der Anpassungsfähigkeit führen könnte. Zusätzlich dazu reduzierte eine Neuronen-spezifische Deletion von GlyT1 im Vorderhirn die Empfindlichkeit gegenüber den stimulierenden Effekten psychomimetischer Substanzen (Appendix I and II), was auf einen antipsychotischen Charakter der Mutation hinweist.


Die vorliegenden Ergebnisse zeigen, dass Inhibition des GlyT1 ein nützlicher Ansatz ist, um Lernen und Gedächtnis zu modulieren sowie die verschiedenen Auswirkungen aufzuziehen, welche selektive (neuronale vs generelle) Manipulationen am GlyT1 auf kognitive Prozesse haben. Sie könnten auch ein Hinweis darauf sein, dass es eine funktionelle Abgrenzung zwischen neuronalem und nicht-neuronalem GlyT1 in Bezug auf die Regulierung höheren kognitiven Verhaltens gibt, was äusserst aufschlussreich für das Design neuer GlyT1 Inhibitoren wäre, die auf eine Verbesserung kognitiver Funktionen ausgerichtet sind.

Mit dem Ziel, die kognitiven Effekte eines neuronalen oder generellen Verlusts von GlyT1 im Vorderhirn weiter aufzuschlüsseln, sind zusätzliche Tests durchgeführt worden, die weitere phänotypische Unterschiede zwischen den beiden GlyT1 knockout-Linien aufzeigten. Erstens war die Expression von prepulse inhibition (PPI), einem Mass für sensorimotorische Verarbeitung, abgeschwächt, wenn die Deletion von GlyT1 auf Neuronen des Vorderhirns beschränkt war; sie war aber nicht beeinträchtigt, wenn die Deletion alle Zelletypen des Vorderhirns umfasste (Kapitel 4). Zweitens hatte der Neuronen-spezifische Ausfall von GlyT1 im Vorderhirn vielfache Auswirkungen auf assoziatives Lernen in mehreren conditioned freezing Experimenten nach Pavlov. Die Resultate dieser Experimente zeigen, dass Neuronen-spezifische Deletion von GlyT1 im Vorderhirn sowohl das
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Ausmaß als auch die Dauer des freezing-Verhaltens gegenüber einem Ton-Stimulus steigerte, wenn dieser Ton (conditioned stimulus, CS) zuvor mit einem elektrischen Fussschock (unconditioned stimulus, US) gepaart worden war. Zudem war das kontextbedingte freezing grösser, wenn die Tiere explizit auf einen neuen Kontext in Abwesenheit eines CS konditioniert wurden. Die Sensitivität zum sogenannten trace conditioning Effekt sogar noch verstärkt war. Bei diesem Effekt verringert ein zunehmendes Zeitintervall zwischen der Präsentation des CS und der Verabreichung des US die spätere konditionierte Reaktion zum CS. Diese Resultate unterstützen den Vorschlag weiter, wonach eine spezifische GlyT1 Deletion in Neuronen des Vorderhirns die Selektivität assoziativen Lernens erleichtern kann, und dass sie ein Index erhöhter kognitiver Flexibilität sein kann, anstatt lediglich den assoziativen Link zwischen zwei möglichen Stimuli zu verstärken.

Die letzte Serie von Experimenten wurde entworfen, um die Konsequenzen einer systemischen Blockade von GlyT1 durch SSR504734 in Bezug auf das Verhalten zu untersuchen. SSR504734 ist ein selektiver GlyT1 Inhibitor, der nachweislich die Funktion der NMDAR fördert (Depoortère et al., 2005). Die leistungssteigernde Wirkung, die SSR504734 auf das Kurzzeitgedächtnis ausübt, wurde in einem sog. continuous delayed alternation Test untersucht (Kapitel 7). Die Resultate zeigen, dass SSR504734 in Abhängigkeit sowohl von der Dosis als auch vom Zeitintervall die Leistung des Kurzzeitgedächtnisses verbessert. Dies ist in Übereinstimmung mit den ebenfalls leistungssteigernden Effekten, die in jenen Mäusen beobachtet wurden, die eine komplette, Vorderhirn-spezifische Deletion von GlyT1 aufweisen, was bedeutet, dass eine generelle Inhibition von GlyT1 ein sinnvoller Ansatz darstellt, um die Leistung des Kurzzeitgedächtnisses zu steigern.

Der Effekt von SSR504734 auf die Empfindlichkeit gegenüber psychomimetischen Substanzen wurde in den Kapiteln 8 bis 9 untersucht. Dabei wurde gezeigt, dass SSR504734 den Lokomotions-stimulierenden Effekt von PCP reduziert, jenen von Dopamin-Agonisten aber verstärkt (Kapitel 7 & 8). Dies kann als Hinweis darauf gewertet werden, dass die viel direktere Wirkung einer GlyT1 Blockade auf NMDAR-abhängige glutamaterge Neurotransmission zu Veränderungen in der dopaminergen Aktivität führen könnte, was dann wiederum zu den beobachteten Effekten von SSR504734 beitragen könnte.

Zusammenfassend bilden die in dieser Doktorarbeit präsentierten Untersuchungen einen klaren Hinweis dafür, dass Inhibition von GlyT1 zu gewissen Formen von kognitiver Verbesserung führen kann; aber auch, dass das Auftreten dieser Effekte zu einem Grossteil davon abhängig ist, ob die GlyT1-Deletion Neuronen-spezifisch ist oder alle Zelltypen des Vorderhirns umfasst. Die Effekte einer Deletion auf neuronaler Ebene des Vorderhirns gehen über eine bloße Verbesserung der Erinnerung an vergangene Ereignisse hinaus, vielmehr finden sie im Zusammenspiel mit anhaltendem, zielorientiertem Verhalten statt. Das wiederum unterstützt den Vorschlag, dass eine Inhibition des GlyT1 eine mögliche, vielversprechende Strategie darstellen könnte, um kognitives Verhalten zu modulieren, was mögliche Anwendungen in der Behandlung kognitiver Beeinträchtigungen in einer
Zusammenfassung


Andererseits kann die beobachtete Abschwächung des PPI-Effekts sowie die Verstärkung der Wirkung von Dopamin-Agonisten in Mäusen mit Neuron-spezifischer Deletion des GlyT1 im Vorderhirn als Zeichen pro-psychotischer Eigenschaften gewertet werden, was allerdings im Widerspruch zum antipsychotischen Profil einer GlyT1-Inhibition steht, welche in anderen Tests gezeigt werden konnte. Dies zeigt allerdings auch, dass jede Veränderung im kognitiven Verhalten sowohl vorteilhafte als auch nachteilige Folgen haben kann, inklusiver möglicher Interaktionen, da die Verbesserung der einen Funktion zur Störung einer anderen Funktion führen kann. Der Gewinn und die Kosten einer solchen Manipulation hängen von der gegebenen Situation ab, und ihr Verhältnis zueinander muss empirischen Tests unterworfen werden.

Die genauen Mechanismen, durch welche die oben beschriebenen Effekte im Hinblick auf einzelne Verschaltungen im Gehirn entstehen, sind immer noch weitgehend unbekannt. Es ist deshalb nötig, die Selektivität der GlyT1 Deletion weiter zu verfeinern, um in diesem Bereich weitere Fortschritte zu erzielen.

REFERENCES


Journal of Neuroscience, 26, 8702–8706.

Chapter 1

General introduction
Preface

Enormous resources have recently been invested in the development of treatments and cognition enhancing drugs for people with degenerative brain diseases, cognitive malfunction, and neuropsychiatric diseases that involve cognitive impairments. ‘Cognitive enhancement' thereby refers to the use of various strategies to boost cognitive functions including learning and memory, attention, or selective forgetting. Cognitive enhancement may also be desirable in non-pathological situations such as normal ageing or to optimize mental capabilities in otherwise healthy people.

The central mechanism thought to underpin learning and memory is synaptic plasticity, referring to changes in the strength of synaptic connections that increase or decrease neurotransmission. On the other hand, processes such as the formation of new synapses (synaptogenesis), proliferation and survival of new neurons (neurogenesis), and neuronal cell death (neurotoxicity and apoptosis) are also implicated in cognition and in its impairment in various diseases. Each of these mechanisms provides possible targets for potential cognitive enhancers. However, the relationship between the expression of cognitive behaviour and changes in synapses, cells and brain circuits remain poorly understood. This lack of understanding limits the translation of laboratory findings into effective interventions for human use and is mainly responsible for the poor efficiency of currently available cognitive enhancers.

Hence, pharmacological research is challenged to find novel avenues for the development of efficient cognitive enhancers for clinical and non-clinical use.

The present thesis was designed to evaluate and validate one possible approach to enhance cognitive behaviour by enhancing neurotransmission through \(N\)-methyl-D-aspartate receptors (NMDAR) which are known to play a central role in neuroplastic events and learning and memory. A potentiation of NMDAR mediated transmission is thereby achieved indirectly via interference with the glycine transporter 1 (GlyT1) which tightly regulates the synaptic availability of glycine, an obligatory NMDAR co-agonist. The present approach will be explained in more detail in the following review paper which also provides an overview of the relevant findings in the field.
Chapter 1

Interference of Glycine Transporter 1: Modulation of Cognitive Functions Via Activation of Glycine-B Site of the NMDA Receptor

With Joram Feldon and Benjamin K. Yee


[Personal contribution to the work: writing the manuscript]
Chapter 1

Abstract

The high-affinity glycine transporter 1 (GlyT1) is the primary endogenous regulator of glycine levels in the vicinity of the N-methyl-D-aspartate receptor (NMDAR). As a co-agonist, glycine can allosterically modulate NMDAR functions through its binding to the glycine binding site (glycine-B site). Under homeostatic conditions, GlyT1 mediated re-uptake is believed to maintain the synaptic glycine concentration below the saturation level of the glycine-B site. Given that glycine-B site occupation is obligatory for glutamatergic activation of the NMDAR, increased availability of glycine in the vicinity of NMDAR’s glycine-B site has been suggested as an alternate strategy to enhance NMDAR functions. Because exogenously administered glycine shows poor blood-brain barrier penetration and must overcome potent regulatory brain mechanisms in order to efficiently enhance NMDAR function, one currently favored strategy is to target the glycine clearance mechanism through inhibition of GlyT1 mediated re-uptake. Numerous studies have demonstrated that pharmacological blockade or molecular down-regulation of GlyT1 leads to enhanced NMDAR functions and thus may provide novel therapeutic avenues in the treatment of neurological and psychiatric disorders in which NMDAR hypofunction has been implicated, including schizophrenia. Several modulatory agents targeted at the glycine-B site are currently undergoing pre-clinical and clinical development as potential antipsychotic drugs. Parallel research in animals with pharmacological inhibition of GlyT1 or GlyT1 knock-out mice has also generated promising results, reinforcing the hypothesis that disruption of glycine reuptake via GlyT1 may entail therapeutic value against primarily negative and cognitive symptoms of schizophrenia.
BACKGROUND

The Glycine Transporter 1

The glycine transporter 1 (GlyT1) belongs to the large family of Na+/Cl- dependent ionotropic transporter proteins [1, 2] and mediates uptake of the neuromodulator glycine from the extracellular space into the cytosol. This polytopic membrane protein is encoded by the human gene SLC6A9 [3] and exists in multiple splice variants generated by three N-terminal (a, b, c) [4] and two C-terminal (d, e) [5] exons. In the mammalian brain, GlyT1 is distributed throughout most regions of the CNS [6] with a high level of expression in the spinal cord, brainstem and cerebellum and a more moderate expression in the cerebral cortex and hippocampus. As a plasma-membrane transporter of the neuromodulator glycine GlyT1 exhibits regulatory functions in both excitatory and inhibitory neurotransmission. In the caudal area of the brain, where GlyT1 is expressed in astrocytes adjacent to glycineergic neurons [1] it is believed to contribute to the termination of inhibitory neurotransmission by removing glycine from glycineergic synapses [7]. In forebrain regions, particularly in the neocortex and hippocampus, GlyT1 is found in both glial cells and in synapses of subpopulations of glutamatergic neurons [7] and its pattern of expression closely corresponds to the distribution of NMDA receptors (NMDAR) [7, 8, 9]. Here, GlyT1 is primarily involved in the regulation of the extra-cellular glycine concentration.

Modulatory Function of GlyT1 at the NMDAR Glycine-B Site

Glycine is an obligatory co-agonist of glutamate at NMDAR [10, 11] thus its binding to the NMDAR glycine binding site (glycine-B site) is necessary for both ion channel opening and receptor internalization. GlyT1 is therefore implicated in the modulation of NMDAR function via a control over glycine availability at the glycine-B site. Evidence for the modulatory function of GlyT1 at NMDA receptors has been provided by a number of biochemical and electrophysiological studies. It has been shown that blockade of GlyT1 by GlyT1 inhibitors results in an up-regulation in the extra-cellular glycine concentration, which is accompanied by a selective increase in the amplitude of NMDAR mediated currents without affecting AMPA receptor mediated currents [12, 13, 14]. Structures and formulae of the GlyT1 inhibitors discussed in the present review are summarized in Table 1. Furthermore, the GlyT1 inhibitors NFPS [15] and CP802,079 [14], enhance NMDAR dependent long-term potentiation (LTP) in vivo. Further support for an involvement of GlyT1 in the regulation of NMDAR function stems from molecular deletion studies. Notably, the amplitude of NMDAR-evoked postsynaptic currents in brain slice preparations of global heterozygous [16] and homozygous forebrain and neuron specific [17] GlyT1 knock-out mice are significantly elevated in comparison with controls.
However, blockade or down-regulation of GlyT1 is only expected to enhance NMDAR mediated neurotransmission if the glycine-B site is not saturated under physiological conditions. The affinity of glycine for the glycine-B site lies in the low micro-molar (0.1-3.0 μM) range depending on the NR2 subtype of NMDAR [10, 18, 19, 20]. Because glycine levels in the cerebrospinal fluid are in the range of 5 to 10 μM [21, 22], it has previously been postulated that the glycine-B site may be saturated at physiological conditions [23]. However, more recent studies revealed that the glycine-B site is normally not saturated under different experimental preparations [9, 13, 24, 25, 26]. Based on these findings it has been suggested that synaptic glycine levels in the vicinity of NMDAR are held below the saturation concentration of the glycine-B site by the GlyT1 re-uptake system [24, 26, 27, 28, 29];
although other small neutral amino acid transporters, called SNATs, may also be involved in the regulation of extra-cellular glycine levels [30]. Hence, elevating extracellular glycine concentration has been suggested as a novel approach to enhance NMDAR function, which may be of therapeutic value in the treatment of diseases related to impaired NMDAR function, including schizophrenia and dementia (as discussed below). GlyT1 is therefore considered a prominent target for the development of novel therapeutic drugs.

**Inhibition of GlyT1 as a Novel Strategy to Enhance NMDAR Function Via the Glycine-B Site**

A number of neurological and psychiatric disorders have been associated with a NMDAR hypofunction, and therefore restoration of the hypoglutamatergic state may be of therapeutic value. However, the use of direct NMDAR agonists has limited clinical benefits due to severe convulsive and neurotoxic side effects [31]. Alternatively, a facilitation of NMDAR mediated neurotransmission can be achieved by increased activation of the glycine-B site. Treatment with glycine-B site agonists such as glycine and D-serine minimizes the risk for excitotoxicity [32] and displays some antipsychotic properties in rodents and humans [33, 34, 35]. Additionally, glycine, D-serine and the partial agonist Dcycloserine are therapeutically beneficial against negative and cognitive symptoms in schizophrenic patients when administered as add-ons to current antipsychotic medications [36, 37, 38]. However, exogenously administered glycine and D-serine are extensively metabolized and must overcome potent regulatory mechanisms to efficiently facilitate NMDAR function. Furthermore, due to poor blood-brainbarrier penetration, systemic glycine must be administered in high doses (~15-60g/day for 6 weeks) to achieve significant elevations in CSF levels of glycine in humans [38, 39, 40]. Although a number of chemically diverse selective glycine-B site agonists have been synthesized [41,42], all of them showed a lower efficacy than the endogenous glycine-B site agonists glycine and D-serine. This may imply that the scope for the development of novel selective ligands may be limited due to the simple molecular structure of glycine [42]. Instead, enhanced activation of the glycine-B site can be achieved indirectly by inhibition of GlyT1 mediated glycine uptake, which results in the elevation of extra-cellular glycine levels at glutamatergic synapses. Hence, significant effort has recently been directed to the development of different classes of GlyT1 inhibitors [43, 44, 45, 46]; and some of them are currently undergoing pre-clinical and clinical investigations [46]. Concerning the antipsychotic potential of glycine and of the endogenous GlyT1 inhibitor sacrosine [32, 36, 37, 38, 47], the main emphasis has been on the evaluation of their potential as a novel class of antipsychotic drugs especially in the treatment of negative and cognitive symptoms of schizophrenia.

The latest developments in the pharmacotherapy of schizophrenia have been extensively discussed in a number of excellent reviews [46, 48, 49]. Given that the NMDAR neurotransmitter system plays a fundamental role in learning and memory [50, 51, 52] and that cognitive and mnemonic impairments in various diseases have been attributed to deficient NMDAR function [48, 53, 54], this review aims to provide an overview on the possible effects of interventions targeted at the GlyT1 system on cognition. In particular, we want to address whether such manipulations may offer novel therapeutic avenues in the treatment of learning and memory disorders. The latest achievements from electrophysiological, behavioural and clinical research will be reviewed concisely in the following sections.
GLYCINE TRANSPORTER 1 AND COGNITION

Electrophysiological Evidence

In this section, we will first review the potential role of the GlyT1 system in learning and memory on the basis of available electrophysiological data. At the neuronal level, memory formation is thought to involve changes in synaptic strength resulting from chemical and structural modification in the synaptic network that is regulated by complex arrays of coordinated cellular and molecular mechanisms [55]. Long-term potentiation (LTP) refers to a form of long-lasting increases in the synaptic strength that is widely considered to be one of the major physiological mechanisms underlying learning and memory [56].

Given that increased LTP has been associated with enhanced mnemonic performance [57, 58] interventions that enhance LTP have been proposed as a potential strategy to improve learning and memory functions [51]. Due to the pivotal role of the NMDAR in LTP induction as well as in learning and memory, interventions targeting NMDAR mediated neurotransmission have been extensively investigated to evaluate the causal link between NMDAR-dependent LTP and mnemonic performance in a multitude of electrophysiological and behavioural studies [51, 57, 59, 60].

As outlined in the previous section, pharmacological blockade or molecular down-regulation of GlyT1 facilitates NMDAR mediated neurotransmission and/or enhances LTP [12, 13, 14, 15, 16, 17, 36]. However, it has been reported that applications of high doses of glycine or GlyT1 inhibitor CP802,079 reduces NMDAR currents and exerts no effect on LTP [14]. One possible explanation for these findings is that under conditions in which synaptic glycine levels are elevated above the saturation concentration of the glycine-B site, nearly all NMDARs are in the active state due to cooccupancy of glycine and glutamate. Since simultaneous binding of glycine and glutamate also primes the NMDAR for endocytosis [61], a notable number of NMDARs undergoes endocytosis at high glycine levels, and thus fewer receptors are available for neurotransmission than at subsaturating glycine levels [14]. These findings indicate that blockade of GlyT1 can enhance NMDAR neurotransmission and LTP so long as the extra-cellular glycine concentration does not exceed the saturation point of the glycine-B site.

Consequently, the use of GlyT1 inhibitors is expected to potentiate NMDAR functions only up to a certain dosage that holds the glycine concentration at sub-saturating levels.

In accordance with this hypothesis that enhanced LTP may be associated with improved mnemonic functions, recent findings of potentiated LTP following pharmacological blockade of GlyT1 provide some evidence for a potential promnesic effect of interventions designed to reduce GlyT1 transport capacity. However, the potential pro-cognitive efficacy of such interventions may be limited by the saturation level of the glycine-B site at which internalization of NMDARs primed by glycine is expected.

Behavioural Evidence from Animal Models of Learning and Memory

Associative Learning and Latent Inhibition

Associative learning in the form of classical and instrumental conditioning is fundamental to adaptive behavioural change in the animal kingdom. Recent studies from our laboratory have shown that mice with a specific knockout of the GlyT1 gene in forebrain neurons have exhibited clear improvement across multiple paradigms of associative learning, including conditioned freezing and conditioned taste aversion – both are examples of Pavlovian conditioning, and conditioned two-way active avoidance is a task that depends on both Pavlovian and instrumental learning [17]. In each case, the expression of learned behaviour is either more strongly expressed or more rapidly acquired. This is in line with the reported promnesic effect of the partial
glycine-B site agonist D-cycloserine on associative learning [18, 62], highlighting the functional importance of expected glycine-B site activation following GlyT1 blockade. Most importantly, such enhanced learning does not appear to be at a cost of selectivity in learning. Selectivity allows adaptive learning and flexible expression of learned behaviour. Associative learning shows property of selectivity. One example is latent inhibition [63], which refers to the observation that repeated pre-exposures of a stimulus without any significant consequence can retard that stimulus’ ability to enter into association with a significant event (i.e., an unconditioned stimulus – US) when this pre-exposed stimulus is subsequently paired with any US (see Fig. 1). In other words, prior experience with [CS → nothing] impedes the impact of subsequent [CS → US] experience in gaining control over the animals’ response to the CS. Although forebrain neuronal GlyT1 deletion enhances [CS → US] learning, it does not impair latent inhibition (LI). Instead, this genetic knockout enhances the ability to show LI [17]. Thus, the mutant mice exhibited LI, when control mice failed to show it because of an insufficient number of pre-exposures. This finding can also be interpreted as enhanced learning of [CS → nothing] as well as [CS → US]. Alternatively, according to the theoretical account of LI in terms of learning to pay less attention to an irrelevant CS [64, 65], the mutant mice are exhibiting superior control of selective attention based on past experience – i.e., learned inattention. Similarly, pharmacological inhibition of GlyT1 achieved by ALX-5407 has also been reported to potentiate the expression of LI [66]. However, unlike forebrain neurons GlyT1 deletion, ALX-5407 treatment is devoid of an enhancing effect on conditioning to a novel stimulus. Hence, ALX-5407 treatment only interferes with the pre-exposure effect, leading to retarded conditioned response following pre-exposure of the CS that is insufficient to generate LI in control subjects.
Fig. (1). A) Latent inhibition refers to the phenomenon that non-reinforced exposure to the to-be-conditioned stimulus (CS) prior to conditioning results in a decrement of the conditioned response (CR). One attentional model [65] of latent inhibition focuses on the effects of CS pre-exposure on the conditioning phase when CS-US pairing takes place. It suggests that non-reinforced CS pre-exposure reduces the salience or surprise of the CS at the time of conditioning [65], so that the CS fails to gain associative strength with the US. This explains the observed reduction in CR to the CS later. An alternative account posits that during CS pre-exposure, the CS has acquired the ability to elicit inattention [64]. Hence, during subsequent conditioning, the CS commands less attention resulting in weaker conditioning to the US. According to such attentional accounts, the LI effect stems primarily from a failure to acquire the critical [CS → US] association – i.e., LI reflects an “acquisition failure”. B) The LI procedure involves the acquisition of two independent and conflicting contingencies, namely a [CS → nothing] association (developed in pre-exposure) and a [CS → US] (developed during conditioning). The two learned contingencies compete for the control of the ‘PE’ animals’ response to the CS (i.e., CR) during test in which the CS is presented on its own. The competition between the acquired [CS → nothing] and [CS → US] associations leads to a reduction in the vigour of the CR in the ‘PE’ animals in comparison to the ‘nPE’ animals that are not under the influence of such competition over the control of CR expression, because their response to the CS is primarily determined by their previous experience of [CS → US] only. This difference in the vigor of CR between PE and nPE subjects constitutes the LI effect. According to this switching model based on proactive interference [69] the LI effect is therefore not an acquisition deficit (as suggested by attentional theories); but rather an ineffective expression of the acquired of [CS → US] association resulting from a competition with the [CS → nothing] association during test. The LI effect is disrupted in actively psychotic schizophrenia patients, thus lending strong support for the face validity to the animal LI model for schizophrenia [97].

Hence, although ALX-5407 cannot differentiate between neuronal and glial GlyT1, it seems to be associated with only part of the effect seen following selective neuronal GlyT1 disruption. This is in keeping with the observation that the glycine-B site agonist D-serine also exerts a similar impact on LI [66], again highlighting that the glycine-B site is likely to be the critical downstream event following GlyT1 inhibition. This may paradoxically suggest that non-selective GlyT1 blockade may reduce some of the pro-cognitive potential of GlyT1 inhibition – a theme that we shall return to later.
In addition, ALX-5407 can also nullify the abnormal expression of LI induced by the NMDAR channel blocker MK-801 [66]. Namely, MK-801 administration at a highly specific dose (0.15mg/kg i.p. in mice) can result in the persistence of LI expression even when pre-exposure and conditioning are conducted in two distinct contexts. This is abnormal because such a change typically abolishes the LI effect in normal subjects. This reflects the context dependency of the LI effect and illustrates that the expression of the LI effect is additionally commanded by contextual cues [67, 68], indicating that the animals are clearly adaptive to ignore or not attend to a stimulus only in the context in which it is irrelevant.

NMDAR blockade by MK-801 eliminates this contextual control and results in the indiscriminate expression of LI, but ALX-5407 pre-treatment restores this contextual control. According to [66] MK-801 treatment results in a failure to switch to responding according to [CS → US] as prompted by the fact that prior [CS → nothing] pre-exposure has been conducted in a different context. Given that behavioral switching has been related to cognitive flexibility [69], this interpretation suggests a role of GlyT1 in maintaining cognitive flexibility. This is in agreement with the interpretation that GlyT1 can modulate selective attention in bringing about selectivity in learning. Unfortunately, an equivalent test has not been conducted in the forebrain neuronal GlyT1 knockout mice. The outcome of such an experiment would yield valuable data regarding not only the control of associative learning by response competition and acquired selective attention, but also the control over the expression of learned behaviour by contextual cues. GlyT1 seems to modulate all these critical factors of associative learning that enable effective adaptation to the changing conditions of an organism’s environment.

**Spontaneous Recognition Memory**

Further support for a potential promnesic effect of forebrain neuronal GlyT1 disruption comes from the recent finding that this molecular manipulation also enhances recognition memory [70]. In this test (see Fig. 2), mice confronted with a novel and a familiar object, typically exhibit preferential exploration of the novel object. This spontaneous preference is used as a measure for object recognition memory. Memory retention of the familiarized object can also be evaluated by using different delay intervals between object familiarization and test. Forebrain neuronal specific GlyT1 knockout mice exhibited such a preference at delays when control mice are no longer able to sustain such a preference – i.e., the control mice were indifferent to the novel and familiar objects. At very short delay intervals however, the two groups of mice performed similarly. This demonstration of a delay-dependent enhancement of spontaneous object recognition memory suggests that this selective molecular intervention facilitates not only the development of Pavlovian conditioning, but also retention of complex object representation.

In addition, memory and encoding of the relative spatial location of objects also appears to be enhanced by forebrain neuronal specific GlyT1 knockout. In a modified version of the object
recognition test [71, 72] memory for the location of an object in a specific place (Fig. 2) was evaluated by testing the animals’ response to a spatial switch in the positions of two objects belonging to an array of four different objects. The finding that mutant mice exhibited preferential exploration towards those displaced objects while such an explorative preference was absent in controls is suggestive of an enhancement in object-in-place memory in mutants, lending further support for the assertion that disruption of neuronal GlyT1 in the forebrain can interfere with multiple memory systems.

Whether pharmacological blockade of GlyT1 by currently available GlyT1 inhibitors would similarly enhance recognition memory has not yet been investigated. However, the effect of the GlyT1 inhibitor SSR-504734 on recognition memory in a social context has been evaluated. In a social novelty discrimination paradigm, adult rats are tested for the ability to discriminate between a familiar and a novel juvenile [12, 73]. It was found that SSR-504734 itself had no effect on social discrimination across the tested dose range of 1-10mg/kg, but the drug was able to antagonise the social discrimination deficit induced by neonatal phencyclidine (PCP) treatment. Since social novelty discrimination has also been proposed as a model for selective attention [74] pharmacological inhibition of GlyT1 may be therapeutically beneficial against existing deficits in attention. This is in agreement with the reported effect of forebrain neuronal GlyT1 deletion and pharmacological blockade of GlyT1 on latent inhibition discussed before. The absence of a promnesic effect of SSR-504734 on its own may be attributed to the fact that the delay interval separating the familiarization phase from the test phase was minimal. The memory demand of the task was probably too low under these conditions to demonstrate a promnesic effect. Additional tests using extended delays would therefore be highly warranted and may shed light on the potential promnesic effect of SSR-504734 and other GlyT1 inhibitors on social recognition memory. Moreover, the effect of SSR-504734 on object (non-social) recognition memory remains to be evaluated. This would allow a more direct comparison with the positive effect observed in forebrain neuronal GlyT1 knockout mice.

Any potential differences may point further to a functional distinction between neuronal and glial GlyT1 function.
Paradigms of spontaneous recognition memory take advantage of the naturally occurring preference for novelty in rodents. In the spontaneous object recognition test [72, 98] the animal is familiarized with two identical sample objects. Following a retention interval the animal is re-exposed to a third copy of the now familiarized sample object and a novel object in the test phase. The explorative preference towards the novel over the familiar object serves as index measure to assess recognition memory for the identity of objects. In the object location recognition memory task [71] the animal is familiarized with a spatial arrangement of four different objects. After the termination of the retention interval the positions of two diagonally opposing objects are interchanged and the animal reexposed to the rearranged objects assembly. The preferential exploration of the displaced objects is used to index recognition memory for the conjunction between objects and spatial locations.

**Spatial Reference Memory in the Morris Water Maze**

Spatial reference memory in the Morris water maze [75] is a classical hippocampus dependent paradigm sensitive to a number of interventions targeting the NMDAR neurotransmitter system in the hippocampal formation [76, 77 78]. This test requires the animals to learn about the fixed location of a hidden escape platform in a circular tank of water. Effective learning is reflected in effective escape from the water regardless of the starting position. Hippocampal lesions severely disrupt water maze learning [77] and so does hippocampal blockade of NMDAR receptors [79, 80]. The water maze test has also been shown to be sensitive to the cognitive enhancing effect of NR2B over-expressing mice [51]. Reports of enhanced acquisition and retention of spatial reference memory in the water maze by treatment of glycine-B site agonists such as D-serine and D-cyclosereine [81, 82, 83] readily suggest that GlyT1 inhibition may achieve similar promnesic effects.

However, it was found that global heterozygous knockout of GlyT1 [16] failed to affect acquisition of spatial reference memory in the water maze, and only a mild improvement of memory retention or retrieval was observed in the form of enhanced accuracy in a transfer test when the escape platform was removed from the water maze. This marginal effect of global heterozygous GlyT1 knockout in the water maze test [16] contrasts with the notable promnesic effect of homozygous forebrain neuronal GlyT1 deletion on associative learning and recognition memory [17, 70]. This contrast may be related to the differences in the specificity of the GlyT1 deletion (neuronal vs. global) or it may be attributed to a gene dosage effect within the neuronal GlyT1 population (homozygous vs. hemizygous deletion of the GlyT1 gene). Hence, it is premature to conclude that reduction of GlyT1 exerts no promnesic
effect on water maze learning. Further experiments with various conditioned knockout systems are clearly warranted.

Prepulse Inhibition of the Acoustic Startle Response

Prepulse inhibition (PPI) refers to the phenomenon that the reflexive reaction to a startle-eliciting stimulus can be attenuated if its presentation is shortly preceded by a nonstartling prepulse stimulus [84, 85]. According to the protective hypothesis of Graham [85], PPI reflects the protection of ongoing processing of the antecedent prepulse against interference by the succeeding pulse, which is believed to be of biological relevance as it prevents interference of currently activated neuronal programs. PPI has been increasingly used as an operational measure of sensorimotor gating, which is thought to play an important role in pre-attentional and executive functions [86] that are crucial for normal cognition. A correlation of the PPI magnitude with the mnemonic performance in different learning tasks [87; 88] has led to the suggestion that high levels of PPI may be a predictor of enhanced cognitive functions [87]. Based on the PPI disruptive effect NMDAR antagonists [89], which can be antagonized by activating the glycine-B site [66], it is suggested that manipulations targeted at the GlyT1 system may also be expected to modulate PPI.

The effect of GlyT1 inhibition on the expression of PPI was evaluated in several pharmacological and molecular studies. The GlyT1 inhibitors SSR-504734 [12] and NFPS [15] were found to enhance the level of PPI in DBA/J2 mice, a mouse strain with a low basal level of PPI [15, 99] and the GlyT1 inhibitor ORG-24598 partially restored the PPI impairment induced by neonatal ventral hippocampal lesions in rats [15]. PPI disruption induced by the NMDAR channel blocker MK-801 was also reversed by systemic administration of the GlyT1 inhibitor ALX-5407 [66]. However, the effect of ALX-5407 in PPI is in sharp contrast with the observation that global heterozygous knockout of GlyT1 exacerbates the PPI disruption induced by MK-801 [16]. This difference may be attributed to the limited impact of the hemizygous GlyT1 knockout in comparison with systemic ALX-5407 treatment. The possible explanation is also consistent with the observation that pre-treatment with NFPS, the racemic form of ALX-5407, is effective against PCP induced motor stimulation [91] while hemizygous GlyT1 knockout is not [16]. Interestingly, forebrain neuronal specific homozygous deletion of the same gene is sufficient to attenuate PCP-induced motor stimulation [17].

In summary, pharmacological inhibition of GlyT1 can reverse PPI deficits in different animal models of PPI deficiency, including the specific mouse strain of DBA/J2. Hence, blockade of GlyT1 may be of therapeutic value in the treatment of diseases with clear sensorimotor gating deficits. In contrast, there is little evidence that genetic knockout of GlyT1 can potentiate PPI beyond the levels observed in wild type mice. This is however in line with the failure of pharmacological GlyT1 inhibition to enhance PPI in the general population, and thus the potential of GlyT1 inhibitors to enhance early attentional control or sensorimotor gating mechanisms in healthy subjects appears limited.
Clinical Evidence from Schizophrenic Patients

Although an increasing number of novel high-affinity GlyT1 inhibitors has been shown to possess varying degrees of antipsychotic-like properties in animal models of schizophrenia [e.g., 12, 15, 91, 66], direct clinical evidence is sparse. Existing data typically involves the use of GlyT1 inhibitor as an adjunctive treatment to conventional pharmacotherapy. For example, the weak endogenous GlyT1 inhibitor, sacrosine, has been reported to facilitate control over negative and cognitive symptoms in schizophrenic patients who are concurrently receiving classical antipsychotic medication [36, 92, 93].

To an extent, the absence of clinical data on high-affinity GlyT1 inhibitors may be related to safety concerns for the sacrosine-based compounds ALX-5407 and Org-24461 [46]. NFPS induced convulsions and elicited akathisia-like behaviour (motor restlessness), in mice [46], a side effect that is also common among existing antipsychotic drugs especially when they are chronically administered [94]. In addition, systemic ALX-5407 and Org-24461 caused neuronal death in various brain regions including hippocampus, striatum and cerebellum [95]. These shortcomings will possibly be overcome by more recently developed non-sacrosine based inhibitors that may fulfil all necessary requirements to enter clinical phase evaluation.

So far, sacrosine as well as glycine and D-serine have been evaluated as an adjunctive treatment to classical antipsychotic drug treatments, and it is therefore unclear if pharmacological GlyT1 inhibition and the resulting activation of the glycine-B site on its own are associated with clear therapeutic efficacy. One caveat is that the apparent efficacy may stem from their ability to counter the NMDAR hypofunction induced by conventional antipsychotic drugs, rather than a genuine attenuation of the negative and cognitive deficits intrinsic to the disease. Support for this possibility is provided by the finding that the typical antipsychotic haloperidol exhibits antagonist activity at the NMDAR which was attenuated by increasing the extra-cellular glycine concentration [96] indicating that haloperidol possibly interacts with the glycine-B-site and that these interactions may be antagonized by the activation of the NMDAR’s glycine-B site as a consequence of GlyT1 inhibition. In order to clarify the extent to which this may be responsible for the therapeutic efficacy of GlyT1 inhibitor co-administration with antipsychotics, further investigations on the potential interactions between other typical/atypical antipsychotics and GlyT1 inhibitors or glycine-B-site agonists need to be carried out.

In summary, the existing clinical data on the efficacy of GlyT1 inhibition for the negative and cognitive schizophrenia symptoms remain preliminary, although there are promising outcomes. On the other hand, the potential of this avenue of pharmacological intervention against other neuropsychiatric conditions has yet to be explored in humans. Data from animal experiments also support the hypothesis that a down-regulation of GlyT1 function may also possess therapeutic value in the treatment of cognitive and mnemonic disorders such as dementia, and cognitive enhancing property in healthy individuals.
CONCLUSIONS AND FUTURE OUTLOOK

On the basis of existing pre-clinical electrophysiological and behavioral studies (summarized in Table 2), it is reasonable to conclude that inhibition of GlyT1 constitutes a feasible approach to modulate NMDAR functions and cognition including learning and memory. Thus, interventions that reduce GlyT1 function may prove useful for the treatment of cognitive disorders related to impaired NMDAR functions and potentially improve learning and memory in nonpathological conditions. Some of the most convincing behavioural evidence in animals for a potential promnesic effect is derived from a series of studies with forebrain neuronal specific GlyT1 knockout mice [17]. These mutant animals exhibit enhanced associative learning across multiple paradigms and facilitated recognition memory. Furthermore, restoration of cognitive deficits in pre-clinical models of neuropsychiatric conditions such as PPI disruption and impaired social recognition was demonstrated by both pharmacological blockade and molecular deletion of GlyT1 [12, 15, 16, 36, 66]. In clinical trials, the endogenous GlyT1 inhibitor, sacrosine, improves cognition in schizophrenia patients when it is administered adjunctively with antipsychotic medication [92,93]. At the neurophysiological levels, both pharmacological and molecular inhibition of GlyT1 can increase NMDAR current and potentiate LTP, thus lending some support for the hypothesis that the potentiation of NMDAR functions by glycine-B site activation may underlie at least some of the observed pro-cognitive effects of GlyT1 inhibition. However, existing evidence remains circumstantial and a causal relationship linking GlyT1 inhibition, enhanced glycine-B site occupancy and NMDAR-mediated neurotransmission to cognitive enhancement is far from firmly established. Necessary experiments are needed to exclude potential contributions by other central actions of GlyT1 inhibitors. First, glycine-B site blocker should be able to nullify the observed promnesic effects of GlyT1 inhibitors, and to normalize the behavioral phenotype of GlyT1 knockout mice to wild type levels. Second, the efficacy of GlyT1 inhibition to enhance cognition should be limited when glycine-B site occupancy reaches saturation. Indeed, further activation beyond saturation would be expected to result in a negative impact on NMDAR-mediated neurotransmission and may even impair NMDAR dependent cognitive processes. Validation of these characteristics would represent significant advances towards a better understanding of the regulatory function of GlyT1 on NMDAR mediated processes. It has been hypothesized that GlyT1 inhibition can cause augmentation or reduction of NMDAR functions via modulation of the synaptic glycine concentration. This is mainly based on the finding that high doses of the GlyT1 inhibitor CP-802,079 not only failed to enhance LTP, but also led to slight reduction in the NMDAR current [14, 61]. A similar outcome seen after exogenous glycine application [14] strengthens this hypothesis and further suggests that moderate levels of GlyT1 inhibition should be more favourable than high doses. The optimal level would be if the synaptic glycine concentration approaches the glycine-B site saturation point but does not exceed it. The therapeutic value of further GlyT1 inhibition is also limited by severe respiratory depression, which can be fatal [46]. This side effect is the primary cause of early death in constitutive homozygous
GlyT1 knockout mice on the first postnatal day [7]. These limitations highlight the difficulty in defining the optimal dosage that can maximize the clinical potential of GlyT1 inhibition while avoiding the potential severe detrimental side effects.

At present, available GlyT1 inhibitors cannot distinguish between glial and neuronal associated GlyT1. Compounds with a selective preference for one or the other type of GlyT1 system may prove valuable. The presence of a more robust and profound promnesic effect following forebrain and neuron specific GlyT1 knockout in comparison to the limited effect seen on learning following constitutive GlyT1 knockout (see Table 2) may suggest that it is desirable to achieve a selective inhibition of neuron-associated GlyT1, and to avoid blockade of glial-associated GlyT1. Insufficient selectivity for neuronal GlyT1 may offer a potential explanation to the apparent lack of any convincing promnesic properties by existing GlyT1 inhibitors apart from some improvement of some cognitive deficits in pre-clinical models of schizophrenia (see Table 2). To fully explore this hypothesis, the development of a new generation of GlyT1 inhibitors with a clear selectivity for neuronal specific GlyT1 populations would be highly relevant.
Table 2. Summary of Electrophysiological, Behavioral and Clinical Evidence for the Potential Role of GlyT1 in Cognitive and Mnemonic Processes

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<td>Spatial learning</td>
<td>Global heterozygous GlyT1 knockout weakly improved reference memory in the Morris water maze</td>
<td>16</td>
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<td>Clinical tasks</td>
<td>The endogenous GlyT1 inhibitor carbazine improved cognitive symptoms of schizophrenia in patients receiving antipsychotic medications</td>
<td>16, 82, 93</td>
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ACKNOWLEDGEMENTS
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REFERENCES
Chapter 1: General introduction

[68] De la Casa, L.G.; Lobow, R.E. Neuropsychology, 2001, 15, 244.
Chapter 2

Enhanced recognition memory following glycine transporter 1 deletion in forebrain neurons

With Joram Feldon, Detlev Boison, Hanns Mohler, Joram Feldon and Benjamin K. Yee

[Personal contribution to the work: Designing and performing the research, analysis of the behavioural data, and writing the manuscript]
Abstract

Selective deletion of glycine transporter 1 (GlyT1) in forebrain neurons enhances N-methyl-D-aspartate receptor (NMDAR) dependent neurotransmission and facilitates associative learning. These effects are attributable to increases in extracellular glycine availability in forebrain neurons due to reduced glycine re-uptake. Here, we investigated whether this molecular intervention can affect recognition memory using our forebrain and neuron specific GlyT1 knock-out mouse line (CamKIIαCre;GlyT1tm1.2fl/fl). In a spontaneous object recognition memory test, enhanced preference to a novel object was demonstrated in mutant mice relative to littermate controls at a retention interval of 2h but not at 2min. Furthermore, mutants were responsive to a switch in the relative spatial positions of objects, whilst controls were not. We went on to show that these potential pro-cognitive effects were demonstrated against a lack of difference in contextual novelty detection: Mutants and controls showed equivalent preference for a novel over a familiar context. Our results therefore extended the possible range of potential mnemonic effects of specific forebrain neuronal GlyT1 deletion from associative learning to recognition memory, and lend further support to the possibility that mnemonic functions can be enhanced by reducing GlyT1 function.
INTRODUCTION

Increased \(N\)-methyl-\(D\)-aspartate receptor (NMDAR) activity has been suggested to enhance cognitive functions by facilitating NMDAR-dependent neural plastic events (Tang et al., 1999). However, direct interventions at the NMDAR with competitive agonists are accompanied by potential undesirable effects including the induction of neurotoxicity and seizures (Danysz & Parsons, 1998). Given that successful activation of the NMDARs by the endogenous neurotransmitter glutamate requires the obligatory binding of the co-agonist glycine to the NMDAR glycine binding site (glycine-\(B\)-site), an alternative strategy to increase NMDAR activity is to enhance the availability of glycine in the vicinity of NMDARs. However, the efficacy of systemic administration of glycine itself is limited due to poor blood-brain barrier penetration and glycine must be administered in high doses (~15-60g/day for 6 weeks) in order to significantly increase brain glycine levels in humans (D’Souza et al., 2000, Heresco-Levy et al., 2000, 2004). Furthermore, the simple structure of glycine allows little scope for the development of novel high-affinity glycine-\(B\)-site agonists (Milan 2002). Instead, glycine site occupancy at NMDARs can be enhanced by elevating the endogenous glycine concentration, which is normally maintained at sub-saturation levels by glycine transporter 1 (GlyT1) (Aragon et al., 2005, Berger et al., 1998, Eulenburg et al., 2005, Javitt 2006). It has been shown that inhibition or down-regulation of GlyT1 can enhance NMDAR functions and may offer a superior approach to enhance NMDAR functions than the use of direct NMDAR agonists (Depoortère et al., 2005, Martina et al., 2005, Bergeron et al., 1998). Although there is evidence that several GlyT1 inhibitors may be effective against psychotic-related cognitive deficits (e.g., Kinney et al., 2003; Lipina et al., 2005, Lindsley et al., 2006), systemic GlyT1 antagonist treatment has not yet revealed any convincing promnesic effects (Depoortère et al., 2005). This may be partly attributable to the inability to distinguish between neuron- and glial-associated GlyT1 by available GlyT1 inhibitors. Similarly, mice with partial deletion of both neuron- and glial-associated GlyT1 through a constitutively targeted disruption of the GlyT1 gene only exhibited limited enhancement in learning and memory (Tsai et al., 2004). The most convincing data has thus far been derived from a novel mouse line (CamKII\(\alpha\)Cre;GlyT1tm1.2fl/fI) with a selective deletion of GlyT1 in forebrain neurons (Yee et al., 2006). These animals exhibited clear facilitation in classical conditioning across multiple paradigms. The possibility that this mouse line may also show enhanced learning and memory function beyond associative learning has not yet been explored. It is the aim of the present study to evaluate this suggestion using a series of recognition memory tests, developed on the basis of the spontaneous explorative preference for novelty in rodents (Aggleton et al., 1999, Ennaceur et al., 1988 & 1994). A number of molecular as well as pharmacological manipulations targeting at the NMDAR have been shown to affect memory performance in object recognition and related paradigms (e.g. Rampon et al., 2000, Tang et al., 1999, de Lima et al., 2005, Baker & Kim, 2002, Hashimoto et al., 2005). In particular, enhanced object recognition memory has been reported following over-expression of the NR2B subunit of the NMDAR in the mouse forebrain which concomitantly led to an elevation in NMDAR activity (Tang et
al., 1999). Like the forebrain neuronal knockout of GlyT1, forebrain overexpression of NR2B subunit also enhanced Pavlovian conditioning. These results suggest that increases in NMDAR activity is a viable strategy to enhance cognitive function, and lend support to the hypothesis that enhanced NMDAR function expected due to forebrain neuronal specific GlyT1 disruption (Yee et al., 2006) may result in a comparable promnesic effect on recognition memory. A positive outcome would extend our previous finding and provide additional support for the suggestion that disruption of GlyT1 function may represent a viable promnesic intervention for multiple mnemonic functions. The experiments were conducted in the same cohort of animals with a genotype × sex factorial design.

MATERIALS AND METHODS

Generation and characterization of CamKIIαCre;GlyT1tm1.2fl/fl mice
A full description of the generation of the CamKIIαCre;GlyT1tm1.2fl/fl mouse line has been previously provided (See Gabernet et al., 2005; Yee et al., 2006). Biochemical validation of the neuron and forebrain specific deletion of the GlyT1 gene, and electrophysiological data on NMDAR functions have been reported elsewhere (Yee et al., 2006).

Subjects
The experimental subjects were obtained by crossing CamKIIαCre;Glyt1tm1.2fl/fl mice with Glyt1tm1.2fl/fl mice which resulted in a mixture of offspring comprising the CamKIIαCre;Glyt1tm1.2fl/fl (hereafter denoted as “mutant”) and Glyt1tm1.2fl/fl (hereon denoted as “control”) genotype in a 1:1 ratio. Using this breeding strategy the CamKIIαCre allele was maintained in a heterozygous state, while all animals were homozygous for the Glyt1tm1.2fl/fl allele. Genotype was determined by standard PCR on postnatal days 21-30 as previously described (Yee et al., 2006).

Breeding was performed in a specific-pathogen free (SPF) breeding facility (ETH Zurich Laboratory of Behavioural Neurobiology, Schwerzenbach, Switzerland). Litters were weaned at postnatal day 21. A mixture of mutant and control littermates of the same sex was kept in groups of four to six in Macrolon Type-III cages (Techniplast, Milan, Italy). At the age of 12 weeks, the animals were transferred to a separate animal vivarium (21±1 C°, relative humidity: 55±5%) for the duration of behavioural testing. Animals always were maintained under ad libitum water and food (Kliba 3430, Klibamuhlen, Kaiseraugst, Switzerland), and kept under a reversed light-dark cycle with lights off from 0800-2000hrs. All behavioural experiments were conducted in the dark phase of the light cycle.

The experimental cohort consisted of both male (11 mutant and 9 control) and female (11 mutant and 11 control) subjects. The animals were approximately 24 weeks old at the time of testing. Previously, at 12 weeks of age, all animals had been used in a classical conditioning experiment with an aversive shock unconditioned stimulus. All animals were employed in all three experiments described here, but only subjects that had successfully completed the sample phases of the experiment were included in the final analyses presented here (see procedures below).
All experimental procedures described had previously been approved by the Zürich Veterinary Office; they also conformed to the ethical standards stipulated by the Swiss Act and Ordinance on Animal Protection and were in accordance to the European Council Directive 86/609/EEC.

**Apparatus**

The apparatus consisted of a rectangular arena measuring 40 x 61 cm and was surrounded by 22 cm high transparent Plexiglas walls. Partition walls of the same material could be installed to allow different compartmentalization of the arena and to achieve the desired configuration (see Figure 1). The arena rested on a table top elevated 100 cm from the floor in the centre of a testing room with an ambient light level of ~100 lux.

In Experiment 1, the arena was divided into three equal compartments (see Figure 1) by the addition of two walls. Each wall had a circular opening (5 cm in diameter) in the middle, positioned at 1 cm above the floor of the arena providing access to the side compartments from the central compartment. The circular opening could be blocked by a sliding door made of Plexiglas. In order to prevent excessive interferences from extra-maze cues, an outer 25-cm high perimeter wall made of white Plexiglas was installed around the entire arena at a distance of 15 cm (see Hale and Good, 2005).

**Figure 1**

![Figure 1: Schematic representation of the experimental procedures (not to scale). Each experiment consisted of a sample and a test phase separated by a predetermined retention interval and took place in a rectangular test arena ‘A’. In Experiments 1 & 2, the test arena was additionally surrounded by an outer perimeter walls ‘B’. Experiment 1: In the sample phase, the animals were familiarized with the sample object C in the left and the right compartment of the test arena. In the test phase, the animals could freely explore the now familiar sample object C and a novel object D. Experiment 2: In the sample phase, the animals were familiarized with the sample object configuration {E, F, G, H}. In the test phase, the animals could freely explore the novel object configuration when the positions of two diagonally opposing objects were switched (objects E and F). Experiment 3: In the sample phase, the animals were familiarized with the sample compartment (I). In the test phase, the animals could freely explore the now familiar sample compartment (I) and the “novel” compartment (J) that was inaccessible to the animals in the sample phase.]


In Experiment 2, the arena was used without any compartmentalization (see Figure 1).

In Experiment 3, the arena was set up similar to the configuration used in Experiment 1, except that two additional walls were installed so that the central compartment was reduced to a 10-cm wide corridor linking the left and the right compartments. The arena was used without the outer opaque perimeter wall (see Figure 1).

A digital camera was mounted directly above the arena. Video records of all sessions were stored for subsequent data extraction. In addition, images were transmitted to a PC running the Ethovision tracking system (Noldus, NL) in Experiment 3 to allow the extraction of additional data.

Various distinct three-dimensional objects, differing in form, size, texture, materials and smell, were employed as discriminanda for Experiments 1 and 2. They were randomized between groups, and were never repeated across tests.

**Behavioural Procedures**

**Habituation**

The animals were first habituated to the apparatus in the configuration described in Experiment 1. The sliding doors were removed allowing free access to all three compartments. The animals were allowed to explore freely for 5 min over two consecutive days.

Next, the animals were familiarized with the placement of objects into the arena. The familiarization (or habituation) procedure was required to minimize potential neophobia that might inhibit object exploration in general. Two identical objects were placed into the arena: one in each of the side compartments. The animals were initially placed into the central compartment with both sliding doors closed. One of the two doors was open, and the animals were allowed to explore for 5 min. This was repeated immediately afterwards by returning the mouse to the central compartment; the other door was then open and the animals were allowed access to the other compartment containing an identical object. This was conducted on two consecutive days, each time with a new pair of identical objects.

**Experiment 1: Object recognition memory with variable retention intervals**

An object recognition trial comprised two phases: a sample phase and a test phase. The sample phase consisted of two consecutive exposures to the apparatus. The subject was first exposed to the sample object placed in one compartment for 5 min, and then immediately afterwards to a similar sample object placed in the other compartment (see Figure 1). It was then removed from the apparatus and was either kept in a holding cage (for 2-min and 2-h) or returned to the home cage (for 1-d) for the retention interval prior to the test phase. The apparatus was then cleansed with 50% ethanol and dried before the test phase commenced. In the test phase, access to both compartments was available. One compartment contained a third copy of the sample object, and the other a novel object. The placement of the novel objects (left or right compartment) was counterbalanced between groups and across the
two trials of a given retention condition. The animal was allowed to explore freely for 5 min.

The entire experiment spanned across a period of 13 days. On days 1 and 3, one trial of object recognition with a 2-min retention interval was performed per day. On days 5 and 7, one trial of object recognition with a 2-h retention interval was performed per day. On days 9–10 and 12–13, one trial of object recognition with a 1-d retention interval was performed every 2 consecutive days. There were altogether six trials with an inter-trial interval of 1 day and 2 trials per retention interval (at 2min, 2h or 1d). All trials were conducted in the same testing room and a new set of objects were used for each trial.

At the end of Experiment 1, the animals were left undisturbed in their home cages for 4 days before the next experiment began.

**Experiment 2: Object-location recognition memory**

To begin the sample phase, the animals were exposed to the apparatus (without any internal walls) containing a configuration of four discrete objects for 10 min. The four objects were positioned in the four corners: 10cm from the long wall, and 15cm from the short wall of the arena. Next, the animals were removed from the apparatus and kept in a holding cage for the 2 min retention interval. The apparatus was then cleansed with 50% ethanol, each object replaced by an identical copy and the positions of two diagonally opposing objects were interchanged. The animals were then returned to the apparatus and allowed to explore the new configuration of objects for 5 min. Two trials were conducted in the same testing room, separated by 24h, each with a different set of four objects.

At the end of Experiment 2, the animals were left undisturbed in their home cages for 4 days before the next experiment began.

**Experiment 3: Spontaneous place preference**

The apparatus was configured as depicted in Figure 1. In the sample phase, the animals were exposed to one of the two compartments, pseudo-randomly selected, for a period of 10 min. The animals were first placed into the central area and the door to one of the compartments was lifted. This was followed by a 2-min retention interval when the animals were kept in a holding cage and the apparatus was cleansed. Next, the test phase commenced when the animals were returned to the apparatus with access to both compartments now available. The animals were allowed to explore freely for 5min. Two such trials, each conducted in a different room, were performed, separated by a 24-h interval. The animal’s trajectory in the arena was recorded and analysed by Ethovision (Noldus, NL). The explorative preference for the unexplored compartment over the familiarized/explored compartment provided a measure of contextual novelty detection.
Dependent variables
Object exploration time was the chief dependent variable extracted from examination of the recorded tapes in Experiments 1 and 2. This was performed with the assistance of Noldus Observer® under blind conditions: the experimenter was blind to the identities (genotype and sex) of the subjects and the identities of the novel/displaced objects. Object exploration was defined as active interaction of the animal (e.g., sniffing, gnawing) with the object at a distance of less than 1 cm (Ennaceur and Delacour, 1988; Aggleton 1993). Object recognition was not scored when an animal made a contact with the objects without facing it, or when it climbed on top of the objects.

The time spent in object exploration in the sample phase and in the test phase were analysed separately. In the latter, a discrimination ratio was also calculated to measure the preferential exploration of the novel (Experiment 1) or displaced objects (Experiment 2). The discrimination ratio always contrasted the differential exploration for the target (novel or displaced) objects with the total amount of exploration (to target and non-target objects).

All data were averaged across the two trials (replications) of each test condition, before being submitted to statistical analysis.

Statistical analysis
All statistical analyses were conducted using SPSS for Windows (version 13) implemented on a PC running the Windows XP (SP2) operating system. Parametric analyses of variance (ANOVA) were performed with the between-subject factors of genotype and sex. Additional within-subject factor included the retention intervals for Experiment 1. Performance in the sample phase and test phase were separately analysed, but additional analysis of covariance (ANCOVA) were conducted to examine the impact of possible confound between sampling phase performance on test phase performance.

RESULTS
Experiment 1: Object recognition memory with variable retention intervals
Object recognition memory was assessed at three different delays: 2 min, 2h or 24h. Two separate tests were conducted for each delay condition to enhance reliability (Hale & Good, 2005). The data were averaged across the two tests of each delay condition before being submitted to statistical analysis. Only animals (mutant: 8 male & 6 female; control: 7 male & 6 female) that exhibited non-zero object exploration in the sampling phase in all six tests were included in the final analysis (excluded animals: mutant: 3 male & 5 female; control: 2 male 5 female).

Preferential exploration of the novel objects in the tests was expressed using a discrimination ratio: (exploration time of novel object - exploration time of familiar object) / sum of both novel and familiar object exploration times, as described by Ennaceur & Delacour (1988). As illustrated in Figure 2, both mutant and control mice exhibited a positive preference for the novel object at the
shortest retention delay of 2 min, but only the mutant mice were able to maintain this up to a delay of 2 h. At a delay of 1 day, both mutant and control mice failed to discriminate between a novel and a familiar object. This phenotype was similarly observed in male and female mutant mice. These impressions were confirmed by a $2 \times 2 \times 3$ (genotype $\times$ sex $\times$ delays) ANOVA of the discrimination ratio, which yielded a significant effect of delays [$F(2,46)=9.55$, $p<0.05$], and of genotype [$F(1,23)=4.67$, $p<0.05$]. No significant interaction was observed. Separate ANOVA’s restricted to each delay condition indicated that the mutant and control mice differed significantly only in the 2-h delay condition [$F(1,23)=11.44$, $p=0.003$]. Neither the factor sex nor its interaction attained statistical significance.

Examination of object exploration time during the sample phase indicated that mutant and control mice also differed in object familiarization (see Table 1). An $2 \times 2 \times 3$ (genotype $\times$ sex $\times$ delays) ANOVA indicated that mutant mice spent more time exploring the object presented during the sample phase than did control mice [$F(1,23)=6.60$, $p<0.05$]. Given that increased familiarization may be expected to lead to enhanced memory retention, we evaluated whether the apparent promnesic effect observed in the test phase could be solely attributed to the increase in object familiarization seen in the mutant mice. To this end, an ANCOVA of the discrimination ratio (used to index preferential exploration of novel object) was conducted using the sample phase object exploration time as...
This was performed for the object recognition tests at 2h retention interval when the mutant mice showed a significant enhancement in object memory. The genotype effect remained significant [F(1,22)=5.74, p<0.05] without a significant effect of covariate [p=0.54]. This lends support to the interpretation that the mutant mice showed enhanced discrimination between novel and familiar objects, and this effect was solely not explicable by their concomitant increased levels of object exploration in the sample phase.

Table 1

<table>
<thead>
<tr>
<th>Retention test at 2min</th>
<th>Mutant Male (n=8)</th>
<th>Female (n=6)</th>
<th>Control Male (n=7)</th>
<th>Female (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sample phase:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample object exploration [sec]</td>
<td>16.7±3.3</td>
<td>21.7±3.2</td>
<td>16.8±3.2</td>
<td>10.1±2.7</td>
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<tr>
<td><strong>Test phase:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Novel object (N) exploration [sec]</td>
<td>13.5±2.4</td>
<td>10.6±2.4</td>
<td>11.0±2.4</td>
<td>5.2±1.9</td>
</tr>
<tr>
<td>Familiar object (F) exploration [sec]</td>
<td>9.7±2.3</td>
<td>4.0±0.4</td>
<td>4.3±0.9</td>
<td>3.7±1.3</td>
</tr>
<tr>
<td>Discrimination ratio* [(N-F)/(N+F)]</td>
<td>0.19±0.07</td>
<td>0.35±0.11</td>
<td>0.33±0.10</td>
<td>0.25±0.15</td>
</tr>
<tr>
<td><strong>Retention test at 2h</strong></td>
<td></td>
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<tr>
<td><strong>Sample phase:</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Sample object exploration [sec]</td>
<td>16.7±2.9</td>
<td>19.7±4.0</td>
<td>11.9±2.5</td>
<td>8.9±2.5</td>
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<td><strong>Test phase:</strong></td>
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<td></td>
</tr>
<tr>
<td>Novel object (N) exploration [sec]</td>
<td>10.6±2.9</td>
<td>7.9±1.3</td>
<td>4.7±1.1</td>
<td>2.5±0.8</td>
</tr>
<tr>
<td>Familiar object (F) exploration [sec]</td>
<td>3.7±0.8</td>
<td>4.9±0.7</td>
<td>4.5±0.9</td>
<td>2.9±0.7</td>
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<tr>
<td>Discrimination ratio* [(N-F)/(N+F)]</td>
<td>0.46±0.07</td>
<td>0.23±0.08</td>
<td>0.04±0.11</td>
<td>-0.02±0.17</td>
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<tr>
<td><strong>Retention test at 1d</strong></td>
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<tr>
<td><strong>Sample phase:</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Sample object exploration [sec]</td>
<td>15.7±2.6</td>
<td>14.8±2.1</td>
<td>11.7±3.5</td>
<td>9.9±1.8</td>
</tr>
<tr>
<td><strong>Test phase:</strong></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Novel object (N) exploration [sec]</td>
<td>6.1±1.2</td>
<td>7.7±1.9</td>
<td>4.2±1.2</td>
<td>2.0±0.8</td>
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<tr>
<td>Familiar object (F) exploration [sec]</td>
<td>4.8±1.21</td>
<td>5.1±0.8</td>
<td>4.0±1.3</td>
<td>2.5±0.9</td>
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<tr>
<td>Discrimination ratio* [(N-F)/(N+F)]</td>
<td>0.10±0.07</td>
<td>0.10±0.14</td>
<td>0.01±0.17</td>
<td>-0.14±0.12</td>
</tr>
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</table>

Table 1: Object exploration in the sample phase (object familiarization) and in the test phase (novelty discrimination) for each delay condition (2min, 2h, 1day) in experiment 1. A discrimination ratio was used to index object recognition memory performance. A positive ratio indicates a preference towards the novel over the familiar objects. All values refer to the mean (± SEM) collapsed across two replications at each delay condition.

Similar to the sample phase, the overall object exploration time (devoted to both novel and familiar objects) was higher in the mutant mice than controls during the test phase. We therefore further investigated if this arose from a difference in time spent in the central compartments (therefore away from where the objects were placed) as suggested by one anonymous reviewer. To this end, the time spent and the distance travelled in the centre were subjected to a 2 × 2 × 3 (genotype × sex × delays)
ANOVA. Neither time spent [F(1,23)=2.50, p=0.13] nor distance travelled in central compartment [F(1,25)=0.01, p>0.9] yielded any significant difference between mutant and control mice. On the critical 2-h delay test, the mean (±SEM) duration spent in the central compartment was: mutant = 66.5 ±11.3s, control = 84.3±16.5s, and covering a mean distance travelled of: 285.1±35.1cm and 298.3±37.0cm, respectively. Hence, the observed poor performance in the control mice is unlikely to stem from a preferential exploration of the central compartment, perhaps arising from the dishabituation (viz., an increase) of spatial exploration that was lacking in the mutant.

Next, we also consider if dishabituation of exploration to the familiar objects may offer an alternative explanation of our results, especially under condition in which the controls failed to show any novelty preference. Accordingly, the presence of a positive preference towards the novel object at 2h delay in mutants but not in controls could potentially be due to impaired dishabituation in mutants rather than facilitated learning. To gauge if the mutant mice had a general impairment in dishabituation of object exploration as such, we examined the object exploration data obtained across the two consecutive sample periods, with each sample period further divided into two time bins. This enabled us to evaluate habituation to a distinct object over time (within a period) as well as dishabituation (due to a change of access to the sample object from one peripheral compartment to another) that took place from the end of the first sample period to the beginning of the second sample period. As illustrated in Figure 3, habituation of object exploration was present in both mutant and controls as evidenced by the decrease in object exploration time from the first to second bins within each period. In spite of the difference in overall exploration time, the habituation effect was highly comparable between the two groups. A 2 × 2 × 3 × 2 × 2 (sex × genotype × delay × periods × 150-s bins) of object exploration time yielded a highly significant effect of bins [F(1,23)=12.24, p<0.005] and of genotype [F(1,23)=5.28, p<0.05], but not of their interaction. The dishabituation process was more specifically analysed by contrasting the last bin of the first period with the first bin of the second period (see Figure 3) using a 2 × 2 × 3 × 2 (sex × genotype × delay × periods) ANOVA. Renewed exploration towards the object was evidenced by the emergence of a significant main effect of periods [F(1,23)=9.12, p<0.01]. Again the dishabituation seen was highly comparable between groups, consistent with the lack of a significant genotype × periods interaction in this analysis.
Enhanced recognition memory following glycine transporter 1 deletion in forebrain neurons

Table 2: Index measures of object exploration and discrimination in experiment 2.

<table>
<thead>
<tr>
<th>Sample phase:</th>
<th>Mutant (Male n=11)</th>
<th>Female (n=11)</th>
<th>Control (Male n=9)</th>
<th>Female (n=10)</th>
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<tr>
<td>Sample object exploration [sec]</td>
<td>37.3±4.8</td>
<td>19.1±13</td>
<td>46.6±4.9</td>
<td>29.8±4.4</td>
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</table>

<table>
<thead>
<tr>
<th>Test phase:</th>
<th>Mutant (Male n=9)</th>
<th>Female (n=10)</th>
<th>Control (Male n=9)</th>
<th>Female (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exploration displaced objects (D) [sec]</td>
<td>10.15±2.0</td>
<td>5.5±1.1</td>
<td>9.9±1.4</td>
<td>6.5±1.7</td>
</tr>
<tr>
<td>Exploration non-displaced objects (N) [sec]</td>
<td>7.3±1.1</td>
<td>3.4±0.9</td>
<td>10.2±1.1</td>
<td>7.1±2.1</td>
</tr>
<tr>
<td>Discrimination Ratio* [(D-N)/(D+N)]</td>
<td>0.21±0.05</td>
<td>0.24±0.09</td>
<td>-0.01±0.05</td>
<td>-0.02±0.09</td>
</tr>
</tbody>
</table>

Table 2: Object exploration in the sample (familiarization) and in the test phase (object-location discrimination) in experiment 2. A discrimination ratio was used to index object recognition memory performance. A positive ratio indicates a preference towards the displaced over the non-displaced objects. Values are means (±SEM) collapsed across two replications.

Experiment 2: Object-location recognition memory

Evaluation of object-location recognition memory was carried out at the minimal delay (2min) condition employed in experiment 1 when performance between mutant and control mice was comparable. Two tests were conducted on separate days, and the data pooled for the purpose of analysis. Again, only animals that exhibited non-zero object exploration to each of the four sample objects were included in the final analysis (mutant: 11 male and 11 female; control: 9 male and 10 female); one female control failed to explore the objects in the sample run and was dropped. Preferential exploration of the displaced objects in the tests was expressed using a discrimination ratio: (exploration time of displaced objects - exploration time of non-displaced objects) / total exploration time of all objects (also see Table 2).
As illustrated in Figure 4, the mutant mice but not the control animals exhibited a distinct preference for the displaced objects during the test. This effect was seen in both male and female mice (see Table 2). A two-way (genotype × sex) ANOVA of the discrimination ratio confirmed this interpretation yielding a significant main effect of genotype [F(1,37)=10.65, p<0.005]. Neither the effect of sex nor the interaction term attained statistical significance.

Examination of object exploration time in the sampling phase revealed a genotype effect [F(1,37)=6.11, p<0.05] opposite in direction to that seen in Experiment 1. This was further accompanied by a highly significant main effect of sex [F(1,37)=18.57, p<0.001] indicating increased object familiarization time in the male regardless of genotype (see Table 2). A reduction of object sampling time might at first sight appear incompatible with enhanced performance in the test phase. However, the nature of this particular version of the test might actually favour sampling regarding the spatial configuration of the objects (which requires examination from afar) rather than individual object identity (which demands closer inspection) as such. Hence, this could still be a potential confounding factor of our results in the test phase. To test this possibility, an ANCOVA of the discrimination ratio was conducted using the object exploration time in the sample phase as the covariate, just as we did in Experiment 1. Again, the main effect of genotype remained statistically significant [F(1,36)=11.35, p<0.005] which was accompanied by a non-significant effect of the covariate [F(1,36)=1.26, p=0.27]. Hence, the decreased object familiarisation in mutants cannot solely account for their superior performance in the test phase.

**Figure 4:** Object-place recognition memory was assessed at a single delay condition (2min) and was indexed by the differential ratio defined as difference in exploration time towards the objects in displaced and objects in non-displaced positions divided by the total object exploration time. A positive ratio therefore denotes an explorative preference towards displaced over non-displaced objects. Mutants exhibited a clear preference towards the displaced objects whereas controls failed to do so. This impression was confirmed by the emergence of a significant main effect of genotype (p<0.005) in genotype x sex ANOVA of the discrimination ratio. * denotes a significant difference from control mice performance. # denotes that the differential ratio significantly differs from chance level (i.e., zero) in a one-sample t-test. All values refer to mean (±SEM) of two replications.
Chapter 2: Enhanced recognition memory following glycine transporter 1 deletion in forebrain neurons

**Experiment 3: Spontaneous place preference**

In this experiment, the differential preference between a familiar and a novel spatial context, instead of between discrete objects differing in familiarity, was examined. As before, the retention interval was 2 min and two replications were conducted on separate days that were averaged for the purpose of analysis. Animals not entering the sample context in the sample phase (2 male control) were excluded in the final analysis, which included the following number of animals: mutant = 11 male and 11 female, control = 7 male and 11 female. A two-way (genotype × sex) ANOVA of three separate discrimination ratios calculated based on time spent in, distance travelled in, and number of entries into, the familiar and the novel compartments failed to yield any significant effect (see Table 3). One-sample t-tests were further conducted for each sex/genotype group to evaluate if the group mean significantly differed from zero. Female/mutant, female/control and male/mutant exhibited a significant preference for the novel context in all three discrimination measures (all p’s<0.05), whereas male control displayed only a non-significant trend towards a positive preference for the novel context in the time, distance and entry measures (p=0.17, p=0.19, and p=0.45, respectively) (see Table 3). Although significant place discrimination was observed in all groups except in the male controls, Fisher’ LSD comparisons indicated that there was not any significant difference between male controls and any other group. Activity during sample phase as indexed by distance travelled also did not reveal any effect (see Table 3).

