Dual approach to study the impact of glycine-mediated modulation of glutamatergic transmission on behaviour

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Publication Date:
2005

Permanent Link:
https://doi.org/10.3929/ethz-a-004960270

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Dual approach to study the impact of glycine-mediated modulation of glutamatergic transmission on behaviour

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY, ZURICH
For the degree of
Doctor of Natural Science

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2005
I am very grateful to Prof. Dr. Isabelle Mansuy who accepted me in her laboratory at the ETH Zürich, supervised and supported my work. These four years have been extremely stimulating, intensive and challenging.

My best thanks are due to Prof. Dr. Wof Singer and Prof. Dr. Hanns Möhler who supported this thesis by agreeing to co-examine it.

I would also like to thank very sincerely our collaborators Prof. Dr. Heinrich Betz and Dr. Volker Eulenburg for contributing the Glyt1 +/- mice, Dr. Detlev Boison for contributing the Glyt1a cDNA and the excellent home-made anti-Glyt1 antibody, and Dr. Dietmar Benke for sharing his competence about glycine uptake assay and receptor binding autoradiography.

I would like to thank with warmth and affection all the colleagues who shared with me their knowledge, their experience and advices, their enthusiasm for science, and the very nice and friendly atmosphere in the lab. Dr. Isabelle Raineteau-Weiss, who trained me on mouse behaviour, and Dr. Gaël Hédou, always present late at night for endless scientific discussions shaped a large part of my scientific abilities, Dr. Ursula Haditsch and Dr. David Genoux who helped me to start off well the thesis, Dr. Kyoko Koshibu and Dr. Richard Munton for advices and help with the manuscript, and Karsten Baumgaertel, Marlen Knobloch, Dr. Sandor Vizi, Magda Livingstone, Steffi Lehmann, Dr. Thomas Mueggler, Torbjörn Johansson, Dr. Christina Fernandez, Melanie Kollen, the past, present and future members of the lab for permanent good atmosphere. Makeba Kampara, Aswin Pyakurel and Dominique Spirig, the trainees who helped with some experiments deserve special thanks.

Dear family and dear friends, you know that it was not easy everyday, and your constant support and affection was a real help. You deserve all my gratitude!
Summary

Glutamate is the most important neurotransmitter in the brain, mediating up to 60% of all synaptic neurotransmission. Among the different types of glutamate receptors, the N-methyl D-aspartate (NMDA) receptors, which are present in almost all glutamatergic synapses, have very specific properties conferring them critical roles in multiple brain processes. NMDA receptors are able to detect coincident neuronal activity and are strongly connected to intracellular signalling. In addition, NMDA receptors play a major role in synaptic plasticity, supporting most cognitive processes like learning and memory, what suggested that NMDA receptor malfunction could be involved in the pathophysiology of neuropsychiatric disorders. In this respect, the hypothesis of NMDA receptor hypofunction has gained much favour as one of the causative agents in schizophrenia.

It was recently discovered that the regulation of NMDA receptor activity could occur in vivo by modulating synaptic glycine concentration, a process mediated by glycine transporter 1 (GLYT1). To better understand this specific mechanism and its implication on cognitive processes and behaviour, we proposed a dual genetic approach to up- and down-regulate GLYT1 activity in vivo, and evaluate the impact on behaviour.

To up-regulate GLYT1 activity, we generated transgenic mice overexpressing GLYT1 specifically in forebrain neurons, using the tetracycline controlled transactivator system (tTA) to regulate GLYT1 expression. This genetic manipulation was expected to reduce synaptic glycine concentration leading to a reduction in NMDA receptor activation. Because NMDA receptor hypofunction is possibly involved in the etiopathology of schizophrenia, GLYT1 overexpressing mice, or tTA-Glyt1 mice, were evaluated on a series of behavioural tests assessing exploratory activity, anxiety, learning and memory and sensorimotor gating abilities. However, in all these tests, namely the open field, elevated plus maze, Morris water maze, novel object recognition task and prepulse inhibition paradigm, control and tTA-Glyt1 mice displayed similar performances. To understand this absence of phenotype, we undertook a biochemical investigation, which revealed that synaptic glycine transport was not augmented in tTA-Glyt1 mice, and that the amount of GLYT1 protein was not increased in cell-body and synaptosomal fractions. These observations suggested that the mRNA of the transgenic Glyt1 gene, whose expression was detected in forebrain structures, was not translated into active GLYT1 protein.
To investigate GLYT1 down-regulation, we obtained by collaboration mice heterozygous for the Glyt1 knockout allele (Glyt1 +/- mice). Because of gene dosage effect, these mice displayed 50% reduction in GLYT1 activity, suggesting that synaptic glycine concentration and NMDA receptor activity would be augmented. The behaviour of Glyt1 +/- mice was evaluated in the open field, elevated plus maze, home-cage and prepulse inhibition tests. The sensorimotor gating ability of Glyt1 +/- mice was significantly reduced by 38% compared to wild-type mice, the anxiety level was normal, and the spontaneous locomotor activity was increased in the open field, decreased in the elevated plus maze, and normal in the home-cage. Complementary electrophysiological experiments would be necessary to determine the mechanisms that impaired the sensorimotor gating ability.

Finally, a transgenic mouse line was generated, which expressed the new reverse tetracycline controlled transactivator (rtTA2) in forebrain neurons. This mouse line that permits inducible gene expression, is now being used in our laboratory and by collaborators.
Résumé

Le glutamate, le neurotransmetteur le plus abondant dans le cerveau, est responsable de plus de 60 % des transmissions synaptiques. Les récepteurs au glutamate du type N-methyl D-aspartate (NMDA) sont présents dans la grande majorité des synapses glutamatergiques et possèdent des propriétés très spécifiques qui en font les acteurs majeurs de nombreux processus neuronaux. Par exemple, grâce à leur capacité à détecter l’activité synchrone des neurones et leur perméabilité aux ions calcium, les récepteurs au NMDA ont un rôle pivot dans les processus de plasticité synaptique, l’apprentissage et la mémoire et la cognition. Du fait de la multiplicité des fonctions dans lesquelles les récepteurs au NMDA sont impliqués, tout disfonctionnement est susceptible de provoquer des troubles neurologiques. Ainsi, il a été suggéré qu’une réduction de l’activité des récepteurs au NMDA participe à l’étiologie de la schizophrénie.

