Doctoral Thesis

Forebrain neuronal glycine transporter 1 AS a neurocognitive modulator
behavioural analysis of an engineered mouse model

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FOREBRAIN NEURONAL GLYCINE TRANSPORTER 1 AS A NEUROCOGNITIVE MODULATOR: BEHAVIOURAL ANALYSIS OF AN ENGINEERED MOUSE MODEL

A dissertation submitted to

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for the degree of
Doctor of Sciences

presented by

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Efficient N-methyl-D-aspartate receptor (NMDAR) signalling requires binding of the co-agonist glycine at the NR1 subunit glycine-B site. The availability of glycine at the synaptic cleft is tightly regulated by the glycine transporter-1 (GlyT1), which is expressed in both neurons and astrocytes. Pharmacological or genetic disruption of GlyT1-mediated glycine-reuptake near excitatory synapses is believed to enhance NMDAR function and may consequently enhance cognitive performance. GlyT1 is therefore regarded as a potential pharmacological target to ameliorate memory deficits in humans, including the schizophrenia-related cognitive symptoms believed to stem from NMDAR hypofunction. Genetic deletion of GlyT1 in forebrain neurons of mice appears to be associated with enhanced memory performance across multiple cognitive tasks. However, the interpretation of these findings is open to question on several accounts, which have not been satisfactorily addressed until now. This thesis therefore aims to provide an in-depth comparative analysis of the behavioural phenotypes expressed by these forebrain neuronal specific GlyT1 disrupted mutant mice and to test the stability and robustness of the enhanced cognitive phenotype previously described. The first series of experiments provides novel qualifications on the sex-dependent nature of the Pavlovian phenotype shown by the mutant mice (Chapter 3). More specifically, gender critically determines the magnitude and direction of Pavlovian conditioning in both aversive and appetitive conditioning paradigms. These findings are relevant to psychiatric illnesses, such as schizophrenia, that show notable sex differences with regard to symptomatology, onset, and course of the disease. In Chapter 2 it was investigated whether the enhanced Pavlovian (aversive) conditioning phenotype observed previously might be modified by age, since lifelong elevation of glycine synaptic levels might result in excitotoxic effects and consequently cell death. Although, both adult and aged mutants exhibited enhanced Pavlovian conditioning, the temporal expression of this phenotype (in terms of within-session extinction) was notably absent in the aged mutants. Furthermore, a noticeable increase in adult hippocampus neurogenesis was seen in the mutant mice, suggesting a possible neuroanatomical contribution to the modulation of the cognitive phenotype. The experiments carried out in a third study (Chapter 4) examined the notion that enhanced Pavlovian conditioning exhibited by the forebrain neuronal specific GlyT1 mutant mice stemmed from alterations in the selectivity of learning as opposed to a broad strengthening of the associative strength between the conditioned and unconditioned stimulus. The outcome suggests a bidirectional effect of forebrain neuronal GlyT1 deletion on Pavlovian associative learning: the conditioned response was stronger in the mutant mice when the conditioned stimulus was a good predictor of the unconditioned stimulus. Conversely, the conditioned response was
weaker when the conditioned stimulus-unconditioned stimulus (CS-US) contiguity or contingency was degraded. It appears that alterations in the selectivity of learning may account for the diverse phenotypes in the regulation of Pavlovian learning. These findings bear clear relevance to the proposed therapeutic potential of GlyT1-inhibiting drugs for schizophrenia-related cognitive deficits that might stem from an underlying lack of selectivity (or inflexibility) in learning. Finally, a last series of experiments examined the impact of forebrain neuronal GlyT1 deletion on working memory function by using a within-subject multi-paradigm approach (Chapter 5). A null effect of the manipulation was seen across three different working memory paradigms. The behavioural effects of the forebrain neuronal GlyT1 deletion thus appear to be restricted to associative learning. These negative outcomes clearly contrasted the improved working performance shown previously by mutant mice with GlyT1 deletion extending into cortical glial cells. These complementary results might thus point to a potential functional divergence between neuronal and glial GlyT1 populations in mediating cognitive function, with a more prominent role for glial-based GlyT1 in regulating working memory function. Hence, the present thesis has provided valuable novel insights into neuronal forebrain specific GlyT1 function and its contribution to memory. Furthermore, the data presented here demonstrate that an in-depth comprehension of the phenotypic expression of forebrain and neuronal GlyT1 deletion, or of any genetic manipulation, is an important step forward in the proof of concept, and constitute a critical component in the pre-clinical evaluation of potential drug targets.
Résumé

La bonne transduction du message nerveux via le récepteur N-methyl-D-aspartate (NMDAR) nécessite la fixation d’un co-agoniste, la glycine, sur le site glycine-B de la sous-unité NR1. La disponibilité de la glycine au niveau de la fente synaptique est régulée par le transporteur 1 de la glycine (GlyT1), exprimé par les neurones et les astrocytes. Un blocage de la recapture de la glycine via GlyT1 par une approche génétique ou pharmacologique est donc supposé augmenter la fonction des NMDAR et ainsi induire une amélioration des fonctions cognitives. Par conséquent GlyT1 est une cible pharmacologique potentielle pour l’amélioration des troubles mnésiques, comme par exemple ceux liés à la schizophrénie et supposés provenir d’une hypofonction des NMDAR. Ainsi une délétion génétique de GlyT1 dans les neurones du prosencéphale de souris est associée à une amélioration des performances mnésiques dans de nombreuses taches cognitives. Cependant l’origine de ces effets reste encore actuellement sujette à interprétation sur de nombreux points et nécessite des études plus approfondies. Les travaux présentés dans cette thèse avaient pour but de fournir une analyse poussée du phénotype comportemental exprimé par des souris mutantes ayant une perturbation des GlyT1 exprimés par les neurones du prosencéphales en testant la stabilité et la fiabilité des résultats précédemment obtenus. La première série d’expériences présentée ici met en évidence que le phénotype observé chez les souris mutant dans une tache de conditionnement de type "Pavlovien" est différent entre le male et la femelle (chapitre 2). Plus précisément, le genre est un déterminant majeur du sens des effets induits par la mutation ainsi que de leur ampleur, lors d’apprentissages Pavloviens aversifs mais aussi appétitifs. Cette découverte est particulièrement pertinente dans le cadre de l’étude de certaines pathologies psychiatriques comme la schizophrénie qui présentent des différences notables en fonction du sexe du patient comme par exemple, la symptomatologie, le moment d’apparition et le développement de la maladie. Les séries d’expériences présentées dans le chapitre 3 visent à déterminer si l’amélioration des performances lors d’un apprentissage Pavlovien (aversif) est modifiée par l’âge, puisque l’augmentation de la concentration en glycine au niveau synaptique tout au long de la vie de l’animal pourrait avoir un effet excito-toxique entrainant une perte neuronale. Cependant, les souris adultes et les souris âgées présentent une amélioration de conditionnement Pavlovien comparable, l’expression temporelle de ce phénotype (en termes d’extinction au cours d’une session) est notamment absente chez les souris mutantes âgées. De plus une augmentation de neurogénèse hippocampique est observable chez les souris mutantes, ce qui suggère une contribution possible de ces nouvelles cellules dans la modulation des phénotypes observé dans les taches d’apprentissage et de mémoire. Les expériences menées
lors d’une troisième étude (chapitre 4) visent à déterminer si l’amélioration de conditionnement pavlovien développée par les souris mutantes ne repose pas en fait sur une altération de la sélectivité de l’apprentissage plutôt que sur un renforcement de la force associative entre le stimulus conditionnel et le stimulus inconditionnel. Les résultats obtenus suggèrent un effet bidirectionnel de la délétion de GlyT1 dans les neurones du prosencéphale sur l’apprentissage associatif Pavlovien: la réponse conditionnée étant plus forte chez les souris mutantes lorsque le stimulus conditionnel prédit bien l’occurrence du stimulus inconditionnel, alors qu’à l’inverse la réponse conditionnée de ces même souris est plus faible quand la contiguïté temporelle ou la contingence entre stimulus conditionnel et stimulus inconditionnel sont dégradées. Il apparait donc qu’une altération de la sélectivité de l’apprentissage pourrait être en partie responsable des modifications des phénotypes Pavlovien observées. Ces travaux apportent donc des preuves claires vis-à-vis du potentiel thérapeutique de drogues permettant d’inhiber sélectivement les GlyT1 dans le cadre du traitement de certains des déficits cognitifs observables chez les patients schizophrènes pouvant résulter d’un déficit de flexibilité (ou rigidité comportementale) lors d’apprentissages. Enfin, une dernière série d’expérience examine l’effet sur la mémoire de travail de la délétion de GlyT1 dans les neurones du prosencéphale en utilisant pour cela une approche intra-sujets dans plusieurs paradigmes expérimentaux (chapitre 5). Ces expériences mettent en évidence une absence d’effet de la manipulation génétique dans trois paradigmes différents testant la mémoire de travail. Les effets comportementaux induits par la manipulation génétique étudiée semblent de plus restreints au domaine non spatial. Cette absence d’effet contraste avec l’amélioration de mémoire de travail observée précédemment chez des souris mutantes pour lesquelles la délétion de GlyT1 s’étendait aux cellules gliales corticales. Ces résultats complémentaires suggèrent donc qu’il pourrait exister une divergence fonctionnelle entre les GlyT1 exprimés par les neurones et ceux exprimés par les cellules gliales dans le contrôle des fonctions cognitives, les GlyT1 des cellules gliales pouvant être plus fortement impliqué dans la modulation des processus de mémoire de travail. Ainsi, la présente thèse fournie de nouvelles connaissances quand aux fonctions spécifiques des GlyT1 exprimés par les neurones du prosencéphale ainsi que sur leur rôle dans les processus mnésique. De plus, les données présentées démontrent qu’une compréhension approfondie, de l’expression phénotypique de la délétion en GlyT1 au niveau des neurones du prosencéphale, mais aussi pour n’importe quelle manipulation génétique, est un important pas en avant dans la validation d’un concept, et constitue un éléments majeur de l’évaluation préclinique d’une potentielle cible pharmacologique.
CHAPTER 1

General Introduction
This chapter chronicles the milestones in research that led to discovery that facilitated N-methyl-D-aspartate receptor (NMDAR) signalling and enhanced cognitive function could be achieved through increased glycine occupation on the NMDAR glycine-B site (See table 1). More specifically, it aims to give an overview of the findings showing that this could be accomplished via Glycine Transporter 1 (GlyT1) inhibition or genetic disruption. The significance of glycine’s modulation of NMDAR function is highlighted by studies utilizing glycine inhibitor drugs. In addition, the availability of GlyT1 conditional knockout mouse models has provided further understanding of GlyT disruption and its impact on cognition. In particular, the behavioural screening of a mouse model with a conditional GlyT1 knockout in forebrain neurons has yielded promising findings. However, at the same time it revealed several limitations that have not been satisfactorily addressed until present. Comprehensive phenotyping of this mouse line within an integrated behavioural framework is a prerequisite for understanding the cognitive aspects involved and/or affected by this manipulation. This would also provide the basis towards translating the experimental findings into therapeutic targets aimed specifically at these changes. The studies presented in this dissertation were designed towards such an approach, and this is elaborated further in the Thesis objectives and Thesis outline at the end of this Chapter.

**Glycine modulation of N-methyl-D-aspartate receptor (NMDAR) function**

Due to its strong implication in several brain disorders and its predominant role in various forms of synaptic plasticity, the NMDAR has been a prime candidate for extensive study (e.g. (Collingridge and Bliss, 1995; Malenka et al., 1989; Tonkiss et al., 1988); see Box 1). Facilitation of NMDAR function is considered a viable strategy to overcome cognitive deficiency associated with a large number of psychiatric disorders. Ever since the pioneer study by Johnson and Ascher (1987) showing that glycine could enhance NMDAR-mediated electrophysiological responses, the glycinerergic modulation of NMDAR function has received considerable interest (for review see Johnson and Ascher, 1987; Monaghan et al., 1988; Thomson, 1989). Their discovery followed previous indications that glycine might play an additional neuromodulatory function besides its well-documented role as an inhibitory neurotransmitter, due to the distribution of glycine binding sites in mammalian brain (e.g. Bristow et al., 1986; Kishimoto et al., 1981). It was observed that inhibitory glycine receptors (radioactively labelled with $[^3H]$ strychnine) were strongly expressed in the pons and spinal cord. In contrast, $[^3H]$ glycine not only labelled these receptors, but also identified the existence of high affinity binding sites in the forebrain (Bristow et al., 1986). Shortly after, Bowery (1987) noted that the distribution pattern of high affinity glycine binding sites corresponded to the NMDA binding profile in the brain. For simplification purposes, the strychnine-sensitive binding site is referred to as the GlyA-site, whereas the strychnine-insensitive glycine recognition site is referred to as the GlyB-site hereafter.
Table 1: Chronology of milestones leading to the development that GlyT1 disruption can facilitate NMDAR function and subsequently modulate cognitive function

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<th>Year</th>
<th>Experimental observations</th>
<th>References</th>
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<tr>
<td>late 1940s</td>
<td>Coincidence detection rule (a.k.a. Hebb's rule): a synapse linking two cells is strengthened if the cells are activated concurrently</td>
<td>(Konorski, 1948)</td>
</tr>
<tr>
<td>1973</td>
<td>Brief trains of high-frequency stimulation applied to monosynaptic excitatory pathways in the hippocampus cause an abrupt and sustained increase in the efficiency of synaptic transmission (i.e. long-term potentiation, LTP)</td>
<td>(Bliss and Lømo, 1973)</td>
</tr>
<tr>
<td>1987</td>
<td>Glycine enhances NMDAR-mediated responses in cortical neurons in a strychnine-insensitive manner</td>
<td>(Johnson and Ascher, 1987)</td>
</tr>
<tr>
<td>1988</td>
<td>Occupation of the glycineB-site is necessary for the activation of NMDA receptors (co-agonist concept)</td>
<td>(Kleckner and Dingledine, 1988)</td>
</tr>
<tr>
<td>1989</td>
<td>Activation of the glycineB-site enhances cognitive function</td>
<td>(Handelmann et al., 1989; Monahan et al., 1989)</td>
</tr>
<tr>
<td>1990</td>
<td>Proposal of the 'NMDAR hypofunction' hypothesis of schizophrenia</td>
<td>(Carlsson and Carlsson, 1990)</td>
</tr>
<tr>
<td>1992</td>
<td>Cloning studies reveal the existence of Glycine transporter 1</td>
<td>(Guastella et al., 1992; Smith et al., 1992)</td>
</tr>
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<td>1994</td>
<td>Amelioration of negative symptoms in schizophrenia by glycine treatment</td>
<td>(Goff et al., 1995; Javitt et al., 1994)</td>
</tr>
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<td>1995</td>
<td>GlyT1 is expressed predominately in spinal cord, brain stem, hindbrain, and forebrain Immunohistochemical analysis reveal glial expression of GlyT1</td>
<td>(Zafra et al., 1995a; Zafra et al., 1995b)</td>
</tr>
<tr>
<td>1997</td>
<td>GlyT1 re-uptake mechanisms can efficiently regulate local glycine levels</td>
<td>(Supplisson and Bergman, 1997)</td>
</tr>
<tr>
<td>2003</td>
<td>Availability of the first GlyT1 knockout mouse line GlyT1 disruption is lethal shortly after birth</td>
<td>(Gomez et al., 2003)</td>
</tr>
<tr>
<td>2004</td>
<td>GlyT1 heterozygous knockout mouse line is viable Behavioural phenotyping reveals its potential as antipsychotic and cognitive enhancer</td>
<td>(Tsai et al., 2004)</td>
</tr>
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<td>2005</td>
<td>GLYT1 is expressed in neurons and is associated with glutamatergic synapses</td>
<td>(Cubelos et al., 2005)</td>
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<td>2006</td>
<td>Neuronal forebrain GlyT1 knockout Start of intensive behavioural screening (see Table 2)</td>
<td>(Yee et al., 2006)</td>
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In 1988, Kleckner and Dingledine revealed that binding of glycine to glycine-B site is indeed a prerequisite for the activation of the NMDAR and proposed the term "co-agonist". This opened alternative avenues to facilitate NMDAR neurotransmission, which could: (i) potentially alleviate NMDAR-associated brain pathological conditions, (ii) augment synaptic plasticity and subsequently enhance cognitive functions under normal conditions. Such an approach could potentially circumvent the severe side-effects associated with direct stimulation of NMDARs, such as neurotoxicity and seizures (Mayer and Westbrook, 1987; Rothman and Olney, 1995). Indeed, one year later, evidence emerged that activation of the glycine regulatory site on the NMDA receptor could enhance cognitive function (Handelmann et al., 1989; Monahan et al., 1989).

One major limitation associated with glycine’s modulatory role on NMDAR function was the apparent lack of sufficient knowledge in the regulatory mechanisms (re-uptake, release, and metabolism) of glycine. It was, for instance, assumed that high affinity (in nM) NMDA-associated glycine sites were saturated due to higher micromolar (μM) concentrations of glycine in the extracellular fluid (Fletcher and Lodge, 1988; Matsiu et al., 1995). This limitation in the knowledge was overcome following the discovery of sodium/chloride (Na\(^{+}/\)Cl\(^{-}\))-dependent transporters for glycine in the brain (Fedele and Foster, 1992). Furthermore, cloning studies uncovered the existence of two families of glycine transporters: glycine transporter 1 (GlyT1, Guastella et al., 1992; Smith et al., 1992) and glycine transporter 2 (GlyT2, Liu et al., 1992). Both glycine transporters differ in terms of their expression profile. GlyT2 is expressed in areas where glycine acts as an inhibitory neurotransmitter, i.e. mainly in the spinal cord, brain stem and hindbrain (Jursky and Nelson, 1996; Zafra et al., 1995a). GlyT1 shares overlapping regional expression patterns with GlyT2 (Jursky and Nelson, 1996; Zafra et al., 1995a), but is furthermore expressed in some regions with low levels of GlyA receptors, such as the forebrain (Zafra et al., 1995a), where it co-localizes with the NMDAR (Smith et al., 1992). Although GlyT1 mRNA was detected in glia as well as in neurons, initial immunohistochemical analysis failed to confirm the presence of GlyT1 in neurons (Zafra et al., 1995a). Therefore, GlyT1 was considered to be localized only on astrocytes (Zafra et al., 1995b), until Cubelos and co-workers (2005) confirmed pre- and post-synaptic GlyT1 expression in a sub-set of glutamatergic neurons closely associated with NMDARs.
Box 1: The NMDA receptor: its properties, role in synaptic plasticity and learning and memory

Characteristics of the NMDAR

The NMDAR belongs to the ionotropic glutamate receptor sub-group, and exists as a heterotetrameric complex consisting of two NR1 and two NR2 (A-D) subunits. Combinations of different NR2 subunits determine the pharmacological and physiological profile of the receptor in terms of channel kinetics, and its affinity for agonists and antagonists (Cull-Candy et al., 2001). Besides glutamate binding at the NR2 subunit, glycine co-agonist binding at the NR1 glycine-B site is essential for effective channel opening (see Danyz and Parsons, 1998). Furthermore, the receptor complex harbours several endogenous allosteric ligand binding sites that modify its physiological properties (See Figure 1).

Figure 1: Schematic illustration of the NMDAR complex. The receptor complex comprises various binding sites, which are responsible for interactions with agonists, antagonists, or modulatory ligands. These sites, aid the facilitatory (polyamine) or inhibitory (Zn+) actions of the receptor. The glutamate/ligand recognition site is critical for receptor activation. Yet, glycine co-agonist binding at the strychnine sensitive glycine-B site of the NR1 subunit is necessary to facilitate channel opening. The receptor channel contains a voltage-dependent Mg2+ binding site, as well as binding sites for dissociative anaesthetics such as PCP, ketamine and MK-801. (Image obtained from www.frca.co.uk/images/NMDA.jpg)

NMDARs are principally neuronal, and whilst they are diffusely distributed throughout the brain, the highest density is detected in the CA1 hippocampal subfield and in layers I and III of the cerebral cortex (Monaghan and Cotman, 1985). The expression and distribution of NMDARs in the CNS is highly dependent on the combination of subunits that constitute the complex as well as the developmental stage of the organism (reviewed by Danyz and Parsons, 1998; Nakanishi, 1992). Consequently, the receptor is known to play a prominent role in a number of neurobiological events during brain development (Ewald and Cline, 2009; Haberny et al., 2002).

The notion that learning and memory depends upon changes in the efficacy of chemical synapses (as discussed elsewhere) has led to a primary focus on the NMDAR as the molecular correlate of synaptic plasticity— the neurochemical substrate presumed to underpin some forms of learning and memory. In this regard, the NMDAR has several unique properties that make it the ideal candidate for synaptic plasticity. Besides being a ligand-gated ion channel, the receptor is subject to a voltage-dependent block by magnesium (Mg2+), which can be removed via AMPA receptor-mediated membrane depolarization (Dingledine et al., 1999). Thus, it acts as a coincident detector for pre-synaptic activity (glutamate release) and post-synaptic activity (adequate depolarization of the post-synaptic membrane). Second, once activated, the NMDAR channel permits ion flow (Ca2+, Na+ and K+), which can give rise to an excitatory postsynaptic current (EPSC). The high Ca2+ permeability is a key feature of the NMDAR given that it is accompanied by a cascade of intracellular events that lead to long-term synaptic changes (Long-term potentiation; Collingridge and Bliss, 1995) and gene transcription (Bading et al., 1993).

Its role in synaptic modifications and memory

Cajal (1893) originally hypothesized that changes in the strength of synaptic connections between active neurons are necessary for information storage. This was subsequently supported by Hebb (1949), who proposed that changes in the strength of synaptic connections require concurrent activation of the pre- and post-synaptic neurons. In accordance with the Hebbian principle, it was later shown that activity-dependent synaptic changes play a vital role in some forms of learning and memory (e.g. Lynch, 2004). Consequently, studies aimed to understand the mechanism for synaptic changes led to the discovery that bi-directional activity-dependent forms of synaptic plasticity, such as long-term potentiation (LTP), required NMDA glutamate receptor channel opening (Bliss and Collingridge, 1993; Bliss and Lømo, 1973; Collingridge et al., 1983; Zalutsk and Nicoll, 1990). As mentioned above, the NMDAR is an ideal ‘Hebbian’ candidate for synaptic changes due to its voltage-dependent magnesium block (Mayer and Westbrook, 1987) and “coincidence detection” mechanism. Notably, NMDAR activation is critical for the induction of LTP, whereas maintenance/expression of LTP is dependent on the recruitment and expression of AMPA receptors at the synapse (Malinow and Malenka, 2002; Martin et al., 2000).

NMDAR-dependent LTP is universally considered relevant to the formation of learning and memory (Lynch, 2004; Martin et al., 2000). This is supported by studies showing that selective pharmacological blockade of NMDARs disrupt the induction of LTP and impair learning and memory function in a variety of paradigms (Bolhuis and Reid, 1992; Kawabe et al., 1998; McHugh et al., 2008; Morris, 1989; Morris et al., 1986). Furthermore, it has been demonstrated that specific hippocampal NMDAR subunits, as well as their expression in different hippocampal subfields, make a unique and differential contribution to learning and memory (Banerman et al., 2008; Nakazawa et al., 2002; Nakazawa et al., 2003; Niewoehner et al., 2007; Sakimura et al., 1995; Tsien et al., 1996).
The discovery that glycine transporters are expressed in the brain (Guastella et al., 1992; Liu et al., 1992; Smith et al., 1992) suggests that these transporters regulate and maintain glycine concentrations below saturation levels in the synaptic cleft. Indeed, Supplisson and Bergman (1997) using an experimental model co-expressing GlyT1 and NMDAR in *Xenopus oocytes* showed that GlyT1 efficiently reduces glycine near the membrane to concentrations lower than the saturating concentration (nM range) for neuronal NMDARs when the bath concentration (µM range) is similar to that in the cerebral spinal fluid. One year later, an *in vitro* study confirmed GlyT1’s efficacy in maintaining unsaturated glycine concentration at the synaptic cleft (Bergeron et al., 1998). The study revealed that blockade of GlyT1 by the inhibitor NFPS (*N*-[3- (4’-fluorophenyl)-3-(4’-phenylphenoxy) propyl] sarcosine) led to increased glycine concentrations and a subsequent boost of NMDAR function by nearly a 50% increase in evoked EPSCs. These findings were replicated by others *in vitro* (e.g. Chen et al., 2003; Martina et al., 2004), and *in vivo* (e.g. Kinney et al., 2003; Whitehead et al., 2004). Hence, since Johnson and Ascher's (1987) initial hypothesis of glycinergic modulation of NMDAR function, nearly two decades of research has corroborated this notion, with further revelations that GlyT1 constitutes an optimal target to regulate glycine availability in the vicinity of NMDARs.

**GlyT1 function and schizophrenia**

In parallel to the findings on NMDAR function, the 'NMDAR hypofunction' hypothesis of schizophrenia emerged as an additional concept to the widely accepted hyperdopaminergic theory on schizophrenia pathophysiology (Carlsson and Carlsson, 1990; also see Box 2; Olney and Farber, 1995). Consequently, it has been suggested that enhanced glutamatergic transmission could potentially have beneficial effects in schizophrenia (Carlsson and Carlsson, 1990). However, given that direct NMDAR activation is associated with neurotoxic and convulsive effects, stimulation of the GlyB-site on the NMDAR might be considered an alternative and favourable approach (Danysz and Parsons, 1998). Studies have indeed shown that glycine, or D-cycloserine (a partial agonist at the GlyB-site that more readily crosses the blood-brain barrier) significantly reduces the negative and cognitive symptoms in schizophrenic patients who are only partially responsive to neuroleptics (Goff et al., 1995; Javitt, 2004). However, exogenous glycine administration may not be an optimal approach towards increasing NMDAR glycine B-site occupancy due to poor blood-brain barrier penetration, extensive metabolism (enzymatic degradation) and potent regulatory mechanisms (glycine transporter mediated re-uptake). Hence, elevating local concentrations of glycine by blocking its re-uptake might be a better alternative to normalize NMDAR function (Javitt et al., 1997). For instance, the GlyT1 inhibitor, glyclyldodecylamide (GDA), antagonizes the hyperactivity induced by phencyclidine (PCP) more efficiently that glycine itself (Javitt et al., 1997). This has led to the production of various selective and high-affinity GlyT1 inhibitors and has prompted the behavioural
assessment of the impact of GlyT1 inhibition. These inhibitors have been shown to up-regulate extracellular glycine concentrations (e.g. Boulay et al., 2008; Depoortere et al., 2005; Pinard et al., 2010). In addition, based on their ability to facilitate NMDAR-mediated transmission and enhance LTP/synaptic plasticity (e.g. Bergeron et al., 1998; Chen et al., 2003; Kinney et al., 2003; Martina et al., 2004), GlyT1 inhibitors have been associated with improved mnemonic function across multiple cognitive domains. For instance, NFPS and SSR504734 enhance working memory and social recognition function (Manahan-Vaughan et al., 2008; Möhler et al., 2011; Shimazaki et al., 2010; Singer et al., 2009b), suggesting that GlyT1 inhibitors may act as potential cognitive enhancers. Furthermore, GlyT1 inhibitors normalize the behavioural effects induced by NMDAR antagonists (such as MK-801 and PCP; Black et al., 2009; Boulay et al., 2008; Depoortere et al., 2005; Hashimoto et al., 2008). The fact that several compounds can reverse several cognitive deficits induced by NMDAR antagonists (Black et al., 2009; Boulay et al., 2008; Depoortere et al., 2005), suggests that GlyT1 inhibition might be relevant and useful to the cognitive deficiency associated with a number of psychiatric disorders, such as schizophrenia.

**Box 2: Schizophrenia and the NMDAR hypofunction hypothesis**

Schizophrenia is a major psychotic disorder, which is characterized by profound disturbances in cognitive function, emotion and behaviour. Its symptomatology is divided into three categories: (i) positive (hallucinations, delusions and thought disturbances), (ii) negative (flat affect, social withdrawal and anhedonia) and (iii) cognitive symptoms (working memory deficits, inability to sustain attention and poor executive function). Schizophrenia is suggested to have a developmental origin, whereby genetic susceptibility and environmental factors both contribute to changes in the developing neural circuitry. In adulthood the disease is manifested in the form of altered brain neurochemistry and aberrant behaviour.

Initially, dopaminergic dysfunction was primarily implicated in schizophrenia pathology due to the capacity of amphetamine (an indirect dopamine agonist) to induce psychoses (Snyder, 1976). In addition, typical and atypical antipsychotics were shown to act by blocking D2/D3 receptors (Creese et al., 1976; Seeman et al., 1975). Moreover, the brains of schizophrenic patients showed increased D2/D3 receptor expression and elevated dopamine levels (Abi-Dargham et al., 2000; Abi-Dargham et al., 2009). Whereas antipsychotic medication is effective in normalizing the positive symptoms, the negative and cognitive symptoms are poorly responsive (Kapur and Remington, 2001; Tamminga, 1999), suggesting that perhaps other neurotransmitter systems contribute to schizophrenia pathology.

Inquiries into the potential role of the NMDA receptor in schizophrenia emerged after the discovery that phencyclidine (PCP) led to a schizophrenic-like psychotic state in humans (Luby et al., 1959). This finding was validated by showing that NMDAR antagonism precipitated schizophrenic-like symptoms in normal subjects and aggravated the symptoms in schizophrenics (Adler et al., 1999; Jentsch and Roth, 1999; Krystal et al., 1994; Lai et al., 1995a; Lahti et al., 1995b). In animal studies, administration of NMDAR antagonists (Gainetdinov et al., 2001) and decreased NMDAR subunit expression (Mohn et al., 1999) leads to multiple behavioural abnormalities, which highly resemble schizophrenia symptoms. These findings led to the formation of the NMDAR hypofunction hypothesis (Carlsson and Carlsson, 1990; Olney and Farber, 1995; Olney et al., 1999), which suggests that schizophrenia pathology stems from decreased NMDAR function. In fact, dopaminergic alterations might be secondary to NMDA receptor dysfunction in schizophrenia, given that these receptors are located in brain circuits regulating dopamine release (Carlsson and Carlsson, 1990). Hence, the NMDAR offers an alternative opportunity for new therapeutic strategies, which can offer a wider range of action, especially with regards to normalizing the negative and cognitive symptoms of the disorder.
Chapter 1: General Introduction

**Genetic knockout mouse models for GlyT1 function**

Advances in molecular biology and genetic engineering techniques have provided a unique and powerful tool to discern GlyT1 function at the behavioural level, which cannot be readily achieved by pharmacological manipulation alone. Gene-targeted GlyT1 deletion corroborates the rationale behind GlyT1 disruption and affords several advantages over the conventional systemic drug studies, such as the capacity to achieve GlyT1 deletion restricted to a particular brain region, or a particular cell type.

In 2003, the first GlyT1 knockout mouse line was generated (Gomeza *et al.*, 2003). GlyT1 was inactivated by homologous recombination disrupting the *Slc6a9* gene (previously known as *GlyT1*). Although these mice were anatomically normal, complete GlyT1 inactivation proved fatal soon after birth, probably due to disturbances in the respiratory system arising from excessive inhibitory glycinergic neurotransmission (Gomeza *et al.*, 2003). The lethality of homozygous mice precluded any behavioural assessment. This was achievable, however, in viable heterozygous knockout (GlyT1 \(+/-\)) mice, which showed a 50% reduction of GlyT1 mRNA expression (Tsai *et al.*, 2004). For instance, by using a one-day protocol in the water maze task, they showed that GlyT1 \(+/-\) mice performed comparably to controls in locating an escape platform during acquisition. However, upon removal of the platform GlyT1 \(+/-\) mice showed a tendency to search more in the former platform location compared to controls, leading the authors to conclude that they had improved spatial retention (Tsai *et al.*, 2004). This effect could be considered relatively weak, given that this effect was only seen during the retention probe test.