<table>
<thead>
<tr>
<th></th>
<th>Mutant</th>
<th></th>
<th>Control</th>
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<tbody>
<tr>
<td></td>
<td>Male (n=11)</td>
<td>Female (n=11)</td>
<td>Male (n=7)</td>
<td>Female (n=11)</td>
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<tr>
<td><strong>Sample phase: locomotor activity</strong></td>
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<tr>
<td>Distance travelled in the sample context [cm]</td>
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<td>1697±84</td>
<td>1708±110</td>
<td>1648±71</td>
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<tr>
<td><strong>Test phase: differential ratio</strong></td>
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<tr>
<td>Dependent variable</td>
<td></td>
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<tr>
<td>Time</td>
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<td>0.18±0.05 *</td>
<td>0.06±0.04</td>
<td>0.16±0.07 *</td>
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<tr>
<td>Distance</td>
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<td>0.24±0.05 *</td>
<td>0.10±0.07</td>
<td>0.21±0.04 *</td>
</tr>
<tr>
<td>Entries</td>
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<td>0.15±0.04 *</td>
<td>0.05±0.05</td>
<td>0.10±0.04 *</td>
</tr>
</tbody>
</table>

**Table 3**: Locomotor activity in and place discrimination in experiment 3. The differential ratio indexes place discrimination and it was calculated based on the time spent, the distance travelled, or the number of entries into, the familiar and the novel compartment. A positive ratio indicates a preference towards the novel over the familiar compartment. All values refer to the mean (± SEM) over two replications. * denotes that the differentia ratio significantly (p<0.05) differs from zero in a one-sample t-test.
DISCUSSION

Here, we show for the first time that a disruption of GlyT1 can lead to an enhancement in recognition memory using a genetically modified mouse line (CamKIIaCre;Glyt1tm1.2f/f) with forebrain neuronal specific deletion of GlyT1. In Experiment 1, the mutant mice showed superior object recognition memory relative to controls at a delay of 2 h but not at the shorter retention interval of 2 min when the two groups of mice performed at similar levels. With the longest delay of 1 day, both groups failed to show any significant discrimination from chance level. In Experiment 2, the mutant mice were able to detect a change in the relative spatial position of objects and modified their explorative behaviour accordingly whilst controls failed to do so. These findings add to our previous demonstration of enhanced associative learning in CamKIIaCre;Glyt1tm1.2f/f mice, and strengthen the hypothesis that forebrain and neuron specific deletion of GlyT1 may be associated with promnesic effects across multiple memory systems, presumably via enhanced NMDAR function (Yee et al., 2006).

It is notable that the potential promnesic effect reported here and in our previous study (Yee et al., 2006) was demonstrated against the relatively poor performance by the control group. This may be attributed to the genetic manipulations (Glyt1tm1.2f/f allele) introduced into both groups of mice as to optimally control for the evaluation of the behavioral effects of the forebrain and neuron specific GlyT1 disruption. This raises the possibility that our controls may differ in general from the wild type mouse line of the same genetic background (namely, C57BL/6), but this is not too surprising as they are not wild type animals. Nonetheless, our adapted test paradigms demonstrated the presence of a novelty preference in controls at 2min delay in Experiments 1 and 3. Such parametric adaptations are not uncommon in the phenotypic evaluation of genetically engineered animals. The future development of similar GlyT1 knockouts in multiple backgrounds by other groups would provide the most stringent tests to evaluate the generality of our present (and previous) findings and may at the same time also shed light on the reasons for the relatively poor performance of our control animals.

The present study focuses on recognition memory and specifically its detection by the spontaneous recognition paradigm that utilizes the naturally occurring explorative curiosity for novelty in rodents. It is therefore imperative to clarify whether the mutation exerts potential non-mnemonic effects that could potentially interfere with the animals’ explorative behaviour. We have previously shown that the mutation affected neither anxiety related behaviour in the elevated plus maze nor locomotor activity under a variety of experimental conditions (Yee et al., 2006). Likewise, locomotor activity in terms of distance travelled was comparable between mutants and controls in Experiment 3. The possibility that the weak object recognition performance in controls may be attributed to an increased explorative activity in the central compartment in Experiment 1 was addressed by an analysis explicitly comparing time spent as well as distance travelled in the central compartment between the two groups. This failed to reveal any significant difference. Hence the possibility of a preferential dishabituation of spatial exploration (and thereby competes with object exploration) in the
control group cannot account for our observation.

The spontaneous object recognition paradigm used here is critically dependent on the interplay of habituation and dishabituation processes related to exploration (Honey and Good 2000). Factors interacting with these processes may therefore potentially influence the object recognition performance. Consequently, the absence of a positive preference towards the novel over the familiar object in controls at longer delays could potentially be due to dishabituation to the familiar object and thus reinstating exploration to the familiarized object in the test phase. This possibility is particularly relevant here at the critical delay of 2h when only mutants but not controls preferentially explored the novel object. Hence, the poor discrimination performance in controls may be due to dishabituation and not the result of memory loss. If so, the enhanced performance in mutants may be described as impaired dishabituation rather than as a promnesic effect. Hence, the cause for the poor performance in controls is critical in distinguishing between the two potential effects of the mutation: enhanced memory vs impaired dishabituation. Evidence for relatively poor learning in controls is provided by previous data collected in three Pavlovian conditioning paradigms (Yee et al., 2006). However, in the same study, there is also evidence for superior dishabituation in controls, as this can also account for the lack of a latent inhibition effect in the controls when the pre-exposure to the to-be-conditioned stimulus led to a significant presence of the latent inhibition effect in the mutant mice (Yee et al., 2006). Is it possible that the mutant mice suffer from a general deficit in dishabituation relative to the controls? This is unlikely because habituation of the explorative activity towards a distinct object over time as well as dishabituation taking place from one object familiarization period to the next did not differ between mutants and controls (see Figure 3).

In Experiment 2, the presence of a positive preference towards the displaced objects in mutants but not in controls may provide some evidence for enhanced memory for the spatial location of objects in mutants as the displaced objects are detectable with reference to external cues (“object-in-place” memory). However, the displacement of two out of four objects also involves a change in the entire configuration of the object assembly, which introduces an additional type of novelty – namely, “configural” novelty. In this experiment, the mutant mice displayed less direct object exploration than controls in the sample phase. This is unlike in Experiment 1 when the object exploration in the sample phase was higher in the mutant mice. Enhanced discrimination between novel and familiar objects in the test phase may either reflect enhanced mnemonic function, or enhanced memorability of the familiar objects. The latter may be expected simply because the sample objects have been more intensely explored. Hence, the mutant mice’s superior discrimination performance in Experiment 1 might potentially be attributed to their concomitant enhanced exploration of the sample objects prior to the test phase. We examined this potential confound using analysis of covariance (ANCOVA), which indicated that superior performance in the mutant remained evident when the level of sample object exploration was controlled for statistically. This is consistent with the general lack of statistical evidence for a positive correlation between sample object exploration time and discrimination
performance in the test phase in the present data sets (data not show).

Hence, when the mutant mice were observed to exhibit less (rather than more) exploration in the sample phase in Experiment 2, it may seem at first that this confounding difference would if anything hinder rather than facilitate familiarity judgement, and therefore would not be expected to be responsible for the enhanced performance in the subsequent test phase. However, a reduction in direct object exploration or contacts might imply the mutant mice were sampling the entire object configuration from afar, and this could facilitate subsequent discrimination between displaced and non-displaced objects in the test. To address this possibility, a covariance analysis was conducted in a manner similar to that in Experiment 1. However, it failed to lend support for this suggestion that difference in direct object exploration might explain the subsequent test performance. These results of the covariance analyses in Experiment 1 as well as Experiment 2 suggest that genotype specific differences in the object familiarization phase could not solely account for the performance difference in the test phase observed between mutant and control in both experiments. This is also in keeping with the observation that although the mutation can produce changes in sample object exploration in either direction, it consistently enhanced recognition memory performance across both experiments. The different levels of object exploration in mutants and controls in the sample phase of the two experiments might be related to the dissimilar arrangement and presentation of objects in the sampling procedure of the two experiments. In Experiment 1, there were two sampling periods, with one object presented in one or the other peripheral compartment within the test box. In Experiment 2, the test box was not divided into discrete compartments, and all four objects were presented in the same sampling period. This is likely to affect the pattern of object investigation, but somehow the mutant mice were preferentially affected by this difference.

On the other hand, one also needs to consider the alternative view that “configural” novelty by itself would predict little or no preferential exploration of the displaced over the non-displaced objects, because the change of configuration is defined by all four objects’ positions relative to each other. Indeed, the configurational change here was subtle: topologically, it is akin to a reflection, with the “neighbourhood” of each object essentially remaining the same. This view would explain why control subjects did not show any differential exploration between displaced and non-displaced objects. And against this, the preferential exploration of displaced objects by the mutant might indicate that they processed the scene experienced during the sample period differently from controls, such that the mutant mice were more sensitive to the location of each object in absolute space as defined by distal room cues. The possibility that the mutant and control mice may differ in their processing or representation of objects in space would need to be carefully considered before the claim for memory enhancement in the mutant mice. Although this claim may represent a parsimonious account of the present (Experiment 1 here) and previous data set (Yee et al., 2006), further experiments would be required to test it, and/or to provide additional characterizations of the possible alterations in information processing induced by the mutation. As a step towards this,
Experiment 3 was conducted to test whether the mutation has an effect on place recognition as such in the absence of any discrete object stimuli, and it yielded no evidence for a genotype effect. This suggests that the phenotype revealed in Experiment 2 was unique to the object-in-place paradigm, and that mnemonic processing of space as defined by distal room cues was comparable between the two groups of mice.

A multitude of similar recognition memory paradigms have proven their worth as effective tools to reveal both promnesic and amnesic properties in genetically engineered mice (e.g. Hale and Good 2005, Tang et al., 1999). Lesion studies in rodents and monkeys have indicated that object recognition memory requires the integrity of the temporal lobe structures including perirhinal, entorhinal and inferior temporal cortices (e.g. Bussey et al., 1999 & 2001, Gilbert et al., 2003; Murray et al., 1999 & 2001, Aggleton et al., 1997, Brown et al., 2001). Thus, object recognition memory as evaluated by the present paradigms may allow the assessment of mnemonic functions beyond hippocampal control. The precise contribution of the hippocampus to object recognition memory, however, remains a matter of debate (Clark et al., 2000, Gaskin et al., 2003). It has been suggested that hippocampal activity is only required for the spatial aspects of object recognition memory (e.g. Brown and Aggleton 2001, Mumby et al., 2002, Wan et al., 1999), and several lesion studies consistently report a critical involvement of the hippocampus in object location memory (e. g. Gaffan 1994, Gilbert & Kesner 2002, Parkinson et al. 1988, Sziklas et al. 1998). Another suggestion posits that the hippocampus is only necessary for recognition memory at long retention intervals (Clark et al., 2000 Hammond et al., 2004). These hypothesized roles of the hippocampus in recognition memory in normal animals therefore match the phenotypes observed in the CamKIIaCre:Glyt1tm1.2fl/fl mice, which exhibited enhanced memory retention at longer intervals, and superior detection of a mismatch in object-place or objects configural relationship. Nevertheless, the possibility that GlyT1 disruption outside the hippocampus has contributed to the observed promnesic phenotypes observed here cannot be excluded. The forebrain GlyT1 disruption used here is expected to affect extra-hippocampal structures including the perirhinal and entorhinal cortices that are also critically involved in object recognition memory.

Recognition memory is also known to be susceptible to a number of molecular and pharmacological interventions targeting the NMDAR. Impaired object recognition memory was found in CA1-specific NMDAR subunit knockout mice (Rampon et al., 2000) whereas over-expression of NMDAR subunit NR2B in the forebrain of transgenic mice led to an improvement on recognition memory (Tang et al., 1999). Pharmacologically, object recognition memory deficits were induced by several NMDAR antagonists such as APV (de Lima et al., 2005), MK-801 (Baker & Kim, 2002) and PCP (Hashimoto et al., 2005). Furthermore, NMDAR populations in different brain areas were reported to have distinct effects on recognition memory. Selective lesions of the major glutamatergic afferents to nucleus accumbens in rats were found to differently affect spatial and non-spatial recognition memory (Sargolini et al., 1999). These findings suggest that an augmentation of NMDAR
activity can translate into enhanced recognition memory performance. This is in keeping with the observation that the CamKIIaCre:Glyt1tm1.2fl/fl mice showed a selective increase in NMDAR (but not AMPA receptor) mediated current (Yee et al., 2006), which may be responsible for the improvement in recognition memory reported here as well as in Pavlovian-type associative memory revealed previously (Yee et al., 2006).

In summary, our results lend some support for a promnesic interpretation of the enhanced recognition memory performance observed in mutants. However, the promnesic phenotype demonstrated in our mutant mice here and elsewhere (Yee et al., 2006) contrasted with the limited effect seen in the watermaze following constitutive heterozygous knockout of the GlyT1 gene (Tsai et al., 2004). This dissimilarity may be related to the high level of specificity of the GlyT1 disruption in our mutant mice – namely GlyT1 was completely deleted (homozygosity of the mutant allele) selectively in forebrain neurons - in contrast to the non-specific reduction (heterozygosity of the mutant allele) of GlyT1 following the constitutive GlyT1 knockout. This molecular distinction between the two mutant lines can also be described as a gene dosage effect (at least within the neuronal GlyT1 population) that is consistent with the contrast between their respective behavioural phenotype in terms of their cognitive enhancing potential.

On the other hand, pharmacological blockade of GlyT1 has not yet revealed any convincing promnesic effects. This may be explained by the fact that existing GlyT1 inhibitors have not been primarily tested for promnesic properties or if such experiments had been conducted, their lack of discrimination between neuronal and glial associated GlyT1 may have limited their promnesic efficacy. The latter explanation would further underlie that the ability to discriminate between neuronal and glial associated GlyT1 may be a requisite to enhance mnemonic functions by interventions designed to reduce GlyT1 function. Despite the extensive amount of resources that has recently been invested in the development of high-affinity GlyT1 inhibitors, the currently available compounds have not attained a level of specificity comparable to the molecular disruption of GlyT1 in forebrain neurons in our mutant mice. Hence, a potential therapeutic use of current GlyT1 inhibitors against mnemonic deficits may be limited due to their insufficient specificity and the development of more specific inhibitors may be warranted.

CONCLUSIONS

There is increasing behavioural evidence to suggest that neuron and forebrain specific GlyT1 disruption can potentially lead to enhanced mnemonic functions in associative learning as well as in recognition memory, possibly as a result of increased NMDAR activity. This suggests that interventions reducing GlyT1 dependent glycine re-uptake may be of therapeutic value in the treatment of learning deficits associated with forebrain NMDAR hypofunction. Furthermore, the present study underscores that the ability to discriminate between neuronal and glial GlyT1
populations may play a critical role. Regarding the development of novel GlyT1 inhibitors a higher level of selectivity for cell-type specific GlyT1 populations may be required, which remains a major challenge to be attained by pharmacological means.
REFERENCES


Chapter 2: Enhanced recognition memory following glycine transporter 1 deletion in forebrain neurons


Chapter 2: Enhanced recognition memory following glycine transporter 1 deletion in forebrain neurons


Chapter 3

Deletion of glycine transporter 1 (GlyT1) in forebrain neurons facilitates reversal learning: Enhanced cognitive adaptability?

With Detlev Boison, Hanns Mohler, Joram Feldon and Benjamin K. Yee
As Submitted in Learning & Memory (revised)

[Personal contribution to the work: Designing and performing the research, analysis of the behavioural data, and writing the manuscript]
Chapter 3: Deletion of glycine transporter 1 (GlyT1) in forebrain neurons facilitates reversal learning: Enhanced cognitive adaptability?

Chapter 3

Abstract

Glycine assumes an important role in the regulation of glutamatergic neurotransmission via modulation of N-methyl-D-aspartate receptor (NMDAR) activity. The binding of glycine to NMDAR’s glycine-B site is mandatory for NMDAR activation by glutamate. The availability of glycine in the vicinity of glutamatergic synapses is normally tightly regulated by glycine transporter 1 (GlyT1), which maintains glycine concentration at sub-saturation levels. We have shown that deletion of GlyT1 in forebrain neurons is sufficient to enhance NMDAR-mediated current and facilitate associative learning as well as object recognition memory. Here, mutant (CamKIIα-Cre;Glyt1tm1.2fl/fl) mice with forebrain neuronal specific GlyT1 disruption were compared with littermate controls in both working memory and reference memory learning in the water maze. Although the mutant mice failed to enhance working memory, their performance was not identical to controls and was suggestive of reduced susceptibility to proactive interference. This suggestion was confirmed in the following reference memory test: the mutant mice exhibited normal acquisition in the first test, but facilitated subsequent new learning. This phenotype disappeared, however, when new learning was conducted in a novel testing room, indicating that the mutant mice were resistant to proactive interference from previously acquired platform locations as defined by extra-maze cues. This phenotype was not restricted to spatial information processing because reversal learning was also enhanced in a non-spatial simultaneous two-choice pattern discrimination reversal task. It is concluded that forebrain neuronal specific GlyT1 deletion can enhance learning in conditions of high proactive interference, when suppression of previously learned information is desirable.
Chapter 3: Deletion of glycine transporter 1 (GlyT1) in forebrain neurons facilitates reversal learning: Enhanced cognitive adaptability?

INTRODUCTION
Glycine is an endogenous modulator of glutamatergic neurotransmission via the N-methyl-D-aspartate receptor (NMDAR). The binding to the glycine-B site of the NMDAR by its endogenous ligands, glycine or D-serine, is required for the activation of the NMDAR by glutamate (Berger et al. 1998). Endogenous glycine is normally maintained at sub-saturation levels by glycine transporter 1 (GlyT1) (Aragon and Lopez-Corcuera 2005, Eulenburg et al. 2005, Borowsky and Hoffmann 1998), which is expressed not only in glial cells (Zafra et al. 1995), but also in glutamatergic synapses in the neocortex and hippocampus (Cubelos et al., 2005). In particular, its co-localization with NMDARs in the forebrain (Wood 1995) allows GlyT1 to modulate NMDAR activity by controlling glycine concentration in the vicinity of NMDARs. Enhanced NMDAR activity therefore can be achieved by systemic pharmacological blockade (Depoortère et al. 2005, Martina et al. 2004, Bergeron et al. 1998) or constitutive GlyT1 knockout (GlyT1+/-, Tsai et al. 2004a, Gabernet et al. 2005). A pivotal role of the neuronal GlyT1 component has been demonstrated by the selective deletion of GlyT1 in forebrain neurons by CamKIIα-Cre-mediated recombination of a conditional loxP-flanked GlyT1 allele in mice (CamKIIαCre;GlyT1tm1.2fl/fl mice), which is associated with an increase in the ratio of NMDAR/AMPAR current and resistance to pharmacological blockade of NMDAR (Yee et al. 2006). It therefore represents an effective strategy to enhance NMDAR activity without directly interfering with the composition of the NMDAR (e.g., Tang et al., 1999) or the availability of associated downstream regulatory proteins (for a review, see Wang et al. 2006).

Functionally, disruption of forebrain neuronal GlyT1 is sufficient to facilitate associative learning (Yee et al. 2006) and object recognition memory in a delay-dependent manner (Singer et al., 2007). Furthermore, it also enhances selective learning in the form of latent inhibition: CamKIIαCre;GlyT1tm1.2fl/fl mice exhibited latent inhibition when control mice did not, suggesting that they are more sensitive to the non-reinforcement history of the conditioned stimulus (Yee et al. 2006). This implies that forebrain neuronal GlyT1 deletion does not indiscriminately strengthen the expression of learned behaviour in the expense of the selectivity in learning.

To further characterize this unique balance in cognitive function in these animals, we examined here the effects of forebrain neuronal GlyT1 deletion on working memory function in the water maze, when the escape platform location was changed daily (Morris 1983, 1984). Efficient working memory requires not only rapid learning within a day, but recall of platform locations learned on previous days would be irrelevant or even deleterious to performance. Hence, effective performance demands selective recall of the appropriate learned information. Next, the susceptibility to such negative proactive interference effect was more explicitly tested using the water maze reference memory task with new learning introduced repeatedly following the initial acquisition (Morris 1984). During new learning when the escape platform is re-positioned, interference due to the learning of its previous location would be sufficiently strong to allow us to detect its possible attenuation in the mutant mice. Finally, the hypothesis that forebrain neuronal GlyT1 deletion might
facilitate reversal learning in general was evaluated further in a simultaneous two-choice pattern discrimination paradigm (Sutherland and Mackintosh 1971). Our findings support the hypothesis that the forebrain neuronal GlyT1 deletion has led to some form of reduced proactive interference, which at the same time affected neither the acquisition of reference memory nor of pattern discrimination. The characterization of this unique cognitive profile of these mutant mice will be highly relevant to the proposed use of GlyT1 inhibitor drugs in the pharmacotherapy of memory disorders (Coyle and Tsai 2004, Danysz and Parsons 1998, Javitt 2008, Lechner 2006).

RESULTS
Cued test in the watermaze
First, the animals were familiarized to the water maze procedure in a cued task whereby the location of the escape platform was indicated by a local cue. This pre-training phase also allowed the detection of possible non-specific differences in sensory and motor function.

All animals rapidly acquired the escape response by climbing onto the escape platform whose location was indicated by a local cue mounted directly above it. Performance was already high on the first trial, because the animals were released in the direction towards the platform (Figure 1). There was a slight improvement from trial 1 to 2, but this did not attain statistical significance, based on separate 2 × 2 (genotype × trials) ANOVAs of escape latency and path length. Neither analyses yielded any indication of a genotype effect or its interaction [all F’s<1]. The additional analysis of average swim speed also failed to show any significant effect [all F’s<1]. The mean(±SEM) swim speed (in cm/s) of the two groups was: controls = 12.1±1.5, mutants = 15.5±1.1. The results indicated that escape performance between the mutant and control mice did not differ significantly at this stage of the task when memory of platform location was not explicitly required.

![Figure 1](image)

**Figure 1.** Performance on the cued task in the water maze as indexed by escape latency (A) and path length (B). Error bars refer to ±SEM, mutant (n=7), control (n=5).
Working memory test in the watermaze

Next, working memory function was evaluated across twelve consecutive days. On each day, the animals were required to learn the new location of a hidden platform revealed to them on the first trial, and to quickly escape onto it on the second trial. The platform assumed a novel location on every day, thus demanding the use of working memory – in recalling the appropriate platform location learned on the day. In the first six days, the delay from trial 1 to 2 was limited to 15s (the minimal delay); this was extended to 10 min in the following six days in order to increase the retention demand of the task.

Over the first six days, there was limited improvement in escape performance over trials when the ITI (or delay) between trial 1 and 2 was minimal – both in terms of escape latency and path length (Figures 2A–2D). Separate $2 \times 6 \times 2$ (genotype $\times$ days $\times$ trials) ANOVA of escape latency and path length only yielded a significant effect of days [F(5,50)=3.90, p<0.005; F(5,50)=2.95, p<0.05, respectively]. Therefore there was no statistical support for an improvement of escape performance from trials 1 to 2 within these 6 days of training. No significant effect emerged from the analysis of swim speed. The mean($\pm$SEM) swim speed (in cm/s) of the two groups on trials 1 and 2 was as follows: controls/trial 1 = 17.8±1.7, controls/trial 2 =20.1±1.6, mutants/trial 1 = 18.1±1.3, mutants/trial 2 = 19.8±1.2.

Performance over the next six days (days 7–12), the ITI was increased to 10 min. There was a notable improvement from trial 1 to 2 in the control but not the mutant mice, as illustrated in Figures 2B and 2D with performance averaged days. However, it is also apparent that the two genotypes differed largely on trial 1’s performance when the platform location was essentially unknown. On trial 2, on the other hand, the two groups were closely matched in both escape latency and path length. A $2 \times 6 \times 2$ (genotype $\times$ days $\times$ trials) ANOVA of escape latency yielded a near-significant effect of trials [F(1,10)=4.72, p=0.05], and a suggestive tendency for a genotype effect [F(1,10)=3.84, p=0.08]. These statistical outcomes were paralleled by a main effect of trials [F(1,10)=5.98, p<0.05] and of genotype [F(1,10)=6.41, p<0.05] from the equivalent analysis of path length. To identify more precisely the source of the significant main effect of genotype, analysis restricted to each trial yielded a clear genotype effect only in trial 1 [latency: F(1,10)=6.16, p<0.05; path length: F(1,10)=6.16, p<0.05], but not in trial 2 [corresponding F’s<1]. However, the critical genotype $\times$ trials interaction term failed to reach significance in either performance measure [latency: F(1,10)=1.83, p=0.21; path length: F(1,10)=2.12, p=0.18]. Hence, it cannot be concluded that the saving from trial 1 to 2 differed statistically between mutant and control mice.

Parallel analyses of swim speed equivalent to all those described above never yielded any suggestion of a significant effect or interaction. The mean($\pm$SEM) swim speed (in cm/s) of the two groups on trials 1 and 2 was as follows: controls/trial 1 = 19.4±1.7, controls/trial 2 =18.9±0.8, mutants/trial 1 = 16.3±1.3, mutants/trial 2 = 15.0±0.6.

The results of the working memory test indicated that mutant mice behaved differently in the 10min delay condition in comparison to the controls on trial 1. The data clearly do not support the hypothesis that forebrain neuronal GlyT1 deletion would be associated with enhanced working
memory, but the pattern of the results also did not readily conform to the typical impression of impaired working memory in the mutant mice. The unexpected effect observed on trial 1 has prevented a straightforward interpretation of the data. As discussed later (see Discussion), this might be taken as evidence that the mutant mice had resorted to a non-mnemonic search strategy independent of extra maze cues to the solution of the task that protected them against the negative impact of proactive interference, which at the same time failed to allow them to improve performance on trial 2.

In contrast, the control mice were relying on spatial mnemonic, and therefore under some degree of negative proactive interference leading to lower trial 1 performance relative to controls, but allowing a degree of saving to be observed from trial 1 to 2. Next, we further adopted the reference memory procedure of the water maze task with additional test conditions that maximized the influence of proactive interference.

**Figure 2.** Performance on the working memory task in the water maze as indexed by escape latency (A & B) and path length (C & D), across trials and days (A & C), or averaged across days as a function of trials (B & D). Error bars refer to ±SEM, mutant (n=7), control (n=5). *p<0.05 based on restricted analyses, contrasting mutant and control performance on trial 1 (collapsed across days), and contrasting between trials 1 and 2 in the control mice.
Reference memory: From initial acquisition to multiple new learning

Next, we changed the demand of the water maze task to evaluate reference memory function. Here, the platform location no longer changed from one day to the next, but remained in the same position across an extended period of days. This therefore encouraged the solution by reference memory (in contrast to working memory), because information acquired on a day would be relevant to the solution between as well as with days. However, we also challenged the animals by changing the position of the platform without warning once performance indicated successful acquisition. This was designed to evaluate the impact of proactive interference on new acquisition, and it was repeated in discrete phases, until the last phase when the entire maze was also moved to a new testing room. It was expected that the mutant mice might have shown facilitation only when the position of the escape platform was suddenly changed, and that this effect would be eliminated when new learning took place in a novel testing room such that the control mice would not be negatively influenced by proactive interference.

During the initial acquisition phase, one male control mouse exhibited very weak swimming accompanied by severe floating from day 4 onwards and was therefore excluded from this experiment. The sample size of the control group was therefore reduced to 4. The four phases of reference memory learning are separately analysed and detailed below as problems 1 to 4.

**Problem 1:** First, the initial acquisition over the first eight days was examined. Performance showed a progressive improvement over test days as evidenced by both escape latency (Figure 3A) and path length (Figure 3C), and this was equivalently observed in both mutant and control mice. Consistent with this impression, separate $2 \times 8 \times 4$ (genotype $\times$ days $\times$ trials) ANOVAs of escape latency and path length yielded a main effect of days [latency: $F(7,63)=5.15$, $p<0.0005$; path length: $F(7,63)=3.7$, $p<0.005$]; but neither the main effect of genotype nor its interactions attained statistical significance. To facilitate comparison with later analyses that focused on the first day of new learning, we also performed such a $2 \times 4$ (genotype $\times$ trials) ANOVA of the first day of acquisition. This revealed no statistically significant effects. The results on the first day of the initial learning phase (and subsequent phases of new learning) are depicted as a function of trials separately in Figures 3B and 3D.

**Problem 2:** Over days 10 to 13, the animals were trained using a new platform location. As expected, performance reduced drastically on the initial day. It took the control mice much longer to locate the platform in comparison to the initial day of the first acquisition. This constitutes the reversal effect. In contrast, although the mutant mice also experienced an increase in search time and path length, they re-bounded to a level comparable to their performance on the first day of the first acquisition. This led to the divergence of performance on day 1 of new learning, but the two groups eventually converged by the fourth day of new learning. These impressions were confirmed by separate $2 \times 4 \times 4$ (genotype $\times$ days $\times$ trials) ANOVAs of escape latency and path length. Both analyses revealed a significant genotype by days interaction [latency: $F(3,27)=2.98$, $p<0.05$; path
length: F(3,27)=3.85, p<0.05] which was accompanied by a significant days effect [latency: F(3,27)=21.27, p<0.0001; path length: F(3,27)=24.51, p<0.0001]. The main effect of genotype also attained significance in the analysis of path length [F(1,9)=10.67, p<0.01]. Separate analyses on each of the four days confirmed that the mutant and control only differed on the first day of new learning, yielding a main effect of genotype [latency: F(1,9)=7.28, p<0.05; path length: F(1,9)=14.68, p<0.005]. And, this effect was apparent across the 4 trials on the first day (see Figures 3B and 3D).

Problem 3: Over the next four days, the animals were trained with yet another platform location. A very similar pattern of results emerged (Figure 3). The control mice exhibited a pronounced reversal effect on the first day of new learning, whereas the mutant mice were hardly showing such an effect. Again the two groups largely converged in the subsequent three days, such that their performance was indistinguishable from each other by the fourth day. Separate 2 × 4 × 4 (genotype × days × trials) ANOVAs of escape latency and path length over these four days again yielded a highly significant genotype by days interaction [F(3,27)=5.83, p<0.005 and F(3,27)=6.03, p<0.005, respectively], which was accompanied by a significant days effect in the escape latency analysis [F(3,27)=3.16, p<0.05] and a near significant days effect in the path length analysis [F(3,27)=2.82, p<0.06]. Additional analyses restricted to each day also indicated that the mutant and control mice only differed significantly from each other on the first day of new learning [latency: F(1,9)=19.41, p<0.005; path length: F(1,9)=16.78, p<0.005], but not on any subsequent days. Again, the difference between mutant and control mice persisted through the four trials on the first day (see Figures 3B and 3D).

Problem 4: On the following four days, new learning was conducted in a novel testing room (Room 2) distinct from the one that was used thus far. This change had led to the disappearance of the mutant mice’s phenotype revealed in the previous two new spatial learning problems (Figure 3). On the first day of this phase of new learning, both groups returned to a level of performance similar to that seen on the first day of the first acquisition phase. Throughout the four days, mutant and control mice behaved highly similarly to each other. ANOVAs of escape latency and path length only yielded a significant effect of days [F(3,27)=3.29, p<0.05 and F(3,27)=5.29, p=0.005, respectively]. No other effects even approached statistical significance [all F’s<1] (see Figures 3B and 3D).

Swim Speed: Analyses of swim speed at each phase of spatial learning failed to reveal any significant effect, except in the last new learning phase (Problem 4) conducted in Room 2, which yielded a significant genotype effect [F(1,9)=9.09, p<0.05], as the mutant mice were swimming at a slower speed than controls [mean±SEM): controls = 19.2±1.1 cm/s and mutants = 15.0±0.8 cm/s]. This however should not compromise the interpretation of the results in this stage, because the impressions obtained from escape latency (which might be expected to be confounded by swim speed) and path length were essentially identical. For comparison, the mean speed (cm/s) of the two groups at the different stages of learning in Room 1 was as follows. First learning: controls = 16.2±0.8 and mutants = 14.8±0.6; second learning: controls = 19.0±1.3 and mutants = 15.7±1.0; third learning:
controls = 19.1±1.2 and mutants = 16.1±0.9. These indicated a stable non-significant tendency that mutant mice were swimming consistently at a marginally slower speed. This would not be expected to contribute to their apparent superior performance on day 1 in successive new learning.

In summary, the results had confirmed our expectation that mutant mice showed an advantage over the controls when the platform assumed a novel position, when control mice were evidently suffering from some form of proactive interference. Information acquired from the preceding phase(s) had impeded acquisition of the new location in the control group, but the mutant mice by contrast were much less affected by this negative proactive interference effect. When the procedure prevented such interference effect from affecting the control mice, the difference between groups essentially disappeared.

**Figure 3.** Performance on the initial reference memory task and subsequent new learning in the water maze as indexed by escape latency (A & B) and path length (C & D). An overview of performance was expressed as a function of days (A and C). To allow a closer inspection of both performance measures [escape latency (B) and path length (D)], performance as a function of trials on day 1, day 10, day 14 and day 18, are illustrated separately. The critical transition from one phase to the next (new) learning is highlighted by the shaded background in A and C (across days); and in B and D (across trials). In each phase of new learning, compared to the final trial in the preceding phase of learning (indicated as 'pre') is also included in the corresponding plot (B and D). Error bars refer to ±SEM, mutant (n=7), control (n=4). * p<0.05, based on restricted analyses contrasting performance on the first day of new learning (New learning 1 and 2) between mutant and control mice. The different locations of the platform used in different phases of the experiment are illustrated in the sketches on top of A.
Reference memory: Probe trials

During the course of the reference memory test, probe tests (in which the escape platform was removed from the maze) were performed. Two probe trials were conducted during the first acquisition phase (1a and 1b). Subsequently, one probe trial was conducted prior to training on the 4th (and the last) day in each successive new learning phase (probe tests 2, 3 and 4). Three main measures were taken from each probe test to index search accuracy: percent time spent in the target quadrant, percent path length (distance) made in the target quadrant and the number of annular crossings made to the relevant platform location at each learning phase.

Figures 4A and 4B illustrate the first two measures across the five successive probe tests. Probe tests 1a and 1b were conducted in the middle and at the end of the first acquisition phase, respectively. Both mutant and control mice showed a clear preference to the target (SE) quadrant. By contrast, performance in probe tests 2 and 3 was relatively low, and close to chance level; and mutant and control mice again behaved similarly. In probe test 4, which was conducted in Room 2 just prior to the last day’s training, performance was somewhat intermediate between probes 1a–1b and probes 2–3. These impressions obtained from these two measures are highly congruent with each other.

Figures 4. Probe trials performance was indexed by percent time in target quadrant (A), percent distance in target quadrant (B) and the number of annular crossings (C). Two probe trials were conducted during the first acquisition phase (1a and 1b). Subsequently, one probe trial was conducted prior to training on the 4th (and the last) day in each successive new learning phase (probe tests 2, 3 and 4). The corresponding target quadrant was also listed below the x-axis. In addition, percent time (A) and percent distance (B) spent in the (underlined) quadrants in which the platform was located in the previous learning phase are also illustrated for probe tests 2 and 3. Error bars refer to ±SEM, mutant (n=7), control (n=4). # indicates that mutants and controls performed significantly above chance level (25%) based one-sample t-tests separately conducted for the five probe tests (p<0.05). * denotes a significant difference (p<0.05) between mutants and controls according to a one-way ANOVA of annular crossings restricted to probe trial 1a.

Separate 2 × 5 (genotype × probes) ANOVAs yielded a near-significant effect of probes in percent time spent [F(4,39)=2.59, p=0.05], and in percent distance in target quadrant [F(4,39)=6.23, p<0.001]. There was no indication of any genotype effect or its interaction [all F’s<1]. To assess whether preference for the target quadrant differed from chance level performance, we conducted separate one-sample t-tests for each of the five probe tests. To this end, mutant and control mice were
pooled for the purpose of analysis because there was no statistical indication of any significant genotype difference in the overall ANOVAs. In agreement with the above impressions: significant above-chance preference (in percent time or percent distance) for the current target quadrant was detected in probe tests 1a and 1b only \[p<0.005\], although a non-significant tendency seemed also to be present in probe test 4 \[p<0.1\].

Next, we also examined whether the two genotypes differed in terms of their search in the quadrants containing the escape platform in the preceding learning phase – on probe tests 2 and 3 (see Figures 4A and 4B). The relatively poor performance on probe tests 2 and 3 might stem from a tendency to search in the target quadrant of the previous learning phase. To this end, we conducted \(2 \times 2\) (genotype \(\times\) quadrants) ANOVA contrasting the two relevant (current vs previous target) quadrants for probe tests 2 and 3 separately. However, none of these analyses (based either on percent time or percent distance per quadrant) yielded any significant effects. Likewise, we also evaluated whether preference for the previous target quadrant might differ from chance level using one-sample t-tests as described before. These suggested a highly significant above-chance preference for the previous target quadrant in probe test 2 \([p<0.005\) and \(p<0.001\) for percent time and percent distance, respectively], but not in probe test 3.

The overall lack of any genotype effects described above was consistent with additional one-way ANOVAs conducted for each probe test (either current or previous target quadrant), and two-way ANOVAs that included all four quadrants within a given probe test.

Finally, the measure of annular crossings was examined as an additional dependent variable of search accuracy. As illustrated in Figure 4C, mutant and control mice performed similarly on this measure, although a transient impairment in the mutant was detected in probe test 1a. A \(2 \times 5\) (genotype \(\times\) probes) ANOVA however failed to yield any significant effect, including the critical interaction term \([F<1]\). Nonetheless, we conducted separate one-way ANOVAs for each successive probe test. These yielded a significant main effect of genotype only in probe test 1a \([F(1,9)=5.31, p<0.05]\), indicating that search accuracy was lower in the mutant mice on this particular probe trial, although they performed similarly to control mice from probe test 1b onwards.

Overall, the various probe tests confirmed that at the end of the first acquisition phase, and by the end of each additional phase of new learning, the mutant and control mice were largely similar in performance, exhibiting similar spatial search in the water maze. This suggests that both groups were relying on extra maze to a large extent in guidance of their spatial search.

**Discrimination reversal**

As an explicit and alternative test of the proactive interference effect on learning, we next compared the mutant and control mice on an appetitive (motivated by food reward) non-spatial two-choice discrimination task on a T-maze. Proactive interference was demonstrated by reversing the reinforcement contingency of the two visual discriminanda upon successful discrimination. In the
initial acquisition, two mutant mice failed to acquire the running response, and they were dropped from the experiment at the end of day 2. The number of subjects in the mutant group was thus reduced to 5. All controls from the reference memory test (n=4) were included here.

As depicted in Figure 5A, mutant and control mice did not differ in the acquisition of a pattern discrimination task in the T-maze. They took a similar number of trials before reaching criterion performance. However, their performance markedly differed in the reversal phase. The control mice displayed a clear reversal effect: they took longer to achieve criterion performance in the reversal phase in comparison to the initial acquisition. By contrast, the reversal effect was absent in the mutant mice. These impressions were confirmed by a 2 × 5 (genotype × phases) ANOVA of the dependent measure, which yielded a significant interaction [F(1,7)=6.99, p<0.05], accompanied also by the emergence of a significant main effect of genotype [F(1,7)=11.41, p<0.05]. Analyses of performance restricted to each phase indicated the presence of a highly significant genotype effect in the reversal phase [F(1,7)=21.00, p<0.005], but not in the acquisition phase [F<1]. Analyses restricted to each genotype confirmed the presence of a reversal effect in the control [F(1,3)=48.76, p<0.01] but not in the mutant mice [F<1].

**Figure 5.** Performance in the acquisition and reversal phase of the discrimination reversal experiment was indexed by the number of trials required to achieve criterion performance (A). Error bars refer to ±SEM, mutant (n=5), control (n=4). * p<0.05, indicates the presence of a reversal effect in the control mice by a restricted analysis contrasting their acquisition and reversal performance; and the attenuation of the impact of reversal in the mutant mice by a contrast between mutant and control mice in their reversal performance. Choice accuracy achieved at the end of acquisition, and in the course of the succeeding reversal phase is expressed in percent correct every 3-day blocks in (B). The horizontal (dotted) reference line refers to chance level. A* refers to the final 3-day block of acquisition phase (of individual mouse) in which criterion performance was achieved. The initial drop below chance level was evident in both mutant and control mice. However, the two groups diverged at about chance level: while the control group showing a characteristic inflexion at around 50% chance levels, the mutant showed a straight linear progression through the 50% barrier. * denotes between-groups difference at p<0.05, based on the pooled variance taken from the error term associated with the significant genotype by blocks interaction.

To allow an examination of the progression of learning in the reversal phase, we performed a separate analysis of choice accuracy as a function of 3-day blocks throughout the course of reversal training (Figure 5B). Animals having achieved criterion performance before the 7th block were assigned a criterions score of 100% on subsequent blocks. There was a clear drop of performance at the onset of reversal compared to the end of acquisition (denoted as “A*” in the x-axis of Figure 5B)
in both mutant and control mice. However, the two groups diverged at about chance level: while the control group showed a characteristic inflexion at around 50% correct trials (when the competition over behavioural control by the old and new reward contingency was expected to be maximal), the mutant showed a straight linear progression through the 50% barrier. A 2 × 7 (genotype × blocks) ANOVA of percent correct over the seven blocks of reversal learning yielded a significant main effect of genotype \([F(1,7)=6.21, p<0.05]\), of blocks \([F(6,42)=61.16, p<0.001]\), and critically of their interaction \([F(6,42)=4.26, p<0.005]\). Consistent with the shape of this interaction, with the two groups matching at the end and beginning of the reversal phase, orthogonal trend analysis indicated that the interaction term was significant specifically in the quadratic trend \([F(1,7)=30.17, p<0.0001]\).

The results of this experiment clearly showed that the reversal effect was attenuated in the mutant mice, which is consistent with the hypothesis based on previous experiments that they were less prone to the negative effect of proactive interference. The phenotype on new learning therefore appeared to be only apparent when previously acquired behaviour is potentially in conflict with current behaviour.

**DISCUSSION**

The present study has provided clear evidence that GlyT1 disruption restricted to forebrain neurons is sufficient to induce anomalous behaviour suggestive of enhanced cognitive flexibility across a variety of learning situations when negative proactive interference is substantially limiting performance in the controls. These novel findings represent significant advances in the cognitive characterisation of CamKIIαCre;GlyT1tm1.2fl/fl mice, far beyond our previous demonstrations of their phenotypes in enhancing Pavlovian associative learning and instrumental active avoidance learning (Yee et al. 2006), and in improving memory retention in an object recognition test (Singer et al. 2007). Hence, forebrain neuronal knockout of GlyT1 does not simply strengthen memory trace or increase the rate of learning. The constellation of behavioural phenotypes identified thus far in CamKIIαCre;GlyT1tm1.2fl/fl mice is suggestive of a general enhancement in behavioural adaptability presumably due to enhanced NMDAR function in multiple forebrain structures. This is highly instructive for the potential use of GlyT1 inhibitors as therapeutic agents in different forms of cognitive inflexibility present in a number of neuropsychiatric conditions, and their potential application as cognitive enhancing agents in non-pathological situations.

The first phenotype here emerged in the working memory test. Over the first six days with minimal delay, learning as indexed by improvement from trial 1 to 2 was weak in all animals. With further training, a statistically significant effect of trails was detected in the next six days with a higher delay. This is likely attributed to the general effect of training rather than an effect of a change in delay intervals. It was against this background that a difference in performance was observed: the mutant mice were performing somewhat better than controls on trial 1, and showed very limited change in performance from trial 1 to 2. The latter does not seem to reflect a floor effect because performance
was still at ~30s and ~5m in escape latency and path length, respectively. Should one therefore interpret this as an indication of poor working memory? Such a conclusion would be premature given that it did not conform to the typical pattern of working memory deficit with comparable trial 1 performance and poor trial 2 performance. A satisfactory explanation would need to accommodate both anomalies observed here in the mutant mice, namely, improved trial 1 performance and lack of trial 1 to 2 saving.

The apparent lack of trial 1 to 2 saving may indicate that the mutant mice were not resorting to memory of the platform location acquired in trial 1 to guide their search in trial 2. This as we discovered later in the reference memory experiment was not due to their inability to use extra maze cues. The mutant mice might have developed some allocentric strategies, the nature of which remained to be delineated. However, if such strategies are non-mnemonic in nature, it would be expected to outperform the controls provided that the trial 1 performance in controls was limited by some forms of proactive interference. In other words, the mutant mice would be less susceptible to potential proactive interference by previous day’s platform location that was affecting performance of the control mice particularly on trial 1 on each day. If so, the present two-trials-per-day paradigm was not sufficiently demanding to tax working memory in the mutant mice. They were able to resort to a non-mnemonic strategy without losing out to the controls in terms of overall escape performance: the gain of performance from trial 1 to 2 by the controls failed to compensate for the gain achieved by the mutant’s non-mnemonic strategy on trial 1. It would therefore be very interesting to assess the impact of additional trials, when continual use of such a strategy by the mutant should eventually lose out to the controls who would be expected to make further gain on performance if there were more trials to follow on the same day. Would the mutant mice maintain such a strategy, or would they begin to rely on recollection of the platform location learned from previous trials? Although we cannot address this in our working memory test since only two trials were performed per day, the first day of the reference memory test provided such an opportunity (even though the ITI was switched back to 15s) as the constancy of platform location across days had yet to be made known to the animals.

As illustrated in Figures 3B and 3D, there was hardly any indication in the mutant mice of an improvement in performance in both dependent measures across trials on the first day of initial acquisition in the reference memory test. At the same time, the controls were displaying a non-significant trend towards improvement across trials. Although this contrast failed to achieve statistical significance here, it certainly deserves further evaluation. As training on the reference memory task progressed, however, the mutant mice apparently had gained on performance, displaying both within-day as well as between-days improvement that was highly comparable to the control mice. Thus, overall performance of the two groups of mice was closely matched across the eight days of first reference memory test (Figures 3A and 3C) in spite of the anomaly on the first day described above. Results of the two probe tests (conducted in the middle and at the end of the initial acquisition) are in full agreement with this impression (see results of probe tests 1a and 1b depicted in Figures 4A and
4B), including the observation of poor spatial search in the first probe test suggested by the measure of annular crossings. The latter probably suggested that the mutant mice were still under the influence of a non-mnemonic strategy (adopted since the working memory test) in the early phase of acquisition. Nonetheless, our results clearly indicated that the mutant mice could acquire a reference memory solution in the water maze test in a manner that was essentially indistinguishable from the controls by the end of acquisition training. It was following this that the second clear phenotype emerged.

Next, when the platform location was moved to a new location, performance in all subjects reduced as expected. However, the mutant mice were substantially less affected by this manipulation. The control mice, on the other hand, exhibited a clear reversal effect – performance at the beginning of new learning was poorer than at the beginning of the first acquisition. This reflected a negative proactive interference effect, when memory of the first platform location came to interfere with the learning of the second location. The mutant mice on the other hand reverted to a level of performance not dissimilar to the beginning of their first learning; they thus essentially did not show a reversal effect. The absence of a reversal effect (or reduced proactive interference) in the mutant mice observed here cannot be attributed to poorer learning in comparison to the control in the first phase, because performance of the two groups remained highly comparable throughout the first phase of learning, and in particular with respect to their probe test performance at the end.

Following another 4 days of training with the second platform location, the escape platform was once again moved, and a very similar pattern of results emerged. The initial negative impact of platform re-location was notably weaker in the mutant mice. To test the idea that this advantage enjoyed by the mutant over the control mice was dependent on a proactive interference effect operating in the controls, we introduced the next new learning not only by moving the platform to a new location as defined by the maze itself, but the entire maze was moved to a novel and distinct testing room. By re-setting the extra maze cues, and thereby removing the source of proactive interference, it was predicted that the two groups should no longer be responding differently to the initiation of new learning. The efficacy of this move from Room 1 to Room 2 was confirmed by the elimination of the reversal effect previously seen in the control mice when new learning took place in Room 1. Initial performance in this phase by the control mice matched their initial performance in the first acquisition. The impact of the change in rooms on the mutant mice, on the other hand, was somewhat limited, and the difference in initial new learning between mutant and control mice seen previously in Room 1 was thereby eliminated in Room 2.

Further examination of the probe tests conducted in Room 1 on the last day of each new learning phase indicated that preference for the most recent target platform was weak in both mutant and control mice (see Figures 4A and 4B). This likely suggests that when the platform was removed from the water maze (in a probe test), and therefore escape was not possible, both mutants and controls did not focus their search solely to the more recent target quadrant. It can be seen that they significantly more time (and proportion distance) as expected by chance searching in the preceding
target quadrant. The fact that this was equivalently seen in both groups again suggested that the anomalous response exhibited by the mutant mice at the initiation of new learning did not stem from a memory retention deficit per se. This leads to the conclusion that the phenotype observed in the mutant was directly related to the presence of the escape platform in a new location. They detected the new location more effectively and they were able to translate this newly acquired knowledge quickly in subsequent trials. The mutant mice might be more effective in detecting the new location of the platform because they were less persistent in searching at where the platform used to be located. This may also suggest that they were more responsive to the absence of the platform in the predicted location. Thus, they were thus less hindered in comparison to the controls. This facilitation should therefore be understood strictly in contrast to the presence of persistent search exhibited by the control mice on the first day of new learning. The mutant mice thereby were able to detect the new location of the platform earlier than controls; and as a consequence perhaps, shifted more readily their search preference to the vicinity of the new location. On the first day of new learning, the mutant mice consistently outperformed the control animals across all four trials, leading to a significant genotype effect on such days. However, the advantage of the mutant was temporary as the control mice were able to catch up in subsequent training days.

In summary, we were able to show that forebrain neuronal deletion of GlyT1 can lead to some forms of learning enhancement, but the emergence of such effects is highly specific to the test situations. Its presence is linked to conditions in which control mice were negatively affected by proactive interference. Although whether this explanation may fit the results of the working memory test awaits further evaluation, its applicability to the reference memory (and repeated new learning) results represents a simple and parsimonious explanation. We adopted therefore a paradigm befitting as a direct test for this hypothesis. To this end, we conducted a two-choice simultaneous discrimination reversal task to maximize the proactive interference effect: not only were the animals required to stop visiting the previously rewarded arms, they needed to choose the previously non-rewarded arms in order to obtain food reward. In addition, we implemented this test in a non-spatial setting to further assess the generality of the findings obtained in the water maze. The results of the discrimination reversal experiment (see Figure 5A) are strongly supportive for the hypothesis that the CamKIIαCre;GlyT1tm1.2fl/fl mice were resistant to the negative impact of proactive interference on learning, especially when competition between the old and new reward contingency over behaviour was expected to be maximal (see Figure 5B). Hence, although the present study yielded little support for a cognitive enhancing effect in these animals on the acquisition and retention of spatial reference memory as reported by Tsai et al. (2004a) in constitutive GlyT1 +/- mice, we have provided specifications (of the cognitive demands) as to when inhibition of GlyT1 activity may prove particularly effective in facilitating learning. The nature of this specificity suggests that the cognitive effects of our genetic manipulation on GlyT1 expression has particular impact on the executive functions of memory – i.e., the selective expression of learned information, and the readiness to adopt
and entertain possible alternative strategies in problem solving (e.g., escaping from water) in a given test situation. This specification is highly relevant for the possible clinical application of GlyT1 inhibitors in the treatment of cognitive deficits. Indeed, this profile fits the suggested therapeutic efficacy of GlyT1 inhibitors against the negative and cognitive symptoms of schizophrenia, which are characterized by overt preservative behaviour and cognitive inflexibility (e.g., Lane et al. 2005, Shimm et al. 2008; Tsai et al. 2004b).

The GlyT1 deletion achieved in our mutant mice is expected to enhance the availability of glycine in the vicinity of the NMDAR and thereby to affect the activity of this receptor. A recent report has shown that systemic D-serine treatment (600mg/kg/day) also enhances reversal learning in mice (Duffy et al. 2008). However, it should be pointed out that the effect of D-serine reported by Duffy et al. (2008) is qualitatively rather different from the enhancement of the new learning demonstrated here in the CamKIIαCre;GlyT1tm1.2fl/fl mice. D-serine pre-treatment did not facilitate learning in the initial phase of new learning (in a reference memory task design; see Figure 3a of Duffy et al. 2008) as our mutant mice did, when proactive interference was expected to be at its strongest. Instead, D-serine was only effective when new learning was already into its third day. Moreover, D-serine treated mice demonstrated a clear preference for the new platform location in the probe test conducted following reversal learning, against a lack of such preference in the vehicle-treated animals (Duffy et al. 2008). In spite of this finer difference, it may be tempting to speculate that modulation of NMDAR function via the glycine-B site is particularly implicated in reversal learning and similar situations when proactive interference is pronounced.

In the same study, Duffy et al. (2008) also reported an enhancement effect of D-serine on working memory as evaluated in the water maze, similar in design to the first experiment here. The effect however appeared weak and transient: (a) it was only apparent on days 5-8 in the test, but not the four days before or after that, (b) this effect coincided with a time when the control failed to improve from trial 1 to 2, and (c) it was only reported in the measure of path length but not escape latency. One may rather conclude that D-serine enhances the consistency of working memory performance rather than mnemonic power in terms of increased memory load and resistance to temporal decay. However, even when D-serine’s effect on working memory is far from convincing, its impact on behaviour is still markedly dissimilar to that of forebrain neuronal GlyT1 deletion revealed here. As discussed above, we cannot be certain that the mutant mice employed a matching-to-position (demanding spatial working memory) strategy in the test. This strategy is prone to proactive interference and thus an alternative non-mnemonic strategy can nonetheless enable effective performance, especially when only two trials were administered. Indeed, one can envisage that although a matching-to-position strategy would allow improvement from trial 1 to 2, this may not be sufficient to outpace the overall performance produced by a non-mnemonic strategy. In this respect, it should be noted that the test design employed by Duffy et al. (2008) employed four consecutive trials, and this might promote the competitive edge associated with a matching-to-position strategy over any
non-mnemonic strategy.

What seems to be a recurring feature is that regardless of whether it was D-serine treatment or the genetic disruption of GlyT1 studied here, the enhancing effect of either manipulation was most clearly seen when the control groups were performing particularly poorly. The latter may be by design, as in the discrimination reversal experiment here, or perhaps accidental as the results by Duffy et al. (2008) seem to suggest. This is noteworthy because our previous demonstration of enhanced object recognition memory in the CamKIIαCre;GlyT1tm1.2fl/fl mice was also against the background of a complete lack of novelty preference in the control mice (Singer et al., 2007). This was achieved at a delay retention interval of two hours, which should still yield appreciable novelty preference in wild type C57BL/6 mice according to some studies (e.g., Hale and Good 2005). Similarly, we reported that CamKIIαCre;GlyT1tm1.2fl/fl mice exhibited the latent inhibition effect when control mice failed to do so (Yee et al. 2006). The number of stimulus pre-exposures employed was typically sufficient to generate latent inhibition in wild type C57BL/6 mice. Although the results here did not suggest that our control mice behaved differently from wild type mice of the same genetic background, yet the emergence of apparent cognitive enhancing effects tended to coincide with relatively weak performance in the controls. Weak performance may not only be the result of task difficulty, but it may also arise when the solution to the task is inconsistent with previous experience – leading to some form of cognitive dissonance or conflict, as in the case of reversal learning. Thus, although proactive interference is a prominent source of cognitive conflict in the experiments reported here, the possibility remains that GlyT1 inhibition or glycine-B site activation may promote the resolution of such conflict whenever a similar cognitive demand is elicited. An obvious step forward is to evaluate the situation under retroactive interference. One argument that reduction in proactive interference may not be the key task demand to solicit the memory/learning enhancing effect of GlyT1 disruption is the finding of enhanced latent inhibition in CamKIIαCre;GlyT1tm1.2fl/fl mice (Yee et al. 2006).

One theoretical account of the latent inhibition effect is precisely in terms of proactive interference (e.g., Bouton 1993; Kraemer and Spear, 1991; Weiner 1990), such that non-reinforced exposures to a neutral conditioned stimulus (CS) leads to formation of an [CS-nothing] association, which competes or interferes with the expression of a conditioned response to the CS following the pairing between the CS and an unconditioned stimulus (US). If GlyT1 disruption reduces proactive interference per se, it should attenuate rather than enhance the latent inhibition effect. One alternative account we recently suggested (Singer et al. 2008) is that CamKIIαCre;GlyT1tm1.2fl/fl mice learned both [CS→nothing] (during pre-exposure) and [CS→US] (during conditioning) more effectively, and hence allowing the former association to reveal its impact on the expression of conditioned response resulting from the latter association. In contrast, the test parameters were insufficient to generate latent inhibition in the control mice because the impact of both [CS→nothing] and [CS→US] on subsequent non-responding and responding to the CS, respectively, was weaker than in the mutant mice (Yee et al. 2006). In this manner, the enhancement of latent inhibition in CamKIIαCre;GlyT1tm1.2fl/fl mice
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reported earlier (Yee et al., 2006) can be explained solely in terms of enhanced associative learning as a result of enhanced NMDAR activity. Indeed, it has been shown that NMDA blockade by MK-801 (0.1-0.2mg/kg i.p. in rats) produces the exact symmetrical pattern of results: MK-801 abolished the latent inhibition effect by reducing conditioning in the non-pre-exposure subjects (see Gaisler-Salomon and Weiner, 2003; their Figure 1).

Hence, although the diverse phenotypes emerged in this mutant mouse line may be, to varying degrees, understood as a form of cognitive enhancement across different experimental preparations, they do not necessarily conform to a single psychological description. This may not be too surprising given that the mutation affected the whole forebrain and thus would exerting an effect on multiple psychological functions. Given that the NMDAR is generally involved in synaptic plasticity, the phenotypic profile of our mutant mice is likely to stem from altered NMDAR function across multiple forebrain structures. The current knockout system does not allow one to differentiate the relative contributions of different forebrain brain regions. To satisfactorily address this, either region-specific molecular intervention or local pharmacological blockade would be necessary.

Here, we have emphasized here the importance of fine behavioural analysis to pinpoint more precisely the nature of potential cognitive enhancing effects associated with our specific genetic manipulation targeting GlyT1. A similar approach would also benefit evaluation of other manipulations with similar pro-cognitive potential. In this respect, one should not overlook the possible caveats in the present study. The fact that we performed the reference memory test after the working memory test in the same cohort of subjects may introduce possible transfer effects, and thus might confound the interpretation of the findings emerging from the latter test. This potential shortcoming led us to perform the two-choice simultaneous discrimination reversal experiment as a further confirmation of our major finding. Despite the reduced number of subjects, the effect was very clear, suggesting that when the condition is conducive the associated statistical effect size can be considerable. The observed phenotype therefore seems very robust.

In terms of the nature of this cognitive enhancement, our results may bring about the following afterthought: Just as cognitive disability is context-specific (Durkin et al. 2006), cognitive enhancement should be similarly considered (e.g., Sandberg and Bostrom 2006). Hence, any given therapeutic manipulation that can bring about biases or shifts in a given cognitive function which may arguably lead to both benefits as well as harms, depending on the precise circumstances. Learning (as an exemplary cognitive function) has evolved as it bestows the ability to exploit predictive cues in one’s environment so as to enhance adaptability through appropriate behavioural adjustment or modification. Thus, flexibility is a pre-requisite to effective learning, and any extreme or rigid form of cognitive bias is likely to be detrimental and counterproductive. The behavioural profile of the CamKIIαCre;GlyT1tm1.2fl/fl mouse line does not seem to fall into this trapping, and it may suggest that pharmacological interference of GlyT1-mediated glycine re-uptake may represent a promising strategy to enhance adaptive cognitive behaviour, and to correct maladaptive cognitive deficits in a
number of neuropsychiatric conditions including schizophrenia (Coyle and Tsai 2004, Danysz and Parsons 1998, Javitt 2008, Lechner 2006).

MATERIALS AND METHODS

Generation of CamKIIαCre;GlyT1tm1.2fl/fl mice

Briefly, the neuron and forebrain specific deletion of the GlyT1 gene was accomplished by CamKIIα-Cre mediated recombination of a conditional loxP-flanked GlyT1 allele (Glyt1tm1.2fl/fl). A full description of the generation of the animals has been reported elsewhere (see Gabernet et al., 2005; Yee et al., 2006). The impacts of the specific deletion of the GlyT1 gene at the biochemical and electrophysiological levels have been described elsewhere (Yee et al. 2006).

Subjects

The subjects were obtained by crossing CamKIIαCre;Glyt1tm1.2fl/fl mice with Glyt1tm1.2fl/fl mice (both on a pure C57BL/6J background). This allowed us to generate litters with a 1:1 mixture of CamKIIαCre;Glyt1tm1.2fl/fl (hereafter simply referred to as “mutant”) and Glyt1tm1.2fl/fl (“control”) mice and to maintain the Cre gene heterozygous in the mutants. Breeding took place in a specific-pathogen free (SPF) breeding facility. Genotyping was performed on postnatal days 21-30 by standard PCR (Yee et al. 2006). Litters were weaned at postnatal day 21. Only male mice (7 mutant and 5 controls) were employed in the present study. At the age of 11 weeks, the animals were transferred to a separate controlled animal vivarium (21±1°C, relative humidity at 55±5%) with a reversed light–dark cycle (lights off: 0800–2000). They were kept in groups of four in Makrolon® Type-III cages (Techniplast, Milan, Italy), and maintained under ad libitum water and food (Kliba 3430, Klibamuhlen, Kaiseraugst, Switzerland) unless otherwise stated. Prior to the last experiment described here, the animals were switched to single housing in Makrolon® Type-II cages (Techniplast), they were switched to a 22h food-deprivation diet with their body weight reduced to not less than 85% of their ad lib weight.

Behavioural experimentation commenced when they were about 12 weeks old, and was always conducted in the dark phase of the cycle. The experimental manipulations and procedures described here had been previously approved by the Swiss Cantonal Veterinary Office; they conformed to the ethical standards required by the Swiss Act and Ordinance on Animal Protection and the European Council Directive 86/609/EEC.

Apparatus

Water maze. The water maze consisted of a white circular tank made of fibreglass, 102 cm in diameter and 36 cm deep. It was filled with a mixture of hot and cold fresh tap water on each test day to a depth of 19 cm and a temperature of 24±1°C. A transparent cylinder made of Plexiglas (diameter 7cm, high 18.5 cm) was used as escape platform. Its surface submerged 0.5 cm below the
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water surface and was therefore invisible to the animals. Its location however could be made visible by mounting a white circular disk (diameter 12 cm) that stood 12 cm directly above the platform. A digital camera was installed above the water maze, capturing and transmitting images at a rate of 5Hz to a PC running the Ethovision tracking system (Noldus, The Netherlands). Ethovision computed the escape latency and distance travelled on each trial, and the additional dependent measures on probe tests (see procedures below).

The watermaze could be positioned in the centre of one of two possible well-illuminated testing rooms (referred as Room 1 and Room 2), each enriched with unique distal spatial cues. The working memory experiment was conducted in Room 1 throughout. In the reference memory experiment with serial new learning, the final stage of new learning was conducted in Room 2.

The T-maze was modified from an elevated plus maze. It was elevated at a height of 50cm on a table-top that was 65 cm above floor level. It was made of transparent Plexiglas, comprising four arms (5 x 30 cm) joined at a central square (5 x 5 cm). One open arm was blocked permanently to give a “T” configuration with the remaining open arm serving as the start stem and the two enclosed arms as choice stems. The maze floor was painted black throughout. The two choice arms (180˚ apart) were surrounded by double-sided transparent Plexiglas walls (15 cm high), except the side adjoining the central square. The start arm was surrounded by a 3-mm high perimeter rim to prevent slipping.

Papers of either vertically or horizontally oriented black-white alternating stripes (1cm wide) pattern printed on both sides could be inserted into double-sided Plexiglas walls of the enclosed arms. The patterns were interchanged according to a pseudorandom sequence across trials to prevent a spatial solution to the discrimination task. The food reward (25mg Noyes sucrose pellet) was hidden behind a 5mm high barrier positioned 5 cm from the arm’s distal end, so as to prevent visual detection of the food reward. Exit from the chosen arm was blocked by an opaque plastic barrier wall.

**Behavioural Procedures**

The animals were first tested on the working memory test in the water maze followed next by the reference memory test in the same apparatus. Although both versions of water maze tests followed a matching rule within-day (see procedural details below), the design of the working memory task demanded a non-matching rule between days while the reference memory task also followed a matching rule between-days. The latter difference might therefore imply some forms of negative transfer effects regardless of the test order selected. We elected to perform the working memory test first in all animals because the primary interest here was on repeated new learning, which was initiated when performance between groups in the initial acquisition converged. Hence, the potential negative impact of prior working memory training on new learning in the reference memory test should be minimal. It would have been difficult to counterbalance the test order between the two water maze tests because the duration of each test was not decided beforehand. Following the conclusion of the
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The second water maze test, the animals were then evaluated in two-choice discrimination reversal procedure conducted with a T-maze.

\[ \text{Cued test in the watermaze.} \] On the first day, the animals were pre-trained using a cued (i.e., visible) platform in order to familiarize them to the apparatus and to swimming in the water maze. It further served as a screen of any non-specific sensorimotor impairment. The platform was positioned in the centre of the maze. Each animal underwent two consecutive trials. The starting positions were randomly selected from the four possible release points (N, E, S, and W). In the first trial, the subject was gently released from the start point with its head facing the platform location. In the second trial, it was released from the start point facing the wall of the maze. The animals were allowed to locate the escape platform within 60s. Upon reaching the platform they spent an inter-trail interval (ITI) of 15s on it before the second trial commenced. If an animal failed to locate the platform within the time limit of 60s, a maximal escape latency of 60s was recorded and it was guided to the platform by the experimenter and allowed to stay on it for 15s.

\[ \text{Working memory test.} \] This lasted for 12 days. The platform was now always hidden from the animals’ view, and assumed a new position on each day, but it remained in that position from trial 1 to 2 on any given day. Working memory was indexed by the improvement from trial 1 when the platform location was essentially unknown to trial 2. As described before, when an animal failed to locate the platform within 60s, it was guided to the platform. The ITIs were always extended to 10 min in the next 6 days. During the 10 min ITI the animals were kept in an opaque waiting box in the testing room. Twelve platform positions were defined: six were located at a distance of 35cm (in the N, S, NE, SE, SW, and NW directions), six more at a distance of 15cm from the centre (in the N, E, S, W, NW and SE directions). The start position always differed from one trial to the next, and was selected randomly from eight possible release points (N, E, S, W, NE, NW, SE and SW) for each mouse.

\[ \text{Reference memory & Serial new learning} \] This commenced five days following the working memory test. First, the animals were trained to locate the escape platform, which was now hidden at a constant location. There were 4 trials per daily session, conducted at an ITI of 15s, and lasted 8 days. Testing was conducted in the testing room where the working memory test had taken place (denoted as Room 1). Here, the position of the platform was fixed in the middle of quadrant SE, 35cm from the maze centre. The starting position varied among eight possible release points (N, E, S, W, NE, NW, SE and SW), and was determined for each animal separately as described above.

A probe test was conducted prior to training on day 5, and again on day 9 (not followed by any training with platform) – these are referred to as probe test 1a and 1b, respectively. The start positions of NW and SW were used on these two probe trials, respectively. During a probe test, the platform was removed and the animals were allowed to swim freely in the maze for 45s. The animals’ spatial
Next the animals were tested with 3 consecutive new spatial reference memory problems as described above, in blocks of 4 days. On days 10-13, the platform was hidden in quadrant NW (35cm from maze centre) in Room 1. On days 14-17, the platform was hidden in quadrant SW (35cm from maze centre) in Room 1. On days 18-21, the platform was hidden in quadrant NE (35cm from maze centre) but the maze was moved to Room 2 (a novel testing room). Prior to the beginning of the last day’s training in each block of new learning (i.e., on days 13, 17 and 21), a probe test was performed (they are referred to as probe test 2, 3, and 4) as described above. The starting positions for these three additional probe tests were: SE, NE, and SW, respectively.

Acquisition and reversal of a simultaneous two-choice discrimination A month following the conclusion of the water maze experiments, the animals were switched to single caging, and introduced gradually to a food deprivation diet, with a progressive reduction of feeding time across five days (12h, 6h, 4h, 2h, 2h). Discrimination learning commenced on the next day, and the animals were maintained with 2-h feeding per day throughout the experimental period. The animals’ body weight was monitored daily and was not permitted to fall below 86% of their ad lib weight.

First, the animals were habituated to the T-maze. For three consecutive days, they were individually placed in the T-maze and allowed to explore freely for 5 min. On day 1, fifteen reward pellets were randomly scattered over the entire maze surface. On day 2, five pellets were scattered in each of the two choice arms. On day 3, five pellets were placed in the goal area at the end of each of the two choice arms. During habituation, the two choice arms were identical in appearance: white papers were inserted into the double-sided Plexiglas walls of both choice arms.

Discrimination learning between vertical (V) and horizontal (H) visual patterns commenced on the next day. Half of the animals within each group were assigned the [V+ vs H–] contingency, and the other [V+ vs H–] contingency, with ‘+’ and ‘–’ referring to whether the corresponding discriminandum was associated with food reward or not. The relative positions (i.e., left vs right choice arms) of the vertical and horizontal discriminanda were randomized across trials.

On days 1 and 2 of training, the animals were given 4 and 8 trials, respectively. Twelve trials were given on all subsequent days. The ITI was 20s in which the animals were placed inside a cardboard waiting box, and the maze was cleansed with 70% alcohol. To begin a trial, the animal was released at the end of the start arm, facing the central square, and allowed to travel down to the choice point to enter one of the two choice arms. A choice was deemed to have been made when the animal’s head crossed halfway into a choice arm, upon which the exit was blocked. A correct choice was rewarded by 2 reward pellets, and an incorrect choice was followed by confinement to the chosen arm for 15s. The animals were allowed make a choice within one minute; if failed, the trial was aborted. There were no aborted trials from day 2 onwards.

When an animal scored ≥10 correct choices per day on three consecutive days, it was
considered to have reached the criterion for successful acquisition of the initial discrimination. It will then be subjected to reversal learning the next day when the reward contingency of the two discriminanda was reversed. Training continued until the animals had achieved criterion performance in the reversal phase. The number of trials required to attain criterion performance in the acquisition and the reversal phase was taken as the dependent measures. The presence of a reversal effect is indicated by a higher number of trials to criterion in the reversal than in the acquisition phase.

**Statistical Analysis**

All data were subjected to parametric analysis of variance (ANOVA) with the between-subject factor genotype and the addition of any relevant within-subject factors (e.g., days, trials, quadrants and probes). The ANOVAs were performed using SPSS® for Windows (Release 13.0) on a personal computer running the Microsoft Window XP Professional SP2 operating system. Significant main effects and interaction terms were further investigated by appropriate restricted ANOVAs. A priori restricted ANOVAs were also conducted to assist interpretation of the data whenever necessary.