Un nouveau mécanisme de régulation de l’activité des récepteurs au NMDA par la glycine a été mis en évidence récemment, et il y a été démontré que les transporteurs de la glycine 1 (GLYT1) contrôlent la concentration synaptique en glycine. Afin d’étudier ce mécanisme de régulation ainsi que son implication dans les processus cognitifs et le contrôle du comportement, nous avons utilisé une double approche génétique chez la souris permettant dans un cas d’augmenter, et dans l’autre cas de diminuer l’activité de GLYT1.

Afin d’augmenter l’activité de GLYT1 in vivo, nous avons généré des souris transgéniques (nommées tTA-Glytl) qui surexpriment GLYT1 de manière spécifique et contrôlée dans les neurones du cerveau antérieur. Il était anticipé que l’accroissement de l’activité de GLYT1 diminuerait la concentration synaptique en glycine, et ainsi diminuerait l’activité des récepteurs au NMDA. En rapport avec l’hypothèse d’une faible activité des récepteurs au NMDA comme cause participant à la schizophrénie, le comportement des souris tTA-Glytl a été évalué sur des tests qui mesurent la locomotion spontanée, l’anxiété, l’apprentissage et la mémoire, et la capacité de filtration sensorielle. Dans les tests de l’open field, du labyrinthe en croix surélevé, de la piscine de Morris, de la reconnaissance d’objet nouveau et de l’inhibition du sursaut, les souris tTA-Glytl se sont comportées de manière identique aux souris contrôles. Pour comprendre cette absence de phénotype, nous avons fait des mesures biochimiques qui ont révélées que l’expression du transgène codant pour GLYT1, démontrée par RT-
PCR, n’induisait pas d’augmentation de la quantité de GLYT1, et donc pas d’augmentation de son activité.

Afin d’étudier l’effet d’une diminution de l’activité de GLYT1, des souris hétérozygotes pour la délétion du gène Glyt1 (nommées Glyt1 +/-) ont été obtenues par collaboration. Dans le test d’inhibition du sursaut, les souris Glyt1 +/-, qui arborent une diminution de 50 % de l’activité de GLYT1, ont montré une réduction de 38 % de la capacité de filtration sensorielle. Dans l’open field et le labyrinthe en croix surélevé, les niveaux d’anxiété étaient normaux. L’activité locomotrice était augmentée dans l’open field et diminuée dans le labyrinthe en croix surélevé, mais normale dans la cage. Des expériences complémentaires d’électrophysiologie seraient nécessaires pour analyser plus en détail le mécanisme de cette diminution de la capacité de filtration sensorielle.

Enfin, une lignée de souris transgéniques a été générée, qui exprime le nouvel activateur de transcription contrôlé par la tétracycline (rtTA2) de manière spécifique dans les neurones du cerveau antérieur. Ces souris permettent le contrôle précis de l’expression d’un transgène par administration de doxycycline et sont désormais utilisées dans notre laboratoire et par des collaborateurs.
## List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>129/SvEvTac</td>
<td>mouse line name</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine</td>
</tr>
<tr>
<td>AMPA</td>
<td>alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate</td>
</tr>
<tr>
<td>bgal</td>
<td>beta-galactosidase</td>
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<tr>
<td>BSTC</td>
<td>brain stem trigeminal complex</td>
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<tr>
<td>C57BL/6J</td>
<td>mouse strain name</td>
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<tr>
<td>CA1,2,3</td>
<td>cornu ammonis area 1,2,3</td>
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<td>CaMKIIa</td>
<td>calcium-calmodulin dependent kinase 2 alpha</td>
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<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CN</td>
<td>calcineurin</td>
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<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>Cre</td>
<td>causes recombination</td>
</tr>
<tr>
<td>CS</td>
<td>conditioned stimulus</td>
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<td>Cx</td>
<td>cortex</td>
</tr>
<tr>
<td>D</td>
<td>acid aspartic</td>
</tr>
<tr>
<td>DA</td>
<td>dopamine</td>
</tr>
<tr>
<td>DBA2</td>
<td>mouse strain name</td>
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<tr>
<td>DG</td>
<td>dentate gyrus</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>Dox</td>
<td>doxycycline</td>
</tr>
<tr>
<td>DSM</td>
<td>diagnostic and statistical manual of mental disorders</td>
</tr>
<tr>
<td>EC50</td>
<td></td>
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<tr>
<td>FCx</td>
<td>frontal cortex</td>
</tr>
<tr>
<td>fEPSP</td>
<td>field excitatory post-synaptic potential</td>
</tr>
<tr>
<td>fNR1</td>
<td>floxed (flanked by loxP) site allele</td>
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<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>GlyB</td>
<td>glycine-binding site on NMDAR</td>
</tr>
<tr>
<td>GLYT1</td>
<td>glycine transporter 1</td>
</tr>
<tr>
<td>Glyt1a-802R</td>
<td>primer name</td>
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<td>GLYT2</td>
<td>glycine transporter 2</td>
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<tr>
<td>Grin1,2,3</td>
<td>name of the gene encoding the NR1,2,3 subunits</td>
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<td>Hi</td>
<td>hippocampus</td>
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<tr>
<td>HSV</td>
<td>herpes simplex virus</td>
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<tr>
<td>ICV</td>
<td>intra-cerebro ventricular</td>
</tr>
<tr>
<td>I-O</td>
<td>input-output relationship</td>
</tr>
<tr>
<td>KO</td>
<td>knock-out</td>
</tr>
<tr>
<td>loxP</td>
<td>locus of recombination</td>
</tr>
<tr>
<td>LTP</td>
<td>long-term potentiation</td>
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<tr>
<td>MAP</td>
<td>metamphetamine</td>
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<tr>
<td>mCPP</td>
<td>meta-chlorophenylpiperazine</td>
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<tr>
<td>MOR</td>
<td>morphine</td>
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<tr>
<td>MPP</td>
<td>medial perforant path</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MWM</td>
<td>Morris water maze</td>
</tr>
<tr>
<td>N</td>
<td>asparagine</td>
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<tr>
<td>NE</td>
<td>norepinephrine</td>
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<tr>
<td>NFPS</td>
<td>N[3-(49-fluorophenyl)-3-(49-phenylphenoxy)-propyl]-sarcosine</td>
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</table>
**List of abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>nM</td>
<td>nanomolar</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NMDAR</td>
<td>NMDA receptor</td>
</tr>
<tr>
<td>NR1, 2, 3</td>
<td>NMDAR subunit 1, 2, 3</td>
</tr>
<tr>
<td>Org 24461</td>
<td>R,S-(+/-)N-methyl-N-[(4-trifluoromethyl)-phenoxy]-3-phenyl-propylglycine</td>
</tr>
<tr>
<td>P</td>
<td>postnatal day</td>
</tr>
<tr>
<td>PCP</td>
<td>phencyclidine</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PPI</td>
<td>prepulse inhibition</td>
</tr>
<tr>
<td>Q</td>
<td>glutamine</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription PCR</td>
</tr>
<tr>
<td>rtTA</td>
<td>reverse tetracycline-controlled transactivator</td>
</tr>
<tr>
<td>SB</td>
<td>Southern-blot</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamid gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SI</td>
<td>primary sensory cortex</td>
</tr>
<tr>
<td>St</td>
<td>striatum</td>
</tr>
<tr>
<td>tetO</td>
<td>tetracycline operator</td>
</tr>
<tr>
<td>Th</td>
<td>thalamus</td>
</tr>
<tr>
<td>tTA</td>
<td>tetracycline-controlled transactivator</td>
</tr>
<tr>
<td>US</td>
<td>unconditioned stimulus</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
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Part 1: Introduction
I- INTRODUCTION