The discovery of pre-and post-synaptic GlyT1 expression in a sub-set of glutamatergic neurons closely associated with NMDARs (Cubelos *et al.*, 2005), provided the rationale to create a mouse line bearing a forebrain- and neuronal-specific GlyT1 knockout (Yee *et al.*, 2006). Due to the nature of GlyT1 knockout, this mouse line is free from developmental respiratory complications linked with excessive glycinergic inhibition. Accordingly, a conditional GlyT1 transgenic (GlyT1tm1.2fl/fl (Slc6a9tm1.1Bois)) mouse line, which harbours two loxP sites flanking the sequence between exons 5 to 11 of the GlyT1 gene was created (Gabernet *et al.*, 2005). This mouse line was backcrossed and maintained on a C57BL/6 background, was viable and devoid of any known phenotypic abnormalities. In order to circumvent developmental compensatory changes and to achieve forebrain-specific neuronal conditional deletion, excision of the GlyT1 coding sequence was accomplished by crossing GlyT1tm1.2fl/fl mice with another C57BL/6 mouse line that expressed a transgene encoding Cre recombinase under the control of the α-CamKII subunit CamKIIαCre2834[Tg(Camk2a-cre)2834Lusc/0]]. This promoter is known to
drive expression in excitatory forebrain neurons beginning at approximately 20 days after birth (Dragatsis and Zeitlin, 2000). The resulting progeny (CamKIIαCre2834:GlyT1tm1.2fl/fl) were characterized by a neuron-selective disruption of GlyT1 restricted to the forebrain (and are henceforth designated as “GlyT1ΔFB-neuron mice”). These mice exhibit a 30% reduction of GlyT1 expression in the forebrain, which is accompanied by a 35% reduction of GlyT1-dependent [3H] glycine uptake. Notably, NMDAR expression levels remains unchanged, although GlyT1ΔFB-neuron mice display enhanced NMDAR-mediated synaptic currents. Furthermore, electrophysiological recordings from GlyT1ΔFB-neuron mice brain slices revealed a significant increase (+120%) in the NMDA/AMPA response ratio relative to controls (Yee et al., 2006). Unlike the heterozygous knockout mice (Tsai et al., 2004), this mouse line has been subjected to comprehensive behavioural phenotyping, which is summarised in Table 2. The mice were initially tested for possible physical and neurological impairments, which could potentially act as confounding factors in subsequent behavioural tests. These results revealed no abnormalities in locomotor activity or anxiety-related behaviour. Subsequently, the mice were assessed for their responsiveness to psychomotor stimulant drugs and a battery of cognitive behavioural tests relevant for schizophrenia-related cognitive deficiency (Table 2).
Table 2: Summary of the behavioural phenotypes exhibited by the GlyT1 forebrain- and neuronal-specific knockout mice across multiple experimental paradigms.

<table>
<thead>
<tr>
<th>Behavioural Function</th>
<th>Forebrain neurons GlyT1^{ΔFB-neuron}</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Associative Learning:</strong></td>
<td></td>
</tr>
<tr>
<td>Conditioned freezing</td>
<td>enhanced</td>
</tr>
<tr>
<td>Active avoidance learning</td>
<td>enhanced</td>
</tr>
<tr>
<td>Conditioned taste aversion</td>
<td>enhanced</td>
</tr>
<tr>
<td><strong>Spatial Learning:</strong></td>
<td></td>
</tr>
<tr>
<td>Working memory</td>
<td>ambiguous</td>
</tr>
<tr>
<td>Reference memory</td>
<td>null effect</td>
</tr>
<tr>
<td><strong>Recognition Memory:</strong></td>
<td></td>
</tr>
<tr>
<td>Object recognition</td>
<td>enhanced</td>
</tr>
<tr>
<td><strong>Attentional Processes:</strong></td>
<td></td>
</tr>
<tr>
<td>Latent inhibition (learned inattention)</td>
<td>enhanced</td>
</tr>
<tr>
<td>Prepulse inhibition (early sensory gating)</td>
<td>impaired</td>
</tr>
<tr>
<td>MK-801 induced PPI disruption</td>
<td>null effect</td>
</tr>
<tr>
<td>Amphetamine induced PPI disruption</td>
<td>(not tested)</td>
</tr>
<tr>
<td><strong>Behavioural Flexibility:</strong></td>
<td></td>
</tr>
<tr>
<td>Two-choice discrimination reversal</td>
<td>enhanced</td>
</tr>
<tr>
<td>Spatial reversal learning</td>
<td>enhanced</td>
</tr>
<tr>
<td><strong>Motor effects of psychostimulant drugs:</strong></td>
<td></td>
</tr>
<tr>
<td>Motor stimulation by PCP or MK-801</td>
<td>resistant</td>
</tr>
<tr>
<td>Motor stimulation by amphetamine</td>
<td>delayed</td>
</tr>
<tr>
<td><strong>Other behaviour:</strong></td>
<td></td>
</tr>
<tr>
<td>Accelerating rotarod</td>
<td>null effect</td>
</tr>
<tr>
<td>Elevated plus maze test of anxiety</td>
<td>null effect</td>
</tr>
<tr>
<td>Spontaneous open field activity and exploration</td>
<td>null effect</td>
</tr>
</tbody>
</table>

**Pro-cognitive phenotype in GlyT1^{ΔFB-neuron} mice?**

Forebrain neuronal GlyT1 knockout mice were examined for changes in non-associative learning by studying habituation in an object recognition task (Singer et al., 2007). The authors used three spontaneous exploration tasks, in which memory for a stimulus was examined by measuring the preference for novel over familiar (previously experienced) stimuli. These tests capitalize on the preference of mice to explore novel stimuli, which is sensitive to habituation.
when the same stimulus is repeatedly presented. The procedure comprises two consecutive phases: (i) a sample phase- in which a stimulus (i.e. an object) is presented and (ii) a test phase, which is conducted following a defined retention interval. Here, a novel stimulus is introduced along with the stimulus presented during the sample phase. In the object recognition memory test, Singer et al. (2007) observed that both mutants and controls showed a preference for the novel over the familiar object in a 2 min delay interval. In contrast, only the GlyT1\textsuperscript{ΔFB-neuron} mutant mice demonstrated a strong preference for the novel object when the retention interval between the sample and test phase was increased to 2 hours. The same study also demonstrated that only the mutant mice were responsive to a change in the spatial configuration of four objects. When examined for contextual novelty, the mutants and controls showed equivalent preference for a novel over a familiar spatial environment. The authors argued that delay-dependent enhanced recognition memory in GlyT1\textsuperscript{ΔFB-neuron} mice was suggestive of a stronger memory trace and/or resistance to memory decay. Hence, they referred to this phenotype as “pro-cognitive”. As discussed later by the authors (Singer et al., 2009c), cognitive enhancement should be put in perspective to the context in which it is observed (e.g. Sandberg and Bostrom, 2006). Thus, more generally speaking, any manipulation that can alter a given cognitive function may possibly lead to beneficial or harmful effects depending on the precise circumstances. For example, a stronger memory trace might be beneficial in the context of a Ph.D. examination, yet detrimental in the context of a traumatic event. This issue was also addressed by Sanderson and Bannerman (2007) when they questioned why evolutionary pressures had not produced a mouse with clear selective advantages such as better learning capacities by now. They argued that perhaps there is a price to pay for better recognition memory. Interestingly, the emergence a cognitive enhancing phenotype in GlyT1\textsuperscript{ΔFB-neuron} mice, such as enhanced latent inhibition effect (Yee \textit{et al.}, 2006) or enhanced recognition memory (Singer \textit{et al.}, 2007) was demonstrated against the background of poor performance in the controls. Therefore, as an alternative interpretation to the “pro-cognitive” phenotype, it seems that the aforementioned effects might reflect inappropriate responding in the mutants under conditions where a response is absent in the controls (see also Sanderson and Bannerman, 2007). For instance, the fact that only the mutants show a novelty preference at the 2 hr delay interval may indicate impaired dishabituation. The control mice are considered to dishabituate because they show renewed response to the previously presented object, probably due to a decrease in familiarity of the stimulus over time. On the contrary, in the mutants the familiarity of the previously presented object does not appear to decay over time. This notion thus disagrees with the stronger memory trace and/or resistance to memory decay view suggested by Singer et al. (2007).
The GlyT1\(^{ΔFB}\)-neuron mouse line was also examined for working memory. Working memory disruption is one of the core symptoms in schizophrenics, and therefore represents an important issue for rehabilitation. Working memory function requires the short-term storage and expression of information that is relevant for effective guidance and execution of goal-directed behaviour (Olton and Samuelson, 1976). This is particularly important in situations whereby the relevant information is constantly changing and requires regular updating. Working memory is therefore, especially prone to interference by previously acquired memory traces. Given that GlyT1\(^{ΔFB}\)-neuron mice are thought to exhibit a stronger memory trace and/or resistance to memory decay, they might be prone to deficiencies in working memory performance.

In the water maze paradigm working memory is assessed by changing the escape platform location daily, yet it remains fixed across trials within a given day. The animals are therefore required to learn the new platform location on the first trial of each day. The working memory performance of GlyT1\(^{ΔFB}\)-neuron mutant mice in the water maze test is ambiguous (Singer et al., 2009a). The GlyT1\(^{ΔFB}\)-neuron mutant mice outperformed control mice in the first trial when the escape platform was unknown. Yet, they failed to show a general improvement of escape performance from trial 1 to 2. Nonetheless, it was suggested that the mutants might be less prone to the negative impact of proactive interference. According to the authors, this argument was corroborated by a facilitation of reversal learning in GlyT1\(^{ΔFB}\)-neuron mutant mice (Singer et al., 2009a). In the reference memory version of the same task, when the platform position is kept constant across days, performance of the control and mutant mice did not differ. However, when the location of the platform was reversed, GlyT1\(^{ΔFB}\)-neuron mice acquired the new platform location more rapidly, and spent less time searching in the previous target location. Such facilitation of reversal learning was also shown in an appetitive non-spatial discrimination learning paradigm, suggesting that GlyT1\(^{ΔFB}\)-neuron mice might be resistant to proactive interference. This interpretation is difficult to reconcile with the notion that the same mice have a stronger memory trace and/or resistance to memory decay. Therefore, the lack of a clear interpretative outcome, and the unresolved issue as to the direction in which working memory function is altered by forebrain neuronal-specific GlyT1 knockout needs to be satisfactorily addressed.

### Impact of GlyT1 disruption on associative learning

Another phenotype displayed by GlyT1\(^{ΔFB}\)-neuron mice is in the form of enhanced associative learning (Yee et al., 2006) as they consistently showed stronger conditioned response. According to associative learning theories, an organism adapts its behaviour to its environment...
by exploiting environmental contingency amongst external stimuli and learn about the predictive significance of a stimulus. This can be directly related to Hebb’s rule of coincidence detection where the animals perceive two coincident events and learn to form an association between them (Hebb, 1949) such that presentation of one can lead to the anticipation of the other. This can be readily demonstrated in Pavlovian conditioning, whereby a neutral conditioned stimulus (CS) is paired with a biologically significant unconditioned stimulus (US). Successful associative learning results in the CS eliciting a conditioned response (CR). Evidence for enhanced associative learning in GlyT1ΔFB-neuron mice, in the form of stronger CR to the CS was shown across multiple paradigms, including conditioned freezing, conditioned taste aversion, and conditioned active avoidance learning (Yee et al., 2006). The authors suggested that neuronal forebrain GlyT1 knockout might lead to a stronger memory trace because of a stronger association between the CS and the US. This phenotype was observed in aversive paradigms involving either foot shock or gastric malaise (illness). However, it remains to be determined whether a similar phenotype might emerge in appetitively motivated paradigms. This is addressed experimentally in Chapter 2.

Conditioning leads to the formation of an association between representations of two events. Experimental findings in the field of animal learning have demonstrated that the formation of associations cannot solely be explained by the principle of contiguity, i.e. conditioning is effective when two events are paired in time and space. Consequently, learning theorists have tried to identify circumstances which determine the effectiveness of a stimulus to enter an association (i.e. its "associability"). The latent inhibition (LI) effect represents an important demonstration of changes in the associability of a stimulus, which is caused by an animal’s past experience of the predictive value of that stimulus for a reinforcer. The LI effect refers to the observation that prior non-reinforced (without consequence) exposures of a stimulus can retard that stimulus’ ability to enter into association with a significant event that is presented subsequently (see Figure 2, Lubow and Moore, 1959). In associative learning, some theories proposed that the CS pre-exposure (Phase 1) retards subsequent development of the conditioned response (CR), which follows pairings between the same CS and a significant event (the US, Phase 2). Different theoretical accounts for LI have been proposed. The dominant concept is that LI reflects a loss of attention to the CS. However, these theories differ in the rules that are believed to govern changes in attention. For instance, attention to the CS is diminished because the animal learns from the non-reinforced CS pre-exposures (in Phase 1) that (i) the stimulus is a relatively poor predictor of reinforcement (Mackintosh, 1975), (ii) that the stimulus is followed by a predicted consequence (Pearce and Hall, 1980), i.e. there was no surprise subsequent to the stimulus presentation, or (iii) because of habituation to the CS.
(Wagner, 1981). The loss of attention leads to slower processing of the CS during subsequent conditioning (Phase 2). Alternatively, LI can be considered as a form of proactive interference, in which the association between the CS and the absence of significant consequence (noUS) during Phase 1 compete or interfere with subsequent expression (Weiner, 1990) or retrieval (Bouton, 1993) of the CS-US association that occurs during Phase 2. Consequently, the two inconsistent associations (CS-noUS and CS-US) will compete for the behavioural control in response to the CS.

*Figure 2*: Schematic representation of an experimental procedure designed to assess the latent inhibition effect.

Yee et al. (2006) showed that GlyT1ΔFB-neuron mice exhibited LI under experimental conditions in which the controls failed to do so, thereby reflecting an enhanced LI effect. The authors, thus, argued that the impact of the molecular deletion could be understood as bi-directional because the CR developed in non-pre-exposed conditions was enhanced, whereas the CR in pre-exposed conditions was reduced. Two possible psychological mechanisms could explain these results: first, stronger associative learning (as seen before), and/or secondly, facilitation in selectivity of learning. The latter could be interpreted as a form of enhanced learned inattention (Gray *et al.*, 1991) or suppression of behavioural switching (Weiner, 1990). No matter which position one may take, it appears that disruption of GlyT1 does not indiscriminately strengthen all
associations at the cost of selectivity in learning. Bannerman and Sanderson (2007) offered an alternative account for the findings of Yee et al. (2006) by extending their suggestion that the mutant mice might be impaired in dishabituation. This was developed in their critique of the observation of enhanced recognition memory reported in these mice by Singer et al. (2007). Bannerman and Sanderson (2007) reckon that the lack of latent inhibition in the control mice could be due to dishabituation to the pre-exposed stimulus (thus attention to it is renewed when it was presented in conjunction to a US after pre-exposure). Accordingly, the presence of LI in the mutants could be understood as stemming from an impairment in dishabituation; the animals remained habituated to the pre-exposed CS. Habituation to the CS extended into Phase 2 when the CS became predictive of the US, which, therefore yielded the latent inhibition effect. Finally, Singer et al. (2009c) offered another account for the LI effect in GlyT1<sub>ΔFB-neuron</sub> mice by disregarding the possible involvement of the two different mechanisms proposed by Yee et al. (2006). They suggested, based on the “switching” account (Weiner, 1990), that because GlyT1<sub>ΔFB-neuron</sub> mice learned both CS-nothing link (during Phase 1) and CS-US link (during Phase 2) more effectively, the stronger CS-nothing link might imply a stronger impact on the subsequent expression of the CR, commanded by the CS-US link. In contrast, the test parameters were insufficient to generate latent inhibition in the control mice, because the impact of the learned CS-nothing association on the expression of the CR was weaker compared to the mutant mice. Hence, given the divergence of possible explanations offered in the literature, the possibility that GlyT1 disruption can result in general facilitation in selectivity of learning warrants further examination. In Chapter 4, this possibility is addressed by introducing additional tests that might be similarly conceived as examples demonstrative of selectivity (not all potential CSs and USs invariably enter into association) in Pavlovian learning.

Further considerations
Although GlyT1 might be beneficial for cognitive functions in the short-term, chronically elevated glycine concentration levels in the vicinity of NMDARs might be detrimental in the long run (see Danysz and Parsons, 1998). It has been argued that this approach might exacerbate, rather than ameliorate, the pathophysiology of conditions like Alzheimer’s disease (Javitt, 2004). Hence, it is worth addressing the impact of enhanced NMDAR excitability, and potential excitotoxicity and neurodegeneration in aged GlyT1<sub>ΔFB-neuron</sub> mice.
Thesis objectives

As detailed above, deletion or inhibition of GlyT1 appears to be effective in modifying cognitive functions. However, the behavioural assessment of GlyT1 inhibition/disruption was neither extensive nor thorough, and several outstanding issues are yet to be addressed. The emphasis in behavioural neuroscience on high-throughput approaches inadvertently favours the sole reliance on the most efficiently implemented tests, such as the water maze or the pre-pulse inhibition (PPI) paradigm, and often at a cost of in-depth analysis. This can lead to premature claims involving hazy terms such as “pro-cognitive effect” or “enhanced flexibility”. Indeed, misinterpretations made from hasty behavioural phenotyping can impede research progress in the long run (Crabbe and Morris, 2004). As correctly put forward by Crabbe and Morris (2004), this echoes the classical adage Festina lente, which highlights the fact that sometimes, if not always, making haste does not necessarily achieve more speed. An accurate understanding of the phenotypic expression of any genetic manipulation is an important step forward in the proof of concept, and constitutes a critical component in the preclinical evaluation of potential drug targets. It is crucial, therefore, to gauge the robustness and perseverance of any given behavioural phenotype to avoid simplistic and biased interpretations that may be counterproductive in advancing translational research. Likewise, it is of the utmost importance that the different findings are considered under a theoretical framework that allows for a coherent integration of different results and aid interpretation.

The experimental studies presented in this thesis aim to dissect the psychological mechanisms modulated by GlyT1 disruption, more specifically, the impact of forebrain neuronal GlyT1 knockout on multiple cognitive functions. They were designed with an emphasis on the use of an accurate and comprehensive behavioural assessment using an integrative approach.
Chapter 1: General Introduction

Thesis outline

The first part of this thesis investigates the generality of the key phenotypes identified previously in the GlyT1ΔFB-neuron mouse line. The experiments described here aimed at extending and building on previous findings in two main directions. First, a study was conducted to examine whether one of the key robust phenotypes in GlyT1ΔFB-neuron mice, namely enhanced Pavlovian conditioning, is compromised by aging. Concurrently, it was investigated whether the genetic manipulation in the aging brain might be associated with neuroanatomical and morphological changes (Chapter 2). Both experimental questions were based on concerns over possible harmful excitotoxic effects linked to chronic elevation of synaptic glycine levels arising from GlyT1 deletion, such as accelerated aging process, weakened and/or reversal of the pro-cognitive phenotypes identified in adulthood. Second, a study was designed (Chapter 3) to include two critical confounds, which had been omitted in the original study by Yee et al. (2006): Pavlovian conditioning was only investigated using (i) aversive paradigms and (ii) only in female mice. Here, the consistency of the enhanced Pavlovian conditioning phenotype across sexes, and the occurrence of this phenotype in an appetitive (non-aversive) paradigm was investigated. This study offered further opportunity to examine the stability of the phenotype across both the light and dark phases of the circadian rhythm. This comparison between the two diurnal phases represents a critical consideration that is often overlooked in the majority of phenotypic assays of genetically modified animals.

The second part of this thesis addressed the psychological nature of the Pavlovian phenotype induced by GlyT1 deletion by focusing on its possible impact on the selectivity of learning. The impression that GlyT1ΔFB-neuron mice might exhibit aberrant selective learning during the formation of associative memory was initially based on their phenotype in the latent inhibition paradigm (Yee et al., 2006). Accordingly, a study was conducted (Chapter 4) which focused on the selectivity of learning by incorporating two major determinants of Pavlovian associative learning: (i) Contiguity, and (ii) Contingency. In light of the hitherto outstanding issues concerning the potential resistance to proactive interference shown by the GlyT1ΔFB-neuron mice (Singer et al., 2009a), Chapter 5 investigates the impact of this mutation across multiple behavioural paradigms designed to assess working memory function.

To conclude, the general discussion in Chapter 6 integrates the findings presented here with the current literature on GlyT1 function, with the aim of providing a detailed understanding of the potential, as well as limitation, of forebrain- and neuronal- specific GlyT1 inhibition in modifying cognitive functions.
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CHAPTER 2

Impacts of forebrain neuronal glycine transporter 1 disruption in the senescent brain:

Evidence for age-dependent phenotypes in Pavlovian learning

With Philipp Singer, Detlev Boison, Joram Feldon, Hanns Möhler, and Benjamin K. Yee

ABSTRACT

Genetic deletion of glycine transporter 1 (GlyT1) in forebrain neurons gives rise to multiple pro-cognitive phenotypes, presumably due to enhanced N-methyl-D-aspartate receptor (NMDAR) functions. However, concerns over possible harmful excitotoxic effects under life-long elevation of synaptic glycine have been raised. Such effects might accelerate the aging process, weakening or even reversing the pro-cognitive phenotypes identified in adulthood. Here, we examined if one of the most robust phenotypes in the mutant mouse line (CamKIaαCre;GlyT1tm1.2fl/fl), namely, enhanced aversive Pavlovian conditioning, might be modified by age. Comparison between 3-month-old (adult) and 22-month-old (aged) mutants confirmed the presence of this phenotype at both ages. However, the temporal expression of the Pavlovian phenotype was modified in senescence; while adult mutants showed a pronounced within-session extinction, aged mutants did not. Expression of NR2B subunits of NMDAR and neural proliferation were examined in the same animals by immunohistochemistry. These were reduced in the aged mice as expected, but not exacerbated by the mutation. Thus, our results do not substantiate the concerns of neurotoxic effects through life-long GlyT1 disruption in forebrain neurons, but provide evidence for a modification of phenotypic expression as a function of age. The latter points raise the need to further investigate other pro-cognitive phenotypes identified at adulthood in this mutant line. In addition, we revealed here for the first time a clear increase in the number of immature neurons in the hippocampus of the mutants, although the behavioural significance of this phenotype remains to be determined.

INTRODUCTION

N-methyl-d-aspartate receptor (NMDAR) -dependent synaptic plasticity has been implicated in various forms of learning and memory processes (Morris, 1989; Morris, Anderson, Lynch, & Baudry, 1986). Its hypofunction may be associated with the cognitive deficiency characteristic of a number of psychiatric disorders, such as schizophrenia, as well as with the course of normal aging (Rosenzweig & Barnes, 2003). Enhancing NMDAR function therefore represents a possible strategy to ameliorate such deficits (Martin, Grimwood, & Morris, 2000). While direct activation of NMDARs is prone to severe side effects including neurotoxicity and seizures (Rothman & Olney, 1995), modulation of NMDAR function via allosteric sites offers an opportunity for alternative pharmacological targets (Yang & Svensson, 2008). One strategy is to increase the availability of glycine in the vicinity of NMDARs, and therefore the occupancy of the co-agonist glycine-B site of the NMDAR complex. This can be effectively achieved by pharmacological inhibition of glycine re-uptake via glycine transporter 1 (GlyT1) which co-localizes with NMDARs in neurons. Such inhibition has been reported to enhance NMDAR excitability, confer resistance to behavioural deficits induced by NMDAR antagonists, and lead to some forms of enhanced cognitive performance (Black et al., 2009; Depoortere et al., 2005; Singer, Boison, Mohler, Feldon, & Yee, 2009).
We have recently shown that disruption of GlyT1 restricted to forebrain neurons is sufficient to facilitate performance on various cognitive processes, including Pavlovian conditioning, reversal learning, latent inhibition and memory for object identity as well as object location (Singer, Boison, Mohler, Feldon, & Yee, 2007; Singer et al., 2009; Yee et al., 2006). When GlyT1 is knocked out in the entire forebrain, working memory function is also enhanced (Singer et al., 2009), extending an earlier report of improved reference memory retention in constitutive heterozygous GlyT1 knockout mice (Tsai et al., 2004). Although direct demonstration of GlyT1 deletion or blockade alone being sufficient to induce excitotoxicity is lacking, Sanderson and Bannerman (2007) have raised concern over possible negative impacts of this manipulation in the long term. The deletion of the GlyT1 gene, in spite of its potential cognitive gain, may carry a putative ‘cost’. More specifically, GlyT1 deletion may carry a greater risk of neurodegeneration or excitotoxicity, especially in aged animals, outweighing any potential cognitive gain from enhanced NMDAR activation (Javitt, 2004). Increasing Ca2+ influx through NMDARs and perhaps AMPA receptors may lead to excitotoxicity and neuronal death implicated in neurodegenerative disorders such as Alzheimer’s disease (Javitt, 2004). A life-long elevation of glycine concentration in the vicinity of NMDARs might be apoptotic. This hypothesis is highly relevant to the potential clinical application of GlyT1-inhibitors as cognitive enhancers or antipsychotic drugs. The present study is designed to address empirically two critical implications raised by Sanderson and Bannerman (2007).

In the present study we first examined if the cognitive enhancing effects of GlyT1 disruption demonstrated in adult animals might be modified in senescence, as would be expected if the genetic disruption did carry long-term negative impact. A diminution, absence or even reversal of such an effect in aged subjects would lend some credence to Sanderson and Bannerman's (2007) concern. To this end, we compared the behavioural effects seen in mutant mice with forebrain neuronal GlyT1 knockout to those observed in control mice at two ages: 3 months (“adult”) vs. 22 months old (“aged”). We focused on one of the most robust pro-cognitive traits of this conditional genetic knockout, namely enhanced associative learning as exemplified by Pavlovian conditioned freezing (Yee et al., 2006). Prior to the conditioning experiment, possible relevant confounding effects that might interfere with the freezing response were assessed by the elevated-plus maze test of anxiety and the open field test of spontaneous locomotors activity.

Following behavioural assessments, selected anatomical markers were assessed to gauge the putative negative impacts of long-term GlyT1 disruption in the brain. First, apoptosis related to neurotoxicity or accelerated neurodegeneration in the hippocampus was evaluated by activated caspase-3 immunohistochemistry (Budihardjo, Oliver, Lutter, Luo, & Wang, 1999). Second, density of the immuno-signals against the NR1 and NR2B subunits of the NMDAR in...
amygdala and prefrontal cortices in addition to hippocampus was measured. NMDARs are pertinent to the normal functioning of these brain regions, which are implicated in the acquisition and extinction of fear conditioning (Maren, 2001). Given that binding to glycine-B site primes the internalization and degradation of NMDARs (Nong et al., 2003), persistent and excessive glycine-B site activation might reduce NMDAR expression. Finally, the immature neuronal marker doublecortin (DCX) was used to index neural proliferation in the dentate gyrus, which is known to be regulated by NMDAR activity, and down-regulated in senescence (Bizon & Gallagher, 2003).

**METHODS**

**Subjects**
The subjects were offspring obtained by crossing CamKIIαCre:Glyt1tm1.2fl/fl mice with Glyt1tm1.2fl/fl mice (both maintained on a pure C57BL/6J background). This breeding strategy yielded 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 (Laboratory of Behavioural Neurobiology, ETH Zurich, Schwerzenbach, Switzerland). Litters were weaned at postnatal day 21. Genotypes were determined by standard PCR on tail biopsies obtained within 10 days after weaning on postnatal day 21 as previously described (Yee et al., 2006). At the age of 11 weeks, the animals were transferred to a separate climatized animal vivarium (21±1°C, relative humidity at 55±5%) under 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 constant ad libitum water and food (Kliba 3430, Klibamuhlen, Kaiseraugst, Switzerland). The ‘adult’ mice were approximately three-months old at the time of testing, comprising 5 male and 6 female mutant mice, and 4 male and 5 female littermate controls. The ‘aged’ mice were approximately 22-24 months of age at the time of testing, and comprised 5 male and 4 female mutant mice, and 7 male and 6 female littermate controls. Animals from both age groups were tested together in a single experiment. All tests were conducted in the dark phase of the light-dark cycle. The experimental manipulations and procedures described here had been previously approved by the Swiss Cantonal Veterinary Office, in accordance to the ethical standards required by the Swiss Act and Ordinance on Animal Protection and the European Council Directive 86/609/EEC, which are in accordance with the NIH Guide for the Care and Use of Laboratory Animals (1996).

The animals were subjected to the three behavioural tests as described below in the order of their implementation.
Elevated plus maze test of anxiety

General anxiety can be affected by senescence and can therefore be a potential confounding factor in the assessment of learned fear. In order to evaluate this, the elevated plus maze test was used as an ethologically relevant test of anxiety, induced by exposed spaces and height. The apparatus has been fully described elsewhere (Yee et al., 2004). Briefly, it consisted of a set of four equally spaced arms (30 cm long and 5 cm wide) joined to a central square platform (5 cm × 5 cm); two opposing arms were exposed and unprotected whilst the other two arms were protected by opaque side walls and an end wall at the distal end of the arm. The test began when the animal was released into the central area of the maze with its head facing an open arm, and allowed to explore freely for 5 min before being removed and returned to the home cage. A digital camera mounted above the maze captured and transmitted images at a rate of 5 Hz to a PC running the Ethovision tracking system (Noldus Technology Wageningen, The Netherlands). This allowed the computation of two standard anxiety-related measures: (i) percent time spent in the open arms = time in open arms / time in all four arms × 100%, and (ii) percent number of entries into open arms = number of open arm entries / number of all arm entries × 100%. In addition, locomotor activity was indexed by the total distance travelled on the entire maze surface during the test period. A rest period of 48 h was allowed before the assessment of the next test.

Open field test of spontaneous locomotor activity

Spontaneous activity can also be altered with senescence and become another potential confound in the measurement of immobility in the subsequent conditioned freezing experiment. This was assessed next in an open field. Four identical 40 × 40 cm² white open field arenas with 35 cm high sidewalls and a water-proof inlay were used. The animals were tested in squads of four, under diffused dim lighting at 30 lux. Allocation to the four test arenas was counterbalanced. Each mouse was gently placed in the centre of the appropriate open field and allowed to explore undisturbed for 1 h. The open fields were cleansed with water and dried after each animal. Locomotor activity was indexed by spatial displacement tracked by the EthoVision® tracking system (Version 3.1, Noldus Technology, Wageningen, The Netherlands), expressed as distance travelled (in m) across successive 10-min bins. The conditioned freezing was assessed 4 days after the open field test.