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REFERENCES


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transporters are differentially expressed among CNS cells. J. Neurosci 15: 3952-3969.
Chapter 4

Learning and memory following conditional knockout of glycine transporter 1 (GlyT1) in neurons and glia restricted to the forebrain

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Abstract

Molecular disruption of the glycine transporter 1 (GlyT1) restricted to forebrain neurons is sufficient to induce specific biases in cognitive information processing, suggesting that increased availability of glycine in the vicinity of N-methyl-D-aspartate receptors (NMDARs) located in glutamatergic synapses may be associated with potential pro-cognitive effects. However, GlyT1 is also abundantly expressed in glia cells, yet their contribution to the regulation of learning and memory has not been systematically evaluated. Due to the lack of a satisfactory system to achieve a glia-specific gene knockout restricted to the forebrain, we have generated mice lacking GlyT1 in neurons and glia of forebrain, thus allowing a comparison with previous electrophysiological, biochemical and behavioral data collected in mice with GlyT1 knockout restricted to forebrain neurons. Here, we demonstrate reduced motor responses to the NMDAR blocker phencyclidine, and enhanced spatial working memory and object recognition memory in these mutant mice. Neither hippocampal AMPAR nor NMDAR currents were altered. In contrast, neither spatial reference memory nor associative learning was affected. Thus, the phenotype generated by the disruption of GlyT1 in both neurons and glia corresponded only partly to the phenotype previously seen in mice with selective neuronal GlyT1 deletion. Novel phenotypes unique to the disruption of GlyT1 in neurons and glia of the forebrain also emerged. The two subpopulations of GlyT1 in forebrain may therefore assume dissociable regulatory functions over learning and memory. This distinction should be considered in the future design of pharmacological interventions against cognitive dysfunction on the basis of reducing GlyT1-mediated glycine re-uptake.
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INTRODUCTION

Glycine is an obligatory co-agonist of glutamate at the glycine-B site of the N-methyl-D-aspartate receptor (NMDAR). Local glycine levels in the vicinity of glutamatergic synapses containing NMDARs are tightly regulated by the glycine transporter 1 (GlyT1), which mediates glycine re-uptake from the synaptic cleft into neurons and adjacent glia cells (Eulenburg et al., 2005; Betz et al., 2006). GlyT1 therefore constitutes an important up-stream modulator of NMDAR activity (Bergeron et al., 1998) and as such represents a potential target for interventions aimed to modulate NMDAR function. An increasing body of evidence indicates that pharmacological blockade or molecular disruption of GlyT1 potentiates glutamatergic transmission and exhibits activity in animal models of neuropsychiatric conditions (Kinney et al., 2003; Tsai et al., 2004; Aragon and Lopez-Corcuera, 2005; Gabernet et al., 2005; Martina et al., 2005; Lindsley et al., 2006; Yee et al., 2006; Singer et al., 2007; Sur and Kinney, 2007). Given that NMDARs assume important roles in cognitive processes underlying learning and memory (Tang et al., 1999), increased availability of glycine at glutamatergic synapses through inhibition of GlyT1 mediated re-uptake may provide novel therapeutic avenues to treat cognitive impairments in a number of psychiatric conditions and may even prove useful in enhancing cognitive functions in non-pathological conditions.

Until now, the most convincing behavioral evidence for a pro-cognitive profile is derived from a GlyT1 knockout mouse line lacking GlyT1 in forebrain neurons. This molecular manipulation was sufficient to improve cognition in multiple learning mechanisms including associative learning (Yee et al., 2006), familiarity judgment (Singer et al., 2007) and reversal learning (Singer et al., submitted). Neurophysiologically, the mutation increased the amplitude of NMDAR currents, which has been associated with pro-cognitive efficacy (Kinney et al., 2003; Martina et al., 2004; Yee et al., 2006; Singer et al., 2007). A causal link between enhanced NMDAR function and improved cognition is not firmly established as shown in heterozygous GlyT1 KO mice (Coyle and Tsai, 2004a, 2004b). Systemic treatment with several GlyT1 inhibitors produced some pro-cognitive effects in preclinical models of cognitive deficits linked to schizophrenia (Atkinson et al., 2001; Gadea and Lopez-Colome, 2001; Chen et al., 2003). To provide a more critical assessment of the potential therapeutic value of GlyT1 blockade, we characterized here the impacts of concomitant neuronal and glial GlyT1 disruption in the forebrain on cognitive functions. To this end, we generated knockout mice lacking GlyT1 in the entire forebrain, and evaluated them through a battery of neurobiological and cognitive assays in order to identify the functional divergence between neuronal (Yee et al., 2006; Singer et al., 2007; Singer et al., submitted(a)) versus neuron-plus-glial knockout of GlyT1 in forebrain. Phenotypic differences between these two conditional GlyT1 knockout systems would constitute evidence for a functional dissociation between the two subpopulations of GlyT1. This would be highly relevant for the critical evaluation of GlyT1 inhibition as a feasible pharmacological route of action to achieve potential therapeutics against cognitive impairments.
METHODS

Generation of Emx1Cre:GlyT1tm1.2fl/fl mice

A strategy similar to the creation of the neuronal forebrain selective GlyT1 knockout CamKIIαCre:GlyT1tm1.2fl/fl mice (CamKII/GlyT1-KO) (Yee et al., 2006) was employed here to create the neuron plus glia forebrain selective GlyT1 knockout mice Emx1Cre:GlyT1tm1.2fl/fl (EMX/GlyT1-KO) used for the experiments described here. Briefly, to achieve neuron plus glia forebrain-selective recombination of the floxed GlyT1 allele, conditional GlyT1 knockout mice GlyT1tm1.2fl/fl (Yee et al., 2006) were bred with Emx1CreCre mice, which contain a Cre-recombinase gene “knocked in” into the endogenous Emx1 locus (Iwasato et al., 2000) resulting in ubiquitous dorsal telencephalon specific expression of Cre in both neurons and astrocytes and minor ectopic expression of Cre beyond the forebrain. Further breeding resulted in Emx1Cre:GlyT1tm1.2fl/fl mice (referred to in the following as “mutant”). Subjects used in this study were generated by breeding Emx1Cre:GlyT1tm1.2fl/fl mice with GlyT1tm1.2fl/fl mice (referred to in the following as “control”) thus producing mutants and controls in a 1:1 ratio as littermates and keeping mutants homozygous for the GlyT1tm1.2fl allele and heterozygous for the Emx1Cre allele. All subjects were generated and maintained on a pure C57BL/6 background. Genotyping was performed as described previously (Yee et al., 2006) using the following primers: o139: 5’-CCTAACCCATGGCCAGGACC-3’, GlyT1 specific antisense primer flanking the PGK-neomycin cassette; o184: 5’-CATCGCCTTCTATCGCCTTCTTGACG-3’, sense primer specific for the PGK-neomycin cassette; o228: 5’-GTCAACCTGACTCCTAGCCCTGTACC-3’, GlyT1 specific antisense primer 3’ to PGK-neomycin-cassette; o232: 5’-AGAAGATCTGAGAGGGTGCATCCC-3’, antisense primer specific to the loxP-flanked region; o250: 5’-CCCATGCCAGATCCATGC-3’, sense primer 5’ of left loxP-site. o234: 5’-TGACAGCAATGCTGTTTCACTGG-3’, sense primer specific for Cre-recombinase; o235: 5’-GCATGATCTCCGGTATTGAAACTCC-3’, antisense primer specific for Cre-recombinase. The thermocycle of the PCR was 35 cycles at 95°C (15s), 68°C (20s), and 70°C (90s).

Preparation of crude synaptosomal membranes for Western blotting and [3H]glycine uptake

Mice were killed by cervical dislocation and the hippocampal formation as well as the cerebral cortex was rapidly dissected on ice. The tissue was homogenized in 20 volumes of ice-cold 10mM Tris, pH 7.4, 0.32M sucrose and centrifuged for 10min at 1000g. The resulting supernatant was centrifuged for 10 min at 27,000 g to obtain the crude synaptosomal membranes.

Western blotting

Crude synaptosomal membranes derived from two behaviorally naïve 8 weeks old male mutant and two control mice per experiment were re-suspended at a protein concentration of about 2mg/ml, supplemented with an equal volume of 125mM Tris/HCl pH6.8, 20% glycerol, 0.002% bromphenol blue, 10% β-mercaptoethanol, 4% SDS and incubated for 15min at 60°C. Aliquots with
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Increasing protein content (2.5, 5, 7.5, 10, 15, and 20µg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5% mini-gels (Mini Protean II, Bio-Rad) and resolved proteins were transferred onto nitrocellulose membranes using a Trans Blot Mini Cell (Bio Rad). The blots were blocked for 1-2h in TBST (10mM Tris/HCl pH8, 0.15M NaCl, 0.05% Tween20) containing 5% non-fat dry milk at RT, followed by incubation with affinity purified GlyT1 antiserum (1:1000, Gabernet et al., 2005) together with a monoclonal antibody directed against the NMDA receptor NR1 subunit (1:1000, Affinity BioReagents) and a monoclonal antibody directed against β-actin (1:40,000, Chemicon International) overnight at 4°C in TBST/5% blocker. The blots were washed once with 20mM Tris pH7.5, 60mM NaCl, 2mM EDTA, 0.4% SDS, 0.4% Triton-X 100, 0.4% deoxycholate and 3 times with TBST. Incubation with the appropriate HRP-conjugated secondary antibodies was carried out for 1h at RT. Following extensive washing immunoreactivity was detected by the enhanced chemoluminescence method (Super Signal West Pico Chemoluminescence, Pierce). Images were captured using a Fuji film LAS-1000 Plus Gel Documentation System, and immunoreactive bands were quantified with the AIDA software (Version 3.25, Raytest, Pforzheim, Germany). Actin immunoreactivity was used to monitor equal sample loading.

Glycine uptake

Freshly prepared crude synaptosomal membranes derived from two to three behaviorally naïve 8 weeks old male mutant and two to three control mice per experiment were immediately washed twice with 25mM HEPES, pH7.4, 125mM NaCl, 5mM KCl, 2.7mM CaCl₂, 1.3mM MgCl₂, 10mM glucose (KH buffer) and subjected to [³H]glycine uptake studies. Crude synaptosomal membranes (~100 µg of protein in KH buffer) containing increasing concentrations of unlabeled glycine (5–250µM) were preincubated for 5min at 30°C. Subsequently, 50µl [³H]glycine (0.1µM final concentration; 60Ci/mmol; Perkin-Elmer, Emeryville, CA) in KH buffer was added and uptake was terminated after 15min at 30°C by rapid vacuum filtration using a semiautomatic cell harvester (Skatron Instruments, Lier, Norway). The filters were washed with ice-cold 10mM Tris, pH7.4, 150mM NaCl, dried, and subjected to liquid scintillation counting. Non-specific [³H]glycine uptake was determined in the presence of 10mM glycine, and GlyT1 specific [³H]glycine uptake was measured in the presence of 10µM ALX5407 (Sigma-Aldrich). V_max values were determined by nonlinear regression using the GraphPad PRISM 4 software (GraphPad Software, San Diego, CA).

Electrophysiology

All experiments were performed under blind conditions, and genotypes were determined retrospectively. All subjects were male. There were a total of 13 slice preparations derived from 7 control mice, and a total of 8 slice preparations from 6 mutant mice included in the final analysis. Brains were taken from P21-P30 mice and prepared as described previously (Gabernet et al., 2005).
Whole cell patch-clamp techniques were used to study synaptic responses of CA1 pyramidal neurons in response to Schaffer collaterals stimulation delivered via a bipolar stimulating electrode (0.05ms, 1-10V, at 0.1Hz). Whole cell patch-clamp experiments were performed on CA1 pyramidal cells in parasagittal brain slices. After decapitation of the mice, brains were quickly removed and placed in aerated (95% O$_2$, 5% CO$_2$) artificial cerebrospinal fluid (aCSF) solution at 4°C. The aCSF solution was composed of (mM): 124 NaCl, 2.5 KCl, 1.3 MgCl$_2$, 26 NaHCO$_3$, 1.25 NaH$_2$PO$_4$, 2.5 CaCl$_2$, and 20 glucose. Between experiments the slices were maintained at room temperature of 24°C. Synaptic AMPA responses were evoked from CA1 pyramidal cells every ten seconds with a bipolar platinum electrode and recorded in the voltage clamp configuration with a 4-5 MΩ patch pipette at a holding potential of -60 mV. All experiments were done at room temperature. Baseline recordings were performed with 100mM picrotoxin in the aCSF. With picrotoxin in the recording solution, a surgical cut was made between CA1 and CA3 to prevent the propagation of epileptic discharges between the two subfields. After establishing a stable synaptic response, 20μM NBQX was added to the bath solution; recording then continued for an additional 3 to 4 minutes until the AMPA response was clearly abolished. Any non-AMPA residual current observed during this phase was averaged and subtracted from the averaged synaptic baseline response previously recorded to obtain the values of pure AMPA current for experimental analysis. The holding voltage of the cell was then shifted to +40mV to release the magnesium block of NMDA receptors and recording continued under the same external stimulus strength as before. Due to the tendency of the NMDA amplitude to decay after reaching a short stable plateau, only the maximal response was used in the subsequent analysis of NMDA/AMPA ratio.

**Behavioral assays**

Three separate cohorts of experimentally naïve mice were used for behavioral and cognitive phenotyping. The test sequence, duration of each test, number of rest days between tests, and number of subjects accepted in the final analyses of each test are all summarized in Table 1. The presentation of the behavioral data later will instead follow a logical sequence allowing us to highlight the functional relevance of the findings, and therefore does not necessarily adhere to their chronological sequence. All mice were 11-12 weeks old at the beginning of behavioral testing. They were kept under controlled conditions (21°C at relative humidity of 55%) and a 12/12h reversed light/dark cycle (lights on at 2000hrs). All tests were conducted in the dark phase. The mice were caged in groups of six or less in Macrolon Type-III cages (Techniplast, Milan, Italy), and maintained under ad libitum food and water unless stated otherwise.

All manipulations described here had been approved previously by the Cantonal Veterinary Office of Zurich; they conformed to the ethical standards required by the Swiss Act and Ordinance on Animal Protection and the European Council Directive 86/609/EEC. All behavioral data were subjected to parametric ANOVA of the appropriate design conducted using SPSS for Windows.
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Statistically significant outcomes were further evaluated by Fisher’s least significant difference (LSD) post hoc comparisons and restricted ANOVAs whenever appropriate.

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Sequence of Experiments</th>
<th>Duration</th>
<th>Post-test rest</th>
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Table 1

Elevated plus maze. Unconditioned fear and anxiety related behavior was first evaluated in the elevated plus maze when all animals were behaviorally naive. The maze consisted of two exposed and two enclosed arms extending from a central square platform. Its construction and dimensions have been fully described elsewhere (Yee et al., 2004). To begin the test, the mouse was gently placed on the central platform with its head facing one of the two opening arms. It was then allowed to explore freely for 5 min before being removed and returned to the home cage. A digital camera connected to a PC running the Ethovision tracking system (Noldus Technology Wageningen, The Netherlands) continuously tracked the animal at a rate of 5Hz. Two anxiety-related measures were computed: (a) percentage of time spent in the open arms = time in open arms / times in all arms \( \times 100\% \), and (b) percent number of entries into open arms = entries into open arms / all arm entries \( \times 100\% \). To index general motor activity level, the cumulative spatial displacement of the animal’s center of gravity was also computed.

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Hanging wire test. Neuromuscular strength was evaluated by the hanging wire test (Crawley, 1996). The apparatus was a rigid wired frame modified from a stainless steel cage top. The frame measured 24 × 29cm, consisted of parallel wires 1.5mm thick, spaced 9.5cm apart (center to center). The wires were exposed only through a central region of 13.7 × 16.5cm so that the mouse could not
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reach the edges of the frame. The frame was held by a test tube clamp, which in turn was firmly fixed to a metal support stand 40cm above the bench top in a quiet room. The clamp also allowed the frame to be rotated. A layer of sawdust bedding was positioned directly below the frame to allow a soft landing when the animals fell off. At first, the frame was in a horizontal position, and the mouse was placed gently on top of it. The wired frame was then rotated 180° with the mouse now hanging up-side down, and remained so until 5min had elapsed or when the animal fell down. The latency to fall was recorded, and a criterion of 5min was assigned to animals that did not fall.

Rotarod test. The rotarod test of motor coordination was conducted with a standard electrical accelerating rotarod apparatus for mice (Model 7650, Ugo Basile, Comerio, VA, Italy). To begin, the mouse was placed on the rotating drum that was turning at the baseline speed of 4rpm. During the 5-min observation period, the speed of rotation increased linearly to 40rpm. A trial ended when the mouse fell from the apparatus or when 5min had elapsed. The latency to fall from the rotating drum was recorded. Animals that did not fall within the time limit were assigned a criterion score of 5min. The animals were tested once per day for three consecutive days, thus further allowing the evaluation of motor skill learning.

Latent inhibition of conditioned freezing. The apparatus and procedures have been fully described before (Meyer et al., 2005; Yee et al., 2006). Briefly, there were 8 conditioning chambers equipped with a sonalert to deliver a 30s, 86dB_A tone serving as the conditioned stimulus (CS), and a metal grid floor via which a 1s, 0.25mA electric foot shock could be delivered and served as the unconditioned stimulus (US). A digital camera was mounted 30cm directly above the area of interest in each chamber. Successive images captured at 1s intervals were compared to allow the indexation of freezing according to the algorithm described by (Richmond et al., 1998). The pre-exposure and conditioning parameters here followed our previously established protocol (Yee et al., 2006), which tended to generate a weak latent inhibition (LI) effect in the control mice. This approach is commonly adopted to assess treatments (e.g., antipsychotic drugs) expected to enhance or potentiate LI (Feldon and Weiner, 1991). Briefly, the procedure comprised four phases: pre-exposure, conditioning, context test, and CS test. Mice of each genotype were randomly subdivided into two conditions: the pre-exposed (PE) and non-pre-exposed (nPE) conditions.

On the first day, PE subjects received 40 presentations of the tone CS at a variable inter-stimulus interval of 40±30s, while nPE subjects were confined to the chamber for an equivalent period of time without any discrete stimulus presentation. Next, the conditioning phase followed immediately without removing the animals from the chambers, and it consisted of three conditioning trials – each began with the tone CS (30s) followed immediately by the shock US (1s). Each trial was preceded and followed by a 180s interval in which no discrete stimuli were presented.

The test of contextual freezing took place 24h later when the subjects were returned to the
same chambers and observed for a period of 480s in the absence of any discrete stimulus. The test of conditioned response to the tone CS was conducted another 24h later, when the animals were returned to the same chambers once again. Following an initial acclimatization period lasting 90s, the tone CS was turned on for 480s.

Latent inhibition of conditioned active avoidance. Signaled conditioned avoidance involves elements of both classical and instrumental conditioning, in which the subjects learn to perform a specific operant act in response to a discrete signal to prevent or avoid the delivery of an aversive foot shock. The apparatus and procedures have been fully described before (Meyer et al., 2005; Yee et al., 2006). Briefly, four identical Coulbourn Instruments two-way shuttle boxes (model H10-11M-SC) were used. Each box comprised two identical compartments separated by a metal wall. An opening (6.5 × 8 cm) in the wall allowed the mouse to shuttle freely between the two compartments. Shuttles were detected by an array of infrared photo sensors positioned on the back wall of the shuttle box. Each box was equipped with a speaker to generate an 83dB white noise that served as the warning signal and a stainless steel grid floor capable of delivering an electric foot shock at 0.25mA. Again, the procedural parameters were identical to our previous study (Yee et al., 2006) that had enabled the detection of an LI-enhancing effect in forebrain neuron specific GlyT1 conditional knockout mice.

The animals were subdivided into PE and nPE conditions, with their PE/nPE experience in the previous (conditioned freezing) experiment counterbalanced. On the first day, after the animals were placed in the shuttle boxes: the PE subjects received 50 pre-exposures of the noise stimulus (5s, 83dB in magnitude against a background noise of 63dB) presented at a variable inter-stimulus-interval of 40±15s, and the nPE subjects spent an equivalent period of time in the chamber without any stimulus presentation. On the next day, the animals returned to the shuttle boxes and underwent one hundred conditioned avoidance trials administered at variable inter-trial intervals of 40±15s. A trial began with the onset of the noise stimulus. If the animal shuttled within 5s of stimulus onset, the noise stimulus was terminated and the trial ended without any shock delivery, and the animal had made a successful “avoidance response”. Avoidance failure led immediately to an electric foot shock presented in coincidence to the noise stimulus. During the noise-shock presentation, a shuttle response would terminate both stimuli and the trial ended with the subject scoring an “escape response”. The trial was ended with the termination of both noise and shock if the subjects failed to escape in 2s, and an “escape failure” was scored. Conditioned avoidance learning was indexed by the number of avoidance response across successive 10-trial blocks. Avoidance and escape responses were separately analyzed.

Latent inhibition of conditioned taste aversion. Conditioned taste aversion is a one-trial classical conditioning paradigm, in which a single pairing of a taste CS and gastric malaise leads to a long lasting aversion to the taste. Pre-exposure to the taste prior to conditioning would retard the generation of the subsequent conditioned taste aversion response, thereby constituting another method
to demonstrate the LI effect. We employed here the minimal number of pre-exposure, which was expected to yield a marginal LI effect in the controls. Here, 10% (w/v) D-sucrose solution served as the taste CS, and gastric malaise induced by an intraperitoneal injection of lithium chloride (LiCl) solution served as the US.

Throughout the experiment, the animals were housed singly in Macrolon cages (1291H, Euro standard type III, Techniplast S.p.a., Milan, Italy) measuring 425 × 266 × 185 mm. After 2 days of habituation to single housing, access to water was gradually restricted over a 5d period as described before (Meyer et al., 2004). On the 5th day, the water restriction was reduced to 1 h. Thereafter, and until the end of experiment, the animals were allowed two 30min-drinking periods per day, 4h apart. Two drinking tubes were inserted into the cage in each of the two drinking periods, and the animals could freely consume liquid from either tube as described before (Yee et al., 2006). Manipulations of the content of the drinking tubes were always conducted in the first drinking period of a day. In the second period, both tubes were always filled with plain tap water.

The experimental procedure consisted of four phases: baseline, pre-exposure, conditioning, and test. Baseline: Over the first 3d, both tubes contained water in both drinking periods to acclimatize the animals to drinking from the water tubes. The allocation of subjects into PE and nPE conditions was counterbalanced according to the animals’ performance in the first drinking period across the three baseline days. Pre-exposure: On the next day, PE subjects were provided with 10%(w/v) D-sucrose solution in both tubes during the first drinking period, whereas nPE subjects had access to water only in both tubes. Conditioning: On the next day, all subjects were provided with sucrose solution in both tubes during the first drinking period, followed 5min later by an intraperitoneal injection of 0.25M LiCl at 2% v/w of the bodyweight. Test: On the next day, conditioned taste aversion to the sucrose solution was measured in a two-choice test in which one tube contained sucrose solution and the other water, thus allowing the animals a free choice between the two.

Water maze. The water maze consisted of a white circular tank made of fiberglass (diameter 102 cm, height 36 cm) filled with water (at 24±1°C) to a depth of 19 cm. Fresh tap water was used on each testing day. A transparent Plexiglas cylinder (diameter 7 cm, height 18.5 cm) was used as the escape platform. It was submerged 0.5 cm below the surface of the water, remaining invisible to the animals. It could be made visible by mounting a white circular disk (diameter 12 cm), 15 cm directly above the platform. A digital camera was installed above the water maze, capturing images at a rate of 5 Hz and transmitting them to a PC running the Ethovision (Noldus Technology) tracking system. Ethovision computed the escape latency, distance traveled (i.e., path length), and average swim speed on each trial, and the additional dependent measures on probe tests in the reference memory task (see below). The water maze could be positioned in the center of one of two possible well-illuminated testing rooms (referred to as Room 1 and Room 2), each enriched with unique distal spatial cues. The pre-training phase and the working memory experiment were conducted in Room 1, and the reference
memory experiment in Room 2.

**n Cued Test (Day 1):** On the first day, the animals were pre-trained using a cued (i.e., visible) platform located in the centre of the maze in order to familiarize them to the apparatus and to swimming in the pool. It further served as a test of any non-specific sensory and motor disturbance. The platform was positioned in the center of the maze. Each animal underwent two consecutive trials. The starting position varied randomly among four possible release points (N, E, S, and W). To begin a trial, the subject was gently released from the start point, facing the wall of the maze. The animals were allowed to locate the escape platform within 60s. Upon reaching the platform they spent an inter-trial interval (ITI) of 15s on it before the second trial commenced. If an animal failed to locate the platform within the time limit of 60s, it was guided to the platform by the experimenter and allowed to stay on it for 15s, and a maximal escape latency of 60s was scored.

**n Working Memory (Days 2-28):** The working memory task was based on the matching-to-sample paradigm, in which the animals were required to learn the novel position of the platform revealed to them on trial 1 of each day in order to navigate effectively to the same location (i.e., matching) on subsequent trials on the same day (Hodges et al., 1995). The daily change of platform location ensured that the solution on a given day was irrelevant for the next day, thus taxing the flexible use of (short-term) working memory (Honig, 1978). The procedures were similar to the cued test, except that the platform was now hidden from the animals’ view and assumed a novel location on each day, but remained in that position from trial 1 to 2. In all trials, the animals were allowed a maximum of 60s to locate the platform, upon then it would be guided to the platform by the experimenter.

To manipulate the retention demand of the task in the temporal domain, the inter-trial interval (ITI) between trials 1 and 2 was also varied. First, the minimal ITI of 15s was employed – this referred to the time animals spent on the platform at the end of trial 1 to the beginning of trial 2. This lasted for six days. Following a break of one day, the ITI was increased to 10min over the next six days. This was followed by another six days with an ITI of 15min, and then a final six days with again an ITI of 10 min. During the extended ITIs of 10 or 15min, the animals spent the first 15s on the platform, and the remaining time in an opaque waiting box located in the testing room.

The six platform locations used in the first six days with the minimal ITI were: 35 cm off center in the NE, SW and NW directions, and 15 cm off center in the N, E and S directions. Across subsequent days, a total of 16 platform locations were defined. These were located at either 15 cm or 35 cm away from the center in the following eight directions: N, E, S, W, NE, NW, SE and SW directions. All positions were used once only in every 16 trials, and then a new random sequence was generated. The start positions also varied between trails among eight possible release points alone the maze wall: N, E, S, W, NE, NW, SE and SW. Each animal followed its own unique random non-repetitive sequence; and a new sequence was generated for every block of 8 trials.

**n Reference memory learning and its reversal (Days 33-46):** To assess long-term spatial
reference memory, the animals were next trained with a procedure in which the platform remained fixed across trials and across training days. Reference memory learning was conducted in another testing room, and commenced five days following the working memory test. The initial acquisition lasted for 8 days, with 4 trials per day and an ITI of 15s. The escape platform was positioned 25 cm from the maze center in one of the four quadrants – the target quadrant. Assignment of the target quadrant was counterbalanced, so that all four quadrants were used in each group. The start position varied among eight possible release points (N, E, S, W, NE, NW, SE and SW), and was determined as described above. A probe test was conducted prior to reference memory training on the eighth acquisition day. During a probe test, the platform was removed and the animals were allowed to swim freely in the maze for 45s after being released from the point opposite to the target quadrant. The time and distance spent in each of the four quadrants were subjected to analysis for search accuracy. Reversal testing began on the next day as described above except that the platform was moved to the opposite quadrant. Following 4 days of reversal learning, another probe test was performed on the following day as described above.

**Object recognition memory.** The apparatus consisted of a rectangular arena measuring 40 × 61 cm and was surrounded by 22 cm high transparent Plexiglas walls (Singer et al., 2007). An outer perimeter wall of white opaque Plexiglas was positioned 15 cm around the entire arena to minimize interference by extra-maze cues. The arena was divided into three equal compartments by the addition of two partition walls. Each wall had a circular opening (5 cm in diameter) in the middle, positioned at 1 cm above the floor of the arena providing access to the side compartments from the central compartment. The circular opening could be blocked by a Plexiglas sliding door. A collection of distinct three-dimensional objects (in triplicates) differing in form, size, texture, materials and smell served as trial-unique discriminanda. A digital camera was mounted directly above the arena. Video records of all sessions were stored for subsequent data extraction. Prior to object recognition tests, the animals were first habituated to the arena for two days as described by Singer et al. (2007).

An object recognition trial comprised a sample phase and a test phase. To begin the mouse was placed in the middle compartment and two copies of the to-be-familiarized objects (A1 and A2) were positioned in the middle of the two side compartments. First, access to one compartment was closed, and the animal was allowed to explore object A1 (in the center of the accessible compartment) for 5min. Next, the animal was again placed in the central compartment, and it was allowed to explore object A2 (located in the center of the other side compartment) for 5 min by closing the door to the compartment containing object A1. This completed the sample phase and the animals was removed from the arena and kept in a holding cage for the retention interval (2min or 2h), except for retention of 1d when the animals were return to the home cage. Before commencement of the choice phase, the arena was cleansed with 50% ethanol and dried. In the test phase, access to both compartments was available. One compartment contained a third copy of the sample object (A3), and the other a novel
object (B). The relative placement of the two objects was counterbalanced across subjects at each retention condition. The animal was allowed to explore freely for 5 min.

Only one trial was performed in a day. A total of six object trials were run, spanning across a period of 13 days, with a 24 h test-free period separating consecutive trials. On days 1 and 3, the retention interval was 2 min. On days 5 and 7, the retention interval was 2 h. On days 9-10 and 12-13, the retention interval was 1 d. All trials were conducted in the same testing room, and a fresh set of objects was used for each trial.

Object exploration was collected manually by an experimenter who was blind to all conditions (grouping and object identities) with the assistance of Noldus Observer®. Object exploration was defined as active interaction of the animal (e.g., sniffing, gnawing) with the object at a distance of less than 1 cm (Singer et al., 2007). Object recognition was not scored when an animal made a contact with the objects without facing it, or when it climbed on top of the objects. Data collected from the sample and test phases were separately analyzed. In addition, a discrimination ratio based on exploration time was calculated: \((\text{novel} - \text{sample})/(\text{novel} + \text{sample})\), to index the preferential exploration of the novel objects. Data across two trials of the same retention condition were averaged before being submitted to statistical analysis.

**Locomotor response to systemic amphetamine and phencyclidine challenge.** Motor activity was measured using four white open fields measuring \((40 \times 40 \text{ cm})\) as described before (Hauser et al., 2005). A digital camera mounted above the open fields captured images at a rate of 5 Hz and transmitted them to a PC running the Ethovision tracking system (Noldus Technology), which calculated a mobility score defined as cumulative displacement of the animal’s center of gravity in successive 10-min bins. First, the response to phencyclidine (PCP) was tested using a between subject design: the mice were injected with 5 mg/kg PCP or vehicle saline solution (0.9% NaCl) via the intraperitoneal route (at an injection volume of 5 ml/kg) immediately before being put inside the open field, and observed for 1 h. Following one week of washout period, the reaction to systemic amphetamine was assessed in the same animals, with previous drug or vehicle experience counterbalanced. Again, animals were injected with amphetamine (2.5 mg/kg, i.p.) or vehicle saline solution (0.9% NaCl) immediately before the test, and were observed for an extended period of 90 min.

**RESULTS**

**Deficient GlyT1- but not NMDAR-expression in the forebrain of EMX/GlyT1-KO mice leads to reduced glycine-re-uptake**

To determine the consequences of a forebrain-specific GlyT1 disruption on GlyT1 protein expression, crude synaptosomal membranes, prepared from the hippocampus and cortex of 8-week old male mutant and control mice, were analyzed by Western blotting using GlyT1-selective antibodies. The
expression of GlyT1 protein levels in mutant mice was substantially reduced. At high protein concentrations, faint GlyT1 immunoreactivity was detectable in mutant mice, whereas samples from control mice showed intense signals (Fig. 1A). Quantification of the blots to the GlyT1 protein signal intensity at 20 micrograms in controls (100%) revealed a 79±2% (±SEM, n=4) reduction of GlyT1 protein levels in mutant as compared to control mice (Fig. 1B). This confirms the efficacy of the mutation to significantly reduce GlyT1 protein expression in the hippocampus/cortex of mutant mice. The functional consequence of the reduced GlyT1 protein levels was assessed in [3H]glycine uptake studies. As expected from the strongly diminished protein levels, GlyT1-specific [3H]glycine uptake was likewise reduced by 77±1% in mutant mice (Fig. 1D; control: V_max = 79±22 pmol/min/mg protein, mutant: V_max = 18±0.7 pmol/min/mg prot.; ±SEM, n=3). These results indicate that GlyT1 protein levels and GlyT1-specific glycine uptake were affected to a similar extent in mutant mice. To rule out that the reduced GlyT1-activity affects NMDAR protein expression, the membrane preparations were probed with NMDA-R1 specific antibodies to quantify NMDAR protein levels. Western blot analysis confirmed that the level of NMDAR expression (Fig. 1C) was not affected by the reduction of GlyT1.

![Figure 1. GlyT1 and NMDA-N1 protein expression and [3H]glycine uptake in the forebrain of mutant and control mice. GlyT1 and NMDA-R1 protein expressions were analyzed by Western blotting using increasing amounts of hippocampus/cortex membrane protein and GlyT1 as well as NMDA-R1 specific antibodies. A representative blot simultaneously probed for GlyT1 and NMDA-R1 immunoreactivity is depicted in A. Expression of GlyT1, but NMDA-R1, was strongly reduced in the mutant mice. Quantification of the Western blots normalized to the GlyT1 (B) and NMDA-R1 (C) protein signal intensity at 20 micrograms in controls (100%). Data represent the mean +/- SD of four independent experiments. GlyT1-specific glycine transport into crude synaptosomal membranes prepared from hippocampus/cortex tissue of wild type and mutant mice was determined by measuring ALX5407-specific [3H]-glycine uptake at increasing glycine concentrations (D). GlyT1-specific glycine uptake was markedly reduced in mutant mice. Data represent the mean +/- SEM of three independent experiments.]
The reduction in GlyT1-mediated glycine reuptake in EMX/GlyT1-KO mice does not affect NMDAR-mediated neurotransmission

Reduced GlyT1 specific re-uptake of glycine is expected to increase extra-cellular glycine levels at glutamatergic synapses, and therefore NMDAR function may be affected. The efficacy of NMDAR-mediated neurotransmission was indexed by the ratio of evoked NMDAR to AMPAR-mediated excitatory postsynaptic currents (eEPSCs) in parasagittal brain slices on CA1 pyramidal cells of 6 mutants and 7 control male mice (postnatal day 17-20). The average peak amplitude of evoked AMPAR and NMDAR-mediated eEPSCs did not differ significantly between mutants and controls (Fig. 2A-2B). Likewise, the ratio of the peak amplitudes of NMDAR to AMPAR-mediated EPSCs based on the obtained EPSCs was comparable between the two groups of mice (mutants=0.64±0.09; controls=0.56±0.08) indicating that the mutation did not significantly alter NMDAR mediated neurotransmission.

The motor stimulant effect of phencyclidine, but not of amphetamine, is attenuated in EMX/GlyT1-KO mice

The mutation led to a substantial reduction in forebrain GlyT1 expression and markedly reduced glycine re-uptake, which is assumed to increase the extra-cellular glycine levels. The extent to which this would alter behavioral control directly linked to NMDARs was assessed via selective pharmacological blockade of NMDAR by an acute challenge with the non-competitive NMDAR antagonist phencyclidine (PCP).

As illustrated in Fig. 3A, systemic PCP (5mg/kg, i.p.) injection led to an immediate increase in activity in control mice over 3-fold of that seen following saline injection, which subsided by the end of the 60-min observation period. The motor stimulant effect of PCP was, however, substantially
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reduced in the mutant mice. This drug-induced phenotype was observed in both male and female mutants. Furthermore, female generally were more active than male mice (data not shown). A $2 \times 2 \times 2 \times 6$ (genotype $\times$ sex $\times$ drug $\times$ 10-min bins) ANOVA of distance moved per bin confirmed the above impressions in revealing a significant main effect of sex [$F(1,28)=20.87$, $p<0.001$] and a highly significant genotype $\times$ drug $\times$ bins interaction [$F(5,140)=4.79$, $p<0.001$]. The latter interaction indicated that the drug’s effect on locomotor activity over time in comparison to saline vehicle injection significantly differed between mutant and control groups. This three-way interaction was further accompanied by a highly significant effect of drug [$F(1,28)=22.54$, $p<0.001$], bins [$F(5,140)=7.61$, $p<0.001$], and their interaction [$F(5,140)=9.48$, $p<0.001$]. Further analyses restricted to either injection condition confirmed that mutant and control mice did not significantly differ under saline condition: neither the genotype effect nor its interaction was close to significance. In contrast, the analysis restricted to the PCP condition yielded a clear genotype by bins interaction [$F(5,70)=6.80$, $p<0.005$], which was further accompanied by a near-significant main effect of genotype [$F(1.14)=3.95$, $p=0.067$] indicating that the two genotypes only differed in the PCP condition. This clearly demonstrates that the EMX/GlyT1-KO was behaviorally effective against blockade of NMDAR achieved by PCP. This drug-induced phenotype in the EMX/GlyT1-KO mice also resembles what has been reported in the more restricted CamKII/GlyT1-KO mice, which lack GlyT1 specifically in forebrain neurons (Yee et al., 2006).

**Figure 3.** The locomotor response to systemic phencyclidine (A) and amphetamine (B). Locomotor activity was measured by distance moved of the animal’s distance travelled in meter and summed into successive 10 min bins. (A) The motor stimulant effect of PCP was notably attenuated in mutants relative to controls in the first 30 min. Locomotor activity was comparable in mutants and controls after saline injection. Data gave rise to a significant Bins x Genotype x Drug interaction in a three-way ANOVA of the distance traveled per bin ($p<0.05$). (B) The mutation did not alter the motor stimulant effect of amphetamine. Similarly, mutants and controls did not differ in the saline condition. * Denotes that the distance traveled was significantly less ($p<0.05$) in the saline condition as compared to the PCP (A) and the amphetamine (B) condition, respectively All values refer to mean +/- SEM.
PCP and other NMDAR blockers are well-known psychomimetic drugs, because such compounds can lead to the emergence of psychotic like behaviour in humans (Farber, 2003). To assess the possibility that the antagonism of PCP induced activity by EMX/GlyT1-KO might be generalized to other psychomimetic drugs, we went on to evaluate the animals’ response to amphetamine (2.5mg/kg, i.p.), which is a dopamine releaser. As shown in Fig. 3B, amphetamine led to a drastic increase in locomotor activity as expected, reaching a peak at 30min into the test. By the end of the 90-min observation period, the motor stimulant effect had largely subsided. This profile of drug action was highly comparable between groups. This impression was supported by a $2 \times 2 \times 2 \times 9$ (genotype $\times$ sex $\times$ drug $\times$ 10-min bins) ANOVA of distance moved per bin, yielding a main effect of drug $F(1,28)=79.96, p<0.001$ and its interaction with bins $[F(8,224)=32.53, p<0.001]$. Similar to the PCP experiment, a main effect of sex emerged $[F(1,28)=10.10, p<0.005]$, because female mice again were generally more active than male (data not shown). There was no statistical evidence for any difference between genotype. This absence of an effect somewhat contrasted with an effect of forebrain neuronal GlyT1 knockout which delayed the peak response to amphetamine challenge (Yee et al., 2006).

Although the animals used in test of amphetamine-induced hyperactivity here were not drug naïve, we have since repeated the experiment with completely naïve animals, and have obtained the same null effect in the EMX/GlyT1-KO mice (data not shown).

**EMX/GlyT1-KO mice show a delay-dependent enhancement in object recognition memory**

To investigate the promnesic potential of the mutation on memory systems sensitive to extra-hippocampal brain structures we assessed spontaneous object recognition memory, which critically involves the contribution of rhinal cortices (Davachi and Goldman-Rakic, 2001; Bartko et al., 2007). Singer et al. (2007) have shown that GlyT1 deletion restricted to forebrain neurons resulted in an enhancement of object recognition memory when the delay was sufficiently long such that control mice fell to chance performance. Here, we adopted a similar test design to assess the EMX/GlyT1-KO mice. Each test of object recognition comprised a sample phase and a test phase, and they were separately analyzed.

**Sample phase:** The amount of exploration directed towards the sample objects in the familiarization phase constitutes a critical variable as it is expected to influence the animals’ familiarity judgment in the test phase (see Singer et al., 2007). It is therefore imperative to examine whether the mutation alters exploration behavior as such. It was found that mutant and control mice spent a similar amount of time exploring the sample objects in the sample phase across the different delay conditions. However, male mice tended to spend more time in sample exploration than female mice. This was in line with a $2 \times 2 \times 3$ (genotype $\times$ sex $\times$ delays) ANOVA of object exploration time per sample run yielding only a significant main effect of sex $[F(1,30)=25.14, p<0.001]$. The mean exploration time (in sec, ±SEM) per sample run collapsed across all delay conditions was: ♂ control=$21.44\pm2.49$, ♂ mutant=$23.43\pm2.87$; ♀ control=$8.64\pm1.16$, ♀ mutant=$11.21\pm1.73$. From this it
follows that a potential effect of the mutation on object recognition memory cannot be attributed to changes in the object familiarization phase.

**Figure 4.** Object recognition memory. Object recognition memory was assessed at three delay conditions (2min, 2h, 1day) and was indexed by the differential ratio defined as difference in exploration time towards the novel and the familiar objects divided by the total object exploration time. A positive differential ratio therefore denotes an explorative preference towards the novel over the familiar object. Mutant mice showed an improved object recognition memory performance relative to controls at the 2h delay condition. This impression was confirmed by the occurrence of a significant main effect of genotype and of its interaction with delays (all p’s<0.05) in a Genotype x Sex x Delays ANOVA. Moreover, separated ANOVA’s restricted to each delay revealed significant difference between mutant and controls on the 2h delay condition. * denotes a significant difference from control mice performance. # denotes that the differential ratio significantly differs from zero.

**Test phase:** The object recognition test relies on the natural tendency of rodents to explore novel elements in their environment. In the test phase, preferential exploration of the novel over the familiar object was taken as a measure of object recognition, and its magnitude was indexed by a discrimination ratio [time of novel object exploration in excess of the familiar object / total time of object exploration]. Indifference corresponds to a value of 0. As illustrated in Fig. 4, both mutants and controls exhibited a positive and comparable preference towards the novel object when the delay from sample to test phase was 2min. With increasing delay to 2h, a preference for the novel object remained detectable in the mutant mice but not in the controls. At 24h delay, both groups were essentially indifferent between the novel and familiar objects. Despite the sex difference observed in the sample phase, sex did not affect performance in the test phase, and the mutation’s effect on object recognition was similarly observed in both sexes. The above impressions were supported by the emergence of a significant main effect of genotype [F(1,30)=4.32, p<0.05] and delays [F(2,60)=6.20, p<0.005] as well as their interaction [F(2,60)=6.93 p<0.005] from a 2 x 2 x 3 (genotype x sex x delays) ANOVA of the discrimination ratio. Further analyses restricted to each delay condition indicated that mutant and control mice differed significantly at 2h delay [F(1,30)=17.30, p<0.001], but not in the shorter or longer delay [Fs<1]. The delay-dependency of this phenotype is suggestive of a specific enhancement in memory retention, and it is in line with what we had previously observed in mice with forebrain neuronal GlyT1 knockout mice (Singer et al., 2007). This common phenotype therefore appears to be independent of the unique difference in cell-type selectivity of GlyT1 knockout between EMX/GlyT1-KO and CamKII/GlyT1-KO mice.
EMX/GlyT1-KO mice exhibited enhanced spatial working memory in the water maze

Spatial working memory is sensitive to a number of glutamatergic interventions within forebrain structures including hippocampus and prefrontal cortex (Jones 2002) and is therefore highly relevant regarding the anticipated role of GlyT1 in NMDAR mediated processes. Previous attempts to assess working memory function in mice with selective forebrain neuronal GlyT1 deletion did not yield any evidence for an enhancing effect (Singer et al., submitted). Here, a similar task design was adopted to compare Emx1Cre:GlyT1tm1.2fl/fl mice with littermate controls. Only male mice were used due to excessive floating exhibited by female mice.

Cued (visible platform) task: All animals acquired the swimming response and learned to escape onto the platform. Performance in both latency and distance to escape was comparable between groups. Parallel 2 × 2 (genotype × trials) ANOVA of the escape latency and the path length yielded only a significant effect of trials [escape latency: F(1,16)=17.18, p=0.001; path length: F(1,16)=24.20, p<0.001]. The mean (±SEM) escape latency across the two trials was as follows: control: trial 1 = 44.3±7.9s, trial 2 = 22.6± 6.4s, and mutant: trial 1 = 40.9±7.1s, trial 2 = 18.2±4.6s. Separate analysis of swim speed did not reveal any effect of genotype or its interaction.

Working memory: First, to assess whether the two groups of mice were able to learn the matching rule underlying the working memory task, working memory was assessed under minimal retention demand with a 15s ITI separating trial 1 and 2. Escape latency and path length were averaged across the six test days and subjected to separate 2 × 2 (genotype × trials) ANOVAs. Mutant and control mice showed comparable improvement form trial 1 to 2, suggesting that moth groups had acquired the matching rule underlying this spatial working memory task (Fig. 5A) and were able to reach the platform effectively on trial 2 based on information acquired on trial 1. This impression was supported by the emergence of a significant main effect of trials in both measures [escape latency: F(1,16)=23.68, p<0.001; path length: F(1,16)=13.86, p<0.005]. Neither the effect of genotype nor its interaction with trials reached statistical significance, in spite of the faster swim speed of mutant mice [F(1,16)=6.02, p<0.05]. The mean swim speed (in cm/s) was as follows (±SEM): control=16.8±1.0, mutant=19.5±0.5.

Next, the temporal retention demand was increased by extending the ITI between trials 1 and 2. Over 18 days, the ITI was extended to 10min in the first block of 6 days, then to 15min in the next block, and returned to 10min in the final blocks.

The average escape latency and path length on trials 1 and 2 were first averaged across days (within each block of training) before submitting to separate 2 × 2 × 3 (genotype × trials × blocks) ANOVAs. These yielded no statistical support for a difference between successive blocks of different trials 1-to-2 delays (10-min vs 15-min vs 10-min). As illustrated in Fig. 5B, the improvement from trials 1 to 2 trials (collapsed over blocks) was more prominent in the mutant mice than controls in both performance measures. This gave rise to a significant main effect of trials [escape latency: F(1,16)=9.80, p<0.01; path length: F(1,16)=16.38, p<0.001] and its interaction with genotype [escape
latency: $F(1,16)=12.33$, $p<0.005$; path length: $F(1,16)=12.07$, $p<0.005$. Additional analyses restricted to either mutant or control groups confirmed the presence of a significant trials effect in the mutant mice [escape latency: $F(1,9)=22.64$, $p<0.005$; path length: $F(1,9)=25.91$, $p<0.005$], but not in the control group [$F$s<1].

Figure 5. Spatial working memory with variable delays in the Morris water maze. A, at the minimal delay, both groups of mice showed a comparable decrease in both the escape latency and the path length from trial 1 to trial 2 (*, $p<0.05$) reflecting the presence of working memory in mutants and controls. B, under extended delay conditions, the working memory performance was clearly improved in mutant mice relative to the controls. This was statistically supported by the emergence of a significant interaction between genotype and trials in $2 \times 2 \times 3$ (Genotype x Trial x Delay) ANOVA of the escape latency as well as path length ($p$'s <0.05). Separate analysis of mutant and controls confirmed the presence of working memory in mutants but not in controls. # refers to a significant difference ($p<0.05$) between mutants and controls. All values refer to mean +/- SEM.

Separate analysis of swim speed again suggested that mutant mice were swimming marginally faster [$F(1,16)=3.98$, $p=0.06$]. The mean (±SEM) swim speed (in cm/s) across the three blocks was: control=15.8±1.2, mutant=18.2±0.5. An additional analysis of covariance (ANCOVA) with swim speed as covariate was therefore performed to assess the impact of the statistical outcome in escape latency described above. The critical genotype × trials interaction remained significant [$F(1,15)=7.28$, $p<0.05$], and the effect of covariate did not reach statistical significance [$F(1,15)=2.86$, $p=0.11$]. The possibility that the mutation’s effect on working memory performance stems solely from an increase in swim speed is therefore highly unlikely, especially when the results of path length closely conformed to the results based on escape latency.

**EMX/GlyT1-KO mice display normal acquisition of spatial reference memory and reversal learning**

Next, the animals were evaluated on the spatial reference memory version of the Morris water maze, which represents another paradigm highly sensitive to manipulations targeting NMDARs especially in the hippocampal formation (McHugh et al., 1996; Tsien et al., 1996; Tonegawa et al., 2003). Unlike the working memory procedure, the location of the platform now remained constant across days as well as across the 4 trials within a day. The relative constant location of the platform allows the use of long-term reference memory to guide navigation to reach the escape platform.

First, acquisition developed progressively over the eight days of training as indicated by a reduction in escape latency and path length as a function of days (Fig. 6A-6B). The acquisition rate was similar between mutant and control mice. Within a day, the performance generally improved
across trials. Separate $2 \times 8 \times 4$ (genotype $\times$ days $\times$ trials) ANOVAs of the escape latency and path length both yielded a highly significant effect of days [escape latency: $F(7,112)=6.95$, $p<0.001$; path length: $F(7,112)=8.42$, $p<0.001$] and trials [escape latency: $F(3,48)=4.98$, $p<0.005$; path length: $F(3,48)=4.35$, $p<0.01$]. Consistent with the impression above, neither the main effect of genotype nor its interactions attained statistical significance. Moreover, mutant and control mice no longer differed in swim speed [$F(1,16)=1.82$, $p=0.196$]. The mean ($\pm$SEM) swim speed (cm/s) was: control=$15.4\pm1.2$, mutant=$18.2\pm1.7$.

### Figure 6.

Spatial reference memory and reversal learning. The mutation did not affect the reference memory learning in the initial acquisition session when the level of pro-active interference is expected to be minimal. This was equally seen in terms of escape latency (A) and path length (B). Similarly, mutants and control animals re-learned the novel platform position at a similar rate in the second acquisition session when the animals were under the influence of proactive interference from the preceding reference memory session. Retention of reference memory was analyzed by the search preference for the trained quadrant in two probe tests, respectively. Probe test 1 (C) was conducted on day 8 prior to the acquisition training and probe test 2 (D) was conducted 24h after acquisition training on day 12. Both mutants and controls preferably searched in the trained quadrant in both probe tests suggesting that the mutation did not affect memory retention. The asterisk denotes that a significant difference from chance level according to Student’s $t$-test ($p<0.05$). All values refer to mean $\pm$SEM.

Two days after the end of acquisition training, reversal learning commenced. The training was identical except that the platform was moved to the diagonally opposite quadrant. The animals therefore were required to approach where they had previously learned to avoid, and vice versa. At the beginning of the reversal phase, performance was drastically reduced in all animals (Fig. 6A-6B). However, they quickly adapted to the change and by the fourth day had achieved a rapid escape to the
platform. There was no apparent difference between groups as indicated by separate 2 × 4 × 4 (genotype × days × trials) ANOVAs of the escape latency and path length, which yielded only a main effect of days [escape latency: F(3,48)=18.34, p<0.001; path length: F(3,48)=17.83, p<0.001]. Furthermore, the animals’ performance generally improved over trials [escape latency: F(3,48)=8.14, p=0.001; path length: F(3,48)=8.06, p<0.001]. No significant difference in swim speed was observed [F<1]. The mean (±SEM) swim speed (cm/s) was: control=16.5±1.4, mutant=17.8±1.0.

Two probe tests were conducted: 24h after acquisition training, and likewise after reversal learning. These allowed an evaluation of spatial search in the absence of the platform. The results were consistent with the above analyses that no difference between groups was apparent. As illustrated in Fig. 6C, both mutant and control mice showed a clear bias in their search in the target quadrant, well above the chance level performance, in the probe test following acquisition training. At the same time, they also avoided in particular the opposite quadrant. A 2 × 4 (genotype × quadrants) ANOVA of percent time spent per quadrant of the first probe test yielded only a highly significant effect of quadrants [F(3,48)=27.79, p<0.001], and similarly so when percent path length per quadrant was analyzed [F(3,48)=27.03, p<0.001] (data not shown). In the second probe test, the spatial distribution of search behavior was again highly similar between groups (Fig. 6D). However, the bias towards the new target quadrant was less pronounced, and there was not a tendency to avoid the opposite (former target) quadrant. Again, the analysis yielded a highly significant quadrants effect [percent time per quadrant: F(3,48)=10.97 p<0.001; percent path length per quadrant: F(3,48)=11.89, p<0.001].

These results suggested that the mutation affected neither the acquisition nor the retention of spatial reference memory; this lack of an effect is consistent with our previous experiment with forebrain neuronal GlyT1 knockout (Singer et al., submitted). On the other hand, the lack of a phenotype of the present mutation on reversal learning when behavior was influenced by conflicting information contrasted with the observation that forebrain neuronal GlyT1 knockout enhanced reversal learning (Singer et al., submitted).

**Lack of an effect on associative learning and failure to potentiate latent inhibition in EMX/GlyT1-KO mice**

Our previous experiments showed that mice with conditional forebrain neuronal specific GlyT1 knockout consistently showed enhanced associative learning and exhibited latent inhibition (LI) under conditions insufficient to induce LI in control mice (Yee et al., 2006). LI refers to the negative impact of non-reinforced pre-exposure of the conditioned stimulus (CS) on subsequent learning about the predictive significance of the same CS when it is paired with an unconditioned stimulus (US) (Lubow and Moore, 1959). LI is a demonstration of selective learning; it is considered to depend on selective attentional processes sensitive to the associative history of CSs (Mackintosh, 1973; Lubow 1989). Here, we adopted the same three associative paradigms and identical test parameters as before (Yee et al., 2006) to evaluate whether a global defect of GlyT1 in forebrain would similarly result in the
potentiation of LI. To this end, the parameters selected for the first two tests tended to yield relatively weak LI in controls.

Figure 7. Latent inhibition in the conditioned freezing paradigm. A, Expression of freezing behavior toward the tone-CS across the three conditioning trials immediately after pre-exposure. The amount of freezing was significantly reduced (p<0.05) in pre-exposed (PE) relative to non-pre-exposed (nPE) animals reflecting the LI effect. B, Freezing to the context 24h after conditioning expressed as a function of 1 min bins on the left and as the overall means on the right. C, Freezing to the tone CS 48h after conditioning. Freezing in the PE subjects tended to be lower than in the nPE subjects, which was more pronounced in the beginning of the CS-phase, which was equally seen in mutants and controls. This impression was confirmed by the appearance of a significant pre-exposure by bins interaction (p<0.05) in a 2 x 10 (pre-exposure x 1-min bins) ANOVA of the percent freezing. The histogram on the right illustrates the mean levels of freezing averaged across the entire 480s CS period. All values refer to mean ± SEM.

Conditioned Freezing: First, associative learning was assessed using the conditioned freezing paradigm with or without prior CS pre-exposure. Conditioning took place immediately following CS pre-exposure (PE subjects) or context pre-exposure (nPE subjects). Over the three trials of tone-shock pairings, an increase in freezing to the CS tone was observed in all groups (Fig. 7A). However, the rate of increase was retarded in the PE subjects relative to the nPE subjects, constituting the LI effect. This was similarly seen in both mutant and control mice. These impressions were confirmed by a 2 x 2 x 2 x 3 (genotype x pre-exposure x sex x trials) ANOVA of percent time freezing which yielded a significant effect of trials [F(2,64)=37.36, p<0.001] and of pre-exposure [F(1,31)=5.10, p<0.05]. No other effect attained statistical significance. Next, the conditioned freezing developed to the context was assessed by returning the animals to the conditioning chamber 24h following tone-shock pairing. As illustrated in Fig. 7B, the expression of conditioned freezing over the 480s test period was comparable across groups. A 2 x 2 x 2 x 8 (genotype x pre-exposure x sex x 1-min bins) ANOVA of percent time freezing yielded only a significant main effect of bins [F(7,224)=3.44, p<0.01]. Another 24h later, conditioned freezing to the CS was evaluated. Ninety seconds after the animals returned to the conditioning chamber, the CS was turned on continuously for 480s and the expression of freezing in response to the CS was examined (see Fig. 7C). In both mutant and control mice, nPE subjects exhibited somewhat higher levels of freezing than PE subjects. This difference diminished over time.
as the overall levels of freezing gradually reduced due to extinction. A 4-way 2 x 2 x 2 x 8 (genotype \times pre-exposure \times sex \times 1-min bins) ANOVA of percent time freezing per bins yielded a significant main effect of bins \([F(7,224)=33.67, \ p<0.001]\) and a significant pre-exposure by bins interaction \([F(7,224)=3.52, \ p<0.05]\). Restricted analyses indicated that the presence of a main pre-exposure effect only in the first bin \([F(1,32)=15.94, \ p<0.001]\). There was no statistical evidence for any difference between groups – either in the magnitude of conditioned freezing or the expression of the LI effect.

**Conditioned active avoidance:** The second test of associative learning was conditioned two-way active avoidance and again the effect of CS pre-exposure was examined. In the pre-exposure phase, PE subjects exhibited somewhat less spontaneous shuttles than nPE subjects in both mutant and control mice \([PE: \ control=43.3\pm5.4, \ mutant=47.6\pm9.8; \ nPE: \ control=57.5\pm8.0, \ mutant=71.8\pm10.6]\). A 2 x 2 (genotype \times pre-exposure \times sex) ANOVA of total spontaneous shuttles yielded a main effect of pre-exposure that just failed to attain significance \([F(1,33)=4.00, \ p=0.05]\). Active avoidance learning was indexed by number of avoided trials over successive 10-trial blocks (Fig. 8). All groups exhibited a similar increase over blocks reaching asymptotic level by the second half of the session. Neither genotype nor pre-exposure condition appeared to affect the rate of conditioned avoidance learning. These impressions were confirmed by a 2 x 2 x 2 (genotype \times pre-exposure \times sex \times 10-trial blocks) ANOVA, which only yielded a significant main effect of blocks \([F(9,297)=92.60, \ p<0.001]\). Despite the absence of an overall LI effect, the results of this experiment are in agreement with the previous conditioned freezing experiment: the mutation did not enhance avoidance learning as such, nor did it lead to the expression of LI under conditions insufficient to generate LI in control mice (c.f., Yee et al., 2006).

![Figure 8. Latent inhibition in conditioned active avoidance learning.](image)

**Conditioned taste aversion:** The third paradigm of associative learning was conditioned taste aversion, which is achieved by pairing the sweet taste of sucrose solution with gastric malaise produced by LiCl injection. Again, animals were either pre-exposed (PE subjects) or non-pre-exposed (nPE subjects) to the sucrose solution prior to conditioning. As expected, consumption of liquid was higher in the PE than nPE subjects due to the rewarding taste of sucrose in the pre-exposure phase.
(Table 2). A $2 \times 2 \times 2$ (genotype × sex × pre-exposure) ANOVA of liquid consumption during pre-exposure session yielded a significant effect of pre-exposure [$F(1,38)=21.14$, $p<0.001$]. Conditioning took place 24h later, and it was uneventful (Table 2). A similar analysis of liquid consumption yielded no significant outcome. In the conditioned taste aversion test on the next day, aversion was indexed by percent consumption of sucrose solution. LI was evident by increased aversion in the nPE relative to the PE subjects. This was largely similar in both mutant and control mice (Fig. 9). A 3-way ANOVA (genotype × sex × pre-exposure) ANOVA of percent consumption of sucrose solution confirmed the overall presence of LI by yielding a significant pre-exposure effect [$F(1,38)=5.43$, $p<0.05$]. Neither the main effect of genotype nor its interaction attained statistical significance. Additional analysis of total liquid consumption (water plus sucrose solution) did not yield any significant effects (Table 2). These results provided further support to the conclusion of the previous two associative learning experiments that neither conditioning as such nor LI expression differed significantly between mutant and control mice. This contrasts with the results obtained with the more selective deletion of GlyT1 restricted to forebrain neurons (Yee et al., 2006).

<table>
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Table 2. Summary of liquid consumption on the days of pre-exposure, conditioning, and test phases of the conditioned taste aversion experiment.

Figure 9. Latent inhibition in conditioned taste aversion. The expression of conditioned taste aversion on the test day (24h after sucrose-LiCl pairing) was indexed by the percentage of sucrose solution consumption in the 30 min test session. The lower the value the greater is the taste aversion. Weaker conditioned aversion in the pre-exposed (PE) relative to the non-pre-exposed (nPE) condition constitutes the LI effect (*$p < 0.05$). All values refer to mean ± SEM.

Normal anxiety-like behavior in $EMX/GlyT1$-KO mice

The standard elevated plus maze was used to assess the expression of unconditioned anxiety-like behavior by measuring the animals’ reluctance to enter the open arms of the maze. This was indexed by percentage time spent in, or entries into, the open arms relative to the total time spent in, or entries, into all arms, respectively. Mutant and control mice were comparable in terms of both measures [% time in open arms: control = 27.6±5.7%, mutant = 35.7±8.0%; % open arms entries: control =
37.2±4.7%, mutant = 35.0±6.7%]. They also did not differ in terms of locomotor activity in the elevated plus maze based on the measure of cumulative distance traveled [control = 7.55±0.50m, mutant = 7.24±0.60m]. Separate 2 × 2 (genotype × sex) ANOVAs of all three measures failed to yield any significant effect.

Hence, the behavioral outcomes reported above are not confounded by changes in potential change in emotionality in the form of anxiety or generalized fear to aversive environmental stimulus.

**Normal sensory motor coordination in EMX/GlyT1-KO mice**

Motor response and coordination are potential confounding variables for most behavioral paradigms. Two standard tests were therefore employed to ascertain if the mutation had affected motor control.

First, mutant and control mice performed similarly in the accelerating rotarod test of motor coordination and motor skill development (Fig. 10A). The latency to fall showed an increase over days in both groups. However, a sex difference was observed: male mice fell earlier than female mice, although this difference disappeared by the third test day. These impressions were confirmed by a 2 × 2 × 3 (genotype × sex × days) ANOVA of the latency to fall (in sec), which yielded a main effect of days \( F(2,74)=13.27, \ p<0.005 \), of sex \( F(1,37)=6.50, \ p<0.02 \), and their interaction \( F(2,74)= 5.33, \ p<0.01 \). No other main effect, including the genotype effect (see Fig. 10B), or interaction terms attained statistical significance. Because of the significant difference in body weight between sexes [♀ control=28.0±0.7g, ♀ mutant=28.2±0.6g, ♂ control=38.7±0.7g, ♂ mutant: 39.5±0.9g], additional analysis of covariance (ANCOVA) was performed on the mean latency to fall across the three days with body weight as the covariate. The ANCOVA indicated that the main effect of sex was no longer significant \( F<1 \), suggesting that the observed difference between sexes could be statistically accounted for by individual differences in body weight.

Second, muscular strength was assessed in the hanging wire test by measuring the latency to fall from the wire grid. Again, mutant and control mice were highly comparable, but male mice [52.8±6.6s] were in general performing poorer than female [289.3±10.4s]. A 2 × 2 (genotype × sex)
ANOVA of the latency to fall (in sec) yielded only a main effect of sex \( [F(1,37)=341.90, p<0.001] \). Similarly, an additional ANCOVA was performed with body weight as covariate, and the main effect of sex remained statistically significant \( [F(1,36)= 69.90, p < 0.001] \). Hence, it is unlikely that the sex difference observed in the hanging wire test could be solely attributed to body between-sex weight difference.

Therefore, the cognitive effects reported above cannot be attributed to possible confounding changes in general motor function or coordination.

**DISCUSSION**

Over the past several years GlyT1 has emerged as a promising pharmacological target to treat cognitive dysfunctions in schizophrenia or to enhance general cognition function (Atkinson et al., 2001; Gadea and Lopez-Colome, 2001; Chen et al., 2003). Following the discovery of neuronal GlyT1 (Cubelos et al., 2005), the cell-type specific roles of GlyT1 (neuronal vs. glial) in modifying cognitive functions have not been addressed. Development of GlyT1-inhibitors for clinical application depends on a more thorough understanding of the cell-type specific roles of GlyT1 in regulating cognitive functions. Previous genetic models were based on heterozygosity of GlyT1 (50% reduction of GlyT1 in neurons and astrocytes) providing a general model of reduced GlyT1 function (Gomeza et al., 2003; Tsai et al., 2004; Martina et al., 2005), or forebrain-selective deletion of GlyT1 in neurons (Yee et al., 2006), designed to exert a more selective effect on NMDAR functions. However, non-selective pharmacological treatment would imply blockade of GlyT1-mediated glycine transport in neurons as well as astrocytes. It is therefore imperative to study GlyT1 function in an animal model, in which GlyT1 has been deleted in both cell types. Since homozygous global deletion of GlyT1 is lethal (Gomeza et al., 2003), we decided to knockout GlyT1 specifically in neurons and astrocytes of the forebrain (EMX/GlyT1-KO). Biochemical analysis of EMX/GlyT1-KO mice confirmed that the resulting GlyT1-deficiency in forebrain was associated with a clear disruption in glycine transport.

**EMX/GlyT1-KO confers a resistance to acute phencyclidine challenge in the absence of enhanced NMDAR-mediated currents**

A direct consequence of forebrain-selective (in neurons and glia) GlyT1 disruption was the near-complete absence of response to the acute phencyclidine (PCP) challenge. This suggests that NMDAR function in EMX/GlyT1-KO mice, was altered – being more resistant to systemic pharmacological blockade, presumably due to increased levels of synaptic glycine. The lack of an effect on NMDAR- or AMPAR-evoked EPSCs in the hippocampus of EMX/GlyT1-KO mice indicates that the resistance to the PCP challenge does not necessarily require increased hippocampal NMDAR-mediated currents under non-challenged conditions. Thus, the altered properties of NMDARs in EMX/GlyT1-KO (confirmed by reduced PCP response) do not translate functionally into EPSC enhancement. This
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contrasts with the enhanced NMDAR-dependent EPSCs seen in CamKII/GlyT1-KO mice (Yee et al., 2006), in which GlyT1 is lacking selectively in forebrain neurons.

Reduced GlyT1 expression and the resulting elevation of extracellular glycine is not only highly effective in enhancing glycine-B site occupancy, but can also lead to additional cellular events, which in turn may affect NMDAR function at the network level in different directions. Thus, it has been shown that pharmacological blockade of GlyT1 can enhance as well as impair NMDAR function. Martina et al. (2004) reported that NMDAR-mediated currents were significantly enhanced in the presence of 25nM of the GlyT1 inhibitor CP802079, but were reduced at higher concentrations (50-1000nM). These authors attributed the latter negative impact on NMDAR-mediated currents to NMDAR internalization. Indeed, direct application of glycine beyond the saturation threshold of glycine-B site primes NMDARs for endocytosis (Nong et al., 2003; Martina et al., 2005). Thus, moderate sub-threshold increases in extracellular glycine may be more effective in achieving an enhanced contribution of NMDAR-mediated currents to neuronal network functioning. Our data may be taken as support of this view, because the elevation in extracellular glycine should be higher and more widespread in EMX/GlyT1-KO than in CamKII/GlyT1-KO mice. However, there was no evidence that glycine-primed NMDAR internalization had led to a reduction in the abundance of NMDARs in EMX/GlyT1-KO mice, because these mice showed a normal expression level of the obligatory NR1 subunit of the NMDAR.

In addition, glycine also produces direct neuronal inhibition via activation of strychnine-sensitive glycine receptors (GlyAR). GlyARs are present in forebrain, although they are most abundant in the brain stem and spinal cord (Béchade et al., 1994; Rajendra et al. 1997). GlyT1 expressed in glia cells adjacent to GlyAR-containing inhibitory glycinergic neurons (Aragon and Lopez-Corcuera, 2003) contributes to the termination of inhibitory neurotransmission by removing glycine from the synaptic cleft (Gomeza et al., 2003). Because glia-associated GlyT1 is disrupted in EMX/GlyT1-KO but not in CamKII/GlyT1-KO mice, GlyAR-mediated neuronal inhibition may be enhanced in the former but not the latter mutant mice. This represents another mechanism whereby hippocampal network NMDAR-mediated currents were only substantially enhanced in CamKII/GlyT1-KO but not EMX/GlyT1-KO mice.

These possible regulatory mechanisms are not mutually exclusive, and they may collectively underlie the difference in NMDAR-mediated currents existing between these two mutant mouse lines, which clearly diverge in terms of several critical behavioral phenotypes related to different forms of memory processes as outlined below.
EMX/GlyT1-KO does not enhance associative learning and reversal learning, but CamKII/GlyT1-KO does

CamKII/GlyT1-KO consistently enhanced associative learning including the control of selective learning by stimulus reinforcement history, namely in the form of latent inhibition (LI; Yee et al., 2006). By contrast, neither effect was apparent in the EMX/GlyT1 mice. The presence or absence of these phenotypes therefore may be linked to whether hippocampal NMDAR-mediated currents were enhanced or not, because hippocampal NMDARs are known to assume an important role in associative learning including the selective property of associative learning as exemplified by LI (Gruart and Delgado-Garcia, 2007; Martinez et al., 2007; Sahun et al., 2007; Valenzuela-Harrington et al., 2007). One parsimonious hypothesis posits that the selective enhancement of NMDAR-mediated currents in CamKII/GlyT1-KO mice facilitates not only the formation of [CS→US] associative links but also [CS→nothing] association (see Möhler et al., 2008), thus leading to the dual phenotypes of enhanced conditioning and LI potentiation.

Secondly, the unique NMDAR-mediated current-properties in the hippocampus of CamKII/GlyT1-KO mice may also facilitate water maze reversal learning seen in these animals (Singer et al., submitted(a)) – a phenotype that is also absent in EMX/GlyT1-KO mice. Damage to hippocampus is known to result in persistent responding and preservation in goal-directed behavior indicative of impaired behavioral inhibition (see Gray, 1982). Our results may support the hypothesis that NMDAR-dependent currents are involved in the flexible expression of alternative (even incompatible) learned responses necessary for the maintenance of adaptive goal-directed behavior against changing environmental contingency.

Object recognition is enhanced by EMX/GlyT1-KO as well as CamKII/GlyT1-KO

Does the emergence of this common phenotype undermine the pivotal role of hippocampal NMDAR in learning and memory? One interpretation is that hippocampal NMDAR function does not normally contribute to object recognition memory. Instead, the rhinal cortices may be more closely linked to object recognition memory (Aggleton et al., 1997; Brown and Aggleton, 2001), whereas the precise modulatory function of hippocampus in recognition memory remains ill defined (Bowles et al., 2007; Suchan et al., 2008). Functional alternations to extra-hippocampal NMDARs common to EMX/GlyT1-KO and CamKII/GlyT1-KO may be responsible for their shared promnesic effect in object familiarity judgment. It is imperative to directly assess NMDAR function in the cortex in order to identify the critical neuronal mechanisms involved in this shared phenotype.
EMX/GlyT1-KO enhances spatial working memory function, but CamKII/GlyT1-KO does not

Intriguingly, working memory was enhanced by EMX/GlyT1-KO, but not by CamKII/GlyT1-KO that facilitates hippocampal NMDAR-mediated currents (Yee et al., 2006; Singer et al., submitted(a)), because hippocampal glutamatergic neurotransmission seems to play a critical role in this form of memory (Bannerman et al., 2008). However, prefrontal dopaminergic transmission also assumes a significant contribution (Kolb, 1984; Jones, 2002). Systemic treatment with the GlyT1 inhibitor SSR504734 in wild-type animals (which may resemble the global EMX/GlyT1-KO rather than the neuron-specific CamKII/GlyT1-KO) has been shown to increase the prefrontal extra-cellular dopamine levels (Depoortère et al., 2005). The direct response to the dopamine releaser amphetamine was apparently unaltered in EMX/GlyT1-KO mice here. However, GlyT1 inhibition appears uniquely able to potentiate limbic glutamate-mediated facilitation of dopamine release in the mesolimbic dopamine system (Leonetti et al., 2006): this may be selectively achieved in the EMX/GlyT1-KO mice. Such concerted alternations in multiple brain regions following GlyT1 disruption may be responsible for the observed enhanced working memory function demonstrated here, and following systemic SSR504734 administration (Singer et al, submitted(b)).