Laboratory animals are the only tool available to investigate thoroughly the relationship between molecular signalling processes, neuronal activity and behaviour. Despite large differences in terms of complexity of the central nervous system, most of the molecular, structural and computational processes are conserved throughout evolution. This amazing similarity permits the use of animals to explore the mechanisms that sustain brain functions and gain the knowledge necessary to develop new treatments against human beings’ brain disorders. Glutamate is the principal neurotransmitter in our brain, where it mediates up to 60% of all neurotransmission. Deregulation of the glutamatergic system has been involved in many neuropathic and psychiatric diseases, including epilepsy, stroke, Alzheimer, schizophrenia and depression. Thus, understanding the mechanisms that regulate the activity of the glutamatergic system is of critical significance for the development of new therapeutic approaches. The N-methyl-D-aspartate receptors (NMDARs) are a group of glutamate receptors that are permeable to Ca\textsuperscript{2+} and act as detectors of coincident neuronal activation. These two very specific properties explain the major physiological function of NMDARs in triggering the intracellular signalling pathways that control synaptic plasticity. Glycine is a necessary co-agonist of glutamate on NMDARs, and its concentration in the synaptic cleft is regulated by specific glycine transporters (GLYTs). The ability of GLYTs to modulate NMDAR activity via control of glycine concentration, and thus, alter cognitive processes and behaviour is not yet known.

Schizophrenia is a debilitating disorder that affects more than 1% of the population, worldwide. A hypofunction of the glutamatergic system has been proposed for the etiopathology of schizophrenia. Targeting the glycinergetic modulation of NMDAR activity could be a promising and safe approach to improve current treatments of schizophrenia, but little is known about interactions with cognition and behaviour.

Therefore, the aim of this thesis was to use a dual genetic approach to investigate the effects that an increase and a decrease of GLYT activity would have on some of the mouse cognitive abilities and behaviours that can be related to some of the symptoms observed in schizophrenic patients. Regarding human beings’ brain disorders and more specifically the ones that affect cognition, animal models have obvious limitations due to our inability to explore their psyche. The direct investigation of animals’ psychosis, hallucinations or delusions for example, is not possible. However, many other traits of a complex syndrome like schizophrenia can be assessed with animals: motor activity and
exploratory behaviour, fear and anxiety, social abilities and aggression, attention and sensorimotor-gating, learning and memory, can be tested in animals as well as in man.

In the first part of the introduction, we present a summary about NMDARs, their localisation in the brain and their specificities. In the second part, we present some arguments about the relationship between NMDAR activity and schizophrenia, and why a pharmacological approach targeting GLYT could be more successful than the direct targeting of NMDARs. In the third part, we review the literature about the genetic investigation of NMDAR functions in the mouse brain in vivo, and confront these data with the hypothesis of glutamatergic hypofunction in schizophrenia. Finally, we describe the dual genetic approach used to investigate the influence of GLYT activity on behaviour.
II- NMDA RECEPTORS

Glutamate is the major excitatory neurotransmitter in the brain. It is present as a neurotransmitter in almost all brain structures, where it plays an important role in synaptic plasticity, neural development and neurodegeneration. Glutamate receptors are categorised into two groups of receptors, termed ionotropic and metabotropic receptors. Ionotropic glutamate receptors are mainly localised on postsynaptic membranes. They contain an integral cation-selective ion channel that allows a net inward current, resulting in postsynaptic depolarisation. Using synthetic partial agonists of glutamate, three broad classes of ionotropic glutamate receptors have been described: the N-methyl-D-aspartate (NMDA), the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and the kainate receptors, which have different electrophysiological and signalling properties. NMDA receptors (NMDARs) have complex and specific characteristics, and play a key role in synaptic transmission and plasticity. In addition, deficiencies in the regulation of NMDAR activity are associated with multiple pathologies, including schizophrenia, alcoholism and depression.