Conditioned freezing paradigm

The apparatus comprised two distinct sets of test chambers (4 per set), as fully described elsewhere (Meyer, Feldon, Schedlowski, & Yee, 2005). All chambers were equipped with a grid floor composed of stainless steel rods spaced at 10-mm intervals, and through which scrambled
Chapter 2: GlyT1 in the aging brain

electric shocks (unconditioned stimulus, US) could be delivered (Model E13-14, Coulbourn Instruments). C57BL/6 mice are known however to suffer from a progressive deterioration in hearing that begins at about 2 months of age and significant hearing impairment is expected by the age of 18 months (Walton, Barsz, & Wilson, 2008). A clear hearing loss in the aged mutant and control mice was confirmed by the presence of a pronounced deficit in the acoustic startle response in comparison to the adult mice (data not shown). Hence, the use of an auditory conditioned stimulus would have been inappropriate in this test, and instead a tactile stimulus in the form of a high-frequency vibration of the grid floor was used as the conditioned stimulus (CS). This was achieved by fixing a commercial vibrating unit (MiniVibrator Model 558095, Orion Versand AG, Buchs, Switzerland) to the grid floor (Yee, Singer, Chen, Feldon, & Boison, 2007). Transmission of the sinusoidal vibration beyond the grid floor was dampened by insulating the grid from the test chamber floor on four pieces of shock-absorbing sponge. A constant background white noise (60 dB) was provided via a loudspeaker mounted inside each chamber to mask the weak noise emitted from the vibrating unit. A digital camera was mounted 30 cm directly above the area of interest in each chamber. Digital images were captured at a rate of 1 Hz and transmitted to a PC running the Windows XP (SP3) operating system via a PicoloTM frame grabber (Euresys s.a., Liège, Belgium). An image analysis algorithm originally described by Richmond et al (1998) was used to determine freezing (immobility) in real-time. The image analysis was performed using Open eVision 1.1 (Euresys s.a., Liège, Belgium) under the control of a customized Microsoft Visual Basic (version 6) script.

On day 1, the animals received two discrete trials of CS-US pairing. Each trial comprised a 30-s CS followed immediately by a 1-s foot-shock set at 0.25mA. Each trial was preceded and followed by a 180s interval. On day 2, the animals were returned to the same chambers and observed for a period 480s in the absence of any discrete stimulus to assess conditioned freezing to the training context. On day 3, the animals were placed in a novel and distinct conditioning chamber to measure the conditioned freezing response specific to the CS. Following an initial 120-s acclimatization period, the CS was presented continuously for 480s. Freezing behaviour during the pre-CS and CS periods were separately evaluated. A second CS test was repeated 24h later (i.e., on day 4). After successful demonstration of the retention of extinction from the first to the second CS tests (i.e., between-days extinction), we went on to examine spontaneous recovery (re-emergence of an extinguished CR) by conducting a third CS test, 7 days later. Given evidence for an extinction phenotype in our mutant mice, the test of spontaneous recovery allowed the examination of the dissipation of the inhibitory mechanism implicated in extinction learning.
Neuroanatomy
Due to advanced age 3 animals (1 female control and 2 male mutant aged mice) died in the week following completion of behavioural testing. The remaining animals were deeply anesthetized with sodium pentobarbital (Nembutal®; 40 mg/kg, i.p.) and then perfused transcardially: first with 0.9% NaCl saline, followed by a cold 0.15M phosphate buffered (pH 7.4) fixative solution containing 4% paraformaldehyde and 15% saturated picric acid solution. Perfusion of one animal (an adult female mutant) was not satisfactory and as a result the brain was not further processed. The brains extracted from 19 adult mice (control male n=4, control female n=5, mutant male n=5, mutant female n=5) and 19 aged mice (control male n=7, control female n=5, mutant male n=3, mutant female n=4) were post-fixed for 24h before being subjected to microwave-assisted fixation as previously described (Fritschy, Weinmann, Wenzel, & Benke, 1998). The brains were then immersed in 30% sucrose solution for cryoprotection until sectioning. Eight series of free-floating 30µm thick coronal sections, spanning approximately from 2.22mm to 7.08mm anterior to bregma, were cut using a freezing microtome. The sections were then kept at -20°C until immunohistochemical processing.

Immunohistochemistry for each marker was performed on one series of sections per animal. The following primary antibodies were used: goat anti-doublecortin (DCX) (1:1000; Santa Cruz Biotechnology, Santa Cruz, USA), rabbit anti-cleaved Caspase-3 (1:600; Cell Signaling Technology, Beverly, MA), rabbit anti-NMDA Receptor subunit type 1 (NR1) (1:4000; ABR, USA), rabbit anti-NMDA Receptor subunit type 2B (NR2B) (1:1000; ABR, USA). Sections destined for NR1 and NR2B immunostaining were first rinsed in Tris buffer for 10 min x 3, whereas sections destined for Caspase-3 and DCX immunostaining were similarly washed in phosphate buffered saline. Endogenous peroxidases were blocked via incubation in a phosphate buffer containing 10% (v/v) of 33% H2O2 for 10 min. Next, the sections were incubated in 0.3% Triton X-100 containing 10% normal serum for 1h at room temperature (RT). The appropriate primary antibodies were then diluted in a buffer containing 0.3% Triton X-100 and 2% normal serum. The sections were incubated overnight at RT (DCX, caspase3) or 4°C (NR1 and NR2B). After three washes in buffer solution (10 min each), the sections were incubated for 1h with the biotinylated secondary antibody diluted 1:500 in a buffer containing 2% normal serum and 0.3% Triton X-100. Sections were washed three times for 10 min, and then incubated with the Vectastain kit (Vector Laboratories, Burligame, CA) diluted in the same buffer for 1h. After three rinses in 0.1M Tris-HCl buffer (pH 7.4), the sections were stained with 1.25% 3,3-diaminobenzidine (DAB, Fluka, Buchs, Switzerland) and 0.08% H2O2 in 0.1 M Tris-HCl (pH 7.6), for 2–15 min. The DAB reaction was terminated by three washes in Tris-HCl. The sections were then mounted onto gelatine-coated slides, air dried overnight, dehydrated and coverslipped with Eukitt™ (Kindler GmbH & Co, Freiburg, Germany).
All subsequent quantifications were performed blind to the genotype and age of the animals.

**Stereological quantification of DCX-ir and Caspase 3-ir cells**

Immunoreactive (ir) cells, identified by the presence of DAB staining in the cell nucleus, were counted in one randomly chosen hemisphere using the optical fractionator method (Gundersen et al., 1988). Every section within a randomly chosen 1:8 series was counted, allowing an average of five sections per animal spanning approximately from 1.22 to 2.30 mm posterior to bregma encompassing the dorsal hippocampus. Sampling was performed with a fixed counting frame with a width of 30 μm and a length of 30 μm; and a sampling grid size of $200 \times 200 \mu m^2$ in the x-y plane. Counting was performed using a 40× oil lens (N.A. 1.30). Delineation of the region of interest (ROI) was performed under live microscopy (Zeiss Axioplan microscope) with a 2.5× objective (N.A. 0.075). Volume estimates were performed with the assistance of StereoInvestigator (version 6.50.1, Microbrightfield, Colchester, VT, USA), and application of the Cavalieri method with the formula

$$V = \frac{\sum A \times t_{nom}}{ssf}$$

where $\sum A$ = the summed areas of ROIs across sections (computed by StereoInvestigator), $t_{nom}$ = the nominal section thickness of 30 μm, and $ssf$ = the section sampling fraction (1/8). The ROI for the quantification of doublecortin-ir cells was restricted to the subgranular zone (SGZ) of the dentate gyrus, whereas the ROI for caspase-3-ir cells included both dentate gyrus and Ammon’s horn (subfields CA1/2 and CA3). All counts were expressed in units of cell per mm$^3$.

**Densitometry**

Four to six coronal sections, extending across the prefrontal cortex, amygdala or the dorsal hippocampus, per animal were analyzed using a Zeiss Axioplan microscope equipped with a 2.5× objective. Digital images of NR1 or NR2B immuno-stained sections were acquired (Axiocam Zeiss, Germany), with the exposure times held constant during acquisition such that pixel brightness was never saturated. Densitometry measurement was then obtained in one randomly selected hemisphere by ImageJ software (National Institutes of Health, Bethesda, MD, USA). For the amygdala, pixel brightness in the basolateral nucleus (BLA) and the central nucleus (CeA) was separately measured. For the prefrontal cortex, cingulate cortex (Cg1), the prelimbic cortex (PrL) and the infralimbic cortex (IL) were separately analyzed. For the dorsal hippocampus, pixel brightness was measured in CA1/2 stratum radiatum and CA3 stratum lucidum (see Figure 5). In each case, two to three non-overlapping $100 \times 100 \mu m^2$ square grids
were placed randomly in the area of interest, and the background-corrected optical densities (pixel brightness in the region of interest minus pixel brightness of the background) were computed and averaged.

**Statistical analysis**

All data were analyzed by parametric analysis of variance (ANOVA) using the between-subject factors genotype and age. Initial preliminary analyses also included the between-subject factor sex, but there was no evidence for any interaction effect involving sex. This factor was therefore dropped in the final analysis to increase statistical power. Additional within-subjects factors were included as appropriated by the dependent variables in question. Supplementary restricted analyses were also conducted to assist interpretation of statistically significant effects. 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 (SP3) operating system. Because the doublecortin data set deviated substantially from the homoscedasticity assumption of parametric ANOVA, a non-parametric ANOVA was performed on the rank-transformed data (Conover & Iman, 1981).

**RESULTS**

**Anxiety and spontaneous activity**

Anxiety-like behaviour in the elevated plus maze was indexed by the animals' reluctance to venture into the open arms relative to the enclosed arms. This was expressed by the percent time spent in the open arms, or the proportion of arms entries into the open arms (Table 1). Separate $2 \times 2$ (age $\times$ genotype) ANOVAs of the two measures did not yield any significant effect [F's$<1$]. Thus, neither genotype nor age significantly modified the expression of anxiety. Similarly, separate analysis of the total distance travelled in the maze was analysed, which also did not yield any significant effects [F's$<1$] (see Table 1), thus confirming that the test was not confounded by possible group differences in spontaneous locomotor activity.
Table 1: Anxiety-like behaviour in the elevated plus maze (EPM) as indexed by (i) percentage of entries made into the open arms, and (ii) percentage of time spent in the open arms. Spontaneous locomotor activity was indexed by total distance travelled in the elevated plus maze. All values refer to mean ± SEM.

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<tr>
<th>Measure</th>
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<tr>
<td>Percent open arms entries</td>
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<td>Percent time in open arms</td>
<td>21.5±6.0</td>
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<td>Percent distance in open arms</td>
<td>14.6±4.0</td>
<td>24.6±5.1</td>
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Locomotor activity in EPM

| Distance moved [m]   | 6.9±0.7 | 6.5±0.6 | 5.9±0.3 | 6.1±0.8 |

Locomotor activity was further evaluated in the open field for a longer period of time sufficient for the assessment of locomotor habituation effect. Habituation was evident from the monotonic reduction of activity over the course of the 60 min period – an effect that was equivalently seen in all groups (see Figure 1). The overall level of activity was highly comparable across genotype as well as age groups. A $2 \times 2 \times 6$ (age $\times$ genotype $\times$ 10-min bins) ANOVA of distance travelled only yielded a significant effect of bins [F(5,190)=101.3, p<0.001].

Potential difference in general anxiety or baseline locomotor activity might confound the measure of conditioned fear in the form of freezing in the subsequent conditioned freezing experiment. These were excluded by these two separate tests.
Conditioned freezing

Here, we focused on the conditioned freezing paradigm since this test revealed the most robust promnesic effect in adult mutants (Yee et al., 2006). On the day of conditioning, the level of freezing generally increased over successive periods of inter-trial intervals (ITIs, Figures 2A). A similar increase from the first to the second CS presentation in each CU-US pairing was observed in the adult but not the aged mice (Figure 2A). Separate split-plot ANOVAs of freezing recorded during ITIs and during CS presentations yielded only a main effect of ITI periods [F(2,76)=55.07, p<0.001].

Conditioned freezing to the context was tested 24h later, and the levels of freezing obtained did not reveal any effect of genotype [F(1,38)=1.78, p=0.20]. However, there was an increase in freezing level over the course of the 8-min test that was apparent only in the adult but not aged mice (Figure 2B). A 2 × 2 × 2 (genotype × age × half-session) ANOVA of percent freezing revealed a significant age by half-session interaction [F(1,38)=4.39, p<0.05].
Next, conditioned freezing to the CS was assessed in two extinction tests conducted across consecutive days in a neutral context. Each test began with a 2-min pre-CS period allowing the evaluation of baseline freezing levels. Analysis of Pre-CS freezing by a $2 \times 2 \times 2$ (age $\times$ genotype $\times$ days) ANOVA did not reveal any clear significant difference between the two ages [$F(1,38)=1.66, p=0.20$] or genotypes [$F(1,38)=4.07, p=0.05$]. Next, the tactile CS was presented for 8 min. The overall freezing response to the CS was stronger in the mutant mice compared with the moderate freezing levels seen in the controls (Figures 3G, 3J). However, the time course of the freezing response to the CS markedly differed between adult and aged mutant subjects (Figure 3A). 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 as a result of within-session extinction (Figure 3A) – a profile that was similarly seen on both test days. In contrast, the temporal profile of the aged mutants’ freezing response remained relatively stable over time (Figure 3A) – suggesting weaker extinction within a test session compared with adult mutants. The contrasting profile is more readily discernable in Figure 3B, which shows the within-session profile averaged across the two consecutive test days. While within-session extinction appeared weak in aged mutants, between-day extinction was still evident in all groups.

A $2 \times 2 \times 2 \times 4$ (genotype $\times$ age $\times$ days $\times$ 2-min bins) ANOVA of percent freezing revealed a significant effect of genotype [$F(1,38)=7.06, p<0.05$], supporting the overall presence of enhanced conditioned freezing to the CS in the mutants regardless of age. Additionally, the presence of a significant bins [$F(3,114)=7.00, p<0.001$] and days effects [$F(1,38)=21.35,$]
p<0.001] reflected the overall presence of both within- and between-day extinction, respectively. Lastly, evidence for age-dependent phenotypes was revealed when within-session freezing over bins was examined, as supported by the presence of a significant genotype × age × bins interaction [F(3,114)=3.85, p<0.05] (Figure 3H and 3K). Orthogonal contrast analysis indicated that this interaction was predominantly attributable to the linear component of bins [F(1,38)=5.10 p<0.05], which accounted for 80% of the variance explained by the three-way interaction. We therefore calculated the linear component for each group for additional post-hoc comparisons. A downward sloping trend was detectable in all groups, and their mean (±SEM) magnitudes are as follows, adult control = 0.63±0.98, adult mutant = 4.17±0.89, aged control = 0.99±0.82, aged mutant=0.38± 0.98. The significantly higher value for the adult mutant [maximum p=0.01] than the other three groups (Figure 3C and 3F or 3I and 3L), which did not differ from each other, suggested that the linear rate of extinction within-session was the highest in the adult mutants.

Spontaneous recovery of conditioned CS freezing was evaluated by a 2 × 2 × 2 × 4 (genotype × age × days × 2-min bins) ANOVA that allowed a direct comparison of the animals’ freezing response exhibited on the CS tests conducted before and after the 7-day retention period. As shown in Figure 4A, the CR recovered to a somewhat higher level, and mutant mice regardless of age, again, exhibited a stronger response than controls. The analysis yielded a significant effect of days suggestive of a spontaneous recovery effect [F(1,38)=8.36, p<0.01] as well as a significant genotype effect [F(1, 38)=8.02, p<0.01] (Figure 4B). Although aged mutant mice once again appeared to exhibit a weaker within-session extinction profile on this test, there was no evidence for a significant genotype by bins interaction [F<1], which might be due to the lack of a strong overall within-session extinction effect in this test, as suggested by the lack of a statistically significant bins effect [F(3,114)=1.63, p>0.05].

Figure 3 (next page): Freezing to the vibration CS in the first and second CS test sessions (conducted 48 h and 72 h after conditioning, respectively) is depicted in two perspectives: (a) comparison of age of each phenotype, and (b) comparison of genotypes at each age. They are demarcated by the horizontal line in the middle. A, D, G, and J depict the percent time freezing across successive 2-min bins (including the pre-CS bin denoted as “P*” in the x-axis) on the two separate CS tests. B, E, H, and K represent the same data when collapsed across the two test sessions. Following the emergence of the significant Genotype × Age × Bins interaction which was predominantly attributable to the linear trend, the linear components of the Bins effect (collapsed across test sessions) in each group was calculated and depicted in C, F, I, and L. Post-hoc comparisons based on the appropriate error mean square terms taken from the overall ANOVA were performed at successive bins and significant pair-wise differences are denoted by asterisks in the appropriate graphs. All values refer to mean ± SEM.
Figure 4: Freezing to the CS 7 days after the last CS-test (A) and overall freezing for the 3 CS-test (B) are depicted. Percent time freezing is indexed as a function of 2-min bins including the 2 minutes of pre-CS. Sp refer to the CS 7 days after the last CS-test. All values refer to mean ± SEM.

**Immunohistochemistry**

*Visualization of apoptosis by Caspase-3 immunohistochemistry*

The possible effect of forebrain neuronal GlyT1 knockout on apoptotic cell death was assessed by caspase-3 immunostaining. Caspase-3 is a cysteine protease and a key degradative enzyme involved in apoptosis (Rami, Jansen, Giesser, & Winckler, 2003). Quantification of caspase-3-ir cells did not reveal any difference between groups. The mean numbers of cells per unit volume (±SEM) were: control: adult=7.67 ± 2.7, aged=7.55 ± 2.36; mutant: adult=4.42 ± 2.46, aged=8.95 ± 3.09. A 2 × 2 (age × genotype) ANOVA of the number of the caspase-3-ir cells in the granular cell layer of the DG in the dHPC revealed neither a significant main effect of genotype and age nor its interaction.

*NR1 and NR2B immunohistochemistry*

None of the measurements revealed any genotype effect (see Table 2). Separate 2 × 2 (age × genotype) ANOVAs (per marker per region) only yielded a main effect of age in the analysis of CA3 NR2B immunodensity [F(1,35)=7.74, p<0.05]. Representative photographs taken from each group are illustrated in Figure 5.
**Table 2:** Summary of the relative optical densities of NMDA receptor subunit 1 (NR1) and NMDA receptor subunit 2B (NR2B) in the subregions of the dHPC (CA1/2 and CA3), Amygdala (BLA and CeA) and Prefrontal cortex (Cg1, PrL and IL) of adult and aged mutant and control mice. Values refer to mean ± SEM.

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Visualization of immature neurons by doublecortin immunohistochemistry

Evaluation of the stereological estimates by a non-parametric ANOVA revealed a clear reduction in the number of DCX-positive cells in the aged animals as expected [F(1,34)=107.07; p<0.05] in comparison with the adults (Figure 6). Importantly, a significant genotype effect was also detected [F(1,34)=4.47; p<0.05] suggesting an increase in DCX-positive cells in the mutant mice. This effect did not appear to be significantly affected in senescence [Genotype x Age interaction: F(1,34)=0.36, p=0.55] in spite of the substantial age-related decrease.
Figure 6: Representative photomicrographs of doublecortin-positive (DCX) immunostaining (scale bar = 200 µm). Separate box-plots showing the stereological estimates of DCX-positive cells per unit volume in the dentate gyrus granule cell layers. Each box plot captures the data within the inter-quartile range in the box. The group median is represented by the horizontal line inside each box. The upper and lower whiskers represent the boundaries of the largest and lowest values within each group. All data refers to the original untransformed data set, and their depiction is appropriate for the non-parametric (Genotype × Age) ANOVA performed, which yielded a significant main effect of Genotype and of Age.

DISCUSSION

The present study replicated the robust effect of enhanced CS-freezing in young adult mice by GlyT1 deletion in forebrain neurons, and confirmed that this phenotype could also be detected in senescent age. As previously shown, this phenotype cannot be attributed to confounding changes in general anxiety or locomotor activity at either age (Yee et al., 2006). However, examination of the temporal expression of the conditioned freezing response revealed clear differences between adult and aged mutants, while the freezing response was hardly modified by age in the control mice (Figures 3D and 3E). Thus, senescence had modified the behavioural effects of forebrain neuronal GlyT1 deletion. Parallel immunohistological examinations did not reveal any obvious changes in apoptosis in the hippocampus or NR1 and NR2 expression in multiple limbic cortices in the mutant mice. Surprisingly, we observed a pronounced
enhancement of neural proliferation in the mutants which seemed to persist into senescence despite the drastic reduction due to aging.

**The impact of aging in the mutants**

Across the first two CS-test sessions, the temporal profile of CS-freezing in adult mutants is marked by (i) pronounced elevation of freezing close to CS-onset, and (ii) rapid within-session extinction across the continuous 8-min presentation of the CS, with freezing levels returning to adult controls’ levels by session’s end (see Figure 3G). The former was most clearly seen on the first CS-test day, whereas the latter persisted into the second and third test sessions albeit with a weaker impression (see Figures 3G & 4A). In comparison with adult mutants, the initial response near CS-onset was weaker in aged mutant mice, which also lacked the within-session extinction phenotype seen in the adult mutants, such that aged mutants exhibited stronger freezing than the other groups by session’s end on the first two CS-test sessions (see Figure 3J). However, the lack of within-session extinction in the aged mutants might not be considered as a phenotype since within-session extinction was weak in control mice at both ages (see Figure 3D). The latter might be attributed to the reduced number of CS-US pairings adopted in this experiment, but it did not undermine the comparison between adult and aged mutants (Figures 3A, 3B and 3C), which indicated the loss of an important aspect of the adult CS-freezing phenotype, suggesting a (qualitative) diminution of the adult phenotype.

While there was statistical evidence for a within-session extinction phenotype that was affected by age, between-day extinction did not seem to be affected by GlyT1 deletion or age. Long-term extinction learning is believed to involve new learning, which acts to inhibit expression of the conditioned response (CR; Wagner, 1981; Wagner & Brandon, 2001). Dissipation of such inhibition in time revealed the spontaneous recovery effect (Rescorla, 2004) and confirmed that extinction did not lead to the erasure of the original CS-US memory trace. Intact retention of extinction learning from the first to the second CS-tests sessions (separated by 24h), and the re-emergence of the CR seven days later, suggest that the mechanism underlying the control of new learning that underlies long-term extinction was relatively unaffected by GlyT1 deletion. What might be unique to within-session extinction in our paradigm?

**Within-session extinction**

Apart from new learning that directly competes or impedes CR during extinction, an independent process has been proposed by Wagner and Brandon (2001). This could be especially relevant to our test paradigm here, whereby the CS was presented continuously for a protracted period (Haselgrove & Pearce, 2003). According to Wagner & Brandon’s model,
prolonged exposure of a CS is expected to reduce its efficacy to evoke a CR, because perception of a stimulus steady in time and intensity is expected to first intensify (temporal integration) and then decline (adaptation). The model attributes this to a progressive shift of the stimulus representation from a primary active (A1) state to a secondary refractory (A2) state. When the CS representation is at the A2 state, its ability to activate the US representation is weakened and so is the evocation of the associated CR. However, when the CS representation departs from the A2 state and returns to its inactive baseline state, it can be readily re-activated to the A1 state when the stimulus reappears, thus again capable of recalling the US and therefore expression of the CR. This process therefore does not contribute significantly to the cessation of CR between-days. Thus, when the CS was presented in a test session the transition of the CS representation from A1 to A2 states might be facilitated in our adult mutant mice, but this facilitation might be selectively lost when they reached senescence.

This theoretical discourse would lead to the interesting prediction that associative learning might be weaker rather than stronger in the adult mutants (compared with adult controls) when the CS is extended in time during acquisition. It would predict that their CR would be weaker than controls when conditioned with an extended CS, but stronger with shorter CS. Thus far, we have obtained evidence that when CS and US are separated in time (i.e., trace conditioning) our mutant mice exhibited weaker freezing than control mice, which might be supportive of this interpretation (Singer et al., unpublished data). The apparent enhanced sensitivity to CS-US temporal discontinuity might again stem from a facilitated transition of the CS representation from A1 to A2 state.

The account based on Wagner’s model shares some similarity with the attentional theory by Mackintosh (1974, 1975), which predicts that attention to a CS depends on its predictiveness of significant events (US’s). Thus, attention to the CS would grow during acquisition but should decay during extinction. The decline in CR during extinction may then be attributed also to inattention to the CS. Indeed, such inattention to the CS is critical to the expression of latent inhibition (Lubow & Moore, 1959), which is facilitated also by forebrain neuronal GlyT1 deletion (Yee et al., 2006). The present results might predict that the latent inhibition phenotype may be similarly attenuated as a function of age. This certainly warrants verification as it might bear implication on the proposed use of GlyT1 inhibitors as antipsychotic drugs (Javitt, 2009).

**Effects in the brain**

Consistent with our previous western blot analysis (Yee et al., 2006), NR1 expression in the hippocampus of our mutant mice remained unaltered. We extended this null effect here to other
brain structures, namely, the amygdala and prefrontal cortices. Likewise, NR2 expression was unaffected, including hippocampal NR2 expression which was particularly sensitive to aging (Magnusson, Nelson, & Young, 2002). These null findings collectively not only speak against compensatory changes in the NMDAR system and increases in NMDAR internalization resulting from excessive priming by extracellular glycine, but also agree with the lack of changes in apoptosis observed in the hippocampus. Hence, there is little evidence here in support of the concern that forebrain neuronal GlyT1 deletion might induce neurotoxicity and/or undermine the integrity of NMDAR transmission in senescent mice. Indeed, increased activation of neuronal hippocampal glycine receptors (Xu and Gong, 2010) by elevated glycine might exert neuroprotective effects. Further investigations within and beyond the glutamatergic neurotransmission system are warranted to ascertain if loss of forebrain neuronal GlyT1 might accelerate the aging process in the brain. Similarly, examinations of caspase-independent apoptotic pathways would also be instructive (Abraham & Shaham, 2004).

Without any significant impact on NR1 or NR2 expression, the novel finding of enhanced hippocampal neural proliferation was revealed in our mutant mice. This was an unexpected finding since enhanced neurogenesis is linked to NMDAR blockade, and NMDAR activation to decreased neurogenesis (Cameron, McEwen, & Gould, 1995). This novel finding revealed here using doublecortin immunohistochemistry might be suggestive of an increase in the rate of neurogenesis, cell differentiation or survival/integration of new neurons (Li, Mu, & Gage, 2009), and additional markers (e.g., BrdU) are required to distinguish between these possibilities. Nonetheless, we have since replicated this finding in behaviourally naïve mice (S. Dubroqua, O. Raineteau, J. Feldon & B.K. Yee, unpublished data), thus excluding the possibility that prior behavioural testing was responsible for this new finding.

Although outside the scope of the present study, one may question the possible functional relevance of the neurogenesis phenotype. It might be tempting to link this to the conditioned freezing phenotype reported here as well as other learning-related phenotypes identified in this mutant mouse line thus far (Singer et al., 2007, 2009; Yee et al., 2006). Such speculations however would be premature given that the cause-and-effect relationship between neurogenesis and learning remains uncertain (Leuner, Gould, & Shors, 2006). Instead, this transgenic mouse model might be useful in assessing the impact of enhanced neurogenesis on learning, by identifying those phenotypes that might be eliminated specifically by neurogenesis suppressing treatments (e.g. Deng, Saxe, Gallina, & Gage, 2009; Winocur, Wojtowicz, Sekeres, Snyder, & Wang, 2006).
Conclusions
The present study has dispelled some concerns over possible negative side effects of long-term GlyT1 down-regulation, but also identified clear modification of a robust behavioural phenotype previously documented in the adult mutants with forebrain neuronal GlyT1 deletion. These are especially relevant to the potential use of GlyT1 inhibiting drugs in the context of age-dependent cognitive decline. The general importance of assessing genetic effects over the course of life is also highlighted by the present study.

REFERENCES
Chapter 2: GlyT1 in the aging brain


CHAPTER 3

Examining the sex- and circadian dependency of a learning phenotype in mice with glycine transporter 1 deletion in two Pavlovian conditioning paradigms

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

Submitted to Neurobiology of Learning and Memory
ABSTRACT

Behavioural characterisation of transgenic mice has been instrumental in search of therapeutic targets for the modulation of cognitive function. However, little effort has been devoted to phenotypic characterisation across environmental conditions and genomic differences such as sex and strain, which is essential to translational research. The present study is an effort in this direction. It scrutinised the stability and robustness of the phenotype of enhanced Pavlovian conditioning reported in mice with forebrain neuronal deletion of glycine transporter 1 by evaluating the possible presence of sex and circadian dependency, and its consistency across aversive and appetitive conditioning paradigms. The Pavlovian phenotype was essentially unaffected by the time of testing between the two circadian phases, but it was modified by sex in both conditioning paradigms. We observed that the effect size of the phenotype was strongest in female mice tested during the dark phase in the aversive paradigm. Critically, the presence of the phenotype in female mutants was accompanied by an increase in resistance to extinction. Similarly, enhanced conditioned responding once again emerged solely in female mutants in the appetitive conditioning experiment, which was again associated with an increased resistance to extinction across days, but male mutants exhibited an opposite trend towards facilitation of extinction. The present study has thus added hitherto unknown qualifications and specifications of a previously reported memory enhancing phenotype in this mouse line by identifying the determinants of the magnitude and direction of the expressed phenotype. This in-depth comparative approach is of value to the interpretation of behavioural findings in general.

INTRODUCTION

The search for genetic control and regulation of cognitive functions has extensively relied on the use of engineered mouse models in which selected genes and their products are modified. These are valuable and powerful translational tools that are widely available. Characterization of the behavioural and cognitive phenotypes associated with engineered genes deletions or mutations represents an important step in the proof of concept, and is an essential component in the preclinical evaluation of potential drug targets. It is of utmost importance therefore to gauge the robustness and generality of any specific behavioural phenotypes. Often, initial findings are based on a single test paradigm and interpreted without reference to possible sex dependency or environmental modulation. However, phenotypic expression can be labile and dependent on sex (Caldarone et al., 2010; Wiltgen et al., 2005; Yee et al., 2004) or circadian phase (Hossain et al., 2004; Marques & Waterhouse, 1994) even within the same mouse strain. Ignoring such factors can result in a simplistic and biased interpretation which may be counterproductive to translational research. Zucker & Beery (2010) have pointed out the limitations associated with the predominant use of males in animal studies, and several studies have emphasised the ethological inappropriateness of conducting behavioural tests on rodents during the light phase,
which corresponds to their inactive phase (Beeler et al., 2006; Hossain et al., 2004; Marques & Waterhouse, 1994; Roedel et al., 2006).

Generalization across test paradigms is often overlooked. The emphasis on high-throughput approaches inadvertently favours the sole reliance on the most efficiently implemented tests, such as the preferential use of conditioned freezing for the assessment of Pavlovian associative learning. Indeed, the origin of Pavlovian associative learning was predominantly based on appetitive procedures, which obviously shares common associative learning mechanisms with aversive procedures (Pavlov, 1927). Yet, they also diverge significantly in terms of the motivational significance of the unconditioned stimulus (Konorski, 1976). In the past, the extension from aversive to appetitive Pavlovian paradigms has enabled us to clarify the nature of a sex-dependent learning phenotype in genetically modified mice (Yee et al., 2004). Such between-paradigm comparison may also be instructive in revealing underlying mechanisms (Austin & Duka, 2010) and brain regions involved (Knapska et al., 2006).