CONCLUSIONS

Our data demonstrate that GlyT1 inhibition represents a feasible approach to modulate cognitive processes, and specifically illustrate the differential impacts on learning via selective (neuronal versus global) GlyT1-targeted manipulations. This possibly points towards a functional dissociation between neuron- and glia-associated GlyT1 in the regulation of higher cognitive behavior, which may be directly tested when a forebrain glia-specific gene knockout system becomes available. The future development of cell-type as well as region specific GlyT1 inhibition therapies (e.g. by gene therapies using cell-type and regionally restricted expression of antisense RNA directed against GlyT1) may provide novel and highly selective avenues in the treatment of cognitive disorders.
REFERENCES


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Labow RE, Moore AU (1959) Latent inhibition: the effect of nonreinforced pre-exposure to the conditional


Singer P, Boison D, Möhler H, Feldon J, Yee BK (submitted (a)) Deletion of glycine transporter 1 (GlyT1) in forebrain neurons facilitates reversal learning: Enhanced cognitive adaptability? Learn Mem

Singer P, Feldon J, Yee BK (submitted (b)) The glycine transporter 1 inhibitor SSR504734 enhances working memory performance in a continuous delayed alternation task in C57BL/6 mice. Psychopharmacology


Chapter 5

Modulation of prepulse inhibition by forebrain glycine transporter 1 (GlyT1): A molecular dissection between GlyT1’s neuronal and non-neuronal population

With Detlev Boison, Hanns Mohler, Joram Feldon and Benjamin K. Yee

As submitted in Neuropsychopharmacology

[Personal contribution to the work: Designing and performing the research, analysis of the behavioural data, and writing the manuscript]
Chapter 5: Modulation of prepulse inhibition by forebrain glycine transporter 1 (GlyT1): A molecular dissection between GlyT1’s neuronal and non-neuronal population

Chapter 5

Abstract

Deficiency in prepulse inhibition (PPI) of the acoustic startle reflex is considered to be an endophenotype of schizophrenia. In normal humans and animals, PPI is disrupted by acute non-competitive NMDA receptor (NMDAR) antagonists, lending support to the glutamate hypofunction hypothesis of schizophrenia; and this suggests that enhancement of glutamatergic neurotransmission via NMDAR may confer antipsychotic potential. This can be achieved by increased activation of the NMDAR glycine-B site, as suggested by several clinical and preclinical studies. However, there is little consistency in the reported effects of the glycine-B site agonist glycine upon PPI. Similarly, elevation in the synaptic glycine levels achieved by inhibition of the glycine transporter 1 (GlyT1) also yielded contradictory outcomes on PPI. Here, we attempt to address this issue by molecular deletion of GlyT1 restricted either to the forebrain or to the neuronal component in the forebrain. These two approaches have been reported to yield similar as well as non-overlapping phenotypes, suggesting that the neuronal and non-neuronal (mainly glial cells) components of GlyT1 differ somewhat in their modulatory roles over NMDAR function. The present study demonstrated that PPI was disrupted by selective forebrain neuronal GlyT1 deletion, but was spared by forebrain-wide GlyT1 deletion. These results provide further evidence for a functional dissociation between neuron and glial associated GlyT1, and that reduced glycine re-uptake mediated by GlyT1 is not associated with PPI enhancement.
INTRODUCTION

The startle reaction to an intense acoustic (pulse) stimulus can be attenuated when it is shortly preceded by a weak non-startle eliciting (prepulse) stimulus (Graham 1975). This phenomenon is known as prepulse inhibition (PPI), and represents a form of early attentional control or sensorimotor gating (Braff and Geyer, 1990), by which on-going information processing is protected from potential interference by antecedent stimuli (Graham 1975). PPI deficiency has been repeatedly observed in schizophrenia patients (Bolino et al. 1994, Braff et al. 1978, 1992, Grillon et al. 1992, Parwani et al. 2000) and is considered to be an endophenotype of the disease. It can be reliably induced by either dopamine receptor agonists (e.g., apomorphine and amphetamine) as well as NMDA receptor antagonists (e.g., phencyclidine, dizocilpine and ketamine) (Mansbach and Geyer 1989 and 1991, Mansbach 1988, Keith et al. 1991, Johansson et al. 1995, al-Amin and Schwartzkopf 1996, Yee et al., 2004a, 2004b), lending support to both the dopamine hyperfunction hypothesis (Snyder 1976) and the glutamate hypofunction hypotheses of schizophrenia (Coyle 1996, Javitt 2007).

According to the glutamate hypothesis, augmentation of NMDAR function may entail antipsychotic potentials especially against the negative and cognitive symptoms of schizophrenia. Because direct activation of NMDAR is associated with severe side-effects including neurotoxicity, one favoured approach has been to increase activation of the glycine-B site in the NMDAR complex. Binding to NMDAR's glycine-B by endogenous glycine site is required for the activation of the NMDAR by glutamate (Johnson and Ascher 1987, Smith 1992). The co-administration of endogenous glycine-B site agonists, glycine and D-serine, has been reported to enhance the efficacy of conventional antipsychotic medications (Heresco-Levy and Javitt, 2004, Hereso-Levy et al., 1999, 2004, and 2005, Javitt 2006, Tsai et al., 1998 and 1999). Another strategy to enhance the glycine availability in the vicinity of NMDAR, and therefore binding to the glycine-B site, is to disrupt glycine re-uptake mediated by glycine transporter 1 (GlyT1; Aragon et al., 2005, Berger et al., 1998, Eulenburg et al., 2005, Javitt 2006). Pharmacological inhibition (Depoortère et al., 2005, Bergeron et al., 1998, Martina et al., 2004) as well as genetic deletion (Tsai et al., 2004; Yee et al., 2006, Singer et al., in preparation) of GlyT1 is highly effective in increasing synaptic glycine levels in vivo; and different classes of GlyT1 inhibitor are currently undergoing clinical and preclinical investigation as potential antipsychotic drugs (Lechner 2006, Lindsley et al., 2006, Harsing et al., 2006 Coyle 2006, Singer et al., in press).

However, experimental evaluations of direct glycine-B site agonists and of GlyT1 inhibitors have yielded a complex pattern of results regarding their effects on PPI, including data suggesting that elevation of brain glycine levels and/or enhanced glycine-B site activation may be associated with detrimental effects on sensorimotor gating.

D-serine appears to be effective in facilitating PPI in mice, but not in restoring PPI deficit induced by dizocilpine (Lipina et al., 2005). Glycine treatment can reverse PPI deficit following hippocampal lesions in rats (Le Pen et al., 2003), but a glycine-rich diet leads to PPI deficiency in rats
GlyT1 inhibitors, such as NFPS and SSR504734, are effective in enhancing PPI in DBA/2J mice, a strain with low basal PPI levels (Kinney et al., 2003, Depoortère et al., 2005). ALX 5407 is also effective in reversing dizocilpine-induced PPI deficit in the common C57BL/6 mouse strain (Lipina et al., 2005). Likewise, ORG 24598 has been shown to partially restore PPI deficits emerged following hippocampal lesions in rats (Le Pen et al., 2003). Against the expectation based on the clinical efficacy of glycine and D-serine as add-on antipsychotic medication, Heresco-Levy et al. (2007) recently reported that PPI deficits amongst schizophrenia patients are associated with high rather than low levels of glycine in cerebrospinal fluid. Similarly intriguing is the observation that constitutive heterozygous knockout of GlyT1 exacerbates rather than counters the PPI disruptive effect of dizocilpine (Tsai et al., 2004). Indeed, there is also suggestion that the GlyT1 inhibitor, ALX 5407, can attenuate PPI at high doses in normal mice (see Table 1 of Lipina et al., 2005).

A consistent link is therefore lacking between PPI performance and brain glycine availability, and by implication also glycine-B site occupancy. Hence, there is a need to clarify more precisely the relationship between enhanced glycine-B site activation and PPI, especially amongst healthy individuals if glycine-B site agonists and GlyT1 inhibitors are to be further considered as cognitive enhancing agents in the treatment of other mental disorders (Singer et al., in press).

Our recent evaluation of the promnesic and antipsychotic potentials of GlyT1 forebrain deletion in mice has yielded dissociable phenotypes between mice with complete forebrain GlyT1 deletion and mice with restricted forebrain neuronal GlyT1 deletion (Yee et al., 2006, Singer et al., 2007, Singer et al., in preparation). As illustrated in Table 1, there are phenotypes that are unique to each of these two mutant mouse lines, in addition to some common features. We aim to examine in the present study if the two mutant lines may produce distinguishable effects on PPI. Our data so far suggest that the forebrain neuronal GlyT1 disruption is associated with a wider spectrum of behavioural effects, and it may also produce a phenotype on PPI while the complete forebrain GlyT1 deletion may not. If so, this distinction may be useful in the identification of how best to avoid the potential detrimental effects of GlyT1 inhibition and glycine-B site agonists suggested by earlier studies.
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<table>
<thead>
<tr>
<th>Phenotypic variables</th>
<th>Forebrain neuron-specific GlyT1 KO</th>
<th>Forebrain non-specific GlyT1 KO</th>
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<tbody>
<tr>
<td></td>
<td>CamKIIα/GlyT1</td>
<td>EMX/GlyT1</td>
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<tr>
<td><strong>Electrophysiology</strong></td>
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<td>NMDA evoked EPSCs</td>
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<td>AMPA evoked EPSCs</td>
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<td>Conditioned taste aversion</td>
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<tr>
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<td>Not affected</td>
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<tr>
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<td>Dizocilpine (MK-801)</td>
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<td>Phencyclidine (PCP)</td>
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<tr>
<td>Amphetamine</td>
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</table>

Table 1. Major behavioural and electrophysiological effects associated with either forebrain neuronal or complete forebrain specific GlyT1 disruption, which have been fully described elsewhere: CamKIIα/GlyT1: Yee et al., 2006, Singer et al., 2007, Möhler et al. 2008. EMX/GlyT1: Singer et al., submitted.

MATERIAL AND METHODS

Animals

A homozygous Glyt1tm1.2fl/fl colony was established and maintained on a pure C57BL/6 background as described before (Gabernet et al., 2005; Yee et al., 2006). Forebrain neuron specific deletion of GlyT1 was achieved by CamKIIαCre-mediated recombination (Yee et al., 2006). Complete forebrain GlyT1 deletion in neurons and mostly astrocytes was achieved by cortical specific EMX-Cre mediated recombination (Singer et al., in preparation). Appropriate heterozygous Cre mice were mated with Glyt1tm1.2fl/fl mice to generate the desired mutant and control littermates (Yee et al., 2006; Singer et al., in preparation). Animals of both sexes were employed in the present study.

The mice were weaned at 12 days old, and littermates of the same sex were kept in groups of four to six in Macrolon Type-III cages (Techniplast, Milan, Italy), and housed in a temperature- and humidity-controlled (at 22°C and 55% R.H.) animal vivarium under a reversed light-dark cycle with lights off from 0800-2000hrs. The animals were maintained under ad libitum water and food (Kliba 3430, Klibamuhlen, Kaiseraugst, Switzerland) throughout the study.

Behavioural testing began when the animals were approximately 12 weeks old, and all tests were conducted in the dark phase of the light cycle. In Experiment 1, there were 24 (9 male and 15 female) CamKIIα/GlyT1 mutant mice and 33 (15 male and 18 female) control littermates. In Experiment 2, there were 17 (7 male and 10 female) EMX/GlyT1 mutants and 24 (13 male and 11 female) littermate controls. The two experiments were conducted separately. All experimental
procedures described had previously been approved by the Zurich Veterinary Office; they also conformed to the ethical standards stipulated by the Swiss Act and Ordinance on Animal Protection and were in accordance to the European Council Directive 86/609/EEC.

Open field

There were four identical open fields each measuring 40 x 40cm\(^2\) in surface area and surrounded by 35cm high walls. They were made of wood with a white water-proof surface as described before (Yee et al., 2006). Animals were tested in squads of four, under diffused dim lighting at about 30 lux. They were gently placed in the centre of the appropriate open field and allowed to explore undisturbed for one hour. The open fields were cleansed with water and dried prior to the next squad. Locomotor activity was indexed by distance travelled recorded in successive 10-min bins. Data were collected and calculated by the EthoVision\textsuperscript{®} tracking system (Version 3.1, Noldus Technology, Wageningen, Netherlands).

Prepulse inhibition of the acoustic startle reflex

The PPI experiment was carried out in four acoustic startle chambers for mice (SR-LAB, San Diego Instruments, San Diego, CA, USA). The test parameters and design was adopted from the procedures recommended by Yee et al. (2005), which allowed the introduction of multiple pulse intensities. Briefly, a PPI testing session lasted approximately 45min in which a series of four different types of trials were presented to the animals. These included pulse-alone trials, prepulse-\textit{plus}-pulse trials, prepulse-alone trials, and no-stimulus trials in which no discrete stimulus other than the constant background noise (65dB\textsubscript{A}) was presented. The intensity of the pulse stimulus varied amongst 100, 110 and 120dB\textsubscript{A}, with a duration of 40ms. The intensity of the prepulse stimulus varied amongst 71, 77 and 83dB\textsubscript{A}, with a duration of 20ms. In prepulse-\textit{plus}-pulse trials, the stimulus onset asynchrony (SOA) between the two stimuli was 100ms. All stimuli were in the form of white noise, presented against a constant background noise at 65dB\textsubscript{A}. A session began with the animals being placed into a Plexiglas enclosure inside the startle chamber, positioned under a loudspeaker. They were acclimatised to the apparatus for two minutes before the first trial began. The first six trials consisted of 6 pulse-alone trials, comprising two trials of each of the three possible pulse intensities. These trials served to habituate and stabilize the animals’ startle response, and were analysed separately. Subsequently, the animals were presented with 10 blocks of discrete test trials to assess PPI. Each block consisted of the following: three pulse-alone trials (100, 110 or 120dB\textsubscript{A}), three prepulse-alone trials (71, 77 and 83dB\textsubscript{A}), and nine possible combinations of prepulse-\textit{plus}-pulse trials (3 levels of prepulse \times 3 levels of prepulse), and one no-stimulus trial (i.e., background alone). The 16 discrete trials with each block were presented in a pseudorandom order, with a variable inter-trials interval of a mean of 15s (ranging from 10 to 20s). The session was concluded with a final block of six consecutive pulse-alone trials similar that administered at the beginning of the test session.
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Statistical analysis

All data were analysed by parametric analysis of variance ANOVA using genotype (mutant vs. littermate control) and sex (male vs. female) as the between-subjects factors. Additional within-subjects factors (e.g., 10-min bins, pulse intensity, prepulse intensity) were included according to the nature of the considered dependent variables. All statistical analyses were carried out using SPSS for Windows (version 13, SPSS Inc. Chicago IL, USA) implemented on a PC running the Windows XP (SP2) operating system. All ANOVAs performed were of Type III as specified by SPSS.

RESULTS

Prior to the assessment of PPI, we subjected the animals to an open test for spontaneous locomotor activity, because an effect on motor activity as such could represent a confounding variable in the later evaluation of PPI. The two experiments were separately analysed.

Open field activity

*CamKIIα/GlyT1*  
The activity levels as measured by distance travelled per 10-min bins were highly comparable between CamKIIα/GlyT1 mutant mice and littermate controls (Figure 1A). Both groups exhibited clear locomotor habituation as activity levels monotonically decreased over time. This was again highly comparable between groups, although male mice tended to habituate quicker than female mice – an effect that was observed in both CamKIIα/GlyT1 and control mice. These impressions were supported by a 2 x 2 x 6 (genotype x sex x 10-min bins) ANOVA of the distance travelled per 10min bins, which yielded a significant main effect of bins \[F(5,265)=210.83, p<0.001\] and a significant sex by bins interaction \[F(5,265)=3.17, p<0.05\]. No other effects achieved statistical significance.

![Figure 1](image-url). Open field activity recorded over a period of 60min is expressed as distance travelled per 10-min bins. Neither the CamKIIα/GlyT1 mutant mice (A) nor the EMX/GlyT1 mutant mice (B) differed in their spontaneous locomotor activity in comparison to either respective control littersmates. All values refer to biased mean ± S.E.M derived from the overall ANOVA.
EMX/GlyT1 The comparison between EMX/GlyT1 mutant mice and their corresponding littermate controls also did not yield any significant group difference (Figure 1B). Again, both the absolute level of activity and the rate of locomotor habituation were comparable between EMX/GlyT1 mutant mice and controls. A similar sex difference as described above also emerged, with male mice habituating faster than female mice regardless of genotype. These impressions are confirmed by a 2 x 2 x 6 (Genotype x Sex x 10-min bins) ANOVA of the distance travelled per 10-min bins, revealing a significant effect of bins $[F(5,185)=98.53, p<0.001]$ and of its interaction with sex $[F(5,185)=4.48, p<0.05]$. No other effects achieved statistical significance.

Thus, neither neuronal specific nor cell-type non-specific GlyT1 deletion in the forebrain affected spontaneous locomotor activity. Overall activity levels appeared to differ between the two experiments; and this was likely attributable to unintended differences in apparatus settings despite our efforts to equate them between the two experiments.

Reactivity to the pulse stimulus alone

CamKIIα/GlyT1 We evaluated the animals’ startle reactivity to the pulse stimulus as such by focusing on the reactivity scores obtained across all pulse-alone trials presented between the first and last blocks of pulse-alone trials. Individual mean reactivity to the pulse stimulus at the three intensities was subjected to a 2 x 2 x 3 (Genotype x Sex x Pulse intensity) ANOVA. As expected, higher reaction was elicited by more intense pulse stimulus, leading to a highly significant main effect of pulse intensity $[F(2,106)=27.72, p<0.001]$. There were no other significant effects (see Table 2).

<table>
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<tr>
<th></th>
<th>CamKIIα/GlyT1</th>
<th>EMX/GlyT1</th>
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<tbody>
<tr>
<td></td>
<td>Mutant</td>
<td>Control</td>
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<tr>
<td></td>
<td>(9m and 15f)</td>
<td>(15m and 18f)</td>
</tr>
<tr>
<td><strong>Pulse-alone trials:</strong></td>
<td></td>
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<tr>
<td>100dB$_A$</td>
<td>26.45±3.24</td>
<td>25.47±2.77</td>
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<tr>
<td>110dB$_A$</td>
<td>49.43±5.10</td>
<td>58.19±7.20</td>
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<tr>
<td>120dB$_A$</td>
<td>79.01±9.33</td>
<td>9.51±17.63</td>
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<tr>
<td><strong>Prepulse-alone trials:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No-stimulus*</td>
<td>6.32±0.57</td>
<td>5.71±0.46</td>
</tr>
<tr>
<td>71dB$_A$</td>
<td>7.33±0.70</td>
<td>6.22±0.55</td>
</tr>
<tr>
<td>77dB$_A$</td>
<td>7.29±0.81</td>
<td>7.51±0.83</td>
</tr>
<tr>
<td>83dB$_A$</td>
<td>8.36±0.71</td>
<td>12.78±2.34</td>
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<tr>
<td>First block</td>
<td>56.42±6.73</td>
<td>57.79±9.31</td>
</tr>
<tr>
<td>Final block</td>
<td>47.80±4.82</td>
<td>59.26±9.48</td>
</tr>
</tbody>
</table>

Table 2. Mean reactivity obtained on pulse-alone and prepulse-alone trials presented in the middle portion of the test – i.e., between the first and last blocks of pulse-alone trials. The mean reactivity obtained in the first and last blocks of pulse-alone trials (collapsed across the three pulse intensities) is presented in the last row of the table; comparison between the two allows an evaluation of startle habituation. All values refer to biased mean±S.E.M (in arbitrary units) derived from the appropriate overall ANOVA. * “no-stimulus” refer to trials in which no discrete stimulus except the background noise (at 69dB$_A$) was presented.
EMX/GlyT1  The analysis of this dataset yielded a similar pattern of results. There was no apparent difference between EMX/GlyT1 mutant and control mice (see Table 2), nor between male and female mice. A 2 x 2 x 3 (Genotype x Sex x Pulse intensity) ANOVA only revealed a main effect of pulse intensity as expected [F(2,74)=42.90, p<0.001].

Reactivity to the prepulse stimulus alone
CamKIIα/GlyT1  Direct reaction to the prepulse stimulus is evaluated for possible differences in the processing of the prepulse stimulus as such (Yee et al., 2004a,b). A 2 x 2 x 4 (Genotype x Sex x Prepulse intensity) ANOVA of the reactivity to the three prepulse stimuli as well as the baseline reactivity obtained on ‘no-stimulus’ trials was performed. This yielded a main effect of prepulse intensity [F(3,159)= 7.63, p<0.005]. CamKIIα/GlyT1 mutant mice and controls exhibited a monotonic increase in reactivity with increasing prepulse intensity (see Table 2). There was no indication for any difference between genotypes or between the two sexes.

EMX/GlyT1  Equivalent analysis of the dataset obtained in this experiment yielded a very similar pattern of results, without any suggestion for any difference between genotypes or between the two sexes. A 2 x 2 x 4 (Genotype x Sex x Prepulse intensity) ANOVA of the reactivity on prepulse-alone and no-stimulus trials only revealed a significant effect of prepulse [F(3,111)=13.40, p<0.001]. This again corresponded to a monotonic increase in reaction to the prepulse stimulus as a function of stimulus intensity (see Table 2).

Within-session startle habituation
We attempted to evaluate habituation of the startle reaction by comparing the reaction on pulse-alone trials in the first block with that of the last block of the test session (see Table 2). There was however no statistical support for a habituation effect in either experiment based on separate 2 × 2 × 2 × 3 (Genotype x Sex x Blocks x Pulse intensity) ANOVAs.

Prepulse inhibition
CamKIIα/GlyT1  The reduction in startle reactivity to the pulse stimulus on pulse-plus-pulse trials relative to pulse-alone trials constitutes the PPI effect. This reduction was indexed here by the conventional measure of percent inhibition, as defined by the expression: %PPI = [ 1 − (mean reaction to prepulse(x)-plus-pulse(y) trials) / (mean reaction on pulse(y)-alone trials) ] × 100%. This was calculated for each subject at each of the nine possible prepulse-pulse combinations. Figure 2A depicts the comparison of %PPI between CamKIIα/GlyT1 mutant and littermate controls as a function of pulse and prepulse intensity. PPI was attenuated in the CamKIIα/GlyT1 mutant mice, and this was consistently seen across all pulse-prepulse condition except at prepulse=77dB and pulse=100dB condition. A 2 × 3 × 3 (Genotype × Prepulse intensity × Pulse intensity) of %PPI revealed a significant main effect of genotype [F(1,53)=5.40, p<0.05]. The main effect of prepulse was also highly
significant \( [F(2,108)=66.73, p<0.001] \) indicating that \%PPI tended to increase as a function of prepulse intensity. No other effects attained statistical significance.

**EMX/GlyT1** As illustrated in Figure 2B, the expression of prepulse inhibition was highly comparable between EMX/GlyT1 mutant mice and their littermate controls across all prepulse-pulse conditions. Statistical analysis of \%PPI yielded only a significant effect of prepulse intensity \( [F(2,74)=37.38, p<0.001] \).

![Figure 2](image)

**Figure 2.** The magnitude of the prepulse inhibition (PPI) effect was indexed by percent inhibition (\%PPI), calculated relative to the appropriate pulse-alone trials in which no prepulse stimulus was presented. \%PPI was expressed as here as a function of prepulse intensity, and separately at each pulse intensity. (A) and (B) depict the effect of CamKII\(\alpha\)/GlyT1 deletion or EMX/GlyT1 deletion on PPI expression, respectively. All values refer to biased mean±S.E.M derived from the appropriate overall ANOVA.

**DISCUSSION**

The present study evaluated the impact of complete and neuron specific GlyT1 deletion in the forebrain on the expression of PPI. Comparison between the two experiments suggested that PPI was attenuated when GlyT1 deletion was restricted to forebrain neurons (CamKII\(\alpha\)/GlyT1), but was unaffected when the same gene was knocked out in a cell-type non-specific manner throughout the forebrain (EMX/GlyT1). At the same time neither knockout affected spontaneous locomotor activity. Furthermore, neither mutation affected startle reaction to the pulse stimulus, which is in agreement with our previous studies indicating that anxiety-related behaviour was largely unaffected by either knockout examined here (see Yee et al., 2006; Singer et al., in preparation). In addition, the direct reaction to the prepulse stimulus also remained comparable to control littermates, indicating that the deficiency in PPI is unlikely a consequence of insufficient prepulse detection or processing. This is distinctly different from the impairment in PPI typically seen following dopamine receptor agonist treatment in C57BL/6 mice when the deficit in PPI is accompanied by an increase in the direct reaction elicited by the prepulse (Yee et al., 2004).

Although we failed to obtain any startle habituation effect here, this lack of habituation was equally observed in both mutant mouse lines. Startle habituation is not as robustly demonstrated in
mice as it is the case in rats. A habituation effect was nonetheless clearly seen in the open field experiment, suggesting that the lack of a habituation effect in the PPI experiment was paradigm-specific, and it did not represent a general habituation deficit. The present findings regarding GlyT1 forebrain deletion were consistent across sexes, and so the functional significance of forebrain neuron-associated GlyT1 on PPI was largely independent of sex. Unlike the open field study in which the two experiments were marked by a difference in overall activity levels, no such confounding was apparent in the comparison of the two PPI experiments across all measures. This considerably strengthens the conclusion that PPI was differentially sensitive to the two GlyT1 molecular manipulations. The difference in sample size also did not undermine our ability to compare between the contrasting outcomes of the two PPI experiments, because it is highly unlikely that the null effect of EMX/GlyT1 deletion was due to Type II error when the effect size of the PPI deficit induced by CamKIIα/GlyT1 deletion was taken into consideration.

Therefore, sensorimotor gating is sensitive to GlyT1 manipulation, and in the absence of any other treatment, selective deletion of GlyT1 in forebrain neurons has a negative impact on sensorimotor gating. This effect cannot be attributed to non-specific effects relating to locomotor activity (open field experiment here), or changes in anxiety-related traits on the basis of our previous experiments in these mutant mice (Yee et al., 2006, Singer et al., in preparation). The intriguing observation that the PPI disruptive effect of neuron specific GlyT1 disruption was no longer present when non-neuronal GlyT1 was additionally disrupted may reflect a functional dissociation between neuron and glial associated GlyT1 on the mediation of PPI. We have previously shown that there are effects unique to neuron specific GlyT1 disruption, i.e., not associated with EMX/GlyT1 deletion (see Table 1). This is surprising because it appears that the less severe manipulation can produce an effect while the more extensive manipulation does not. One possible explanation is simply in terms of gene dosage effect, such that PPI is only sensitive to the imbalance produced by an incomplete GlyT1 deletion. This can accommodate the finding that PPI also appeared to be more fragile in constitutive GlyT1+/- heterozygous knockouts, such that they were more sensitive to the PPI-disruptive effect of dizocilpine (Tsai et al., 2004). The CamKIIα/GlyT1 deletion may therefore bring about a unique level of imbalance so that PPI was attenuated without any additional pharmacological challenge. This explanation does not take into account the unique cell-specific profile of the CamKIIα/GlyT1 deletion. On the other hand, a less parsimonious account may posit that deletion of neuron- and glial-associated GlyT1 is associated with separate and opposing effects on PPI, and when the two manipulations were combined they nullify each other’s unique effect. This would imply that selective disruption of glial-associated GlyT1 may enhance PPI. This possibility is of potential interest because some glycine-B site agonists (e.g., D-serine, Lipina et al., 2005) or GlyT1 inhibitors (e.g., SSR504734 and NFPS, Depoortère et al., 2005, Kinney et al., 2003) have indeed been reported to augment PPI. This possibility certainly warrants further investigation by selective molecular knockout. However, no sufficiently selective Cre mice are presently available suitable for the induction of complete glial-
specific GlyT1 deletion in the forebrain. When feasible, a direct comparison between neuron and glial specific GlyT1 deletion in the forebrain would also be instrumental in addressing other effects identified as being unique to the CamKIIα/GlyT1 deletion (see Table 1).

Nevertheless, the present results clearly highlight the possibility that sensorimotor gating (as indexed by the PPI paradigm) may be negatively affected by GlyT1 inhibition. Not only is this suggestion supported by the constitutive GlyT1+/- mice (Tsai et al., 2004), but also by the finding that (i) the irreversible GlyT1 inhibitor ALX-5407 significantly attenuated PPI in mice (at 10 and 15mg/kg i.p.; Lipina et al., 2005), and (ii) PPI is disrupted in rats maintained on a glycine-rich diet (Waziri and Baruah, 1999). The present study suggests that such detrimental effect of GlyT1 inhibitor or glycine-B site agonist treatment may stem primarily from an effect on the neuronal element of GlyT1 in the forebrain. A possibility that would be of relevance to the report that PPI deficiency amongst schizophrenia patients was associated with high levels of glycine in the CSF (Heresco-Levy et al., 2007).

What are the potential mechanisms whereby neuronal GlyT1 deletion may attenuate PPI? We have previously demonstrated that CamKIIα/GlyT1, but not EMX/GlyT1, deletion delayed the motor stimulant effects of an acute low dose amphetamine challenge (Yee et al., 2006), suggesting that neuronal GlyT1 may uniquely interfere with dopaminergic transmission, especially in the nucleus accumbens. It has been suggested that interaction between descending glutamatergic inputs from limbic cortices (including hippocampus, amygdale, entorhinal cortex and prefrontal cortex) and the ascending dopaminergic innervations from ventral tegmental area in the nucleus accumbens assume an important role in the regulation of PPI and its disturbance may be implicated in the pathophysiology of schizophrenia (Wan and Swerdlow 1996, Wan et al., 1995). In particular, dopamine release in the accumbens is regulated by limbic glutamatergic activities (Floresco 2007, Floresco et al., 2001 and 2003, Grace 1991). Hence, enhanced glutamatergic (especially NMDAR current) activity caused by forebrain neuronal GlyT1 deletion may result in an increase in dopamine release by ventral tegmental neurons, leading to PPI attenuation. It has been shown that activation of the mesolimbic dopamine system by amphetamine can be blocked by glycine-B site antagonists, including L-701,324 (Bristow et al., 1995) and HA-966 (Huston et al., 1991). Furthermore, L-701,324 could reverse PPI disruption induced by social isolation rearing, which is associated with a hyperactive mesolimbic dopamine system (Bristow et al. 1995). These findings provide some indication that enhanced glycine-B site activation may impair PPI function. However, the precise picture is further complicated by region-specific glycine-B site dependent mechanisms. Kretschner et al. (1997) showed that intra-accumbens application of the glycine-B site antagonist 7-CLKYN impaired PPI, and this effect could be antagonized by systemic pre-treatment with the glycine-B site agonists D-cycloserine. Yet, in vivo microdialysis revealed that accumbal and striatal dopamine release was not affected by glycine-B site blockade with 7-CLKYN, indicating that the glycine-B site in the accumbens may assume an important role in the regulation of PPI, but not via a direct interaction with the dopamine system.
Chapter 5: Modulation of prepulse inhibition by forebrain glycine transporter 1 (GlyT1): A molecular dissection between GlyT1’s neuronal and non-neuronal population

(Kretschner et al. 1997). Clearly, the role of the glycine-B site on dopamine release in the nucleus accumbens remains to be clarified. Nevertheless, the possibility remains that forebrain neuronal GlyT1 deletion may disturb the balance of glutamate-dopamine activity in the mesolimbic dopamine system, leading to PPI deficiency.

Alternatively, besides its role as an excitatory neuromodulator at the NMDAR glycine-B site, glycine also serves as an inhibitory neurotransmitter at strychnine-sensitive glycine receptors (Gly\textsubscript{A}R) (Legendre 2001). Gly\textsubscript{A}R is expressed in the spinal cord and brain stem (Lynch 2004, Webb and Lynch 2007), and Heresco-Levy et al. (2007) suggested that excess binding to glycineric inhibitory interneurons in these areas may interfere with the co-ordination of spinal reflexes and/or the excitability of the acoustic startle reflex circuitry (Fendt et al., 2001), and thereby exerts a negative influence on PPI expression. However, Gly\textsubscript{A}R is also found in the forebrain (Rajendra et al. 1997 Betz and Laube 2006), which may be in a position to modulate cortical functions (Betz 1991, Malosio et al. 1991, Naas et al. 1991, Rampon et al. 1996). Although the affinity of glycine for the glycine-B site is much higher than for the strychnine-sensitive site, the latter represents yet another mechanistic link between enhanced glycine availability in the forebrain and behaviour. Yet, this is unlikely to play a substantial role in the CamKII\textalpha/GlyT1 deletion mediated PPI disruption, because GlyT1 is not expressed in inhibitory neurons and GlyT1 deletion restricted to forebrain neurons is not expected to alter Gly\textsubscript{A}R mediated neurotransmission. On the other hand, inhibitory glycineric transmission in the forebrain ought to be potentiated following EMX/GlyT1 deletion due to the loss of glial-associated GlyT1, which normally contributes to the termination of inhibitory neurotransmission by removing glycine from the synaptic cleft. If this additional effect on glycineric transmission was solely responsible for the lack of an effect on PPI in the EMX/GlyT1 knockout mice, then the application of Gly\textsubscript{A}R antagonists should reveal a PPI-disruptive phenotype in these animals.

In conclusion, the present study demonstrates again, in the PPI paradigm, the importance and value in distinguishing between the neuronal and non-neuronal GlyT1 population in the forebrain in the control of behaviour. This distinction would be instrumental to the development of pharmacotherapy against psychotic-like symptoms as well as the development of cognitive enhancement for schizophrenia and beyond.

ACKNOWLEDGEMENTS
The present study was supported by the NCCR (National centre for competence in research) in neural plasticity and repair, with funding from the Swiss National Science Foundation. We would also like to acknowledge additional support from the Swiss Federal Institute of Technology, and to express our gratitude to Peter Schmid for technical support, Liz Weber for her assistance in PCR genotyping, Dr. Frank Bootz for his veterinary expertise, and the animal technicians for their assistance in animal husbandry.
REFERENCES


Chapter 5: Modulation of prepulse inhibition by forebrain glycine transporter 1 (GlyT1): A molecular dissection between GlyT1’s neuronal and non-neuronal population


Lipina T, Labrie V, Weiner I, Roder J (2005) Modulators of the glycine site on NMDA receptors, D-serine and ALX 5407, display similar beneficial effects to clozapine in mouse models of schizophrenia. Psychopharmacology (Berl) 179:54–67.


Chapter 6

The effects of forebrain GlyT1 disruption, in neurons or in neurons and glia, on aversive fear conditioning and its sensitivity to the temporal CS-US discontiguity

With Deltev Boison, Hanns Mohler, Joram Feldon and Benjamin K. Yee

Manuscript in preparation

[Personal contribution to the work: Designing and performing the research, analysis of the behavioural data, and writing the manuscript]
Abstract

We have recently shown that molecular GlyT1 deletion in forebrain neurons potentiated hippocampal NMDAR currents and enhanced both classical conditioning and latent inhibition in multiple paradigms. By contrast, NMDAR currents as well as associative learning were not affected by completed forebrain specific GlyT1 disruption indicating a functional dissociation between neuronal and non-neuronal GlyT1 populations. To identify further phenotypic differences on associative learning we tested the two GlyT1 knockout mouse lines in a series of conditioned freezing experiments. First, long-term retention of conditioned tone freezing was evaluated by introducing a delay of three weeks between conditioning and CS-test. Second, contextual fear was assessed by conditioning the animals to a novel context in the absence of any discrete stimuli. Finally, the sensitivity to the temporal CS-US discongruity was examined in a trace-conditioning experiment. The results showed that although forebrain neuronal GlyT1 disruption led to enhanced retention of conditioned tone freezing and facilitated contextual conditioning it increased the sensitivity to CS-US discongruity resulting in an attenuation of the conditioned response in the trace condition. By contrast, these effects were absent in complete forebrain specific GlyT1 knockouts lending further support to a functional dissociation between neuronal and non-neuronal GlyT1 in associative learning. The unique phenotype of enhanced conditioning and increased response to the trace-effect in the forebrain neuronal GlyT1 knockouts suggests that selective inhibition/deletion of GlyT1 in forebrain neurons does not simply strengthen associative links but enhances selectivity in associative learning which may be a sign of enhanced cognitive flexibility.
INTRODUCTION

The glycine transporter 1 (GlyT1) tightly regulates the extra-cellular glycine concentration at glutamatergic synapses by mediating glycine re-uptake. In the forebrain, GlyT1 is expressed in glia cells as well as in glutamatergic neurons in which it is co-localized with N-methyl-D-aspartate receptors (NMDARs) (Aragon and Lopez-Corcuera 2005, Cubelos et al., 2005). Glycine acts as an obligatory co-agonist of glutamate at NMDARs and its binding to the NMDAR glycine binding site (glycine-B site) is necessary for ion channel opening (Berger et al., 1998). By controlling glycine availability at the glycine-B site GlyT1 constitutes a pivotal up-stream regulator of NMDAR activity, which is closely involved in the mediation of NMDAR-dependent neuroplastic events implicated in various forms of learning and memory (Collingridge and Bliss 1995, Tang et al., 1999). Reduction of GlyT1 via pharmacological blockade or molecular disruption of its expression is an efficient means to facilitate NMDAR-mediated transmission (e.g. Tsai et al., 2004, Martina et al., 2004, Depoortère et al., 2005) and presumably thereby to enhance cognitive functions (Lipina et al., 2005, Depoortère et al., 2005, Singer et al. 2007, Yee et al., 2006). Hence, interventions targeting GlyT1 may provide novel avenues in the treatment of cognitive deficits and may even improve cognition in non-pathological situations.

We have recently shown that genetically modified mice lacking GlyT1 in forebrain neurons (CamKIIαCre/GlyT1, Yee et al., 2006) exhibited enhanced classical conditioning across multiple paradigms including conditioned freezing and taste aversion, as well as active avoidance learning, which taxes both Pavlovian and instrumental learning (Yee et al., 2006). Importantly, this phenotype is not associated with a loss in selectivity in associative learning as indicated by enhanced latent inhibition (LI) in the mutants (Yee et al., 2006). LI refers to a form of selectivity in learning whereby inconsequential or non-reinforced exposure to the to-be-conditioned stimulus (CS) prior to its pairing with the unconditioned stimulus (US) results in a decrement of the generation of the conditioned response to that CS (Lubow, 1959).

In contrast, mice lacking GlyT1 in the entire forebrain (EMX-1Cre/GlyT1, Singer et al., submitted) exhibited normal associative learning and LI under similar experimental conditions (Singer et al., submitted). Thus, the impact on associative learning and its selective control by the CS reinforcement history (LI) can fundamentally differ depending on the specificity of the GlyT1 targeted mutation (neuronal vs. complete). These phenotypic differences between the two GlyT1 knockout mouse lines may point towards a functional dissociation between neuronal and non-neuronal GlyT1 on associative learning.

The present study was designed to extend the assessment of associative learning in the two GlyT1 knockout mouse lines in order to identify further functional differences between neuronal and complete GlyT1 deletion in the forebrain. To this end, three conditioned freezing experiments were conducted. First, we evaluated whether enhanced tone-freezing in the CamKIIαCre/GlyT1 mice may persist over a prolonged period of retention by extending the delay interval between conditioning and
Chapter 6: The effects of forebrain GlyT1 disruption, in neurons or in neurons and glia, on aversive fear conditioning and its sensitivity to the temporal CS-US discontiguity

CS-test to 21 days. In the same experiment we also assessed whether the facilitatory effect on conditioning may exert a negative impact on extinction learning by repeatedly exposing the animals to the CS without the antecedent US. Second, we evaluated conditioning to the context in the absence of a discrete CS. Given that contextual conditioning closely involves NMDARs in the dorsal hippocampus (Anagnostaras et al., 2001; Gale et al., 2001) we anticipated that neuronal but not complete GlyT1 disruption might enhance contextual fear conditioning. Third, to seek further evidence for the hypothesis that forebrain neuronal GlyT1 deletion may also enhance selectivity in associative learning, as suggested by enhanced LI in CamKIIαCre/GlyT1 knockouts (Yee et al., 2006), we tested the animals’ sensitivity to temporal CS-US disconguitity using a trace conditioning paradigm.

METHODS

Animals

A homozygous Glyt1tm1.2fl/fl colony was established and maintained on a pure C57BL/6 background as described before (Gabernet et al., 2005). Forebrain neuron specific deletion of GlyT1 was achieved by CamKIIαCre-mediated recombination as described elsewhere (Yee et al., 2006). Complete forebrain GlyT1 deletion in neurons and astrocytes was achieved by cortical specific EMX1-Cre mediated recombination (Singer et al., submitted). Appropriate heterozygous Cre mice were mated with Glyt1tm1.2fl/fl mice to generate the desired mutant and control littermates. Animals of both sexes were employed in the present study. The mice were weaned at 21 days old, and littermates of the same sex were kept in groups of four to six in Macrolon Type-III cages (Techniplast, Milan, Italy), and housed in a temperature- and humidity-controlled (at 22°C and 55% R.H.) animal vivarium under a reversed light-dark cycle with lights off from 0800-2000hrs. The animals were maintained under ad libitum water and food (Kliba 3430, Klibamuhlen, Kaiseraugst, Switzerland) throughout the study. Behavioral testing began when the animals were approximately 12 weeks old, and all tests were conducted in the dark phase. The allocation of subjects employed in each of the experiments is summarized in Table 1. All experimental procedures described had previously been approved by the Zurich Veterinary Office; they also conformed to the ethical standards stipulated by the Swiss Act and Ordinance on Animal Protection and were in accordance to the European Council Directive 86/609/EEC.
TABLE 1. Sample sizes for the three fear conditioning paradigms

<table>
<thead>
<tr>
<th>Groups</th>
<th>Context foreground conditioning</th>
<th>Cued conditioning</th>
<th>Trace conditioning</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 shock</td>
<td>3 shocks</td>
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</tr>
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<td>Male mutant</td>
<td>8</td>
<td>6</td>
<td>6 (0s-trace)</td>
</tr>
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</tr>
<tr>
<td>Female mutant</td>
<td>8</td>
<td>6</td>
<td>6 (0s-trace)</td>
</tr>
<tr>
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</table>

Table 1. The numbers of subjects employed in each of three conditioning experiments are summarized here according to the between-subjects factors: genotype (mutant vs. control), sex (male vs. female) and trace interval (0s vs. 20s).

Apparatus

The apparatus has been fully described before (Pietropaolo et al., 2007). Briefly, two sets of four conditioning chambers installed in separate testing rooms were used to provide two distinct contexts. The first set of chambers (context ‘A’) comprised four Coulbourn Instruments (Allentown, PA, USA) operant chambers (Model E10-10) each equipped with a grid floor made of stainless steel rods (4 mm in diameter) spaced at an interval of 10 mm centre to centre, and through which scrambled electric shocks [unconditioned stimulus (US)] could be delivered (model E13–14; Coulbourn Instruments, Allentown, PA). A transparent Plexiglas enclosure confined the animals to a rectangular region (17.5×13 cm). The inside of the chambers was illuminated by a house light (2.8 W) positioned on the panel wall, 21 cm above the grid floor. The second set of chambers (context ‘B’) comprised four cylindrical (19 cm in diameter) enclosures made of clear Plexiglas resting on a metal mesh floor. Illumination inside the chamber was provided by an infrared light source instead of a visible light. The conditioned stimulus (CS) in experiment 2 and 3 was an 86 dBA tone provided by a sonalert (model SC628; Mallory, Indianapolis, IN). Each of the eight chambers contained a miniature digital camera mounted 30 cm directly above the centre of the area of interest. A digital camera was mounted 30 cm directly above the area of interest. The algorithm of the freezing response detection procedure has been validated and fully described before (Richmond et al., 1998). If the number of pixel difference
between adjacent frames was less than 0.05% of the total number of pixels in a frame, the animal was considered to be freezing in that 1-s interval.

Experiment 1: Delayed conditioning with extended retention interval

The experiment consisted of three phases: conditioning, context-test and CS-test. On day 1, conditioning took place in context ‘A’ and comprised three discrete trials of CS-US pairing. Each trial began with a 30s tone CS followed immediately by the delivery of a 1s foot-shock set at 0.3mA. Each trial was preceded and followed by a 180-s ITI. The test of contextual freezing took place on day 2 when the subjects were returned to context ‘A’ for a period of 8min in the absence of any discrete stimulus. The test of conditioned response (CR) to the CS was conducted on day 3 in the neutral context ‘B’. Following a 120-s acclimatization period, the CS was presented for 8min; freezing behaviour during this period of CS presentation was evaluated. The pre-CS and CS periods were analyzed separately. To assess between-day extinction of CS freezing, the CS test was repeated on day 4 and 5.

Experiment 2: Context foreground conditioning

The procedure has been fully described elsewhere (Pietropaolo et al., 2007). The test comprised three stages: (i) conditioning to context ‘A’, (ii) assessment of conditioned responding to the shocked context ‘A’ and (iii) evaluation of freezing behaviour to the neutral context ‘B’. On day 1, the animals received either one or three unsignaled electrical foot shocks (1s, 0.3 mA) in context ‘A’. The first shock-delivery was administered 3 minutes after the animals were placed into the chambers. Successive shocks deliveries were administered every 3 min. The conditioning session was concluded with a final shock-free 3-min interval. On day 2, all mice were returned to context ‘A’. They were placed in the chambers for 4 min and freezing levels were recorded in the absence of any further stimulus. On day 3, mice were given a similar 4 min non-stimulus testing session in the neutral context ‘B’. A similar ‘A’-‘B’ test block was conducted on days 4 and 5, respectively. This procedure served the purpose of analyzing the freezing response specifically directed towards the conditioned context ‘A’ versus the freezing response to the neutral context ‘B’ induced by generalization.

Experiment 3: Trace conditioning

For the assessment of trace fear conditioning, the animals of each of the four groups (genotype x sex) were randomly allocated into one of two conditions (see also Yee et al., 2004): 0s-trace and 20-s trace. On day 1, all animals were given three trials of CS–US pairings in context ‘A’. In the ‘0s-trace’ condition, offset of the CS (30s-tone) was immediately followed by the foot-shock US (1s, 0.3mA). In the’ 20-s trace’ condition, the CS and US were separated by a 20-s trace interval. Each trial was preceded and followed by a 180 s inter-trial interval (ITI). On day 2, the animals were returned to context ‘A’ for a period of 8 min in order to examine conditioned responding to the context. On day 3,
CS freezing to the tone stimulus was assessed in the neutral context ‘B’. After a 2 min acclimatization period in absence of any discrete stimulus, the tone-CS was presented for 8 min and freezing behaviour to the tone stimulus during this period was evaluated.

Statistical analysis

All data were analysed by parametric analysis of variance ANOVA using genotype (mutant vs. littermate control), sex (male vs. female) and trace interval (0s vs. 20s) as the between-subjects factors. Additional within-subjects factors (e.g. blocks, days, ISIs, 1-min bins) were included according to the nature of the considered dependent variables. Data interpretation was further assisted by Fisher’s least significant difference (LSD) post hoc comparisons and the use of restricted ANOVAs whenever appropriate. All statistical analyses were carried out using SPSS for Windows (version 13, SPSS Inc. Chicago IL, USA) implemented on a PC running the Windows XP (SP2) operating system.

RESULTS

Delayed conditioning with extended retention interval.

CamKIIαCre/GlyT1: During conditioning, freezing times in the presence of the tone-CS steadily increased across the three pairings (Figure 1a). Mutants appeared to freeze more than controls, especially in the second and third pairings when the tone-CS was expected to generate conditioned responding. A 2 x 2 x 3 (genotype x sex x pairing) ANOVA of percent freezing revealed a significant main effect of pairing \[F(2,42) = 15.80; \ p < 0.001\] but there was only a suggestive tendency for a genotype effect \[F(1,21) = 3.23; \ p = 0.087\]. Similarly, restricted analysis at each paring failed to reveal any significant difference between the two genotypes. The factor sex and any of its interactions never attained statistical significance.

As illustrated in Figure 1b, assessment of context freezing on day 2 revealed comparable freezing times between mutants and controls. There was a monotonic increase in the freezing level that peaked at the second bin, which then gradually subsided across the following bins until the end of the test period. ANOVA of percent freezing across the eight 1-min bins yielded a significant main effect of bins \[F(7,147) = 12.73; \ p < 0.001\]. Neither the effect of genotype nor its interaction with bins attained statistical significance. Furthermore, male subjects seemed to freeze less than female subjects. This was however not supported statistically. There was only a non-significant trend towards sex effect \[F(1,21) = 3.84; \ p = 0.063\].

Conditioned responding to the tone-CS and the extinction of the CR was examined on days 3-5, which is depicted in Figure 1c. Irrespective of genotype and sex, baseline freezing behaviour in the pre-CS phase was generally low and did not change across days. A 2 x 2 x 3 x 2 (genotype x sex x day x 1-min bins) ANOVA of percent freezing failed to generate any significant effects. The mean percent freezing values (±SEM) in the pre-CS phase were: mutant: day1 = 2.9 ± 1.3%, day2 = 5.1 ± 1.3%, day3 = 4.6 ± 1.7%; control: day1 = 3.1 ± 1.1%, day2 = 3.1 ± 0.9%, day3 = 2.8 ± 0.9%. CS-freezing
was consistently increased in the mutants relative to controls across the three tone tests. The reduction of freezing over days indicates extinction of CR, and it was seen in both mutants and controls. Freezing time also decreased as a function of bins within a day reflecting within-session extinction of the CR. Again, this was equally observed in mutants and controls. These impressions were consistent with a 2 x 2 x 3 x 8 (genotype x sex x day x 1-min bins) ANOVA of percent freezing which gave rise to a significant main effect of genotype \( F(1,21) = 4.62; p = 0.043 \), days \( F(2,42) = 23.02; p < 0.001 \) and bins \( F(7,147) = 24.86; p < 0.001 \) as well as a significant interaction between days and bins \( F(14,294) = 8.93; p < 0.001 \). The factor sex and its interactions failed to attain statistical significance.

Figure 1. Conditioning with 21-day interval in the \textit{CamKII\alpha Cre/GlyT1} line. The acquisition of conditioned freezing to the tone-CS on day 1 (a), context freezing on day 2 (b) and test of CS freezing on days 3, 4 (c) are depicted. (a) The freezing levels generally increased across the three CU-US pairings. Mutants appeared to freeze more than controls which was however not supported statistically. ANOVA of freezing revealed only a non-significant tendency towards a genotype effect \( p > 0.08 \). (b) Conditioned responding to the context was comparable between mutants and controls. (c) CS-freezing was consistently increased in the mutants relative to controls across the three tone tests. The freezing levels gradually deceased between days as well as across bins within a day, reflecting extinction of the conditioned tone freezing. However, the rate of within-day and between-day extinction was comparable between mutants and controls indicating that enhanced tone freezing in the mutants was no compromised by an extinction deficit. These impressions were supported by the appearance of a significant main effect of genotype \( p < 0.05 \) in a 2 x 2 x 3 x 8 (genotype x sex x day x 1-min bins) ANOVA of percent freezing. All values refer to mean ± SEM. * denotes a significant difference between mutants and controls \( p < 0.05 \).
**EMX-1αCre/GlyT1**: The level of freezing gradually increased across the three CS-US pairings which was equally seen in mutants and controls (Figure 2a). A 2 x 2 x 3 (genotype x sex x pairing) ANOVA of percent freezing revealed only a significant main effect of pairing \[F(2,50) = 25.63; p < 0.001\].

Similarly, context freezing did not differ between the two genotypes. The freezing levels reached its peak at the 4th bins and then continually decreased till the end of the 8 min test period (Figure 2b). This gave rise to a significant main effect of bins \[F(7,175) = 2.63 ; p < 0.05\] in a 2 x 2 x 8 (genotype x sex x bins) ANOVA of percent freezing.

![Figure 2](image)

**Figure 2**: Conditioning with 21-day interval in the EMX-1αCre/GlyT1 line. The acquisition of conditioned freezing to the tone-CS on day 1 (a), context freezing on day 2 (b) and test of CS freezing on days 3.4 (c) are depicted. (a) The freezing levels generally increased across the three CU-US pairings (b) There was not genotype specific difference on context freezing. (c) Mutants and controls showed highly similar freezing behaviour across the three CS-tests. The freezing level generally decreased across days reflecting extinction of the freezing response. The freezing level also gradually decreased across bins in the first two CS-tests but it rebounded at the end of the third CS-test which is likely to reflect habituation of locomotor/exploratory activity. All values refer to mean ± SEM.
Conditioned freezing to the tone-CS across the three CS-tests was assessed next. Baseline freezing in the pre-CS phase was generally very low but slightly increased across the two 1 min bins within a day as well as between days. This was supported by a significant main effect of bins and days \([F(2,50) = 12.06; p < 0.005]\) in a 2 x 2 x 3 x 2 (genotype x sex x day x 1-min bins) ANOVA. The mean percent freezing values (±SEM) in the pre-CS phase were: mutant: day1 = 1.6 ± 0.9%, day2 = 2.2 ± 0.8%, day3 = 4.0 ± 1.2%; control: day1 = 1.5 ± 0.6%, day2 = 2.1 ± 0.6%, day3 = 3.8 ± 1.0%. The freezing response across the three CS-tests is depicted in Figure 2c. Mutants and controls showed highly similar freezing behaviour. The freezing level generally decreased across days reflecting extinction of the freezing response. The freezing level also gradually decreased across bins in the first two CS-tests but rebounded at the end of the third CS-test which is might reflect habituation of locomotor/exploratory activity. This was in line with a 2 x 2 x 3 x 8 (genotype x sex x day x 1-min bins) ANOVA of percent freezing yielding a significant main effect of days \([F(2,50) = 3.97; p < 0.05]\) and bins \([F(7,175) = 28.67; p < 0.001]\) as well as the interaction between the two factors \([F(14,350) = 4.70; p < 0.001]\). There was no indication of a significant effect of sex or its interactions throughout the experiment.

**Context foreground conditioning**

**CamKIIαCre/GlyT1**

*One-shock paradigm:* Freezing behaviour in the inter-stimulus-interval (ISI) before and after the foot shock in the conditioning phase is depicted in Figure 3a. As expected, the freezing increased after the presentation of the foot shock which was supported by the emergence of a significant effect of ISI \([F(1,27) = 42.09; p < 0.001]\) in a 2 x 2 x 2 (genotype x sex x ISI) ANOVA of percent freezing. Although mutants appeared to freeze more than controls in the post-shock ISI restricted analysis to the critical ISI revealed only a non-significant trend towards a genotype effect (p=0.11).

The animals’ freezing response to the conditioning context ‘A’ and the neutral context ‘B’ was evaluated on days 2 to 5. A 2 x 2 x 2 x 2 (genotype x sex x block x context) ANOVA of percent time freezing was carried out to analyze the data. Irrespective of genotype and sex, the animals showed a much stronger freezing response to the shocked context ‘A’ than to the neutral (non-shocked) context ‘B’ (see Figure 3b). Subsequent re-exposure to the context ‘A’ on day 4 led to a re-emergence of the freezing response while re-exposure to context ‘B’ on days 5 again elicited little freezing in the animals. This pattern of results indicates that the freezing response to context ‘A’ represents associative learning between this specific context and its pairing with the unconditioned foot shock stimulus (US) and is unlikely to stem from a non-specific fear response. However, the conditioned freezing response to context ‘A’ was clearly enhanced in the mutants as compared to controls while the two groups of mice exhibited a comparable level of freezing in context ‘B’. This gave rise to a significant main effect of genotype \([F(1,27) = 4.53; p < 0.05]\) and context \([F(1,27) = 75.80; p < 0.001]\), as well as a significant interaction of these two factors \([F(1,27)=5.75; p<0.05.].\) Separate ANOVAs of
freezing for each context confirmed that the mutants froze significantly more than controls in the shocked context ‘A’ [F(1,27)=5.24; p=0.03.] but not in the neutral context ‘B’ [F(1,27)=0.20; p>0.6].

**Three-shock paradigm:** In the conditioning phase, freezing time monotonically increased across successive ISI periods, which was equally seen in mutants and controls (Figure 3a). This was confined by a 2 x 2 x 4 (genotype x sex x ISI) ANOVA of percent time freezing yielding a significant main effect of ISI [F(3,60)=44.16; p<0.001].

Context freezing on days 2-5 did not differ between genotypes (Figure 3b). Both groups showed a higher level of freezing to the shocked context ‘A’ relative to the neutral context ‘B’ yielding a highly significant main effect of context [F(1,20) = 53.91; p < 0.001] in a 2 x 2 x 2 x 2 (genotype x sex x block x context) ANOVA of percent freezing. The factor sex yielded no significant effects or interactions throughout the experiment.

![Figure 3](image-url)

**Figure 3:** Context foreground conditioning in the CamKIIαCre/GlyT1 line. (a) Freezing time steadily increased in mutants and controls across ISI-periods in the conditioning phase, and this was seen in the 1-shock and the 3-shock condition, respectively. (b) When freezing to the context was evaluated (days 2 to 5), mutants exhibited an increased freezing response to the shocked context ‘A’ relative to controls in the 1-shock condition but not in the 3-shock conditions. The freezing levels in the neutral context ‘B’ never differed between the two genotypes and were generally much lower than in context ‘A’. All values refer to mean ± S.E.M. * denotes a significant difference between mutants and controls (p<0.05).
**EMX-1Cre/GlyT1**

**One-shock paradigm:** During conditioning, the level of freezing was generally low in the ISI before the shock (Figure 4a). After the shock, the animals spent more time immobile which was more pronounced in the controls. ANOVA of percent freezing across the two ISIs yielded a significant effect of ISI \([F(1,27)=13.77; \ p=0.001]\) but only a non-significant trend towards an effect of genotype \([F(1,27)=3.39; \ p=0.077]\). Restricted analysis to the post-shock ISI also failed to reveal a significant difference between mutants and controls \((p=0.088)\).

Context freezing days 2-5 did not differ between mutants and controls (Figure 4b). Irrespective of genotype and sex, the animals froze more in the shocked context ‘A’ than in the neutral context ‘B’. This was in line with a highly significant main effect of context \([F(1,27)=32.73; \ p<0.001]\). Neither the effect of genotype nor any of its interactions attained statistical significance throughout.

**Figure 4:** Context foreground conditioning in the EMX-1Cre/GlyT1 line. Freezing time steadily increased in mutants and controls across ISI-periods in the conditioning phase, and this was seen in the 1-shock (a) and the 3-shock (b) condition, respectively. Similarly, context freezing (days 2-5) did not differ between the two genotypes independent of whether the animals received 1 (c) or 3 (d) unsignaled shocks during conditioning. The freezing levels in the neutral context ‘B’ never differed between the two genotypes and were generally much lower than in context ‘A’. All values refer to mean ± S.E.M.
Three-shock paradigm: In the conditioning phase, the amount of freezing increased as a function of ISI [F(3,60)=43.06; p<0.001] and this phenomenon was similarly observed in mutants and controls (Figure 4a). Similarly, context freezing on days 2-5 did not differ between the two genotypes (Figure 4b). Both mutants and controls froze more in the shocked context ‘A’ as compared to the neutral context ‘B’ [F(1,60)= 90.79; p<0.001].

Trace conditioning

CamKIIαCre/GlyT1: First, we examined the amount of freezing in the presence of the CS across the three trials of CS-US pairings, which is depicted in Figure 5a. The level of freezing to the tone-CS increased as a function of trials and this increment in the freezing time was slower in the 20s-trace relative to the 0s-trace condition which was equally observed in mutants and controls. These impressions were confirmed by a 2 x 2 x 2 x 3 (genotype x sex x trace interval x pairing) ANOVA of percent freezing which revealed a significant main effect of trace interval [F(1,50) = 10.39; p = 0.002] and pairing [F(2,100) = 35.38; p < 0.001] and its interaction [F(2,100) = 8.03; p = 0.001]. Restricted analysis at each pairing indicated that the trace effect only attained significance on trial 3 [F(1,50) = 13.38; p = 0.001]. In addition, male subjects generally spent more time immobile than female subjects [F(1,50) = 12.41; p = 0.001].

The animals’ freezing response to the context was evaluated 24h after conditioning. There was little difference in the amount of freezing amongst groups throughout the duration of the test (Figure 5b). The animals exhibited a relatively strong freezing response at the beginning of the test which steadily decreased across the 8 min testing period. This was consistent with a 2 x 2 x 2 x 8 (genotype x sex x trace-interval x 1-min bins) ANOVA of percent freezing which revealed only a significant main effect of bins [F(7,350) = 9.39; p < 0.001]. There were no indications for a significant sex effect or its interactions.

Conditioned response to the tone was evaluated in a novel context (context ‘B’) another 24 h later. During the first 2 min, baseline freezing behaviours were evaluated in the absence of any discrete stimuli (Figure 5c). Freezing levels were generally low and did not differ much across days. A 2 x 2 x 2 x 2 (genotype x sex x trace interval x 1-min bins) ANOVA of percent freezing yielded only a significant main effect of bins [F(1,50) = 43.94; p < 0.001]. CS freezing to the tone was examined over the next 8 min. As illustrated in Figure 5c, the amount of freezing was clearly reduced in the 20s-trace relative to the 0s-trace condition reflecting the trace effect. This effect of trace interval upon conditioned tone freezing was more pronounced in the mutants, which exhibited more intense freezing in the 0s-trace condition but spent less time immobile in the 20s-trace condition as compared to the controls. These observations were supported by A 2 x 2 x 2 x 8 (genotype x sex x trace interval x 1-min bins) ANOVA which revealed a significant main effect of trace interval [F(1,50) = 33.22; p < 0.001] and bins [F(7,350) = 6.41; p < 0.001] and as well as a significant interaction between genotype and trace interval [F(1,50) = 4.60; p = 0.037]. This was accompanied by a significant three-way interaction of genotype, trace intervals and bins [F(7,350) = 2.32; p = 0.03]. Post hoc comparisons revealed a significant difference between mutant/0s-trace and mutant/20s-trace as well as between
control/0s-trace and control/20s-trace (p’s < 0.05) indicating the presence of the trace effect in both genotypes. However, mutant/0s-trace and control/0s-trace as well mutant/20-trace and control/20s-did not significantly differ.

Figure 5: Trace conditioning in the CamKIIαCre/GlyT1 line. The acquisition of conditioned freezing to the tone-CS on day 1 (a), context freezing on day 2 (b) and test of CS freezing on day 3 (c) are depicted. (a) Percent freezing in the presence of the tone-CS generally monotonically increased across the three CU-US pairings. The freezing level increased at a higher rate in the 0s-trace relative to the 20s-trace condition reflecting the ‘trace effect’. (b) The animals were returned to the conditioning context in the absence of the CS 24 h later. The level of percent freezing is expressed as a function of 1-min bins, which was comparable amongst groups. (c) After a 2 min CS-free period, the CS was presented for 8 min. Percent freezing was expressed as a function of 1-min bins. Baseline freezing behaviour in the pre-CS phase did not differ between groups. In the CS-phase, percent freezing was markedly reduced in animals conditioned with a 20s-trace interval. This phenomenon was more pronounced in the mutants, which showed increased freezing levels in the 0s-trace condition but reduced freezing times in the 20s-trace condition relative to controls. This gave rise to a significant genotype by trace interval interaction (p<0.05) in an ANOVA of percent freezing across the eight 1-min bins. All values refer to mean ± SEM.
**EMX-1αCre/GlyT1:** The development of the conditioned freezing response across the three CS-US pairings is depicted in Figure 6A. The amount of freezing to the tone-CS steadily increased across pairings, which was less evident in animals conditioned with a 20s- than with a 0s-trace interval. Mutants showed increased freezing levels in the 0s-trace condition relative to controls. ANOVA of percent freezing across the 3 pairings yielded a significant main effect of trace interval \[F(1,43) = 21.07; \ p < 0.001\] and pairing \[F(2,86) = 16.18; \ p < 0.001\] This was accompanied by a significant interaction of genotype and trace interval \[F(1,43) = 6.78; \ p < 0.05\], as well as genotype, trace interval and pairing \[F(2,86) = 10.53; \ p < 0.005\]. Post hoc comparison revealed that mutant/0s-trace mice exhibited a significantly higher level of freezing than control/0s-trace mice \(p < 0.05\).

The context freezing test on day 2 was uneventful. The freezing levels were comparable between groups over the 8 min test period (Figure 6B). ANOVA of percent freezing revealed only a significant main effect of bins \[F(7,301) = 3.99; \ p < 0.05\].

Assessment of CS freezing took place on the next day (Figure 6C). The amount of baseline freezing in the 2 min before CS onset was generally very low. Nevertheless, 20s-trace animals spent more time freezing than 0s-trace animals, which was seen in both mutants and controls. This was confirmed by a significant main effect of trace interval \[F(1,43) = 4.30; \ p < 0.05\] in a 2 x 2 x 2 x 2 (genotype x sex x trace interval x 1-min bins) ANOVA of percent freezing. Conditioned responding to the tone CS was evaluated over the next 8 min. Similar to the previous experiment, the amount of freezing was substantially reduced in the 20s-trace condition relative to the 0s-trace condition. However, there was no indication for any difference between mutants and controls. ANOVA of percent freezing across the 8min test period revealed a significant main effect of trace interval \[F(1,43) = 8.72 \ p=0.005\] and bins \[F(7,301) = 12.19; \ p < 0.001\] as well as a significant interaction of the two factors \[F(7,301) = 7.22; \ p < 0.001\].
Figure 6: Trace conditioning in the EMX-1aCre/GlyT1 line. The acquisition of conditioned freezing to the tone-CS on day 1 (a), context freezing on day 2 (b) and test of CS freezing on day 3 (c) are depicted. (a) The freezing levels steadily increased across the three CU-US pairings, which was less evident in the 20s-trace condition than in the 0s-trace condition. This phenomenon was more evident in the mutants which showed increased freezing in the 0s-trace condition but reduced freezing in the 20s-trace condition as compared to controls. This gave rise to a significant genotype x trace interval interaction (p<0.005) in an ANOVA of percent freezing. (b) Context freezing across the 8 min period was not affected by the factors genotype and trace interval. (c) Baseline freezing levels in the pre-CS phase were rather low and did not differ between mutants and controls. However, 20s-trace animals spent more time freezing than 0s-trace animals which was consistent with a significant main effect of trace interval (p<0.05) in a 2 x 2 x 2 x 2 (genotype x sex x trace interval x 1-min bins) ANOVA of percent freezing. In the CS-phase, percent freezing was markedly reduced in animals conditioned with a 20s-trace interval relative to 0s-trace interval conditioned animals, which was equally seen in both genotypes. ANOVA of percent freezing across the 8-min period revealed a significant effect of trace interval (p<0.05). All values refer to mean ± SEM.
DISCUSSION

The present study provides clear evidence that restricted GlyT1 deletion to forebrain neurons exerts multiple effects on Pavlovian fear conditioning. The mutation enhanced both the magnitude and persistence to decay of conditioned tone freezing and facilitated conditioning to a novel context in the absence of a discrete CS. However, such enhanced conditioned responding was not at the expense of selectivity in associative learning as evidenced by an increased sensitivity to the trace conditioning effect in the CamKIIαCre/GlyT1 knockout mice. By contrast, these effects were completely absent in the EMX-1Cre/GlyT1 mice which lack GlyT1 in neurons and glia thus providing further evidence for a functional dissociation between neuronal and non-neuronal GlyT1 populations in associative learning. Importantly, the emergence of these effects is unlikely to be due to potential changes in locomotor activity or the expression of unconditioned fear in the two GlyT1 knockout mouse lines. We have previously shown that both locomotor activity in the open field and anxiety related behaviour in the elevated plus maze were not altered by neuron-specific or complete GlyT1 deletion in the forebrain (Yee et al., 2006, Singer et al., submitted)

Forebrain neuronal GlyT1 disruption leads to enhanced long-term retention of cued fear conditioning

We have previously demonstrated CamKIIαCre/GlyT1 knockouts showed enhanced freezing to a tone-CS and they learned to avoid a foot shock US signalled by a noise-CS at a faster pace than the controls in a two-way active avoidance paradigm (Yee et al., 2006) indicating the mutation enhanced the magnitude of the CR as well as its acquisition. Here we found that the mutation also enhanced long term retention of the conditioned tone freezing. Given that complete forebrain specific GlyT1 deletion was completely devoid of an effect in these tests the present findings strongly indicate that specific GlyT1 deletion in forebrain neurons as opposed to cell-type non-specific loss of GlyT1 is critical to achieve enhanced conditioned responding to discrete stimuli, such as a tone. Given that forebrain neuronal (Yee et al., 2006) but not complete forebrain specific GlyT1 disruption (Singer et al., submitted) potentiated hippocampal NMDAR currents it has been argued that the presence or absence of enhanced conditioning may be linked to whether hippocampal NMDAR-mediated currents were enhanced or not, because hippocampal NMDARs are known play an important role in associative learning processes (e.g. Gruart and Delgado-Garcia, 2007, Valenzuela-Harrington et al., 2007). However, although there is convincing evidence for an involvement of the hippocampus, in particular the ventral part, in simple cued fear conditioning (Bast et al., 2001, 2003) it has been suggested that hippocampal mechanisms are particularly important for the formation of complex representation of stimuli and their spatial and/or temporal relation which is not essential for the formation of simple associative links between an US and a discrete stimulus (Anagnostaras et al., 2001). A key structure in cued fear conditioning is the amygdala which is central in the procession of emotionally significant stimuli (Weinberger 1993; LeDoux 1998; Morris et al. 1999). Given the strong connectivity between...
the ventral hippocampus and the amygdala (e.g. Petrovich et al., 2001) it is tempting to propose that enhanced conditioned responding to a discrete CS in the CamKIIαCre/GlyT1 mice may be due to glutamatergic changes in both the ventral hippocampus and the amygdala.

**Forebrain neuronal GlyT1 disruption marginally enhances contextual fear conditioning**

There is convincing evidence that fear conditioning to the environmental context is closely regulated by NMDAR mediated processes in the dorsal hippocampus (Fanselow et al., 1994; Young et al., 1994; Anagnostaras et al., 2001; Gale et al., 2001) which are essential for context representation. Enhanced contextual conditioning in the absence of any discrete stimuli has also been observed in the CamKIIαCre/GlyT1 knockouts but this was only seen in the one-shock paradigm and not in the three-shock paradigm. In addition, the effect only reached significance on the first re-exposure to the conditioning context (day 2, in Figure 3) but not on the second re-exposure on day 4. Although not statistically supported, but a similar pattern of results emerged in the EMX-1Cre/GlyT1 mice on the 3-shock paradigm (Figure 4). Actually, the magnitude of the effect was even higher in the EMX-1Cre/GlyT1 knockouts as compared to the CamKIIαCre/GlyT1 mice. Furthermore, when comparing the control groups of the two knockout lines it appears that CamKIIαCre/GlyT1 controls froze less than the EMX-1Cre/GlyT1 controls particularly on the day when the CamKIIαCre/GlyT1 knockouts showed an enhancement. Although statistical analysis of the two control groups did not reveal any statistical significant difference on the critical day (data not shown) the apparent difference between the two control groups constitutes a potential confound when comparing the two GlyT1 knockout lines. From this it follows that the effect of forebrain specific GlyT1 deletion on contextual condition is weak which contrasts with the robust enhancement of cued conditioning. This discrepancy also supports the hypothesis that effects of forebrain neuronal GlyT1 deletion may not be solely mediated via enhanced NMDAR function in the hippocampus but that effects in other brain regions may contribute to the observed facilitation on cued conditioning. To further advance in this direction selective GlyT1 disruption restricted to the critical brain regions would be required.