1) Structure and localisation of NMDA receptors

NMDARs are hetero-tetrameric proteins, composed of at least two different types of subunits (Figure 2) (Behe et al., 1995; Laube et al., 1998). To date, three main families of NMDAR subunits (NR) have been identified: NR1, NR2 and NR3. NR1 is a fundamental subunit that is obligatory for channel activity of NMDAR, whereas the NR2 subunit confers specificity of function. The NR3 subunit is also a regulatory subunit expressed mostly during development.

a) The NR1 subunits

NR1 subunits are encoded by a single gene, but posttranslational modifications can occur at three independent splice sites on that gene, generating eight functional isoforms (NR1-1 to -4) (Bigge, 1999; Nakanishi et al., 1998). NR1 subunits are expressed throughout the entire central nervous system (CNS) (Figure 1), but the different NR1 splice variants are differentially distributed. NR1 entails the channel forming properties and can form homomeric functional channels in heterologous
expression systems (Moriyoshi et al., 1991; Yamazaki et al., 1992). However, in mammalian cells, NR1 must be coexpressed with NR2 subunits to form functional receptors (Dingledine et al., 1999; Nakanishi et al., 1998). In fact, all native NMDARs are thought to contain both subunits. The type of NR1 isoform involved in receptor formation is of importance in determining various properties such as their sensitivity to pH changes (Cull–Candy, 2000). For example, the presence of the exon 5 determines the pH sensitivity of NR1 subunit. At physiological pH, splice variants that include exon 5 are fully active, whereas those that lack exon 5 are partially blocked (Traynelis et al., 1995).

b) The NR2 subunits

The NR2 family of subunits is represented by four members: NR2A, 2B, 2C and 2D arising from separate gene (Hollmann and Heinemann, 1994). NR2 are modulatory subunits that alone do not exhibit NMDA receptor activity but, in heteromeric formation with NR1, potentiate and differentiate NMDA receptor activity (Monyer et al., 1994). NR2 subunit expression exhibits both developmental and regional variations, with particular NR2 subunits restricted to defined neuronal populations. NR2A and NR2B are the predominant NR2 subunits in the adult forebrain. NR2A expression increases with aging, while NR2B subunit is most abundant in young animals. NR2C is expressed mainly in the cerebellum and select nuclei, whereas NR2D is confined to the diencephalon and midbrain (Wenzel et al., 1997) (Figure 1).

The type of subunits involved in the formation of hetero-tetrameric NMDAR is critical for determining the receptor-channel properties. In NMDARs composed of two copies of NR1 and two copies of NR2, the single-channel conductance provides a “signature” for the type of NR2 subunit involved. NR2A and NR2B subunits give rise to channels with high-conductance states, whereas NR2C and NR2D containing NMDARs are characterised by low-conductance openings (Cull-Candy et al., 2001; Cull-Candy and Leszkiewicz, 2004). The deactivation kinetics of NMDARs is also strongly dependent on the NR2 subtype. In response to a 1 ms pulse of 1 mM glutamate, the time constants of deactivation were estimated to be around 100 ms for NR2A containing NMDARs, 250 ms for NR2B and NR2C, and 4 s for NR2D (Vicini et al., 1998). Finally, the identity of the NR2 subunit is also critical for determining the affinity for glutamate, modulation by glycine, and sensitivity to Mg$^{2+}$ (Brimecombe et al., 1997).
c) The NR3 subunits

The NR3 family of subunits has two gene members (NR3A, 3B). NR3A and NR3B subunits cannot form homomeric NMDARs. They give rise to functional NMDARs only when coexpressed with NR1 and NR2 subunits (Ciabarra et al., 1995; Matsuda et al., 2002; Nishi et al., 2001; Sucher et al., 1995). NR3A is expressed ubiquitously during development and its expression reaches a maximum at approximately the first postnatal week. Thereafter, the level gradually decreases, and in adult animals, NR3A is confined to selected nuclei in the thalamus, amygdala, and nucleus of the lateral olfactory tract (Al Hallaq et al., 2002; Ciabarra et al., 1995; Sucher et al., 1995; Wong et al., 2002). NR3B expression is restricted to somatic motoneurons of the brainstem and spinal chord (Matsuda et al., 2002; Nishi et al., 2001). Like NR2 subunits, NR3 subunits have a major influence on the properties of NMDARs.

**Figure 1:** Regional distribution of mRNAs encoding the NR1 and NR2 subunits of the NMDA receptor, revealed by *in situ* hybridization on parasagittal sections of adult rat brain. OB, olfactory bulb; Cx, cortex; Hi, hippocampus; Cb, cerebellum; Th, thalamus; St, striatum. Adapted from Nakanishi S. et al. 1992
2) **Properties of NMDA receptors**

NMDARs exhibit remarkable properties that distinguish them from other types of ligand-gated ionotropic receptors. Firstly, NMDAR channels display a high permeability to Ca$^{2+}$ ions, what couple them with intracellular signalling. Secondly, they are subject to a voltage-dependent block by physiological levels of extracellular Mg$^{2+}$, what confers to NMDARs the capacity to act as a molecular coincidence detector. Thirdly, the receptor’s response to neurotransmitter is modulated by glycine, a necessary co-agonist of glutamate (Figure 2). Other factors encountered in the extracellular milieu also modify NMDAR properties, like pH and concentrations of Zn$^{2+}$ and polyamines, but their effects are not described here.