The present study provided the necessary scrutiny of a procognitive phenotype in Pavlovian learning identified in mice carrying a genetic deletion of glycine transporter 1 in forebrain neurons (GlyT1∆FBNeuron). This phenotype is presumably attributed to enhanced N-methyl-d-aspartate receptor (NMDAR) function due to elevated synaptic availability of glycine, an obligatory co-agonist of NMDAR activation. This has been taken as support of the general thesis that NMDAR-dependent neural plasticity is central to learning and memory (Morris, Anderson, Lynch, & Baudry, 1986; Morris, 1989), and the suggestion that its augmentation could be beneficial for cognitive deficiency in a number of psychiatric disorders (Black et al., 2009; Depoortere et al., 2005; Ingram et al., 1996; Martin, Grimwood, & Morris, 2000; Singer et al., 2009). However, although the initial report of an enhanced Pavlovian conditioning phenotype in GlyT1∆FBNeuron mice has been demonstrated across three conditioning paradigms, the paradigms were all aversive in nature and the reported data were exclusively derived from female subjects maintained in a reversed light-dark cycle to allow testing in the dark phase (Yee et al., 2006). Although there is evidence that this phenotype is present in both sexes and persisted into old age (Dubroqua et al., 2010), some data suggested that the phenotype might be somewhat weaker statistically in the male sex (Philipp Singer, personal communication).

The present study therefore explicitly examined the possibility of a sex-dependent phenotypic expression and further incorporated the time of testing (light vs. dark phases) as an additional factor. This design was repeated across two separate experiments for comparison between aversive (conditioned freezing) and appetitive (conditioned approach response) conditioning paradigms. Spontaneous locomotor activity, anxiety-related behaviour, and shock
sensitivity were assessed in order to control for potential confounding effects to assist interpretation of the conditioning results.

**METHODS**

**Subjects**

All mice were derived from a pure C57BL/6J background as fully described before (see Yee et al., 2006). The experimental subjects were bred by pairing CamKIIαCre;Glyt1tm1.2fl/fl mice with Glyt1tm1.2fl/fl mice to yield litters of an expected 1:1 ratio of CamKIIαCre;Glyt1tm1.2fl/fl (carrying one copy of CamKIIα-driven Cre-expression with homozygously floxed GlyT1 gene) and Glyt1tm1.2fl/fl (with only homozygously floxed GlyT1 gene and no Cre expression) genotypes. The former CamKIIαCre;Glyt1tm1.2fl/fl genotype is referred to as GlyT1AFBNeuron and denoted as the "mutant", with the latter Glyt1tm1.2fl/fl genotype serving as comparison "control" littersmates. Breeding took place in a specific-pathogen free (SPF) breeding facility (Laboratory of Behavioural Neurobiology, ETH Zurich, Schwerzenbach, Switzerland), and litters were weaned and sexed on postnatal day 21. Genotypes were determined by standard PCR on tail biopsies obtained within 10 days after weaning as previously described (Yee et al., 2006).

All manipulations and procedures described here had been previously approved by the Swiss Cantonal Veterinary Office as required by the Swiss Act and Ordinance on Animal Protection, which conforms with the ethical standards stipulated in the European Council Directive 86/609/EEC and the NIH publication no. 86-23 (revised 1985) on animal experimentation.

**Housing under different circadian cycle**

At the age of 11 weeks, a cohort of 87 mice (cohort A in Table 1) was subdivided into two balanced groups, with respect to genotype and sex, for separate housing between two identical climatized (21±1°C, relative humidity at 55±5%) vivaria differing only in their circadian rhythm. One vivarium was maintained under a "normal" 12/12h light-dark cycle (lights off: 1900–0700h), while the other was under a "reversed" light-dark cycle (lights off: 0700–1900hrs). It thus allowed the direct behavioural comparison between light and dark phases in a between-subjects manner. All behavioural experiments were conducted between 0800 and 1800 h, thus falling within the light phase of animals kept in the "normal" cycle and the dark phase of those kept under the "reversed" cycle. Henceforth, we refer to this between-subject factor as "light-dark phase". Mice of the same sex were housed in groups of 4 to 6 in Makrolon® Type-III cages (Techniplast, Milan, Italy) with constant provision of water and food (Kliba 3430, Klibamuhlen,
Kaiseraugst, Switzerland) unless otherwise specified. The mice were approximately 12-16 weeks old at the time of testing.

Four cages (one for each genotype/sex group) each housing four mice of the same sex and genotype, were arranged in each animal vivarium; and a miniature digital camera operating in the visible and infrared spectrum was mounted above the grid cage to allow monitoring of home cage activities. Video outputs of the four cameras in each animal vivarium were fed to a multiplexer (YSQ-430, Sony, Japan) before being transmitted to a PC for digital storage and cage-by-cage analysis. Five-minute 8-bit gray-scale video footages were sampled at 30 min past every o’clock over the 11-day acclimatization period. An image analysis algorithm calculated the number of pixels changed after appropriate thresholding (Open eVision 1.1, Euresys s.a., Belgium) between successive (360 x 288 pixels) binary images 1s apart. The number of pixels changed was expressed as percentage of total pixels per frame, averaged across each hourly 5-min sample. This number was then normalized and expressed as z-scores with respect to each cage’s average second-by-second percent pixels changed across the 11-day period). For each cage the 5-h running average time series was calculated and depicted in Figure 1.

Because our mice were bred under a reversed light-dark cycle, animals allocated to the vivarium with the same circadian rhythm adapted quickly and exhibited the expected diurnal variation in activity, namely, higher in the dark phase and lower in the light phase (Figure 1A,C). In contrast, animals switched to the normal light-dark cycle experienced a shift of circadian rhythm, and therefore their previous diurnal activity pattern persisted in the first 48h. Thereafter, they underwent a period of adjustment for about two days before the emergence of a new stable diurnal activity rhythm matching the light-dark rhythm (Figure 1B,C). As shown in the average activity profiles obtained from the two rooms (Figure 1C), their activity cycles were 12-h out-of-phase with each other for the last 7-8 days prior to commencement of experiments. Because all behavioural tests were conducted between 0800 to 1800h, mice kept in the reversed cycle would be tested in the dark phase, and those in the normal cycle in the light phase. The comparison between cycles effectively served as a contrast between light and dark phases while keeping the time and conditions of testing identical.
**Figure 1:** Acclimatization to separate housing conditions with opposite light-dark (12h/12h) cycle. Activity levels were determined by imaging analysis per cage. The hourly time series data obtained from each cage lasted for 11 days. The data from each cage were normalized and expressed as z-scores (with respect to the cage’s overall average and standard deviation) and then smoothed by a moving average operator with a 5-h window (-2h to +2h). (A) shows the four cages kept under reversed cycle, and (B) the four cages in the normal cycle. The lighting conditions in each room are indicated by appropriate shading of the background (gray = dark phase, white = light phase). (C) illustrates the average profile obtained in the two vivaria. The time-shift between light and dark phases are indicated by the vertical gray lines. By the end of the acclimatization period, the diurnal activity patterns between the two housing conditions were perfectly out-of-phase with each other.

**Experimental Plan**

The cohort of 87 mice, prepared as described above, was first evaluated in an elevated plus maze test of anxiety and an open field test of locomotor activity before being split randomly into two subsets: one destined for the aversive conditioning experiment, and the other for the appetitive conditioning experiment (see Table 1). A separate cohort of naive mice (cohort B), which comprised only female mice kept in reversed light-dark cycle, was prepared especially for test of shock sensitivity.
Table 1: Number and distribution of the separate cohorts of animals used in the present study. Animals in cohort A were all tested initially in the elevated plus maze (EPM) test of anxiety and in the open field test of locomotor activity. The animals were then subdivided for Pavlovian conditioning using either an aversive (conditioned freezing) or appetitive (conditioned approach) paradigm. Cohort B was behaviourally naive at the time of testing.

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Elevated plus maze
The elevated plus maze test of anxiety has been fully described before (Hagenbuch et al., 2006). Briefly, the maze was made of acrylic and consisted of four equally spaced arms radiating from a central square measuring 5 × 5 cm. Each arm was 30 cm long and 5 cm wide. A gray removable plastic floor in-lay was placed in the entire maze surface. One pair of opposing arms was enclosed with opaque walls 14 cm high. The remaining two arms were exposed with a 3-mm-high perimeter border along the outer edges. The maze was elevated 70 cm above floor level, and positioned in the middle of a testing room with diffuse dim lighting (25 lux in the centre of the maze). A digital camera was mounted above the maze and images were captured at a rate of 5 Hz and transmitted to a PC running the Ethovision (Version 3.1, Noldus Technology, The Netherlands) tracking system. A test trial began with the mouse being placed in the central square with its head facing one of the open arms. It was allowed to explore freely and undisturbed for 5 min. The percent time in open arms = [time in open arms/time in all arms] × 100% was used to index anxiety. In addition, the total distance travelled in the entire maze (i.e., arms and central platform) was recorded.
Open Field Test for spontaneous locomotor activity

This was conducted 48 h after the elevated plus maze experiment. The apparatus consisted of four identical square arenas (40 × 40 cm) surrounded by 25 cm high walls. They were made of wood with a white waterproof plastic surface. The four arenas were arranged in a 2-by-2 configuration, located in a testing room under diffused dim lighting (25 lux). A digital camera was mounted directly above the four arenas, transmitting images to a PC running the Ethovision (Version 3.1, Noldus Technology, The Netherlands) software which tracked the animals with a temporal resolution of 5Hz. Four mice were tested simultaneously. They were placed in the centre of the appropriate arena and allowed to explore undisturbed for 60 min. Afterwards, they were returned to their home cage and the arenas were cleansed with water and dried before the next squad of four mice. Locomotor activity was indexed by distance travelled (in meter) across consecutive 10-min bins.

Pavlovian aversive conditioning

Five animals from each of the eight experimental groups were selected for this experiment (see Table 1), which commenced 48h after the open field test.

Two sets of chambers were used to provide two distinct contexts. The first set of chambers (context A) comprised four operant chambers (30 × 25 × 29 (high) cm, model E10-10, Coulbourn Instruments, Allentown, PA) individually installed in a ventilated, sound insulated chest. Each chamber was 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 shock could be delivered by a shock generator (Model E13-14). The animal was confined to a rectangular region (17.5 × 13 cm) in the centre by a clear Plexiglas enclosure. Illumination inside the chamber was provided by a house light (2.8 W) positioned on the right 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, and located in ventilated, sound-insulated, wooden cabinets. They were illuminated by an infrared light source instead of visible light. Each chamber also contained a sonalert tone module (Model SC628, Mallory), which provided an 86 dB, tone, and a miniature digital camera (sensitive to visible and infrared range) mounted 30 cm directly above the centre of the area of interest. The output of the camera was fed to a multiplexer (YSQ-430, Sony, Japan) before being transmitted to a computer running the Open eVision 1.1 (Euresys, Liège, Belgium) software under the control of a customized Microsoft Visual Basic (version 6) script. The algorithm of the freezing response detection procedure has been validated and fully described before (Richmond et al., 1998). In brief, successive digitized images (192 × 144, at 8-bit gray scale) obtained at a rate of 1 Hz were compared. The difference in number of pixels between adjacent frames was
then computed. If this 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. The procedures comprised three distinct phases: (i) conditioning, (ii) test of conditioned context freezing, and (iii) test of conditioned tone freezing across days.

On Day 1, all animals were given three conditioning trials in context A. Each trial consisted of a 30s tone stimulus (conditioned stimulus, CS) followed immediately by a 1-s 0.25 mA foot shock (unconditioned stimulus, US). The first trial was administered 3 minutes after the animals were placed into the chambers. Successive trials were administered every 3 minutes. The conditioning session was concluded with a final 3-min interval.

On Day 2, the animals were returned to context A. They were placed in the test chamber for a period of 8 min.

On Days 3 to 10, conditioned freezing to the tone stimulus was assessed in context B. The tone stimulus was administered 2 min after the animals were placed into the test chamber. The tone remained on for a period of 8 minutes. The expression of freezing or immobility was expressed as percent time freezing.

The three phases were separately analyzed.

**Appetitive Pavlovian conditioning**

The animals from cohort A not included in the aversive conditioning experiment (see Table 1) were used in the appetitive conditioning experiment, which commenced 12 days after conclusion of the open field experiment. Five days prior to conditioning, the animals were gradually introduced and acclimatized to a food-restricted diet until they were finally maintained on free feeding 2h per day throughout the experimental period. Their body weight was closely monitored and prevented from falling below 85% of their ad libitum weight.

The apparatus consisted of eight Habitest System operant chambers (29 × 25.5 × 28 high cm, Model E10-10, Coulbourn Instruments Allentown, PA), each located in a ventilated, sound-insulated chamber. Illumination inside the chamber was provided by a house light (2.8 W) positioned on the right panel wall, 21 cm above the grid floor. A partition wall (parallel to the panel wall) was installed to reduce the floor area to 17 × 25.5 cm. A magazine tray was positioned in the middle of the panel wall and 2 cm above floor level. Nose-poke responses to the magazine were detected by an infrared photocell beam (Model H14-01M) placed at the entrance. Liquid reward (0.01 ml of a 20% dilution of a commercial condensed milk, Milch Lait®, Switzerland) was delivered in the magazine by an automated dipper (Model H14-05R) attached to it. Each chamber also contained a sonalert (Model SC628, Mallory), which provided an identical tone (86 dB) to that used in the Pavlovian aversive conditioning experiment. Each set of four chambers were connected via an interface to a PC running the Graphic State software.
The animals were first familiarized with consuming the milk reward from the liquid dispenser in a 15-min session, during which the liquid dipper was programmed to be raised (for 5s at a time) at random intervals (mean=30s, range = 5 to 60s). All animals learned to access the magazine and to consume the liquid reward. Conditioning commenced the next day and continued for 14 consecutive days. On each daily conditioning session, the animals were placed in the same test chamber with house light on throughout. Ten discrete conditioning trials, each consisting of a 5-s tone stimulus (the conditioned stimulus, CS) followed immediately by the delivery of the liquid food reward available for 5s (the unconditioned stimulus, US), were presented at random intervals (mean=120s, range=60–180s).

Eight days of extinction immediately followed the last conditioning session. An extinction session was identical to that of the conditioning session except that no reward was available (although the dipper still underwent the motion of delivery).

The approach response was measured by magazine nose pokes. Conditioned approach response was indexed by comparing the frequency of nose pokes during CS presentation with the 5-s pre CS period to control for baseline difference using the ratio: $\frac{\text{CS nose pokes}}{\text{CS nose pokes} + \text{Pre-CS nose pokes}}$, calculated daily. A value of 0.5 refers to chance performance such that the nose poking frequency does not differ between the pre-CS and the CS periods. Data collected in the conditioning and extinction phases were separately analysed.

**Shock sensitivity**

To evaluate if differences in sensitivity to shock between mutant and controls might contribute to the conditioning phenotypes observed in the aversive Pavlovian conditioning experiment, a separate cohort of naive mice (mutant: n=8, control: n=7) was prepared. Only female mice kept in the reversed cycle (therefore tested in the dark phase) were used here since it was the combination exhibiting the strongest effect size of enhanced conditioned freezing (see Discussion). Four acoustic startle chambers for mice (SR-LAB, San Diego Instruments, San Diego, CA, USA) were used to measure the direct whole body motor response to electric foot shock. Each startle chamber comprised a cylindrical enclosure made of clear Plexiglas attached horizontally on a lightweight mobile platform, which in turn was resting on a solid base inside a sound-attenuated isolation cubicle. A foot shock grid was located along the length of the animal enclosure and was remotely controlled by a programmable shocker. Whole body motion was converted into analogue signals by a piezoelectric unit attached underneath the platform. These signals (in arbitrary units proportional to the force produced) were digitized and stored by a computer. Testing was performed under a constant background noise of 65 dB_A produced by a
high-frequency loudspeaker mounted directly above the animal enclosure inside each test chamber. The mice were first acclimatized to the apparatus for 2 min after being placed inside the enclosure; 0.5-s foot shocks were then administered in either ascending-descending or descending-ascending sequence (counter-balanced across animals) of the following intensity: 0 (baseline), 0.045, 0.075, 0.105, 0.135, 0.165, 0.195, 0.225 and 0.255 mA. A total of 18 readings were obtained. A 0.5-s response window was defined starting from the shock onset. The force (in arbitrary units) produced by whole body movements was sampled at a rate of 1kHz. The peak response and the latency to peak response were taken to index the reaction to the shock.

**Statistical analysis**

All data were analysed by parametric analysis of variance (ANOVA) using the between-subject factors genotype, light-dark phase and sex. Additional within-subject factors were included as determined by the experimental design, such as days, bins, blocks and shock intensity, with polynomial orthogonal contrasts. Statistical significant main effects and interaction terms were further examined by post-hoc pair-wise analysis (based on the pooled error variance) and supplementary restricted analyses to assist interpretation. All statistical analyses were carried out using SPSS for Windows (version 18, SPSS Inc. Chicago IL, USA) implemented on a PC running the Microsoft Windows 7 operating system.

**RESULTS**

**Elevated plus maze test of anxiety**

All animals in cohort A were first evaluated in the elevated plus maze (see Table 1). This revealed that the presence or absence of a genotype effect depended on sex as much as on light-dark phase (Figure 1). First of all, a sex difference across light and dark phase was seen in control mice; male controls were more anxious than female controls. Against this background, a pronounced anxiolytic phenotype was observed in male mice tested in the light phase, which was in contrast to the opposite trend (i.e., anxiogenic effect) observed in the female tested also in the light phase. These impressions were supported by a \(2 \times 2 \times 2\) (genotype \(\times\) sex \(\times\) light-dark phase) ANOVA of percentage time spent in the open arms, which yielded a significant 3-way interaction \([F_{(1,79)}=5.20, p<0.05]\). Post-hoc pair-wise comparisons revealed a significant genotype difference in male mice tested in the light phase \([p=0.03]\), but the opposite trend between female mutant and controls housed in the same condition did not achieve statistical significance \([p=0.09]\). Nonetheless, the appearance of a sex-dependent phenotype in the opposite direction gave rise to a significant genotype \(\times\) sex interaction \([F_{(1,39)}=6.35, p<0.05]\) in a restricted ANOVA confined to animals tested in the light phase. No evidence for such an
interaction was found in the ANOVA restricted to mice tested in the dark phase \( [F<1] \), which revealed only a significant sex effect \( [F_{(1,40)}=4.85, p<0.05] \). The main effect of sex, however, did not achieve significance in the overall ANOVA.

Separate analysis of locomotor activity confirmed that the observed effect on anxiety-related behaviour described above was not confounded by any group differences in spontaneous locomotor activity. Parallel analysis of the total distance travelled on the entire maze surface during the test did not reveal any significant effect. The animals on average (±SEM) had covered a total distance of 9.01±0.19 m within the 5-min test period.

Figure 2: The elevated plus maze test. Anxiety-like behaviour was indexed by the percentage of time spent in the open arms. A significant anxiolytic effect was observed in male mutant mice compared with male controls tested during the light phase \( (*p<0.005) \). No genotypic difference was apparent in animals tested in the dark phase, although a sex effect was revealed in a restricted ANOVA performed in these animals. \( \bigcirc \) = light phase, \( \bigcirc \) = dark phase, \( \bigcirc \) = male, \( \bigcirc \) = female. Data refer to mean ± SEM.

**Locomotor activity in the open field**

Locomotor activity was further evaluated in the open field for an extended period of time to allow for the assessment of locomotor habituation effect. Habituation was evident by the monotonic reduction of activity over the course of the 60 min period which resulted in a highly significant bins effect \( [F_{(5,395)}=336.08, p<0.001] \) in a 2 × 2 × 2 × 6 (genotype × sex × light-dark phase × 10-min bins) ANOVA of distance travelled. Open field activity was independently affected by light-dark phase (Figure 3A) and sex (Figure 3B). These were confirmed by the presence of a significant sex effect \( [F_{(1,79)}=14.54, p<0.001] \), its interaction with bins \( [F_{(5,395)}=5.01, p<0.001] \), and the light-dark phase by bins interaction \( [F_{(5,395)}=2.55, p<0.05] \). Post-hoc pair-wise comparisons at successive bins revealed that testing in the dark phase facilitated habituation transiently, leading to lower activity in bins 2-4 when compared to mice tested in the light phase. Thus, the phase by bins interaction was predominantly explained by its quadratic component \( [F_{(1,79)}=5.83, p<0.02] \). On the other hand, female mice were consistently more active than male mice. But this effect was visually most pronounced towards the session’s end, suggesting that habituation was stronger in male mice. Consistent with this impression, the sex
by bins interaction was strongest in its linear component \( [F_{(1,79)}=8.95, p<0.005] \). However, there was no evidence of any genotype effect [all \( F \)'s <1] (Figure 3C).

**Figure 3:** Locomotor activity in the open field test. Activity was indexed by distance travelled across successive 10 min-bins. The reduction of activity over time constituted locomotor habituation. The effects of light-dark phase (A) and sex (B) are shown with data collapsed across the other factors. * = \( p<0.05 \) based on pair-wise comparisons between phases (in A) or sexes (in B). The genotype effect is separately plotted according to sex and light-dark phase (C). ♂ = light phase, ♀ = dark phase, ♂♂ = male, ♀♀ = female. Data refer to mean ± SEM.

**Pavlovian aversive conditioning**

*Conditioning session (day 1):*

- **CS-freezing:** The amount of freezing observed in the presence of the tone increased across the three tone-shock pairings (Figure 4). This was not affected by genotype or light-dark phase (Figures 4A,C). However, female mice exhibited a stronger freezing response by the last trial (Figure 4B), which was independent of the other factors. A \( 2 \times 2 \times 2 \times 3 \) (genotype × sex × light-dark phase × conditioning trials) ANOVA revealed a significant trials effect \( [F_{(2,64)}=57.3, p<0.001] \), and its interaction with sex \( [F_{(2,64)}=4.70, p<0.05] \). Post-hoc pair-wise comparisons at successive trials indicated that this interaction stemmed from a sex difference specific to the last CS presentation \( [t_{(64)}=4.10; p<0.01] \).

- **ITI-freezing:** A similar pattern emerged in the analysis of between-trials (ITI) freezing (Figure 4D-F), such that female mice again showed stronger freezing, which emerged over successive ITI-periods (Figure 4E). In addition, an effect of genotype was observed with mutant mice displaying a stronger progressive increase in freezing across ITI’s (Figure 4F). These impressions were confirmed by a \( 2 \times 2 \times 2 \times 4 \) (genotype × sex × light-dark phase × ITI-periods) ANOVA, which yielded an effect of ITI-periods \( [F_{(3,96)}=49.99, p<0.001] \) and of sex \( [F_{(1,32)}=5.94, p<0.05] \), with the genotype effect just missing the criterion for significance \( [F_{(1,32)}=4.09, p=0.05] \). The temporal dependency of the sex and genotype effects was evident by
the presence of the highly significant sex by ITI-periods interaction [$F_{(3,96)}=8.47$, $p<0.001$] and genotype by ITI-periods interaction [$F_{(3,96)}=4.54$, $p=0.005$]. Post-hoc pair-wise comparisons at successive ITI’s indicated that the sex and genotype effects were essentially restricted to the last two ITI periods. A significant sex difference was only detected in the last two ITI's [$t_{(96)}=3.56$ and 5.97, respectively; both $p's<0.01$]; and a significant genotype difference was likewise detectable only in the last two ITI's [$t_{(96)}=2.67$ and 4.68, respectively; $p's<0.01$].

Figure 4: Conditioning session. Freezing is quantified as percent time during tone presentation across three tone-shock trials (A-B), or during the inter-trials intervals (ITIs) period (D-F). * refers to significant differences ($p<0.01$) indicated by post-hoc comparison at specific trial or ITIs following emergence of the corresponding interaction effect depicted. Data refer to mean ± SEM.
Test of context freezing (day 2):
Conditioned fear to the context acquired on the first day was assessed by re-exposing the animals to the same context 24h later. The levels of freezing were generally low during the 8-min test and were not affected by sex, light-dark phases or genotype (Figure 5A,B,C). A $2 \times 2 \times 2$ (genotype $\times$ sex $\times$ light-dark phase) ANOVA of percent time freezing did not yield any significant effects.

![Figure 5](image)

**Figure 5:** Test of context freezing. Overall levels of conditioned freezing to the context across the entire 8-min test were not affected by sex (A), light-dark phase (B) or genotype (C). Data refer to mean ± SEM.

CS freezing (days 3 – 10):
Expression of conditioned freezing to the tone CS was assessed across the next 8 days in a neutral context. Each daily test began with a 2-min “pre-CS” period followed by the presentation of the CS continuously for 8 min. The weakening of the conditioned response (CR) over time (within and across days) further provided a measure of extinction learning, since the CS was now no longer followed by any US.

- **Pre-CS freezing:** The levels of freezing observed in the pre-CS period remained low and stable over days (Figure 6). Animals tested in the light phase exhibited a significantly higher level of Pre-CS freezing (2.02±0.31%) than those tested in the dark phase (0.87±0.31%). A $2 \times 2 \times 2$ (genotype $\times$ sex $\times$ light-dark phase) ANOVA of percent time freezing during the 2-min pre-CS period yielded a significant light-dark phase effect [$F_{(1,32)}=7.56$, $p<0.01$], and a marginal sex effect [$F_{(1,32)}=3.79$, $p=0.06$] with female mice also showing higher levels of Pre-CS freezing (1.98±0.31%) compared with male mice (0.98±0.31%). The analysis revealed no other significant effects.
- **CS freezing:** Presentation of the CS induced a conditioned freezing response due to its previous pairing with the shock US. Because the shock no longer followed the CS, the level of freezing fell across days, as well as across bins (within-day); and the latter effect was progressively less pronounced across days. A 5-way $2 \times 2 \times 8 \times 4$ (genotype $\times$ sex $\times$ light-dark phase $\times$ days $\times$ 2-min bins) ANOVA of percent time freezing revealed highly significant effects of days [$F(7,224)=51.18$, $p<0.001$], bins [$F(3,96)=32.83$, $p<0.001$] as well as their interaction [$F(21,672)=10.40$, $p<0.001$].

The overall level of conditioned freezing to the CS was significantly modified by genotype [$F(1,32)=4.69$, $p<0.05$], sex [$F(1,32)=10.33$, $p<0.005$] and light-dark phase [$F(1,32)=7.60$, $p<0.01$]. Freezing was generally higher in the mutants, amongst female mice tested in their light phase (see Figure 6). The genotype and sex effects were visibly stronger in the early phase of the test in terms of days, as well as time bins, leading to the emergence of these factors’ interactions with days [genotype $\times$ days: $F(7,224)=2.07$, $p<0.05$; sex $\times$ days: $F(7,224)=7.79$, $p<0.001$], with bins [genotype $\times$ bins: $F(3,96)=3.56$, $p<0.05$; sex $\times$ bins: $F(3,96)=7.47$, $p<0.005$], and with bins across days [genotype $\times$ days $\times$ bins: $F(21,672)=1.68$, $p<0.05$; sex $\times$ days $\times$ bins: $F(21,672)=2.99$, $p<0.001$].
Figure 6 (next page): Test of CS freezing (across 8 CS-test days). The levels of freezing (indexed as percent time freezing) is expressed as a function of 2-min bins across the 8 consecutive CS-test days, illustrating the comparison between mutant and control mice (A) or between male and female mice (B), collapsed across the different time of testing. The effect of bins, collapsed across days (B and E), and the effect of days, collapsed across bins (C and F) are also illustrated, again either comparing between genotypes (B and C) or between sexes (E and F). * refers to significant differences (p<0.05) obtained by post hoc pair-wise comparisons based on the error variance associated with the illustrated interactions. ’Pre’ refers to the 2-min bins preceding the CS presentation at the beginning of each CS-test session. Data refer to mean ± SEM.

The 4-way genotype × sex × days × 2-min bins (2 × 2 × 8 × 4) interaction also attained significance [F(21,672)=1.69, p<0.05], which was attributable to a sex-dependent genotype effect on the extinction of CS-freezing across bins that was most pronounced on the first CS test day. Additional analyses restricted to each test day confirmed the emergence of a significant genotype × sex × bins interaction only on the first CS test day [F(3,96)=3.59, p<0.05], which was further accompanied by a significant effect of genotype [F(1,32)=5.07, p<0.05] and of sex [F(1,32)=19.20, p<0.001], but not of light-dark phase [F(1,32)=2.03, p=0.16]. As shown in Figure 7, the enhanced freezing observed in the female mutants (compared to female controls) was the strongest in the last two bins (Figure 7A), whereas it was the clearest in the first bin in the males (Figure 7B).

Figure 7: Test of CS freezing (during first CS-test day). The genotype effects of conditioned freezing to the CS on the first CS test conducted 48h after CS-US conditioning are illustrated separately in the male (A) and female (B) mice. * refers to significant differences (p<0.05) obtained from post hoc comparisons between genotypes at the specific 2-min bins based on the error variance associated with the significant genotype × sex × 2-min-bins interaction effect. ♂ = male, ♀ = female. 'Pre’ refers to the 2-min bins preceding the CS presentation at the beginning of each CS-test session. Data refer to mean ± SEM.
Figure 8: Modification of CS freezing by sex and light-dark phase across the 8 CS-test days. The effects of sex, collapsed across other factors, was illustrated separately for animals tested in the light phase (A) and in the dark phase (B). * refers to significant differences (p<0.05) based on the error variance associated with the significant light-dark phase × sex × days interaction. ◦ = light phase, □ = dark phase. Data refer to mean ± SEM.

Evidence for an interaction between sex and light-dark phase was supported by a significant light-dark phase × sex × days interaction [F(7,224)=2.10, p<0.05], which was attributed to the sex effect across days being stronger in animals tested in the dark phase than those tested in the light phase (Figure 8). Consistent with this interpretation, a significant sex × days interaction was only detected in a supplementary analysis restricted to the dark phase [F(7,112)=12.65 p<0.001], but not when restricted to the light phase [p=0.2].

- Spontaneous Recovery of CS freezing between consecutive CS sessions: The freezing response to the CS in the initial bin tended to be higher to that seen in the last bin of the previous CS test (see Figure 6A). This effect represents a partial recovery from the previous day’s extinction learning, and is commonly referred to as ‘spontaneous recovery’. As extinction was progressively consolidated over test days, the magnitude of spontaneous recovery also weakened. However, it appeared that the spontaneous recovery effect was somewhat stronger and more persistent in mutant mice. To specifically gauge spontaneous recovery between days, a difference score was computed contrasting the magnitude of freezing of the first CS bin of a given test day to the last CS bin of the preceding day. The difference scores were then subjected to a 2 × 2 × 2 × 7 (genotype × sex × light-dark phase × days) ANOVA, which yielded a main effect of days [F(1,32)=12.04, p<0.01] (see Figure 9A) and of genotype [F(1,32)=4.66, p<0.05] (see Figure 9B), thus confirming the initial visual impression derived from Figure 6A. No other effects attained or approached statistical significance.
Chapter 3: Pavlovian learning & GlyT1 deletion

Figure 9: Spontaneous Recovery effect from the second to the last CS-test day. Comparison between the spontaneous recovery of the mutant and control mice from the second CS-test and onwards, collapsed across the other factors as a function of days (A), or averaged across CS-test days 2 to 8 (B). Spontaneous recovery between successive CS-tests was indexed by a difference score = (levels of freezing during the first 2-min bins of day$_N$ − levels of freezing during last 2-min bin of day$_{N-1}$). A positive value refers to the spontaneous recovery effect. Data refer to mean± SEM.

Pavlovian appetitive conditioning

- **Acquisition:** The animals were subjected to CS-US conditioning for 14 days. Conditioning was evident in all groups as indicated by the progressive increase in magazine approaches during the CS presentation relative to the pre-CS period (Figure 10). A $2 \times 2 \times 2 \times 7$ (genotype $\times$ sex $\times$ light-dark phase $\times$ 2-day blocks) revealed only a significant effect of blocks $[F(6,234)=37.05, p<0.001]$. 