CamKIIαCre/GlyT1 mice also showed a tendency towards increased context freezing when the context was in the background (experiment 1 and 3) because the discrete tone-CS was the best predictor of the US in these experiments. In contrast, such a tendency was completely absent in the EMX-1Cre/GlyT1. This observation may indicate that CamKIIαCre/GlyT1 mice are indeed more responsive to contextual cues in Pavlovian conditioning and certainly deserves further investigation.

**GlyT1 disruption in forebrain does not affect extinction learning**

A frequently used approach to assess extinction learning is to repeatedly present the CS without the antecedent US (Pavlov 1927). Extinction learning is commonly understood as a form of learning implying the acquisition of new information, namely that the CS is no longer a predictor of the US whereby the previously formed CS-US association remains intact. This mechanism has been
incorporated into a large number and variety of theories (e.g., Bouton 1993, Pearce 1987, Wagner 1981). In addition to associative mechanisms, non-associative mechanisms, such as habituation processes (McSweeney and Swindell 2002), may also contribute to extinction. NMDAR-mediated processes in the hippocampus, especially in CA1 region (Szapiro et al., 2003) as well as in the basolateral amygdala (Falls 1992) play an important role in extinction learning. In addition, long term potentiation in the prefrontal cortex is also critically involved in extinction of conditioned fear (Herry and Garcia 2002). While NMDAR blockade impairs extinction learning (Baker and Azorlosa 1996) stimulation of NMDAR by the partial NMDAR glycine-B site agonist D-cycloserine facilitated extinction learning (Myers and Davis 2007). On this basis one may expect that CamKIIαCre/GlyT1 mice would also show enhanced extinction learning. However, our results demonstrated that neither complete nor neuron specific GlyT1 deletion in forebrain affected extinction learning. Both extinction of the CS-US association in the conditioned tone freezing paradigm as well as extinction to the conditioning context in the context foreground conditioning paradigm was highly comparable between mutant and controls in both knockout lines. This indicates that inhibition/deletion of GlyT1 in the forebrain facilitates the CS-US formation but does not affect its extinction.

**GlyT1 disruption in forebrain enhances the sensitivity to temporal CS-US discontiguity**

We have previously shown that neuron but not complete forebrain specific GlyT1 deletion led to the expression of LI under pre-exposure conditions that were insufficient to yield LI in the controls (Yee et al., 2006, Singer et al., submitted) indicating an increased sensitivity to the reinforcement history of the CS in these mutant mice. This unique phenotype demonstrates that enhanced conditioning in the CamKIIαCre/GlyT1 mice did not lead to an indiscriminate strengthening in associative links thereby compromising selectivity in associative learning. Selectivity with respect to the associative history of the CS was enhanced in the CamKIIαCre/GlyT1 mice. Another way to demonstrate selectivity in associative learning is the trace conditioning paradigm in which the introduction of a time interval - a so-called trace interval – between the offset of the CS and the onset of the US normally reduces conditioning. It has been found that the CamKIIαCre/GlyT1 mice were more sensitive to the CS-US disconguity than the controls and this was again observed against the background of enhanced conditioning in the 0s-trace condition. Hence forebrain neuronal GlyT1 deletion enhances tone-freezing as well as its non-freezing implying that enhanced freezing at the 0s-trace condition does not represent an indiscriminate enhancement of associative strength. The results provide evidence of increased sensitivity to parameters affecting the generation of the conditioned response which could be interpreted as enhanced flexibility in learning. This is consistent with our most recent data showing that forebrain neuronal GlyT1 deletion also led to enhanced spatial and non-spatial reversal learning (Singer et al., submitted) which may be interpreted as enhanced adaptability to changes in the conditions that lead to a current goal. In contrast, none of these effects was observed in the EMX-
CRE/GlyT1 knockouts, which highlight the importance and value in distinguishing between neuronal and non-neuronal GlyT1 in the modulation of cognitive behaviour.

CONCLUSIONS

The present findings demonstrate that specific deletion of GlyT1 in forebrain neurons enhances aversive conditioning to discrete stimuli and to the context but did not affect extinction learning. In addition, sensitivity to the CS-US discontiguity was increased in forebrain neuronal GlyT1 knockout mice indicating that the mutation did not lead to mere strengthening in any associative links but also enhances selectivity in associative learning. The finding that these effects were not observed following complete (neuronal and glia) forebrain specific GlyT1 points towards a functional dissociation of neuronal and non-neuronal GlyT1 populations in the regulation of associative learning. This is highly instructive for the development of cognition modulating agents for the clinical and non-clinical use.
REFERENCES


Lipina T, Labrie V, Weiner I, Roder J (2005) Modulators of the glycine site on NMDA receptors, D-serine and ALX 5407, display similar beneficial effects to clozapine in mouse models of schizophrenia. Psychopharmacology (Berl) 179:54–67.
Chapter 6: The effects of forebrain GlyT1 disruption, in neurons or in neurons and glia, on aversive fear conditioning and its sensitivity to the temporal CS-US discontiguity


Chapter 7

The glycine transporter 1 inhibitor SSR504734 enhances working memory performance in a continuous delayed alternation task in C57BL/6 mice

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As submitted in Psychopharmacology (revised)

[Personal contribution to the work: Designing and performing the research, analysis of the behavioural data, and writing the manuscript]
Abstract

Rationale: Inhibition of the glycine transporter 1 (GlyT1) activity increases extracellular glycine availability in the CNS. At glutamatergic synapses, increased binding to the glycine-B site located in the N-methyl-D-aspartate receptor (NMDAR) can enhance neurotransmission via NMDARs. Systemic treatment of 2-chloro-N-[(S)-phenyl[(2S)-piperidin-2-yl]methyl]-3-trifluoromethyl benzamide, monohydrochloride (SSR504734), a selective GlyT1 inhibitor is effective against social recognition impairment induced by neonatal phencyclidine treatment, and enhances prepulse inhibition in a mouse strain (DBA/2) with intrinsic sensorimotor gating deficiency, suggesting that SSR504734 may be an effective cognitive enhancer.

Objective: To examine if SSR504734 exhibits a promnesic effect on working memory function in wild type C57BL/6 mice using an automatic continuous alternation task.

Methods: Hungry mice were trained to alternate their nose pokes between two food magazines across successive discrete trials in an operant chamber, in order to obtain food reward. Correct choice on a given trial thus followed a non-matching or win-shift rule in relation to the preceding trial, with manipulation of the demand on memory retention, by varying the delay between successive trials.

Results: Pre-treatment with SSR504734 (30mg/kg, i.p.) improved choice accuracy when the delay from the previous trial was extended to 12-16s. Furthermore, a dose-response analysis (3, 10, 30mg/kg) revealed a clear dose-dependent efficacy of the drug: 3mg/kg was without effect, whilst 10mg/kg led to an intermediate enhancement in performance.

Conclusion: The present findings represent the first demonstration of the promnesic effects of SSR504734 under normal physiological conditions, lending further support to the suggestion of its potential as a cognitive enhancer.
Chapter 7: The glycine transporter 1 inhibitor SSR504734 enhances working memory performance in a continuous delayed alternation task in C57BL/6 mice

INTRODUCTION

The hypothesis that N-methyl-D-aspartate receptor (NMDAR) activation plays a central role in the neural plasticity underlying learning and memory (Morris 1989, Collingridge and Bliss 1995, Tang et al. 1999) has generated growing interest in agents that can enhance NMDAR activation as potential therapeutics against cognitive deficits caused by diseases, and their potential use as cognitive enhancers in otherwise healthy individuals (Sahakian and Morein-Zamir, 2007). Because the simultaneous binding of both glutamate and glycine to NMDAR is mandatory for NMDAR ion channel opening (McBain and Mayer 1994), both the glutamate binding site located on the NMDAR NR2 subunit, and the glycine binding site (glycine-B site) on the NR1 subunit constitute potential targets to enhance NMDAR activation. Due to a number of undesirable side effects associated with competitive NMDAR agonists acting at the glutamate site (Danysz and Parson 1998), a current focus is on targeting the glycine-B site. It has long been suggested that the glycine-B sites are not saturated under physiological conditions (Danysz et al., 1987, 1989), and the subsequent confirmation of this hypothesis (Berger et al., 1998; Bergeron et al. 1998, Chen et al. 2003) has led to the suggestion that NMDAR activation can be facilitated by increasing glycine-B site occupancy. In support of this hypothesis, glycine-B site agonist (glycine and D-serine) as well as partial agonist (D-cycloserine) treatment, can improve the therapeutic efficacy of conventional antipsychotic medications against negative and cognitive symptoms of schizophrenia (Coyle and Tsai 2004, Heresco-Levy 2000) that are closely linked to NMDAR hypofunction (Olney and Farber 1995). Whilst there is no convincing evidence that glycine-B site agonists or partial agonists improve cognitive functions in healthy humans, this has however been demonstrated in laboratory animals in tests of associative learning, working memory, reversal learning and familiarity judgment (Monahan et al. 1989, Pussinen and Sirviö 1999, Duffy et al. 2008, Nunnink et al. 2007, Zlomuzica et al. 2007).

One potential limit to the use of glycine or D-serine to potentiate NMDAR activation is that high systemic doses are required to significantly elevate their availability within the CNS, because they are extensively metabolised and pass poorly through the blood-brain barrier (D’Souza et al. 2000). Furthermore, existing synthetic glycine-B site agonists have relatively low affinity in comparison to the endogenous agonists, glycine and D-serine (Milan 2002, Bräuner-Osborne et al. 2000). Indeed, it has been argued that the simple molecular structure of glycine in itself poses a limitation on the development of novel synthetic analogues (Milan 2002).

Alternatively, glycine-B site activation can be enhanced indirectly through pharmacological blockade of glycine transporter 1 (GlyT1). GlyT1 is a major reuptake regulator of glycine concentration at glutamatergic synapses (Aragon and Lopez-Corcuera 2005), and is co-localized with NMDARs (Attwell et al. 1993). Its normal physiological role and anatomical localization indicate that pharmacological inhibition of GlyT1 would be a highly effective strategy to increase glycine-B site occupancy, overcoming the limitations of systemic treatment with glycine-B site agonists/partial agonists as described above. Amongst the growing number of GlyT1 inhibitors available, some have
been shown to possess the preclinical profile of putative antipsychotics and are currently undergoing clinical evaluation (Lechner et al. 2006). However, there is until now only limited evidence for their potential pro-cognitive effects in non-pathological conditions. The most promising GlyT1 inhibitor in this regard is SSR504734, which has been shown to reverse the social recognition impairment in rats induced by neonatal phencyclidine treatment (Deportèere et al. 2005, Harich et al. 2007), and to enhance prepulse inhibition (PPI) in a mouse strain (DBA/2), with intrinsic sensorimotor gating deficiency (Depoortère et al., 2005). PPI is often taken as a measure of sensorimotor gating, which refers to a form of early-attentional control in selective information processing, central to the prevention of sensory overload (Graham 1975, Swerdlow et al., 2000). Impaired PPI may be linked to other cognitive deficits and therefore restoration of deficient PPI may alleviate such associated cognitive dysfunctions. There is also recent evidence suggesting that the PPI magnitude correlates with mnemonic performance in various learning tasks (Giakoumaki et al. 2006, Bitsios et al. 2006), suggesting that PPI enhancement may be a predictor of pro-cognitive properties. This lends further support to the hypothesis that SSR504734 may possess pro-cognitive potential.

The present study aimed to extend the behavioural assessment of SSR504734 in cognitive function (Depoortère et al., 2005) to working memory function in normal mice as a test of the drug’s promnesic potential in non-pathological conditions. Here, working memory was assessed using an automated operant continuous delayed alternation procedure that readily allows the manipulation of delay intervals between successive trials, thus varying the retention demand on working memory function.

**METHODS & MATERIALS**

**Subjects**
The subjects were 31 naïve adult male C57BL/6 mice bred in a specific-pathogen free (SPF) breeding facility (ETH Zurich Laboratory of Behavioural Neurobiology, Schwerzenbach, Switzerland). They were weaned at postnatal day 21, and caged in groups of unisex littermates until 10 weeks old, when they were transferred to single caging in Type II cages (Techniplast, Milan, Italy). The animals were maintained in a temperature (21°C) and humidity (55%) controlled vivarium under a 12:12h reversed light-dark cycle (lights on at 1900hr). Food and water were provided ad libitum until one week prior to behavioural training. Behavioural testing took place in the dark phase of the cycle, and commenced when the animals were 12 weeks old. During the course of the experiment, the animals were maintained on a 23h food deprivation regime with their body weight reduced to not less than 85% of their ad lib weight. Water was available throughout.

The experimental manipulations and procedures described here had been previously approved by the Zurich Cantonal Veterinary Office. All procedures conformed fully to the ethical standards required by the Swiss Act and Ordinance on Animal Protection and the European Council Directives 86/609/EEC.
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Apparatus
The experiments were conducted using four Habitest System (Coulbourn Instruments, Allentown, PA) operant chambers (Model E10-10), each placed inside a ventilated and sound-attenuated chest. The internal dimensions of each chamber were 31 cm × 25 cm × 33 cm, but an opaque Plexiglas partition wall was installed to reduce the available floor area to 15 cm × 25 cm. Illumination inside the chamber was provided by a house-light (2.8 W) positioned on the panel wall, 21 cm above the grid floor. Two food magazine trays were mounted on the panel wall, each equipped with a 1.4 W light mounted inside. They were separately connected to two independent food pellet dispensers (Model H14-22M-20) using 20 mg sucrose pellets (BioServ, Frenchtown, NJ) as food reward. Nose poke responses (magazine entries) were detected by an infrared beam positioned at the entrance to each food magazine. The magazine trays were positioned on either side of a retractable lever mounted in the middle of the panel wall (Model H21-03M). A 2.8 W stimulus light was mounted 10 cm directly above the lever. Two additional transparent partitioning walls (5 cm long and 6 cm apart) were mounted on either side of the retractable lever, so that the animal was required to take a detour to move between the lever and either magazine tray.

Drugs
SSR504734 (2-chloro-N-[(S)-phenyl [(2S)-piperidin-2-yl] methyl]-3-trifluoromethyl benzamide, monohydrochloride) was donated by Sanofi-Aventis (Paris, France). The compound SSR504734 was suspended in distilled water containing 5% Tween 80 (see Deportèere et al. 2005). Both SSR504734 and vehicle-control (5% Tween) solutions were freshly prepared daily and injected via the intraperitoneal (i.p.) route 30 min before testing. The injected volume was 10 ml/kg.

Acquisition of the delayed alternation task

Introduction of food deprivation
At the age of 10 weeks, the animals were singly housed and maintained on an ad lib food regime for 1 week prior to the commencement of the food deprivation. This was introduced gradually, with a progressive reduction of feeding time across six days (12 h, 6 h, 4 h, 2 h, 1 h, 1 h). Water was constantly available. At the same time, the animals were familiarized with the reward pellets in the home cage to minimize food neophobia when operant training began. Operant training commenced on the next day, and the animals were maintained with 1 h free access to food per day throughout the experimental period. The animals’ body weight was monitored daily and was not permitted to fall below 85% of their ad lib weight.

Magazine training
The animals were first habituated to the operant chambers and allowed to consume food pellets freely from the food magazines. Each session began with 10 food pellets already placed in each magazine tray. The house light and magazine lights were switched on throughout the 20 min of the session.
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Once all mice were reliably consuming the reward pellets, they were trained to earn reward by alternating their successive nose poke responses between the two magazines.

**Nose pokes training with signalled alternation**

Under the constant illumination of the house light, the session (once per day) began with the illumination of one of the two magazine trays. A nose poke into this magazine resulted in the delivery of one reward pellet. This magazine stayed illuminated for two additional seconds before being turned off, and at the same time the alternative magazine was then illuminated awaiting for a nose poke response. Nose pokes directed at an unlit magazine tray did not yield any programmed consequence. The session ended with the cessation of the house light, either when the animal had made 16 correct nose poke responses, or when 10 min had elapsed. When an animal had completed the 16 correct responses within the maximum time allowed on two consecutive days, it was trained to press a lever following each rewarded nose poke response, in order to trigger the illumination of the next appropriate food magazine (see below).

**Lever press training with trial-discrete signalled nose pokes alternation**

With the house light on throughout, this training session (once per day) began with the presentation of the lever, which coincided with the illumination of the lever stimulus light directly above. One lever press resulted in the retraction of the lever, cessation of the lever stimulus light, and illumination of one of the two food magazines. Upon a nose poke to the illuminated magazine, one reward pellet was delivered to that magazine tray. The magazine light was then switched off after 2s, and the next trial commenced with the presentation of the lever signalled by the illumination of the lever stimulus light. Nose pokes into an unlit magazine tray would not yield any programmed consequence. The session was terminated with the cessation of the house light when either 16 trials had been completed, or 10 min had elapsed. Delayed alternation commenced the day after the animals had completed the session within the maximum time on two consecutive days.

**Baseline delayed alternation training**

In the delayed alternation task, the animals were required to alternate successive nose pokes between the two food magazines to earn a reward. This essentially followed a “win-shift” rule, by which the correct response on a given trial was to shift away from the previous rewarded response. Choice accuracy thus depended on memory of the last rewarded response. The information relevant to the solution was trial-dependent, hence the task taxed one key aspect of working memory function (Honig 1978). The retention demand on working memory was manipulated here by varying the delays (i.e., the inter-trial intervals) between a (rewarded) response to the next choice.

The present delayed alternation task adopted a “two food magazines and one lever” configuration which differs its design from the conventional trial-discrete nonmatching-to-sample
(DNMS) operant working memory task based on a “two levers and 1 food magazine” configuration (e.g., Dunnett 1993). However, this set-up was deliberately chosen because our intention to mimic the T-maze continuous delayed alternation procedure (e.g., see Marquis et al. 2007) whereby the animals were required to alternate their successive arm entries between the left and right choice stems on a T-maze to obtain food reward. The continuous nature of this task design was preserved in our operant procedure such that the response on a given choice trial served effectively as the sample run of the following choice in accordance with the non-matching rule. This design has also been adopted previously in delayed nonmatching-to-sample tests (e.g., Aggleton et al. 1986, 1992, Yee and Rawlins, 1994). Here, the use of a correction trial (see below) further ensured that correct choice behaviour would also be consistently guided by a “win-shift” strategy.

A schematic of the delayed alternation test procedures is provided in Figure 1. With the house light on, each daily session began with an initial “forced” trial in which a randomly selected magazine was illuminated. A nose poke into this magazine resulted in the delivery of a reward pellet. The magazine light remained on for two additional seconds, followed by the initiation of the inter-trial interval (ITI) preceding the first “choice” trial. A choice trial began with the presentation of the lever and the simultaneous illumination of the lever stimulus light, while both magazine lights remained off. A lever press resulted in the retraction of the lever, cessation of the lever light, and the illumination of both magazines. A correct nose poke response was followed by the immediate delivery of a reward pellet, cessation of the alternative magazine light, and the continual illumination of the chosen magazine light for 2s, followed then by the next ITI. On the other hand, an incorrect choice resulted immediately in the cessation of all magazine lights, and a 5s time-out period during which the house light was switched off and the lever remained retracted. At the end of the time-out period, a “forced” trial using the previous correct magazine was administered as described above (also see Figure 1). This was then followed by the appropriate ITI prior to the initiation of the next choice trial.

At the beginning of baseline training, all animals were trained using an ITI of 1s, with each session consisting of a maximum of 12 choice trials to be completed within a maximum of 10 min. Animals that consistently failed to complete the 12 trials within the allotted time were excluded from further testing. Otherwise, when choice accuracy achieved ≥90% correct on two consecutive daily sessions, a variable ITI procedure was introduced. Within a session, the ITI varied between one of two possible delays counterbalanced amongst the 12 trials. Over the first 4 days, the ITI was either 1s or 4s. Over the next 4 days, the ITI was either 1s or 8s. This allowed us to confirm and to identify the sensitivity of the task to increasing ITI within sessions. A total of 17 mice reached this stage of training, and they were then given 2 days of rest before the next phase of testing with compound SSR504735.
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Figure 1. Schematic illustration of the delayed alternation task. The session began with the presentation of the centrally located lever, the depression of which led to the start of a “forced trial” in which one of the two food magazines was illuminated waiting for a nose poke response. Upon detection of a nose poke into one of the two magazines, a food pellet was delivered into this magazine, and the first inter-trial interval (ITI) delay was initiated. During the ITI period, the lever remained retracted and the magazine lights off. At the end of the ITI, the lever was presented and its depression was followed by its retraction and illumination of both magazines. Nose poke into the magazine alternative to the one that yielded a reward now resulted in the delivery of a reward (correct response). Nose poke into the magazine that previously yielded a reward (incorrect response) was followed by a 5-min “time-out” and then the presentation of a “forced trial” in which only the correct magazine was illuminated. Upon a nose poke into this magazine, the programme resumed to the next appropriate ITI period, followed then by a choice trial. Response latency was defined as the time between lever press that triggered the magazine lights, and the magazine entry (nose poke) which constituted to an explicit choice.

Experiment 1: Effects of SSR504734 on delayed alternation (1s vs 4s vs 8s delays)
We started by evaluating the effect of systemic SSR504734 on performance in the delayed alternation task in two delay function experiments. We elected to focus first on the specific dose of 30mg/kg i.p., because this was found to be behaviourally effective by Depoortère et al. (2005), and because it is also consistent with our impression obtained from initial pilot studies with this compound (unpublished data by P. Singer, J. Feldon and B.K. Yee).

In Experiment 1, the delay function included three possible ITI delays: 1s, 4s and 8s, intermixed across the 12 choice trials per session. On day 1, all animals were given a vehicle injection 30 min before delayed alternation training to familiarize them with the injection procedure. The animals were subdivided into two groups – balanced with respect to their baseline performance (on
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day 1). Over the next three days, one group received daily pre-treatment of SSR504734 at 30mg/kg (n=8) and the other of vehicle injection (n=9). One SSR504734-treated subject was excluded because it failed to complete the 12 trials on the first two tests days. At the end of this experiment, the animals were given two days of rest before they were tested again in Experiment 2. However, another mouse (from the vehicle group) died in this period, leaving a total of 15 mice available, before Experiment 2 commenced.

Experiment 2: Effects of SSR504734 on delayed alternation (1s vs 8s vs 12s vs 16s delays)

In Experiment 2, the delay function included four possible ITI delays: 1s, 8s, 12s and 16s, intermixed across the 16 choice trials per session. First, animals were given one session of pre-training in which they all received a vehicle injection 30 min before the test (as in Experiment 1). The animals (n=15) were then subdivided into two new subgroups in order to counterbalance their previous drug experience in Experiment 1. Namely, half of SSR504734-treated animals in Experiment 2 had received SSR504734 in Experiment 1 and the other half vehicle in Experiment 1; and half of vehicle-treated animals in Experiment 2 had received SSR504734 in and the other half vehicle in Experiment 1. This allowed us to gauge the possible effects of previous experience with the drug. The two new groups (SSR504734: n=7, vehicle: n=8) received either daily pre-treatment of SSR504734 (30mg/kg, i.p.) or vehicle solution as described above. Drug testing lasted for three consecutive days, after one day of training with vehicle injection in all animals (as described in Experiment 1). After this, the animals were again given a break of 2 days prior to the next experiment.

Experiment 3: Dose response analysis of SSR504734’s effect in delayed alternation

We then conducted a dose response analysis of SSR504734 (0, 3, 10 and 30 mg/kg) focusing on the minimal delay of 1s and the extended delay of 12s. All subjects had completed Experiments 1 and 2 before. On day 1, all animals received a vehicle injection as described before for a practice session. Over the following four days, a Latin square design was adopted to balance the order of drug doses across subjects (n=15). Each daily session consisted of 12 choice trials, with half of the trials following a 1s ITI delay, and the other half a 12s delay. A maximum time of 10 min per session was allowed. No further animals were excluded for not completing sessions.

Dependent measures and statistical analysis

Working memory performance was indexed by percent correct choices, at each delay condition on each day. In addition, the number of nose pokes recorded during the ITI delays on a given day was also calculated and separately analysed. All statistical analyses were carried out using SPSS® for Windows™ (version 13) on a PC running the Windows XP (SP2) operating system. Parametric analyses of variance (ANOVA) were performed with the between-subjects factors, drug and previous drug experience, and the within-subjects factors, delays and days (in Experiments 1 and 2).
Experiment 3, the effect of drug dosage was investigated using a Latin square design, and the factor of drug doses was analysed as a within-subjects factor. Data interpretation was further assisted by Fisher’s least significant difference (LSD) post hoc comparisons and the use of restricted ANOVAs for determining the locus of the significant effects. A critical level of $p < 0.05$ was set for all statistical analyses.

RESULTS

Delayed alternation baseline training

Baseline training on the continuous delayed alternation task began after the animals had successfully completed the pre-training of trial-discrete signalled nose poke alternation in which no mnemonic demand was required. The baseline training on the continuous delayed alternation procedure consisted of three consecutive phases, lasting for 15d, 4d and 4d, respectively. In the first phase, all ITI’s were 1s in duration. As illustrated in Figure 2A, choice accuracy indexed by percentage of correct trials per daily session was already above chance on day 1. Afterwards performance continued to improve steadily over days, achieving criterion performance by the 15th days. Repeated measures ANOVA of percent correct over the 15 days yielded a main effect of days $[F(14,238)=11.96, p<0.001]$ (n=18).

In the second phase of baseline training, the ITI’s varied between 1s and 4s. One animal failed repeatedly to complete the 12 trials within the time limit and was therefore excluded from the data analysis and from further testing. The total number of mice was thereby reduced to 17. As shown in Figure 2B, increasing the ITI to 4s did not result in any observable impact on choice accuracy in comparison with the minimal 1s delay condition. A $2 \times 4$ (delays $\times$ days) repeated measures ANOVA of percent correct response failed to yielded any significant effects.

In the third phase, the ITI within each daily session was either 1s or 8s. Choice accuracy was substantially reduced by the extension of ITI to 8s in comparison with the high choice accuracy maintained at the 1s delay condition (Figure 2C). A $2 \times 4$ (delays $\times$ days) repeated measures ANOVA of percent correct response yielded a highly significant effect of delays $[F(1,17) = 36.69, p < 0.001]$. Neither the main effect of days nor its interaction with delays achieved statistical significance.

The results from the baseline phase indicated that the 4s delay condition may be useful for assessing treatment expected to impair working memory function. On the other hand, delays of 8s and above were sufficiently taxing for normal animals and would therefore be suitable for the detection of performance enhancing agents. The results of phases 2 and 3 here also verified the stability of performance at the minimal 1s delay condition, even when it was intermixed with trials of a higher mnemonic demand as in phase 3. This design of varying delays within-session enjoys a potential advantage over a between-session design, because the latter may lead to profound reduction of choice accuracy, such that the subjects might give up on the task.
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Figure 2. Baseline delayed alternation training
(A) Acquisition of the delayed alternation task under the minimal (1s ITI) delay condition. Performance was indexed by percent correct responses expressed as a function of daily sessions. (B) Within-session comparison between trials following a 1-s ITI and those following a 4-s ITI indicated little evidence for an impact by this limited increase in retention demand. (C) Within-session comparison between trials following a 1-s ITI and those following a 4-s ITI yielded a significant main effect of delays (* p<0.005) as confirmed by a 2 x 4 (delays x days) repeated measures ANOVA of percent correct per day (n=17). All values refer to mean ±SEM.

Experiment 1: Delay function of 1s, 4s, and 8s

One animal belonging to the SR504734 condition repeatedly failed to complete the 12 trials within the time limit and was therefore excluded from the data analysis of Experiment 1 and discontinued from further testing. The final number of subjects was therefore: SSR504734 n=7 and vehicle n=9. As shown in Figure 3A, performance of both SSR504734 and vehicle-treated animals was maintained at a high (~90%) and comparable level at delays of 1s and 4s across the three days of drug test. Choice accuracy expressed as mean percentage correct at different delays is depicted in Figure 3A. A group difference emerged at the longest delay of 8s with SSR504734-treated animals outperforming the vehicle controls. SSR504734 thus improved working memory performance in a delay-dependent manner, i.e., only when the demand on temporal retention was high.

These interpretations of the data were supported by a 2 x 3 x 3 (drug x delays x days) ANOVA of percentage of correct across the three days of test, which yielded a significant main effect of delays [F(2,28)=21.01, p<0.001] and its interaction with drug [F(2,28)=7.39, p<0.005]. Separate analyses restricted to each delay condition indicated that SSR504734 significantly enhanced choice accuracy only at 8s delay [F(1,14)=6.88, p=0.02] but not at the two shorter delays. Furthermore, at the critical delay of 8s when SSR504734 treatment exhibited a positive effect on performance, the controls were still able to maintain performance above chance (p<0.05 based on one sample t-test). This suggested that the superior performance by the SSR504734 group could not be attributed to non-performance in the control group at this particular delay condition. The effects of the drug on performance did not appear to depend on days: neither the main effect of days nor any of its interactions achieved statistical significance. Indeed, analysis of data derived from day 1 only (when the SSR504734-treated mice were receiving the drug for the first time) were sufficient to yield a near-significant effect of drug by delays interaction [F(2,28)=3.15, p=0.058] and a main effect of delays [F(2,28)=5.60, p<0.05]. When the comparison was restricted to the 8s delay condition on day 1 the drug effect again approached statistical significance [F(1,14)=4.43, p=0.055], but did not do so at the shorter delay conditions [F<1].
In addition, a separate analysis was made of the number of nose pokes recorded during all ITI’s (when both magazine trays were not lit). Such nose pokes were a potential source of retroactive interference that might affect working memory performance. A $2 \times 3$ (drug $\times$ days) ANOVA of this measure did not yield any significant effects. The frequency of nose pokes during ITI’s was stable across days and comparable between treatment groups. The mean numbers of ITI nose pokes ($\pm$SEM) per daily session were: SSR504734 = 59.3$\pm$7.1, vehicle control = 62.1$\pm$4.7.

Figure 3. Effects of systemic SSR504734 on the delayed alternation task. Two separate delay-function experiments (Experiments 1 and 2, depicted in A and B respectively) were conducted to examine the effects of SSR504734 on delayed alternation performance. Animals used in Experiment 2 were previously tested in Experiment 1. Drug experience was fully counterbalanced (see Methods). Choice accuracy in each experiment was indexed by percent correct (average across three drug test days) and illustrated here as a function of inter-trial interval (ITI) delays based on data obtained on three testing days. In both experiments, SSR504734 significantly improved choice accuracy in comparison to vehicle treatment at longer delays. Significant between-groups difference at $p<0.05$ is indicated by ‘*’. Error bars refer to $\pm$SEM.

Experiment 2: Delay function of 1s, 8s, 12s and 16s

In the second delay function experiment, the temporal demand of retention was increased by extending the delay up to 16s. Again, three days of drug test were conducted following one day of vehicle injection practice session in all subjects. The subjects’ previous drug experience (in Experiment 1) was counterbalanced in the allocation of drug condition in Experiment 2. Thus in each drug condition here, there were subjects that had received the drug in Experiment 1 and subjects that did not. Prior drug experience in Experiment 1 was included as a between-subject factor in the analysis of the present data set, but this factor was excluded in the final analysis because it did not achieve statistical significance either as a main effect or interaction.

As depicted in Figure 3B, SSR504734 again selectively enhanced choice accuracy at longer delays of 12s and 16s. The drug was, however, no longer effective at the 8s delay condition (c.f. Experiment 1), yet the general impression of a performance enhancing effect by SSR504734 was essentially replicated. Under the more extended delays of 12s and 16s, the vehicle group was no longer able to sustain above chance performance (based on one sample t-test), whereas the SSR504734 group maintained performance at about 70% correct.

These interpretations of the data were supported by a $2 \times 4 \times 3$ (drug $\times$ delays $\times$ days) ANOVA of percent correct responses, which yielded a significant main effect of drug [$F(1,13)=8.50$, $p<0.02$], delays [$F(3,39)=19.10$, $p<0.001$], and their interaction [$F(3,39)=3.45$, $p<0.05$]. Again, there was no statistical evidence that performance varied significantly across days: neither the main effect of
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days nor its interactions achieved statistical significance. Additional restricted analyses at each delay condition confirmed that SSR504734 treatment significantly enhanced choice accuracy when the delay was extended to 12s [F(1,13)=8.48, p<0.02] and 16s [F(1,13)=7.39, p<0.02].

Analysis of the number of nose pokes made during ITI delays per daily session by a 2 x 3 (drug × days) ANOVA again yielded no significant effects. The mean numbers of nose pokes (±SEM) per daily session were: SSR504734 = 106.9±10.8, vehicle control = 102.4±9.8.

Experiment 3: Dose response analysis

Finally, we conducted a dose response analysis across doses: 0, 3, 10 and 30 mg/kg by focusing on the minimal delay of 1s and the extended delay of 12s to ascertain whether a positive drug effect might also be identified at lower doses. The animals from Experiments 1 and 2 (n=15) were employed here in a Latin square design to best counterbalance across drug doses in a within-subject manner.

SSR504734 dose-dependently enhanced choice accuracy, but this effect was most clearly demonstrated in the 12s delay condition (Figure 4). The efficacy of the highest dose (30 mg/kg) was again confirmed here, with the 10mg/kg dose producing an intermediate level of enhancement. These contrasted with the lack of effect at the lowest dose of 3 mg/kg.

A 4 × 2 (drug doses × delays) repeated measures ANOVA of choice accuracy yielded a significant main effect of drug doses [F(3,42)=5.76, p<0.005] and delays [F(1,14)=241.43, p<0.001]. Despite the tendency apparent in Figure 4, the interaction term failed to attain statistical significance. However, separate analyses restricted to each delay condition revealed a significant effect of drug doses only in the 12s delay condition [F(3,42)=3.93, p<0.05]. Planned contrast analysis of this significant effect indicated that only the highest dose (30mg/kg) significantly enhanced performance in comparison with vehicle treatment (p<0.05).

Separate one-way repeated measures ANOVAs of nose poke frequency during ITI delays yielded no significant effect of drug doses. The mean numbers of nose pokes (±SEM) per daily session were: vehicle-control = 60.2± 6.0, 3mg/kg = 63.1 ± 6.1, 10mg/kg = 50.0 ± 6.1, 30mg/kg =65.6 ± 7.1.

As a further test of the robustness of our results here, which were obtained using a Latin square design and where the factor dose was evaluated in a within-subject manner, we performed an additional analysis restricted to the first test day, in which the same factor was analysed as a between-subject factor. In spite of the anticipated reduction in statistical power, this nonetheless would allow an assessment of whether the overall pattern of results described above was already apparent on the first day. To this end, a 4 x 2 (drug doses x delays) ANOVA was performed on this restricted data set, which revealed a pattern of results almost identical to the overall analysis across the 4 test days. The analysis yielded a significant effect of delay F(1,11)=86.3, p<0.001] and of dose [F(3,11)=3.83, p<0.05]. Post-hoc LSD comparisons further confirmed the efficacy of SSR504734 to enhance performance at the highest dose (30mg/kg) in comparison with vehicle treatment [p=0.006]. In the
minimal 1s delay condition, choice accuracy across increasing dose (0, 3, 10 and 30 mg/kg) was: 83.3±3.9, 95.8±3.9, 91.7±3.9, 94.4±4.5%, respectively. In the 12s delay condition, the corresponding percent correct scores were: 41.7±7.3, 50.0±7.3, 54.2±7.3, 77.8±8.4%, respectively.

DISCUSSION
The present study sought to evaluate the promnesic potential of SSR504734 on working memory functions using an operant delayed alternation paradigm. Across three experiments, the results revealed that SSR504734 reliably enhanced working memory performance at a dose of 30mg/kg. The robustness of this particular dose demonstrated here is in agreement with the report by Depoortère et al. (2005) who also showed that the dose of 30mg/kg was consistently active across a number of behavioural paradigms relevant for schizophrenia-related cognitive deficits. In contrast, the lowest dose of 3mg/kg was essentially devoid of any efficacy in the present task.

The promnesic effect of SSR504734 probably stems from the acute effect of the drug, although in each experiment animals received multiple injections of the drug across daily sessions. The overall pattern of results in Experiment 1 was evident when animals were first administered the drug on the first day of the experiment. The outcome of Experiment 2 did not depend on whether the subjects were naïve to SSR504734 before the experiment. In Experiment 3, dose was varied over 4 days and a response was clear. In addition, the overall pattern of results of Experiment 3 was clearly evident on the first day of test. Thus, the robustness of the dose-response analysis was supported in that it did not depend on the uniqueness of the Latin square design.

Our results therefore implied that systemic treatment of SSR504734 had very limited long-term effect on the present memory task. This may represent an advantage from the perspective of a cognitive enhancing agent for the non-clinical population, but could also be a disadvantage as a
therapeutic agent against chronic and persistent cognitive deficiency.

The positive effect of SSR504734 on choice accuracy expressed itself in a delay-dependent manner. This suggests that the drug did not indiscriminately elevate performance but was most effective when the delay condition was sufficiently taxing. We manipulated task difficulty primarily by varying the task’s retention demand. In Experiment 1, the drug was only effective at 8s delays when performance of the controls began to deteriorate drastically while remaining above the level of performance expected by chance alone. In Experiment 2, although the drug appeared ineffective under the same delay conditions, its ability to improve performance was clearly evident at extended delays of 12s and 16s and at a range at which the control failed to exceed the level of performance expected by chance alone. We have no explanation for the drug’s lack of efficacy in Experiment 2 at the 8s delay condition, which contrasted with its demonstrated effect in Experiment 1. However, we can exclude the possibility that repeated dosing was the cause because the impact of prior drug exposure in Experiment 1 did not affect the outcome of Experiment 2. We can also exclude the introduction of the 12s and 16s delays in Experiment 2 as a likely reason because the results obtained on the first test day (when the novelty of these test conditions was maximal) conformed to the outcome at 8s delays in Experiment 1. Thus, previous experience of Experiment 1 probably is important. Alternatively, the 8s delay condition was not functionally identical in the two experiments because its presentation was intermixed with different delay conditions. Nonetheless, this does not detract from our general conclusion that SSR504734 affects working memory function under varying retention demands.

From the end of the baseline training onwards, the animals had maintained a consistently high level of choice accuracy at performance at the minimal delay condition in all three experiments. It suggests that the animals had maintained the motivation to perform the task throughout the session in spite of increasing task difficulty imposed by the inclusion of more extended delays. This further strengthens our conclusion that SSR504734 can enhance mnemonic capacity with respect to memory retention and retrieval. It would be highly relevant and interesting to assess if the drug also possesses such potential when task difficulty is achieved by other task parameters, for example by varying the levels of interference.

One critical determinant of working memory function is the ability to overcome or resolve interference (Jonides and Nee 2006), and the present working memory task is no exception. Indeed, the levels of interference might be considerable. Every trial involved the same two choices (or discriminanda), namely the two magazine trays, which were identical in appearance except for their location in the operant chamber. The repeated use of a single pair of discriminanda in delayed matching-to-sample task (another working memory test) is considerably more difficult in comparison with the use of trial-unique discriminanda (e.g., Yee and Rawlins 1998). It is because memories acquired from preceding trials potentially interfere with the retrieval of the relevant information on a given trial. This source of (proactive) interference is, however, expected to reduce as the delay interval between trials is lengthened.
Furthermore, the animals made additional nose pokes into the inactive magazine trays (i.e., when the magazine lights were off) during the retention intervals. These responses represented another potential source of (retroactive) interference, making accurate recall of the relevant past information (location of the previous nose poke that generated reward) more difficult. It is not possible to prevent such responses in the present task design unless switchable magazine doors are installed. An alternative to overcome this interference is the “two levers and one magazine” configuration where the discriminanda (i.e., the levers) can be retracted during the inter-trial intervals. In contrast to proactive interference resulting from preceding trials, retroactive interference is expected to increase as the delay interval between trials widens.

Our data indicated that such retroactive interference was largely similar for both drug and vehicle conditions, thus the possibility that SSR504734 enhanced performance here solely by reducing the occurrence of interfering inter-trial nose pokes could be excluded. This assertion, however, does not repudiate the possibility that SSR504734 may act by reducing the functional impact of retroactive interference on working memory function. For example, it may enhance the perceptual distinctiveness or discriminability between memory traces, thereby facilitating their selective retrieval. If so, SSR504734 may be generally effective in facilitating cognitive flexibility. This interesting possibility would certainly require additional tests in which interference is explicitly manipulated (e.g., Yee and Rawlins 1998, Sanderson et al. 2007).

The demonstrated delay-dependent effect of SSR504734 readily suggests that the drug has a promnesic impact on the animal’s working memory capacity. It enhances the ability to retain and retrieve information across the inter-trial intervals in order to respond correctly on the next trial. This characteristic of trial dependency is the hallmark of working memory according to the relevant theories by Honig (1978) and Olton (1979). The alternative “win-shift” strategy would also be expected to require recall of trial-dependent memory because throughout the sessions both magazine trays would be associated with rewards. In addition, the task can be solved based on relative familiarity (or recency) judgement, because accurate solution can be achieved by always selecting the relatively less familiar magazine tray (Rawlins and Tsaltas 1983). This particular strategy would be most prone to the accumulation of interference and would predict that trials at the beginning of the session are easier than those at the end. We attempted to address this possibility by comparing performance between the two halves within a session using our current data sets. No significant difference in performance was however evident between the two halves of a test session (data not shown), although a consistent trend of lower accuracy in the last half (by approximately 5% points) relative to the first half was noted in all three experiments. This trend was similarly observed regardless of drug conditions suggesting that the drug did not enhance performance primarily through a reduction in within-session proactive interference.

One potential problem associated with sequential alternation task (especially in an automated setup) is the possible adoption of a motor mediating strategy (Olton et al. 1979), which may allow a
solution that is essentially free from any working memory demand. Here, this possibility had been reduced by requiring the subjects to press a centrally located lever to activate the availability of a choice (illumination of both magazine lights), thus ensuring that the mouse would always be in the same location when the choice was presented. Furthermore, the installation of partition walls between the lever and magazines also reduced the use of simple motor pattern as mediating strategy, although this possibility can never be completely eliminated (see Stanhope et al., 1995). Most critically, however, the use of mediating behaviour is expected to assist performance at short rather than at long delays (Rawlins and Tsaltas 1983). Given that SSR504734 was primarily effective at longer delays, the mnemonic conclusion of this outcome is less likely to be weakened by the interpretative problems associated with the potential use of mediating strategy in such tasks.

Another relevant performance measure, as suggested by an anonymous reviewer, is the animals’ response speed or latency to make a decision in the context of task difficulty as a function increasing delays. In our task, this corresponds to the time between the lever press that triggered the magazine lights, and the nose poke that constituted the choice selection (see Figure 1). We elected to focus on Experiment 2, because it covered the widest spectrum of delays (Figure 5). A distinction between correct and incorrect response was made, but there was insufficient data to allow such a distinction at 1s delays because of the high level of performance in this condition. This analysis revealed that response latency for correct response was significantly higher than for incorrect response across delays of 8-16s (see Figure 5B) \[F(1,13)=12.70, \ p>0.005\]. Response latency for correct responses showed an inverted U-shaped function against increasing delays. This led to the emergence of a significant main effect of delays \[F(3,39)=3.55, \ p<0.05\] in a repeated measure ANOVA of the latency of correct responses across the four delay conditions, which was solely attributed to the quadratic component \[F(1,13)=15.92, \ p<0.005\] (see Figure 5A). Taken together, response latency may therefore reflect effortful retrieval, which was more demanding with increasing delays; but the animals eventually shifted from such effortful recall when the delay was too high. In this respect, the non-significant protraction of response latency by SSR504734 may be relevant to the psychological impact of the drug on cognitive functioning in general, even though this tendency in itself may not fully account for the significant delay-dependent enhancement of choice accuracy by SSR504734.

The present demonstration of a promnesic effect following GlyT1 inhibition is of critical importance, because until now, clinical evidence on the pro-cognitive potential of GlyT1 inhibition is limited to the endogenous GlyT1 inhibitor sarcosine in the treatment of schizophrenia (Tsai et al. 2004, Lane et al. 2005). Moreover, although adjunct glycine therapy was found to improve negative as well as cognitive symptoms in schizophrenia patients (Heresco-Levy et al. 1999, 2004, Javitt et al. 2001), studies in healthy volunteers had questioned the ability of acute high-dose glycine treatment to improve cognitive function in non-pathological conditions. Recently, Palmer et al. (2008) failed to obtain any evidence of cognitive enhancement in healthy subjects following treatment with high doses of glycine (0.8g/kg, *per os*) in measures of working memory, declarative memory, attention and perceptual
processing. Interpretation is further complicated by reports that a single oral dose of glycine (0.8g/kg, *per os*) can produce a negative impact on cognition as demonstrated in the test of mismatch negativity (Leung et al. 2008), in which schizophrenia patients also exhibit a deficit (e.g., Umbricht and Krljes 2005). Given that acute glycine administration at 0.1 to 0.2mg/kg via the intraperitoneal route is highly effective in substantially elevating plasma and CSF levels of glycine within two hours after injection (D’Souza et al. 2000), the oral dose of 0.8mg/kg described above is considerably high in spite of the difference in the route of administration.

**Figure 5. Response latency as a function of delay and response in Experiment 2.** (A) Response latency (in seconds followed by ln-transformation) as a function of delays in Experiment 2 is represented by separate curves, according to drug treatment conditions and whether the response was correct or incorrect. In the 1-s delay condition, 4 mice did not make any error and therefore representative group means for incorrect response latency cannot be calculated. Error bars refer to ±SEM. Analysis of correct responses across all four delay conditions by a 2 × 4 (drug × delays) ANOVA yielded a main effect of delays [F(3,39)=3.55, p<0.05], which was associative with a significant effect exclusively in the quadratic trend [F(1,13)=15.92, p<0.005] according to an orthogonal contrast analysis, supporting the presence of an inverted U-shape effect. To examine the difference in response latency between correct and incorrect response, the analysis was restricted to the three higher delay conditions by a 2 × 2 × 3 (drug × response type × delays) ANOVA. This yielded a main effect of delays [F(2,26)=4.52, p<0.05] and response type [F(1,13)=12.70, p<0.005], while the main effect of drug and its interaction terms did not achieve statistical significance. (B) This depicts the mean response latency averaged across the delays conditions of 8s, 12s, and 16s, illustrating the significant difference in response latency between correct and incorrect responses (* p<0.05) Error bars refer to ±SEM.

Why may high concentrations of glycine fail to enhance and even impair cognition? First, excessive elevations of glycine above the saturation threshold of glycine-B site can result in NMDAR internalization and may thereby lead to a negative impact on overall NMDAR functions at the network levels. This can result from either endogenous glycine treatment (Nong et al. 2003) or GlyT1 blockade (Martina et al. 2004). Secondly, elevated glycine levels could also potentiate NMDARs located on inhibitory γ-aminobutyric acid (GABA) interneurons (Javitt et al. 2005), which in turn would dampen overall excitatory neurotransmission at the network levels. Thirdly, neuronal inhibition can also result from the action of excessive glycine on low-affinity inhibitory glycine receptors on pyramidal neurons (Laube et al. 2002, Betz and Laube 2006, Rajendra et al. 1997). These possible physiological responses to global elevation of glycine concentration may imply that a moderate increase in the glycine levels is more favourable (Singer et al., 2007).

Interestingly, Palmer et al. (2008) suggested that the lack of a pro-cognitive effect following acute glycine treatment may be due to individual variability in endogenous glycine concentration. Given that the optimal elevation of glycine for improving cognitive functions may differ considerably
between subjects, individual titration would be necessary to show a consistent effect. The ability to adjust for the optimal glycine concentration is further hindered by the poor blood-brain barrier passage of glycine, which necessitates massive dosing: a typical dose of 0.8g/kg implies a daily intake of glycine exceeding 50g for an adult human (Heresco-Levy et al. 1996, 1999, Javitt et al. 2001). The potential side effects under such conditions must be seriously considered. Besides the obvious respiratory problems associated with exposure to high doses of glycine, morphological changes in the cerebellum (e.g., a decrease in density of astrocytic fibres) have been reported in rats following a 2-week glycine-rich diet (Shoham et al. 1999).

In view of the above limitations associated with direct glycine intake, blockade of GlyT1 represents a superior pharmacological approach to achieving selective potentiation of NMDAR activation aimed at enhancing cognitive functions. First of all, GlyT1 inhibitors are expected to readily pass through the blood-brain barrier (e.g. Molander et al. 2007), and therefore do not require exceptionally high doses to reach their targets. Secondly, given the close anatomical proximity between GlyT1 and NMDARs (Cubelos et al., 2005a,b), GlyT1 inhibition is ideally suited to achieve elevation of the functionally most relevant local concentration of glycine – that is, in the vicinity of the NMDAR’s glycine-B site. Hence, this would maximize the functional impact in terms of its desired action on NMDAR function, without inducing excessive changes to the global concentration of glycine in the brain, which as explained above, could be counterproductive to the desired aim to enhance NMDAR activities.

There is considerable evidence to suggest that effective working memory function depends on both hippocampus and prefrontal cortex. However, the sensitivity of the present task to hippocampal or prefrontal cortex damage has not been validated in mice. We have thus far validated its sensitivity to systemic scopolamine treatment retrospectively, using the same animals examined here: A clear dose dependent effect of scopolamine was observed (Figure 6), with significant impairment seen at 1mg/kg (i.p.) and the effect appeared to be independent of delays (c.f., Dunnett et al., 1990). The pivotal contribution of NMDAR activity and glutamatergic transmission in the hippocampus to working memory has been suggested by Bannerman and colleagues (e.g. McHugh et al. 2008; Bannerman et al., 2006, 2008). SSR50473 has been shown to potentiate NMDAR-mediated excitatory postsynaptic currents in rat hippocampal slice preparations (Depoortère et al., 2005), and this is consistent with the suggestion that enhanced NMDAR activation in the hippocampus had contributed to the working memory enhancement seen in the present study. Concerning the contribution of prefrontal cortex to working memory function, the primary focus is on dopaminergic activity, especially via dopamine D1 receptors (for a review, see Bach, 2004; Mehta and Riedel 2006), which may at first glance seem unrelated to the pharmacology of SSR50473. However, consistent with the relevance of prefrontal dopamine activity to working memory function, SSR50473 has been reported to increase the extra-cellular dopamine levels in prefrontal cortex in vivo (Depoortère et al., 2005), and to facilitate dopamine release in the nucleus accumbens evoked by electrical stimulation of the
basolateral amygdala (Leonetti et al., 2006). It is therefore possible that the more direct impact of SSR504734 on NMDAR-mediated glutamatergic neurotransmission within limbic cortices (e.g., hippocampus as well as amygdala) can subsequently lead to the potentiation of dopaminergic activity in prefrontal cortex. Such concerted alternations in multiple brain regions following systemic SSR504734 administration may be responsible for the enhancement in working memory function demonstrated here. Further dissections of the relative contributions between different brain structures would necessitate additional experiments involving local intracerebral drug infusion.

Figure 6. Dose-dependent sensitivity of the delayed alternation test to systemic scopolamine. A dose response analysis of scopolamine (0, 0.5 and 1mg/kg i.p.) was conducted on the same animals that had completed the three SSR experiments reported here (n=15). The animals were given 2 weeks for recovery after Experiment 3, before commencement of the scopolamine experiment. The experiment lasted for 5 days. On day 1, all animals received one day of practise training on the 1s-4s-8s delay function. On day 2, a similar practise session took place with all animals receiving a saline (0.9% NaCl) vehicle pre-treatment. The effect of scopolamine was evaluated over the next 3 days. A Latin square design was employed to balance individual drug experience. The higher dose (1mg/kg) clearly reduced choice accuracy as compared to the control, which was seen across all delays conditions. Choice accuracy following treatment at the lower dose (0.5mg/kg) was intermediate between control and the higher dose, indicating that scopolamine disrupted performance in a dose-dependent manner. This gave rise to a significant main effect of dose \( F(2,28)=3.50, p<0.05 \) and of delays \( F(2,28)=6.56, p<0.01 \) based on a 3 × 3 (dose × delays) ANOVA of percent correct. The impairment produced by 1mg/kg scopolamine relative to vehicle treatment was confirmed by supplementary planned contrast analysis \( F(1,14)=9.88, p<0.01 \), but the deficit produced by 0.5mg/kg scopolamine did not reach statistical significance \( p>0.3 \). The impairment produced by 1mg/kg scopolamine did not appear to be delay-dependent, given the absence of a significant dose × delays interaction. * \( p<0.05 \) based on planned contrast analysis applied to the within-subject factor of dose. Error bars refer to ±SEM.

CONCLUSIONS

The present report demonstrated, for the first time, that the enhancement of glycine availability in brain by inhibition of its uptake via the GlyT1 transporter can lead to a clear mnemonic improvement in non-pathological conditions. In particular, it adds to previous reports of SSR504734’s efficacy to restore the social recognition deficits in rats induced by neonatal exposures to phencyclidine, and to alleviate the sensorimotor gating deficit intrinsic to the DBA/2 mouse strain (Depoortère et al. 2005, Harich et al. 2007). The current novel finding thus substantiates the potential use of GlyT1 inhibitors, and specifically SSR504734, as adjunctive medication in the treatment of cognitive and negative schizophrenia symptoms (Kinney et al. 2003; Kinney and Sur 2005), and further extends its potential application in other domains of cognitive well-being (Lechner 2006) as highlighted by recent studies of GlyT1 knockout mice (Yee et al. 2006; Singer et al. 2007).

DISCLOSURE
Chapter 7: The glycine transporter 1 inhibitor SSR504734 enhances working memory performance in a continuous delayed alternation task in C57BL/6 mice

The experimental manipulations and procedures described here had been previously approved by the Swiss Cantonal Veterinary Office, and conform to the ethical standards required by the Swiss Act and Ordinance on Animal Protection and the European Council Directives 86/609/EEC. No conflicts of interest are to be declared by any of the authors of the present article.
REFERENCES


Coyle JT, Tsai G (2004) NMDA receptor function, neuroplasticity, and the pathophysiology of schizophrenia. *Int Rev Neurobiol* 59:491–515


Danysz W, Parsons AC (1998) Glycine and N-methyl-D-aspartate receptors: physiological significance and


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Chapter 8

General Discussion
Overview

The central question of the present thesis was to evaluate whether indirect interference with the NMDA receptor via inhibition of GlyT1 would be a feasible approach to modulate cognitive behaviour and whether such interventions may provide novel therapeutic avenues to rectify cognitive impairments or to enhance cognition in the normal population. The studies built on previous findings showing that molecular down-regulation or pharmacological blockade of GlyT1 efficiently potentiated NMDA mediated transmission (Tsai et al., 2004, Bergeron et al., 1998, Depoortère et al., 2005); and they aimed to evaluate the potential mechanistic links between GlyT1 function, NMDA activation and the expression of cognitive behaviour. Two this end, two molecular-based approaches were adopted by generating conditional GlyT1 knockout mice with differing specificity in the deletion of the GlyT1 gene in the forebrain (complete or neuronal); thereby achieving a distinction that current GlyT1 inhibitors cannot yet achieve. Comparison of the two GlyT1 knockout systems thus provided an experimental approach to dissect the cognitive effects of neuronal from complete GlyT1 deletion in forebrain. This was complemented by a pharmacological approach which focused on the acute short-term effects of systemic GlyT1 blockade achieved by the GlyT1 inhibitor SSR504734 as opposed to an irreversible defect of GlyT1 due to molecular gene deletion.

The first series of experiments included in Appendix I (see Appendix I) confirmed that GlyT1 deletion restricted to forebrain neurons drastically reduced GlyT1-specific glycine uptake implying an elevation in extra-cellular glycine concentration. This was accompanied by a potentiation of NMDA function as evidenced by an increase in hippocampal NMDA currents and a substantial reduction in the hyperlocomotor response to NMDA blockade by PCP or MK-801 (see Appendix II). At the behavioural level, the mutation improved the mnemonic performance in multiple paradigms including associative learning, object recognition memory and reversal learning (see Chapters 2-3). Furthermore, the wide range of pro-cognitive effects was not limited to a mere strengthening of learned memory in terms of retention and expression but also led to enhanced selectivity and flexibility in learning which was realized in tune with goal-directed behaviour in problem solving tasks. Hence, these results demonstrate that GlyT1 deletion restricted to forebrain neurons is sufficient to potentiate NMDA mediated transmission and to enhance cognitive capabilities in various learning situations suggestive of a general enhancement in cognitive flexibility or adaptability.

The experiments presented in Chapter 4 evaluated the functional impact of complete forebrain specific GlyT1 deletion on NMDA mediated transmission and cognitive behaviour. Evidence for enhanced NMDA function was provided by the near-complete nullification of NMDA blockade by PCP presumably due to increased availability of glycine in the vicinity of NMDA’s glycine-B site which was supported by a drastic reduction in GlyT1 mediated glycine re-uptake implying an increase in extra-cellular glycine. This, however, was not accompanied by an increase in hippocampal NMDA currents as observed following more restricted forebrain neuronal GlyT1 deletion, thus constituting a notable physiological difference between the two GlyT1 knockout systems. Behavioural assessment
revealed that complete forebrain specific GlyT1 deletion led to enhanced object recognition memory and working memory but affected neither associative learning nor reversal learning, indicating that there were unique phenotypes to each of these two GlyT1 knockout mouse lines, in addition to some common phenotypes. The data also show that forebrain neuronal GlyT1 disruption is associated with a wider spectrum of pro-cognitive effects than complete forebrain specific GlyT1 disruption. On the other hand, forebrain neuronal GlyT1 deletion attenuated prepulse inhibition (PPI), a measure of sensory motor gating, which was not altered by complete forebrain GlyT1 disruption (see Chapter 5). These phenotypic differences demonstrate the importance and value in distinguishing between neuronal and non-neuronal GlyT1 populations in the control of cognitive behaviour and may point towards a functional dissociation between neuron and glia-associated GlyT1.

The experiments described in Chapter 6 further evaluated the effects of the two GlyT1 knockout systems on associative learning in a series of conditioned freezing paradigms. The results revealed that forebrain neuronal GlyT1 disruption increased both the magnitude and the persistence of the conditioned freezing response to a discrete conditioned tone stimulus (CS) previously having been paired with a foot shock unconditioned stimulus (US) and enhanced contextual fear when the animals were explicitly conditioned to a novel context in the absence of any discrete CS. This was accompanied by increased sensitivity to CS-US discontinuity in a trace conditioning experiment, in which a protracted interval between CS and shock presentation normally attenuates conditioned response, thus lending further support to the suggestion that specific GlyT1 deletion in forebrain neurons does not simply strengthen any potential associative links between stimuli at the cost of selectivity in learning; selectivity was again enhanced in these mutant mice. In contrast, complete GlyT1 disruption never affected conditioned response which confirms the null-effect seen in Chapter 4.

The next series of experiments aimed at exploring the effects of systemic GlyT1 blockade by the GlyT1 inhibitor SSR504734 on memory function and the reaction to psychomimetic drug challenge. Chapter 7 demonstrates that systemic SSR504734 dose- and delay-dependently improved working memory performance in a continuous delayed alternation task when the demand on memory retention was high. In addition, SSR504734 attenuated the motor responsiveness to NMDAR blockade by PCP (see Appendix IV). These results closely match the working memory enhancement and the reduced responsiveness to PCP following complete forebrain specific GlyT1 knockout suggesting that blockade or deletion of GlyT1 in a cell-type non-specific manner may be useful to enhance working memory function and to counter NMDAR hypofunction. This may be indicative of an antipsychotic profile against negative and cognitive symptoms of schizophrenia according to the glutamate hypothesis of schizophrenia (Javitt 2007). On the other hand, the experiments described in Appendix IV and V also revealed that the SSR504734 potentiated the behavioural effects of dopaminergic agonists. Given that dopamine agonist induced behaviours are closely related to positive symptoms of schizophrenia (i.e. Arguello and Gogos, 2006) this may suggest that the potential beneficial effects of
GlyT1 inhibition against negative and cognitive symptoms of schizophrenia may be compromised by an exacerbation of the positive symptoms of the disease which would have major implications on the proposed therapeutic use of GlyT1 inhibitors as antipsychotic drugs (e.g. Lechner et al., 2006). Together, the results presented in this thesis clearly illustrate that inhibition of GlyT1 provides a feasible approach to modulate cognitive behaviour. The cognitive effects exhibited by forebrain neuronal GlyT1 disruption were not limited to a mere strengthening of learned memory or facilitation in its acquisition, but also led to enhanced selectivity and flexibility in learning, two core features of effective learning, thus providing convincing evidence that cognitive functions can be enhanced via interference with GlyT1. However, the emergence of such effects was largely dependent on the selective deletion/inhibition of GlyT1 in forebrain neurons as opposed to a cell-type non-specific loss of GlyT1 in forebrain. This highlights the importance and value to distinguishing between neuron and non-neuronal GlyT1 which is highly instructive for the development of cognition modulating agents for therapeutic use against cognitive impairments or for non-clinical enhancement in otherwise healthy people. However, the finding that forebrain neuronal GlyT1 deletion also attenuated the expression of PPI, which may be interpreted as a deficit in pre-attentional information processing (Arguello and Gogos, 2006), demonstrates that the beneficial effects of the GlyT1 targeted manipulation are likely to be context-dependent and that the induced bias in cognitive behaviour could be counterproductive or even harmful in certain situations.

**Functional dissociation between complete and neuronal GlyT1 deletion in forebrain**

The rationale behind molecularly deleting GlyT1 is to up-regulate the extra-cellular glycine levels aiming at potentiating NMDAR-mediated transmission by enhancing NMDAR’s glycine-B site occupancy. Evidene for a potentiation of NMDAR function is the drastic reduction of a response to NMDAR blockade by PCP which was consistently observed independent of the specificity of the GlyT1 targeted manipulation (complete or neuronal). However, while this was accompanied by an enhancement in hippocampal NMDAR currents in the forebrain neuronal GlyT1 knockout mice (in the following referred to as ‘CamKII/GlyT1-KO mice’), NMDAR currents were not altered in the complete forebrain specific GlyT1 knockouts (in the following referred to as ‘EMX/GlyT1-KO mice’) indicating that the impact on NMDAR function critically depends on the specificity of the GlyT1 targeted intervention. Apart from activating the glycine-B site at individual NMDARs GlyT1 inhibition and the resulting increase in extra cellular glycine concentration can trigger a number of cellular processes regulating NMDAR function at the networks level which may counter the enhancing effects of glycine at individual NMDARs as discussed in detail in Chapters 1 and 7. These possible regulatory mechanisms are not mutually exclusive, and they may collectively contribute to the presence and absence of enhanced NMDAR currents in the CamKII/GlyT1-KO and EMX/GlyT1-
KO mice, respectively. Specific deletion or inhibition of GlyT1 in forebrain neurons may therefore provide a feasible strategy to minimize the negative impact of increased glycine levels on overall NMDAR function at the networks levels. This may open novel possibilities in the design of agents aiming at enhancing NMDAR function.

However, although the placing of the functional GlyT1 deletion under the control of the forebrain neuron-specific CamKIIa promoter constitutes an important approach in distinguishing the roles of neuronal and non-neuronal GlyT1 in brain function it does not allow one to attribute any effects as being specific to the removal of this gene in neurons. The possibility that removal of the same gene in the other major cell type that expresses it, namely glia (astrocytes), may also produce similar effects cannot be excluded. As such, based on the present results one cannot conclude with certainty that the unique effect of forebrain neuronal GlyT1 disruption are specific to the function of GlyT1 in one cell type (neurons) as opposed to an effect of generally elevated extracellular levels of glycine. Hence, the demonstrated functional relevance of the neuronal GlyT1 component should not be understood as an exclusion of the possible importance of the other (astrocytic) components. In order to reveal the precise contribution of the two subsets of GlyT1 restricted knockout of GlyT1 in glia cells would be required which would allow a comparison with the effects associated with neuronal loss of GlyT1. A number of different conditional Cre systems designed to enable inducible, astrocyte specific recombination of floxed genes have been described in a recent study by Casper et al. (2007). However, none of those achieves the required efficacy and specificity to selectively target astrocytes in forebrain areas such that a meaningful comparison with the CamKII/GlyT1-KO system is not at present feasible. Hence, until a satisfactory glia-specific knockout system is available, comparing the effects of neuronal and complete GlyT1 deletion therefore constitutes one possible approach to dissect the in vivo functions of neuron and glia-specific GlyT1.

Although the underlying mechanisms are poorly understood, the difference on NMDAR currents constitutes a notable physiological difference between the two GlyT1 knockout systems which may account for the observed phenotypic differences and may explain the wider spectrum of pro-cognitive effects associated with forebrain neuronal GlyT1 disruption which uniquely potentiated NMDAR currents. Given the pivotal role of hippocampal NMDAR activity in associative learning (Gruart and Delgado-Garcia, 2007; Martinez et al., 2007; Sahun et al., 2007; Valenzuela-Harrington et al., 2007) the presence or absence of enhanced hippocampal NMDAR currents therefore provides a plausible parsimonious explanation for the multiple effects on associative learning in the CamKII/GlyT1-KO mice but not in the EMX/GlyT1-KO mice. However, excessive neural signalling through NMDARs on its own does not necessarily lead to enhanced learning and memory function. Behavioural learning and the formation of memories is a highly dynamic process which requires a flexible neuronal network. The central mechanism thought to underpin memory is synaptic plasticity (Silva 2003), regulating changes in the strength and size of synapses that increase or decrease efficiency of transmission. The fundamental model of synaptic plasticity is long-term potentiation (LTP) referring
to an increase in synaptic signalling after the synchronised stimulation of connected neurons. LTP and its opposing process, long-term depression (LTD) are therefore widely considered as the major cellular mechanisms underlying learning and memory (Cooke and Bliss 2006). Since NMDAR is critically involved in the induction of different types of LTP (Lisman 2003) and NMDAR dependent LTP is assumed to play a central role in the formation of hippocampus-dependent memories (Tang et al., 2001) it would be interesting to assess whether the hippocampal LTP/LTD would also differ between the two GlyT1 knockout lines.

Not only do hippocampal NMDARs play an important role in associative learning but are also critically involved in the expression of working memory (e.g. Bannerman et al., 2008). At first glance, it is therefore intriguing that spatial working memory performance in the Morris water maze was enhanced in EMX/GlyT1-KO mice (see Chapter 4) but not in CamKII/GlyT1-KO mice (see Chapter 3) in which NMDAR currents were enhanced. However, there is evidence that the employed two-trials-per-day paradigm may not be sufficiently demanding to tax working memory in the CamKII/GlyT1-KO mice as discussed in detail in Chapter 3, which complicates the interpretation of working memory function in these mutant mice. Further investigations using more challenging working memory paradigms would therefore be required to resolve the question whether working memory might be enhanced by forebrain neuronal GlyT1 deletion or not. Secondly, working memory performance was also improved by the GlyT1 inhibitor SSR504734 (see Chapter 7) which has been found to potentiate NMDAR currents (Depoortère et al. 2005). It follows that enhanced working memory function achieved by deletion or blockade of GlyT1 does not necessarily depend on the presence of increased NMDAR currents in the hippocampus. This raises the question as to what is the mechanism underlying the working memory enhancement in the EMX/GlyT1-KO mice.

One critical determinant of working memory function is the ability to overcome or resolve interference (Jonides and Nee 2006). Thus, it may be possible that the working memory enhancement in the EMX/GlyT1-KO mice resulted from a reduced susceptibility to proactive interference rather than represent an increase in strength and/or persistence of memory trace. In the employed two-trials-per-day working memory task the source of pro-active interference was derived from platform locations of previous days which were irrelevant to the goal platform location on the current trial and thus potentially interfered with the acquisition and recall of the current goal location. However, such a facilitatory effect was not generalized to reversal learning, when proactive interference was much more severe. By contrast, CamKII/GlyT1-KO mice showed a clear performance enhancement under increased levels of pro-active interference on repeated new-learning in the water maze and on two-choice discrimination reversal learning in the T-maze (see Chapter 3). It is therefore rather unlikely that reduced susceptibility to pro-active interference can explain the working memory enhancement of the EMX/GlyT1-KO mice.

Another possibility is that changes in other forebrain regions may be responsible. Synaptic plasticity is known to occur in several forebrain structures which are implicated in different types of
learning. A potential candidate might be the prefrontal cortex which is known to play an important role in working memory processes. However, prefrontal modulation of working memory is rather mediated via dopaminergic than via glutamatergic processes (for a review, see Barch, 2004; Mehta and Riedel 2006) which may suggest that reduced GlyT1 function possibly indirectly activates prefrontal dopamine activity. Direct evidence that GlyT1 inhibition increases prefrontal dopamine activity comes from a study by Depoortère et al., (2005) showing that SSR504734 significantly increased the extra-cellular dopamine levels in the prefrontal cortex. However, whether increased dopaminergic activity in the prefrontal cortex may indeed account for the working memory enhancement in the EMX/GlyT1-KO mice remains to be further elucidated. One possible way is to test whether the observed working memory effects could be reversed by local blockade of dopamine receptors in the prefrontal cortex via intracerebral infusion of dopamine antagonists. In order to further dissect the relative contributions of different brain structures additional experiments are necessary with restricted deletion or local pharmacological blockade of GlyT1 in the critical brain regions. Nevertheless, these results support the possibility that the behavioural changes induced by interference with GlyT1 may not be solely mediated via NMDAR related processes but that concomitant alternations in the dopamine system may contribute to cognitive effects depending on the memory system involved.