**a) NMDARs are coupled via Ca$^{2+}$ to intracellular signalling.**

The pore of NMDARs is selective for cations and, more specifically, is highly permeable to Ca$^{2+}$ ions (Dingledine et al., 1999). As calcium is a major intracellular secondary messenger, the opening of NMDARs triggers the activation of multiple signalling cascades, which can ultimately lead to changes in synaptic connectivity through activation of various kinases and phosphatases. For example, the kinases calcium-calmodulin dependent kinase II alpha (CaMKIIα) and protein kinase A (PKA), and the phosphatase calcineurin (CN) are Ca$^{2+}$ dependent enzymes that have a major role in synaptic plasticity. Their activity at the post-synaptic level is triggered by Ca$^{2+}$ flux through NMDAR. In the case of the intense NMDAR activation that occurs in ischemia, the extreme influx of Ca$^{2+}$ leads to cell death by apoptosis.

**b) NMDARs are coincidence detectors.**

Extracellular Mg$^{2+}$ ions can enter and bind within the pore of NMDARs, and prevent ion flux through the channel. Binding of Mg$^{2+}$ is strongly voltage-dependent, so that most subtypes of NMDARs undergo rapid channel block at resting membrane potentials. However, upon depolarisation, for example by intense activation of colocalised postsynaptic AMPA receptors, the voltage-dependent block by Mg$^{2+}$ is partially relieved, allowing ion influx through activated NMDARs (Figure 2). Thus, the Ca$^{2+}$ flux through NMDARs occurs only upon coincident depolarisation and synaptic
release of glutamate.

Figure 2: Structure of the NMDA receptor. Two NR1 and two NR2 or NR3 subunits are associated to form the selective cation-permeable channel. (a) At resting cell-membrane potential, the channel pore is blocked by Mg\(^{2+}\) ions, preventing Ca\(^{2+}\) and Na\(^{+}\) flow. (b) Binding of both glutamate and glycine and coincident depolarisation of the membrane are required to open NMDA receptor channel and permit the inward flow of Ca\(^{2+}\) and Na\(^{+}\). NMDA receptor is anchored in the post-synaptic density by a PDZ domain, and its activity is strongly coupled to intracellular signalling via Ca\(^{2+}\)-depending enzymes like PKA, CaMKII and calcineurin

c) NMDARs are regulated by synaptic glycine concentration.

i- Glycine binding site

NMDARs possess a binding site for glycine, which must be occupied before NMDARs are activated by agonist binding (Johnson and Ascher, 1987, 1992; Mayer et al., 1989). The glycine-binding site lies on the NR1 subunit, and was named glycine-B site (Gly-B). All NR1 splice variants exhibit similar affinities for glycine, whereas the identity of the NR2 subunit exerts a major influence on receptor glycine affinity. Receptors containing NR2D appear to exhibit the highest affinity for both glutamate and glycine (Ikeda et al., 1992), whereas receptors containing NR2A exhibit a markedly lower affinity for glycine (Kutsuwada et al., 1992). In individual cortical neurons of young adult rats, three populations of NMDARs differing by their affinity for glycine were reported. The proportion of each of these populations of NMDARs was correlated
with the changes in NR2A and NR2B expression during development. It suggested that receptors exhibiting high affinity for glycine and slow glycine dissociation kinetics contained NR2B as the sole NR2 subunit, whereas receptors exhibiting low affinity for glycine and fast dissociation contained NR2A as the sole NR2 subunit (Kew et al., 1998).

The EC50 is the molar concentration of an agonist, which produces 50% of the maximum possible response for that agonist. In cultured neurons and in heteromeric NMDARs expressed in Xenopus oocytes, the EC50 for glycine was measured between 100 and 300 nM (Ikeda et al., 1992; Johnson and Ascher, 1992; Kutsuwada et al., 1992; Mayer et al., 1989). In the cerebrospinal fluid and in microdialysis samples, the glycine concentration was measured in the low micromolar range, what would permanently saturate the Gly-B site (Kemp and Leeson, 1993; McGale et al., 1977; Westergren et al., 1994). Therefore, a key issue was whether glycine is a significant modulator of NMDAR activity or is it always present at supramaximal amounts and therefore incapable of modulating NMDAR synaptic activity.

**ii- Glycine transporters**

Synaptic glycine concentration is regulated by two glycine transporters, GLYT1 and GLYT2, which differ mainly by their expression pattern (Figure 3). GLYT2 is expressed predominantly by neurons of the brain stem and spinal cord, and to a lesser extent in the cerebellum, which are regions rich in glycineric synapses (Jursky and Nelson, 1995, 1996; Jursky et al., 1994; Zafra et al., 1995a; Zafra et al., 1995b). In addition, GLYT2 was shown to be concentrated in the plasma membrane directly apposed to glycine receptors, and was therefore proposed to be the main reuptake mechanism at inhibitory glycineric synapses (Jursky and Nelson, 1995; Spike et al., 1997). In contrast, GLYT1 is expressed strongly in glial cells of the brain stem and spinal cord, and weakly in glial cells of the hippocampus and cortex (Adams et al., 1995; Jursky and Nelson, 1996; Zafra et al., 1995a; Zafra et al., 1995b). Considering the expression pattern, it was proposed that GLYT1 would have two functions. At inhibitory glycineric synapses in the spinal cord, brain stem and cerebellum, GLYT1 would primarily participate to the glycine reuptake mechanism. Then, in forebrain structures deprived of glycineric neurotransmission, like the striatum, hippocampus and cortex, GLYT1 would be associated with NMDAR mediated glutamatergic neurotransmission, where it would regulate synaptic glycine concentration (Smith et al., 1992).
iii- Synaptic glycine concentration is not saturating