Figure 10: The acquisition and extinction of Pavlovian appetitive conditioning. Conditioned approach response was indexed by comparing the frequency of nose pokes during each 5-s CS presentation with the 5-s pre-CS period using the ratio: CS nose pokes / (CS nose pokes + Pre-CS nose pokes). The genotype effect across 2-day blocks is separately illustrated for the factor sex and light-dark phase (A-D). The 7 blocks of acquisition are demarcated by a vertical line from the last 4 blocks of extinction. The dotted line represents the level of chance performance. $\bigcirc$ = light phase, $\mathcal{D}$ = dark phase, ♂ = male, ♀ = female. Data refer to mean ± SEM.
Next, the animals were subjected to eight daily extinction sessions when the tone CS was no longer followed by the reward US. There was a progressive reduction in the conditioned approach response, constituting extinction learning (Figure 10). A $2 \times 2 \times 2 \times 4$ (genotype $\times$ sex $\times$ light-dark phase $\times$ 2-day blocks) ANOVA revealed a significant blocks effect [$F(3,117)=31.42$, $p<0.001$]. A sex-dependent genotype effect was evident by the emergence of a genotype $\times$ sex $\times$ blocks interaction [$F(3,117)=3.10$, $p<0.05$]. As shown in Figures 11A-B, female mutants showed a trend towards resistance to extinction compared with female controls, while a weak and opposite trend was observed in the male mutants. Given that the critical 3-way interaction was solely attributed to the linear component of blocks [$F(1,39)=10.37$, $p<0.005$], accounting for 97% of the variance explained by the interaction, further post hoc comparisons of the linear rate of extinction were conducted, which confirmed the impression that the mutation resulted in opposite effects on the linear rate of extinction across days between sexes (see Figure 11C).

**Shock sensitivity**

A separate cohort of female mice maintained under reversed cycle was used for the assessment of shock sensitivity. This was chosen because this particular sex/light-dark cycle combination resulted in the largest effect size of genotype in the conditioned freezing experiment (see Discussion later). Analysis of peak response (Figure 12A) as well as time to reach the peak response (Figure 12B) recorded in the startle chambers did not yield any genotype effect across...
the entire range of electric shocks (0.045 – 0.255mA) examined. A separate 2 × 9 (Genotype × Shock intensity) ANOVA only revealed a main effect of shock intensity [peak response magnitude: F(8,104)=17.37, p<0.001; time to peak response: F(8,104)=12.20, p<0.001].

**Figure 12:** Reactivity to shock. Reactivity to electric foot shock measured by peak response of whole body movement (A) and time to reach the peak response (B) as a function of the shock intensity in a separate naive cohort of female mutant and control mice tested during the dark phase. Data refer to mean± SEM.

**DISCUSSION**

In our attempt to scrutinize the reported enhanced Pavlovian learning phenotype following forebrain neuronal GlyT1 deletion, we uncovered that the phenotypic expression was distinguishable between the sexes as demonstrated in the aversive as well as in the appetitive paradigms of Pavlovian conditioning employed here. These findings were free from any confounding genotypic effect on pain sensitivity (Figure 12) and spontaneous activity measured in the open field (Figure 3) or in the elevated plus maze. In the elevated plus maze test, we further identified a hitherto unknown phenotype in anxiety-related behaviour through extending the test to the light phase. This finding agrees with the anxiolytic profile of the GlyT1 inhibitor SSR504734 which was obtained in male animals tested during the light phase (Depoortère et al., 2005). At the same time, the lack of such anxiety effects in the dark phase agrees with previous reports based solely on results obtained in this phase (Yee et al., 2006), and therefore anxiety did not confound the previously established phenotypes (Dubroqua et al., 2010; Singer et al., 2007, 2009; Yee et al., 2006). This new finding in the elevated plus maze may be interpreted as a state-dependent genotypic effect, revealed here by the explicit comparison between mice kept in opposite light-dark cycles but tested at the same time under identical test conditions. In contrast, the Pavlovian phenotypes were essentially unaffected by the light-dark cycle, and against which evidence for sex-dependency was obtained.
**Crossing the Sex Boundary**

Jazin and Cahill (2010) have summarized many sex differences in the phenotypic expression of a host of genetically manipulated mice. Here, the sex comparison allowed us to specify the form and the extent to which the Pavlovian phenotype associated with forebrain neuronal GlyT1 deletion might differ between sexes. In the aversive paradigm, the phenotypic dependency on sex was primarily one of magnitude: the phenotype of enhanced conditioned freezing appeared stronger in the female mutants (Figure 7). As reported before (Yee et al., 2006), this phenotype was specific to CS-freezing, without any significant impact on context-freezing. Indeed, neither sex nor light-dark cycle exerted significant effect (Figure 5). However, given that the CS was a good predictor of shock US here, context-freezing was predictably low in the range of 12%, hence interpretation of these null effects should be exercised with caution due to possible floor effects. Lack of statistical power was unlikely a concern here because the effective sample size in each of these contrasts was large (with sample size around 20 per sex, genotype or light-dark cycle). By contrast, sex critically determined the direction of the Pavlovian learning phenotype in the appetitive paradigm. The conditioned approach response was similarly enhanced in the mutant females, but was surprisingly somewhat weakened in male mutants (Figure 11C). Overall our results suggest that the enhanced Pavlovian learning phenotype appeared more consistent and robust in females than males.

Evidence for sex-dependent phenotypic expression was obtained regardless of whether sex exerted an effect by itself on performance. Across the different stages (acquisition, context-test and extinction) of aversive conditioning, female mice were consistently showing a stronger freezing response than male mice (Figure 6D; in keeping with previous mice studies e.g., Wiltgen et al., 2005; Yee et al., 2004; but also see Dalla & Shors, 2009). Our data therefore concurred with the faster extinction rate in the female rats in fear conditioning reported by Milad et al. (2009), but did not replicate their observation of weaker conditioning in female rats during acquisition. Notably, the latter effect was weak and indeed non-significant. We observed instead stronger conditioning in females although this emerged only on the 3rd CS presentation and this minor divergence might stem from species differences. By contrast, no such sex difference was apparent throughout the appetitive conditioning experiment. The apparent sex effect in conditioned freezing experiment (i.e. generally stronger freezing in female mice) cannot be attributed to sex differences in spontaneous activity given that the female mice were more active than the male mice (Figure 3B). If anything, this sex difference in spontaneous activity would under- rather than over-estimate freezing performance.

Another possible confound that might be expected to influence expression of the conditioned freezing response, but not the conditioned approach responses was general
anxiety. The anxiety phenotype demonstrated in the elevated plus maze test was clearly determined by the combined influence of sex and light-dark cycle. Forebrain neuronal GlyT1 deletion appeared to be anxiolytic in males but anxiogenic in females, thus effectively reversing the sex-difference seen in control mice (Figure 2); and this cross interaction was only evident in the light phase. To what extent could this pattern of results in anxiety-related behaviour explain the sex-dependent outcomes seen in the Pavlovian aversive conditioning experiment?

Based on the premise that the expression of conditioned freezing depends on the development of conditioned fear in response to the CS, anxiousness to threatening stimuli in general might promote aversive associative learning. Hence, an anxious phenotype in female mutants might be linked to the conditioned freezing phenotype in the same animals. However, this account was undermined by two considerations. First, the conditioned freezing phenotype did not significantly depend on light-dark cycle; and if anything, it was the strongest in female mice tested in the dark phase of the cycle (kept in reversed light-dark cycle). Second, this account should also predict an opposite conditioned freezing phenotype in the male mutants, which was obviously not the case even though the magnitude of the enhanced freezing phenotype was visually weaker in the male than the female mutants. As summarized in Table 2, the critical effect sizes associated with the relevant genotype effect in the aversive conditioning experiment was highest in the female/dark-phase combination, when the comparison between mutants and controls yielded no difference in elevated plus maze behaviour (Figure 2). Indeed, the combination of a null effect in anxiety and enhanced conditioned freezing replicated our previous report (Yee et al., 2006). Re-calculation of the effect size from the original data reported by Yee et al. (2006) yielded a value that is closer to that obtained here in animals of the same sex and tested in the same phase (see Table 2).
Table 2. Effect size ($\eta^2_p$) of the genotype derived from the restricted analysis for each combination of sex and light-dark phase in the aversive Pavlovian paradigms and from the non pre-exposed group of the previous aversive conditioning experiment (Yee et al., 2006). Effect size provides a measure of the magnitude of a treatment effect, this index is independent of sample size and allowed us to directly compare each of the sex and phase of testing combination used. The largest effect size was found in the female mice tested during the dark phase of the light-dark cycle. The effect size of the previously reported genotype effect on aversive conditioning, with female mice tested during the dark phase was also, larger than the other sex/phase combinations in the present study.

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<th>Experiments</th>
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<tr>
<td>Aversive conditioning</td>
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<td>Aversive conditioning (Yee et al., 2006)</td>
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The evidence thus did not support an emotional account of the conditioned freezing phenotype, but favoured instead a cognitive interpretation. Importantly, the cognitive perspective can readily accommodate the enhanced conditioned approach phenotype seen in the female mutants in the appetitive Pavlovian paradigm, which shared a similar associative learning mechanism (CS-US association), but did not involve any fear-related emotion.

**Lost and Found in Translation across Paradigms**

The inclusion of the appetitive conditioning experiment was highly instructive and meaningful, because it provided an alternative test with similar cognitive demand but differed in several psychological as well as procedural aspects. Appetitive conditioning is based on a motivational system antagonistic to that in aversive conditioning (Konorski, 1976) and is therefore ideally suited to test the generality of the reported Pavlovian phenotype that, so far, has been demonstrated solely in associative learning paradigms using aversive unconditional stimulus (Yee et al., 2006).

While this phenotype of enhanced conditioning was translatable to the current appetitive paradigm in the female sex, the results obtained in the male sex were surprising, as they yielded somewhat opposite effects between paradigms. The latter does defy an interpretation simply based on enhanced associative strength. Indeed, such a simple account is already insufficient to explain the original finding that forebrain neuronal GlyT1 deletion...
enhanced the latent inhibition effect (i.e., the sensitivity to a CS’s history of non-reinforcement prior to conditioning; Yee et al., 2006). Thus, there are situations in which the mutant mice were more sensitive to the negative modulation of learning and/or expression of learned behaviour.

There was no evidence here to suggest that acquisition of the CS-US association was facilitated across the three tone-shock pairing trials in the aversive conditioning experiment (Figure 4C). However, freezing recorded during the inter-trial intervals was enhanced in the mutants, with a notable difference emerging in the last two intervals – i.e., after the second and third tone-shock pairing (Figure 4F). Unlike the development of conditioned freezing, the acquisition of the (appetitively motivated) conditioned approach response is a relatively protracted process allowing a closer examination of its development over many CS-US trials conducted across days. The appetitive conditioning experiment here consolidated the lack of an effect on conditioned responding throughout the entire acquisition phase (Figures 10 & 11).

The present study also showed that, irrespective of conditioning paradigm, the Pavlovian phenotype was confined to the extinction phase – when the animals were confronted with extended/repeated CS’s that was no longer followed by any US. This result suggests that forebrain neuronal GlyT1 deletion robustly modified the expression rather than the acquisition of the critical CS-US association. In the appetitive paradigm, evidence for enhanced conditioned responding seen in the female mutants only emerged across daily extinction tests (Figure 11B,C). Although the direction of the weaker effect seen in male mutants was in the opposite direction here (Figure 11A,C), the phenotypic expression was also confined to the extinction phase. In the conditioned freezing experiment, the phenotype remained visible across the eight daily extinction tests (Figure 6A), and the sex-dependent phenotypic expression was a quantitative one as shown in the first extinction test (Figure 7). Hence, the unique phenotypic element that is translatable across paradigms refers to the increased resistance to extinction in female mutants. This element was detected across days in the appetitive conditioning experiment, but solely on the first test day in the aversive conditioning experiment. Although not seemingly a sex-dependent effect, evidence that extinction learning across extinction days was also modified by GlyT1 deletion was obtained when we examined spontaneous recovery of the conditioned freezing response between successive tests (Figure 9). Overall, spontaneous recovery, attributed to the dissipation of inhibitory mechanisms responsible for extinction (Rescorla, 2004), was stronger in the mutants – the daily re-emergence of the CS evoked a stronger initial response in the mutants.

Taken together, extinction learning is most robustly affected by GlyT1 deletion in the female sex. Although we cannot explain why this phenotype might be weaker or somewhat reversed in the male sex when switched to the appetitive paradigm, this conclusion may be considered as being consistent with the previous finding in the female mutants that latent
inhibition (LI) was enhanced (Yee et al., 2006). The demonstration of LI requires two distinct phases with contrasting CS-US relationships: the pre-exposure phase in which CS is not followed by any significant event \([CS \rightarrow \text{nothing}]\) precedes the conditioning phase. LI refers to the observation that CS pre-exposed subjects show weaker conditioned response (CR) to the CS after CS-US pairing compared to non-pre-exposed subjects. Several theories suggest that the weaker CR does not stem from a failure to acquire the critical CS-US association but reflects the \([CS \rightarrow \text{nothing}]\) memory trace competing over the control of behaviour during testing (Bouton, 1993; Kraemer and Spear, 1991, 1993; Weiner, 1990). Thus, a stronger influence of the prior experience of non-reinforced CS exposures leads to stronger LI. Procedurally, extinction is exactly the reverse of LI, whereby \([CS \rightarrow \text{US}]\) follows \([CS \rightarrow \text{nothing}]\). Thus, the phenotype of increased resistance to extinction could be similarly understood as a stronger influence of the prior experience of \([CS \rightarrow \text{US}]\) over current control of responding. The mutant mice appeared to be biased towards what they have learned first about the CS, and this might be described as a stronger primacy effect or proactive interference in memory terms. This contradicts however the finding that forebrain neuronal GlyT1 disruption enhanced rather than impaired reversal learning (Singer et al., 2009), as it has been taken to suggest resistance rather than vulnerability to proactive interference.

In discrimination learning, animals not only learn the opposing reward valence associated with the discriminanda provided by the experimenter, but they also learn to dichotomize their responses (approach vs avoidance) efficiently by focusing attention on one or few distinguishable dimensions/features separating the discriminanda. Achieving the latter would facilitate reversal learning, because the animals need only to reverse the contingency related to the few attended dimensions (Sutherland and Mackintosh, 1971). This has been offered as an explanation of the over-training reversal effect, whereby over-training paradoxically enhances subsequent reversal learning (Reid, 1953). Hence, it is conceivable that the reversal phenotype reported by Singer et al. (2009) reflects primarily enhanced selective attention learning, whereas the extinction phenotype here reflects a stronger primacy effect in memory recall.

The hypothesized impact on memory expression and selective attention are not mutually exclusive, even though they might be expected to yield opposing effects on specific paradigms, e.g., in discrimination reversal. When the separate effects act in the same direction then the observed behavioural effect would be substantial. The LI effect which is expected to be potentiated by a susceptibility to the primacy effect (Postman and Phillips, 1965) as well as enhanced attentional learning (e.g. Sutherland and Mackintosh, 1971), was highly sensitive to forebrain neuronal GlyT1 deletion (Yee et al., 2006). However, the possible sex and paradigm dependency of the LI phenotype awaits further evaluation.
**Separating the light from the dark**

Sex is strictly a between-subject factor. The factor referring to the contrast between light and dark phases, on the other hand, ought to be interpreted as a potential within-subject factor referring to the different states (of wakefulness) phase-locked with the diurnal cycle. For practical reasons, it was evaluated here in a between-subject design by shifting the light-dark cycles between two animal keeping rooms by 12h, thus enabling us to conduct all tests at the same absolute time. This approach allowed a more effective approximation to the contrast between laboratories that systematically keep their animals either in one or the other cycle. This contrast did not yield any substantial impact on the measures of conditioning behaviour here. Notably, even when it was effective in eliciting a sex-dependent phenotype in the elevated plus maze in the mutant mice, it was without an effect in the controls (Figure 2). Contrary to some previous studies, we did not observe an increase in spontaneous locomotor activity in the open field in mice tested in the dark phase in comparison with those tested in the light phase. If anything, a transient effect in the opposite direction was recorded (Figure 3A). However, increased activity was evident in the animals tested in the dark phase during the CS test of the aversive conditioning experiment in the form of reduced freezing, and this was already evident in the pre-CS phase. When the subsequent CS-freezing score on each day was corrected for baseline pre-CS freezing, the main effect of light-dark phases on CS-freezing remained significant \( F_{(1,32)}=6.94, p<0.02 \), without altering any other significant effects in the original analysis. Therefore, the contrast between light-dark phases did appear to exert an effect on conditioned freezing rather than affecting the general propensity to freeze as such. Finally, the contrast between light-dark phases did not reveal any impact on behaviour in the appetitive conditioning test. Thus, Pavlovian learning of specific CS-US association in general seems to be relatively stable across the light-dark phases, with limited impact on the magnitude (effect size) of the conditioned freezing phenotype identified in our mutant mice. Our results do not readily support the general assertion that behavioural testing in the dark phase is generally more appropriate than in the light phase solely on the basis that the former represents the active phase in rodents (Marques & Waterhouse, 1994). Yet, caution should be exercised when interpreting ethologically based behavioural tests, such as the elevated plus maze test of anxiety, and other such tests that might be highly sensitive to wakefulness or circadian fluctuation of hormonal activity.
Chapter 3: Pavlovian learning & GlyT1 deletion

Conclusions

In our attempt to test the generality and the robustness of a phenotype on conditioned freezing behaviour previously reported in mice with forebrain neuronal GlyT1 deletion, the present study has revealed (i) an hitherto unknown anxiety-related phenotype, and (ii) determinants that may influence the magnitude and perhaps even the direction of a Pavlovian learning phenotype in this particular genetic mouse model. Our results suggest the interaction between genomic (sex difference) and environmental (light-dark cycle) factors critically determined the expression of the anxiety phenotype, but sex-dependency predominates over the Pavlovian phenotype. A satisfactory neurobiological account of the emergence of these phenotypes must therefore take into account such specifications revealed here.

REFERENCES


Chapter 3: Pavlovian learning & GlyT1 deletion

CHAPTER 4

Glycine transporter 1 deletion enhances sensitivity to CS-US contiguity and CS-US contingency:

Two phenotypes, one psychology?

With Philipp Singer, Detlev Boison, Hanns Möhler, Joram Feldon and Benjamin K. Yee

Submitted to Learning & Memory
ABSTRACT

Mutant mice with conditional deletion of glycine transporter 1 (GlyT1) in forebrain neurons exhibit enhanced Pavlovian conditioning. This phenotype seems to reflect an alteration in the selectivity of learning rather than a broad strengthening of CS-US associative strength. Evidence against the latter is derived from the observation that the latent inhibition effect was potentiated in this mutant mouse line, suggesting that these mice were more sensitive to a history of non-reinforcement of a potential CS. Here, we extended this hypothesis by examining the response of these engineered mice to two other major determinants of Pavlovian associative learning. To compare with the conditioning procedure in which the US always followed the CS immediately, we either (i) inserted a 'trace' interval between CS and US presentations to reduce the temporal contiguity between CS and US, or (ii) added CS-only presentations that were not followed by US to degrade CS-US contingency. While the impact of these manipulations on the subsequent conditioned response (CR) to the CS was weak or absent in the control mice, they strongly reduced CR in the mutant mice. These outcomes are attributable to the bidirectional effects of the GlyT1 deletion in forebrain neurons on Pavlovian associative learning: the CR was stronger than in controls when CS was a good predictor of US, but weaker than in controls when the CS-US contiguity or contingency was degraded. Hence, an interpretation based on simple modulation of associative strength again failed to account for these new data sets. Instead, a stronger bias in the selectivity of learning may offer a unifying account of these diverse phenotypes in the regulation of Pavlovian learning. These results bear clear relevance to the proposed therapeutic potential of GlyT1-inhibiting drugs for schizophrenia-related cognitive deficits that might stem from an underlying lack of selectivity (or inflexibility) in learning.

INTRODUCTION

The N-methyl-d-aspartate receptor (NMDAR) is believed to be central to neuroplastic events underlying at least some forms of learning and memory (e.g. Collingridge & Bliss, 1995; Morris, 1989; Morris, Anderson, Lynch, & Baudry, 1986), including Pavlovian associative learning. Molecular interventions designed to boost NMDAR function have been reported to enhance simple Pavlovian conditioning. A selective increase in NMDAR-mediated (as opposed to AMPA receptor-mediated) currents can be achieved by altering directly the molecular composition of the NMDAR (e.g. Tang et al., 1999), or indirectly by disrupting glycine transporter 1 (GlyT1) and thereby increasing the availability of glycine, an obligatory NMDAR co-agonist, in glutamatergic synapses (Yee et al., 2006).

Evidence for enhanced Pavlovian learning by GlyT1 disruption restricted to forebrain neurons is supported by stronger conditioned responding (CR) to a conditioned stimulus (CS) following its pairing with an unconditioned stimulus (US) across a variety of Pavlovian learning paradigms, including conditioned freezing, conditioned taste aversion and conditioned active
avoidance learning (Yee et al., 2006). However, in each case this effect was coupled with an increased sensitivity to the CS pre-exposure effect. Prior non-reinforced exposure to the CS is known to reduce the associability of the CS, reducing the effectiveness of subsequent CS-US conditioning – a phenomenon known as latent inhibition (LI). Mutant mice with forebrain neuronal GlyT1 disruption exhibited strong LI under a pre-exposure condition that was insufficient to generate clear LI in controls. Thus, the impact of the molecular deletion appeared to be bi-directional. The CR developed under the non-pre-exposed condition was somewhat enhanced, whereas it was reduced under the pre-exposed condition. Hence, it is apparent that the phenotype could not satisfactorily be understood as a broad (uni-directional) modulation of associative strength.

The LI effect represents an important demonstration of selectivity in associative learning, in the sense that not all potential CSs have an equivalent capacity to form an effective CS→US association. Learning selectively avoids the acquisition of potential spurious associations that might not be adaptive, thus affording animals with the ability to focus on the more informative environmental cues relevant in predicting future outcomes. Learning to anticipate and to be prepared for future events is the key adaptive value of learning. The possibility that GlyT1 disruption might generally facilitate selectivity in learning thus warrants further examination, since this might underlie the anti-psychotic potential of GlyT1 inhibition against the cognitive inflexibility and deficiency characteristic of schizophrenia.

While LI reflects a modulation of CS associability due to its prior non-reinforced history, the efficacy of CS-US association is also regulated by other factors. Here, we examined two such critical determinants by manipulating the contiguity (Experiment 1) or contingency (Experiment 2) between CS and US during conditioning. Contiguity refers to the temporal proximity between the antecedent CS and the to-be-predicted US. When they are interspersed by a "trace" interval, and are therefore discontiguous, the subsequent CR is weakened (Pavlov, 1927). According to the Rescorla-Wagner model (R.A. Rescorla & Wagner, 1972), the trace conditioning effect is partly attributable to competition from context-US association promoted during the trace intervals. Alternatively, the trace intervals may further render the CS to be perceived as a safety signal (Seligman & Binik, 1977). The CS effectively serves as a signal predicting the temporary absence of US, fostering inhibitory CS-US association that counters the excitatory associative link that underlies the generation of the CR.

Similarly, CS-US contingency also emphasizes the informativeness of CS in predicting the US, and it is typically formulated in probabilistic terms (Rescorla, 1968). CS-US contingency is strongly positive when the probability of US occurring following the CS [P(US|CS)] exceeds the probability of US occurrence in the absence of an antecedent CS [P(US|~CS)]. Under such conditions, the CS-US association would be excitatory. Lowering P(US|CS), ceteris paribus,
should degrade the CS-US contingency, for instance by embedding CS-alone trials amongst CS-US trials (without altering the number or the density of US occurrence in time, see Figure 1). The interspersed CS-alone trials constitute omissions of anticipated US, which according to the Rescorla-Wagner model would produce a negative change to the CS-US associative strength by promoting inhibitory CS-US association (Konorski, 1948), thus weakening the overall associative strength and the intensity of the CR.

The present study examined the impact of forebrain neuronal GlyT1 deletion on the expression of the trace conditioning effect and contingency degradation in two separate conditioned freezing experiments. The trace intervals and the degree of CS-US contingency degradation were deliberately chosen here to yield relatively weak effects in control mice, so as to allow the demonstration of the enhanced sensitivity to CS-US discontiguity and contingency anticipated in the mutant mice. In each case, the effect was gauged in comparison to a standard procedure adopted from Yee et al. (2006) employing three discrete tone-shock pairings in which the shock US always immediately followed the cessation of the tone CS. Finally, additional experiments of foreground contextual conditioned freezing (i.e., in the absence of any competing discrete CS) were also conducted (Experiments 3A and 3B) to allow comparison with contextual freezing obtained in Experiments 1 and 2 when the context (in the background) was competing with a discrete CS for potential associative strength to the US.

**Figure 1:** Schematic representation of the arrangements of tone CS and shock US in the conditioning phase of the different experiments. The "no-trace/full contingency" conditioning was the control condition, with which the "trace conditioning" (Experiment 1) and the "partial contingency" conditioning (Experiment 2) was compared. In "foreground contextual conditioning", no discrete CS was presented, and either a three-shock (Experiment 3A) or one-shock (Experiment 3B) was investigated here. The horizontal line illustrated is not to scale. See methods for full descriptions.
METHODS

Subjects
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 (hereafter designated as “GlyT1Δfb-neuron”) was achieved by CamKIIαCre-mediated recombination (see Yee et al., 2006). 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 Makrolon Type-III cages (Techniplast, Milan, Italy). The subjects were 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 0700-1900hrs. Testing was always conducted in the dark phase of the cycle. The animals were maintained under ad libitum water and food (Kliba 3430, Klibamuhlen, Kaiseraugst, Switzerland) throughout the study. Separate cohorts of naïve mice were used for each experiment according to a 2 × 2 factorial design (genotype × sex) (see 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: Allocation and number of subjects for each experiment. Experiments were conducted separately, with controls littersmates derived from the same breeding cycle for comparison. Both male and female mice were included. Experiment 1 and 2 involved an additional between-subject factor referring to the test condition (see Procedures below).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Condition</th>
<th>Mutants</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Trace conditioning</td>
<td>no-trace</td>
<td>12 (6♀ + 6♂)</td>
<td>16 (8♀ + 8♂)</td>
</tr>
<tr>
<td>1 Trace conditioning</td>
<td>trace</td>
<td>13 (7♀ + 6♂)</td>
<td>17 (10♀ + 7♂)</td>
</tr>
<tr>
<td>2 Contingency degradation</td>
<td>full contingency</td>
<td>17 (12♀ + 5♂)</td>
<td>17 (12♀ + 5♂)</td>
</tr>
<tr>
<td>2 Contingency degradation</td>
<td>partial contingency</td>
<td>18 (13♀ + 5♂)</td>
<td>18 (13♀ + 5♂)</td>
</tr>
<tr>
<td>3A 3-shock contextual conditioning</td>
<td></td>
<td>12 (6♀ + 6♂)</td>
<td>12 (6♀ + 6♂)</td>
</tr>
<tr>
<td>3B 1-shock contextual conditioning</td>
<td></td>
<td>16 (8♀ + 8♂)</td>
<td>15 (7♀ + 8♂)</td>
</tr>
</tbody>
</table>
**Apparatus**

The apparatus consisted of two sets of four conditioning chambers. The two sets were distinct from each other, and were installed in separate testing rooms, providing two distinct contexts as fully described before (Pietropaolo, Mintz, Feldon, & Yee, 2007; Yee et al., 2006). 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 (the unconditioned stimulus, US, set at 0.3 mA) could be delivered (model E13–14; Coulbourn Instruments). 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 visible light. The conditioned stimulus (CS) in all the experiments was an 86-dBA tone provided by a sonalert (model SC628; Mallory, Indianapolis, IN, USA). Each of the eight chambers contained a miniature digital camera mounted 30 cm directly above the centre of the area of interest. The algorithm of the freezing response detection procedure has been validated and fully described before (Richmond et al., 1998).

**Experiment 1: Trace conditioning**

The procedure for trace conditioning followed that described earlier (Yee et al., 2004). Subjects of each of the four groups (genotype × sex) were randomly allocated into one of two conditions of CS-US pairing differing in the (trace) interval between CS offset and US-onset: either 0 or 20 s (‘no-trace’ and ‘trace’ conditions, respectively; Figure 1). The animals were given three trials of CS–US pairings (CS: 30 sec, US: 1 sec, 0.3mA) in context A on day 1. Each trial was preceded and followed by a 120 s interval. Context test was performed on the next day by returning the animals to the training context for a period of 8min. CS freezing to the tone stimulus was evaluated on the next day (day 3) in the neutral context B where the CS was presented for 8 min following a 2 min acclimatization period.

**Experiment 2: Contingency degradation**

Animals of each of the four groups (genotype × sex) were randomly allocated into one of two conditions of CS-US pairing differing in overall CS-US contingency (full contingency vs. partial contingency). In the full contingency condition, the shock US always followed immediately at the end of tone CS, and three such CS-US pairings were administered. Thus, the probability of US given CS, P(US|CS), is one. In the partial contingency condition, three additional CS-only
presentations were intermixed so as to reduce P(US|CS) to 0.5. Figure 1 compares schematized the contrast between these two CS-US arrangements.

On the day of conditioning (Day 1), three discrete trials of CS-US pairings were administered at 3, 6.3 and 10 min into the session that lasted for a total of 13 min 33 s. In each such trial, the CS and US were serially arranged with the termination of the 30-s CS coinciding with the onset of the 1-s US. Animals in the partial contingency condition received in addition three CS-alone trials at 4.3, 9 and 11.3 min into the session. On the next day (Day 2), a context-freezing test was conducted by returning the animals to the training context A for a period of 8 min in the absence of any discrete stimuli. On Day 3, the freezing to the tone was evaluated in the neutral context B during when the CS was presented for 8 min following an initial acclimatization period of 2 min without CS.

**Experiments 3A and 3B: Context foreground conditioning**

The procedures have been fully described by Pietropaolo et al. (2007). Following conditioning in context A on Day 1, test of context freezing was assessed by placing the animals in contexts A and B on alternate days (in the order of A-B-A-B across Days 2 to 5), thus allowing the context-specificity of the freezing response to be evaluated. Each context test lasted for 4 min. Experiments 3A and 3B differed only with respect to the number of un-signalled shock (0.3mA for 1s) employed on the conditioning day (see Figure 1). Three shocks were delivered in Experiment 3A, and only one shock in Experiment 3B. Each shock delivery was preceded and followed by a 3-min interval.

**Statistical analysis**

All data were analysed by parametric analysis of variance (ANOVA) with the between-subject factor Genotype (mutant vs. littermate control). Preliminary analyses also included the between-subject factor sex; this was dropped from the final analysis to increase statistical power given that there was no evidence for any interaction effect involving sex. An additional factor between subject of Trace interval (0s vs. 20s) and Contingency (full contingency vs. partial contingency) was introduced in the analysis of Experiments 1 and 2, respectively. Appropriate within-subject factors (time bins, trials, and days) were included according to the nature of the considered dependent variables. To assist interpretation of the statistical outcomes, significant effects were further investigated by pair-wise comparisons based on the associated error terms taken from the overall ANOVA or supplementary restricted analyses applied to a subset of data. All statistical analyses were carried out using SPSS for Windows (version 18, SPSS Inc. Chicago IL, USA) implemented on a PC running the Windows 7 operating system.
RESULTS

Experiment 1: Trace conditioning

• CS-freezing (Day 1): The level of freezing during the tone-CS presentation increased progressively across trials and this increment was noticeably weaker in the ‘trace’ relative to the ‘no-trace’ condition regardless of the genotype (Figure 2A). These impressions were confirmed by a $2 \times 2 \times 3$ (genotype × trace × conditioning trials) ANOVA of percent freezing, which revealed a significant main effect of trace $[F(1,54)=10.21; p<0.01]$, trials $[F(2,108)=34.86; p < 0.01]$, and their interaction $[F(2,108)=8.28; p<0.01]$. Post hoc pair-wise comparison (collapsed across genotype) indicated that the contrast between the trace and the no-trace condition attained statistical significance in the last two CS presentations [at the second CS-presentation: $t(108)=2.75, p<.001$; at the third CS-presentation: $t(108)=8.16, p<.0001$].