Overall, the present findings demonstrate that both neuronal and complete GlyT1 deletion in forebrain effectively counter NMDAR blockade presumably due to increased glycine-B site occupancy by glycine. However, only the former potentiated NMDAR currents in the hippocampus suggesting that both GlyT1 knockout systems ‘enhance’ the properties of individual NMDARs in a similar way, but they differentially interfere with the regulation of NMDAR function at the network levels which may account for the difference on NMDAR currents. Given that hippocampal NMDARs play an important role in learning and memory the unique enhancement in NMDAR current in the CamKII/GlyT1-KO mice may explain the wider spectrum of pro-cognitive effects in these mutant mice. The precise mechanisms whereby these changes in cognitive functions are realized at the system and in terms of brain-circuitry remain poorly understood. Selectivity of the GlyT1 molecular deletion therefore requires further refinement in its expression to advance on this direction. The unique constellation of behavioural phenotypes demonstrated in the CamKII/GlyT1-KO mice provides convincing evidence that specific inhibition/deletion of GlyT1 in (forebrain) neurons may provide a feasible approach to enhance cognitive functions. The way in which this may prove useful to achieve cognitive therapeutic or non-clinical enhancement in humans is addressed in the following sections.
**GlyT1 disruption restricted to forebrain neurons enhances the formation of associative links without compromising selectivity is associative learning**

There is strong evidence that GlyT1 deletion restricted to forebrain neurons leads to enhanced associative learning as evidenced by enhanced conditioned responding of the CamKII/GlyT1-KO mice in various Pavlovian conditioning paradigms. First, the mutant mice exhibited an enhanced conditioned freezing response to a tone conditioned stimulus (CS) previously having been paired with a foot shock unconditioned stimulus (US) and learned to avoid a foot shock US signalled by a noise-CS at a faster pace than the controls indicating that the mutation improved acquisition as well as retention of associative learning (see Appendix I). Second, the experiments in Chapter 6 revealed that enhanced tone freezing in the mutant mice persisted over extended retention intervals up to 3 weeks and this effect remained apparent even in aged mice over 24 months old (Appendix III). In addition, the mutation also enhanced contextual fear when the animals were explicitly conditioned to a novel context in the absence of any discrete CS. Hence, this pattern of results indicates that the CamKII/GlyT1-KO mice generally showed a more intense conditioned response (CR) to the best predictors of the aversive US. This phenotype clearly points to a mnemonic effect of the mutation, however, in terms of ‘cognitive enhancement’, the question also arises whether it would be (always) desirable to facilitate the formation of associative links and to enhance memory retention of such associations? Associative learning in the form of classical conditioning is likely to develop whenever one event reliably precedes another and plays an important role in numerous causal judgments we make in course of our daily lives (e.g. Shanks and Dickinson 1987). Thus, an enhancement in the formation and/or expression of potential associations might improve the ability to identify significant relations between events which certainly would be beneficial in many occasions but it might be counterproductive in certain situations such as the development of drug tolerance, addiction or phobias. It follows that an indiscriminate strengthening of associative links might not be desirable in terms of the persistence of formed associations (forgetting) and in terms of selectivity in associative learning. It is therefore important to address whether enhanced associative learning in the CamKII/GlyT1-KO mice leads to abnormally persistent conditioned response or possibly compromises selectivity in associative learning.

A common approach to evaluate the persistence of conditioned responding is extinction learning (Pavlov 1927). In classical Pavlovian conditioning, extinction refers to phenomenon that any procedure in which the predictive value of the CS with respect to the occurrence of the US is reduced will result in a decrement of the conditioned response to the CS. A widely used approach to assess extinction learning is to repeatedly present the CS without the antecedent US (see for a review Myers and Davis 2007). Extinction learning is commonly understood as a form of ‘learning learning’ implying the acquisition of new information, namely that the CS is no longer a predictor of the US whereby the previously formed CS-US association remains intact. This mechanism has been
incorporated into a large number and variety of theories (e.g., Bouton 1993, Pearce 1987, Wagner 1981). Extinction learning cannot be explained by forgetting because its demonstration requires the exposure to the CS in the absence of the US as opposed to the simple passage of time in forgetting. Resistance to extinction is indicative of impaired extinction learning which can result in abnormally persistent memories of aversive events as observed in the Post-traumatic Stress Disorder (PTSD) (see for a review de Quervain 2008). The impact of the mutation on long-term extinction learning was evaluated in Chapter 6. The results showed that the rate of extinction across subsequent CS-tests conducted on three consecutive days was comparable between mutants and controls although CS-freezing was consistently increased in the mutants. Similarly, the cessation of the freezing response within a day did not differ between the two groups of mice. This demonstrates that enhanced conditioned responding in the mutants was not accompanied by a resistance to extinction providing evidence that enhanced conditioning in the mutants did not lead to reduced extinction learning. Likewise, extinction learning was not affected by complete forebrain specific GlyT1 knockout, indicating that GlyT1 deletion in forebrain does not interfere with extinction learning independent of the specificity of the GlyT1 targeted mutation.

A widely used paradigm to assess selectivity in associative learning is latent inhibition (LI) which refers to the phenomenon that (non-reinforced) exposure to the to-be-conditioned stimulus (CS) prior to conditioning results in a decrement of the conditioned response (Lubow 1959) demonstrating that the associative history of the CS critically influences its associative strength. According to some theoretical accounts (Mackintosh, 1973, Lubow, 1989) LI is considered as a form of learned inattention describing the ability to ignore or reduce attention to stimuli that appear irrelevant. Alternatively, LI has also been characterized as a form of proactive interference in which the CS-no consequence relationship experienced by subjects during the preexposure phase interferes with the retrieval of an effective CS-US association acquired subsequently as the two stimuli are explicitly paired (Bouton, 1993; Kraemer and Spear, 1991, 1993). As a result, the inconsistent associative histories of the CS can compete for behavioural control in response to the CS leading to a reduction in the vigour of the CR. Although the precise mechanisms underlying LI are still a matter of debate it is important to note that LI constitutes a paradigm in which the generation of the CR is normally attenuated and LI deficiency is associated with an enhancement of the CR. LI therefore constitutes an suitable paradigm to assess whether enhanced conditioning in the CamKII/GlyT1-KO mice may negatively influence selectivity in associative learning by reducing the sensitivity to the reinforcement history of a given CS. Impaired LI is widely considered as endophenotype of schizophrenia reflecting impaired attention or the inability to compare and express stored associations (Weiner 1990, 2003). The effect of the mutation on the expression of LI was assessed in multiple conditioning paradigms as described in Appendix III. The results showed that in conditions that were insufficient to yield LI in the controls, a clear expression of LI was revealed in the CamKIIα/GlyT1 KO mice. LI in the mutant mice was expressed against the presence of enhanced associative learning in the non-preexposed (nPE)
condition. This pattern of results indicates that the mutation did not impair selectivity of learning resulting from CS non-reinforcement prior to CS-US contingency; in contrast selectivity was actually enhanced.

Another way to demonstrate selectivity in associative learning in which conditioned responding is normally reduced is the trace conditioning paradigm. In classical conditioning paradigms, the US is normally presented immediately after the offset of the CS. The introduction of a time interval – a so-called trace interval – between the offset of the CS and the onset of the US normally attenuates the CR. This reflects a temporal control over Pavlovian learning and shows that the temporal CS-US contiguity is a critical determinant for the expression of the CR. The effect of forebrain neuronal GlyT1 disruption on the insertion of a 20sec CS-US trace interval in conditioned tone-freezing was evaluated in Chapter 6. It was found that the mutants were more sensitive to the trace effect. Again, this was observed against the presence of enhanced conditioning in the 0sec-trace condition. It follows that the mutation enhanced tone-freezing as well as its non-freezing providing further evidence that enhanced conditioning in the CamKII/GlyT1-KO mice does not indiscriminately strengthen associative links.

In summary, the present findings indicate that enhanced associative learning in the CamKII/GlyT1-KO mice in terms of strength of CS, and resistance to temporal decay did not indiscriminately strengthen the formation and/or expression of any potential links. Moreover, there is evidence that selectivity in learning was actually enhanced by the mutation as evidenced by an increased sensitivity to the CS reinforcement history as well as the CS-US temporal discontiguity. However, the emergence of these effects was largely dependent on the selective deletion of GlyT1 in forebrain neurons because LI as well as trace-conditioning was not affected by complete forebrain specific GlyT1 disruption.

**GlyT1 disruption in forebrain neurons is associated with enhanced cognitive flexibility/adaptability**

In associative learning, restricted deletion of GlyT1 in forebrain neurons provided evidence of increased sensitivity to parameters affecting the generation of the conditioned response. This could be interpreted as a sign of enhanced flexibility in learning. In terms of cognitive enhancement, the question arises whether this can be translated to the domain of goal-direct behaviour. Would the mutant mice be more adaptive to changes in the conditions that lead to a goal? This was tested in a number of problem solving tasks which required the flexible use of memories in order to adapt to changes in the task-response contingencies.

One of the tasks that requires highly flexible use of learned information is “working memory” as defined by Olton (1979) which requires the animals to employ context/situation specific information from the relevant past in obtaining the goal or finding the solution to a problem effectively. Spatial working memory was assessed using the standard two-trials-per-day water maze paradigm in which
the presence of working memory was indicated by an improvement in the performance from trial 1 to trial 2 (see Chapter 3). The CamKII/GlyT1-KO mice showed an enhanced performance on trial 1 when the ‘goal’ (platform location) was essentially unknown to the animals but they showed very limited improvement form trial 1 to trial 2 as opposed to a clear enhancement across trials in the controls. At first glance, the mutants’ failure to improve across trials may be considered as a working memory deficit which would undermine the hypothesis that forebrain neuronal GlyT1 disruption might lead to enhanced cognitive flexibility. However, the overall performance across the two trials was actually enhanced in the CamKII/GlyT1-KO mice and the lack of trial 1 to 2 improvement was mainly due to an enhanced performance on trial 1. This anomalous working memory performance does not readily conform to a classical working deficit associated with comparable trial 1 performance and poor trial 2 performance. As discussed in Chapter 3, one possible explanation is that the mutants were using a non-mnemonic search strategy and thereby eliminated the negative influence of proactive interference from previous days’ platform locations affecting the controls particularly on trial 1. This may have enabled the mutants to outperform the controls on trial 1 without losing out to the controls on trial 2. As a result, in terms of overall escape performance, the mutants’ non-mnemonic strategy was more successful to achieve the goal (escape from the water) than the use of working memory in the controls which were suffering form pro-active interference. This may be a sign of reduced susceptibility to proactive interference in the CamKII/GlyT1-KO mice.

In contrast, a clear enhancement on working memory was observed in the EMX/GlyT1-KO mice, which has been discussed before. Since the CamKII/GlyT1-KO mice were obviously not relying on the recollection of the platform location learned in trial 1 to guide their search on trial 2 the employed working memory paradigm is not suitable to compare working memory function between the two GlyT1 knockout mouse lines. Other working memory paradigms, such as the operant delayed alternation task described in Chapter 7, might be better suited to tax working memory in the CamKII/GlyT1-KO mice and to compare the working memory performance between the two GlyT1 knockout mouse lines. Nevertheless, the dissociable phenotype emerged on the present working memory experiment underlines that the impact on cognitive behaviour in a given learning situation is largely dependent on whether GlyT1 is deleted in the entire forebrain or specifically in forebrain neurons.

The clearest effect in the CamKII/GlyT1-KO mice was the substantial enhancement on trial 1 which may be associated with a reduced susceptibility to pro-active interference. Whether this may constitute a general trait of the mutation was further explored by testing the animals’ learning capabilities under increased levels of pro-active interference. First, the animals were tested to adopt their search to a sudden change in the platform location after being familiarized with that location across several days. The CamKII/GlyT1-KO mice were found to be substantially less affected by this manipulation than the controls; they more efficiently detected novel platform location on the first day of new-learning when the influence of pro-active interference from the previous platform location was expected to be
maximal but they eventually converged to the control level by the fourth day of new learning. The robustness of this effect was confirmed by a second new-learning session with yet another platform location which revealed a highly similar pattern of results. Furthermore, the superior performance of the mutants on the first day of new learning was no longer observed when the source of pro-active interference was removed by moving the maze to a novel room thereby re-setting the extra-mice cues. These results strongly indicate that the mutants adapted faster to changes in the conditions that that lead to the goal under increased levels of pro-active supporting that hypothesis that the mutation reduces the negative impact of pro-active interference. To further assess the generality of this finding a two-choice simultaneous discrimination reversal was designed task in which the pro-active interference effects was maximized. Again the mutation did not affect the initial learning (acquisition) but facilitated learning in the reversal phase when the response-outcome contingency was reversed. All together, these results are strongly supportive of the hypothesis that forebrain neuronal GlyT1 deletion reduces the negative influence of pro-active interference which is realized in goal-directed behaviour leading to an enhanced adaptability to changes in the conditions that lead to a current goal. However, there is evidence that the forebrain neuronal GlyT1 disruption does not reduce proactive interference per se, which is derived from the finding that the mutation increased the sensitivity to the LI effect. As discussed before, one theoretical account of the LI effect is precisely in terms of proactive interference (e.g., Bouton 1993; Kraemer and Spear, 1991; Weiner 1990), such that non-reinforced CS exposure leads to the formation of an CS-nothing association, which competes with the more recently formed CS-US association for behavioural control in the CS-test. From this it follow that reduced susceptibility to pro-active interference is expected to attenuate rather than enhance the LI effect. As mentioned in Appendix II, one possible explanation for this discrepancy is that the mutation led to more efficient learning of both the CS-nothing (during pre-exposure) and the CS-US (during conditioning) associations which allowed the former association to exert a bigger impact on the expression of CR in the mutants than in the controls under pre-exposure conditions that were insufficient to yield LI in the controls. In this manner, the enhancement of LI can be explained solely in terms of enhanced associative learning lending further support to the hypothesis that the mutation facilitates the formation of associative links. This further highlights that reduction in pro-active interference as such does not guarantee enhanced adaptability. Pro-active interference can positively or negatively affect the achievement of a goal. It follows that the appropriateness or adaptiveness of the response selection when confronted with changes in test conditions is to be judged in terms of the realization of the current goal. In classical conditioning, the goal behind the resulting change in behavioural response is less specific. However, a goal was clearly defined in both the water maze (escape from water) and the T-maze (obtain food reward) paradigms when the mutants exhibited a clear enhancement to achieve the goal when the task contingencies were changed. Hence the constellation of cognitive phenotypes identified in the CamKII/GlyT1-KO mice thus far is suggestive of an enhancement in cognitive flexibility and behavioural adaptability in the sense that the
mutation enhances the selective expression of learned information, and the readiness to adopt and entertain possible alternative strategies in problem solving in a given test situation. This also underlines that forebrain neuronal specific GlyT1 deletion does not simply strengthen memory traces or increase the rate of learning. These properties may be of therapeutic value since different forms of cognitive inflexibility are present in a number of neuropsychiatric diseases including schizophrenia. A general enhancement in cognitive flexibility/adaptability may also be desired by the non-clinical population in various situations; it may improve the ability to solve problems which in turn may enhance performance in school or at work. Hence, interference with GlyT1 may be useful for therapeutic as well as for non-clinical enhancement. However, the translation of such effects to humans remains an uncertain question which will be addressed in the next sections.

Deletion/inhibition of GlyT1 in forebrain neurons: A potential approach to achieve cognitive enhancement?

'cognition enhancement' refers to possible approaches aiming at boosting cognitive functions that underpin information processing including attention, learning and memory. The term was originally used for the treatment of cognitive impairments associated with diseases such as dementia and schizophrenia. Nowadays, the term 'cognition enhancement' is applied to the use of interventions for normal ageing and for non-clinical purposes. It follows that cognition-enhancing technologies have three potential applications: diseases with severe cognitive impairments, normal ageing and mild cognitive deficits; and non-clinical enhancement (e.g. Caplan and Elliott 2004, Whitehouse 2004, Farah et al., 2004)

Forebrain specific GlyT1 disruption led to a number of biases and shifts in the expression of higher cognitive behaviour which may be associated with 'cognitive enhancement'. Not only did the mutation facilitate the formation of associative links in classical conditioning, it also increased the sensitivity to changes in the temporal CS-US contiguity (trace effect) and the reinforcement history of the to-be-conditioned CS (LI effect) implying that excessive conditioning in the mutants was not at the expense of selectivity in associative learning; selectively was actually enhanced by the mutation which could be interpreted as enhanced flexibility in learning. Furthermore, this was not limited to simple associative learning; it could be translated into the domain of goal-directed behaviour. The mutant mice more rapidly adapted to changes that lead to a current goal, as evidenced by enhanced performance under conditions of high levels of proactive interference. This reduced susceptibility to the negative influence of the past in the form of pro-active interference was not due to weaker memory retention or forgetting. In contrast, there is evidence that memory retention was enhanced in the mutants as further demonstrated by a spontaneous object recognition memory task (see Chapter 2) which revealed that the mutation led to enhanced memory for the identity of objects when the demand
on retention was high. Furthermore, CamKII/GlyT1-KO mice did also not discount the relevance of past information. They showed enhanced retention as well as expression of the CR in aversive conditioning, to both discrete CS and the environmental context. In addition, they exhibited LI when the past non-reinforcing history of the CS failed to produce LI in the controls. This constellation of phenotypes indentified in the CamKII/GlyT1-KO mice is suggestive of a general enhancement in behavioural adaptability and flexibility due to an increased sensitivity or alertness to events indicating potential changes in environmental contingency. In discrimination reversal learning, and repeated new-learning in the water maze, the mutants were thus better prepared for environmental changes in the condition that led to the current goal. In classical conditioning, in which the resulting change in behavioural response is less specific, enhanced associative learning may enhance the animals’ ability to make predictions about environmental contingency, thereby enriching its representation of the ‘world’, such that the organism is better prepared for an adaptive behavioural response. This is also in keeping with the finding that the mutants were able to detect a change in the relative spatial position of objects and modified their explorative behaviour accordingly whilst controls were insensitive to this environmental change, thus providing further evidence for an increased sensitivity and response to changes in the environment. In summary, the behavioral profile of the CamKII/GlxT1-KO mice is suggestive of enhanced selectivity and flexibility in learning accompanied by a general increase in the alertness of environmental changes. This anomalous cognitive behaviour may be interpreted as form of ‘cognitive enhancement’. Learning - as probably the most important cognitive function - has evolved from the ability to exploit predictive environmental cues so as to enhance adaptability through appropriate behavioural adjustment or modification which implies that flexibility is a pre-requisite for effective learning. Learning relies on the acquisition of knowledge supported by perceived information leading to the development of new capacities, skills, understanding, and preferences. Given the incredible number of relevant and irrelevant environmental cues and stimuli that are constantly perceived, selectivity in learning is central to cognitive information processing because it allows to detect and relate relevant cues. Thus, effective learning is selective. On this basis one may suspect that reduced GlyT1 function in forebrain neurons may indeed exert a ‘cognitive enhancing’ effect by improving core features of learning which may be therapeutically important as to correct cognitive inflexibilities present in a number of neuropsychiatric diseases and may provide a feasible means to boost mental function in the normal population in various learning situations.

Although the diverse phenotypes emerged in the CamKIIα/GlyT1-KO mice may be associated with some form of ‘cognitive enhancement’, they cannot easily be subsumed under a single psychological description. It is likely that the observed phenotypic profile of the CamKIIα/GlyT1-KO mouse line stems from altered brain function across multiple forebrain structures. The current knockout system does not allow one to differentiate the relative contributions of different forebrain regions. To satisfactorily address this, either region-specific molecular intervention or local pharmacological...
blockade would be necessary. This also underlines the needs for regulatory DNA elements that simultaneously control cell-type and region-specific gene expression. Hence, the mechanisms whereby these changes in cognitive functions are realized remain to be identified. It may be possible that the specificity of the mutation enables a selective modulation on NMDAR function contributing to this unique behavioural profile. Furthermore, since the majority of the cognition enhancing effects associated with GlyT1 inhibition/deletion are largely dependent on its selective inhibition in neurons as opposed to cell-type non-specific inhibition this also highlights the importance of discriminating between neuron and glia-specific GlyT1 in the modulation of cognitive functions and opens novel possibilities for the development of cognition-enhancing technologies. On the other hand, this would imply that current GlyT1 inhibitors, which cannot distinguish between the two populations of GlyT1, might exert limited efficacy as ‘cognitive enhancers’. This was, however, not supported by the finding that the GlyT1 inhibitor SSR504734 (see Chapter 7) led to a clear enhancement on working memory function when the demand on retention was high. It follows that the in vivo functions of neuronal and non-neuronal (glia) GlyT1 populations warrant further investigations.

**Is there a price to pay for the cognitive bias in information processing achieved by forebrain neuronal GlyT1 disruption?**

Forebrain neuronal GlyT1 disruption increases the alertness and the adaptability to environmental changes and enhances flexibility and selectivity in learning. This change in cognitive core capabilities may be understood as ‘cognitive enhancement’. However, any biases and shifts in cognition are likely to result in multiple effects, reflecting a disturbance or deviation from the balance achieved by evolution which may not be necessarily ideal and possibly leads to both benefits and harms, depending on the precise circumstances. Flexibility and selectivity may be essential for effective learning but any extreme or rigid form is likely to be detrimental or counterproductive. This raises the intriguing question whether the cognitive bias induced by forebrain neuronal GlyT1 deletion may be accompanied by a negative impact under certain circumstances.

Assessment of prepulse inhibition (PPI) of the acoustic startle reflex revealed that PPI was clearly present in CamKII/GlyT1-KO mice but was weaker than in the controls. In contrast, EMX/GlyT1-KO mice showed normal PPI indicating that PPI reduction in the CamKII/GlyT1-KO mice is closely related to the specificity of the mutation and does not stem from a general non-specific GlyT1 inhibition. PPI represents a form of pre-attentive control or sensorimotor gating (Brann and Geyer, 1990), by which on-going information processing is protected from potential interference by antecedent stimuli (Graham 1975). In the CamKII/GlyT1-KO mice, the protection or ‘gating’ of the on-going processing of the prepulse against a distraction by the more salient pulse stimulus appears to be diminished. This cannot be attributed to changes in the ability to perceive or process the pulse and
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the pre-pulse stimulus as such, because the reaction to either stimulus when presented on its own was not affected by the mutation. Although the precise mechanism is presently unknown this nevertheless provides some evidence that the mutation may exert a negative impact on pre-attentive information processing operating outside of conscious awareness. Impaired PPI is consistently reported in acute and chronic schizophrenia patients (e.g. Braff et al., 2001) and is widely considered as an endophenotype of schizophrenia reflecting a loss of sensorimotor gating abilities in schizophrenia. Deficits in the brain's sensory gating mechanisms lead to an inability to filter out irrelevant information causing sensory overload which contributes to the emergence of schizophrenia symptoms including thought disorder or impaired attention. Is it therefore possible that forebrain neuronal GlyT1 disruption may increase the risk for schizophrenia or leads to an exacerbation of certain symptoms in schizophrenia patients? Is this a potential price to pay for the cognition enhancing effects of the mutation? Such an interpretation is contrary to the finding that the mutation attenuated the motor-stimulant effect of psychostimulant drugs which is associated with antipsychotic properties. Likewise, the enhancement of LI demonstrated in the CamKII/GlyT1-KO mice has led to the suggestion that forebrain neuronal GlyT1 disruption may exert therapeutically beneficial effects against antienational and cognitive impairments characteristic of schizophrenia (Appendix I, Yee et al., 2006). On the other hand, certain manipulations can lead to the expression of LI under conditions when LI is normally not observed (Weiner et al., 1990, 2003). Such abnormally persistent LI has been associated with impaired set shifting, the ability to update or ‘shift’ cognitive strategies in response to changes in the environment, and has therefore been considered as a model of cognitive and negative symptoms of schizophrenia (Weiner 1990, 2003) while LI disruption has been linked to positive symptoms of schizophrenia (Weiner 1990, 2003, Moser 2000). This illustrates that whether a cognitive change, such as enhanced LI in the CamKII/GlyT1-KO mice, is considered to be a ‘cognitive enhancement’ is context specific which also illustrates that the ‘therapeutic’ and ‘enhancing’ potential of a given intervention do not necessarily correspond.

To come back to the initial question as to whether there is a price to pay for the pro-cognitive effects induced by forebrain neuronal GlyT1 deletion one may come to the following conclusion. The potential costs and benefits of any manipulation are situation-dependent and their balance needs to be subjected to empirical evaluation. However, a simple trial and error strategy using a wide variety of behavioural tests of uncertain validity without drawing attention to the specific psychological and neurobiological mechanisms underlying behavioural enhancement is not desirable in search of clinically effective cognitive enhancers. For example, an improved performance in a specific behavioural task may be due to enhanced mnemonic function but could also reflect changes in motivational and attentional components which play an important role in many behavioural paradigms. It follows that a more successful strategy requires certainly more than the indiscriminate accumulation of apparently beneficial effects in a variety of behavioural tasks and animal models. Good construct and face validity are therefore essential for screening tests with predictive validity for
cognition enhancing properties. This however does not diminish the pro-cognitive effects observed in the CamKII/GlyT1-KO mice but indicates that a straightforward interpretation of the data is not possible and that further investigations are required to indentify what in psychological terms may have changed in these mutant animals.

Considering the cognitive capabilities of CamKII/GlyT1-KO mice demonstrated in different tests it is tempting to assume that such superior cognition should provide an evolutionary advantage which immediately raises the question why a mouse with similar cognitive capabilities has not already been created by selection pressure (see also Sanderson and Bannerman 2007). As discussed before, at least part of the pro-cognitive effects of the mutation are attributed to increased NMDAR activity such as enhanced associative learning. However, excessive NMDAR mediated transmission is also known to increase excitotoxicity (see for a review Albensi 2007) which plays an important role in neurodegenerative conditions such as Alzheimer’s disease (Javitt 2004). It is therefore possible that the promnesic effects of the mutation in the short-term may be at the expense of increased neurodegeneration in the long run.

In order to evaluate this hypothesis we tested whether the enhancement on associate learning demonstrated in adult CamKII/GlyT1-KO mice would be still be present in aged mice. To this end, we compared 3 and 24 month old mice in the conditioned freezing paradigm using a vibratory CS in order to circumvent the progressive hearing loss in the C57BL/6 mouse strain (Appendix III). It was found that the conditioned freezing response was clearly enhanced in both adult and aged mutant mice in comparison to the controls, which demonstrates that the enhancement on associative learning was conserved in aged animals. However, the time course of the freezing response markedly differed between adult and aged mutant mice. Namely, adult mutants reacted with a pronounced freezing response to CS presentation which quickly extinguished reaching control level at the end of the CS test. In contrast, aged mutants also reacted to the CS with a strong freezing response which, however, remained relatively stable over the entire period of testing. Is this a sign of resistance to extinction of the CR in aged mutant mice? The finding that all groups showed a comparable decrease in freezing response when re-exposed to the CS 24h later does not support a general deficit in extinction learning in aged mutant mice. This pattern of results indicates that enhanced associative learning in the mutants is not simply a short term effect but represents a robust phenotype. On the hand, the mutation’s impact on associative learning did change in aged animals as suggested by their more persistent freezing response which may be understood as reduced extinction. Hence, the appearance of an age-dependent phenotype in the CamKII/GlyT1-KO mice underlines the possibility that direct cognitive effects of forebrain neuronal GlyT1 disruption may change in the long-term. Whether this may be due to changes in brain function resulting from excessive NMDAR-mediated transmission remains highly speculative. Nevertheless this is highly instructive for the potential use of GlyT1 inhibitors - or any other agent - as potential therapeutics for cognitive impairments which most likely require chronic treatment over longer time periods in order to correct or alleviate cognitive malfunction.
Concluding remarks

Taken together, deletion/inhibition of GlyT1 provides a feasible approach to modulate and study cognitive behaviour in animals highlighting the importance and value of genetically engineered animals in neuropharmacological research attempting to expand the understanding of cognitive behaviour and its impairment in diseases or normal ageing. The constellation of behavioural phenotypes demonstrated in GlyT1 knockout mice in the present thesis strongly indicate that interference with the GlyT1 glycine re-uptake system can lead to enhanced cognitive functions in various learning situations. GlyT1 may therefore constitute a promising target for the development of cognition enhancing agents - as therapeutics against cognitive impairments or for non-clinical purposes to optimize mental function in healthy humans or halt cognitive decline in normal ageing. However, approaching possible caveats should not be overlooked. Any biases and shifts in the expression of cognitive behaviour can lead to benefit or harm depending on the actual situation or context. Tradeoffs between enhancement of certain functions and impairment are likely to occur. This implies that the therapeutic and the enhancing potentials of an intervention designed to boost cognitive functions do not necessarily correspond. Empirical evaluation using paradigms of relevance to different domains of cognitive functions is therefore indispensable to evaluate the potential costs and benefits of a specific class of interventions. Furthermore, the underlying mechanisms by which these changes in cognitive functions are realized remain largely unknown which may hinder the translations of laboratory findings into effective interventions for human use. A multidisciplinary approach is therefore required in order to deepen our understanding of the relationship between molecules, cells and brain circuits and the expression of cognitive behaviour. A combination of molecular approaches to study behaviour in genetically modified animals, imaging techniques to investigate brain structure and functional activity (Morris et al., 2005) as well as advanced neuropsychological testing of cognitive functions (Sahakian et al., 2005) may help to reveal mechanistic links between cognition and brain functions and to apply them for the design of novel therapeutically efficient cognitive enhancers.

Finally, the use of cognitive enhancers also raises social and ethical issues. Cognitive enhancement could bring great social benefits especially in the context of ageing populations, which bring with them a vast increase in both neurodegenerative diseases and normal decline in cognitive function. On the other hand, the use of cognition enhancement in the normal population produces 'medicalising' conditions in people that have not previously been regarded as needing treatment which can cause high socioeconomic costs to society. The availability of cognitive enhancers will also raise the question about where boundaries lie between different targets for modification (disease, normal aging, non-clinical enhancement) and in what situations treatment may be ethically justifiable. It follows that when evaluating the costs and benefits of cognitive enhancers socioeconomic and ethical accounts should also be considered.
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References


Graham F.K., 1975. The more or less startling effects of weak prestimulation. Psychophysiology 12, 238–248.

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Appendix I

Disruption of Glycine Transporter 1 Restricted to Forebrain Neurons Is Associated with a Procognitive and Antipsychotic Phenotypic Profile

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[Personal contribution to the work: Designing and performing the research, analysis of the behavioral data, and writing the manuscript]
Abstract

The NMDA receptor is thought to play a central role in some forms of neuronal plasticity, including the induction of long-term potentiation. NMDA receptor hypofunction can result in mnemonic impairment and has been implicated in the cognitive symptoms of schizophrenia. The activity of NMDA receptors is controlled by its endogenous coagonist glycine, and a local elevation of glycine levels is expected to enhance NMDA receptor function. Here, we achieved this by the generation of a novel mouse line (CamKII Cre;Glyt1tm1.2fl/fl) with a neuron and forebrain selective disruption of glycine transporter 1 (GlyT1). The mutation led to a significant reduction of GlyT1 and a corresponding reduction of glycine reuptake in forebrain samples, without affecting NMDA receptor expression. NMDA (but not AMPA) receptor-evoked EPSCs recorded in hippocampal slices of mutant mice were 2.5 times of those recorded in littermate controls, suggesting that neuronal GlyT1 normally assumes a specific role in the regulation of NMDA receptor responses. Concomitantly, the mutants were less responsive to phencyclidine than controls. The mutation enhanced aversive Pavlovian conditioning without affecting spontaneous anxiety-like behavior in the elevated plus maze and augmented a form of attentional learning called latent inhibition in three different experimental paradigms: conditioned freezing, conditioned active avoidance, conditioned taste aversion. The CamKII Cre;Glyt1tm1.2fl/fl mouse model thus suggests that augmentation of forebrain neuronal glycine transmission is promnesic and may also offer an effective therapeutic intervention against the cognitive and attentional impairments characteristic of schizophrenia.
INTRODUCTION
According to the glutamate hypothesis of schizophrenia, reduced NMDA receptor (NMRAR) function may contribute to the cognitive impairments of schizophrenia (Farber, 2003; Coyle and Tsai, 2004). Blockade of NMDAR is known to impair the induction of long-term potentiation and learning in animals (Morris et al., 1986; Morris, 1989; Davis et al., 1992). However, NMDAR blockade can also give rise to behavioral dysfunction such as impulsivity (Tonkiss et al., 1988) and psychotic-like behavior: phencyclidine and ketamine (two noncompetitive NMDAR antagonists classified as dissociative anesthetics) are well known psychomimetics (Farber, 2003). In contrast, coagonists of the NMDAR, such as D-serine and glycine, improve cognition and negative symptoms in schizophrenia (Coyle and Tsai, 2004). The pharmacological characterization of glycine site ligands was, however, confounded by side effects and insufficient selectivity (Danysz and Parsons, 1998). Thus, the characterization of the glycine site of NMDARs as antipsychotic drug target has remained unsatisfactory.

The action of glycine is essentially terminated by rapid reuptake, mediated by two glycine transporters, glycine transporter 1 (GlyT1) and GlyT2 (Aragon and Lopez-Corcuera, 2005; Eulenburg et al., 2005). Three variants of GlyT1 (GlyT1a, b, and c) are encoded from the same gene. Their expression is restricted to the CNS, distributed widely in the spinal cord, brainstem, cerebellum, and, to a lesser degree, in the cerebral cortex and hippocampus. Autoradiography and immunoelectron microscopy revealed that glial cells around both glycinergic and nonglycinergic neurons contain GlyT1 (Zafra et al., 1995). Novel sequence-specific antibodies, however, have helped to reveal the presence of GlyT1 in glutamatergic synapses of neurons, particularly in the neocortex and hippocampus (Cubelos et al., 2005a). Recently, GlyT1 was also shown to interact with the scaffolding protein PSD-95 (postsynaptic density-95) (Cubelos et al., 2005b). These findings suggest that GlyT1 regulates the concentrations of glycine in the micro-environment of NMDARs. Thus, the selective GlyT1 antagonist N-[3-(4’-fluorophenyl)-3-(4’-phenylphenoxy)] propylsarcosine (NFPS) can enhance NMDAR responses in CA1 pyramidal cells after Schaffer collateral stimulation in acute hippocampal slice preparations (Bergeron et al., 1998) and augment long-term potentiation in the dentate gyrus in vivo (Kinney et al., 2003).

Behavioral analysis of GlyT1 antagonists has thus far failed to yield any convincing promnesic effects, but there is evidence that several GlyT1 inhibitors [NFPS (Kinney et al., 2003), (R)-(N-[3-(4’-fluorophenyl)-3-(4’-phenylphenoxy)propyl])sarcosine (ALX 5407) (Lipina et al., 2005), N-methyl-N-[3-[4-trifluoromethyl]phenoxy]-3-phenylpropyl]glycine (ORG 24598) (Le Pen et al., 2003)] may possess antipsychotic property. Similar findings have emerged in mice with a constitutively targeted disruption of the GlyT1 gene. Although homozygous deletion of GlyT1 was lethal (Gomeza et al., 2003), heterozygous mutant mice survived, and these animals showed an enhanced NMDA/AMPA response ratio, a resistance to disruptive effect of amphetamine on prepulse inhibition, and a tendency for improved memory retention (Tsai et al., 2004; Gabernet et al., 2005; Martina et al., 2005).
To molecularly dissect in vivo GlyT1 functions specific to forebrain neurons, from brain-wide mostly astrocytic GlyT1, the selective deletion of GlyT1 in cortical and hippocampal neurons was achieved here by CamKIIa Cre-mediated recombination of a conditional loxP-flanked Glyt1 allele in mice.

MATERIAL AND METHODS

Generation of Glyt1tm1.2 mice
ES cells carrying the conditional Glyt1tm1 allele (Gabernet et al., 2005) were subjected to a second electroporation with an FLP-expression plasmid to remove the frt-flanked neomycin resistance gene. This new Glyt1 allele was designated as Glyt1tm1.2 (see Fig. 1A). Glyt1tm1.2 ES cells were injected into C57BL/6 blastocysts, which were implanted into pseudopregnant mothers. Offspring positive for the Glyt1tm1.2 allele were bred with C57BL/6 mice and selected for germline transmission of the mutation. Finally, a homozygous Glyt1tm1.2f/f colony was established and maintained on a pure C57BL/6J background (Glyt1tm1.2Blt).

Genotyping
PCR primers for genotyping were as follows: o139, 5’-CCTA-ACCCATGGCCA GAGACC-3’, Glyt1-specific antisense primer flanking the PGK (phosphoglycerate kinase)-neomycin cassette; o184, 5’-CAT-CGCCCTTCTATCGCCTTCTTGACG-3’, sense primer specific for the PGK-neomycin cassette; o228, 5’-GTCAACCTGACTCCTAGCCC-TGTACC-3’, Glyt1-specific antisense primer 3’ to PGK-neomycin-cassette; o232, 5’-AGAAGATCTGTAGGCGGTGCATCCC-3’, antisense primer specific to the loxP-flanked region; o250, 5’-CCCAT-GCCCATCCATGC-3’, sense primer 5’ of left loxP-site; o234, 5’-TGACAGCATTGCTGTTTCAGTCC-3’, sense primer specific for Cre-recombinase; o235, 5’-GCATGATCTCCTCGGATTTGACCTCC-3’, antisense primer specific for Cre-recombinase. The thermocycle of the PCR was 35 cycles at 95°C (15 s), 68°C (20 s), and 70°C (90 s).

Glycine uptake
Adult mice (8 weeks of age) were killed by cervical dislocation and hippocampal formation, and the cerebral cortex was rapidly dissected on ice. Tissue was homogenized in 20 volumes of ice-cold 10 mM Tris, pH 7.4, 0.32 M sucrose (four strokes at 1500 rpm; Braun Potter S Homogenizer) and centrifuged for 10 min at 1000 x g. The resulting supernatant was centrifuged for 10 min at 27,000 x g to obtain the crude synaptosomal membranes. The membranes were washed twice with 25 mM HEPES, pH 7.4, 125 mM NaCl, 5 mM KCl, 2.7 mM CaCl2, 1.3 mM MgCl2, 10 mM glucose (KH buffer) and subjected immediately to [3H]glycine uptake studies. For kinetic studies, 150 µl of crude synaptosomal membranes (100 µg of protein) in KH buffer containing increasing concentrations of unlabeled glycine (5–250 µM) were incubated for 5 min at 30°C before addition of 50 µl [3H]glycine (0.1 µM final concentration; 60Ci/mmol; PerkinElmer, Emeryville, CA) in KH buffer. After 15 min at 30°C, the uptake was terminated by rapid vacuum filtration using a semiautomatic cell harvester.
(Skatron Instruments, Lier, Norway). The filters were washed with ice-cold 10 mM Tris, pH 7.4, 150 mM NaCl, dried, and subjected to liquid scintillation counting. Nonspecific [3H]glycine uptake was determined in the presence of 10 mM cold glycine, and GlyT1-specific [3H]glycine uptake was measured in the presence of 10 µM NFPS. Km and Vmax values were determined by nonlinear regression using the GraphPad PRISM 4 software (GraphPad Software, San Diego, CA).

**Western blotting**

Tissue of the hippocampus and cortex of adult mice (8 weeks of age) was homogenized in 10 vol of 5 mM Tris/HCl, pH 7.4, containing 0.32 M sucrose and centrifuged for 10 min at 10,000 x g. The resulting supernatant was recentrifuged for 10 min at 40,000 x g to obtain the crude membrane fraction. The pellet was washed twice in buffer and resuspended at a protein concentration of 5 mg/ml. Aliquots of the membranes with increasing protein content (2.5, 5, 7.5, 10, 15, and 20 µg) were incubated for 15 min at 60°C with an equal volume of 125 mM Tris/HCl, pH 6.8, 20% glycerol, 0.002% bromphenol blue, 10% -mercaptoethanol, 4% SDS, and subjected to SDS-PAGE using 10 or 7.5% mini-gels (Mini Protean II; Bio-Rad, Hercules, CA). Proteins were transferred onto nitrocellulose membranes using a Trans Blot Mini Cell (Bio-Rad). For immunodetection, the blots were blocked for 1–2 h in TBST (10 mM Tris/HCl, pH 8, 0.15 M NaCl, 0.05% Tween 20) containing 5% nonfat dry milk at room temperature (RT), followed by incubation with affinity purified GlyT1 antiserum (Gabernet et al., 2005) together with monoclonal actin antibodies (Chemicon, Temecula, CA) overnight at 4°C in TBST/5% blocker. The blots were washed once with 20 mM Tris, pH 7.5, 60 mM NaCl, 2 mM EDTA, 0.4% SDS, 0.4% Triton X-100, 0.4% deoxycholate, and three times with TBST. Incubation with the appropriate secondary antibodies was performed for 1 h at RT. After extensive washing, immunoreactivity was detected by chemiluminescence (Super Signal West Pico Chemoluminescence; Pierce, Rockford, IL). Images were captured using a Fuji LAS-1000 plus imaging system (Fujifilm, Tokyo, Japan), and immunoreactive bands were quantified with the AIDA software (version 3.25; Raytest, Pforzheim, Germany). Actin immunoreactivity was used to monitor equal sample loading.

**Receptor autoradiography**

Adult mouse brains (8 weeks of age) were rapidly dissected and frozen. Slide-mounted parasagittal sections (12 µm) were thawed and preincubated four times for 15 min in 50 mM Tris-HCl, pH 7.5, at 4°C, followed by incubation for 120 min with a saturating concentration of [3H]L-689,560 (15 nM, 23.6Ci/mmol; Tocris Cookson, Bristol, UK). After washing in buffer (five times for 1 min) and a quick dip in distilled water, the sections were dried and exposed to a tritium-sensitive phosphor-imaging screen (Packard Cyclone Storage Phosphor System). Nonspecific [3H]L-689,560 binding was assessed in parallel by including 1 mM glycine in the assay buffer. The screen was digitized with a Packard Cyclone Scanner (Packard, Meridian, CT). Specific [3H]L-689,560 binding was determined
using the Optiquant software (Packard) and an autoradiographic [3H]micro-scale standard (Amersham Biosciences, Piscataway, NJ) exposed in parallel. The analysis included four animals for each genotype and four to eight sections per animal.

Electrophysiology
All experiments were performed blinded, and genotypes were determined retrospectively. Brains were taken from postnatal day 21 (P21) to P30 mice and prepared as described previously (Gabernet et al., 2005). Whole-cell patch-clamp techniques were used to study synaptic responses of CA1 pyramidal neurons in response to Schaffer collateral stimulation delivered via a bipolar stimulating electrode (0.05 ms, 1–10 V, 10 s interstimulus intervals). CA1 pyramidal cells were recorded in the voltage-clamp configuration with 3–6 MΩ patch pipettes containing the following (in mM): 130 cesium gluconate, 10 HEPES, 5 NaCl, 1 EGTA, 5 ATP-Mg, and 0.5 GTP-Na. AMPA receptor-mediated EPSCs (AMPA EPSCs) were evoked in artificial CSF (ACSF) with picrotoxin (100 µM) at Vm –70 mV. The stimulus amplitude was adjusted to evoke average current amplitude of ~100 pA. AMPA EPSCs were abolished in ACSF containing 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide (NBQX; 20 µM). Next, the potential of the cell was clamped at +40 mV to release the Mg2+ block and permit the recording of isolated NMDA receptor-mediated EPSCs (NMDA EPSCs). In some experiments, the NMDA receptor blocker 3-((R)-2-carboxypiperazine-4-yl)-propyl-1-phosphonic acid (R-CPP), 20 µM was added at the end of the recording to ensure that all outward currents were NMDA receptor mediated. AMPA receptor-mediated miniature EPSC events (AMPA mEPSCs) were recorded at Vm –70 mV in normal ACSF containing picrotoxin (100 µM), tetrodotoxin (TTX; 1 µM), and R-CPP (20 µM) with patch pipettes containing the following (in mM): 130 potassium gluconate, 10 HEPES, 1 EGTA, 5 NaCl, 5 ATP-Mg, and 0.5 GTP-Na. Voltage-clamp recordings were obtained with an Axopatch 200B (Molecular Devices, Foster City, CA) amplifier. Signals were sampled at 2–5 kHz and acquired and analyzed using IgorPro (Wavemetrics, Lake Oswego, OR) software. The NMDA/AMPA ratio was given as the ratio of the NMDA peak amplitude to the AMPA peak amplitude (INMDA at +40 mV/IAMPA at –70mV). AMPA mEPSCs were analyzed using Mini Analysis Program (Synaptosoft, Decatur, GA). Average values are expressed as mean ± SEM. Student’s t test was used for statistical comparisons. Picrotoxin, NBQX, R-CPP, and TTX were from Tocris Cookson (Bristol, UK).

Behavioral assays
All behavioral experiments were conducted in the Laboratory of Behavioral Neurobiology, Schwerzenbach, which maintained eight independent CamKIIa Cre:Glyt1tm1.2fl/fl x Glyt1tm1.2fl/fl breeding pairs. The offspring were sexed and genotyped at 3 weeks of age by standard PCR. CamKIIa Cre:Glyt1tm1.2fl/fl (hereafter simply referred to as "mutant") mice were compared with Glyt1tm1.2fl/fl littermate controls. Mice were caged in groups of five or less and kept under
temperature-controlled (21°C) and humidity-controlled (55%) conditions with a 12 h reversed light/dark cycle (lights on at 8:00 P.M.). They were maintained under ad libitum food and water unless stated otherwise, and testing always took place in the dark phase of the light/dark cycle.

Three separate cohorts of adult mice were used in the behavioral experiments, and the mice were all at the age of 11–12 weeks at the beginning of behavioral testing. The first consisted of 15 mutant and 18 control female mice. They were all tested in experiment 1 before being further evaluated in experiments 2 and 3. One control mouse became ill after experiment 1, and one mutant mouse also appeared unfit after experiment 2; these mice were not included in the subsequent tests. The second cohort, comprising 17 mutant and 13 control female mice, was used in experiment 4. These animals were used previously in another operant conditioning experiment (not reported here). Prior pre-exposed (PE) and non-pre-exposed (nPE) experiences in preceding tests were always counterbalanced.

The third cohort comprised nine mutant and eight control male mice that had been used previously in two pilot tests of associative learning. All manipulations described here had been approved previously by the Cantonal Veterinary Office of Zurich; they conformed to the ethical standards required by the Swiss Act and Ordinance on Animal Protection and the European Council Directive 86/609/EEC.

All behavioral data were subjected to parametric ANOVA of the appropriate design conducted using SPSS (Chicago, IL) for Windows (release 13.0). Data interpretation was further assisted by a priori contrast comparison, Fisher's least significant difference (LSD) post hoc comparisons and the use of restricted ANOVAs whenever appropriate.

Experiment 1: elevated plus maze. The expression of spontaneous anxiety-like behavior was assessed in this test, and the animals were behaviorally naive at the time of testing. The maze consisted of two exposed and two enclosed arms joined to a central square platform. Its construction has been described fully (Yee et al., 2004). Each test session began by placing the animal into the central area facing one of the open arms. It was then left to explore freely for 5 min before being removed and returned to the home cage. A digital camera was mounted above the maze. Images were captured at a rate of 5 Hz and transmitted to a personal computer running the Ethovision tracking system (Noldus Technology, Wageningen, The Netherlands). Two anxiety-related measures were computed: (1) percentage of time spent in the open arms [open/(open + closed) x 100%]; (2) percentage of the number of entries made to the open arms [open/(open + closed) x 100%]. In addition, the total mobility score (defined as the cumulative spatial displacement of the animal’s center of gravity, and expressed in centimeters) was computed.

Experiment 2: latent inhibition in the conditioned freezing paradigm. This test allows the evaluation of Pavlovian conditioning. We adopted a design that further allowed the assessment of latent inhibition effect, whereby pre-exposures of the to-be-conditioned conditioned stimulus (CS) before conditioning retard the development and subsequent expression of the conditioned response. In this and all subsequent latent inhibition experiments, we adopted a design that, in general, tended to generate
weak or only marginal latent inhibition effect in the control. This approach is common for the
demonstration of treatment expected to enhance the latent inhibition effect (Feldon and Weiner, 1991).
In the conditioned freezing experiment, the apparatus comprised eight conditioning chambers as
described previously (Meyer et al., 2005). All chambers were equipped with a grid floor made of
stainless-steel rods spaced at 10 mm intervals, and through which scrambled electric shocks
[unconditioned stimulus (US)] could be delivered (model E13–14; Coulbourn Instruments, Allentown,
PA). The CS was an 86 dBA tone provided by a sonalert (model SC628; Mallory, Indianapolis, IN). A
digital camera was mounted 30 cm directly above the area of interest in each chamber, which captured
images at a rate of 1Hz. Successive frames were compared to evaluate freezing behavior (Richmond et
al., 1998).

The test procedures of the latent inhibition (LI) test consisted of four phases: Pre-exposure,
conditioning, context-test, and CS-test. Within each genotype group, the animals were randomly
allocated to either the PE condition (mutant/PE, n = 8; control/PE, n = 9) or the nPE condition
(mutant/nPE, n = 7; control/nPE, n = 8). PE subjects received 40 presentations of a 30 s tone CS at a
variable interstimulus interval of 40 ± 30 s; nPE subjects were confined to the chamber for an
equivalent period of time. Conditioning commenced immediately at the end of pre-exposure without
removing the animals from the chambers. Conditioning comprised three discrete trials of CS-US
pairing. Each trial began with the 30 s tone stimulus (identical to the one used during pre-exposure)
followed immediately by the delivery of a 1 s foot shock set at 0.25 mA. Each trial was preceded and
followed by a 180 s interval. The test of contextual freezing took place 24 h later when the subjects
were returned to the same chambers and observed for a period of 480 s in the absence of any discrete
stimulus. The test of conditioned response to the tone CS was conducted another 24 h later, when the
animals were returned to the chambers. After a 180 s acclimatization period, the tone CS was turned
on for 480 s; freezing behavior to the tone stimulus during this period was evaluated.

Experiment 3: latent inhibition in the conditioned active avoidance paradigm. This task captures both
elements of classical and instrumental conditioning, in which the animals learned to perform a specific
operant act in response to a noise stimulus to avoid the delivery of an aversive foot shock. Again, we
adopted a design to allow the assessment of latent inhibition in this learning paradigm.
The apparatus consisted of four identical two-way shuttle boxes (model H10–11M-SC; Coulbourn
Instruments). The internal dimensions of each box were 35.5 x 18 x 32.5 cm. The box was separated
into two identical compartments by an aluminum wall with an interconnecting opening (6.5 x 8 cm),
allowing the animal to move freely from one compartment to the other (i.e., a shuttle response). The
grid floor was made of stainless-steel rods (diameter, 0.4 cm; spaced, 0.7 cm) and connected to a
constant current shock generator (model H10–1M-XX-SF; Coulbourn Instruments). Through the grids,
electric shocks (0.3 mA) could be delivered. The CS was an 83 dBA white noise. Shuttle response was
detected by a series of photocells (H20–95X; Coulbourn Instruments) mounted on the side of both
shuttle compartments.
The animals were subdivided into two conditions, PE and nPE, with their pre-exposure experience in the preceding LI experiment counter-balanced (mutant/PE, n = 8; control/PE, n = 9; mutant/nPE, n = 7; control/nPE, n = 8).

On the first day, the animals were placed in the shuttle chambers: PE animals received 50 presentations of a 5 s white noise stimulus (83 dBA in magnitude against a background noise of 63 dBA) presented at a variable interstimulus-interval (mean of 40 s, ranging from 25 to 55 s); the nPE animals spent an equivalent period of time in the chamber without any stimulus presentation.

On the second day, the animals underwent 100 conditioned avoidance trials administered at variable intertrial intervals (mean of 40 s, ranging from 25 to 55 s). A trial began with the onset of the noise CS. If the animal shuttled within 5 s of CS onset, the CS was terminated and the animal avoided the electric shock on that trial. Avoidance failure led immediately to an electric foot shock presented in coincidence to the CS. This could last for a maximum of 2 s but could be terminated by a shuttle response during this period (i.e., an escape response). To index conditioned avoidance learning, the mean latency to response across successive 10-trial blocks was submitted to statistical analysis. The latency was transformed to speed (1/s) for the purpose of analysis.

**Experiment 4: latent inhibition in the conditioned taste aversion paradigm.** This paradigm is a one-trial classical conditioning paradigm, in which a single pairing of a taste CS and gastric malaise leads to a lasting aversion to the taste. However, the efficacy to acquire the conditioned aversion would be reduced if the subjects experienced the same taste CS without any consequence. This effect of prior CS exposures constitutes another demonstration of the latent inhibition effect and was also assessed here.

Throughout the experiment, the animals were housed singly in Makrolon cages (1291H, Eurostandard type III, H:425 x 266 x 185 mm; Tecniplast S.p.a., Milan, Italy). The two drinking tubes were made from 15 ml polypropylene test tubes (Cellstars; Greiner Bio-One, Frickenhausen, Germany) equipped with an air-tight screwed top. An opening, 2.5 mm in diameter, was made in the bottom of the tubes to allow access to the liquid within the animals. Two acrylic rings (20 mm in inner diameter) were affixed to the metal grids of the cage to provide a stable anchor for the drinking tubes. When the tubes were inserted lumen of the rings, the two drinking holes were 40 mm apart, at a level of 50 mm above the cage floor. With this arrangement of the tubes, the animals could easily switch drinking form one tube or the other. The liquid consumption of a drinking tube was assessed by calculating the weight difference of each drinking tube before and after a drinking session.

A 10% (w/v) D-sucrose solution served as the taste CS. Gastric malaise, induced by systemic injection of lithium chloride (LiCl) solution, served as the US. LiCl (Sigma-Aldrich, Buchs, Switzerland) was dissolved in 0.9% NaCl solution to a final concentration of 0.25 M on the day of conditioning. The volume of LiCl injection was 2% v/w of the body weight and was administered via the intraperitoneal route at the end of the first drinking period on the conditioning day.
After habituation to single housing for 2 d, access to water was gradually restricted over a 5 d period as described by Meyer et al. (2004). On the last of these 5 d, the water restriction was reduced to 1 h. Thereafter and until the end of experiment, the animals were allowed two 30 min drinking periods per day, separated by 4 h. Two drinking tubes were inserted into the cage in each of the two drinking periods, and the animals could freely consume liquid from either tube. Manipulations of the content of the drinking tubes were also conducted in the first drinking period of a day (see below). In the second period, both tubes were always filled with plain tap water.

As described below, the experimental procedure consisted of four phases: baseline, pre-exposure, conditioning, and test of conditioned aversion.

Baseline. Over the first 3 d, both tubes contained water in both drinking periods to acclimatize the animals to drinking from the water tubes. The allocation of subjects into PE (nine mutant and seven control mice) and nPE (eight mutant and six control mice) conditions was counterbalanced according to the animals’ performance in the first drinking period across the three baseline days.

Pre-exposure. On the next day, PE subjects were provided with 10% (w/v) D-sucrose solution in both tubes during the first drinking period. nPE subjects had access to water in both tubes during the first drinking period.

Conditioning. The next day, PE and nPE subjects were provided with sucrose solution in both tubes during the first drinking period. All animals received an LiCl injection 5 min afterward.

Test. The next day, conditioned taste aversion to the sucrose solution was measured in a two-choice test in which one tube contained sucrose solution and the other water, thus allowing the animals a free choice between the two.

Experiment 5: locomotor reaction to systemic amphetamine and phencyclidine. The apparatus consisted of four open-field white wooden square arenas (40 x 40 cm) as described previously (Hauser et al., 2005). Amphetamine (2.5 mg/kg), phencyclidine (5 mg/kg), or vehicle saline solution (0.9% NaCl) was administered via the intraperitoneal route (at an injection volume of 5 ml/kg) immediately before testing. The animals were observed for 2 h. A digital camera mounted above the open fields captured images at a rate of 5 Hz and transmitted them to a personal computer running the Ethovision tracking system (Noldus Technology), which calculated a mobility score defined as distance traveled per bin at successive 5 min bins. A square-root transformation was performed before statistical analysis.

RESULTS

Generation of CamKIIa Cre:Glyt1tm1.2fl/fl mice
To achieve forebrain-specific neuronal recombination of the floxed Glyt1 allele, conditional Glyt1 knock-out mice (Glyt1tm1.2fl/fl) (Fig. 1A) were generated and bred with CamKIIa Cre2834 mice. CamKIIa Cre2834 mice were produced by pronuclear injection of a construct carrying an 8.5 kb fragment of the CamKIIa promoter (Mayford et al., 1996) fused to the Cre gene. The spatiotemporal
expression pattern of the CamKIIα Cre2834 transgene has been described previously (Schweizer et al., 2003). According to studies of CamKIIα Cre2834 expression in Z/AP reporter mice and in floxed 2-GABAA receptor knock-out mice, CamKIIα Cre2834-induced recombination was first detected in the hippocampus at P17 and then gradually increased to near adult levels by P34, concomitant with a spread to other forebrain regions (Schweizer et al., 2003). Whereas the onset and final pattern of recombination in CamKIIα Cre2834 mice was very similar to that observed in T29–1 CamKIIα Cre mice (Tsien et al., 1996), recombination of the floxed 2-GABAA receptor locus by the T29–1 transgene progressed more slowly than recombination by the CamKIIα Cre2834 transgene (Schweizer et al., 2003). Thus, CamKIIα Cre2834 mice constitute an ideal tool to study Cre-mediated GlyT1 ablation in the hippocampus after the third postnatal week and in the forebrain of adult mice.

Both parent lines were on a pure C57BL/6J background, and additional breeding gave rise to CamKIIα Cre:Glyt1tm1.2fl/fl mice. The mouse colony was maintained by breeding CamKIIα Cre:Glyt1tm1.2fl/fl mice with Glyt1tm1.2fl/fl mice, maintaining the colony heterozygous for the Cre-gene. Therefore, we obtained mixed litters of CamKIIα Cre:Glyt1tm1.2fl/fl and Glyt1tm1.2fl/fl mice in a 1:1 ratio, the latter genotype being used as control. Genotypes were determined by PCR analyses of mouse-tail biopsies (Fig. 1A,B). Both CamKIIα Cre:Glyt1tm1.2fl/fl mice and Glyt1tm1.2fl/fl mice were characterized by the lack of the neomycin resistance cassette, which was not amplified using primers o184/o139, whereas a 450 bp amplification product was obtained using DNA from Glyt1tm1 mice (Fig. 1). The presence of the loxP flanked region was verified in all three genotypes (Glyt1tm1, CamKIIα Cre:Glyt1tm1.2fl/fl, and Glyt1tm1.2fl/fl) by a 1050 bp amplification product using primers o232/o250 (Fig. 1). In tail biopsies from these mice, recombination of the loxP sites and deletion of the intervening sequence was never observed (primers o228/250) (Fig. 1). However, when DNA isolated from the forebrain of CamKIIα Cre:Glyt1tm1.2fl/fl mice was amplified with primers o228/o250, a characteristic 500 bp band resulted, indicative of a forebrain-specific deletion of the loxP-flanked region (Fig. 1). These mice (tail and forebrain DNA) were also characterized by a 650 bp amplification product from primers o234/o235, indicative for the presence of CamKIIα Cre. Because CamKIIα Cre is selectively expressed in neurons (Mayford et al., 1996), the o228/o250-dependent 500 bp amplification product from forebrain samples (Fig. 1B) most likely results from neuronal recombination of the Glyt1tm1.2fl allele. The presence of the o232/o250-dependent 1050 bp amplification product from forebrain samples (Fig. 1B) is indicative of a non-recombined Glyt1tm1.2fl allele, most likely derived from astrocytes or other non-neuronal cells. The PCR analysis presented here thus documents Cre-mediated recombination in the forebrain of CamKIIα Cre:Glyt1tm1.2fl/fl mice.
CamKIIa Cre:Glyt1tm1.2fl/fl mice display reductions of GlyT1 levels and glycine uptake but no changes in NMDA receptor expression in the forebrain

To assess the consequences of a forebrain-specific neuronal disruption of the Glyt1 gene on GlyT1 protein expression, membrane preparations of hippocampus/cortex of adult (8 weeks of age) wild-type and mutant mice were analyzed by Western blotting using GlyT1-selective antibodies. In adult animals CamKIIa Cre2834-mediated recombination in the forebrain is considered to be complete (Schweizer et al., 2003). GlyT1 protein levels in mutants were reduced by 30 ± 8%, indicating a significant neuronal pool of GlyT1 (Fig. 2B). Functionally, the reduced GlyT1 protein level resulted in a 35 ± 4% decrease of GlyT1-specific [3H]glycine uptake as determined in synaptosomal membrane preparations from hippocampus/cortex in the presence and absence of the GlyT1-selective inhibitor NFPS (Fig. 2A). Saturation experiments revealed a significant reduction of the Vmax value in mutants from 26 ± 1 to 17 ± 1 pmol/min/mg protein with no significant change in the Km value (wild type, 26 ± 4 µM; mutant, 21 ± 1 µM). These results demonstrate that the neuron-specific disruption of GlyT1 in the forebrain translates into a severe deficit of NFPS-specific glycine uptake. The diminished GlyT1-selective glycine uptake suggests elevated extracellular glycine levels in mutant mice, which may affect NMDA receptor expression. Therefore, potential changes in NMDA receptor expression were analyzed with a high spatial resolution by receptor autoradiography in the hippocampal formation using the glycine site antagonist [3H]L-689,560. No significant difference in [3H]L-689,560 binding was detected in various areas of the hippocampal formation (Fig. 2C).
Appendix I

Figure 2. Reduction of GlyT1 and NFPS-specific [3H] glycine uptake but not of NMDA receptor in mutant mice. A. Reduced glycine uptake in mutant mice. Glycine transport in synaptosomal membrane preparations from hippocampus/cortex of mutant and wild-type mice was analyzed by measuring NFPS-specific [3H] glycine uptake at different glycine concentrations. The $V_{\text{max}}$ value was reduced in mutants from 26 ± 1 pmol/min/mg (control) to 17 ± 1 pmol/min/mg protein with no change in the $K_m$ value (control, 26 ± 4 µM; mutant, 21 ± 1 µM). Data represent the mean ± SD of three independent experiments. B. Reduced GlyT1 levels in mutant mice. Expression levels of GlyT1 in wild-type and mutant mice were analyzed by probing Western blots containing increasing protein concentrations from hippocampus/cortex with GlyT1-specific antiserum. The top panels depict representative Western blots probed with GlyT1 antibodies and simultaneously with actin antibodies to control for equal sample loading. The bottom panels show the quantification of the Western blots normalized to the GlyT1 signal at 20 µg in control mice (100%). Data represent the mean ± SD of four experiments. C. Unaltered [3H]L-689,560 binding to NMDA receptors in hippocampal formation in mutant mice. The level of NMDA receptors in the hippocampal formations was assessed by radioligand binding to brain sections and autoradiography using [3H]L-689,560, an antagonist of the glycine site. Results are given as mean ± SD of four mice for each genotype. Inset, Representative autoradiograms depicting [3H]L-689,560 binding to the hippocampal formation in the presence or absence of 1 mM glycine. CA1ori, stratum oriens layer of CA1; CA1pyr, stratum pyramidale of CA1; CA1rad, stratum radiatum of CA1; CA1lac, stratum lacunosum moleculare of CA1; CA3ori, stratum oriens of CA3; CA3pyr, stratum pyramidale of CA3; CA3rad, stratum radiatum of CA3; DGgcl, stratum granulosum dentate gyrus; DGpol, stratum polymorphe of dentate gyrus; DGMol, stratum moleculare of dentate gyrus.

Enhancement of hippocampal NMDA receptor function by disruption of GlyT1 in forebrain neurons

The lack of GlyT1 in hippocampal neurons was expected to lead to an increase of glycine at glutamatergic synapses and to an enhancement of NMDA receptor function. The experiments described here were performed on slightly older brain slices (P21–P30) compared with our previous publication (P17–P24) (Gabernet et al., 2005) to ensure that CamKIIa Cre2834-mediated recombination in the hippocampus (beginning from P17 onwards) had taken place. The ratio of NMDA to AMPA-mediated currents was chosen to assess the modification of NMDA receptor function in CamKIIa Cre:Glyt1tm1.2fl/fl mice. The average peak amplitude of evoked AMPA EPSCs in CamKIIa Cre:Glyt1tm1.2fl/fl and Glyt1tm1.2fl/fl mice amounted to 85.9 ± 16.4 pA (n = 6) in Glyt1tm1.2fl/fl mice and to 90.6 ± 11.1 pA (n = 8) in CamKIIa Cre:Glyt1tm1.2fl/fl mice (Fig. 3A). After blocking the AMPA EPSCs by the addition of NBQX, NMDA EPSCs were isolated in the same cells at Vm +40 mV and displayed average peak amplitude of 44.5 ± 13.0 pA (n = 6) in Glyt1tm1.2fl/fl mice and 81.5 ± 7.4 pA (n = 8) in CamKIIa Cre:Glyt1tm1.2fl/fl mice (Fig. 3A).
NMDA component of EPSCs was completely blocked by the addition of R-CPP (data not shown) as described previously (Gabernet et al., 2005). The ratio of the peak amplitudes of NMDA to AMPA-mediated EPSCs amounted to 0.46 ± 0.06 in Glyt1tm1.2fl/fl mice (n = 6) and 1.01 ± 0.16 in CamKIIa Cre;Glyt1tm1.2fl/fl mice (n = 8), corresponding to a 2.15-fold enhancement of the NMDA/AMPA response ratio in the mutants (p < 0.05). To exclude the possibility that the increased NMDA/AMPA ratio is attributable to a reduction of the AMPA response in the mutants, the kinetics of the NMDA and AMPA components of EPSCs was compared and found not to differ significantly between genotypes. The properties of AMPA mEPSCs, which reflect the postsynaptic response to the presynaptic release of single vesicles, were recorded at Vm –70 mV in the presence of TTX, R-CPP, and picrotoxin. Average AMPA EPSCs decay time constants were 13.2 ± 1.1 ms (n = 6) (Glyt1tm1.2fl/fl) and 15.6 ± 2.0 ms (n = 8) (CamKIIa Cre;Glyt1tm1.2fl/fl). Likewise, the rise times of AMPA EPSCs were similar (2.0 ± 0.2 ms for the control and 2.0 ± 0.1 ms for the mutants). The average NMDA EPSC decay time constant was 105.2 ± 18.4 ms (n = 6) (Glyt1tm1.2fl/fl) and 86.4 ± 8.7 ms (n = 8) (CamKIIa Cre;Glyt1tm1.2fl/fl), whereas the rise times were 4.2 ± 0.4 ms and 4.2 ± 0.2 ms, respectively (Fig. 3B). We conclude that the NMDA receptor activity is enhanced in CamKIIaCre;Glyt1tm1.2fl/fl mice, whereas kinetic properties of AMPA and NMDA EPSCs remained unaltered.

Figure 3. AMPA/NMDA EPSCs peak amplitude and mEPSCs properties of hippocampal CA1 pyramidal cells of CamKIIaCre;Glyt1tm1.2fl/fl mice. A, Averaged EPSCs peak amplitude (amp) in CA1 pyramidal neurons of Glyt1tm1.2fl/fl (con) and CamKIIaCre;Glyt1tm1.2fl/fl (mut) mice. B, AMPA and NMDA mEPSCs properties are not affected in CamKIIaCre;Glyt1tm1.2fl/fl cells. Average AMPA and NMDA mEPSCs decay time and rise time constants from Glyt1tm1.2fl/fl (con) and CamKIIaCre;Glyt1tm1.2fl/fl (mut) cells indicating no significant change in the mEPSC kinetics (two-tailed independent Student's t test).

Elevated plus maze behavior remains unaltered in the CamKIIa Cre;Glyt1tm1.2fl/fl mice

For the interpretation of data derived from the subsequent aversive conditioning paradigms, it is essential to ascertain whether the mutation had any effects on unconditioned anxiety-like behavior and spontaneous locomotor activity. The elevated plus maze test was therefore conducted in a cohort of adult mice (15 mutants and 19 littermate controls) when the animals were totally behavioral naive. The measures of percentage of open arm entries and percentage of time spent in open arms conformed closely to each other and suggested that the expression of anxiety-like behavior as indexed by the
Appendix I

animals' reluctance to venture into the open arms was highly comparable between the mutant and control mice. The mutant and control mice spent, respectively, 29.0 ± 6.5 and 24.9 ± 3.5% time in the open arms. Based on the total number of arm entries, the mean percentage of entries into the open arms by the mutant and control mice are 40.0 ± 5.5 and 35.4 ± 3.7%, respectively. Examination of locomotion as measured by total distance traversed in the maze also indicated that the two groups behaved similarly to each other (mutant, 907.4 ± 62.6 cm; control, 942.2 ± 29.1 cm). Separate one-way ANOVAs of the three variables failed to yield any significant group effect (all F values <1).

CamKIIa Cre:Glyt1tm1.2fl/fl mice show enhanced expression of conditioned tone-freezing with an augmentation of the latent inhibition effect

To investigate the consequences of the forebrain and neuron-selective disruption of GlyT1 on associative learning and its control by selective attention, we compared mutant (n = 15) and littermate controls (n = 17) in a conditioned tone-freezing paradigm that included the assessment of LI. LI is demonstrated when subjects having been pre-exposed to the to-be-conditioned CS exhibited less learning than non-pre-exposed subjects.

First, the development of conditioned freezing was evaluated across the three CS-US pairings by the amount of freezing exhibited over three successive tone-CS presentations (Fig. 4A). LI was evident by increased freezing in the nPE animals relative to the PE animals (F(1,28) = 6.05; p < 0.05). This relative retardation in learning was evident in both the mutant and control mice, especially in trials 2 and 3 when the tone-CS was expected to generate conditioned responding. Although the LI effect appeared somewhat stronger in the mutant relative to the controls, the interaction between pre-exposure and genotype was not significant (F(1,28) = 1.76; p = 0.20). Given the overall presence of LI across the three trials of CS-US pairing, we conducted a priori contrast to ascertain the statistical presence of LI in the mutant and control groups separately, which revealed a significant presence of LI in the mutant (p = 0.015) but not in the controls (p = 0.414). The comparison between mutant and control in the nPE condition did not yield any significant group difference (p = 0.16).

Twenty-four hours later, the animals were returned to the conditioning context for the assessment of conditioned freezing to the context over a period of 8 min (Fig. 4B). Although the PE control and nPE control showed a significant difference in their freezing to the context, no such difference was detected in the mutant. This led to the emergence of a significant interaction between genotype and pre-exposures (F(1,28) = 4.86; p < 0.05). Post hoc comparison confirmed that a statistically significant difference between PE and nPE conditions was exclusively found in the control animals (p = 0.012). Therefore, it showed that the control mice were not entirely indifferent to the effects of CS pre-exposure before conditioning. The direction of this effect is also in accordance with elemental associative learning theories, suggesting a competition between discrete CS and contextual cues for associative strength with the US. The absence of this effect in the mutant was further accompanied by
a tentative increase in contextual freezing in the nPE condition (Fig. 4B): a direct comparison by a restricted ANOVA between nPE mutant and nPE control revealed that the increase in conditioned freezing to the context seen in the mutant mice approached statistical significance ($F(1,13) = 4.02; \ p = 0.066$).

**Figure 4.** Latent inhibition in the conditioned freezing paradigm. **A**, Expression of freezing behavior toward the tone-CS across the three conditioning trials immediately after pre-exposure. The histogram to the right illustrates the level of freezing averaged across the three trials. Freezing in the PE subjects tended to be lower than in the nPE subjects, constituting the latent inhibition effect. Mutant/PE, $n = 8$; mutant/nPE, $n = 7$; control/PE, $n = 9$; control/nPE, $n = 8$. **B**, Freezing to the context 24 h after conditioning. This is expressed as a function of 1 min bins on the left and as the overall means on the right. The error bar on the left refers to twice the SE of difference derived from the mean square error term associated with the three-way (genotype x pre-exposures x bins) interaction. Error bars on the right correspond to the SEM associated with the respective mean values. The asterisks denote a significant difference between PE controls and nPE controls, as indicated by Fisher’s LSD comparison, following the emergence of the significant interaction between genotype and pre-exposures ($p < 0.01$). **C**, Freezing to the tone CS 48 h after conditioning. Error bars in the line plots refer to twice the SE of difference derived from the appropriate mean square error term taken from the ANOVA of the 3 min pre-CS period or the 8 min CS period. The histogram on the right illustrates the mean levels of freezing averaged across the entire 8 min CS period. The presence of the latent inhibition effect in the mutant mice is evident by significant increase in conditioned freezing in the mutant/nPE than in the mutant/PE group ($p < 0.05$ based on a priori contrast). The control group did not exhibit a significant latent inhibition effect. This contrast led to the interaction between genotype and pre-exposures attained significance ($p < 0.05$). Comparison between mutant/nPE and control/nPE also indicated a significant difference ($p < 0.05$). Additional analysis restricted to nPE subjects indicates increased conditioned freezing in the mutant compared with the control group between the third and seventh bins in the CS period. All values refer to mean ± SEM.
The generation of the conditioned response to the tone CS was assessed 48 h after conditioning, when the animals were returned to the conditioning chamber. The tone was presented 3 min into the session and remained continuously for 8 min (Fig. 4C). In the 3 min before CS onset, freezing was generally low and the mutant and control mice did not differ in their overall level of freezing. Analysis over the 8 min CS period revealed a significant interaction between genotype and pre-exposure ($F(1,28) = 5.41; p < 0.05$). Fisher's LSD comparisons confirmed the significant presence of LI in the mutant ($p < 0.05$) but not in the control. It is apparent that the enhancement of the LI effect seen in the mutant was achieved via increased tone-freezing in the nPE condition and decreased tone-freezing in the PE condition, compared with control mice.