They were several attempts to determine whether GLYT1 activity at glutamatergic synapses would decrease synaptic glycine concentration below the saturation point for NMDARs. These studies used a similar approach, and tested if adding exogenous glycine would potentiate NMDAR mediated currents. Unfortunately, they produced contradictory results, showing either an absence of potentiation (Fletcher et al., 1989; Kemp et al., 1988), or a potentiation (Thomson et al., 1989; Watanabe et al., 1992; Wilcox et al., 1996), of the NMDAR component of synaptic currents. The same approach was used to test the effect of D-serine, a potent agonist at the glycine-binding site that is not taken up by glycine transporters (Supplisson and Bergman, 1997). The application of D-serine potentiated NMDAR mediated currents (Berger et al., 1998; Watanabe et al., 1992), suggesting that the glycine–binding site is not saturated but protected from an increase of synaptic glycine concentration by a very powerful uptake system (Berger et al., 1998). Bergeron et al. provided a more elegant demonstration of this mechanism. On rat hippocampal slices, the bath application of 100 nM N[3-(49-fluorophenyl)-3-(49-phenylphenoxy)-propyl]-sarcosine (NFPS), a specific inhibitor of GLYT1, increased NMDAR mediated currents in the same extent than the adjunction of 10 μM glycine (Bergeron et al., 1998). Finally, the deletion of both Glyt1 alleles in mice caused rapid postnatal lethality, but heterozygous knockout mice (Glyt1 +/-) developed normally (Gomeza et al., 2003a; Tsai et al., 2004b). Glycine uptake was reduced by almost 50% in heterozygous knockout mice, and the NMDAR mediated currents could not be potentiated by application of 10 μM glycine or D-serine (Tsai et al., 2004b). This confirmed that in vivo, synaptic glycine concentration is regulated by GLYT1, a powerful uptake mechanism that prevents saturation of NMDARs.

As NMDARs have been involved in multiple brain disorders (described below), the possibility to fine tune NMDAR activity via a modulation of synaptic glycine concentration could be of great pharmacological interest.
Figure 3: Distribution of GLYT1 and GLYT2 immunoreactivities in mice

a-b) Longitudinal sections of E18 embryos. Sc, spinal cord; L, liver; P, pancreas Bar = 2.2 mm

c- d) Transversal sections of adult spinal cords Bar = 32 µm

e-f) Parasagittal sections of adult brains Bar = 1.9 mm

g) Horizontal section of an adult brain Bar = 2 mm

Adapted from Jursky et al. 1996
3) NMDAR are involved in multiple brain disorders

As described above, glutamatergic neurotransmission through NMDARs occurs in almost all brain areas. As glutamate is involved in many processes, including developmental and adult synaptic plasticity, neurogenesis and neurodegeneration, it is plausible that dysregulation of glutamatergic neurotransmission could generate a pathological state. Hyperfunction of NMDARs has been proposed as a causative agent for traumatic and neurodegenerative disorders and neurogenic pain, whereas hypofunction of NMDARs has been proposed for psychiatric disorders with psychosis and negative symptoms.

a) Hyperactivation of NMDARs

Excitotoxicity is a process that occurs when abnormal excessive glutamate release triggers a massive opening of glutamate receptors. Through NMDARs, a disproportionate Ca^{2+} entry initiates a series of cytoplasmic and nuclear processes that promote neuronal cell death (Dirnagl et al., 1999). For instance, Ca^{2+}-activated proteolytic enzymes, like calpains, can degrade essential proteins, and Ca^{2+}-dependent endonucleases can degrade DNA. It is well established that excitotoxicity happens during major brain injuries and acute neurological disorders, like ischemic stroke, post-traumatic lesions, and epileptic convulsions. Moreover, a role for excitotoxicity has also been proposed for neurodegenerative disorders like Parkinson’s or Alzheimer disease, Huntington’s chorea or amyotrophic lateral sclerosis (Hynd et al., 2004; Rego and Oliveira, 2003).

Pain is detected by two different types of peripheral nociceptor neurons. C-fibre nociceptors have unmyelinated axons and a slow conduction speed, and A-delta nociceptors have thin myelinated axons and a fast conduction speed. Neuropathic pains result from a central sensitisation of the nociceptive tracks, which occurs via activation of NMDARs and increase of the neuronal response to pain stimuli (Bennett, 2000). Concomitantly, there is a decrease in neuronal sensitivity to opioid receptor agonists, what prevents the effectiveness of classical analgesic treatments (Parsons, 2001).

The crucial role played by NMDARs in the pathophysiology of traumatic, degenerative and neuropathic diseases suggested great therapeutic power for functional inhibition of NMDARs. Unfortunately, antagonists that completely block NMDARs
also impair normal synaptic transmission and cause numerous side effects such as psychosis, ataxia and motor coordination problems, and memory impairment, preventing clinical use. The recent development of drugs like Memantine, which is a weak, activity-dependent and subunit-specific NMDAR antagonist, was a promising breakthrough. This drug is devoid of major debilitating side-effects, and has considerable potential in the clinic.

b) Hypoactivation of NMDARs

A hypofunctional state of glutamatergic neurons has been proposed for psychiatric diseases like schizophrenia (detailed in the next section) and depression. However, the clinical use of direct NMDAR agonists is not possible due to their tendency to trigger seizures and cause neuronal death.

Drugs that modulate NMDAR activity, rather than act directly on the receptor itself, would probably lack the negative effects of direct NMDAR agonists and antagonists, and could prove very useful at the clinical level (Bleich et al., 2003). The regulation of synaptic glycine concentration is one of the promising alternatives to modulate NMDAR activity. However, a better understanding of the physiological processes influenced by synaptic glycine concentration and their interactions with behaviour is necessary. Schizophrenia is a common and debilitating disease for which no optimal treatments are available, and could, in theory, benefit from drugs enhancing NMDAR activity.
III- SCHIZOPHRENIA

Schizophrenia is a devastating, complex and multifactorial mental disorder, which impairs cognitive and psychomotor abilities. It is characterised by episodic psychotic phases comprising positive symptoms such as hallucinations, delusions, disorganised speech and behaviour, followed by phases of negative symptoms such as affective flattening, alogia, avolition, social withdrawal and anhedonia. In the diagnostic and statistical manual of mental disorders (DSM), four different types of schizophrenia have been defined, paranoid, catatonic, disorganised and undifferentiated types, to fit better the diversity of the symptoms, the severity and the time course of the disease (see annexe 1, DSM IV list of diagnostic criteria). The prevalence of schizophrenia in the population is about 1 %, but up to 3 % of the population have a schizotypal personality disorder. Surprisingly, the prevalence seems to be equal in all countries and across social class. Numerous genetic studies have shown that there is a genetic predisposition to schizophrenia. The concordance among homozygotic twins is 48 % whereas it is only 17 % among dizygotic twins (McGue and Gottesman, 1991). Numerous studies have investigated the neuropathology of schizophrenia, but no reliable and specific markers have been found. Similarly, the dopaminergic, serotonergic, and glutamatergic systems have been involved in the aetiology of schizophrenia, but a unifying model is still missing. Current antipsychotic treatments act mainly at the dopaminergic and serotonergic levels, but it is believed that drugs targeting the glutamatergic system would have a better therapeutic efficacy.