• Pre-CS freezing (Day 1): The freezing observed during the 30 s preceding the presentation of the CS consistently increased across the successive CS-US pairings (Figure 2B). This was not affected by either genotype or trace condition. Consistent with these impressions, a $2 \times 2 \times 3$ (genotype × trace × Pre-CS periods) ANOVA of percent freezing only yielded a significant effect of PreCS-periods $[F(2,108)=71.11; p<0.01]$. There was no evidence for a genotype effect $[F<1]$.

• Trace periods (Day 1): Freezing during the 20-s trace intervals (in-between CS-US) which were only relevant for the animals in the trace condition was examined separately (Figure 2C). This variable likewise showed a consistent increase across trials. A $2 \times 3$ (genotype × trials) ANOVA of percent time freezing revealed only a significant effect of trials $[F(2,56)=36.75; p<0.01]$. There was no evidence for a genotype effect $[F<1]$.

Figure 2. Acquisition of the conditioned response across the three CS-US pairing in Experiment 1. Levels of freezing were expressed as percent time during the 30 s presentation of the CS and are depicted for the no-trace and the trace conditions (A). Freezing observed during the 30 s preceding each CS presentation (B) and during the 20 s trace interval for the trace condition only (C) are separately depicted. All group showed an increase in the freezing response during acquisition of the conditioned response. Error bars refer to ±SEM.
• **Context freezing (Day 2):** Expression of conditioned fear developed towards the training context was evaluated 24h after conditioning by re-exposing the animals to the context. No difference was apparent amongst the four groups of mice (Figure 3). A 2 × 2 (genotype × trace) ANOVA of percent time freezing observed in the 8-min period did not reveal any significant effects [all F's<1].

![Figure 3: Test of context freezing in Experiment 1.](image)

- **Pre-CS & CS freezing (Day 3):** Conditioned responding to the tone CS was evaluated in a novel context 48 h after the initial conditioning session. Freezing was relatively low and highly comparable across groups in the first 2 min baseline period prior to the presentation of the CS (Figure 4A). A 2-way (genotype × trace) ANOVA of percent freezing recorded during the 2min Pre-CS period did not yield any significant effect.

Onset of the CS led to a notable rise in freezing that was substantially weaker in the trace condition than in the no-trace condition, constituting the trace conditioning effect, and this effect was visibly more pronounced in the mutant mice (Figures 4A & 4B). The mutant mice exhibited marginally stronger CS-freezing than controls in the no-trace condition. On the other hand, the mutant mice exhibited marginally weaker CS-freezing than controls in the trace condition. The combination of these contrasting impacts of genotype on conditioned freezing (Figure 4B), led to the emergence of a near-significant genotype by trace interaction \([F_{(1,54)}=3.81; \ p=0.056]\) in a 2 × 2 × 4 (genotype × trace × 2-min bins) ANOVA of percent time freezing across successive 2-min bins during the 8-min CS period. This interaction term was accompanied by a significant main effect of trace \([F_{(1,54)}=33.80; \ p<0.01]\), bins \([F_{(3,162)}=10.32; \ p<0.01]\), and the three-way interaction \([F_{(3,162)}=3.01; \ p<0.05]\). Post hoc pair-wise comparisons
were then performed to assess genotypic difference within either the trace or no-trace condition at each successive bins. These indicated that the mutant mice exhibited stronger freezing than controls in the no-trace condition across bins 2 and 3 \(p<0.05\), while the mutant mice showed weaker freezing than controls in the trace condition during the same two bins \(p<0.05\). These confirmed the graphical impression (Figure 4A) that the trace conditioning effect was exacerbated in the mutants solely in bins 2 and 3. Supplementary analyses restricted to each of the four CS bins further revealed the presence of a significant genotype × trace interaction only in bins 2 and 3 \(F_{(1,54)}=5.88\); \(p=0.02\); at bin 3: \(F_{(1,54)}=6.69, p=0.01\).

Figure 4: Test of CS freezing in Experiment 1. The levels of freezing observed in the CS test (day 3) are expressed as a function of 2-min bins. The first bin referred to the initial baseline period prior to CS-onset. The CS was presented throughout the subsequent four bins in A. Baseline freezing behaviour in the pre-CS bin did not differ between groups. Next, the trace conditioning effect was evident across all four bins: animals in the trace condition (red lines) showed weaker freezing than those trained under the no-trace condition (black lines). However, the mutants (filled symbols) exhibited clearly a stronger trace conditioning effect than controls (open symbols) during bins 2 to 3 as indicated by post-hoc comparisons between mutants and controls of either the trace (red # : \(p<0.05\)) or no-trace condition (black # : \(p<0.05\)). The interpretation of a stronger trace conditioning effect was also evident in the average freezing level across all four CS-bins as depicted in B, which was associated with a significant genotype × trace interaction \(p<0.05\). Pair-wise comparison confirmed the overall presence of the trace conditioning effect in both mutant and control mice [*: \(p<0.05\)]. Error bars refer to ±SEM.

Experiment 2: Contingency degradation

- **CS-freezing (Day 1):** Freezing levels during CS presentation obtained in the fully contingent and partial contingent training conditions were separately analysed because the full contingency condition involved three discrete CS presentations (each followed by a US, i.e., they were reinforced) whereas the partial contingency condition involved additional three CS presentations (that were not followed by a US). Separate genotype × CS-presentations ANOVAs indicated that freezing generally increased across successive CS presentations regardless of training condition and genotype [full contingency condition: \(F_{(2,64)}=33.33, p<0.001\); partial contingency condition: \(F_{(5,170)}=14.93, p<0.001\)].
expected, the CS-freezing achieved under the partial contingency training condition was lower than the full contingency condition as training progressed. This impression was confirmed by an additional ANOVA comparing directly the two training conditions by focusing on the three-shared reinforced CS-presentations. This $2 \times 2 \times 3$ (genotype $\times$ contingency $\times$ CS-presentations) ANOVA yielded a significant main effect of contingency [$F_{(1,66)}=11.49$, $p<0.01$]. This was also accompanied by a significant main effect of CS-presentations [$F_{(2,132)}=49.52$, $p<0.001$], and its interaction with contingency [$F_{(2,132)}=12.30$, $p<0.001$]. Post hoc pair-wise comparison indicated that the contrast between full and partial contingency only attained statistical significance in the third reinforced CS-presentation [$t_{(132)}=6.49$, $p<.0001$].

**Figure 5:** Acquisition of the conditioned response across the CS-US pairing in Experiment 2: Percent time freezing at successive CS presentations is illustrated in A. In the full contingency condition (black lines), animals experienced three CS presentations, and they were all followed by a US. These CS presentations are indicated as "CS+". In the partial contingency condition (blue lines), animals experienced six CS presentations: three were followed by shock US (CS+) which were interspersed by CS-alone presentation (CS-). The recorded percent time freezing in the 30-s period preceding each CS presentation is depicted in B. Error bars refer to ±SEM.

- **Pre-CS freezing (Day 1):** The freezing during the 30 sec preceding successive CS presentations steadily increased regardless of training conditions and genotype (Figure 5B). The two training conditions were again separately analysed by using a 2-way (genotype $\times$ Pre-CS periods) ANOVA differing in the levels of the repeated measures factor. The main effect of Pre-CS periods achieved statistical significant in both analyses [full contingency condition: $F(2,64)=6.78$, $p<0.01$; partial contingency condition: $F(5,170)=12.76$, $p<0.01$]. As expected, contingency degradation also had an impact on Pre-CS freezing but in the opposite direction to that observed in terms of CS-freezing. Thus, animals trained under the partial contingency condition exhibited stronger Pre-CS freezing as training progressed. This interpretation was examined by a $2 \times 2 \times 3$ (genotype $\times$ contingency $\times$ Pre-CS periods) ANOVA comparing directly the two training conditions focusing on the three Pre-CS periods preceding the reinforced CS's, which yielded a significant main effect of contingency [$F_{(1,66)}=4.87$,
p<0.05], Pre-CS periods [F(2,132)=35.19, p<0.001], and their interaction [F(2,132)=7.45, p<0.001]. Post hoc pair-wise comparison indicated that the increase Pre-CS freezing observed in the partial contingency condition only achieved statistical significance in the Pre-CS period preceding the third reinforced CS-presentation [t(132)=4.55, p<.0001].

**Context freezing (Day 2):**

Expression of conditioned fear developed towards the training context was evaluated 24h after conditioning by re-exposing the animals to the context. No difference was apparent amongst the four groups of mice (Figure 6) as shown by a 2 × 2 (genotype × contingency) ANOVA of average percent time freezing observed in the 8-min test period which did not yield any significant effect [all F’s<1].

![Figure 6](image)

**Figure 6:** Test of context freezing in Experiment 2. The animals were returned to the conditioning context in the absence of the CS one day after conditioning. The average levels of freezing observed over the 8 min context-test session were largely comparable amongst the four groups (Control/full contingency, Control/partial contingency, Mutant/ full contingency, and Mutant/ partial contingency). Error bars refer to ±SEM.

**Pre-CS & CS freezing (Day 3):**

Expression of conditioned freezing to the tone CS was assessed on the next day in a neutral context, consisting of an initial 2-min pre-CS period followed by a period of 8-min CS presentation. Freezing was low and highly comparable across the four groups in the pre-CS period (Figure 7A). A 2-way (genotype × contingency) ANOVA of percent freezing recorded during the 2min Pre-CS period did not yield any significant effect.

Onset of the CS led to a clear rise in freezing. The increase was substantially weaker in mice trained under the partial contingency condition than those trained under the full contingency condition (Figure 7A). However, this effect of contingency degradation was only visible in the mutant mice (Figure 7B). The mutant mice were freezing more strongly than controls in the full contingency condition, whilst they exhibited marginally weaker CS-freezing than controls in the partial contingency condition (Figure 7B). These bidirectional effects of the mutation contributed to the emergence of a significant genotype by contingency interaction [F(1,66)=5.15, p<0.05] in a 2 × 2 × 4 (genotype × contingency × 2-min bins) ANOVA of percent time freezing across successive 2-min bins during the 8-min CS period. Post-hoc pairwise comparisons of the average freezing levels across the 8-min test indicated a significant effect of
contingency degradation in the mutant (p=0.009) but not the controls, and that this contrast was associated with a significant difference between genotypes in the full contingency condition (p=0.038).

The ANOVA also yielded a significant effect of bins \([F(3,198)=91.94, p<0.01]\) as well as the three-way interaction \([F(3,198)=3.42, p<0.05]\). Post hoc pair-wise comparisons were then performed to assess genotypic difference within either the full contingency or the partial contingency condition at each successive bins. These indicated an increase in conditioned freezing by the mutant relative to the control in the full contingency condition over the first two bins \(p<0.05\). Supplementary analyses restricted to each of the four CS bins revealed a significant genotype × contingency interaction in the first two bins \(F(1,66)=4.52; p=0.04; \) at bin 2: \(F(1,66)=6.34; p=0.01\), confirming the graphical impression (Figure 7A) that the increased sensitivity to contingency degradation in the mutants was most clearly seen in the first four min of the test.

**Figure 7:** Test of CS freezing in Experiment 2. The levels of freezing observed in the CS test (day 3) is expressed as a function of 2-min bins. The first bin referred to the initial baseline period prior to CS onset. The CS was presented throughout the subsequent four bins in A. Baseline freezing behaviour in the pre-CS bin did not differ between groups. Onset of the CS induced a substantial increase in freezing which subsided over the course of the 8-min test period. The contrast between full and partial contingency significantly affected the expression of conditioned CS-freezing in mutants but not control mice. This was associated with an increase in conditioned freezing by the mutant relative to the control in the full contingency condition over the first two bins \(# : p<0.05\). A similar impression was obtained when the average freezing level across all four CS-bins is considered in B. Pair-wise comparisons following emergence of the significant genotype by contingency interaction revealed (i) only the mutant mice exhibited a significant effect of contingency degradation, and (ii) mutant mice exhibited stronger CS-freezing than controls in the full contingency condition (both \(p<0.05\)). Error bars refer to ±SEM.

**Experiment 3A: Context foreground conditioning with 3 shocks**

On the day of conditioning, freezing increased across successive shock USs (Figure 8A) without any apparent difference between genotypes. A \(2 \times 4\) (genotype × ISI’s) ANOVA of freezing across four successive inter-shock intervals (ISI’s) revealed only a significant effect of ISI’s \([F(3,66)=43.63, p<0.01]\). Across the next four test days, freezing was strong in the training context
'A' but remained low in the neutral context 'B' (Figure 8B), confirming the context-specificity of the conditioned freezing response. A $2 \times 2 \times 2$ (genotype $\times$ contexts $\times$ days) ANOVA supported this conclusion with a significant effect of contexts [$F_{(1,22)}=47.47$, $p<0.001$]. Freezing on the second exposure to the training context was visibly lower than the first, reflecting extinction of this conditioned response. A $2 \times 2$ (genotype $\times$ days) ANOVA restricted to context 'A' yielded a significant effect of days [$F_{(1,22)}=7.25$, $p<0.05$]. In all analyses, there was no evidence for any genotype effects.

**Figure 8:** Acquisition and Test of context foreground conditioning in Experiment 3A. Percent time freezing showed a monotonic increase across successive ISI-periods on day 1 as illustrated in A, with the arrows representing the three shock US's. As shown in B, context freezing was assessed by returning the animals to the shocked context (A) on days 2 and 4, in comparison to exposure to a neutral context (B) on days 3 and 5. Higher levels of freezing in context A relative to that in context B constitutes conditioned freezing specific to the shocked context. Mutant and control mice remained indistinguishable throughout the entire experiment. All values refer to mean ± S.E.M.

**Experiment 3B: Context foreground conditioning with 1 shock**

There was a clear increase in freezing before and after the shock delivery on conditioning day (Figure 9A) without any difference between mutants and controls. A $2 \times 2$ (genotype $\times$ ISI's) ANOVA only revealed a significant ISI's effect [$F_{(1,29)}=34.55$, $p<0.01$]. Across the next four test days, freezing was stronger in the training context 'A' but remained low in the neutral context 'B' (Figure 9B). This confirmed again the context-specificity of the conditioned freezing response, which is supported by a $2 \times 2 \times 2$ (genotype $\times$ contexts $\times$ days) ANOVA yielding a highly significant effect of contexts [$F_{(1,29)}=59.29$, $p<0.001$]. The genotype by contexts interaction also emerged significantly [$F_{(1,29)}=5.51$, $p<0.05$] due to stronger freezing exhibited by the mutant mice in context 'A' [$t_{(29)}=2.66$, $p<0.05$] but not context 'B' [$t_{(29)}=0.13$, $p=0.89$] (Figure
9C) as indicated by post hoc pair-wise comparisons. A $2 \times 2$ (genotype × days) ANOVA restricted to context ‘A’ reaffirmed this conclusion, yielding an overall effect of genotype [$F_{(1,29)}=5.29$, $p<0.05$]. The absence of a significant effect of days here was consistent with the graphical impression of the lack of extinction across the two tests in context ‘A’.

Figure 9. Acquisition and Test of context foreground conditioning in Experiment 3B. A. The animals showed a sharp increase in percent time freezing after the delivery of the shock US (indicated by the arrow) in day 1. B. context freezing was assessed by returning the animals to the shocked context (A) on days 2 and 4, in comparison to exposure to a neutral context (B) on days 3 and 5. C. Higher levels of freezing in context A relative to that in context B constitutes conditioned freezing specific to the shocked context. Mutant mice exhibited stronger freezing specifically to context A, although this effect only achieved statistical significance on the first re-exposure test on day 2. * indicates $p<0.05$ based on appropriate post hoc comparison. Values refer to mean ± S.E.M.

DISCUSSION

The present study has extended the previous finding of enhanced latent inhibition (LI) reported in GlyT1Δfb-neuron mice (Yee et al., 2006) by showing that this conditional GlyT1 deletion model was further associated with enhanced sensitivity to CS-US trace (Experiment 1, see Figure 4B) and degradation of CS-US contingency (Experiment 2, see Figure 7B). At the same time, we replicated the phenotype of enhanced conditioned freezing under the "no-trace" or "contingent" test condition, which is operationally identical to the "non-pre-exposed" condition in the original LI experiment reported by Yee et al. (2006). Furthermore, we showed that this phenotype of enhanced conditioned freezing can be extended to foreground contextual conditioning (Experiment 3B, see Figure 7B), but this effect seems fragile by comparison.

The joint presence of phenotypes in trace conditioning and contingency degradation in GlyT1Δfb-neuron mice is of significant relevance to the interpretation of the mutation’s overall impact on associative learning. As mentioned in the Introduction, a description based on stronger conditioning as such is untenable. Such an account is particularly problematic in
dealing with the trace conditioning results here (Experiment 1) whereby GlyT1Δfb-neuron mice showed weaker conditioning under the 20-s trace condition. Similarly, there was no evidence for enhanced conditioning either in the partial contingency condition (Experiment 2) or the CS pre-exposed conditioning (see Yee et al., 2006). Hence, the test condition decides whether one may observe an enhancement or conversely a diminution in the intensity of the conditioned response. We propose here that perceptual learning mechanisms underlying the modulation of the associability of a potential CS may offer a possible unifying account.

**The dynamic of associability**

CS associability refers to the perceived properties of a potential CS that determines its readiness to enter into association with an impending US (Hall, 1991). One critical determinant of CS associability is the CS's salience, which refers to its ability to occupy the subject’s focus of attention. Essentially, formal theories developed in the past accounting for the phenomena of LI, trace conditioning and contingency degradation are, to varying extent, related to attention-mediated selectivity in learning under differing Pavlovian learning situations.

All associative learning theories emphasize that attention to the CS critically governs the rate of CS-US learning. Thus, stronger attention (i.e., CS being more salient) facilitates the formation and growth of CS-US association. In its simplest form, the Rescorla and Wagner (1972) model specifies the change of associative strength after successive CS-US pairing based on a simple error correction rule, and the change in associative strength ($ΔV_n$) after the $n^{th}$ trial is formally described as:

$$ΔV_n = α(\lambda - V),$$

where $α$ denotes the associability of the CS, $λ$ refers to the maximum strength that the CS-US association can achieve and is set by the magnitude of the US, and $V$ stands for the current CS-US associative strength. The resulting (cumulative) associative strength after the $n^{th}$ trial ($V_n$) is therefore:

$$V_n = V_{n-1} + ΔV_n$$

The parameter $α$ is what we are interested in here, and it reflects the intensity, discriminability, or salience of the CS. $α$ is considered as a fixed property of the CS in the Rescorla-Wagner's initial model (1972), but mutant and control might perceived the same stimulus with different effective $α$. Figure 10 depicts the consequence of this difference with $α_{control} = 0.5$ and $α_{mutant} = 0.75$. By the end of three CS-US pairings, the cumulative associative
strength is higher in the mutant than in the control mice. Most interestingly, application of the same parameters yielded the opposite outcome in our partial contingency design according to the Rescorla-Wagner model (1972; Experiment 2): the final associative strength is lower in the mutant than in the control mice.

**Figure 10.** The impact of CS Salience on CS-US associative strength under the partial and Fully contingent conditions in Experiment 2 as predicted by the Rescorla-Wagner model. We applied the Rescorla-Wagner model to calculate the final associative strength according to the partial or full contingency conditions employed in Experiment 2 as a function of CS salience, $\alpha$ (in the range of 0.01 to 0.99). $\lambda$ is set to 1, and the initial value of CS-US associative strength is set to 0 in these simulations. Increasing $\alpha$ leads to a monotonic increase in the final associative strength in the full contingency condition, but increasing $\alpha$ is associated with an inverted U-shape in the partial contingency condition. Hence, beyond a critical value ($\alpha = \frac{1}{3}$ in this specific example), any further increase in $\alpha$ result in opposite impact on the final associative strength. We therefore speculate that the outcome in Experiment 2 might be explicable in similar terms, namely $\alpha_{\text{mutant}} > \alpha_{\text{control}}$. To illustrate this, we assign $\alpha_{\text{mutant}} = 0.75$, and $\alpha_{\text{control}} = 0.5$, and highlighted the corresponding theoretical values for mutant (□) and controls (○) of final associative strength in the graph. The blue arrow refers to the increase in associative strength when comparing mutant/full contingency with controls/full contingency condition. The red arrows refers to the decrease in associative strength when comparing mutant/partial contingency with controls/partial contingency condition.
The impact of CS-US discontiguity, as captured by the trace conditioning procedure (Experiment 1), is not easily conceptualized within the Rescorla-Wagner's model (1972) which does not readily incorporate the temporal dynamic between CS and US. On the other hand, the "Sometimes Opponent Process" (SOP) model developed by Wagner (1981) can readily deal with trace conditioning, and critically, here, offers an interpretation of the trace conditioning effect as a consequence of the modulation of the subject's attention to the CS. The model specifies that the representation of a stimulus can assume different states of activation and this bears direct consequence on the stimulus' associability. Briefly, when a CS is presented, its representation is, at first, strongly activated (A1 state), and can readily form an association with other stimuli at the same state of activation. However, the primary A1 activated state is transient and the representation will decay into a secondary state (A2 state) with low associability. The decay from A1 to A2 states can be understood as a shift from the focus to the periphery of attention. The A1→A2 decay can take place at any time and proceeds particularly rapidly as soon as the stimulus is removed. Hence, imposition of a trace interval between CS and US hinders the formation of effective CS-US association, and weakens subsequent CR expression. Enhanced sensitivity to CS-US trace may imply that the A1→A2 decay is facilitated in the mutant (see Figure 11). As depicted in Figure 11, application of Wagner's model (1981) provides a temporal dimension that incorporates the suggested difference in the perceived salience of the CS, i.e., $\alpha_{\text{mutant}} > \alpha_{\text{control}}$. This translates into a stronger initial primary activation of CS representation, which is responsible for the stronger CR in the mutant trained under the contiguous condition (same as the contingent condition above). Faster A1→A2 decay is responsible for the mutant mice's weaker CR trained in the "trace" condition relative to controls.
Figure 11: Analysis of the phenotype revealed in experiment 1 on trace conditioning based on Wagner’s SOP model (1981). According to Wagner’s SOP model (1981), the internal representation of a stimulus (CS or US) may assume different states of activation, A1 and A2. It is activated at the primary state (A1) upon presentation of the stimulus at a time when its representation is initially inactive (I state). The A1 state can be considered as a stimulus in the centre of attention. However, the activation state will decay in time into a secondary state (A2) when the stimulus is occupying the periphery of attention. Eventually, it would return to the inactive state. The model posits that associations are formed between stimuli that are both in the A1 state, for instance, when CS and US are contiguously presented. When CS and US are separated in time, the ensuing partial decay of CS representation into A2 state during the CS-US trace interval will render the formation of CS-US association more difficult. According to this description, faster A1→A2 decay (as emphasized by the thicker red A1→A2 arrow in A) will give rise to a stronger sensitivity to the trace conditioning effect, as seen in our mutant mice. This mouse line also responded more strongly when CS-US contingency was degraded (Experiment 1), which we proposed may be explicable in terms of the CS as being perceived as more salient as predicted by the Resorla-Wagner model (i.e., $\alpha_{\text{mutant}} > \alpha_{\text{control}}$, see Figure 10). This translates into a stronger initial A1 activation in Wagner’s SOP model and is represented by the red thick I→A1 arrow in A. Since only a limited amount of information held active in A1 at any given time, an initial stronger activation in A1 state may be linked to the faster A1→A2 decay, as depicted in B. The illustrated comparison of the hypothetical temporal function of the CS’s A1-state activation over time between mutant and control mice provides a theoretical account for the bi-direction effect seen in Experiment 1 within the framework of Wagner’s SOP model. The key characteristics of the mutant’s profile (red) in comparison with that of the controls (blue) are: (i) stronger activation of the representation of the CS in A1 upon presentation of the CS, leading to stronger CR in the contiguous training condition; and (ii) a faster transition from A1 to A2 state, leading to weaker CR in the discontiguous training condition.

And, it may not be surprising that the initial elevation of perceived salience is accompanied by a more rapid decay when one takes into account that only a limited amount of stimuli (or their elements) can be maintained under A1 activation. Taken together, the phenotypes emerged in the trace conditioning and the contingency degradation experiments could be attributed to specific changes in the dynamic of attention modulation as described by existing (and compatible) formal theories of associative learning (Mackintosh, 1975; Pearce & Hall, 1980; Wagner, 1981), rather the differences in the overall capacity of information processing. Thus, Figure 11 further emphasizes that the amount (area under the curve) of focal processing as approximated by A1 activation summed across time needs not differ substantially between genotypes.
Learning not to attend

Thus, learning can be facilitated or hindered by appropriate changes in CS associability alone. Rescorla-Wagner’s initial model (1972) considers CS associability ($\alpha$) as a fixed property of the external CS. Mackintosh (1975) and Pearce and Hall (1980) have developed separate theories formally specifying how $\alpha$ may be modified depending on the signal-relation between the CS and US, e.g., as learning progresses. They similarly predict that non-reinforced CS pre-exposures (prior to CS-US pairing) would diminish the effective salience of CS and therefore impede subsequent conditioning, giving rise to LI. GlyT1\textsuperscript{Δfb-neuron} mice were more responsive to the CS pre-exposures effect — these mutant mice thus expressed LI when the pre-exposure condition was insufficient to yield LI in controls (Yee et al., 2006), suggesting a more rapid decay of the pre-exposed CS’s effective salience when the CS had been presented repeatedly without being followed by any significant events. According to Wagner’s SOP model (1981), this once again involves a shift of CS representation into the $A_2$ state, thus rendering it harder to form association with any potential US. The model specifies that pre-exposure of the CS in a given context endows to the context the ability to cue or prime the representation of the CS directly into the $A_2$ state when the animals are subsequently returned to it. The mechanism thus depends on the formation of context-CS association. GlyT1\textsuperscript{Δfb-neuron} mice might learn the context-CS association more rapidly, for reasons similar to those underlying stronger CS-US association explained above, and thus facilitated the expression of LI later.

In Experiments 3A and 3B, we directly tested if contextual conditioning might be facilitated in GlyT1\textsuperscript{Δfb-neuron} mice. The evidence was, however, tentative at best because (i) stronger contextual conditioning was only observed in the one-shock but not the three-shock paradigm, and (ii) its presence in the one-shock paradigm was restricted to the first re-exposure test conducted 24 h following conditioning, when control mice were exhibiting relatively low levels of freezing, but not on the second re-exposure test (Figure 9B). However, it should be noted that learning to associate a given context with a significant event such as foot shocks may not faithfully reflect the processes underlying association with a neutral stimulus. Hence, it is worth noting that the novelty response induced by displacement of objects was stronger in GlyT1\textsuperscript{Δfb-neuron} mice than controls (Singer, Boison, Mohler, Feldon, & Yee, 2007), and this phenotype might be interpreted as evidence for enhanced association between spatial context and discrete object stimuli.

Conclusions

Instead of a broad strengthening of memory trace, we conclude here that the associative learning phenotypes resulting from the selective deletion of forebrain neuronal GlyT1 might stem from an effect on attentional modulation related to the processing of the critical CS. What
is common to these apparently disparate phenotypes is that they all reflect a stronger selectivity in learning, and that they can be attributed to attentional mechanisms specified in existing learning theories. Although the precise physiological mechanisms whereby enhanced NMDAR function (induced by GlyT1 deletion specifically) may bring about such impacts on behaviour and cognition is unknown, our mouse model highlights neuronal GlyT1 in the forebrain as a potential target for diseases with characteristic dysfunction in attentional rigidity, including schizophrenia.

REFERENCES


Working memory performance is unaffected by glycine transporter 1 deletion from forebrain neurons

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ABSTRACT
Glycine transporter 1 (GlyT1) is a potential pharmacological target to ameliorate memory deficits, including schizophrenia-related cognitive symptoms believed to stem from \(N\)-methyl-\(d\)-aspartate receptor (NMDAR) hypofunction. Disruption of glycine-reuptake near excitatory synapses is expected to enhance NMDAR function by increasing glycine-B site occupancy. Although genetic models with conditional GlyT1 deletion restricted to forebrain neurons have yielded several promising promnesic effects, its impact on working memory function remains unclear. Since working memory deficiency represents a prominent cognitive symptom of schizophrenia, the present study aims to clarify this important outstanding lacuna using a within-subject multi-paradigm approach. Here, a consistent lack of effects was convincingly demonstrated across three working memory test paradigms – the radial arm maze, the cheeseboard maze, and the water maze, thus providing valuable specification to the regulation of forebrain neuronal GlyT1 over learning and memory. These null outcomes contrasted with the enhancement in working memory seen in mutant mice with GlyT1 deletion extended to cortical glial cells suggesting that glial-based GlyT1 may be more closely linked to modulation of working memory function. The present study thus points to a possible functional divergence between neuronal and glial GlyT1 populations in the brain amidst evidence that they are both cognitively relevant, but perhaps mediated by dissociable mechanisms.

INTRODUCTION
The \(N\)-methyl-\(d\)-aspartate receptor (NMDAR) is critical for long-term synaptic modifications believed to underlie various forms of learning and memory (Morris, Anderson, Lynch, & Baudry, 1986; Morris, 1989). Its hypofunction is implicated in cognitive deficiency that is characteristic of a number of psychiatric disorders, including schizophrenia (Javitt, 2004). Hence, enhancing NMDAR function might ameliorate such deficits (Martin, Grimwood, & Morris, 2000). However, stimulation by direct NMDAR agonists can lead to severe side effects including neurotoxicity and seizures, and therefore alternative approaches focusing on the modulatory sites on NMDAR have received increasing attention. One such strategy is to increase occupancy of the glycine-B site (located on NR1 subunit). Binding to the glycine-B site is mandatory for receptor activation by the neurotransmitter glutamate, but extracellular glycine levels near glycine-B sites are governed by glycine transporter 1 (GlyT1) co-localized with NMDAR in neurons and also expressed in adjacent glial cells. GlyT1 mediates the re-uptake of glycine from the relevant synaptic clefts thus maintaining levels of glycine at glycine-B sites at sub-saturated levels (Wood, 1995; Danysz & Parsons, 1998; Aragon & Lopez-Corcuera, 2005; Eulenburg, Armisen, Betz, & Gomez, 2005). Disruption of GlyT1-mediated reuptake can therefore facilitate NMDAR function due to the resulting elevation of glycine levels in extracellular space.
Systemic pharmacological blockade (Bergeron, Meyer, Coyle, & Greene, 1998; Depoortere, et al., 2005) or genetic deletion (Tsai, et al., 2004; Gabernet, et al., 2005; Yee, et al., 2006) of the GlyT1 gene enhances NMDAR excitability, reverses behavioural deficits produced by NMDAR antagonists, and confers pro-cognitive effects relevant for the functional deficiency in schizophrenia. In particular, working memory (the deficiency of which is a cardinal feature of cognitive dysfunction in schizophrenia) is effectively enhanced in a delay-dependent manner by the specific GlyT1 inhibitor, SSR504734 (Singer, Feldon, & Yee, 2009b). However, systemic drug action does not differentiate between GlyT1 near NMDARs versus those expressed near inhibitory glycinergic synapses. Hence, the use of genetically engineered mouse models is necessary to consolidate the proof of principle underlying the rationale of GlyT1 inhibition as an effective strategy to boost cognitive functions.