To directly compare the expression of conditioned freezing to the tone in the nPE condition, an additional ANOVA restricted to the nPE subjects was conducted. This revealed a near significant main effect of genotype ($F(1,13) = 4.18; p = 0.06$), which was accompanied by a significant interaction between genotype and bins ($F(7,91) = 2.97; p = 0.02$). Post hoc comparison indicated that mutant/nPE mice exhibited a significantly higher level of freezing than control/nPE from the third to the seventh bins of the tone CS presentation CS ($p$ values $<0.05$).

These results are consistent with the expectation of an enhanced NMDAR function in the mutant mice. The observation that these animals exhibited LI when the pre-exposure/conditioning parameters were insufficient to yield significant LI in the control is suggestive of enhanced selective attention.

**CamKIIa Cre:Glyt1tm1.2fl/fl mice show enhanced acquisition of active avoidance with an augmentation of the latent inhibition effect**

To further test the generality of the results obtained in the conditioned freezing experiment, we next subjected the animals (14 mutant and 17 controls) to another associative learning paradigm (i.e., active avoidance) using a design that again allowed the assessment of the LI effect. Here, LI would be observed if animals that were exposed to the signal for foot shock before acquisition training learned to avoid the foot shock at a slower pace than the non-pre-exposed subjects.

The pre-exposure session was uneventful: a 2 x 2 (genotype x pre-exposure) ANOVA of the total number of spontaneous shuttles recorded on the pre-exposure session yielded no significant effect. The acquisition of the conditioned avoidance response on the following day was indexed by the speed to shuttle (= one/latency to response from CS onset). Learning was evident in all groups as indicated by a reduction in the mean shuttle speed over successive blocks of 10 trials (Fig. 5). This was confirmed by a 2 x 2 x 10 (genotype x pre-exposure x blocks) split-plot ANOVA of response latency, which only yielded a main effect of blocks ($F(9,243) = 27.10; p < 0.001$).

An overall presence of LI was suggested by the near-significant pre-exposure x blocks interaction ($F(9,243) = 1.87; p = 0.058$). However, LI was more clearly seen in the mutant mice, as mutant/nPE showed a shorter latency to shuttle compared with the mutant/PE (Fig. 5A). In contrast, LI was visibly
absent in control mice. We therefore conducted a priori contrast to ascertain the presence of LI in mutant and control mice: a significant presence of LI was detected in the mutant (p = 0.046) but not in the control (p = 0.69). Similar to the pattern of results obtained in the LI conditioned freezing experiment (Fig. 4A,C), the enhancement of LI seen in the mutants was also associated with faster learning in the mutant/nPE relative to the control/nPE. Restricted direct comparison between these two groups yielded a significant genotype x blocks interaction (F(9,126) = 1.98; p < 0.05), suggesting that the mutant had acquired the avoidance response at a faster rate than the control in the same condition.

Figure 5. Latent inhibition in conditioned active avoidance learning. Acquisition of the conditioned avoidance response across 10 blocks of 10 trials conducted 24 h after tone pre-exposure in mutants (left) relative to controls (right). Learning was indexed by response speed (calculated by 1/latency to shuttle from the stimulus noise-onset). A response speed above (0.2 = 1/5) would constitute an avoidance response. Increasing response speed indicated the acquisition of avoidance learning as training progressed. The mean response speed across the acquisition 100 acquisition trials in each group is illustrated in the histogram. *p < 0.05, significant difference between PE mutants and nPE mutants, as indicated by the analysis restricted to the mutant mice. All values refer to mean ± SEM. Mutant/PE, n = 7; mutant/nPE, n = 7; control/PE, n = 9; control/nPE, n = 8.

CamKIIa Cre:Glyt1tm1.2fl/fl mice show an augmentation of the latent inhibition effect in the conditioned taste aversion paradigm

To test the hypothesis that the mutation led to enhanced LI, we compared the expression of LI in mutant mice (n = 17) and littermate controls (n = 13) in yet another associative learning paradigm, namely, conditioned taste aversion, in which reduced conditioned aversion to the taste CS in the pre-exposed subjects relative to non-pre-exposed subject constitutes LI.

Baseline, pre-exposure, and conditioning phases of the experiment were uneventful. All animals readily drank from the drinking tubes by the end of the baseline phase, with the mutant and control mice consuming on average 1.31 ± 0.10 and 1.51 ± 0.15 g of water, respectively, in the first drinking period on the final baseline day. A 2 x 2 (genotype x pre-exposure) ANOVA of liquid consumed in the pre-exposure session yielded no significant effect (Table 1). In the conditioning session, the PE animals consumed a higher amount of liquid (F(1,26) = 7.12; p < 0.05) (Table 1). This effect reflected
a reduction in taste neophobia in the PE subjects and was equivalently seen in both mutant and control mice as indicated by post hoc Fisher's LSD comparisons (p values <0.05).

In the test session, the consumption of sucrose solution in absolute amount and in terms of percentage of total liquid consumed were submitted to 2 x 2 (genotype x pre-exposure) ANOVA. In each case, the overall main effect of pre-exposure approached statistical significance (sucrose solution consumed, $F(1,26) = 3.89, p = 0.06$; percentage of sucrose solution consumption, $F(1,26) = 3.39, p = 0.08$), although they might be considered so if a one-tailed criterion was applied with the alternative hypothesis of HA: $\mu_{PE} > \mu_{NPE}$ (instead of HA: $\mu_{PE} = \mu_{NPE}$) in accordance with the expected direction of the latent inhibition effect.

As illustrated in Figure 6, the latent inhibition effect was again more clearly seen in the mutant than in the control mice. Although the interaction between genotype and pre-exposure failed to attain statistical significance ($F < 1$), this impression was in line with the results of the previous two experiments. We therefore conducted separate restricted ANOVAs to separately evaluate the presence of the latent inhibition effect in the mutant or the control mice. A highly significant effect of pre-exposure, in the direction of latent inhibition, was obtained in the mutant using both measures of sucrose consumption on the test day (absolute amount, $F(1,15) = 7.56, p = 0.015$; percentage, $F(1,15) = 11.57, p = 0.004$) but not in the control animals (both $F$ values <1). This contrast lent support to the interpretation that the expression of latent inhibition was stronger in the mutant animals. However, direct comparisons between mutant and control restricted to the nPE condition did not indicate any difference in the magnitude of taste aversion between the two groups ($F$ values <1).

Table 1. Summary of the liquid consumption on the days of pre-exposure, conditioning, and test days in the conditioned taste aversion experiment.

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<th>Control</th>
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<td>Latent inhibition in conditioned taste aversion</td>
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<td>1.58 ± 0.09</td>
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<td>Pre-exposure sucrose solution consumption (g)</td>
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<tr>
<td>Predoctoral total liquid consumption (g)</td>
<td>1.59 ± 0.3</td>
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*All values refer to mean ± SEM.

Figure 6. Latent inhibition in conditioned taste aversion. The expression of conditioned taste aversion on the test day (24 h after sucrose-LiCl pairing) was indexed by sucrose solution consumption. This was expressed in absolute amount (A) or in the percentage of total liquid consumed in the 30 min test session. The lower the amount of sucrose consumed, the greater the taste aversion. Weaker conditioned aversion in the PE relative to the nPE condition constitutes the LI effect. LI was significantly seen in the mutant mice in both measures (*$p < 0.05$) but not in the control. All values refer to mean ± SEM. Mutant/PE, $n = 9$; mutant/nPE, $n = 8$; control/PE, $n = 7$; control/nPE, $n = 6$. 
CamKIIa Cre:Glyt1tm1.2fl/fl mice exhibit reduced sensitivity to the motor stimulant effect of psychomimetic drugs

To clarify whether the consistent enhancement of LI seen in the CamKIIa Cre:Glyt1tm1.2fl/fl mice implies a "antipsychotic-like" phenotypic profile, we attempted to assess the animals' reaction to psychostimulant drugs, with the expectation that the mutant mice would exhibit some degree of resistance. To this end, we contrasted the motor stimulant effect of amphetamine (an indirect dopamine receptor agonist) and of phencyclidine (a noncompetitive NMDAR antagonist) to vehicle treatment in nine mutant and 14 littermate control mice. Because of the increased baseline activation of NMDAR function in the mutants, we expected that the stimulatory response to phencyclidine would be reduced. Given that the psychostimulant effects of phencyclidine are also partly attributed to dopamine reuptake inhibition (Johnson and Jones, 1990), we included amphetamine as another psychostimulant drug for comparison, allowing us to test whether an anti-psychostimulant effect would be seen equivalently between the two drugs differing in their principal mode of pharmacological action.

The activity profile over the 2 h test period is illustrated in Figure 7. Systemic challenge of either amphetamine or phencyclidine at the selected doses led to comparable elevations of locomotor activity in the control animals. The mutation led to a delay of the action of amphetamine and partially reduced the impact of amphetamine over the first 20 min of the test (Fig. 7A). In contrast, the mutation drastically attenuated the response to phencyclidine that was apparent from 15 min onwards (Fig. 7B). No difference was observed between mutant and control in locomotor activity after vehicle saline injection (Fig. 7C).

The impression that the expression of drug effects over bins differed between the mutant and control mice is confirmed by a 2 x 3 x 24 (genotype x drug x bins) ANOVA of (square-root transformed) distance moved per 5 min bins, which yielded a significant three-way interaction (F(46,414) = 1.69; p < 0.005). This was accompanied by a main effect of drug (F(2,18) = 8.89; p = 0.002), bins (F(21,414) = 15.90; p < 0.001), and their interaction (F(46,414) = 2.18; p < 0.001).

Additional analyses restricted to each drug condition were conducted to ascertain the presence of a genotype effect in each case. When confined to the amphetamine condition, the interaction between genotype and bins attained statistical significance (F(23,128) = 2.24; p = 0.002). Post hoc comparisons indicated that the activity level of the mutant-amphetamine group was significantly below that of the control-amphetamine group in the first four bins (p < 0.05). The main effect of genotype did not attain significance (p = 0.96), indicating that the overall activity level (under amphetamine) did not differ between the two groups. In contrast, the two-way ANOVA restricted to the phencyclidine condition revealed a main effect of genotype (F(1,6) = 6.52; p < 0.05). Finally, the analysis restricted to the vehicle condition did not yield any significant effect of genotype or its interaction with bins (both F values <1).
The preferential efficacy of the mutation against the motor stimulating effect of phencyclidine is consistent with the fact that deletion of neuronal GlyT1 in the forebrain selectively enhances NMDAR functions, with limited direct effects on other neurotransmitter systems.

**Figure 7.** The locomotor effect after systemic amphetamine (A), phencyclidine (B), or saline vehicle (C) treatment. Locomotor activity was measured by distance moved of the animal's center of gravity sampled at a rate of 5 Hz and summed into successive 5 min bins over the 2 h test period. A square-root transformation was performed before statistical analysis to better conform to the homogeneity of variance assumption of parametric ANOVA. The inset embedded within each line graph illustrates the overall mean distance traveled per bin, averaged over the entire test period. As indicated in A, the locomotor effect of amphetamine was delayed in the mutant mice relative to the control; this gave rise to a significant genotype x bins interaction \( p = 0.002 \). In B, the asterisk denotes the significant overall difference between mutant and control \( \ast p < 0.05 \). All values refer to mean ± SEM. Amph, Amphetamine; PCP, phencyclidine. Mutant/Amph, \( n = 3 \); mutant/PCP, \( n = 3 \); mutant/vehicle, \( n = 3 \); control/Amph, \( n = 5 \); control/PCP, \( n = 5 \); control/vehicle, \( n = 4 \).

**DISCUSSION**

The forebrain and neuron-specific deletion of GlyT1 achieved here has enabled us to provide a functional analysis of a highly specific subset of GlyT1. We demonstrate that disruption of GlyT1-mediated glycine reuptake, restricted to forebrain neurons, represents an effective strategy to selectively enhance NMDAR activity, affect associative learning, and modulate an example of selective learning, namely the latent inhibition effect.

**Neuronal specificity of GlyT1 disruption**

Here, we used Cre-mediated recombination of a conditional Glyt1 allele, driven by the forebrain and neuron-specific CamKIIa promoter. CamKIIa is a serine-threonine protein kinase, with its expression restricted to neurons of forebrain structures (Mayford et al., 1996). It is therefore reasonable to conclude that GlyT1 has selectively been deleted from forebrain neurons in our CamKIIa Cre;Glyt1tm1.2fl/fl mice. This has a drastic impact on glycine transport in forebrain as indicated by the comparison of synaptosomal \([3H]\) glycine uptake in CamKIIa Cre;Glyt1tm1.2fl/fl and Glyt1tm1.2fl/fl mice. The observed reduction of Vmax in the mutant mice (Fig. 2B) is striking.
considering that Cre-mediated recombination is expected to essentially spare astrocytic GlyT1-mediated glycine uptake. This implies an elevation of glycine concentration in the microenvironment of forebrain (predominantly glutamatergic) synapses in the CamKIIa Cre:Glyt1tm1.2fl/fl mice and implies that the function of neuronal GlyT1 has been underestimated previously (Tsai et al., 2004). This hypothesis was confirmed by the 2.15-fold increase in the NMDA/AMPA response ratio recorded in hippocampal pyramidal neurons of CamKIIa Cre:Glyt1tm1.2fl/fl mice compared with Glyt1tm1.2fl/fl mice (Fig. 3). Despite the presence of astrocytic GlyT1, the magnitude of the enhancement seen here is in excess of that reported in mice with a heterozygous constitutive disruption of GlyT1, with an increase of 1.36- to 1.71-fold relative to control mice (Tsai et al., 2004; Gabernet et al., 2005). Therefore, the reported elevation of the NMDA/AMPA response ratio in the heterozygous knock-out mice is likely attributable to the partial loss of neuronal GlyT1, implying that the astrocytic GlyT1 may not be a major determinant for the control of NMDAR functions.

**Effects on associative learning and its control by selective attention**

The molecular disruption of forebrain neuronal GlyT1 has achieved a level of specificity that is otherwise unattainable by pharmacological means. It has afforded us the opportunity to evaluate the contributions of this subset of GlyT1 to associative learning and, in particular, to the phenomenon known as latent inhibition (Lubow and Moore, 1959). LI is one of the ways in which selectivity in associative learning can be demonstrated, in the sense that not all potential CSs have an equivalent capacity to form an effective CS US association. A number of animal learning theories explain LI as a form of learned inattention, in which subjects learn to ignore, tune out, or reduce attention to stimuli that appear irrelevant (Mackintosh, 1973; Wagner, 1978; Lubow, 1989). Alternatively, LI has also been characterized as a form of proactive interference in which the CS no consequence relationship experienced by subjects during preexposure phase interferes with the retrieval of an effective CS US association acquired subsequently as the two stimuli are explicitly paired (Bouton, 1991, 1993; Kraemer and Spear, 1991, 1993). As a result, the inconsistent associative histories of the CS can compete for behavioral control in response to the CS, and the readiness for the more recent CS US relationship to command behavior is referred to as "behavioral switching" (Weiner, 1990). Accordingly, disruption and augmentation of LI are considered to reflect facilitation and retardation of behavioral switching, respectively.

LI is disrupted in schizophrenic patients but can be restored after effective neuroleptic treatment (Baruch et al., 1988; Gray et al., 1992). This LI deficiency has been related to the attentional impairment characteristic of schizophrenia (Gray et al., 1991; Moser et al., 2000). It is nonetheless not inconsistent with the alternative view that this represents an indication of enhanced behavioral switching in schizophrenic patients (Weiner, 1990). In animals, LI is abolished by amphetamine and enhanced by both typical and atypical neuroleptics (Weiner and Feldon, 1987; Christison et al., 1988; Feldon and Weiner, 1991; Peters and Joseph, 1993; Dunn et al., 1993), suggesting that LI may
constitute an animal model of the attentional deficits associated with schizophrenia (Solomon et al., 1981; Lubow et al., 1987; Weiner, 1990; Feldon and Weiner, 1991; Gray et al., 1991; Moser et al., 2000). The suggestion that elevated levels of glycine can be effective against schizophrenia-related cognitive symptoms (Coyle and Tsai, 2004) has led us to expect a possible LI-enhancing phenotype in the mutant mice with protocols suitable for the demonstration of such an effect.

We evaluated LI in three associative learning paradigms involving aversive unconditional stimuli. It is therefore imperative to first identify possible effects of the mutation on the unconditioned expression of fear. The mutation exerted no effect fear-related behavior in the elevated plus maze. Spontaneous locomotor activity in the plus maze, the testing boxes in conditioned freezing, the shuttle chambers, and the open field was all unaffected. Hence, any observed changes in conditioned responding are unlikely to be attributed to changes in emotion or changes in spontaneous locomotor activity.

Direct evidence for enhanced associative learning was obtained in conditioned tone-freezing and active avoidance learning. In the nPE condition, mutant mice expressed a higher level of conditioned freezing across the three tone-shock pairings (Fig. 4A) and on the subsequent test of context freezing (Fig. 4B) as well as tone freezing (Fig. 4C); this effect was statistically the clearest in the final tone freezing test. Similarly, mutant mice in the nPE condition learned to avoid foot shock by responding to the noise signal at a faster pace than control mice in the same condition (Fig. 5). Given that the motor requirements of the learned responses are incompatible between the two tests, the increased conditioned responding that is evident in both paradigms cannot be accounted for by an effect of the mutation on motor behavior. Furthermore, a similar trend of enhanced associative learning in mutant/nPE relative to control/nPE was also present in the conditioned taste aversion experiment, in which learning was indexed by a preference test (Fig. 6), although the increase in taste aversion developed in the mutant/nPE relative to the control/nPE animals did not achieve statistical significance. Enhanced memory retention was tentatively suggested in constitutive heterozygous GlyT1 knock-out mice (Tsai et al., 2004). We now show that a deficit of forebrain GlyT1 is sufficient for an improvement of the acquisition as well as retention of associative learning.

Regarding the effect of the mutation on the expression of LI, a highly consistent picture has emerged across all three tests: a robust and clear LI was demonstrated in the mutant against a weak level of LI in the control mice (Figs. 4 –6). In each case, the parameters chosen did not favor the robust expression of LI in the control. This qualitative difference is likely to reflect a quantitative bias in the mutant toward the expression of LI. Psychologically, this may represent an enhancement of learned inattention (Gray et al., 1991) or a suppression of behavioral switching (Weiner, 1990). In combination with the effect of the mutation on associative learning as such (as confirmed by data restricted to the nPE condition), the observed augmentation of LI always appears to be attributed to a difference between mutant and control in the nPE rather than the PE condition. The impact of the mutation on LI therefore does not conform to the LI enhancement typically seen after neuroleptic medication, which was primarily mediated via an effect on the PE condition (Moser et al., 2000). The
combined effect of enhanced associative learning and potentiation of the stimulus pre-exposure effect, the resultant augmentation of LI, thus appears dissimilar to that reported after systemic GlyT1 blockade by ALX 5407 (Lipina et al., 2005). This dissimilarity is not surprising given that the drug did not enhance associative learning, and it cannot discriminate between neuronal and astrocytic GlyT1. Nevertheless, the conclusion that selective forebrain neuronal GlyT1 disruption entails antipsychotic properties appears to be justified. The application of LI in animal models of schizophrenia based on selective brain lesions (Yee et al., 1995; Gal et al., 2005; Pothuizen et al., 2005) or molecular manipulation (Miyakawa et al., 2003) demonstrates that the resulting disruption of LI is often accompanied by reduced learning in the nPE condition. Importantly, the reduced conditioning in the nPE condition seen in these animal models matches the observation made in schizophrenia patients (Baruch et al., 1988), and it may reflect aspects of cognitive impairment. Thus, we propose that the enhancement of NMDAR function and the concomitant enhancement of LI demonstrated in the CamKIIa Cre:Glyt1tm1.2fl/fl mice is indicative for the potential of neuronal GlyT1 blockade in the forebrain as an effective intervention against the dysfunction in attentional control or behavioral switching mechanism and the cognitive impairment characteristic of schizophrenia.

Reduced sensitivity to psychomimetic drugs

To clarify further whether the CamKIIa Cre:Glyt1tm1.2fl/fl mice possess an antipsychotic-like phenotypic profile, we assessed the motor stimulant effect of systemic amphetamine and phencyclidine. Both drugs are known to exacerbate and induce psychotic symptoms in humans. The mutation delayed the onset of the motor stimulant effect of amphetamine and substantially attenuated the response to phencyclidine (Fig. 7). This effect can be attributed to the increased baseline activation of NMDAR function in the mutants and therefore the functional efficacy of a given concentration of phencyclidine to block the NMDAR should be reduced. In contrast, enhanced NMDAR activity in the mutant mice was only effective in delaying the initial impact of amphetamine, and any resistance incurred by the intrinsic elevation of NMDAR activity was eventually overcome by the sustained elevation of dopamine release. This suggests that interaction between glutamate and dopamine transmission was somewhat affected by the specific deletion of GlyT1 from forebrain neurons.

In contrast, in heterozygous constitutive GlyT1 knock-out mice with a deficit of both glial and neuronal GlyT1 in the entire brain (Tsai et al., 2004), the motor stimulating effect of amphetamine or MK-801 (dizocilpine, another noncompetitive NMDAR antagonist) was not affected. At the same time, although this mutation exhibited resistance to the disruptive effect of amphetamine on prepulse inhibition of the acoustic startle reflex, it exacerbated the disruptive effective of MK-801 in the same test. These illustrate that intricate differences do exist between the functional relevance of neuronal and astrocytic GlyT1.
CONCLUSIONS
Mice with neuronal GlyT1 deletion restricted to the forebrain enhance associative learning and its control by experience-dependent selective learning, thus mirroring the psychological deficits characteristic of schizophrenia. Our results further highlight the importance of distinguishing between neuronal and astrocytic GlyT1 in the development of novel compounds designed to interfere with its regulatory function on NMDAR activity.
REFERENCES


Appendix I


Appendix II

Regulation of cognition and symptoms of psychosis: Focus on GABA<sub>A</sub> receptors and glycine transporter 1

*With Hanns Mohler, Uwe Rudolph, Detlev Boison, Philipp Singer, Joram Feldon, and Benjamin K. Yee*


[Personal contribution to the work: Designing and performing the research, analysis of the behavioural data]
Abstract
Adaptive purposeful behaviour depends on appropriate modifications of synaptic connectivity that incorporate an organism's past experience. At least some forms of such synaptic plasticity are believed to be mediated by NMDA receptors (NMDARs). Complementary interaction with inhibitory neurotransmission mediated by GABA_A receptors, and upstream control of the excitability of NMDARs by glycine availability can greatly influence the efficacy of NMDAR mediated neuroplasticity, and thereby exert significant effects on cognition. Memory, selective attention or sensorimotor gating functions can be modified in mice with a reduction of a5GABA_A receptors in the hippocampus or a selective deletion of glycine transporter 1 (GlyT1) in the forebrain. Both genetic manipulations altered the formation or persistence of associative links leading to distinct phenotypes on trace conditioning, extinction learning, latent inhibition, working memory, and object recognition. Behavioural assays of latent inhibition, prepulse inhibition, working memory, and sensitivity to psychostimulants in particular suggest that a3 and a5 subunit-containing GABA_A receptors as well as GlyT1 are potential sites for ameliorating psychotic-like behaviour. Taken together, these results qualify distinct GABA-A receptor subtypes and GlyT1 as molecular targets for the development of a new pharmacology in the treatment of cognitive decline and psychotic symptoms.
1. Introduction: GABAergic control of neuronal rhythms

Various brain functions, such as sensory perception, cognition, emotion and memory, are linked to oscillatory activity of neuronal networks. The disruption of neural oscillations may contribute to brain dysfunctions, including psychiatric disorders. The spike timing of principal cells and the sculpting of neuronal rhythms are largely governed by GABAergic interneurons. To ensure adequate response characteristics of the target neurons, the brain has a bewildering array of inhibitory interneurons at its disposal (Somogyi and Klausberger, 2005) which is complemented by a correspondingly extensive multiplicity of GABA_A receptors. By their strategic positioning in the neuronal networks the GABA_A receptor subtypes affect distinct input–output patterns. The functional analysis of a particular GABA_A receptor subtype is therefore expected to relate to a particular network activity which subserves a particular behaviour. GABA_A receptor subtypes are precision targets for the functional analysis of neuronal circuits in normal and diseased brain.

A case in point is a comparative high-frequency EEG analysis of a visual perception task in normal controls and in schizophrenics (Spencer et al., 2004). Visual Gestalt stimuli elicited a γ-band oscillation generated in the visual cortex. The fact that this oscillation was elicited by the Gestalt pattern and was phase-locked to the reaction time suggests that it could reflect the neuronal mechanism involved in linking the elements of the illusory square into a coherent percept. The occipital response-locked oscillation could be the most direct manifestation of visual feature-binding processes on the macroscopic EEG level. While both controls and schizophrenics display γ-band oscillations in visual Gestalt recognition, the frequency of the oscillation is lower in schizophrenics than in healthy individuals. This pattern may reflect impairments in the formation of neural assemblies, which use γ-band oscillations as a mechanism for synchronization. Although synchronization must occur for the perception of the Gestalt, it occurs at a lower frequency in schizophrenics. The neuronal networks are apparently not able to support high-frequency synchronization. If the disruption of fast neuronal oscillations indeed reflects a basic pathophysiological mechanism in schizophrenia, a greater understanding of the functional role of these brain oscillations in information processing may provide a background upon which to design and test new pharmacological therapeutic interventions (Whittington et al., 2000). γ-Oscillations are known to require the input of particular GABAergic interneurons (Somogyi and Klausberger, 2005) and it is also known that cortical inhibitory neurons are strongly impaired in schizophrenia ([Lewis et al., 2005] and [Benes et al., 2007]). On the molecular level, the pathology of GABA neurons is accompanied by a compensatory upregulation of a2GABA_A receptors on the cortical pyramidal target neurons (Lewis et al., 2005). In addition, a deficit in GABAergic transmission may also contribute to the dopaminergic hyperactivity, which is typical of schizophrenia. Dopamine neurons are under inhibitory control through a2GABA_A receptors (see below). Thus, enhancing GABAergic transmission at a2 and a3GABA_A receptors would be expected to rectify at least some of the pathophysiological deficits in
schizophrenia. It is therefore encouraging that MK-0777, a partial agonist acting at \(a_2\) and \(a_3\)GABA\(_A\) receptors, is presently being tested clinically as a potential therapeutic for schizophrenic patients, especially with regard to cognitive deficits. This example of the development of a new therapeutic strategy illustrates the contribution of GABA\(_A\) receptor research to the understanding and therapy of mental disorders.

2. Dissecting GABA\(_A\) receptor functions

Receptors containing the \(a_1\), \(a_2\), \(a_3\) or \(a_5\) subunit in combination with any of the \(\beta\) subunits and the \(\gamma_2\) subunit are most prevalent in the brain, with the major subtype being \(a_1\beta_2\gamma_2\). These receptors are sensitive to benzodiazepine modulation. The pharmacological relevance of GABA\(_A\) receptor subtypes for the spectrum of benzodiazepine effects was recently identified based on a highly selective genetic approach ([Rudolph et al., 1999], [Löw et al., 2000], [McKernan et al., 2000], [Whiting et al., 2000], [Rudolph et al., 2001], [Crestani et al., 2002], [Möhler et al., 2002], [Whiting, 2003b], [Möhler and Rudolph, 2004], [Rudolph and Möhler, 2006], [Möhler, 2007a] and [Möhler, 2007b]). Experimentally, the GABA\(_A\) receptor subtypes were rendered diazepam-insensitive by replacing a conserved histidine residue with an arginine residue in the benzodiazepine binding site of the respective subunit gene of mice \(a_1(H101R), a_2(H101R), a_3(H126R)\) and \(a_5(H105R)\) ([Rudolph et al., 1999], [Löw et al., 2000] and [Crestani et al., 2002]). A deficit of behavioural drug action in the corresponding mutant mouse pointed to the functional role of the respective GABA\(_A\) receptor subtype. This strategy permitted the allocation of benzodiazepine drug actions to the \(a_1\), \(a_2\), \(a_3\) and \(a_5\)GABA\(_A\) receptor subtypes ([Rudolph et al., 2001] and [Crestani et al., 2002]). In addition, by visualizing GABA\(_A\) receptor subtypes immunohistochemically, the neuronal networks mediating the corresponding drug actions became apparent. This genetic approach to GABA\(_A\) receptor subtype functions was complemented by a medicinal-chemistry approach. Ligands which differentiate between receptor subtypes by efficacy and/or affinity became available for direct behavioural testing ([McKernan et al., 2000], [Whiting, 2003a] and [Whiting, 2003b]). Thus, both genetics and the medicinal-chemistry, led to a unified concept of a new CNS pharmacology based on GABA\(_A\) receptor subtypes as novel targets (see Table 1).

3. Behavioural correlates of GABA\(_A\) receptor subtypes: separating sedation from anxiolysis

Among \(a_1\)-, \(a_2\)- and \(a_3\)-point-mutated mice only the \(a_1(H101R)\) mutants were resistant to the depression of motor activity by diazepam or zolpidem ([Rudolph et al., 1999], [Löw et al., 2000], [McKernan et al., 2000] and [Crestani et al., 2000]) while pentobarbital or a neurosteroid remained as effective in \(a_1(H101R)\) mice as in wild-type mice in inducing sedation. Clearly, diazepam-induced sedation is linked to \(a_1\)GABA\(_A\) receptors and ligands with preferential affinity for \(a_1\) receptors comprise common hypnotic drugs (Table 1).
Table 1

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Parallages</th>
<th>Major results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 (H101R)</td>
<td></td>
<td>Lack of the depressant action on hypothermia</td>
<td>Bürgi et al., 1999</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Elevated escape latencies</td>
<td>Tse et al., 2000</td>
</tr>
<tr>
<td>a2GABAΔ241</td>
<td></td>
<td>Lack of hypothermic effects on locomotion</td>
<td>Löw et al., 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lack of ataxic effects on grooming</td>
<td>Löw et al., 2000</td>
</tr>
<tr>
<td>a2 knockout</td>
<td></td>
<td>Increased spontaneous locomotor activity</td>
<td>Yvert et al., 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increased sensitivity to anxiogenic stimuli</td>
<td>Yvert et al., 2000</td>
</tr>
<tr>
<td>a2 knock out</td>
<td></td>
<td>Increased anxiety</td>
<td>Sellin et al., 2000</td>
</tr>
<tr>
<td>a2H101RΔ241</td>
<td></td>
<td>Reduced locomotor activity</td>
<td>Low et al., 2000</td>
</tr>
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</table>

Summary of the behavioural endophenotypes associated with selective GABA<sub>Λ</sub> receptor subtype mutant mice.

* This point mutation is associated with a partial knock-down of the a5 GABA-Λ receptors.

In contrast, the anxiolytic-like action of diazepam is attributed to the modulation of a2GABA<sub>Λ</sub> receptors as shown by the lack of tranquilizing action of diazepam in a2(H101R) mice (elevated plus maze; light/dark choice test) (Löw et al., 2000). The a2GABA<sub>Λ</sub> receptors, which comprise only about 15% of all diazepam-sensitive GABA<sub>Λ</sub> receptors, are mainly expressed in the amygdala and in principal cells of the cerebral cortex and the hippocampus with particularly high densities on their axon initial segments ([Nusser et al., 1996], [Fritschy et al., 1998a] and [Fritschy et al., 1998b]). Thus, the control of the amygdala and the inhibition of the output of these principal neurons appear to be a major mechanism of anxiolysis. In keeping with this notion, the ligand L-838417 with partial efficacy at a2, a3 and a5 but not on a1GABA<sub>Λ</sub> receptors, was anxiolytic in wild-type rats in the absence of sedation (McKernan et al., 2000) (Table 1). Similar findings applied to TPA023, a partial agonist acting at a2 and a3GABA<sub>Λ</sub> receptors (Attack et al., 2006). Thus, a neurobiological basis was discovered for the development of daytime anxiolytics which are devoid of drowsiness and sedation. This is the more encouraging as these 1-sparing ligands show a much reduced dependence liability compared to classical benzodiazepines (Ayor, 2005).

It had previously been postulated that the anxiolytic action of diazepam is based on the dampening of the reticular activating system which is mainly represented by noradrenergic and serotonergic neurons of the brain stem. These neurons express preponderantly a3GABA<sub>Λ</sub> receptors. The analysis of the 3-point-mutated mice [a3(H126R)] indicated that the anxiolytic effect of benzodiazepine drugs was unaffected (Low et al., 2000). The reticular activating system therefore does not appear to be a major contributor to anxiolysis. Nevertheless, the a3-selective ligand TP003 showed anxiolytic activity, at least at high receptor occupancy (Dias et al., 2005). Thus, under these conditions of a3GABA<sub>Λ</sub> receptors to anxiolytic activity may have to be considered.

4. Associative learning and memory

The acquisition of spatial and temporal memory is associated with excitatory synaptic plasticity involving hippocampal NMDA receptors ([Morris et al., 1986], [Davis et al., 1992], [McHugh et al., 1996], [Tsien et al., 1996], [Tang et al., 1999], [Huerta et al., 2000] and [Nakazawa et al., 2002]). This process was found to be amenable to molecular regulation in vivo. By manipulating genetically either
the inhibitory GABAergic control or the role of the co-transmitter glycine, striking behavioural alterations in learning and memory were found. They point to a5GABA<sub>A</sub> receptors and to the neuronal glycine transporter 1 as promising targets for improving cognitive behaviour.

4.1. a5GABA<sub>A</sub> receptors and cognition

In a5(H105R) mice, the content of a5GABA<sub>A</sub> receptors was reduced by 30–40% exclusively in the hippocampus (Crestani et al., 2002). This is presumably due to an effect of the mutation on receptor assembly or insertion. There was no indication for adaptive changes of other GABA<sub>A</sub> receptors expressed in the same pyramidal cells. Behaviourally, the partial deficit of hippocampal a5GABA<sub>A</sub> receptors resulted in an improved performance in trace fear conditioning, a hippocampus-dependent memory task (Crestani et al., 2002). These results pointed to a role of a5GABA<sub>A</sub> receptors in the function of temporal memory. When the a5GABA<sub>A</sub> receptors were deleted in the entire brain by targeting the a5 subunit gene ([Whiting, 2003a] and [Collinson et al., 2002]) a significant improvement in working memory performance in a water maze spatial learning test was observed. These findings were accompanied by a decrease of the amplitude of hippocampal IPSCs and an increase of the paired-pulse facilitation of field EPSPs amplitudes. These data strongly suggest that a5GABA<sub>A</sub> receptors play a crucial role in cognitive processes of hippocampal learning and memory. Indeed, a partial inverse agonist acting at a5GABA<sub>A</sub> receptors enhanced the performance of wild-type rats in the water maze test (Chambers et al., 2004).

There is further evidence to suggest that the role of a5GABA<sub>A</sub> receptors in the hippocampus extends to the modulation of associative learning in the form of selective attention. The hippocampus together with its allied structure entorhinal cortex plays a crucial role in the expression of a form of selective learning called latent inhibition (Gray et al., 1996). Latent inhibition refers to the retardation of conditioning to a CS that has previously been pre-exposed without consequence in comparison to the conditioning to a novel CS ([Lubow and Moore, 1959] and [Lubow, 1989]). A neutral stimulus with a history of non-reinforced exposures would be perceived as less salient and command less attention ([Mackintosh, 1973], [Mackintosh, 1974] and [Mackintosh, 1975]). Hence, conditioning to a pre-exposed CS typically proceeds more slowly. Partial deficit of hippocampal a5GABA<sub>A</sub> receptors has been shown to disrupt latent inhibition (Gerdjikov et al., 2008). First, this indicates that a partial reduction in neuronal inhibition normally mediated by hippocampal a5GABA<sub>A</sub> receptors is associated with enhanced associative learning under condition that normal animals tend not to learn. This is in line with the result on trace conditioning demonstrated previously ([Crestani et al., 2002] and [Yee et al., 2004]). Second, the loss of the latent inhibition under reduced hippocampal a5GABA<sub>A</sub> receptors suggests a role of these receptors in normal selective attention. Effective and adaptive learning obviously requires the ability to decide when an association ought to be formed and when not. Indeed, loss of latent inhibition has been reported in schizophrenia patients ([Baruch et al., 1988] and [Gray et
and it has been suggested that this may contribute to the emergence of some positive symptoms in schizophrenia (Gray et al., 1991) also see further discussion below).

In support of the hypothesis that $\alpha_{5}\text{GABA}_A$ receptors in the hippocampus may assume a general role in the control of the selectivity of learning and expression of learned behaviour, relevant findings have been reported in extinction learning. In Pavlovian conditioning, extinction training involves the repeated presentations of the CS without the antecedent US. This leads to the cessation of conditioned responding and is an active learning process implying the acquisition of new information (instead of “un-learning”). Partial deficit of hippocampal $\alpha_{5}\text{GABA}_A$ receptors impairs extinction of a conditioned fear response, leading to persistent conditioned responding despite that the CS is no longer predictive of US occurrences (Yee et al., 2004). This confirms the suggestion that the interplay between GABAergic and glutamatergic activities determines the efficacy of extinction training ([Davis and Myers, 2002], [Myers and Davis, 2002] and [Myers and Davis, 2007]). The extent to which the formation and persistence of such learned association may result in pathological conditions, e.g., in PTSD patients suffering from traumatic memory, would point to a potential therapeutic use of $\alpha_{5}$-selective agonist in treating such anxiety-related disorders.

In summary, $\alpha_{5}\text{GABA}_A$ receptors in the hippocampus are in a pivotal position to modulate activity of hippocampal principle neurons, which are assumed to be central to various mnemonic functions. While glutamatergic neurotransmission via NMDA receptors in these neurons is widely believed to mediate long-term potentiation (LTP) that sustains memory functions, inhibitory modulation via $\alpha_{5}\text{GABA}_A$ receptors may regulate the acquisition of new associations and/or the expression learned associations. This gate-keeping function is essential for flexible and adaptive learned behaviour and is a hallmark of higher cognition that goes beyond the conception of memory as a passive registry of past experiences. This is in line with the observation, mentioned above, that in the water maze, it is working memory rather than reference memory that has been reported to be enhanced by either whole-brain $\alpha_{5}\text{GABA}_A$ receptors knockout (Collinson et al., 2002) or $\alpha_{5}\text{GABA}_A$ receptors an inverse agonist ligand ([Chambers et al., 2004] and [Collinson et al., 2006]). Indeed, the mammalian memory system has exploited multiple avenues, other than GABAergic inhibition, to allow additional control over NMDA receptor-mediated synaptic plasticity. Another such control is achieved through the regulation of the obligatory co-agonist of NMDA receptors — glycine.

4.2. Neuronal glycine transporter 1 and cognition

Glycine is an obligatory co-agonist of glutamate at NMDA receptors and its binding to the NMDA receptor glycine site (glycine-B-site) is necessary for ion channel opening ([Johnson and Ascher, 1987] and [Verdoorn et al., 1987]). The concentration of glycine in the vicinity of NMDA receptors is normally maintained at sub-saturation levels by glycine transporter 1 (GlyT1) ([Smith et al., 1992], [Supplisson and Bergman, 1997] and [Bergeron et al., 1998]). An elevation of the synaptic availability of glycine constitutes a potent means to enhance the efficacy of NMDA receptor-mediated
neurotransmission and neural plasticity (Igartua et al., 2007). This can be achieved effectively by the inhibition or down-regulation of GlyT1 activity, either by means of pharmacological blockade (e.g. [Depoortere et al., 2005], [Sur and Kinney, 2007] and [Walter et al., 2007]) or molecular deletion of the transporter ([Tsai et al., 2004a] and [Yee et al., 2006]) (see Table 2). In particular, deletion of neuron-associated GlyT1 in the forebrain has recently been shown to be sufficient to enhance NMDA receptor current and is effective in inducing enhanced mnemonic functions ([Yee et al., 2006] and [Singer et al., 2007]).

Table 2

<table>
<thead>
<tr>
<th>Mutations</th>
<th>Polymorphisms</th>
<th>Results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Knockout</td>
<td>Abnormal neuronal morphology</td>
<td>Reduced neuronal activity</td>
<td>Yee et al., 2006</td>
</tr>
<tr>
<td>Forebrain GlyT1 deletion</td>
<td>Enhanced freezing</td>
<td>Reduced freezing</td>
<td>Sur and Kinney, 2007</td>
</tr>
<tr>
<td>Latent inhibition</td>
<td>Enhanced freezing, active avoidance</td>
<td>Reduced freezing</td>
<td>Walter et al., 2007</td>
</tr>
<tr>
<td>Sociometric response to social stimuli</td>
<td>Enhanced freezing</td>
<td>Reduced freezing</td>
<td>Tsai et al., 2004a</td>
</tr>
<tr>
<td>Recognition memory</td>
<td>Enhanced recognition for the context and the spatial location of objects</td>
<td>Yee et al., 2006</td>
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</table>

Unlike a partial deficit of hippocampal a5GABA A receptors, forebrain neuronal specific deletion of GlyT1 has been found to facilitate Pavlovian conditioning across a variety of paradigms including conditioned freezing, active avoidance learning, and conditioned taste aversion learning (Yee et al., 2006). Moreover, forebrain neuronal GlyT1 deletion enhances the latent inhibition effect (Yee et al., 2006), which is precisely opposite to the effect of hippocampal a5GABA A receptors deficiency (Gerdjikov et al., 2008). These apparent contradictory outcomes may reflect the differing functional modulations exerted by glycine/GlyT1 and VA receptors on NMDA receptor activity.

One parsimonious interpretation is that forebrain neuronal GlyT1 deletion enhances the formation of associative links, not only between CS and an unconditioned stimulus (US), but also the formation of [CS → nothing] association as a result of CS alone presentations during the pre-exposure (prior to CS–US pairing) stage of a latent inhibition experiment. Latent inhibition emerges because of the two opposing associative links: ([CS → nothing] vs [CS → US]) compete with each other over the control of behaviour in response to the CS ([Kraemer and Spear, 1991] and [Kraemer and Spear, 1993]). The response by animals that have not received CS pre-exposure would be totally governed by the [CS → US] association and therefore is stronger than in animals having been through CS pre-exposure. Hence, forebrain neuronal GlyT1 deletion leads to the expression of latent inhibition when the amount of pre-exposures is insufficient to generate latent inhibition in control littermates; and at the same time it also facilitates the expression of conditioned responding in the non-pre-exposed condition relative to control mice. In contrast, hippocampal a5GABA A receptors deficiency abolishes the latent inhibition effect (Gerdjikov et al., 2008), because the inhibition mediated by hippocampal a5GABA A receptors...
normally suppresses the formation of the \([CS \rightarrow US]\) associative link and/or the expression of the \([CS \rightarrow \text{nothing}]\) associative link. A partial deficit in hippocampal \(\alpha 5\text{GABA}_A\) receptors therefore biases the competition over behavioural control towards \([CS \rightarrow US]\) leading to an attenuation of the latent inhibition effect. Thus, while glycine (controlled by GlyT1) may represent an upstream control over NMDA receptor function, extrasynaptic \(\alpha 5\text{GABA}_A\) receptors in the hippocampus may assume a downstream control. Normal cognitive function relies on their coordinated interplay to yield optimal learning performance. Indeed, this may be a widespread strategy adopted by other brain structures, as it has been shown that an extinction deficit of emotional memory induced by intra-amygdala infusion of the \(\text{GABA}_A\) agonist muscimol can be reversed by the administration of the partial glycine-B site agonist d-cycloserine (Akirav, 2007).

Importantly, GlyT1 deletion enhances cognition without compromising the selectivity in learning. Indeed it enhances selectivity by promoting the acquisition of relevant events (or non-event) associated with a given environmental stimulus. In contrast, deficiency of hippocampal \(\text{GABA}_A\) receptors result in inflexible learning performance as outlined previously: (i) reduced sensitivity to the insertion of a CS–US trace interval, (ii) retarded extinction when the CS is no longer followed by the US, and (iii) insensitivity to CS pre-exposure effect leading to a loss of the latent inhibition effect.

Recently, the promnesic effects of GlyT1 deletion have been extended to learning beyond Pavlovian conditioning. Singer et al. (2007) showed that object recognition memory as well as memory of the spatial location associated with specific objects is enhanced following forebrain GlyT1 neuronal deletion. These findings are encouraging because object recognition memory is believed to be mediated largely by rhinal cortices with hippocampus playing a more critical role only when the spatial location of specific objects becomes relevant. Manipulations targeted at GlyT1 may therefore provide a means to modulate multiple memory systems: from the hippocampus to the neocortex.

While the deletion of GlyT1 restricted to forebrain neurons is associated with some clear promnesic effect as described above, brain-wide constitutive heterozygous disruption of GlyT1 only leads to limited and marginal enhancing effect on spatial reference memory learning in the water maze (Tsai et al., 2004a). This may reflect a gene dosage effect due to the heterozygosity of the mutation. Alternatively, GlyT1 disruption restricted to forebrain neurons — and therefore selective inhibition of the neuronal subset of GlyT1 — is somehow functionally more effective than a whole-brain approach. Unfortunately, currently available GlyT1 inhibitors cannot discriminate between glial- and neuron-associated GlyT1. We are therefore in the process of evaluating these two alternative accounts by generating mice with both neuronal and glial GlyT1 deletion in the forebrain. If neuronal specificity is a critical property, this would have important implications with respect to the development of GlyT1 inhibitors as potential cognitive enhancing agents or therapeutics for cognitive impairments in schizophrenia and other relevant psychiatric conditions.

The strategy to inhibit or delete GlyT1 and extrasynaptic \(\alpha 5\text{GABA}_A\) receptors activity is ultimately to enhance NMDA receptors excitability, and thereby to boost NMDA receptor mediated neural plastic
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events that underlie learning and memory. However one potential caveat of this approach may be
excitotoxicity in the longer term. Chronic and sustained elevation of NMDA receptors activity may
lead to cell death and eventual deterioration of function instead of enhancement. Indeed, it has been
argued that such approach might exacerbate, rather than ameliorate, conditions like Alzheimer's
disease (Javitt, 2004). We have preliminary data now to suggest that at least some of the promnesic
effects associated with forebrain neuronal GlyT1 deletion can be demonstrated still in mice over 2
years old (P Singer, D Boison, H Möhler, J Feldon, BK Yee, unpublished data). Examination of the
brains of these animals for the presence or absence of excessive neurotoxic damage is now underway.

5. Ameliorating psychotic symptoms: role of a5GABA\textsubscript{A} receptors and neuronal glycine
transporter 1

A deficit in GABAergic inhibitory control is one of the major hypotheses underlying the
symptomatology of schizophrenia ([Lewis et al., 2005] and [Benes et al., 2007]). A potential
contribution of GABA\textsubscript{A} receptor subtypes was therefore investigated with regard to the functional
hyperactivity of the dopaminergic system, considered to be a major factor in the genesis of
schizophrenia symptoms, especially positive symptoms.

The dopaminergic system in animals and humans is under GABAergic inhibitory control mainly via
3-containing GABA\textsubscript{A} receptors ([Fritschy and Möhler, 1995], [Pirker et al., 2000] and [Waldvogel et
al., 2008]). In mice lacking the 3 subunit gene no adaptive changes in the expression of 1, 2 and 5
subunits was observed (Studer et al., 2006) and anxiety-related behaviour was normal (Yee et al.,
2005). However, the mice displayed a marked deficit in prepulse inhibition of the acoustic startle
reflex, pointing to a deficit in sensorimotor information processing (Yee et al., 2005). This deficit in
prepulse inhibition was normalized by administration of the antipsychotic dopamine D2 receptor
antagonist haloperidol, suggesting that the phenotype is linked to hyperdopaminergia (Yee et al.,
2005).

The hippocampus is also believed to play an important role in the modulation of prepulse inhibition
(Bast and Feldon, 2003). In the a5(H105R) point-mutated mice (see above) prepulse inhibition was
attenuated concomitant with an increase in spontaneous locomotor activity, the latter being tested in a
novel open field and a novel environment (Hauser et al., 2005). Thus, the a5 subunit-containing
GABA\textsubscript{A} receptors which are located extrasynaptically and are thought to mediate tonic inhibition
([Caraiscos et al., 2004], [Scimemi et al., 2005], [Glykys and Mody, 2006] and [Prenosil et al., 2006])
are important regulators of the expression of prepulse inhibition. Attenuation of prepulse inhibition is
a robust phenotype of psychiatric conditions including schizophrenia. These results suggest that a3-
and/or a5-selective agonists may constitute an effective treatment for sensorimotor gating deficits in
various psychiatric conditions.

In addition, the hippocampus and the adjoining entorhinal cortex also assume a critical role in
modulating the expression of latent inhibition ([Gray et al., 1991], [Gray et al., 1996] and [Yee et al.,
These structures project directly to the nucleus accumbens and make excitatory glutamatergic synaptic connections onto neurons that also receive direct dopaminergic innervations from the ventral tegmental area (nucleus A10) ([Totterdell and Smith, 1989] and [Sesack and Pickel, 1990]). Specifically, the interaction between limbic glutamatergic inputs and ascending dopaminergic inputs in the nucleus accumbens underlies the normal expression of latent inhibition ([Gray et al., 1991], [Weiner, 1990] and [Weiner and Feldon, 1997]). Its disturbance is believed to be involved in the reported lack of latent inhibition in at least some subsets of schizophrenic patients ([Baruch et al., 1988] and [Gray et al., 1992]). Hence, the loss of tonic inhibition normally mediated by a5GABA_A receptors in the hippocampus may result in a functional disturbance of the hippocampal–accumbens projection. Loss of latent inhibition represents a severe deficit in selective attention and may contribute to some of the positive and cognitive symptoms observed in schizophrenic patients. In the a5(H105R) point-mutated mice, latent inhibition is severely attenuated. This suggests that a5-selective agonists may represent a potential treatment for the selective attention deficits and related positive symptoms in schizophrenia. In addition, the lack of flexible learning shown by a5(H105R) point-mutated mice in trace conditioning and extinction may also be related to the negative symptoms of schizophrenia, suggesting that 5-selective agonists may also be effective against negative symptoms. The latter is consistent with the glutamate hypothesis of schizophrenia ([Goff and Coyle, 2001], [Tsai and Coyle, 2002], [Coyle and Tsai, 2004] and [Coyle, 2006]) and current findings regarding GlyT1 inhibition or deletion (Lindsley et al., 2006).

Likewise, the efficacy of GlyT1 deletion to enhance latent inhibition also suggests that blockade or inhibition of GlyT1 may possess antipsychotic potential. These findings on the behavioural paradigm of latent inhibition however emphasize in particular positive schizophrenia symptoms. Current thinking on the other hand suggests that glutamatergic (and in particular NMDA receptor) hypofunction is more closely linked to the emergence of negative symptoms ([Supplisson and Bergman, 1997] and [Coyle and Tsai, 2004]). Accordingly, the use of NMDA receptor orientated therapy, including the use of NMDA receptor co-agonists such as d-serine and glycine has been reported to improve cognition and negative symptoms in schizophrenia (Lindsley et al., 2006). Alternatively pharmacological blockade of GlyT1 by the co-administration of sarcosine in combination with conventional antipsychotic treatment has yielded some promising synergistic efficacy against both positive and negative schizophrenia symptoms ([Tsai et al., 2004b], [Lane et al., 2005] and [Hersesco-Levy, 2006]). Recently, synthetic GlyT1 inhibitors with enhanced specificity than the endogenous GlyT1 antagonist sarcosine have been developed ([Lechner, 2006] and [Harsing et al., 2006]), which have been shown to exhibit significant activity in preclinical model predictive of antipsychotic efficacy ([Harsing et al., 2003] and [Depoortère et al., 2005]).

However, it should be noted that NMDA receptor blockade (e.g., by phencyclidine and ketamine) can also give rise to psychotic-like behaviour akin to the positive florid symptoms of schizophrenia (Farber, 2003). It has been shown that deletion of neuron-associated GlyT1 attenuates the locomotor
response to phencyclidine (Yee et al., 2006). We can confirm that this effect stems primarily from a functional antagonism against NMDA receptor blockade by PCP, because neuron-associated GlyT1 deletion was similarly effective against the specific NMDA receptor blockade achieved by systemic MK-801 (Fig. 1). Interestingly, GlyT1 deletion is also efficacious in delaying the motor stimulating effect of the indirect dopamine agonist and potent psychostimulant amphetamine (Yee et al., 2006). One may therefore suspect that GlyT1 can indirectly modulate glutamate–dopamine interaction at the ventral striatum, suggesting that GlyT1 blockade not only can directly counter NMDA receptor hypofunction but also dampen behavioural abnormalities due to functional hyperdopaminergia.

Fig. 1. Acute locomotor response to selective NMDA receptor blockade in the open field is attenuated in mice with forebrain neuron-specific deletion of glycine transporter 1 (GlyT1). Locomotor response to an acute MK-801 challenge was evaluated in the open field using a within-subjects design. First, all animals were injected with saline and exposed to the open field arena (40 × 40 cm) for 30 min in order to habituate them to the novel environment and to allow the assessment of baseline locomotor activity. To evaluate the motor stimulant effects of MK-801, the animals were next injected with MK-801 at either a dose of 0.15 mg/kg [mutant (n=12), control (n=16)] or 0.3 mg/kg [mutant (n=11), control (n=16)]. The animals were then observed for another 60 min. All injections were administered via the i.p. route. Locomotor activity was indexed by the distance traveled in the open field. Baseline activity in the saline phase was comparable between all groups which was supported by the absence of any group differences in a 2×2×6 (Genotype×Dose×5min-bins) ANOVA of the distance traveled in the saline phase. A 2×2×12 (Genotype×Dose×5min-bins) ANOVA of the distance traveled in the drug phase was carried out to assess the motor effect of MK-801. The emergence of a highly significant main effect of dose \([F(1,51)=35.94, \ p<0.001]\) indicated that MK-801 enhanced locomotor activity in a dose dependent manner. However, the motor stimulant effect of MK-801 was clearly reduced in mutants relative to controls, which was observed regardless of MK-801 dosage. This impression was confirmed by the presence of a significant main effect of genotype \([F(1,51)=6.45, \ p<0.05]\). For details on the generation of the mutant mice, the biochemical and electrophysiological properties in these animals please refer to Yee et al. (2006).

In summary, the full antipsychotic potential of the potential receptor sites highlighted here would require further evaluation in terms of their specific efficacy against different symptoms or clusters of symptoms. Given that the hypothesized therapeutic potentials of drugs targeting GABA_A receptor subtypes and GlyT1 are mainly derived from genetically modified animals that are not primarily
created as disease models, the use of specific animals models of schizophrenia would be highly relevant here ([Lipska and Weinberger, 2002] and [Meyer et al., 2005]). This approach allows one to more directly assess whether relevant compounds possess the ability to normalize schizophrenia-related endophenotypes.

Schizophrenia is a disorder with a complex clinical manifestation involving multiple symptoms, which are most likely to involve aetiological mechanisms extending beyond one single brain region or neurotransmitter system. Hence, multiple therapeutic interventions seem necessary for the effective treatment of the disease. Various GABA_A receptor sites and possibly GlyT1 may represent promising drug targets for the development of novel pharmacotherapy as well adjunctive interventions alongside conventional typical and atypical neuroleptics (Gray and Roth, 2007).
REFERENCES


Appendix II


tonic GABA_\textsubscript{A} receptor currents in the hippocampus. J Neurosci 25:10016–19924.
Sesack SR, Pickel VM (1990) In the rat medial nucleus accumbens, hippocampal and catecholaminergic
terminals converge on spiny neurons and are in opposition to each other. Brain Res 527:266-279.
transporter reveal colocalization with NMDA receptors. Neuron 8:927-935.
transporter 1 deletion in forebrain neurons. Behav Neurosci, in press.
Supplisson S, Bergman C (1997) Control of NMDA receptor activation by a glycine transporter co-expressed in
Sur C, Kinney GG (2007) Glycine transporter 1 inhibitors and modulation of NMDA receptor-mediated
Somogyi P, Klausberger T (2005) Defined types of cortical interneurone structure space and spike timing in the
indexes disordered perception and cognition in schizophrenia. Proc Natl Acad Sci USA 101:17288–17293.
synapses and gephyrin clusters in the thalamic reticular nucleus of GABA_\textsubscript{A} receptor alpha3 subunit-null
Totterdell S, Smith AD (1989) Convergence of hippocampal and dopaminergic input onto identified neurons in
Tsai G, Coyle JT. (2002) Glutamatergic mechanisms in schizophrenia. Annu Rev Pharmacol Toxicol. 42:165-
179.
Tsai G, Ralph-Williams RJ, Martina M, Bergeron R, Berger-Sweeney J, Dunham KS, Jiang Z, Caine SB, Coyle
JT. (2004a) Gene knockout of glycine transporter 1: characterization of the behavioral phenotype. Proc Natl
Acad Sci USA. 101:8485-8490.
Tsai G, Lane HY, Yang P, ChongMY, LangeN (2004b) Glycine transporter 1 inhibitor,N-methylglycine
Tsien JZ, Huerta PT, Tonegawa S (1996) The essential role of hippocampal CA1 NMDA receptordependent
Verdoorn TA, Kleckner NW, Dingledine R (1987) Rat brain N-methyl-D-aspartate receptors expressed in


Appendix III

Pavlovian cued fear conditioning in aged forebrain and neuron specific GlyT1 knockout mice

Preliminary data
RATIONALE
The enhancements on associative as well as on non-associative forms of learning previously demonstrated in forebrain and neuron-specific GlyT1 knockout mice lend support to the hypothesis that pharmacological interference of GlyT1-mediated glycine re-uptake may represent a promising strategy to correct cognitive deficits in a number of diseases and may improve cognition in the non-clinical population. However, it has been brought into question (see Sanderson and Bannerman 2007) why a mouse with such superior learning capabilities has not already been evolutionary selected. It has been argued that the anomalous cognitive behaviour in these mutant mice might lead to both benefits as well as harms, depending on the precise circumstances (Sanderson and Bannerman 2007). Furthermore, although a potentiation of NMDAR mediated transmission may improve cognitive functions this could be at the cost of increased excitotoxicity and therefore lead to a greater risk for neurodegeneration in the longer term. There is indeed evidence that excessive glutamatergic signaling possibly contributes to neurodegenerative conditions such as Alzheimer’s disease (Javitt 2004).

To explore the possibility that forebrain neuronal GlyT1 disruption might exert a negative impact on learning and memory function in the long term we tested whether the most robust pro-cognitive phenotype of the mutation, namely enhanced Pavlovian conditioning, would still be observable in aged animals. To this end, we evaluated associative learning in aged (24 months old) as well as adult (3 month old) mutant and controls mice using the conditioned freezing paradigm.

METHODS

Subjects
The subjects were obtained by crossing CamKIIαCre:Glyt1tm1.2fl/fl mice with Glyt1tm1.2fl/fl mice (both on a pure C57BL/6J background). This allowed us to generate litters with a 1:1 mixture of CamKIIαCre:Glyt1tm1.2fl/fl (hereafter simply referred to as “mutant”) and Glyt1tm1.2fl/fl (“control”) mice. Breeding took place in a specific-pathogen free (SPF) breeding facility. Genotyping was performed on postnatal days 21-30 by standard PCR (Yee et al. 2006). Litters were weaned at postnatal day 21. At the age of 11 weeks, the animals were transferred to a separate controlled animal vivarium (21±1°C, relative humidity at 55±5%) with a reversed light–dark cycle (lights off: 0800–2000). They were kept in groups of 3-5 in Makrolon® Type-III cages (Techniplast, Milan, Italy), and maintained under ad libitum water and food (Kliba 3430, Klibamuhlen, Kaiseraugst, Switzerland). Behavioural testing took always place in the dark phase of the cycle. Subjects consisted of 42 mice aged to 3 months (11 Mutants and 9 controls) or 24 months (9 Mutants and 13 controls) at the beginning of the experimentation. The experimental manipulations and procedures described here had been previously approved by the Swiss Cantonal Veterinary Office; they conformed to the ethical standards required by the Swiss Act and Ordinance on Animal Protection and the European Council Directive 86/609/EEC.
Appendix III

**Elevated plus maze**

A standard elevated plus maze test was conducted to assess the expression of anxiety related behavior. The maze consisted of two exposed and two enclosed arms joined to a central square platform. The apparatus has been fully described elsewhere (Yee et al., 2006). The test began by placing the animal into the central area facing one of the open arms. It was then allowed to explore freely for 5 min before being removed and returned to the home cage. A digital camera was mounted above the maze. Images were captured at a rate of 5 Hz and transmitted to a personal computer running the Ethovision tracking system (Noldus Technology Wageningen, The Netherlands). Two anxiety-related measures were computed: (1) percentage of time spent in the open arms \[\text{open}/(\text{open} + \text{closed}) \times 100\%\]; (2) percentage of number of entries made to the open arms \[\text{open}/(\text{open} + \text{closed}) \times 100\%\]. In addition, locomotor activity as indexed by the total distance travelled in cm was calculated.

**Open field paradigm**

There were four identical open fields each measuring 40 x 40cm² in surface area and surrounded by 35cm high walls. They were made of wood with a white water-proof surface as described before (Yee et al., 2006). Animals were tested in squads of four, under diffused dim lighting at about 30 lux. They were gently placed in the centre of the appropriate open field and allowed to explore undisturbed for one hour. The open fields were cleansed with water and dried prior to the next squad. Locomotor activity was indexed by distance travelled in cm recorded in successive 10-min bins. Data were collected and calculated by the EthoVision® tracking system (Version 3.1, Noldus Technology, Wageningen, Netherlands).

**Conditioned freezing paradigm**

The apparatus comprised four conditioning chambers as described before (Yee et al., 2006). All chambers were equipped with a grid floor made of stainless steel rods spaced at 10-mm intervals, and through which scrambled electric shocks (unconditioned stimulus, US) could be delivered (Model E13-14, Coulbourn Instruments). Because C57BL/6 mice carry the Ah1 deafness gene which leads to a sensorineural hearing loss beginning at about 2 months of age and rapidly progresses to total deafness by 18 months (Walton et al., 2008) the use of an acoustic conditioned stimulus (CS) – such as a tone - which is typical for this paradigm was therefore impractical. Hearing loss in aged mutant and control mice was confirmed by a pronounced reduction in the acoustic startle response relative to adult animals (data not shown). We therefore employed a tactile vibration-CS (see Yee et al., 2007) which was generated by a commercial vibrating unit (MiniVibrator Model 558095, Orion Versand AG, Buchs, Switzerland) attached to the grid floor. To avoid transmission of the vibration beyond the grid floor, the grid floor was isolated from the test chamber floor by four pieces of sponge to dampen transmission. A constant background white noise (60 dB) was provided via a loudspeaker mounted inside each chamber to mask over the weak noise emitted from the vibrating unit. A digital camera
was mounted 30 cm directly above the area of interest in each chamber, which captured images at a rate of 1Hz. Successive frames were compared to evaluate freezing behavior (Richmond et al., 1998).

On the first day, the animals received two discrete trials of CS-US pairing. Each trial began with the 30s CS presentation immediately followed by the delivery of a 1-s foot-shock set at 0.25mA. Each trial was preceded and followed by a 180s interval. 24h later, the animals were returned to the same chambers and observed for a period of 480s in the absence of any discrete stimulus in order to evaluate contextual conditioning. Another 24h later, the animals were placed in a different conditioning chamber in order to assess the conditioned freezing response to the CS in a novel context. Following a 120-s acclimatization period, the CS was turned on for 480s; freezing behaviour during this period of CS presentation was evaluated. The pre-CS and CS periods were evaluated separately.

Statistical analysis

All data were analysed by parametric analysis of variance ANOVA using age (adult vs. aged), genotype (mutant vs. littermate control) and sex (male vs. female) as the between-subjects factors. Additional within-subjects factors (e.g., time bins, days, pulse intensity) were included according to the nature of the considered dependent variables. Supplementary restricted analyses were also conducted to assist data interpretation whenever appropriate. All statistical analyses were carried out using SPSS for Windows (version 13, SPSS Inc. Chicago IL, USA) implemented on a PC running the Windows XP (SP2) operating system.

RESULTS

Elevated plus maze

The expression of anxiety like behavior was indexed by the percentage of time spent in the open arms and the percentage of entries made into the open arms. As illustrated in Table 1, the level of anxiety was not affected by the factors genotype and age. Similarly, locomotor activity as indexed by the total distance moved did not differ between groups. These impressions were confirmed by the absence of any significant effects in one-way ANOVAs separately conducted on each of the three index measures.
### Elevated plus maze

<table>
<thead>
<tr>
<th>Measure</th>
<th>Control</th>
<th>Mutant</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Adult</td>
<td>Aged</td>
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<tr>
<td><strong>Anxiety</strong></td>
<td></td>
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</tr>
<tr>
<td>Percent open arms entries</td>
<td>31.1 ± 3.4</td>
<td>35.2 ± 4.6</td>
</tr>
<tr>
<td>Percent time in open arms</td>
<td>21.5 ± 6.0</td>
<td>32.6 ± 7.0</td>
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<tr>
<td>Percent distance in open arms</td>
<td>14.6 ± 4.0</td>
<td>24.6 ± 5.1</td>
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</table>

*Locomotor activity*

<table>
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<th>Measure</th>
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</thead>
<tbody>
<tr>
<td>Distance moved [cm]</td>
<td>693.4 ± 65.3</td>
<td>648.8 ± 60.2</td>
<td>586.4 ± 32</td>
<td>609.8 ± 79.9</td>
</tr>
</tbody>
</table>

Table 1: Anxiety/fear related behaviour and locomotor activity on the elevated plus maze test. The expression of anxiety-like behaviour as indexed by the percentage of entries made into the open arms, the percentage of time spent in the open arms and the percentage of distance travelled in the open arms did not significantly differ amongst groups. Similarly, the level of spontaneous locomotor activity as measured by the total distance travelled was not affected by the factors genotype and age. All values refer to mean +/- SEM.

### Open field

An open field test was conducted to assess the overall level as well habituation of locomotor activity. A 2 x 2 x 2 x 6 (age x genotype x sex x 10min bin) ANOVA of the distance travelled in cm was carried out to analyze the data. The overall level of locomotor activity did not differ between groups (Figure 1). The presence of locomotor habituation was indicated by a monotonic decrease in the level of activity over time [F(5,170)=98.50, p<0.001] which was equally seen in all groups. No other significant effects were observed.

![Figure 1: Locomotor activity was indexed by the distance moved as a function of successive 10-mins bins. The overall mean across the entire observation period of 1h is depicted in the inserted histogram. Locomotor activity both in terms of the overall level and the rate of habituation over time was highly comparable between groups. All values refer to mean +/- SEM.](image)
**Conditioned freezing**

Associative learning was evaluated in the conditioned freezing paradigm. In order to circumvent the deafness in aged C57/BL6 mice we used a tactile CS whose efficacy has been established in a previous study (Yee et al., 2007). Performance in the conditioning phase, context test, and CS test were separately analyzed.

First, the level of freezing in the presence of the CS across the two CS-US pairings was examined (Figure 2A). As expected, the amount of freezing moderately increased from the first to the second paring reflecting the development of the conditioned response. This increase in the amount of freezing across parings appeared more pronounced in the adult as compared to the aged mice. Furthermore, the overall level of freezing in the presence of the CS was somehow higher in the aged controls relative than in aged mutants. However, a $2 \times 2 \times 2$ (age x genotype x sex x paring) ANOVA of percent freezing per paring only yielded a significant sex by pairing interaction [$F(1,34)=6.17, p<0.05$] indicating that the factors age and genotype did not affect the development of the conditioned freezing response.

Next, 24h after conditioning, the animals’ conditioned freezing response to the context was evaluated across the 8min test period (Figure 2B). While there was little difference in the overall level of freezing for the duration of the test, the expression of freezing response across time differed between groups. Mutants showed a relatively stable freezing response throughout, which was equally seen in both adult and aged subjects. In contrast, there was a steady increase in the freezing response in adult controls but not in aged controls whose freezing level remained stable across the entire period of testing. These impressions were in agreement with a $2 \times 2 \times 2 \times 4$ (age x genotype x sex x 2min-bin) ANOVA of percent freezing which gave rise to a significant age by genotype by bins interaction [$F(3,102)=2.73, p<0.05$]. To reveal the nature of the significant three way interaction additional ANOVAs restricted to adult and aged subject was conducted which yielded a significant bins by genotype interaction in the adults [$F(3,54)=3.74, p<0.05$] but not in the aged animals [$F(3,48)=1.43, p>0.24$].
Figure 2: The acquisition of conditioned freezing to the vibration CS on day 1 (A), context freezing on day 2 (B) and test of CS freezing on days 3 and 4 (C) are depicted. (A) Percent time freezing during the 30-s presentation of the CS is expressed as a function of the two consecutive CS-US pairing trials. All groups showed an increase in the freezing response from trial 1 to trial 2, which appeared more pronounced in the adult relative to the aged subjects, irrespective of genotype. There were, however, no significant effects in a 2 x 2 x 2 x 2 (age x genotype x sex x trial) ANOVA. (B) Freezing to the context 24 h after conditioning. Percent time freezing is expressed as a function of 2-min bins on the left and as overall mean on the right. While there was little difference in the overall level of freezing, the expression of freezing response across time differed between groups. Mutants showed a relatively stable freezing response throughout which was equally seen in adult and aged subjects. In the controls, however, there was a steady increase in the freezing response in adults whereas aged subjects showed stable freezing level across the entire period of testing. This gave rise to a significant age by genotype by bin interaction (p<0.05). (C) Freezing to the vibration CS 48h and 72h after conditioning, respectively. Percent time freezing is indexed as a function of 2-min bins on the left. The mean level of freezing averaged across the 8min CS period is depicted in the histogram. CS-freezing was enhanced in the mutant mice, which was seen in both adult and aged animals. However, the time course of CS-freezing markedly differed between adult and aged mutant subjects. Adult mutants reacted to the initiation of the CS with a pronounced freezing response which then quickly extinguished in the course of the 8min test period. In contrast, aged mutants also exhibited a strong freezing response in the beginning which, however, remained relatively stable over the 8min of testing indicating a resistance to within-session extinction of the freezing response. Extinction between days, on the other hand, was equally seen in all groups. This yielded a significant main effect of genotype and an age x genotype x bins interaction (p<0.05). All values refer to mean +/- SEM. * denotes a significant difference between mutant and controls (p<0.05).