1) Neuropathology of schizophrenia

A hundred years ago, Kraepelin, who described first the syndrome now called schizophrenia, was convinced that it was an organic brain disease. Since then, many studies have investigated the neuroanatomy in schizophrenic patients, but few of them have made convincing observations that could be replicated by independent groups. A very good and comprehensive review of this literature was published by Paul J. Harrison in 1999 (Harrison, 1999a). The most consistent findings describe an enlargement of the lateral and third ventricles in schizophrenic patients. In proportion to the brain volume,
the increase of the ventricle size was found between 20 and 75 % (Daniel et al., 1991; Van Horn and McManus, 1992). In agreement with this result, an average 3 % loss of brain tissue was also observed, affecting grey matter more than white matter (Lawrie and Abukmeil, 1998; Zipursky et al., 1998). Ventricular enlargement and cortical volume reduction are both present in first-episode patients, excluding the possibility that these observations are a consequence of chronic illness or its treatment. The cytoarchitecture of neurons in schizophrenic patients has also been examined thoroughly in cortical regions associated with positive symptoms. Various abnormalities of cytoarchitecture and lamination where found in the entorhinal cortex: pre-alpha cells appeared shrunken, misshapen and heterotypic, and formed abnormal clusters (Jakob and Beckmann, 1986). In addition, pyramidal neurons of the hippocampus were found to be smaller and disorganised (Jonsson et al., 1997; Zaidel et al., 1997). Finally, quantitative analyses of synaptic protein content have shown a decrease of some pre- and post-synaptic markers in the hippocampus and the dorsolateral prefrontal cortex (Glantz and Lewis, 1997; Harrison and Eastwood, 1998). However, for all these neuropathological observations, the magnitude of change was usually small, the group means showed considerable overlap between schizophrenia and comparison groups and the findings were rarely specific for schizophrenia, what explains that no reliable and specific marker for schizophrenia has been found so far.

2) Neurochemistry of schizophrenia

a- The dopaminergic hypothesis

Based on the positive correlation between the affinity for the D₂ dopamine receptor and the clinical potency of classical antipsychotic drugs, it has been proposed that schizophrenic patients would suffer from an excess of dopaminergic neurotransmission in the striatum and a deficit in the prefrontal cortex (Lewis and Lieberman, 2000). Accordingly, increased levels of D₂ receptors were measured in cortical areas of patient’s brains, whereas in the mesolimbic system, dysfunctions of presynaptic storage, release, uptake and metabolism of dopamine were reported. However, classical antipsychotic drugs have only a limited impact on schizophrenia and attenuate positive but not negative symptoms, suggesting that additional neurotransmitter systems may be involved.
b- The serotonergic hypothesis

The idea that serotonin, also called 5-hydroxytryptamine (5-HT) is involved in schizophrenia comes from the observation that the hallucinogenic drug lysergic acid diethylamide (LSD) is an agonist at specific 5-HT receptors. Many studies have found lowered 5-HT2A receptor expression in the frontal cortex of schizophrenic patients (Harrison, 1999b). Furthermore, a blunted neuroendocrine response to 5-HT2 agonists was observed in some patient groups (Abi-Dargham et al., 1997). However, other recreational drugs that are not serotonergic induce psychosis with more similarities to schizophrenic symptoms.

c- The glutamatergic hypothesis

It was observed that the drug of addiction phencyclidine (PCP) induces a psychosis very similar to that seen in schizophrenic patients. This includes hallucinations, delusions, disorientation, coordination deficits, stereotypical behaviour, anxiety and paranoia, which are almost indistinguishable from the ones observed in schizophrenic patients. In addition, PCP administration to schizophrenic patients causes a dramatic worsening of the symptoms. PCP is a non-competitive antagonist of glutamate on NMDA receptor, and other NMDAR antagonists like ketamines and MK-801 induce a similar schizophrenia-like psychosis, what suggested that schizophrenic patients may suffer from NMDAR hypofunction (Lewis and Lieberman, 2000; Weinberger, 1997). Accordingly, post-mortem studies measured a reduced level of glutamate in the brain of schizophrenic patients, correlated with a reduction of NAALADase activity, a key enzyme in the glutamate synthesis pathway. In addition, radioligand-binding studies showed an increase of NMDA and AMPA receptor concentrations in the prefrontal cortex, which was interpreted as a compensatory mechanism for reduced glutamatergic activity.

The effects of PCP, ketamines and MK-801 administration have been tested on laboratory animals. It induced behavioural alterations, like hyperactivity, stereotypical behaviour, anxiety and deficits of sensorimotor gating, which resembled deficits observed in human beings after drug intake and some of the symptoms of schizophrenic patients. These drug-induced behaviours were reversed by administration of typical and atypical antipsychotic medications used for the treatment of schizophrenia. Thus, drug-induced NMDAR hypofunction induces behaviours in mammals (including humans).
that resemble some schizophrenic symptoms, providing a tool to investigate the pathophysiological mechanisms of schizophrenia and validate molecules with potential therapeutic efficacy.