A genetic mouse model has confirmed that GlyT1 disruption restricted to the telencephalon is sufficient to enhance working memory function (Singer, Boison, Mohler, Feldon, & Yee, 2009a). However, this genetic model did not discriminate between the neuronal and glial components of GlyT1. The latter would affect inhibitory glycinergic neurotransmission via the strychnine-sensitive glycine-A site located on glycinergic receptors (Eulenburg, et al., 2005; Betz, Gomez, Armes, Scholze, & Eulenburg, 2006). The genetic model based on the CamKIIαCre loxP conditional gene deletion system is selective for forebrain neuronal GlyT1 and offers a more specific model. Our previous attempt to evaluate working memory in this mouse line (CamKIIα/GlyT1-KO mouse) using the water maze paradigm however failed to yield a clearly interpretable outcome (Singer, et al., 2009a). In this test, the hidden escape platform assumes a new position in the water maze on each day, and indexation of working memory relies on within-day performance improvement seen from trial 1 to trial 2. However, CamKII/GlyT1-KO already outperformed controls in trial 1 while not differing from controls in trial 2. This divergence in baseline performance (when the platform location was essentially unknown to the animals) has precluded a simple interpretation in terms of enhancement or impairment.

The present study aimed to clarify this outstanding issue in order to identify the critical cellular/regional specificity of the working memory-enhancing effect of GlyT1 down-regulation. Here, a within-subject multi-paradigm approach was adopted. The animals were first evaluated in the radial arm maze and cheeseboard paradigms of working memory, before being subjected to a replication of the water maze working memory test based on Singer et al. (2009a). We report here a convincing set of null effects indicating that cell-specificity and/or the magnitude of deletion could be critical determinants on the efficacy of GlyT1 inhibition on working memory function.
METHODS

Subjects

A full description of the generation of the CamKIIαCre; GlyT1tm1.2fl/fl mouse line has been provided previously (see Yee, et al., 2006). The experimental subjects were generated by crossing CamKIIαCre;Glyt1tm1.2fl/fl mice with Glyt1tm1.2fl/fl mice (both maintained on a pure C57BL/6J background) to yield a 1:1 mixture of offspring comprising the CamKIIαCre;Glyt1tm1.2fl/fl (“mutant”) and Glyt1tm1.2fl/fl (“control”) genotype. This breeding strategy maintained the CamKIIαCre allele in a heterozygous state for the mutant, whereas all mice were homozygous for the Glyt1tm1.2fl/fl allele. Breeding was performed in a specific-pathogen free breeding facility (Laboratory of Behavioural Neurobiology, Swiss Federal Institute of Technology Zurich, Schwerzenbach, Switzerland). Genotype was determined by standard polymerase chain reaction on postnatal Days 21–30 as described by Yee et al. (2006). Litters were weaned at postnatal day 21, and a mixture of mutant and control littermates of the same sex were housed in groups of 4–6 in Macrolon Type-III cages (Techniplast, Milan, Italy). At the age of 12 weeks, the subjects were transferred to a separate mouse vivarium (21 ± 1 C°; relative humidity, 55 ± 5%) and single caged in Macrolon Type-II cages (Techniplast, Milan, Italy) for the duration of behavioural testing. Mice had ad libitum access to water and food (Kliba 3430, Klibamuhlen, Kaiseraugst, Switzerland), unless otherwise specified, and kept under a reversed light–dark cycle with lights off from 0700 to 1900 hrs. All behavioural experiments were conducted in the dark phase.

A cohort of male mice consisting of 10 mutant and 10 littermates controls was used in the present study. The animals were approximately 24 weeks old at the beginning of behavioural testing, and were subjected to working memory assessment in the radial arm maze, cheeseboard maze and water maze. A separate cohort of naive animals comprising 7 mutant and 7 control male mice was used for the Y-maze test of spatial familiarity judgement.

All experimental procedures described had been previously approved by the Zürich Veterinary Office. All manipulations conformed to the ethical standards stipulated by the Swiss Act and Ordinance on Animal Protection, and were in accordance with the European Council Directive 86/609/EEC as well as the National Institutes of Health publication no. 86-23 (revised 1985) on animal experimentation.
Apparatus & Procedures

Experiment 1: Radial Arm Maze

The apparatus consisted of an automated maze made of grey acrylic (Imetronic, Pessac, France) position in the middle of a well-lit room enriched with extra-maze cues. The maze comprised eight identical arms radiating from a common central platform. The maze was elevated 73 cm above floor level. Each arm measured 55.5 cm long and 12 cm wide, and each was guided by transparent Plexiglas walls (16 cm long and 11 cm high) mounted on either side to prevent mice from jumping across adjacent arms. The entrance to each arm from the central platform was guided by a movable transparent Plexiglas door (12 cm × 16 cm), which could be retracted into the floor of the maze to allow access to specific arms. A food pellet dispenser was located at the far end of each arm delivering 20 mg Noyes pellets (Noyes Precision Pellets PJPPP-0020, Research Diets Inc., New Brunswick, NJ, USA). The entrance doors and pellet dispensers were individually controlled by a PC that was connected to a digital camera mounted directly above the maze for continuously tracking the animal’s position. The test procedures consisted of four consecutive phases as specified below.

- **Habituation:** The animals were gradually introduced to a restricted food diet, reducing access to food in the home cage from 12 h per day (day 1) to 2 h per day (from day 5 onwards). Body weights were monitored daily and prevented from falling below 86% of their ad lib weight with additional feeding whenever necessary. In the same period, habituation to the maze was conducted over six consecutive days. On the first two days, mice were placed in the maze in pairs and allowed to explore freely the entire maze surface for 5 min where food pellets were scattered along the eight arms. On the next two days, mice were placed individually on the maze for 5 min with food pellets scattered only towards the end of each arm. On the last two days, access was restricted to 4 arms forming a “+” configuration to be used in the next stage. The two possible configurations were balanced between mutant and control animals. They were allowed to collect one food pellet at the end of each arm within 5 min. Two mutant mice (out of the initial ten) failed to explore the maze or did not consume any reward, and were dropped from further testing.

- **Working memory test (4-arm):** Working memory performance was first assessed in a 4-arm design with the arms spaced 90° apart. A trial began by confining the mouse in the centre of the maze with all doors closed. Ten seconds later, the doors to the four designated arms were open and the animals were allowed to enter in any one arm. A choice was deemed to be made when the body of the mouse reached 4 cm into the chosen arm. All doors were then closed and they remained so except the door to the chosen arm was re-opened as the mouse reached the food dispenser. Only the first visit to a given arm was rewarded by the delivery of one reward pellet. The mouse returned to the central platform and again confined there for 10 s before the
next choice. The mice were allowed a maximum of 10 min to collect all four rewards, and all mice succeeded in doing so across the 8 days of test.

- **Working memory test (8-arm):** The procedures were identical to the previous phase except that all eight arms were employed and the maximum session time was extended to 30 min. The animals were now required to collect a total of 8 rewards, one from the end of each arm. Re-entries like before were never rewarded. This phase include 5 consecutive daily sessions.

- **Working memory test (8-arms) with an intra-session time-out:** Memory load was increased from the 4-arm to the 8-arm procedures. Here, the retention load was taxed by introducing a time-out following the collection of the fourth reward in a session. When the mouse returned to the central platform after consuming the fourth reward, it was removed from the maze and placed inside an opaque box in the experimental room for the time-out period, which lasted 2 min in the first two sessions, and 15 min in the next two sessions. At the end of the time out period, the mouse was returned to the central platform and allowed to collect the remaining four pellets from the unvisited arms.

Working memory performance was indexed by scoring the number of working memory errors defined as re-entries to any arms. The session time was also recorded.

**Experiment 2: Cheeseboard Maze**
The apparatus has been fully described previously (Llano Lopez, Hauser, Feldon, Gargiulo, & Yee, 2010). It was made from a 3-cm thick circular wooden table measuring 1.1 m in diameter. The maze was elevated 123 cm above the floor and positioned in the same testing room used for the radial arm experiments without any alteration to the extra maze cues. One side of the board was smooth and was used in the habituation phase. The other was prepared with 32 food wells, arranged in a radial pattern resembling the arrangement of arms in the radial arm maze (see Llano Lopez, et al., 2010). There were thus eight rows of food wells radiating from the board centre, each comprising four food wells spaced equally apart, spanning 20 to 45 cm from the board centre. Each food well measured 3.1 cm in diameter and 1.3 cm deep, into which a removable plastic food cup could be fitted. A reward of 75 µl condensed milk solution (25% dilution of the commercial condensed milk manufactured by Alicommerce SAS, Liebefeld-Bern, Switzerland) could be placed in the centre of the removable food cup. A digital camera mounted directly above the maze was connected to a PC running the Ethovision tracking system (Noldus Information Technology, Wageningen, The Netherlands) to record distance travelled at a sampling rate of 5 Hz.
• **Habituation**: The animals were already familiar with the testing room, and they were therefore only habituated to the cheeseboard maze using the smooth side of the cheeseboard. To begin, the mice were individually placed in the centre confined under a semitransparent plastic beaker (diameter: 15cm, height: 25cm) for 5 s before being released and allowed two minutes to explore the maze surface.

• **Working Memory test**: The test procedures resembled that of the water maze working memory protocol and consisted of discrete visits (or trials) to the maze within a day. A different food well was baited every day, but the same food well was repeatedly rewarded across visits within a day. The baited well positions were counterbalanced across days, delays and animals but only the wells located at 37.5 cm from the centre were selected. Each visit began with the mouse placed in the board centre and confined under a beaker for 5 s. If an animal failed to collect the reward within 120s, it was guided to it by the experimenter. After locating the reward, the animals were allowed 20s to consume the reward. Over the first four days of test, animals were given four trials/visits per day with an inter-trial interval (ITI) of 2 min. The ITI delay was extended to 15 min over the next four days and the number of trials reduced to two. The animals were kept in an opaque box during the ITIs.

  Working memory performance was indexed by the improvement in efficiency of collecting the food reward from trial 1 to trial 2 on each day. Further improvement from trials 3 onwards (in the first 4 days) might incorporate elements of reference memory and was not included in the analysis, but they were effective in instilling the matching or ‘win-stay’ rule implicit in this working memory procedure, which in contrast to the ‘win-shift’ strategy intrinsic to the radial arm maze procedure.

**Experiment 3: Water Maze**

The water maze apparatus, procedures and testing room were essentially similar to those employed by Singer et al. (2009a, b) to facilitate comparison with the previous studies. Briefly, the maze was a white circular fibreglass tank, 102 cm in diameter and 36 cm deep. It was filled to a depth of 19 cm with tap water which temperature was strictly maintained at 24±1°C. A transparent Plexiglas cylinder (diameter 7 cm, height 18.5 cm) served as the escape platform, with its surface submerged 0.5 cm below the water surface and therefore invisible to the animals. A local cue (a black wooden arrow measuring 10 × 2 × 2 cm) could be mounted 7 cm directly above the platform to allow visual detection of its location during the cued test. A digital camera was installed above the water maze and was connected to a PC running the Ethovision (version 3.1, Noldus Information Technology, Wageningen, The Netherlands), which tracked the animal's position at a sampling rate of 5 Hz and calculated the escape latency, distance moved and average swim speed on each trial. Four points, spaced equally along the circumference of
the WM, served as the cardinal points: N, E, S and W, dividing the maze surface into four quadrants (NE, SE, NW and SW) of equal size.

- **Cued task:** After the completion of the cheeseboard experiment, the mice were returned to an ad libitum diet and left undisturbed for one day before commencement of the water maze experiment. On the first day, the mice were evaluated on a ‘cued task’ in which a local cue indicated the platform’s location. This served to familiarize the animals with the escape response. The platform was positioned in the maze centre and each animal underwent two consecutive trials, with the starting positions randomly selected from four release points (N, E, S, and W). In the first trial, the mouse was released from the starting point with its head facing the platform location. In the second trial, it was released from the starting point facing the wall of the maze. The two trials were separated by 15s. When an animal failed to locate the platform within the 60s limit, an escape latency of 60s was assigned and the animal was guided to the platform by the experimenter and allowed to stay on it 15s.

- **Working memory test:** The platform now always remained hidden underneath the water surface, and assumed a new position on each test day but remained in the same position across trials within the same day. The animals were first trained on a 4-trial-per-day procedure to facilitate acquisition of the matching rule over the first four days with an ITI of 2 min, which matched the design adopted in Experiment 2 with the cheeseboard maze. Over the next days, the ITI was extended to 15 min, and returned to a 2-trial-per-day procedure. The mice were kept in a waiting cage during the ITI located in the same experimental room. Working memory was indexed by the reduction in time spent and path length in trial 2 in comparison with trial 1 when the platform location was unknown. As in the cheeseboard experiment, the third and fourth trials included in the first four days of training were not included in the analysis. Twelve platform positions were defined: six located at a distance of 35cm (in the N, S, NE, SE, SW, and NW directions), and six more at a distance of 15cm from the centre (in the N, E, S, W, NW and SE directions). These locations were counterbalanced across days and genotype factor. The start position differed between consecutive trials, and was randomly selected from eight possible release points (N, E, S, W, NE, NW, SE and SW) for each mouse.

### Experiment 4: Y-maze

This test was performed in a separate cohort of naïve mice and provided an independent evaluation of spatial recognition by testing the animals’ ability to distinguish between familiar and novel locations as defined by extra maze cues. The Y-maze consisted of a wooden floor surface, elevated 80 cm from the ground level, with three identical stems spaced 120° extending from a triangular central area. Each arm measured 50 cm long and 10.5 cm wide, and the entire maze area was surrounded from all sides by transparent Plexiglas walls 10 cm high. The floor of
the maze was covered with fresh sawdust bedding. A digital camera mounted above the maze was connected to a PC running the Ethovision tracking system (version 3.1, Noldus Information Technology, Wageningen, The Netherlands), which tracked the animal’s position at a sample rate of 5 Hz.

Each mouse was randomly assigned two arms to serve as the ‘start’ and ‘familiar’ arms used in the first (sample) phase of the test. The remaining third arm served as the ‘novel’ arm in the second (test) phase. Allocation of arms (start, familiar, and novel) was counterbalanced between genotype. At the start of the sample phase, access to the novel arm was blocked by an opaque Plexiglas door (12 cm high, 7.5 cm wide and 0.5 cm thick). The mouse was then introduced to the distal end of the start arm and allowed to explore freely both the start and the unblocked (familiar) arm for 5 min before being removed from the maze. Timing of the 5 min sample phase period began when the mouse had left the start arm. At the end of a specified delay period, the animals were returned to the maze with free access to all three arms. The mouse was once again placed in the start arm and allowed to explore the maze for 3 min starting from the moment it left the start arm. The test was performed three times, each conducted in a novel testing room with distinct extra maze cues, with increasing duration of delay between the sample and test phase: 2 min, 2 h and 24 h. Except for the limited delay of 2 min, when the animals were placed inside separate waiting cages kept in the same testing room, the animals were returned to their home cages during the longer delays of 2 and 24 h. To prevent possible use of odour trail, the floor bedding was replaced with fresh sawdust after every single exposure. Time spent as well as distance travelled within each arm of the maze in the sample and test phases of the experiment was separately analysed. Performance in the test phase was indexed by the preferential exploration of the ‘novel’ arm relative to the other two arms.

**Statistical analysis**

All data were analysed by parametric analysis of variance (ANOVA) with the between-subject factor Genotype (mutant vs. littermate control) and any within-subject factors deemed appropriate for the specific experiment. To assist interpretation of the statistical outcomes, significant effects were further investigated by pair-wise comparisons based on the associated error terms taken from the overall ANOVA or supplementary restricted analyses applied to a subset of data. All statistical analyses were carried out using SPSS for Windows (version 18, SPSS Inc. Chicago IL, USA) implemented on a PC running the Windows 7 operating system.
RESULTS

Experiment 1: Working memory in the Radial arm maze

The experiment comprised four separate stages spanning across 17 days. These were separately analyzed. Performance was indexed by working memory errors throughout as defined by any re-entry.

- Four-arm protocol: Learning was reflected by the decrease of working memory errors progressively across days, which was similarly observed in both mutant and control mice (Figure 1A). A 2 × 8 (Genotype × Days) ANOVA of total errors only yielded a significant effect of days \[F(7,112)=2.59; \ p<0.05\]. Neither the effect of genotype nor its interaction was close to statistical significance [F's < 1].

- Eight-arm protocol: As shown in Figure 1B, working memory errors increased when the test was extended to the 8-arm protocol. This was followed by the gradual reduction of working memory across the five test days. Performance was again largely comparable between mutants and controls. A 2 × 5 (Genotype × Days) ANOVA of working memory errors yielded a significant main effect of days \[F(4,64)=10.24; \ p<0.01\] without any statistical evidence for a genotype effect \[F(1,16)=1.76; \ p=0.20\] or its interaction \[F(4,64)=1.32; \ p=0.27\].

- Eight-arm protocol with 2 min intra-session timeout: Consistent with the high level of performance already established by the end of the previous stage, the animals committed very few working memory error in collecting the first four rewards in the first half of the test session. Next, there was as expected an increase in errors when the animals were returned to the maze following the 2-min timeout (Figure 1C). This pattern of outcome was comparably observed in mutant and control mice. A 2 × 2 × 2 (Genotype × Half-session × Days) ANOVA of working memory errors only yielded a significant main effect of Half-session \[F(1,16)=34.42; \ p<0.001\].

- Eight-arm protocol with 15 min intra-session timeout: A similar pattern of results was obtained with the intra-session timeout extended to 15 min (Figure 1D), again without any evidence for any genotypic difference. A 2 × 2 × 2 (Genotype × Half-session × Days) ANOVA of working memory errors again only revealed a significant effect of half-session \[F(1,16)=14.78; \ p<0.01\] without any statistical significance for any genotype effect [all F's <1].

Figure 1 (next page): Working memory performance on the Radial Arm Maze. The total number of errors (re-entry into a previously visited arm) is shown across days for both groups in the four arms and the eight arms configuration (A&B). There is a reduction of the total number of errors across days for both genotypes reflecting learning of the tasks and improvement of working memory performance. In (C&D), working memory performance on the eight arms baited configuration when a time-out is imposed after four collected rewards. For each delay is depicted the total number of errors per half-sessions on each day. In all the tasks (A, B, C & D) the mutation did not affect the performance. Error bars refer to ±SEM, mutant (n=8), control (n=10).
Experiment 2: Working memory (delayed matching-to-place) in the Cheeseboard maze

In this experiment, working memory performance was indexed by the reduction of latency or distance travelled to reach the food reward in trial 2 relative to trial 1 when the location of the food reward was essential not known to the animals.

- **2-min ITI**: Averaged across the four test days, both mutant and control mice were more efficient in reaching the food reward in trial 2 compared to trial 1 (right panel of Figure 2A). Separate $2 \times 2 \times 4$ (Genotype × Trials × Days) ANOVAs of the latency and distance measures yielded a clear trial effect [latency: $F_{(1,16)}=15.61$, p<0.01, distance: $F_{(1,16)}=15.78$, p<0.01]. Although there was no evidence for an interaction between genotype and trials [F<1], closer examination between days suggested that the trial effect was more consistently expressed in the controls than in the mutant mice (left panel of Figure 2A). Mutants exhibited a clear tendency for improved performance across trials on days 3–4, but not days 1–2. This divergent pattern between genotypes led to the emergency of a significant three-way interaction [latency: $F_{(3,48)}=3.33$, p<0.03, distance: $F_{(3,48)}=2.73$, p=0.05].

- **15-min ITI**: As expected, extending the ITI to 15 min imposed additional retention demand and had affected performance accordingly, while the overall improvement from trial 1 to 2 appeared weaker than before (Figure 2B). Separate $2 \times 2 \times 4$ (Genotype × Trials × Days)
ANOVAs distance and time to reach the reward location only yielded near-significant effects of trials [latency: $F_{(1,16)}=4.20; p=0.06$, distance: $F_{(1,16)}=4.45; p=0.05$]. The analysis yielded no support for any genotype effect or its interaction [All $F$'s<1].

The consistency of the latency and distance measures across the two ITI conditions was in agreement with the lack of any genotypic effect on the separate analysis of speed of movement (data not shown).

**Figure 2**: Working memory performance was assessed in the cheeseboard and the watermaze. Performance in the working memory task in the cheeseboard maze indexed by latency to reach the reward location, across days at each trial is depicted in A when the ITI was 2min and B when the ITI was 15 min. Latency averaged across days as a function of trials are depicted at the right of each graphs A and B. The latency to reach the reward was significantly reduced from Trial 1 to Trial 2 when the delay was 2min, but failed to achieve significance when the delay was 15min. The performance never statistically differed between genotypes (A and B). Working memory performance in the water maze indexed by latency to reach the platform location, across days, for each trial is depicted in A when the ITI was 2min and B when the ITI was 15 min. The latency averaged across days as a function of trials is depicted for each ITI in the right of the graphs (C and D). In both delays, mice escaping performance statistically progressed from Trial 1 to Trial 2 without any differences between genotypes. Error bars refer to ±SEM. * refers to statistical significant difference ($p<0.05$) obtained from the pair-wise comparison after significant genotype × trial × day interaction.
**Experiment 3: Working memory in the Water maze**

- **Cued task:** This was uneventful and all mice acquiring the escape response (data not shown) without any suggestion of any genotypic difference, which is consistent with the previous study by Singer et al. (2009b). The outcomes of the subsequent working memory tests approximated closely to those obtained using the cheeseboard maze (Experiment 2) described above.

- **2-min ITI:** Over the first four test days when the ITI was limited to 2 min, both mutant and control mice showed a similar magnitude of overall reduction in escape latency and associated path length from trial 1 to 2 (right panel of Figure 2C). Separate $2 \times 2 \times 4$ (Genotype $\times$ Trials $\times$ Days) ANOVAs of both performance measures yielded a significant effect of trials [escape latency: $F_{(1,16)}=45.49; p<0.01$; path length: $F_{(1,16)}=41.10; p<0.01$]. Evidence for a genotype effect was supported by the three-way interaction in escape latency which was near-significant [$F_{(3,48)}=2.69, p=0.06$] and reaching clear significance in escape latency [$F_{(3,48)}=3.25, p<0.03$]. The emergence of the three-way interaction once again stemmed from the variable expression of the trials effect in the mutants across days in contrast to the stable expression observed in controls (left panel of Figure 2C). Although the mutant mice’s performance approximated the controls on the last two days, there was hardly any suggestion of improved performance from trial 1 to 2 in the mutant mice over the first two test days. The latter, albeit transient, effect was sufficient to produce also a significant genotype by trials interaction in the path length measure [$F_{(1,16)}=4.89; p<0.05$] (data not shown), though not in the parallel analysis of escape latency [$F_{(1,16)}=2.73; p=0.12$].

- **15-min ITI:** When the ITI was extended to 15 min over the next four days, performance had been stabilized in both groups. A consistent trial-dependent reduction in escape latency and path length was detected across all days (Figure 2D), yielding a highly significant trials effect in both performance measures [latency: $F_{(1,16)}=77.51; p<0.0001$, path length: $F_{(1,16)}=26.73; p<0.0001$]. There was no longer any indication for a genotype effect or its interactions [All $F$’s$<1$].

Similar to the cheeseboard experiment, there was no evidence for any significant group difference in speed of movement (swim speed) across all stages of the watermaze experiment (data not shown).

**Experiment 4: Spatial familiarity judgement**

Mutant and controls mice both exhibited similar preferential exploration of the novel arm in the test phase in all three Y-maze tests of spatial familiarity judgement with increasing delay being imposed between sample and test phase (Figure 3). Separate $2 \times 3$ (genotype $\times$ arms) ANOVA of time spent per arm yielded a significant arm effect in all delay conditions [2-min: $F_{(2, 24)}= 12.96$;
There was no statistical indication for any genotype effect whatsoever. The null results obtained in the test phase could not be attributable to any difference in sample phase performance as suggested by separate analysis of exploration time in the familiar arm recorded in the sample phase (data not shown).

**Figure 3:** Time spent in each arm during the Test phase. Mice show a higher exploration time on the novel arm (Nv) compared to the familiar (Fm) and the start (St) arm, independently of their genotype. Error bars refer to ±SEM.

**DISCUSSION**

The present study has succeeded in clarifying that forebrain neuronal GlyT1 disruption does not produce appreciable impact on working memory functions. We demonstrated a consistent null effect of this mutation on working memory function across three different paradigms (Experiments 1–3), and showed that the mutation was free of any confound in spatial familiarity judgement and related spatial perception (Experiment 4). The findings here thus constitute an important qualification to the reported efficacy to enhance working memory functions by other means of GlyT1 deletion or inhibition (Singer, et al., 2009b; Singer, et al., 2009c). The comparison with the null effect of the present conditional GlyT1 deletion model might shed light on how to maximize the therapeutic potential of GlyT1 inhibition against working memory deficiency.

**Meeting previous interpretative challenge**

One impetus of the present study was to clarify the impact of forebrain neuronal GlyT1 deletion on working memory because the data reported by Singer et al. (2009a) did not allow a clear answer. The first challenge of interpretation stems from divergent performance on trial 1 observed between mutant and control mice in the original water maze working memory test (Singer, et al., 2009a). It is hard to explain how the mutants outperformed the controls on trial 1
when the platform location was unbeknownst to the animals. Both groups were expected to locate the platform solely by chance, and thus the reported trial-1 effect is counterintuitive. The second interpretative challenge arose when trial 2 performance was taken into consideration, because the pattern emerged appears (see Figure 2 in Singer, et al., 2009a) to be open to conflicting interpretations. On the one hand, the lack of improvement from trial 1 to 2 in the mutants against the presence of a significant improvement in the controls might speak for impairment in the mutants. On the other hand, as argued by Singer et al. (2009a), the overall escape performance of the mutant mice across the two trials was actually better than that of controls even though overall performance in itself is not a measure of working memory. Acknowledging the latter, Singer et al. (2009a) argued that the superior trial 1 performance in the mutants could be linked to the enhanced reversal learning phenotype observed in the same animals, and therefore the mutants would be better able to cope with conflicting memory. In the working memory test, memory of the previous day’s platform location potentially might harm trial 1 performance. Being able to overcome such negative interference allowed the mutants to outperform controls. Singer et al. (2009a) speculated that this might be promoted by a non-mnemonic search strategy that effectively minimized the negative impact of such proactive interference in mutants, which incidentally might also be responsible for the limited improvement from trial 1 to trial 2 observed in the mutant mice. This in fact could undermine the validity of the task (in the hands of Singer, et al., 2009a) to assess working memory function (see Crabbe & Morris, 2004). As a remedy for this shortcoming, Singer et al. (2009a) proposed that increasing the number of trials within a day (without changing platform location) should encourage the mutants to adopt a working memory strategy operating according to a "win-stay" rule, since this would greatly benefit performance in all trials subsequent to the first. An effective assessment of working memory function can then be realized. This prediction has been partially fulfilled in Experiment 3 here, which allowed us to overcome the interpretative difficulties in Singer et al.’s original report (2009a).

**Now you see it, now you don’t**

We were able to eliminate the trial-1 effect reported by Singer et al. (2009a) and thus overcame a critical interpretative issue in the original study by the end of the first four days test of working memory test when the ITI’s were 2 min. Thus, extension to a 4-trial-per-day protocol had been effective in instilling an adherence to the matching rule underlying the watermaze working memory paradigm. However, it has not escaped our notice that the precise pattern of outcome reported by Singer et al. (2009a) was replicated here also on the first two test days (Figure 2C) even though the critical 3-way interaction term just failed to attain outright statistical significance [p=0.06]. Transient as it might be, its re-appearance is sufficient for us
not to dismiss Singer et al.’s (2009a) original outcome merely as a sampling (Type I) error. Yet, the fact that this transient pattern emerged at the beginning (when the source of proactive interference was low) rather than later into the experiment (when proactive interference would be higher) is at odd with the account offered by Singer et al. (2009a) explained above. Their speculation would have predicted the opposite. Thus, attributing the (transient) divergent trial-1 performance to a resistance to proactive interference enjoyed by the mutants would seem untenable here. The need for an alternative explanation would seem inescapable.

In the parallel evaluation of working memory extended to the cheeseboard maze (Experiment 2), we obtained very comparable outcomes. The experiment again yielded an overall pattern of result free from any significant divergence in trial-1 performance (right panel of Figure 2B), and a similar dependency on days that differentiated mutants from controls (left panel of Figure 2B). Hence, the key findings here including the transient phenotype preferentially expressed at the beginning of training (as originally reported by Singer, et al., 2009a) are not unique to the watermaze paradigm. They are demonstrably extendable to the non-aversive alternative test that relies on positive (as opposed to negative) reinforcement but carried essentially the same spatial and cognitive demand – viz., both the cheeseboard maze and watermaze paradigms followed a matching-to-place (or “win-stay”) rule. When the Olton’s (1976) radial arm maze test (Experiment 1) is also taken into consideration, we can further conclude that the null effect in working memory function also generalized to a task that relied instead on a non-matching-to-place rule, that emphasizes a "shift" rather than a "stay" strategy.

Interference & forgetting
The radial arm maze not only provided us a working memory paradigm based on a win-shift rule, its application in the solution of the task further enables us to examine the capacity of the working memory buffer. In the watermaze and cheeseboard experiments, the task simply required the animals to return to one specific location made known to them a short while ago (hence, 'win-stay'). In the radial arm maze task, however, efficient performance depends on the recollection of multiple locations - i.e., the ability to judge the familiarity of many locations at the same time, so the animals might effectively distinguish novel arms from familiar (visited) arms so as to avoid the latter. A reduction in the capacity of the working memory buffer and/or persistence of the memory trace of the visited arms may therefore impair performance. The ability to avoid entering an arm that the animal has just visited would be less demanding than avoiding an arm visit earlier, and therefore fewer errors of the former kind should be made. This relative advantage can be readily understood as a form of the "recency effect" (Lund, 1925), because more recent experiences have less time for decay (forgetting) and suffers less from retroactive interference (e.g., intervening arm-visits). We attempted to examine this by
classifying working memory errors (i.e., re-entries into already visited arms) by the number of intervening choices preceding the error in question, i.e., the degree of disparity between two visits into the same arm. The frequency of errors is expected to increase with increasing disparity. This was equally observed in mutant and controls mice (Figure 4) based on the data taken from the 8-arm protocol (i.e., those illustrated in Figure 1B). This is consistent with the Y-maze experiment (Experiment 4) examining the persistence of memory trace across increasing delays in familiarity judgement. Taken together, the radial arm maze convincingly showed that forebrain neuronal GlyT1 disruption had no significant impact on the operation of working memory. Given that this was the first test examined in this cohort, one can also be confident that the results were free from any possible confounds due to transfer effects between tests.

Figure 4: Frequency of errors classified as a function of the degree of disparity between two visits in the same arm for control and mutant mice based on the data obtained across the five days of test with the 8-arm protocol (Fig. 1B). The working memory errors were classified depending on the number of arms visited (disparity) between two visits into the same arm. For example, in the sequence of arm entries: \(1 \rightarrow 3 \rightarrow 7 \rightarrow 2 \rightarrow 4 \rightarrow 9 \rightarrow 7 \rightarrow 2 \rightarrow 8\) (where re-entry or working memory errors are underlined), the re-entry into arm 7 is an error with disparity = 0 whereas the re-entry into arm 9 is an error with disparity = 2. With increasing disparity, the frequency of error rises. This monotonic increase of error frequency as a function of the disparity did not differ between genotype. Error bars refer to ±SEM.