Finally, the animals’ conditioned responding to the CS was evaluated in two CS-tests conducted 48 and 72h after conditioning, respectively. The animals were first placed in the test chambers for 2 min in the absence of any CS. This allowed the evaluation of baseline freezing behaviour. A 2 x 2 x 2 x 2 (age x genotype x day) ANOVA of pre-CS freezing failed to reveal any significant effects indicating that baseline freezing did not differ between groups and was stable across days. The mean baseline percent freezing scores collapses across the two days (±SEM) were: control: adult = 5.00 ± 1.49, aged = 3.72 ± 0.88; mutant: adult= 5.01 ± 1.82, aged= 8.47 ± 2.02. CS freezing to the tactile CS
was assessed in the next 8 min. As illustrated in Figure 3C, CS-freezing was enhanced in the mutant mice, which was seen in adult as well as aged animals. However, the time course of CS-freezing markedly differed between adult and aged mutant subjects. Adult mutants reacted to the initiation of the CS with a pronounced freezing response which then quickly subsided in the course of the 8 min test period. In contrast, aged mutants also exhibited a strong freezing response in the beginning which however remained relatively stable over the entire 8 min of testing indicating that they were resistant to within-session extinction. On the other hand, between-day extinction of the conditioned freezing response was equally seen in all groups. These observations were supported by a 2 x 2 x 2 x 2 x 4 (age x genotype x sex x day x 2 min-bin) ANOVA of percent freezing which revealed a significant main effect of bin [F(3,102)=7.30, p<0.005], day [F(1,34)=23.43, p<0.001] and genotype [F(1,34)=6.44, p<0.05]. This was accompanied by a significant interaction between age and bin [F(3,102)=3.53, p<0.05] and between age, genotype and bins, respectively [F(3,102)=3.87, p<0.05].

REFERENCES
Appendix IV

The glycine transporter 1 inhibitor SSR-504734 attenuates motor effects of PCP but potentiates the locomotor response to amphetamine and apomorphine

With Joram Feldon and Benjamin K. Yee
To be submitted in Neuropsychopharmacology

[Personal contribution to the work: Designing and performing the research, analysis of the behavioural data, and writing the manuscript]
Abstract

Glycine transporter 1 (GlyT1) is an attractive target for manipulating N-methyl-D-aspartate receptor (NMDAR) activation, because it is the key transporter controlling extracellular glycine levels in the brain. Glycine is the obligatory co-agonist for NMDAR activation by glutamate, and blockade of GlyT1 by SSR504734 has been shown to enhance NMDAR mediated currents. Hence, it may be effective against conditions attributable to NMDAR hypofunction, including the cognitive and negative symptoms of schizophrenia. Here, we evaluated in C57BL/6 mice the effects of SR504734 on response to two classes of psychostimulant drugs: the NMDA antagonist phencyclidine (PCP; 5mg/kg, i.p.), and dopamine agonists amphetamine (2.5mg/kg, i.p.) and apomorphine (0.75mg/kg, s.c.). PCP and amphetamine are potent motor stimulants at the specified dose, whereas apomorphine at the given dose depresses locomotor activity, presumably via stimulation of presynaptic dopamine autoreceptors. SSR504734 attenuated PCP-induced hyperlocomotion, but potentiated the motor-stimulant and motor-depressant effects of amphetamine and apomorphine, respectively. Hence, SSR504734 treatment not only confers resistance to NMDAR blockade, but also enhances reactivity to presynaptic dopaminergic interventions. The latter is consistent with reports that systemic SSR504734 treatment can augment extra-cellular dopamine levels in the prefrontal cortex as well as glutamate-mediated dopamine release in nucleus accumbens evoked by stimulation of the amygdala. The present study provides a functional demonstration of the impact of GlyT1 (achieved by SSR504734) on dopaminergic function, and therefore represents an important addition to the behavioural assessment of SSR504734, especially in relation to its potential use as an antipsychotic drug.
INTRODUCTION

Glycine acts as a requisite co-agonist of glutamate at the N-methyl-D-aspartate receptor (NMDAR). In the central nervous system, the extra-cellular glycine levels are tightly controlled by two different types of glycine transporters (GlyT1 and GlyT2) which mediate re-uptake of glycine at synapses (Cubelos et al., et al., 2005; Gomez et al., 2003). Glycine transporters therefore assume important roles in NMDAR/glutamatergic neurotransmission. GlyT1 is closely associated with NMDAR (Smith et al., 1992) whereas GlyT2 is co-localized with inhibitory (i.e. strychnine-sensitive) glycine receptors (Aragon and Lopez-Corcuera 2003). By increasing the synaptic concentration of glycine in the vicinity of NMDARs, GlyT1 blockers are expected to potentiate NMDA/glutamatergic transmission, and as such represent a potential therapeutic target for the treatment of disorders characterized by impaired NMDAR function, including schizophrenia (e.g. Lechner 2006; Lindsley et al., 2006, Sur and Kinney 2007).

SSR504734 is a potent and selective inhibitor of native human, rat, and mouse GlyT1 which reversibly inhibits glycine re-uptake (Depoortere et al., 2005) and leads to increases in the extracellular glycine levels in the nucleus accumbens (NAC) and prefrontal cortex (PFC) (Depoortere et al, 2005; Leonetti et al., 2006). SSR504734’s efficacy to elevate the central glycine levels has been closely linked to its facilitatory effect on NMDAR mediated neurotransmission. SSR504734 potentiated NMDA evoked eEPSCs recorded in hippocampal neurons and enhanced long term potentiation in the hippocampus in vivo (Depoortere et al., 2005).

In addition to its NMDAR-modulatory effects SSR504734 also exerted a functional impact on dopaminergic transmission. SSR504734 was found to augment the extracellular dopamine levels in the PFC (Depoortere et al., 2005) and facilitated glutamate dependent dopamine release in the nucleus accumbens (NAC) evoked by electrical stimulation of the basolateral amygdala (BLA) without affecting basal NAC dopamine levels (Leonetti et al., 2006). The findings indicate that SSR504734 modulates subcortical dopamine-glutamate interaction by increasing the central glutamatergic tone.

In animal models related to schizophrenia, SSR504734 improved a spontaneous PPI deficit in DBA/J2 mice and attenuated the hyperlocomotor effect of the NMDAR antagonist MK-801 in male Swiss mice. In neurodevelopmental models of schizophrenia (eWeinberger, 1987; Lieberman et al, 1997), SSR504734 attenuated hypersensitivity to the motor effects of acute amphetamine and restored impaired social recognition in rats neonatally treated with PCP (Depoortere et al., 2005). These finding thus provide convincing evidence that blockade of GlyT1 achieved by SSR504734 may prove useful in treatment of schizophrenia.

In the present study, we attempted to evaluate the functional impact of SR504734 on glutamatergic and dopaminergic pathways at the behavioural level by assessing its effects on the locomotor profile of adult C57/BL6 mice treated with (i) the NMDAR antagonist PCP (5mg/kg), (ii) the indirect dopamine agonist amphetamine (2.5mg/kg) and (iii) the direct dopamine receptor agonist apomorphine (0.75mg/kg). Amphetamine and PCP are potent motor stimulants at the employed dose
levels whereas the used dose of apomorphine is expected to depress locomotor activity by stimulating presynaptic dopamine autoreceptors. By this approach we expect to provide novel insights into the potential used of SR504734 as a novel antipsychotic compound.

**METHODS**

**Subjects**
Naïve adult male C57/BL6 mice were used in the present experiments which were bred in a specific-pathogen free (SPF) breeding facility (ETH Zurich Laboratory of Behavioural Neurobiology, Schwerzenbach, Switzerland). They were weaned at postnatal day 21, and then kept in groups of 3-5 in Makrolon® Type-III cages (Techniplast, Milan, Italy). They were maintained in a temperature (21°C) and humidity (55%) controlled vivarium under a 12:12h reversed light-dark cycle (lights on at 1900hr) and had ad libitum access to food and water throughout. Behavioural testing took place in the dark phase of the cycle, and commenced when the animals were 12 weeks old.

The experimental manipulations and procedures described here had been previously approved by the Swiss Cantonal Veterinary Office, conforming to the ethical standards required by the Swiss Act and Ordinance on Animal Protection and the European Council Directive 86/609/EEC.

**Drugs**
All drug solutions were freshly prepared on the day of testing. SSR-504734 (SSR, obtained as a gift from Sanofi-Synthelabo, France) was suspended in distilled water containing 5% Tween 80 to obtain the required doses: 3, 10, and 30mg/kg. Amphetamine (AMPH, obtained from Sigma-Aldrich, Germany) and Phencyclidine (PCP, obtained from NIDA) were dissolved in saline 0.9% NaCl to achieve the required doses of 2.5 and 5mg/kg, respectively. Apomorphine HCl (APO, obtained from Sigma-Aldrich, Germany) was dissolved in 1% sterile ascorbic acid (VitC, pH 3.2) to achieve the desired concentrations (0.25, 0.5, 1.0 and 2.0mg/kg in experiment 5 and 0.75mg/kg in experiment 6). SSR, AMPH and PCP were injected via the intraperitoneal route, APO was administered subcutaneously. The volume of administration was 10ml/kg body weight for SSR and 5ml/kg for APO, AMPH and PCP, respectively. AMPH, APO and PCP were administered 5min before testing. SSR was administered immediately before testing in experiment 1 and 30min before testing in remaining experiments.

**Open field**

*Apparatus.* The apparatus consisted of four identical open field arenas, each measured 40 × 40 cm in surface area and was surrounded on all sides by a 30cm high wall. The four open field arenas were made of wood with a white waterproof plastic surface. They were located in a testing room under diffused dim lighting (30 lx). A digital camera was mounted directly above the four arenas, capturing images from all four arenas at a rate of 5Hz. The images were transmitted to a PC running the
Ethovision (Noldus Technology, The Netherlands) tracking system. Locomotor activity was indexed by distance travelled in cm recorded in consecutive 10-min bins.

**Procedure.** Experimentally naïve mice were used in each open field experiment and were tested in squads of four counterbalancing across drug conditions. They were gently placed in the centre of the appropriate arena and allowed to explore undisturbed for the entire period of testing. Afterwards, they were returned to the home cage and the arenas cleansed with water and dried prior to the next squad.

**Elevated plus maze**

**Apparatus.** The elevated maze consisted of two exposed and two enclosed arms joined to a central square platform. Its construction has been fully described elsewhere (Yee et al., 2004). A digital camera was mounted above the maze. Images were captured at a rate of 5 Hz and transmitted to a personal computer running the Ethovision tracking system (Noldus Technology, Wageningen, The Netherlands). Two anxiety-related measures were calculated: percent time in open arm = time in open arms/time in all arms×100%, and percent entries into open arms = entries into open arms / entries into open or enclosed arms×100%. In addition, locomotor activity as indexed by the distance moved in cm was computed.

**Procedure.** Each trial began by placing the animal into the central area facing one of the open arms. It was then left to explore freely for 5 min before being removed and returned to the home cage. Afterwards, the maze was cleansed with water and dried prior to the next animal.

**Statistical analysis**

All data were subjected to parametric analysis of variance (ANOVA) with the between-subject factor drug and the within-subject factor time bins. The ANOVAs were performed using SPSS® for Windows (Release 13.0) on a personal computer running the Microsoft Windows XP Professional SP2 operating system. Open field data were submitted to a square-root transformation to better conform to the homogeneity of variance assumption of parametric ANOVA before being submitted to statistical analysis. Data interpretation was further assisted by Fisher’s least significant difference (LSD) post hoc comparisons and the use of restricted ANOVAs to determine the locus of the significant effects. A critical level of \( p < 0.05 \) was set for all statistical analyses.

**RESULTS**

1) **The effects of SSR 504734 on open field locomotor activity**

For the interpretation of the data derived from the subsequent drug experiments it is essential to ascertain whether SSR on its own exerts any effect on the locomotor activity. A dose response analysis was therefore conducted containing 3, 10 and 30mg/kg. Locomotor activity was indexed by the distance travelled in cm and recorded into 10-min bins over a test period of 2.5h.
transformed) were submitted to a 4 x 15 (drug x 10-min bins) split-plot ANOVA. As illustrated in Figure 1, the tested doses of SSR did not significantly affect the overall level of locomotor activity or the rate of locomotor habituation over time. Consequently, neither the effect of drug nor its interaction with bins attained statistical significance. The overall presence of locomotor habituation was confirmed by the appearance of a significant effect of bins \([F(14,392)=27.52, p<0.001]\) (all \(n=8\)).

![Figure 1](image-url)

**Figure 1.** The locomotor profile of SSR in the open field paradigm. Locomotor activity was indexed by square-root transformed distance travelled per 10-min bins. Activity is depicted either as a function of bins (a), or as overall mean across the entire observation period of 2.5h (b). SSR affected neither the overall activity level nor the rate of locomotor habituation over the 2.5h observation period. All values refer to mean ± SEM.

2) Effects of SSR on the expression of anxiety-like behaviour in the elevated plus maze

To evaluate whether SSR may affect anxiety related behaviour we conducted a similar dose response analysis using the elevated plus maze test. Anxiety-like behaviour was indexed by the percent entries into open arms and the percent time spent in open arms. Both measures conformed closely to each other and were comparable between drug conditions (Figure 2B-C) indicating that SSR did not affect the expression of anxiety like behaviour. Similarly, locomotor activity as measured by the total distance travelled did not differ between drug conditions (Figure 2A) which is in agreement with the open field experiment. These observations were supported by the lack of any significant effect of drug condition in separate one-way ANOVA’s of the three variables. Vehicle (\(n=7\)), SSR(3mg) (\(n=8\)), 10mg SSR(10mg) (\(n=8\)), and SSR(30mg) (\(n=8\)).
3) The effect of SSR on PCP-induced hyperlocomotion

Next, we evaluated the efficacy of SSR to antagonize the motor stimulating effect of PCP. Because of SSR’s ability to enhance NMDAR mediated neurotransmission (Dépoortère et al., 2005) we expected that SSR would attenuate PCP induced hyperlocomotion by countering the blockade of the NMDAR ion channel by PCP. The activity dose-response curve over the 2 h test period is depicted in Figure 3. SSR completely abolished the hyperlocomotor effect of PCP (5mg/kg) at the highest dose but not at the two lower doses. The activity levels exhibited by the latter two conditions were intermediate between Veh-Veh control and Veh-PCP subjects indicating that SSR attenuated the locomotor response to PCP in a dose-dependent manner. These impressions were confirmed by the appearance of a significant main effect of drug [F(4,31)=5.46, p<0.005] in a 5 x 12 (drug x 10min-bins) ANOVA of the distance travelled (square root transformed)]. Fisher’s least significant difference (LSD) pair-wise comparisons between groups confirmed that the locomotor activity in the Veh-PCP condition was significantly higher than in the Vehicle-Vehicle (p=0.02) the 30mg SSR-PCP (p<0.001) conditions. Vehicle-Vehicle (n=5), Vehicle-PCP (n=7), SSR(3mg)-PCP (n=8), SSR(10mg)-PCP (n=8), and SSR(30mg)-PCP (n=8).
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Figure 3. The effects of SSR on the motor stimulating effects induced by PCP in the open field. Locomotor activity was indexed by square-root transformed distance travelled per 10-min bins, and is depicted either as a function of bins (a), or as overall mean across the observation period (b). PCP led to an elevation of locomotor activity as indicated by the comparison between Veh-Veh and Veh-PCP group. SSR at 30mg/kg (30mg SSR-PCP) antagonised this effect of PCP, and restored activity level to that comparable to Veh-Veh group. In (b), * denotes significant difference at p<0.05. All values refer to mean ± SEM.

4) The effects of SSR 504734 on amphetamine-induced hyperlocomotor activity

Given that PCP-induced hyperactivity has been most closely linked to NMDAR blockade within subcortical structures leading to enhanced dopamine release in the NAC (e.g. Javitt and Zukin 1991, Iversen, 1995; Steinpreis, 1996; Breier et al., 1998; Smith et al., 1998) one may suspect that SSR not only counters NMDAR hypofunction but may also correct behavioural abnormalities induced by dopaminergic dysregulations. To explore this possibility we tested the effect of SSR on the hyperlocomotor response to an acute challenge with the indirect dopamine agonist amphetamine. The activity profile over the 2h test period is illustrated in Figure 4. As expected, amphetamine enhanced locomotor activity in the open field but rather unexpectedly, pre-treatment with SSR exacerbated the animals’ response to amphetamine in a dose-dependent manner. This interpretation was supported by a 5 x 12 (drug x 10min-bins) ANOVA of the distance travelled (square root transformed), which yielded a highly significant main effect of drug [F(4,32)=6.61, p=0.001] and its interaction with bins [F(44,352)=2.59, p<0.005]. Subsequent Fisher’s LSD comparisons between group means indicated that (i) the Veh-Veh control group differed significantly from all other groups (p<0.001), and (ii) pre-treatment with SSR at 30mg/kg led to a significantly higher level of activity than amphetamine treatment alone (p<0.05). Veh-Veh (n=5), Veh-AMPH (n=8), SSR(3mg)-AMPH (n=8), SSR(10mg)-AMPH (n=8), and SSR(30mg)-AMPH (n=8).
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Figure 4. The effects of SSR on hyperlocomotion induced by amphetamine in the open field. Locomotor activity was indexed by square-root transformed distance travelled per 10-min bins, and is depicted either as a function of bins (a), or as overall mean across the observation period (b). Amphetamine significantly elevated activity as indicated by the comparison between Veh-Veh group against all others (denoted by # p<0.05). SSR dose-dependently potentiated amphetamine-induced hyperlocomotion, with the highest dose (30mg/kg) achieving a significantly higher level of activity in comparison to the Veh-Amph group (indicated by *, p<0.05). All values refer to mean ± SEM.

5) The effect of apomorphine on locomotor activity

While the motor stimulant effects of dopaminergic agonists are considered to result from activation of postsynaptic dopamine receptors the motor depressant response to low doses of direct dopamine agonists has generally been interpreted in terms of presynaptic or 'autoreceptor' dopamine stimulation (Strömbom, 1976, Skirboll et al., 1979, Carlsson 1975, Di Chiara et al., 1976) which exert a negative feedback control on dopamine release/synthesis and thereby reduce, dopaminergic transmission (Tamminga 2002). To address the question whether the observed potentiation of amphetamine induced hyperactivity may represent a general feature of SSR to reinforce the behavioral effects of dopamine agonists we attempted to evaluate the effect of SSR on the motor depressant response to a presynaptically active dose of apomorphine. To this end we first conducted a dose response analysis of apomorphine in order to define the dose range in which apomorphine exerts a motor inhibitory effect (Vehicle (n=5), 0.25, 0.5, 1.0, and 2.0mg/kg APO (n=7)). Locomotor activity was measured by the distance travelled (square root transformed) in the open field arena over a period of 2 hours. As illustrated in Figure 5, APO reduced moderately reduced locomotor activity at 0.5mg/kg and led to a more pronounced reduction at 1mg/kg. In contrast, the activity profile of 0.25 and 2mg/kg condition did not differ from vehicle control. The observed U-shape of the dose response curve gave rise to a significant main effect of dug [F(4,28)=4.48, p<0.01] and its interaction with bins [F(44,308)=1.89,
p<0.05] in a 5 x 12 (drug x 10-min bins) ANOVA of the distance travelled (square root transformed). Fisher’s least significant difference (LSD) pair-wise comparisons between groups revealed that the reduction in activity reached statistical significance at 1mg/kg (p<0.005) and approached significance at 0.5mg/kg (p=0.067). This indicates dose levels in the range of 0.5-1mg/kg are effective to inhibit locomotor activity which is highly similar to previous reports (Sansone et al. 1981). Thus, to assess whether SSR would also potentiate the motor depressant effect of apomorphine we selected a dose level of apomorphine (0.75mg/kg) which was in-between 0.5 and 1mg/kg. This dose is expected to moderately inhibit locomotor activity and is therefore suitable to reveal to reveal a potential enhancing effect of SRR.

Figure 5: Locomotor activity profile of apomorphine in the open field paradigm. Locomotor activity was measured by the distance travelled (square root transformed) in the open field arena over a period of 2 hours, and is depicted either as a function of bins (a), or as overall mean across the observation period (b). Locomotor activity was significantly reduced at a dose of 1mg/kg (indicated by *, p<0.05) as compared to vehicle control. A similar non-significant trend was found at 0.5mg (p=0.067). All values refer to mean ± SEM.

6) The effects of SSR 504734 on apomorphine-induced hypolocomotion
Here, we evaluated the SSR’s ability to enhance the motor depressant effect of a presynaptically active dose of apomorphine (0.75mg/kg). The results showed that the inhibitory effect of apomorphine was further diminished by SSR in a dose-dependent manner (Figure 6). Data were analyzed by 5 x 12 (dose x 10min-bins) ANOVA of the distance travelled (square root transformed), which confirmed our impressions by yielding a significant main effect of drug [F(4,19)=3.22, p<0.04]. Fisher’s least significant difference (LSD) post hoc comparisons showed that the highest dose of SSR significantly exacerbated the hypolocomotor effect of apomorphine (p<0.004). Veh-Veh (n=5), Veh-APO (n=5), SSR(3mg)-APO (n=4), SSR(10mg)-APO. (n=5), and SSR(30mg)-APO (n=5).
DISCUSSION

Our results showed that the GlyT1 inhibitor SSR504734 dose-dependently attenuated the hyperlocomotor effect of PCP but potentiated the motor-stimulating as well as the motor depressing effect of amphetamine and apomorphine, respectively. This unique behavioural profile indicates that blockade of GlyT1 by SSR504734 exerts a functional impact on glutamatergic and dopaminergic pathways, which has important implication regarding its potential therapeutic use as antipsychotic compound. Based on the null effect of SSR504734 in the open field and the elevated plus made paradigms we assume that the observed pattern of results is not confounded by potential effects of SSR504734 on locomotor activity or anxiety-like behaviour per se.

SSR504734’s ability to potentiate NMDAR mediated transmission (Depoortère et al., 2005) therefore provides a plausible parsimonious explanation for observed efficacy of SSR504734 to attenuate the motor stimulating effect of PCP such that an increased number of activated NMDARs reduces the functional efficacy of a given concentration of PCP to block the NMDAR ion channel. Further evidence that this effect stems primarily from a functional antagonism against NMDAR blockade is provided by the finding that SSR504734 also inhibited the motor stimulating effect of specific NMDAR blockade achieved by MK-801 (Depoortère et al., 2005). This is also in keeping with data derived from the GlyT1 inhibitors NFPS and Org24461 which were found to enhance NMDAR function and reduced the motor responsiveness to PCP in a similar way (Harsing et al., 2003 and 2006; Kinney et al., 2003).
PCP has been consistently reported to produce behaviours in healthy individuals that closely mimic positive and negative symptoms of schizophrenia (Javitt and Zukin, 1991, Steinpreis and Salamone 1993, Krystal et al., 1994, Malhotra et al., 1996) and to exacerbate existing psychosis in schizophrenics (Javitt and Zukin, 1991). NMDAR antagonists such as PCP have therefore been extensively used to pharmacologically model neuropsychiatric conditions in animals. Increased dopaminergic transmission has been considered as the major mechanism underlying the behavioural and psychomimetic effects of these drugs (e.g. Javitt and Zukin 1991, Iversen, 1995; Steinpreis, 1996; Breier et al., 1998; Smith et al., 1998). However, more recent evidence indicates that dopaminergic activity is not necessary for evoking the behavioural effects of PCP but that the induced dopaminergic changes may be an indirect consequence of activated glutamatergic transmission at non-NMDA receptors (Takahata and Moghaddam, 2003, Chartoff et al. 2005). This suggests that increased dopamine activity may be a downstream effect of the glutamatergic actions of PCP which is not required but may contribute to the motor stimulating effect of NMDAR blockade. This could also explain why dopamine receptor agonists can impede NMDAR antagonist induced hyperlocomotion (e.g. Chartoff et al. 2005). Since PCP produces both negative and positive symptoms of schizophrenia these findings suggest that it may be more effective to target the critical glutamatergic pathways to antagonize PCP induced behaviours and thereby to treat negative symptoms of schizophrenia than interfering with the dopamine system. Regarding that SSR504734 effectively antagonized PCP and MK-801 induced hyperlocomotion and potentiated NMDAR activity this lends support to the suggestion that SSR504734 may be therapeutically beneficial against both negative and positive symptoms of schizophrenia by restoring hyperglutamatergia which is assumed to be one of the primary causes of schizophrenia (Farber 2003; Coyle and Tsai 2004).

In contrast to its efficacy against PCP and MK-801 SSR504734 facilitated the motor stimulating effect of the indirect dopamine agonist amphetamine. A similar tendency has also been observed in rats in which amphetamine-stimulated hyperactivity was tentatively elevated by much lower doses of SSR504734 (Depoortère et al., 2005). On the other hand, the GlyT1 inhibitor Org24461 was found to reduce hyperlocomotor response to amphetamine whereas the GlyT1 inhibitor NFPS was inactive against amphetamine in the same study (Harsing et al., 2003). This inconsistency has been attributed to differences in the mode of action between these two compounds because of the fluoxetine moiety of Org2446 which potentially interferes with other neurotransmitter systems (Harsing et al., 2003). The motor stimulating effect of amphetamine is assumed to be mediated via increased dopamine release in subcortical structures, particularly in the NAC, leading to increased neuronal firing at postsynaptic dopamine receptors (Kelly et al. 1975; Grace 1991, 2000). This may indicate that SSR50473 leads to a potentiation of amphetamine induced accumbal dopamine release which is in keeping with the finding that SSR504734 facilitated BLA-stimulated accumbal dopamine release (Leonetti et al. 2006). Interestingly, SSR504734 did not alter the basal dopamine levels in the NAC (Leonetti et al., 2006) suggesting that SSR504734 may only increase accumbal dopamine release
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under conditions of enhanced dopaminergic activity but may be devoid of an effect on dopamine efflux under physiological conditions. This is in line with the null-effect of SSR504734 on locomotor activity per se. However, since amphetamine and SSR504734 were given systemically in the present study it cannot be excluded that potential dopaminergic effects of SSR504734 in other brain regions may explain the observed results. The finding that SSR504734 increased the dopamine levels in the PFC (Depoortere et al., 2005), which projects to the NAC and tightly controls subcortical dopamine release (Grace 1991 and 2000), provides some evidence that potential changes in the cortical dopamine pathways may contribute to the obtained pattern of results. Local administration of SSR504734 to specific brain regions would therefore be required in order resolve the precise mode of action.

While the motor stimulating effects of dopamine agonists are attributed to their actions at postsynaptic dopamine receptors the motor depressant response to low doses of dopamine agonists has generally been interpreted in terms of presynaptic or 'autoreceptor' dopamine stimulation (Strömbom, 1976, Skirboll et al., 1979, Carlsson 1975, Di Chiara et al., 1976), which exerts a negative feedback control on dopamine release/synthesis and thereby reduces dopaminergic transmission (Tamminga 2002). In order to investigate whether SSR504734 would also potentiate the inhibitory effects of dopamine agonists we tested SSR504734 in combination with a motor-depressant dose of apomorphine. Interestingly, SSR504734 further deceased apomorphine induced motor depression despite the fact that it enhanced the motor stimulating effect of amphetamine, which was opposite in direction. This indicates that SSR504734 can potentiate the presynaptic as well as the postsynaptic actions of dopamine agonists which can lead to stimulation or depress of locomotor activity. Although the underlying mechanism is presently unknown it appears that SSR504734 produces a hyper-responsivity of the dopamine system such that it over-reacts to homeostatic imbalances resulting in abnormally intensified behavioural responses.

This unique behavioural profile therefore provides a novel addition to the behavioural evaluation of SSR504734 which has important implications regarding the potential therapeutic use of this compound as antipsychotic drug. The dopamine hypothesis of schizophrenia (Carlsson and Iverson 1963) posits that a dopaminergic hyper function contributes to the emergence of positive symptoms of schizophrenia which was supported by several pieces of evidence including the psychotogenic effects of dopamine releasing agents such as amphetamine (for a review see Angrist 1994). Given that the neuronal circuitry by which amphetamine mediates positive symptoms in humans and locomotor activation in rodents may partly overlap amphetamine induced hyperlocomotion in rodents constitutes a widely used animal model to mimic positive symptom of schizophrenia (e.g. Arguello and Gogos 2006). SSR504734 which potentiated the motor effects of amphetamine may therefore bear the risk to exacerbate positive symptoms of schizophrenia. This is in sharp contrast with the finding that this compound attenuated hypersensitivity to acute amphetamine
induced by neonatal treated with PCP which also emphasis the positive symptomatology of schizophrenia (Wang et al., 2001 and 2003; Javitt et al, 2004; Laruelle 2000).

Furthermore, the observation that SSR504734 potentiated the motor-depressant effect of apomorphine presumably by facilitating the presynaptic actions of this drug also supports a antipsychotic profile of SSR504734 which is in keeping with the hypothesis that stimulation of presynaptic autoreceptors may provide a potential approach to normalize excessive dopaminergic transmission in schizophrenia by activating the negative feedback control mechanism (e.g. Tamminga 1978, 2002) which was mainly based on the therapeutically beneficial effect of apomorphine in schizophrenia patients when given at low doses which are assumed to stimulate dopamine autoreceptors (Tamminga et al., 1978). These findings suggest that SSR50473 can interfere with the negative feedback control mechanism of the dopamine system under certain conditions which may contribute to the observed antipsychotic properties of this compound. However, based on the inconsistent results concerning the effects of SSR504734 in dopamine oriented models of schizophrenia we consider it as particularly important to use other preclinical models that emphasise specifically positive symptoms such as the latent inhibition paradigm (e.g. Weiner 2003) to further evaluate the potential impact of SSR50473 on positive schizophrenia symptoms.

In summary, there is increasing evidence that SSR504734 may be therapeutically beneficial against different symptoms or clusters of symptoms of schizophrenia (Depoortère et al., 2005). However, this may be accompanied by a potential risk to exacerbate positive symptoms especially in the acute psychotic phase of the disease. In order to assess the full antipsychotic potential of this compound highlighted here and elsewhere (Depoortère et al., 20005) further evaluations are therefore required in terms of the potential therapeutic consequences on the expression of positive symptoms. Schizophrenia is a neuropsychiatric disorder with an extremely complex clinical manifestation such that multiple therapeutic interventions may be required to effectively treat this disease. SSR50473 may represent promising drug candidate for the development of novel pharmacotherapy as well adjunctive interventions to conventional antipsychotic medication. There is some evidence that combined treatment with SSR504734 and classical neuroleptics might be superior to either treatment alone. Most of the therapeutically efficient antipsychotic drugs exert their therapeutic effects via blockade of dopamine receptors which is associated with a shutdown of phasic dopamine release and an inactivation of dopamine cell firing (Grace 1991, 2000). This mode of action is efficient against positive symptoms but it also forces the dopamine system in an extreme state of imbalance which could also be responsible for the lesser efficacy of these drugs in reducing negative symptoms (Meltzer et al., 1986). Hence, adjuvant SSR504734 treatment may normalize this imbalance by increasing NMDAR mediated tonic dopamine release (Gray 1991, 2000) which possibly leads to improved therapeutic efficacy against negative symptoms. This approach may also allow to lower the effective neuroleptic dosage which may reduce neuroleptic-induced side effects (e.g. Baldessarini and Tarsy 1980). In turn, potential undesired effects of SSR504734 on positive symptoms would be
efficiently prevented by co-treatment with antipsychotics. Support for this hypothesis is provided by recent clinical studies on the GlyT1 inhibitor sacosine added to antipsychotics in the treatment of schizophrenia which revealed significant improvements in positive, negative and cognitive symptoms (Tsai et al., 2004; Heresco-Levy U 2006).

**CONCLUSIONS**

Our result showed that the GlyT1 inhibitor SSR504734 attenuated PCP induced hyperlocomotion but potentiated both the motor-stimulating effect of amphetamine and the motor-inhibitory effect of apomorphine. This pattern of results provides novel insights into the evaluation of SSR504734 as a potential antipsychotic drug suggesting that SSR504734 may be therapeutically efficient in particular against negative symptoms. The unique behavioural profile of SSR504734 further suggests that combined treatment with classical antipsychotics may provide superior therapeutic efficacy against positive and negative symptoms than either treatment alone.
REFERENCES


Appendix IV


Skirboll LR, A.A. Grace AA, Bunney BS (1979) Dopamine auto- and postsynaptic receptors: electrophysiological

280


Strömbom U (1976) Catecholamine receptor agonists effects on motor activity and rate of tyrosine


Tamminga CA, Schaffer MH, Smith RC, Davis JM (1978) Schizophrenic symptoms


Appendix V

Glycine transporter 1 inhibitor SSR504734 moderately increases basal PPI but exacerbates apomorphine induced PPI disruption in C57BL/6 mice

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Manuscript in preparation

[Personal contribution to the work: Designing and performing the research, analysis of the behavioural data, and writing the manuscript]
Abstract

Inhibition of glycine transporter 1 (GlyT1) leads to enhanced NMDA-receptor (NMDAR) function via increases in synaptic glycine and may be therapeutically beneficial in the treatment of schizophrenia disorders related to NMDAR hypofunction. The GlyT1 inhibitor SSR504734 has been found to facilitate NMDAR currents and exerted an antipsychotic profile in animal models of schizophrenia. Furthermore, SSR504734 increased extra-cellular dopamine levels in the prefrontal cortex and enhanced dopamine release in the nucleus accumbens evoked by electrical stimulation of the basolateral amygdala indicating a modulatory effect on the dopamine system. Most recently, we found that SSR504734 enhanced the motor-stimulant effect of the dopamine releaser amphetamine and potentiated the motor-depressant effect of the direct dopamine agonist apomorphine. Given that dopamine agonists are widely used to model schizophrenia endophenotypes emphasising the positive symptoms one may suspect that GlyT1 blockade may exacerbate the positive symptoms.

Here, we evaluated SSR504734’s effect on prepulse inhibition (PPI) in mice and tested its potential to enhance the PPI-disruptive effect of apomorphine, an animal correlate of the positive symptoms of schizophrenia. The results revealed that SSR504734 moderately enhanced basal PPI but exacerbated apomorphine induced PPI disruption. This may suggest that SSR504734 leads to a hyper-responsivity of the dopamine system which in turn enhances behavioural response to dopaminergic stimulation. We therefore propose that co-treatment with conventional antipsychotics may counter the potential negative impact on positive symptoms of GlyT1 inhibitors and at the same time may improve the therapeutic efficacy of antipsychotics against negative and cognitive symptoms of schizophrenia.
INTRODUCTION

The glycine transporter 1 (GlyT1) inhibitor SSR504734 has been shown to increase the extra-cellular glycine levels and led to enhanced N-methyl-D-aspartate receptor (NMDAR) mediated responses (Depoortère et al., 2005). Behavioural assessment revealed that this compound might possess antipsychotic properties. Firstly, SSR504734 attenuated hyperlocomotion in response to NMDAR blockade by the non-competitive antagonists MK-801 (Depoortère et al., 2005) and PCP (Singer et al., in preparation) which has been linked to beneficial effects against negative and cognitive symptoms of schizophrenia according to the glutamate hypothesis of schizophrenia (Javitt, 1987, 2007, Javitt and Zukin 1991). Secondly, SSR504734 reversed impaired social recognition and motor hyper-sensitivity to amphetamine in rats neonatally treated with PCP (Depoortère et al., 2005), a commonly used neurodevelopmental model of schizophrenia (Weinberger, 1987; Lieberman et al, 1997). Hence, SSR504734 does not only counter the acute effect of NMDAR blockade but also improves long-term behavioural abnormalities associated with schizophrenia.

Despite the limitation of the dopamine hypothesis of schizophrenia (Carlsson 1988) to explain the negative and cognitive symptoms of the disease it has had heuristic value in explaining the positive symptoms and in detecting efficient pharmacotherapy against this symptom class. Dopamine agonists are therefore widely used to model endophenotypes of schizophrenia emphasizing the positive symptoms including PPI disruption and hyperlocomotion (e.g. Arguello Gogos 2006). Our most recent data showed that SSR504734 unexpectedly exacerbated the motor effects of dopamine agonists, namely it enhanced the motor-stimulant effect of the dopamine releaser amphetamine and potentiated the motor-depressant effect of the direct dopamine agonist apomorphine, without affecting locomotor activity per se (Singer et al., in preparation). These results therefore raise the important question whether the potential beneficial effects of GlyT1 blockade against negative and cognitive symptoms of schizophrenia might be compromised by an exacerbation of the positive symptoms, which would have major implications concerning the proposed therapeutic use of GlyT1 inhibitors as antipsychotic drugs.

Here, we evaluated the effect of SSR504734 on the expression of prepulse inhibition (PPI) of the acoustic startle response. PPI is defined as the attenuation of the response to a startling stimulus (pulse) that is briefly preceded by a stimulus of subthreshold intensity (prepulse) (Graham 1975) and represents an operational measure of sensorimotor gating (Braff and Geyer 1990). PPI deficits have been consistently reported in acute and chronic schizophrenia patients (Braff et al., 1992, 2001) and although PPI impairment is not unique to schizophrenia (Braff et al, 2001; Castellanos et al, 1996; Swerdlow et al, 1995), the sensitivity of PPI to dopaminergic manipulations is consistent with the dopamine hypothesis of schizophrenia. PPI is reliably disrupted by apomorphine which can be antagonized by antipsychotic drugs (Swerdlow et al, 1994) and is therefore considered as an animal correlate of the positive symptom of schizophrenia (Swerdlow and Geyer, 1998). We therefore tested SSR504734’s effect on PPI on its own and examined its potential to exacerbate apomorphine induced
PPI disruption. The outcome is expected to provide important novel additions to the evaluation of GlyT1 inhibitors as potential antipsychotic drugs.

METHODS

Subjects
Two cohorts of experimentally naïve C57BL6 male mice were used. The mice were bred in a specific-pathogen free (SPF) breeding facility (ETH Zurich Laboratory of Behavioural Neurobiology, Schwerzenbach, Switzerland). They were weaned at postnatal day 21, and then kept in groups of 3-5 in Makrolon® Type-III cages (Techniplast, Milan, Italy). They were maintained in a temperature (21°C) and humidity (55%) controlled vivarium under a 12:12h reversed light-dark cycle (lights on at 1900 hr) and had ad libitum access to food and water throughout. Behavioural testing took place in the dark phase of the cycle, and commenced when the animals were 12 weeks old. Mice from cohort 1 were used in experiments 1, 2 and 4 and mice and mice from cohort 2 were used in experiment 3, respectively.

The experimental manipulations and procedures described here had been previously approved by the Swiss Cantonal Veterinary Office, conforming to the ethical standards required by the Swiss Act and Ordinance on Animal Protection and the European Council Directive 86/609/EEC.

Drugs
All drug solutions were freshly prepared on the day of testing. SSR-504734 (SSR, obtained as a gift from Sanofi-Synthelabo, France) was suspended in distilled water containing 5% Tween 80 to obtain the required doses: 3, 10, and 30 mg/kg. Apomorphine HCl (APO, obtained from Sigma-Aldrich, Germany) was dissolved in 1% sterile ascorbic acid (VitC, pH 3.2) to achieve the desired concentrations of 1 and 1.5 mg/kg in experiments 2 and 4, respectively. SSR was injected via the intraperitoneal route, APO was administered subcutaneously. The volume of administration was 10 ml/kg body weight for SSR and 5 ml/kg for APO, respectively. APO and SSR were administered 5 and 30 min before testing, respectively.

Apparatus
The apparatus consisted of two acoustic startle chambers for mice (SR-LAB, San Diego Instruments, San Diego, CA, USA). Each startle chamber comprised a non-restrictive cylindrical enclosure made of clear Plexiglas attached horizontally on a mobile platform, which was in turn resting on a solid base inside a sound-attenuated isolation cubicle. A high-frequency loudspeaker mounted directly above the animal enclosure inside each cubicle produced a continuous background noise of 65 dBA and the various acoustic stimuli in the form of white noise. Vibrations of the Plexiglas enclosure caused by the whole-body startle response of the animal were converted into analog signals by a piezoelectric unit attached to the platform. These signals were digitized and stored by a computer. A total of 130
readings were taken at 0.5-ms intervals (i.e. spanning across 65 ms), starting at the onset of the startle stimulus in pulse-alone and prepulse-plus-pulse trials, and at the onset of the prepulse stimulus in prepulse-alone trials. The average amplitude over the 65 ms was used to determine the stimulus reactivity. The sensitivity of the stabilimeter was routinely calibrated to ensure consistency between chambers and across sessions.

**Procedure**

Procedures and test parameters have been fully described before (Yee et al., 2004a,b). Briefly, each session began with a 2 min acclimatization period followed by three blocks of discrete test trials. The first block consisted of six consecutive pulse-alone trials in order to stabilize the animals’ startle reactivity. In the intermediate block, the animals were presented with twelve series of discrete test trials comprising a mixture of four types of trials. These included pulse-alone trials, prepulse-plus-pulse trials of each of five possible prepulse intensities, prepulse-alone trials of each of the five intensities, and no-stimulus trials (i.e. background alone). Each series consisted of one trial of each trials type presented in a pseudo-randomized order. This was followed by a final block of six consecutive pulse-alone trials. The interval between successive trials was variable with a mean of 15 s (ranging from 10 to 20 s). The pulse stimulus was a 40 ms pulse of white noise at 120 dBA. Prepulses were 20 ms bursts of white noise at one of five intensities: 69, 73, 77, 81, and 85 dBA, which corresponded to 4, 8, 12, 16, and 20 dBA above background (65 dBA). The stimulus onset asynchrony (SOA) of the prepulse and pulse stimuli on prepulse-plus-pulse trials was 100 ms. A reduction of startle magnitude in prepulse-plus-pulse trials relative to that in pulse-alone trials constitutes the PPI effect. The expression of PPI was indexed as percentage for each of the five prepulse intensities by the formulae: \[(pulse-alone)-(prepulse-plus-pulse)]/(pulse-alone)*100\%\]. Habituation to the startle reaction was assessed by a comparison between the reactivity obtained on the first and the last block of six consecutive pulse-alone trials.

**Data analysis**

All data were subjected to parametric analysis of variance (ANOVA) with the between-subject factor drug and the within-subject factors blocks and prepulse. The ANOVAs were performed using SPSS® for Windows (Release 13.0) implemented on a personal computer running the Microsoft Window XP Professional SP2 operating system. Data interpretation was further assisted by Fisher’s least significant difference (LSD) post hoc comparisons to determine the locus of the significant effects. A critical level of p < 0.05 was set for all statistical analyses.

**RESULTS**

**Dose response analysis of SSR504734 on PPI**

The influence of SSR504734 on the expression of PPI was evaluated by a dose response analysis including 3, 10 and 30 mg/kg SSR in comparison to vehicle solution (5% Tween).
**Reactivity to the pulse stimulus alone:** SSR treatment did not affect the animals’ startle reaction to the pulse stimulus when it was presented in pulse-alone trials. This impression was supported by the absence of a significant main effect of drug (F<1) in a one-way ANOVA of the reactivity scores obtained across the 12 pulse-alone trials presented in the intermediate block of trials. The mean reactivity scores are depicted in Table 1.

**Reactivity to the prepulse stimuli alone:** Reactivity to the prepulse stimulus presented in prepulse-alone trials monotonically increased with increasing prepulse intensity which was equally seen in all drug conditions (Table 1). This was in agreement with a 4 x 5 (drug x prepulse) ANOVA of the reactivity scores which yielded a significant main effect of prepulse [F(4,80)=11.34, p<0.001]. Neither the main effect of drug nor its interaction with prepulse attained statistical significance (all F’s <1). Likewise, SSR did not affect baseline reactivity obtained in the no-stimulus trials in which no discrete stimulus other than the background noise was presented (F<1).

**Within-session startle habituation:** Startle habituation was evaluated by comparing the animals’ startle reactivity from the first to the last block of pulse-alone trials. There was no indication that the startle reaction habituated within the period of testing which was equally seen in all drug conditions. Data were analyzed by a 4 x 2 (drug x blocks) ANOVA of the reactivity scores which yielded no significant effects (all F’s <1). The mean reactivity scores are depicted in Table 1.

<table>
<thead>
<tr>
<th></th>
<th>Vehicle (n=6)</th>
<th>3mg/kg (n=6)</th>
<th>10mg/kg (n=6)</th>
<th>30mg/kg (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulse-alone trials (120dB A)</td>
<td>142.54 ± 43.73</td>
<td>139.57 ± 44.31</td>
<td>146.51 ± 26.39</td>
<td>132.85 ± 14.62</td>
</tr>
<tr>
<td>Prepulse-alone trials:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No-stimulus*</td>
<td>7.57 ± 1.86</td>
<td>8.53 ± 2.45</td>
<td>10.63 ± 2.29</td>
<td>6.28 ± 1.79</td>
</tr>
<tr>
<td>69dB A</td>
<td>7.58 ± 1.87</td>
<td>8.46 ± 2.14</td>
<td>9.81 ± 2.61</td>
<td>6.10 ± 1.65</td>
</tr>
<tr>
<td>73dB A</td>
<td>8.43 ± 2.13</td>
<td>8.74 ± 2.54</td>
<td>10.88 ± 3.64</td>
<td>7.33 ± 1.73</td>
</tr>
<tr>
<td>77dB A</td>
<td>9.10 ± 1.66</td>
<td>8.82 ± 2.42</td>
<td>10.67 ± 3.39</td>
<td>7.93 ± 1.38</td>
</tr>
<tr>
<td>81dB A</td>
<td>9.68 ± 1.60</td>
<td>9.28 ± 2.23</td>
<td>11.88 ± 2.74</td>
<td>10.00 ± 2.64</td>
</tr>
<tr>
<td>88dB A</td>
<td>11.86 ± 2.07</td>
<td>11.13 ± 1.56</td>
<td>15.38 ± 2.29</td>
<td>14.29 ± 3.79</td>
</tr>
</tbody>
</table>

**Startle habituation:**

<table>
<thead>
<tr>
<th></th>
<th>Vehicle (n=6)</th>
<th>3mg/kg (n=6)</th>
<th>10mg/kg (n=6)</th>
<th>30mg/kg (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First block</td>
<td>116.44 ± 31.91</td>
<td>84.83 ± 21.44</td>
<td>126.28 ± 27.12</td>
<td>134.36 ± 45.58</td>
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<tr>
<td>Final block</td>
<td>167.72 ± 60.50</td>
<td>120.03 ± 40.51</td>
<td>118.33 ± 33.96</td>
<td>125.86 ± 10.88</td>
</tr>
</tbody>
</table>

**Table 1:** Mean reactivity obtained on pulse-alone and prepulse-alone trials presented in the middle portion of the test – i.e., between the first and last blocks of pulse-alone trials. The mean reactivity obtained in the first and last blocks of pulse-alone trials is presented in the last row of the table. All values refer to biased mean ± S.E.M (in arbitrary units) derived from the appropriate overall ANOVA. “no-stimulus” refer to trials in which no discrete stimulus except the background noise (at 69dBA) was presented.
Percent prepulse inhibition (%PPI): Since SSR did not exert any effect on the startle reaction per se we followed the convention of indexing PPI in percent prepulse inhibition (%PPI). As expected, the PPI magnitude increased with increasing prepulse intensity. As illustrated in Figure 1, SSR was found to affect PPI in a biphasic way. 3 and 30 mg/kg SSR increased PPI as a function of prepulse intensity. In contrast, 10mg/kg SSR led to a weak but consistent decrease in %PPI as compared to vehicle-control (Figure 1). This gave rise to a significant main effect of prepulse [F(4,80)=36.14, p < 0.01] and its interaction with drug [F(12,80)=2.21, p < 0.05]. Fisher’s LSD comparisons separately conducted on each pre-pulse condition revealed that 3 mg/kg SSR significantly increased PPI at 81 dB\text{A} and 30 mg/kg SSR at 81 and 85 dB\text{A} (all p’s < 0.05). On the other hand, 10 mg/kg never significantly affected the %PPI.

Figure 1: Dose response analysis of SSR504734 on the expression of PPI
The magnitude of PPI was indexed by percentage of prepulse inhibition (%PPI). SSR504734 affected PPI in a bi-phasic way. PPI was enhanced at doses of 3 and 30 mg/kg with increasing prepulse intensity but not at the intermediate dose of 10 mg/kg when PPI levels were, if anything reduced. These impressions were confirmed by a 4 x 5 (drug x prepulse) ANOVA yielding a significant drug by prepulse interaction (p<0.05). Additional post hoc comparisons separately conducted on each pre-pulse level revealed that 3 mg/kg SSR significantly increased PPI at a prepulse intensity of 81 dB\text{A}, and 30 mg/kg SSR at 81 and 85 dB\text{A} (*, p < 0.05) in comparison to vehicle control. All values refer to the mean +/- SEM.

Dose response analysis of SSR504734 on the PPI disruptive effect of apomorphine
We have previously shown that SSR potentiated the motor-depressant effect of apomorphine in a dose dependent manner (data in preparation). Here, we evaluated whether SSR would also enhance the PPI disruptive effect of apomorphine by testing SSR (0, 3, 10 or 30mg/kg) in combination with apomorphine (0 or 1 mg/kg).

Reactivity to the pulse stimulus alone: The startle reactivity to the pulse-alone trials presented in the intermediate block of trials did not differ amongst drug conditions. This impression was consistent with the absence of a significant main effect of drug (F<1) in a one-way ANOVA. The mean reactivity scores are depicted in Table 2.

Reactivity to the prepulse stimuli alone: The reactivity to the prepulse stimulus monotonically increased with increasing prepulse intensity which was equally seen in all drug conditions (see Table 2). A 5 x 5 (drug x prepulse) ANOVA revealed only a significant main effect of prepulse [F(4,176)=15.74, p < 0.001] confirming our impressions. Similarly, baseline reactivity in no-stimulus
trials did not significantly differ between groups (F<0.5).

Within-session startle habituation: None of the tested groups showed any habituation of the startle reaction from the first to the last block of pulse-alone trials (see Table 2). This was consistent with the absence of any statistically significant effects or interactions in a 5 × 2 (drug x blocks) ANOVA.

<table>
<thead>
<tr>
<th>Pulse-alone trials (120dB)</th>
<th>Vehicle (n=10)</th>
<th>0 mg/kg SSR (n=10)</th>
<th>0.75mg/kg s.c. Apomorphine 3 mg/kg SSR (n=8)</th>
<th>10 mg/kg SSR (n=10)</th>
<th>30 mg/kg SSR (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>147.11 ± 24.16</td>
<td>130.64 ± 18.32</td>
<td>132.42 ± 27.94</td>
<td>134.47 ± 15.82</td>
<td>121.24 ± 22.19</td>
<td></td>
</tr>
</tbody>
</table>

**Prepulse-alone trials:**

| No-stimulus | 6.83 ± 1.85 | 8.73 ± 1.84 | 8.89 ± 1.84 | 9.17 ± 1.16 | 7.12 ± 2.11 |
| 69dB | 7.18 ± 1.81 | 8.44 ± 1.79 | 9.34 ± 1.68 | 8.88 ± 1.05 | 7.61 ± 2.16 |
| 73dB | 8.05 ± 1.69 | 10.04 ± 1.66 | 9.63 ± 1.77 | 10.04 ± 1.16 | 9.00 ± 2.60 |
| 77dB | 9.74 ± 1.94 | 9.72 ± 1.67 | 10.81 ± 1.69 | 10.35 ± 1.26 | 9.56 ± 2.36 |
| 81dB | 8.05 ± 1.69 | 10.04 ± 1.66 | 9.63 ± 1.77 | 10.04 ± 1.16 | 9.00 ± 2.60 |
| 88dB | 9.01 ± 1.70 | 10.81 ± 1.71 | 11.64 ± 1.67 | 11.30 ± 1.30 | 10.88 ± 2.69 |

**Startle habituation:**

| First block | 165.81 ± 38.92 | 127.80 ± 21.19 | 131.49 ± 23.48 | 131.60 ± 26.01 | 121.97 ± 37.62 |
| Final block | 118.90 ± 19.63 | 157.48 ± 32.14 | 157.13 ± 29.69 | 180.88 ± 39.18 | 124.25 ± 23.82 |

**Table 2:** Reactivity scores obtained on the pulse-alone, prepulse-alone and no-stimulus trials presented in the intermediate block of trials. The reactivity scores obtained in the first and last blocks of pulse-alone trials are presented in the last row of the table. All values refer to biased mean ± S.E.M (in arbitrary units).

Percent prepulse inhibition (%PPI): The expression of prepulse inhibition as indexed by %PPI is illustrated in Figure 2. Pre-treatment with APO moderately decreased PPI which was potentiated by co-treatment with 3 and 10mg/kg SSR but not with 30 mg/kg. A 4 × 5 (drug x prepulse) ANOVA yielded a significant main effect of prepulse [F(4,172)=84.94, p < 0.001] and drug [F(4,43)=2.80, p < 0.05] and their interaction F(16,172)=1.87, p < 0.05]. Fisher’s LSD Post hoc comparisons revealed that the Veh-Veh group significantly differed from the APO-SSR(3mg) (p<0.05) and the APO-SSR(10mg) groups (p<0.005). There was only a non-significant trend (p = 0.094) towards a PPI reduction in the Veh-APO group.
Appendix V

Figure 2: Dose response analysis of SSR504724 on the PPI-disruptive effect of apomorphine
SSR504734 dose-dependently enhanced the PPI-disruptive effect of apomorphine (1 mg/kg). This was confirmed by a 5 x 5 (drug x prepulse) ANOVA of %PPI which yielded a significant main effect of drug (p <0.05). Fischer’s LSD post hoc comparison revealed that PPI was significantly reduced (*, p<0.05) in the SSR-3mg-APO and SSR-10mg-APO conditions in comparison to the Veh-Veh condition. All values refer to the mean +/- SEM.

Effect of 10mg/kg SSR504734 on PPI

Amongst the tested doses of SSR in experiment 2, 10 mg/kg most efficiently potentiated the PPI-disruptive effect of apomorphine. Although not supported statistically, the same dose also appeared to decrease PPI on its own in experiment 1, which would provide a parsimonious explanation for potentiation of the PPI-disruptive effect of apomorphine. We therefore re-tested this specific dose of SSR on PPI by increasing the sample size to clarify whether PPI was indeed reduced or not by 10 mg/kg SSR.

Reactivity to the pulse stimulus alone: As seen before, 10mg/kg SSR did not alter the startle reactivity which was supported by a non-significant effect of drug [F(3,22)=2.73, p=0.11] in a one-way ANOVA. The mean reactivity scores are depicted in Table 3.

Reactivity to the prepulse stimuli alone: Similar to the first experiment, 10mg/kg SSR did not affect the reactivity to the prepulse stimulus (see Table 3). This was in agreement with a 2 x 5 (drug x prepulse) ANOVA which revealed only a significant main effect of prepulse [F(4,88) = 5.13, p<0.01] (Table 3). Likewise, the baseline reactivity was highly comparable between the two drug conditions (F<0.5).

Within-session startle habituation: Startle reactivity did not habituate from the first to the last blocks of pulse-alone trials. (Table 3). A 3 x 2 (drug x blocks) split-blot ANOVA failed to yield any statistically significant effects or interactions.

Percent prepulse inhibition (%PPI): As illustrated in Figure 3, SSR levels were highly comparable between the SSR and the vehicle conditions. This impression was supported by a 2 x 5 (drug x prepulse) ANOVA which yielded a highly significant main effect of prepulse [F(4,88) =37.60, p<0.001] but neither the main effect of drug nor its interaction with prepulse attained statistical significance (all F’s <1). This clear null-effect therefore provides strong evidence that SSR at a dose of 10 mg/kg does not decrease PPI.
Table 3: Reactivity scores obtained on the pulse-alone, prepulse-alone and no-stimulus trials presented in the intermediate block of trials. The reactivity scores obtained in the first and last blocks of pulse-alone trials are presented in the last row of the table. All values refer to biased mean ± S.E.M (in arbitrary units).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Vehicle (n=12)</th>
<th>SSR5047343 10mg/kg (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulse-alone trials (120dB)</td>
<td>106.92 ± 21.56</td>
<td>164.80 ± 27.64</td>
</tr>
<tr>
<td>Prepulse-alone trials:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No-stimulus</td>
<td>9.99 ± 1.42</td>
<td>9.18 ± 1.86</td>
</tr>
<tr>
<td>69dB_A</td>
<td>8.13 ± 1.23</td>
<td>8.01 ± 1.64</td>
</tr>
<tr>
<td>73dB_A</td>
<td>9.18 ± 1.35</td>
<td>9.08 ± 1.73</td>
</tr>
<tr>
<td>77dB_A</td>
<td>10.45 ± 2.47</td>
<td>9.82 ± 2.43</td>
</tr>
<tr>
<td>81dB_A</td>
<td>10.31 ± 1.87</td>
<td>9.63 ± 2.03</td>
</tr>
<tr>
<td>88dB_A</td>
<td>12.67 ± 2.55</td>
<td>10.13 ± 1.55</td>
</tr>
<tr>
<td>Startle habituation:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>First block</td>
<td>113.31 ± 38.00</td>
<td>164.72 ± 32.10</td>
</tr>
<tr>
<td>Final block</td>
<td>90.78 ± 19.50</td>
<td>134.08 ± 30.75</td>
</tr>
</tbody>
</table>

Figure 3: The effect of 10 mg/kg SSR504734 on PPI
Re-assessment of 10 mg/kg SSR revealed a clear null-effect indicating that PPI was not reduced by this specific dose. No significant effects or interactions emerged in a 2 x 5 (drug x prepulse) ANOVA of %PPI. All values refer to the mean +/- SEM.
Variability between experiments:
Because the mice used here derived from a different cohort than those used in experiments 1, 2 and 4, a comparison with the first experiment may be confounded by variability occurring between cohorts/experiments. In order to statistically control for such variability we analyzed the vehicle-control and the 10 mg/kg SSR504734 conditions of the two experiments by introducing the additional between-subject factor cohort. However, no such confound was revealed across all measures (data not shown).

Effect of 10mg/kg SSR504734 on the PPI disruptive effect of apomorphine
Experiment 2 revealed that SSR potentiated the PPI-disruptive effect of apomorphine. However, this was observed using a dose of apomorphine that did not significantly decrease PPI on its own. We therefore went on to test whether SSR (10 mg/kg) would also further reduce PPI when selecting a dose of apomorphine (1.5 mg/kg) expected to significantly disrupt PPI on its own.

Reactivity to the pulse stimulus alone:
Consistent with our previous findings, the startle reactivity did not significantly differ between groups (F<1). The obtained mean reactivity scores are depicted in Table 4.

Reactivity to the prepulse stimuli alone:
The reactivity to the prepulse stimulus generally increased as a function of prepulse intensity, which was most pronounced in the Veh-APO group. This gave rise to a highly significant main effect of prepulse [F(4,80) = 11.03, p<0.001] and a near-significant drug by prepulse interaction [F(8,80) = 2.05, p=0.05] in a 3 x 5 (drug x prepulse) ANOVA (Table 4). Baseline reactivity in no-stimulus trials did not significantly differ amongst drug conditions (F<1).

Within-session startle habituation:
Again, startle reactivity did not habituate within the test period irrespective of drug condition. A 3 x 2 (drug x blocks) ANOVA revealed no significant effects or interactions (all F’s <1).

Percent prepulse inhibition (%PPI):
As expected, the selected dose of apomorphine substantially decreased PPI which was further decreased by co-treatment with 10mg/kg SSR (Figure 4). These observations were supported by a 2 x 5 (drug x prepulse) ANOVA which revealed a highly significant effect of drug [F(2,20) = 14.08, p<0.001] and prepulse [F(4,80) = 16.54, p<0.001]. Fisher’s LSD Post hoc comparisons revealed that %PPI was significantly reduced in the Veh-APO (p<0.05) and the SSR(10mg)-APO conditions (p<0.001) as compared to vehicle-control. Furthermore, PPI was significantly lower in SSR(10mg)-APO than in SSR(10mg)-APO condition (p < 0.005).
<table>
<thead>
<tr>
<th>Vehicle</th>
<th>0.75 mg/kg s.c.</th>
<th>0 mg/kg 3 mg/kg SSR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulse-alone</td>
<td>(n=7)</td>
<td>(n=8)</td>
</tr>
<tr>
<td>(120dB A)</td>
<td>113.79 ± 25.53</td>
<td>75.98 ± 15.55</td>
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<tr>
<td>Prepulse-alone trials:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No-stimulus</td>
<td>9.96 ± 1.76</td>
<td>11.93 ± 1.07</td>
</tr>
<tr>
<td>69dB A</td>
<td>9.68 ± 1.34</td>
<td>10.99 ± 1.06</td>
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<tr>
<td>73dB A</td>
<td>9.70 ± 1.46</td>
<td>12.97 ± 1.58</td>
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<td>77dB A</td>
<td>9.93 ± 1.72</td>
<td>14.58 ± 1.98</td>
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<td>81dB A</td>
<td>10.69 ± 1.47</td>
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<td>88dB A</td>
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<tr>
<td>First block</td>
<td>193.21 ± 47.32</td>
<td>87.21 ± 15.03</td>
</tr>
<tr>
<td>Final block</td>
<td>96.81 ± 32.45</td>
<td>96.19 ± 21.53</td>
</tr>
</tbody>
</table>

Table 4: Reactivity scores obtained on the pulse-alone, prepulse-alone and no-stimulus trials presented in the intermediate block of trials. The reactivity scores obtained in the first and last blocks of pulse-alone trials are presented in the last row of the table. All values refer to biased mean ± S.E.M (in arbitrary units).

Figure 4: The effect of 10 mg/kg SSR504734 on apomorphine induced PPI disruption.
SSR504734 further reduced PPI when disrupted by a more efficient dose of apomorphine (1.5 mg/kg). This was supported by a 2 x 5 (drug x prepulse) ANOVA of %PPI which yielded a significant main effect of drug (p < 0.05). * denotes that PPI was significantly lower than in the Veh-Veh condition and # denotes a significant difference between the Veh-APO and the SSR-APO condition based on Fisher’s LSD post hoc comparison (p < 0.05). All values refer to the mean +/- SEM.
DISCUSSION
Our results revealed that the GlyT1 inhibitor SSR504734 moderately enhanced basal PPI but exacerbated the PPI-disruptive effect of apomorphine. At the same time, SSR504734 did not alter the startle reaction to the pulse stimulus in pulse-alone trials indicating that any PPI effect of the drug cannot be attributed to changes in startle reactivity. The absence of an effect on startle reactivity is also in agreement with the observation that SSR504734 did not affect anxiety/fear related behaviour in the elevated plus maze test (Singer et al., in preparation). Similarly, SSR504734 did not influence the direct reaction to the prepulse stimulus suggesting that the observed PPI effects of the drug are not explicable by potential changes in the animals’ perception and processing of the prepulse, which is essential to the demonstration of the PPI effect (see Yee et al., 2004b). Furthermore, SSR504734 did not alter baseline reactivity in no-stimulus trials which is consistent with the null-effect of SSR504734 on locomotor activity in the open field paradigm (Singer et al., in preparation). Finally, the animals’ startle reactivity was generally stable across the period of testing excluding a potential confound of the PPI results by an effect of SSR504734 on startle habituation/sensitization. Hence, these findings strongly indicate that SSR504734 directly interferes with the neural elements that contribute to the mediation and/or regulation of PPI.

Dose response analysis of SSR504734 revealed a U-shape dose–response relationship on PPI. SSR504734 enhanced PPI with increasing prepulse intensity at doses of 3 and 30 mg/kg but not at the intermediate dose of 10 mg/kg at which PPI appeared to be reduced, although this was not supported statistically. Re-assessment of this specific dose using higher N revealed a clear null-effect providing convincing evidence that PPI is not reduced by this dose. The dependency of the PPI enhancement on the prepulse intensity is indicative of a subtle effect of the drug. It is unlikely to represent a ceiling effect because PPI can be substantially enhanced in the C57BL/6 mouse strain by typical and atypical antipsychotics (Ouagazzal et al., 2001). On the other hand, Depoortère et al. (2005) reported that SSR504734 increased PPI irrespective of prepulse intensity in DBA/J2 mice, a mouse strain with an innate PPI deficit indicating that SSR504734 may be more efficient to improve PPI impairments than further increase normal PPI.

The biphasic PPI enhancement exhibited by SSR504734 conflicts with a recent study by Lipina et al. (2005) showing that the GlyT1 inhibitor ALX-5407 dose-dependently decreased PPI in C57BL/6 mice. One possible explanation for this discrepancy may be that there are differences in the mode of action of the two GlyT1 inhibitors since SSR504734 blocks GlyT1 reversibly (Depoortère et al. 2005) whereas the binding of ALX-5407 is essentially irreversible (Atkinson et al., 2001). Lipina et al. (2005) argued that the PPI-disruptive effect of ALX-5407 at higher doses might result from a reduction of NMDAR activation based on the observation that NMDAR currents were enhanced by low concentrations of the GlyT1 inhibitor CP-802079 but slightly reduced by higher concentrations of CP-802079 (Martina et al. 2004). This is unlikely to explain the U-shape dose-response curve of SSR504734 indicating that modulation of NMDAR function may not be sole mechanism underlying
the PPI effects of GlyT1 inhibitors. The involvement of other neural processes is also supported by the finding that impaired PPI following blockade of NMDAR by MK-801 was exacerbated by heterozygous knockout of GlyT1 despite the fact that NMDAR mediated transmission was enhanced in these mutant mice (Tsai et al., 2004). SSR504734 was also found to increase the extra-cellular dopamine levels in the prefrontal cortex (PFC) (Deportère et al., 2005) and facilitated dopamine release in the nucleus accumbens (NAC) evoked by electrical stimulation of the basolateral amygdala (BLA) (Leonetti et al., 2006). Given the close involvement of the dopaminergic system in the regulation of PPI (e.g. Geyer et al., 2003) it may follow that the more direct impact of SSR504734 on NMDAR function can subsequently lead to dopaminergic changes which contribute to the PPI effects of the drug. Direct evidence for a mechanistic link between the glutamatergic and dopaminergic effects of SSR54734 is provided by the finding that the local infusion of the NMDAR antagonist APV into NAC hindered SSR504734’s facilitatory effect on dopamine release evoked by BLA stimulation (Leonetti et al., 2006). Here, we found strong evidence that SSR504734 exacerbated the PPI-disruptive effect of apomorphine. Firstly, SSR504734 disrupted PPI when co-administered with a dose of apomorphine (1 mg/kg) that did not significantly disrupt PPI on its own. Secondly, SSR504734 potentiated PPI disruption induced by a more efficient dose of apomorphine (1.5 mg/kg). The PPI-disruptive effect of apomorphine is believed to be mediated at least partly by increased dopamine activity in the NAC (Geyer et a., 2003). Furthermore, it has been shown that low doses of apomorphine that normally do not decrease PPI led to a substantial PPI disruption in rats with ‘supersensitive’ NAC dopamine receptors induced by intracerebral infusion of the neurotoxin 6-hydroxydopamine (6-OHDA) (Swerdlow et al., 1986). Most recently, we found that SSR504734 also enhanced the motor-stimulant effect of amphetamine as well as the motor-depressant effect of apomorphine without affecting locomotor activity per se (Singer et al., in preparation). Since excessive subcortical dopaminergic transmission largely contributes to the motor effects of dopamine agonists, one may suspect that SSR504734 leads to a general hyperresponsivity of the subcortical dopamine system which in turn causes enhanced behavioural response to dopaminergic stimulation but does not affect such behaviours under homeostatic conditions. This is consistent with the observation that SSR504734 facilitated NAC dopamine release when activated via electrical stimulation of the BSA but did not alter basal NAC dopamine levels. On the other hand, increased basal dopamine levels in the PFC may be responsible for the PPI enhancing effect of SS504734 in the absence of dopaminergic stimulation. This is supported by the finding that PPI is decreased under conditions of reduced PFC dopamine activity such as local dopamine depletion by infusion of 6-OHDA (Koch and Bubser 1994) or blockade of PFC dopamine receptors (Ellenbroek et al. 1996). However, a further dissection of SSR504734’s effects in different brain structures would necessitate additional experiments with local intracerebral drug infusion.

Dopamine agonist induced PPI disruption and hyperlocomotion are believed to mimic the positive symptoms of schizophrenia (Arguello and Gogos 2006). The potentiation of these effects by
SSR50473 therefore raises the question whether SSR504734 might exacerbate positive symptoms of schizophrenia which is in sharp contrast to the drug’s antipsychotic profile in glutamatergic and neurodevelopmental models of schizophrenia (Depoortère et al., 2005). According to the dopamine hypothesis of schizophrenia (Carlsson 1988), the positive symptoms are believed to result from a pathological dopaminergic dysfunction causing a decrease in dopamine activity in prefrontal brain regions, along with excessive subcortical dopaminergic transmission. This fundamentally differs from the dopaminergic changes induced by SSR504734, namely elevated dopamine levels in the PFC and no change in accumbal basal dopamine levels (Deportère et al., 2005, Leonetti et al., 2006). Moreover, SSR504734 was found to attenuate amphetamine hypersensitivity induced by PCP treatment in the neonatal stage (Depoortère et al., 2005), which is believed to mimic the dopaminergic imbalances present in schizophrenia (see Weinberger 1986). However, in the same experiment there was a clear tendency that SSR504734 potentiated the reaction to amphetamine in the control animals receiving no PCP pre-treatment which is in keeping with the dose-dependent enhancement of amphetamine induced hyperlocomotion recently found in our lab (Singer et al., in preparation). Hence, this pattern of results may suggest that SSR504734 stabilizes prefrontal dopaminergic dysregulations associated with schizophrenia but at the same time enhances the response to a sudden, acute activation of the subcortical dopamine system. Thus, the impact of GlyT1 inhibition on positive symptoms of schizophrenia may also depend on whether the disease is in the acute or chronic phase. With respect to the proposed therapeutic use of GlyT1 inhibitors as potential antipsychotic drugs (see for a review Lechner et al., 2006) the relationship between GlyT1 function and the emergence of positive symptoms therefore warrants further evaluations.

Alternatively, potential undesirable effects of GlyT1 blockade on positive symptoms may be countered by co-treatment with conventional antipsychotics which efficiently block excessive dopaminergic transmission. In turn, GlyT1 inhibitors might enhance the therapeutic efficacy of antipsychotics against negative and cognitive symptoms. Support for this hypothesis is provided by clinical studies showing that the GlyT1 inhibitor sarcosine administered as add-on to antipsychotic treatment in schizophrenic patients significantly improved positive, negative and cognitive symptoms in comparison to antipsychotic treatment alone (Tsai et al., 2004; Heresco-Levy U 2006).

CONCLUSIONS

Increasing evidence suggests that pharmacological blockade of GlyT1 provides potential novel avenues in the treatment of schizophrenia with enhanced therapeutic efficacy against negative and cognitive symptoms. The present finding highlights that the beneficial effects of GlyT1 inhibitors might be compromised by an exacerbation of the positive symptoms due to a hyper-responsivity of the dopamine system. We therefore propose that combined treatment of GlyT1 inhibitors and classical antipsychotics may enhance therapeutic efficacy against different classes of symptoms than either treatment alone.
REFERENCES


Graham F.K., 1975. The more or less startling effects of weak prestimulation. Psychophysiology 12, 238–248.