What’s wrong with your mouse but not my mouse?
In contrast to previous demonstrations of a working memory enhancing effect by other forms of GlyT1 inhibition or deletion (Singer, et al., 2009b,c), the resounding null effect here in mice lacking specifically GlyT1 in forebrain neurons is intriguing. Our two previous positive demonstrations involved: (i) disruption of GlyT1 from neurons and glial cells in the telencephalon (EXM/GyT1-KO mice, Singer, et al., 2009c), and (ii) systemic injection of the

![Graph showing frequency of errors classified as a function of disparity.](image)
potent GlyT1 inhibitor, SSR504734 (Singer, et al., 2009b). Comparing the similarities and differences amongst the three GlyT1 manipulations may lead to the identification of the critical determinants allowing the pro-cognitive impact of GlyT1 inhibition in working memory to be maximized. The critical elements should be common to the two efficacious GlyT1 manipulations, but at the same time absent from the current CamKIIα/GlyT1 KO model examined here. Given that Cre recombination is restricted to forebrain neurons in the CamKIIα/GlyT1 KO mice, but extended to cells of both neuronal and glial lineages, this deduction would place an emphasis on telencephalonic glial GlyT1 component.

The emphasis on this specific subpopulation of GlyT1 may first imply the importance of achieving a sufficiently strong disruption of GlyT1 in the telencephalon. Because GlyT1 is mainly expressed in glial cells (Gomeza, Ohno, & Betz, 2003a) their inclusion would produce a stronger disruption of glycine uptake. Extending the deletion to glial cells (as in the EXM/GyT1-KO model) doubled the reduction of GlyT1-mediated glycine uptake in the brain in comparison with the current CamKIIα/GlyT1 KO model in which the deletion was confined to the forebrain neuronal component (see Mohler, et al., 2011). At glutamatergic synapses where neuronal GlyT1’s and NMDARs are co-localized, neighbouring glial-GlyT1s provide additional clearance of glycine from the synaptic cleft. Hence, the additional loss of glial-GlyT1 would be expected to further increase glycine-B site occupancy and consequently NMDAR-dependent neuronal activity than that achieved in CamKIIα/GlyT1 mice. However, glial-GlyT1 also assumes a major role in the uptake of glycine in forebrain glycineric synapses (Eulenburg, et al., 2005; Gomeza, Armsen, Betz, & Eulenburg, 2006). One may therefore expect that deletion of brain glial-GlyT1 might additionally potentiate inhibitory glycineric transmission that depends on the binding of glycine to strychnine-sensitive glycine-A site located on glycineric receptors (GlyRs). This consideration thus raises the interesting possibility that enhanced glycineric inhibitory activity might be partly responsible for enhancement in working memory reported before (Singer, et al., 2009b,c). This not only accounts for the positive effect of systemic SSR504734, which likewise may be expected to influence glycineric neurotransmission, but also the lack of an effect in the present CamKIIα/GlyT1 KO model in which the selective loss of neuronal GlyT1 should not result in any appreciable direct impact on glycineric neurotransmission.

Disrupting glial-GlyT1 may therefore lead to complementary changes on neuronal excitation specifically mediated by NMDARs and network excitability via glycineric inhibition (Zhang, Gong, Fei, Xu, & Xu, 2008). The importance of inhibitory regulation in the maintenance of network stability is well recognized (e.g. Mohler, et al., 2008). For instance, the relevance of GABAergic inhibition to learning and memory has been most extensively examined (e.g. Collinson, et al., 2002; Yee, et al., 2004). Glycineric inhibition may assume a similar regulatory role (Zhang, et al., 2008; Xu & Gong, 2010), and this certainly warrants direct investigation. The
two mechanisms (NMDAR-based and GlyR-based) are therefore not mutually exclusive. It is possible that both contribute to the observed effects on working memory reported by Singer et al. (2009b; 2009c). This hypothesis can be directly tested by mouse models with selective disruption of glial-GlyT1. Although glial-GlyT1 function is essential for early life survival, its deletion in adult mice is not associated with any gross abnormality (Eulenburg, Retiounskaia, Papadopoulos, Gomez, & Betz, 2010) and would be congenial for cognitive assays. It would also be instructive, when one day becomes feasible, to target separately glial-GlyT1 associated with NMDAR versus those near glycinergic synapses. The impact of selective glycinergic manipulations in the brain on working memory is presently unknown. However, this possibility may deserve further consideration even though glycine re-uptake at glycinergic synapses depends on GlyT2 as well as glial-GlyT1 (Eulenburg, et al., 2005; Gomez, et al., 2006).

**The multiple yet dissociable effects of GlyT1 disruption**

Comparison between the two conditional GlyT1 knockout models suggests that although both EXM/GlyT1-KO and CamKIIα/GlyT1 KO mice showed functional resistance to a systemic challenge to NMDAR antagonists (Yee, et al., 2006; Singer, et al., 2009c), only the latter has produced a clear physiological phenotype in the form of enhanced NMDAR-mediated current. Possibly due to glycine-primed NMDAR internalization (see Nong et al., 2003) or other functional compensatory mechanisms, EXM/GlyT1-KO mice did not express this phenotype. Yet, the reported enhanced NMDAR function in CamKIIα/GlyT1 KO mice did not translate into enhanced working memory function as shown here, even though these mice have been shown to exhibit other forms of cognitive enhancement (for a review, see Mohler, et al., 2011). Taken together, these findings not only highlight the possible relevance of glycinergic neurotransmission, but may also question the reliability or even validity of the link between NMDAR and memory (see also Bannerman & Sprengel, 2010).

The timing of GlyT1 deletion is another major difference between the two mutant lines. GlyT1 was deleted during embryonic development around gestational day 10 in EXM/GlyT1-KO mice (Iwasato, et al., 2004). This is considerably earlier than in CamKIIα/GlyT1 KO mice, in which GlyT1 was knocked out near postnatal day 21 at a time when brain development was almost complete (Dragatsis & Zeitlin, 2000). One may reason therefore that EXM/GlyT1-KO mice might be more prone to developmental compensation. Although this cannot be ruled out, we can safely conclude that such compensatory changes were unlikely by themselves be responsible for the working memory phenotype. It is because systemic exposure to the GlyT1 inhibiting drug, SSR504734, was able to facilitate working memory in adult wild type mice when given acutely and therefore free from any developmental compensation (Singer, et al.,
However, the drug's efficacy seems to be critically dependent on dosage in a manner that could be relevant here.

Singer et al. (2009b) showed that the positive effect on working memory by SSR504734 only became apparent at high doses (30mg/kg) with lower doses (1mg/kg) completely ineffective. However, such lower doses were sufficient to enhance a form of selective attention, known as latent inhibition (LI, Black, et al., 2009). Such LI-enhancing action is shared by known antipsychotic drugs (Moser, Hitchcock, Lister, & Moran, 2000), and the LI deficit is considered a relevant endophenotype of schizophrenia (Amann, et al., 2010) central to the positive symptomatology of schizophrenia on theoretical grounds (Gray, Feldon, Rawlins, Smith, & Hemsley, 1991). This contrasts with the present emphasis on working memory, the deficiency of which is of cardinal significance to schizophrenia cognitive symptoms. Interestingly, the LI-enhancing effect of low doses of systemic GlyT1 inhibiting drugs (Black, et al., 2009) is captured by CamKI\textsubscript{II\alpha}/GlyT1 KO but not EXM/GlyT1-KO mice. It remains to be tested if GlyT1 inhibitors at high doses that are effective in enhancing working memory (Singer, et al., 2009b) are devoid of a latent inhibition enhancing effect. If so, then the double dissociation between CamKI\textsubscript{II\alpha}/GlyT1-KO and EXM/GlyT1-KO mice with respect to their facilitatory effects on LI and working memory would be effectively mimicked by the contrast between low and high doses of GlyT1 inhibiting drugs, respectively.

The divergent effect on LI between the two conditional knockout mouse lines might be further related to their respective regional specificity of the gene deletion. Critically, the striatal GlyT1 was spared in EXM/GlyT1-KO mice, but striatal GlyT1 in principal neurons were disrupted in CamKI\textsubscript{II\alpha}/GlyT1-KO mice. Hence, striatal GlyT1 disruption might be central to the LI phenotype seen in CamKI\textsubscript{II\alpha}/GlyT1-KO mice or produced by low doses of GlyT1 inhibitors in wild type mice. This possibility warrants serious consideration because LI expression is known to depend on striatal function (Weiner, 1990; Gray, et al., 1991). Our current attempt to generate striatal specific GlyT1 disruption has generated some support for this hypothesis (Yee, et al., 2010). This may strengthen glutamatergic inputs from limbic cortices to the ventral striatum at relevant NMDARs (Gray, et al., 1991; Yee, Feldon, & Rawlins, 1995).

**Postscript**

The present discourse highlights that multiple molecular and pharmacological models are necessary to appreciate fully the potential and limitations of a possible therapeutic target. This approach enables the full and effective exploit of any potential drug target. Here, we show that divergences as much as convergences are instructive towards the building of a more complete mechanistic picture, and the synthesis of further hypotheses taking into account relevant cell biology, anatomy and physiology.
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Chapter 5: GlyT1 & Spatial working memory


CHAPTER 6

General Discussion
As detailed in the General Introduction and the subsequent experimental Chapters, molecular down-regulation of GlyT1 restricted to forebrain neurons is effective in modifying several cognitive functions (e.g. Yee, et al., 2006; Singer, et al., 2007, 2009). This supposedly stems from increased glycine occupancy at NMDARs, which subsequently potentiates NMDAR-mediated neurotransmission. However, careful examination of the enhanced cognitive phenotype displayed by these mice, including the interpretations of the behavioural outcomes offered in the literature, revealed several issues that have not been satisfactorily addressed until now. A detailed screening might therefore be essential in order to avoid simplistic and biased interpretations. Hence, the present thesis aimed towards such an in-depth comparative analysis of the behavioural phenotypes expressed by forebrain neuronal specific GlyT1 genetic disruption.

The application of this in-depth approach affords several advantages. First, this approach favours the examination of the stability and robustness of the previously identified phenotypes in GlyT1\(\Delta^{\text{FB}}\)-neuron mice. Part of the results presented in this thesis replicated previously identified phenotypes, yet novel phenotypes were also identified and provided important qualifications to previous claims. Second, discussing the various findings under a theoretical framework allow for a coherent integration, which not only foster more powerful interpretation, but also facilitates the proposition of new research directions. Finally, comparing the behavioural outcomes with those from other available GlyT1 manipulations might shed light on the neuronal mechanisms and neural substrates involved.

**Overview of behavioural results**

In our attempt to scrutinize the reported enhanced Pavlovian phenotype (Yee, et al., 2006) we uncovered that the phenotypic expression was distinguishable between sexes in both the aversive as well as the appetitive paradigm employed (Chapter 3), and across age in the aversive paradigm (Chapter 2). In the aversive paradigm, the phenotypic dependency on sex was primarily one of magnitude; the enhanced conditioned freezing phenotype appeared stronger in the female mutants (Chapter 3). On the other hand, in the appetitive paradigm, sex critically determined the direction of Pavlovian conditioning phenotype; the conditioned approach response was enhanced in the mutant females but was somewhat weakened in male mutants (Chapter 3). The phenotype of enhanced conditioned in freezing was detected in senescence, but the temporal expression of the Pavlovian phenotype was modified; the adult mutants showed pronounced within-session extinction while aged mutants did not (Chapter 2). This exercise has also allowed further identification of hitherto unknown phenotypes and neuroanatomical changes. In the elevated plus maze test, the explicit comparison between mice kept in opposite light-dark cycles but tested at the same time, revealed changes in anxiety-related behaviour (Chapter 3); an anxiolytic phenotype was observed in male mice tested in the light phase whereas an anxiogenic phenotype was observed in female mice in the light phase. In Chapter 2, we observed that the expression of NR2B and apoptosis were not aggravated by the mutation, limiting thereby the possible concerns of neurotoxic effects through lifelong GlyT1
disruption in forebrain neurons. However, we revealed a clear increase in the number of immature neurons in the dentate gyrus of the adult mutants (Chapter 2).

The previous finding of enhanced latent inhibition (LI) reported in GlyT1ΔFB-neuron mice (Yee, et al., 2006) was extended by showing that this mouse model was further associated with enhanced sensitivity to CS-US trace and degradation of CS-US contingency (Chapter 4). In addition, we showed that the phenotype of enhanced conditioned freezing could be extended to foreground contextual conditioning albeit weaker by comparison. An attempt to unify these results under a theoretical framework identified a possible common perceptual learning mechanism that might underlie the modulation of the associability of a potential CS. Finally, the results from the experiments described in Chapter 5 clarified the null effect of the mutation on working memory function across three different paradigms. Furthermore, the spatial familiarity judgement and related spatial perception were not affected by the mutation. Altogether, the results presented in this thesis are clear illustration of how an in-depth analysis can reveal unknown phenotypes and lead to a better understanding of the limitation and potential of any manipulation.

**Confirmation and extension of GlyT1ΔFB-neuron Associative learning phenotype**

Forebrain neuronal deletion of GlyT1 gives rise to a phenotype in Pavlovian associative learning, whereby a neutral conditioned stimulus (CS) is paired together with a biologically significant unconditioned stimulus (US). Successful associative learning results in the conditioned stimulus eliciting a conditioned response (CR). Evidence for enhanced associative learning in GlyT1ΔFB-neuron mice, in the form of stronger CR to the CS, was shown across multiple paradigms (Yee, et al., 2006). The robustness of the phenotype of enhanced conditioned freezing, originally detected in the “non-pre-exposed” condition in the LI experiment (Yee, et al., 2006), was confirmed in four different experiments using freezing conditioning, including the “no-trace” or “contingent” test condition (Chapter 4). This phenotype was consistent across age, gender, and different light-dark cycle conditions (Chapters 2-3). It was free from any non-specific confounding changes in anxiety, spontaneous activity or pain sensitivity (Chapter 3). Our experiments consistently revealed that the enhanced conditioned freezing was restricted to the test phase, suggesting therefore, that forebrain neuronal GlyT1 deletion robustly modified the expression rather than the acquisition of the CS-US association. One may speculate that the procedure used during conditioning (i.e. two or three CS-US pairing) might be insufficient to allow the emergence of differences, since such a trend was seen during acquisition in the last ITI or the last CS pairing (Chapter 3). This might be the case since initial evaluations indicated that the mutation can improve acquisition when tested in conditioned active avoidance, whereby the phenotype was demonstrated through the acquisition of the response across 100 trials (Yee, et
al., 2006). Nonetheless, the stronger CR to the CS in GlyT1ΔFB-neuron mice suggests that the memory trace of the Pavlovian association was stronger, and that this effect was robust.

In contrast to the consistent phenotype in the expression of the acquired CS-US association, the extinction of this association was unstable. Both within-session and between-day extinction were variably affected. The pattern of within-session extinction was modified only in aged mutants, and resistance to extinction was shown only in female mutants. Moreover, evidence that extinction learning across extinction days was modified by GlyT1 deletion was also obtained when the spontaneous recovery of the CR between successive days was examined in conditioned freezing (Chapter 3). Although increased resistance to extinction was also detected across days in the appetitive conditioning paradigm, the spontaneous recovery, seven days after the last CS-test, was not affected by GlyT1 disruption (Chapter 2). Extinction occurs when the predictive value of the CS with respect to the occurrence of the US is reduced, leading to a decline of the conditioned response (CR) to the CS. Long-term extinction learning is widely accepted as a form of new learning (the CS no longer predicts the US), which acts to inhibit expression of the CR without interfering with the previous CS-US association. Here, dissipation of this inhibition with time revealed the spontaneous recovery effect (Rescorla, 2004) and confirmed that extinction did not lead to the erasure of the original CS-US association.

Apart from the new learning, which that can directly compete with or impede CR during extinction, an independent and not exclusive process which does not involve new learning or rely on non-associative learning could be at play. Wagner and Brandon (2001) proposed that the strength of a CR could be weakened by changing the environment in which the response occurs, through generalization decrement. If an animal is repeatedly presented with the CS that is directly followed by a US, then it will suddenly find itself in a new environment/situation when the US is no longer delivered. This change may weaken responding through generalization decrement, rather than through a change to the association involving the CS. One plausible explanation of the extinction phenotype obtained in mutants could be that these mice are less sensitive to a change in the environment and as a result continue to respond to the CS (see Wagner’s SOP model later in the discussion). This effect could explain the resistance to within-session extinction and might be vulnerable to different factors such as sex or aging. On the other hand, the resistance to long-term extinction in mutant mice might suggest that the mechanism underlying the control of new learning is somehow affected by GlyT1 disruption. In Chapter 3, we attempt to interpret the extinction learning phenotype as reflecting a stronger proactive interference. This explanation assumes that the phenotype of increased resistance to extinction could be understood as a stronger influence of the prior experience of CS-US association over current control of responding. Indeed, this interpretation was initially used by Yee et al. (2006) to explain the enhanced LI effect in the mutant mice. The demonstration of LI requires two
distinct phases with contrasting CS-US relationships: the pre-exposure phase, in which CS is not
followed by any significant event [CS \rightarrow \text{nothing}] precedes the conditioning phase. LI refers to
the observation that CS pre-exposed subjects show weaker CR to the CS after CS-US pairing
compared to non-pre-exposed subjects. Several theories propose that the weaker CR does not
stem from a failure to acquire the critical CS-US association but reflects the [CS \rightarrow \text{nothing}]
memory trace competing over the control of behaviour during testing (Weiner, 1990; Bouton,
1993). Thus, a stronger influence of the prior experience of non-reinforced CS exposures leads
to stronger LI. This view conflicts with a prior report of enhanced reversal learning in GlyT1^{ΔFB-}
neuron mice, which suggested resistance rather than vulnerability proactive interference in these
mutants (Singer, et al., 2009). Another explanation for the reversal effect comes from an
additional study in discrimination learning. During a discrimination, animals learn to pay more
attention to relevant than irrelevant stimuli (Mackintosh, 1974). Hence, the animals learn to
focus their attention to one or few features that allow an optimum discrimination. Reversal
learning would be facilitated because the contingency of only few features has to be reversed
(Sutherland and Mackintosh, 1971). Hence, it is conceivable that the reversal phenotype
reported by Singer et al. (2009) reflects primarily enhanced selective association learning.

Two mechanisms, one explanation?
As mentioned above, GlyT1^{ΔFB-neuron} disruption is effective in enhancing associative strength, as
evidenced in all the conditioning experiments carried out. The mice showed stronger
conditioning in the classical conditioning (Chapter 2 and 3); in the no-trace and the non-
contingent condition (Chapter 4) and in the no-PE condition (Yee, et al., 2006). However, an
increased associative strength per se cannot explain why the imposition of a delay between CS
and US, the presentation of CS-alone trial, or the pre-exposure to the CS, leads to a weaker CR in
the mutants compared to the controls. One might expect that if GlyT1^{ΔFB-neuron} disruption is
effective in enhancing associative strength, it should also lead to a stronger CR under these
conditions. Therefore, the different associative learning phenotypes present in GlyT1^{ΔFB-neuron}
mice are difficult to reconcile under one unique psychological mechanism that only affects the
modulation of associative strength. Here, it is important to address the conditions that can lead
to a stronger or weaker associative link in GlyT1^{ΔFB-neuron} mice.

The majority of associative learning theories emphasize that the attentional processing
of the CS determines its associability; the more attention paid to a CS, the easier it is to enter
association with a US. It was shown in Chapter 4 that a change in the attention perceived by the
mutants could lead to enhanced associative learning in the mutants. A change in the perceived
intensity of the CS would also predict a reduction of associative learning in the degraded
contingent condition schedule used in our experiment (Chapter 4). This can be readily tested
with the prediction that, if the GlyT1ΔFB-neuron mice perceive the salience of a stimulus as stronger, a simple freezing conditioning experiment in which the salience of the CS is low will lead to a stronger CR in the mutants compared to the controls. Furthermore, the Rescorla-Wagner model (1972) used in Chapter 4 would also predict a stronger associative strength at the end of the acquisition in the mutants compared to the controls, if the schedule of contingency degradation used was different (i.e. if the last pairing was an omitted US). This can also be examined, and if in the expected direction, it would support the idea that attention is modulated by GlyT1ΔFB-neuron disruption.

However, a sole increase in attention to the CS is unable to explain why, for instance, the imposition of a trace between the CS and the US diminishes rather than enhances the CR observed in the mutants, or why the pre-exposure of the CS affects the subsequent acquisition of the CS-US association in the mutants but not in the controls. Here, the issue of selectivity in learning can be addressed. Selectivity in learning refers to the notion that some CS-US relationships are learned more readily than others. This comes from studies showing that the effectiveness of conditioning depends on the stimuli paired together (e.g. Shapiro, et al., 1980). One explanation for selective association is that it is due to some stimuli, or stimulus dimension, being more relevant to the occurrence of biologically significant events than others are. For example, tastes are likely to provide more reliable information than auditory-visual stimuli about whether or not a food is poisonous. Although this kind of selective association are considered as innate, the discovery of the so-called “learned irrelevance effect” suggests that an animal’s experience may also contribute to selective association. Learned irrelevance (Mackintosh, 1973) reflects the slower learning that takes place when a CS and US are paired if they have previously been presented randomly with respect to each other. Mackintosh’s theory (1975) claims that the attention paid to a stimulus will increase if it is a good predictor of a significant event and decrease if this stimulus is a bad predictor. In fact, to be fully attended, a CS must not only be a good predictor of the US, but it must be a better predictor than all other stimuli that are present in the trial. Therefore, during the random pairing of a CS and a US, the animals learn that this specific CS is irrelevant to the delivery of this specific US and will decrease the attention paid to the CS. This will result in animals ignoring this stimulus when these CS and US are eventually paired. In the latent inhibition experiment, during the pre-exposure phase, the animals learn that the CS is irrelevant; as a result, the animal will ignore the CS when it is paired with the US in the subsequent acquisition phase. Yee et al. (2006) previously discussed this possibility and proceeded to propose that GlyT1ΔFB-neuron mice might present an enhancement of learned irrelevance because GlyT1 disruption could affect the rules by which the attention is adjusted. For example, the rate of change in attention could be facilitated. Therefore, in GlyT1ΔFB-neuron mice, when a CS is a good predictor, the attention paid to
it is tuned up in such a way that they will pay more attention to the stimulus than the controls will after the same number of trials. This could also predict the bi-directional effect observed in trace conditioning in the mutant mice. We may also hypothesise that the "learned irrelevance" effect would be stronger in GlyT1ΔFB-neuron mice compared with controls.

Wagner’s SOP (1981) model also offers an interpretation of the results that could be understood as a consequence of the modulation of the attention paid to the CS. The theory assumes that the representation, or the memory of a stimulus can be in three different states. The inactive state refers to a state where memory is not modifiable and cannot influence an animal’s behaviour. The two remaining states (called A1 and A2) are considered active because a stimulus in one of these states can influence behaviour. The different activation states can be viewed as the attention allocated to a stimulus. A stimulus in A1 is at the centre of attention whereas a stimulus in A2 is at the periphery of the field of attention. Like in other theories, a stimulus needs to be fully attended to be able to enter association. Thus, it is proposed that excitatory and inhibitory conditioning is only possible when the CS is in the A1 state. Information in the inactive state is believed to last more or less indefinitely, and can be viewed as the long-term memory. On the other hand, the information in an active state is considered as labile; the active states could refer to a short-term memory. This assumption implies that short-term memory has a limited capacity (Wagner, 1976) presumably because of the limited capacity of attention (James, 1890). Hence, the model proposes that a representation of a stimulus in A1 state will rapidly decay to the A2 state, as soon as it is not present anymore, and will then revert more slowly to its inactive state. Importantly, only one route allows a representation to be activated in A1. If a stimulus representation is in the A2 state, further presentation of this stimulus will not activate an A1 representation of it and this stimulus will not be fully attended. By contrast, two routes allow a stimulus to gain access to the A2 state. The first one, also called the self-generated A2 representation route, is the one described earlier when a stimulus representation is first activated from an inactive state to an A1 state that decay to an A2 state before slowly returning to the inactive state. An interesting feature of this proposal is that it can also provide an account for non-associative processes. The orienting response (OR) or the investigatory reflex in the terminology of Pavlov (1927) is the (unconditional) response observed to a (novel) stimulus. Habituation refers to the fading of an unconditioned response, such as an OR, to a stimulus that is repeatedly presented. This phenomenon can be explained by the SOP model. The first occurrence of a stimulus triggers its representation to the A1 state and may elicit a strong response OR. This state will decay to the A2 state and the model predicts that the strength of the response will show a corresponding decline. If the stimulus is presented again while the A2 representation is still active, the stimulus will be unable to activate an A1
representation, and will thus be less able to evoke the relevant response, i.e. response to subsequent presentations of the stimulus will become habituated. The other route by which a stimulus can gain access to the A2 state, and thus influence attention, is made possible by associative learning. The model states that two stimuli (CS and US) can be associated only when the representations of these stimuli are simultaneously in A1, when both are fully attended. After effective conditioning, the presentation of the CS will be able to trigger a representation of the US directly into the A2 state. This is referred to as a retrieval-generated representation, as it is not excited by the direct presentation of the US, but by a CS with which it is associated. In chapter 4, based on the results obtained by GlyT1ΔFB-neuron mice in the trace experiment, we suggested that the rate of activation between the Inactive state and the A1 state is facilitated as well as the rate of decay between A1 and A2. In addition, the model could explain the pattern of results obtained in LI. As explain in Chapter 4, according to Wagner’s SOP model (1981), during pre-exposure the CS is associated with the context. When the animals are returned to this context during subsequent acquisition, the context primes the representation of the CS directly into A2 state, rendering its association with the US more difficult. The mechanism thus depends on the formation of context-CS association. GlyT1Δfb-neuron mice might learn the context-CS association more rapidly, for reasons similar to those underlying stronger CS-US association, and thus facilitate the expression of LI later. An additional explanation, based on the non-associative self-generated A2 route could be drawn to explain the LI effect. During the pre-exposure phase, repeated presentations of the CS leads to its habituation. When it is again presented during acquisition, the CS is still habituated (in A2) and thus cannot be associated readily with the US, leading to the LI effect. Bannerman and Sanderson (2007) proposed that GlyT1Δfb-neuron mice could be impaired in dishabituation, based on the results obtained in recognition memory (see Chapter 1). Therefore, the LI effect could also stem from impaired dishabituation to the pre-exposed CS. This impaired dishabituation could be represented in the Wagner model (1981) by a slower decay rate from the A2 state back into the inactive state. As suggested by Bannerman and Sanderson (2007), this would predict an enhanced novelty preference in a Y-maze task for example. However, as shown in Chapter 5, a null effect was shown in the Y-maze task. Despite this, our proposition that the rate of activation between the Inactive and the A1 state is facilitated could be easily tested in an experiment assessing the orienting response (OR) as employed by Sanderson et al. (2011).

In conclusion, the attempt to apply different associative learning theories to explain our results has led to the proposition of a possible common perceptual learning mechanism that could lead to enhanced association learning and enhanced selectivity in learning. One explanation of the stronger associative strength in GlyT1ΔFB-neuron mice would be that the attention paid to a
stimulus is stronger in the mutants. Whereas one explanation for the enhanced selectivity in learning would be that mutants adjust this attention less efficiently.

**Comparisons with other GlyT1 disruption models: evidence for underlying neural substrates?**

In contrast to the effect of GlyT1ΔFB-neuron on associative learning, no effect was seen in reference memory (Singer, et al., 2009). Moreover, the ambiguous effect on working memory described before (Singer, et al., 2009) was clarified in the Chapter 5 of the present thesis. Working memory function seems not to be influenced by the mutation. Similarly, evaluation of foreground contextual freezing revealed a relatively weak enhancement in GlyT1ΔFB-neuron mice. One might question why the strong and robust effect seen in associative learning does not translate to the spatial and contextual forms of learning. Indeed, they both seem to involve the processing of often large, diffuse, multicue, polymodal and relational stimuli (Bannerman, et al., 2004). It is conceivable therefore, that GlyT1ΔFB-neuron disruption interferes with attentional processes related directly with discrete cues. Nonetheless, these behavioural phenomena are believed to be mediated by distinct neural substrates. The GlyT1 genetic manipulation used in the present thesis affects the entire forebrain and we therefore have no direct evidence for the involvement of a specific brain region in the modulation of GlyT1 in memory function. However, the phenotypic similarities as well as differences between neuronal-specific GlyT1 deletion and another conditional GlyT1 knockout extending into cortical glial cells specifically expressed in the telencephalon, point to the uniqueness of the anatomical and cell-type specific nature of the genetic manipulation examined here (see Chapter 5, see also Mohler et al., 2011). More specifically, contrasting effects were seen in spatial working memory performance between the two regional-specific GlyT1 manipulations, with a predominant role for glial-based GlyT1 in this behavioural function. This might suggest that the behavioural effects mediated by the forebrain neuronal GlyT1 depletion are supported by different neural substrates compared to the glial-specific GlyT1 manipulation. However, this clearly warrants more investigation.

Correspondingly, we recently obtained evidence that viral-mediated neuronal-specific depletion of GlyT1 in the nucleus accumbens also was effective in enhancing the latent inhibition effect (Yee, et al., 2010). Interestingly, this region-specific depletion of GlyT1 was able to isolate only one of the mechanisms via which enhanced latent inhibition can occur. In contrast, to the observed effects in the non-pre-exposed group here, the effects following nucleus accumbens-specific GlyT1 depletion were seen solely in the pre-exposed group. Thus, this appears to suggest that the nucleus accumbens is not mediating the associative learning improvement seen in the GlyT1ΔFB-neuron mice, but might be more relevant for increasing the selectivity in learning. It would therefore be worthwhile to examine the behavioural relevance
of nucleus accumbens-specific GlyT1 depletion on tasks which were affected by GlyT1ΔFB-neuron disruption, such as trace conditioning or contingency degradation conditioning.

Concluding remarks

The present thesis provides evidence for novel critical mediating factors, such as gender and age, which should be taken into account when designing novel drug therapies to ameliorate memory deficits as seen in e.g. schizophrenia. The in-depth characterization of the neuronal forebrain-specific GlyT1 mutant mice has confirmed the importance of GlyT1 in the modulation of cognitive function, with particular focus on its impact on attentional processes, which might be relevant for schizophrenia related cognitive impairments. However, the proposal that GlyT1 disruption might be a potential “cognitive enhancer” is problematic. As discussed in the introduction, enhanced memory has its cost, especially in situations involving traumatic events. The observation that GlyT1ΔFB-neuron alters extinction learning hinders the use of GlyT1 inhibition as a potential pharmacotherapy for post-traumatic stress disorder (PTSD). However, the results presented here reveal the potential for GlyT1 disruption/inhibition in anxiety (Chapter 3). Moreover, the observation of increased neurogenesis in this mouse line might indicate a potential use for GlyT1 inhibition in depression related disorders, which are linked to reduced neurogenesis. This is due to the fact that anti-depressants are thought to exert their effect by increasing neurogenesis. This warrants further investigation which could lead to a potential therapeutic role for GlyT1 inhibition. Finally, the data presented here appears to indicate that the GlyT1-mediated effects might show some degree of region-specificity. This latter facet might open new paths for the development of brain region-specific therapeutic targets.
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