The limitations of current antipsychotic treatments, which mostly target the dopaminergic system, suggests that pharmacological agents enhancing NMDAR activity could have a great potential as antipsychotics. Considering the toxicity of direct NMDAR agonists (presented in the section 3-a p.21), an indirect approach to enhance NMDAR activity is required. Glycine is a necessary co-agonist of glutamate on NMDAR, whose concentration in the synapse is tightly regulated by a reuptake mechanism. Glycine recapture is mediated by Na+/Cl⁻ coupled membrane glycine transporters (Glyt). As shown by two independent experiments on both hippocampal pyramidal neurons and hypoglossal motoneurons, the concentration of glycine in the synaptic cleft is subsaturating and can therefore modulate NMDAR activity (Berger et al., 1998; Bergeron et al., 1998). This property has been used already in clinical trials, where administration of glycine or glycine agonists (cyclo-serine and D-serine) in addition to classical antipsychotic treatments improved the wellness of the patients, reduced the severity of the symptoms and improved the cognitive functions (Heresco-Levy et al., 1999; Tsai et al., 2004a; Tsai et al., 1998; Tuominen et al., 2005). These results suggest that modulation of NMDAR activity by glycine is a promising approach to improve the current treatments available for schizophrenic patients. However, a better understanding of this regulatory mechanism and its influence on behaviour and cognition is still necessary, as it would help the development and validation of drug candidates targeting the glycinerergic modulation of NMDAR activity.

To summarise, similarities between PCP-induced behaviours and symptoms of schizophrenia led to the hypothesis of glutamatergic hypofunction in schizophrenic patients, and permitted the use of animal models to test the potential therapeutic efficacy of new molecules. However, this pharmacological approach may not model correctly the complexity of the aetiology of schizophrenia, as acute drug injection lacks the developmental and long-lasting effects. Thus, mutated mouse lines with NMDAR dysfunction should be more appropriate to investigate NMDAR functions in vivo and evaluate the hypothesis of glutamatergic hypofunction in schizophrenia. We propose to review this literature in the next chapter.
In the previous sections we reported that glutamate is the most prevalent neurotransmitter in the brain, and that NMDARs are expressed throughout the brain and have peculiar properties that confer them an important role for synaptic transmission and plasticity. In addition, hypofunction of NMDAR activity has been proposed to participate to the aetiology of schizophrenia. This section summarises the genetic analysis of NMDAR function in mice, and focus on the role of NMDAR activity for correct brain development and patterning, learning and memory and schizophrenia-like behaviours.

**NMDAR AND BRAIN PATTERNING**

It was proposed that precise patterning of neuronal connectivity results from both activity-dependent and activity-independent mechanisms (Goodman and Shatz, 1993). The very unique properties of NMDARs suggested that they could be a critical component of the interface linking neuronal activity with neuronal connectivity. This specific function of NMDARs was investigated with multiple mouse models of altered NMDAR activity, focusing on whisker-related patterns as a model of somatosensory map formation.

1) **NMDARs are necessary for somatosensory map formation**

Whisker-related sensory inputs are topographically mapped at multiple relay stations from the trigeminal input to the primary sensory cortex (Erzurumlu and Kind, 2001; Molnar and Hannan, 2000). At each level, presynaptic afferents and postsynaptic target cells form discrete cytoarchitectural and functional units that replicate the whisker pattern on the muzzle in a one-to-one relationship (Figure 4). Whisker-related patterns
develop along a peripheral to central temporal gradient. At birth, the precise whisker-related patterning can be detected by staining for cytochrome oxidase at the first central synapses of the pathway, which are located in the brainstem trigeminal complex and are called barrelettes. Pattern formation then progresses to the thalamus, where the whisker-related structures are named barreloids. Later during maturation of the brain, the patterns become visible on the cortex, where they are called barrels.

Figure 4: Schematic representation of the whisker-barrel pathway. On the rodent snout, vibrissae are laid out in a stereotypical pattern. Whisker movements are detected by the sensory neurons of the trigeminal nerve, which relay at the brainstem trigeminal complex (BSTC). The information is then conveyed to the ventral medial posterior thalamus (VPM) and to the primary somatosensory cortex (S1). In these three relay stations, neurons terminate in clusters following the somatotopical organisation of the whiskers, which are named barrels in the S1 (not drawn at scale). Each barrel receives preferentially input from the corresponding whisker.
**NR1 knockout mice**

The genetic analysis of NMDAR function started in 1994 with the publication of two independent mouse lines bearing a deletion of the Grin1 gene that encodes NR1 subunit. Both groups reported perinatal death of homozygous knockout mice (NR1 -/-) due to respiratory distress (Forrest et al., 1994; Li et al., 1994). The absence of NR1 subunit rendered all NMDARs functionally ineffective, because NMDARs are heteromeric assemblies requiring the principal NR1 subunit in combination with the modulatory NR2 and NR3 subunits to form functional receptor channels in vivo. Accordingly, the absence of NMDAR current in NR1 -/- mice at birth was demonstrated in the trigeminal nuclei (Li et al., 1994), and in explant culture of cerebellar granular neurons and hippocampal pyramidal neurons (Forrest et al., 1994). The investigation of whisker-related patterns in NR1 -/- mice showed that, in absence of NMDARs but in the presence of functional excitatory and inhibitory synaptic transmission, neurons failed to develop barrelettes in the brainstem trigeminal complex (Li et al., 1994). This observation led to the conclusion that NMDARs were involved in the detailed patterning of target neurons to reflect the precise arrangement of peripheral sensory organs (Li et al., 1994).

**NR2B knockout mice**

In a similar way, the deletion of Grin2b gene encoding NR2B, the most abundant NR2 subunit, altered brain patterning and mouse survival. Kutsuwada et al. reported that NR2B knockout mice (NR2B -/-) were obtained in Mendelian ratio, had a normal weight at birth but died within one day due to the absence of suckling response (Kutsuwada et al., 1996). The survival of NR2B -/- pups could be extended by hand feeding via implantation of a canula into the stomach. The absence of NR2B subunit prevented the formation of active NMDARs, demonstrated by the absence of measurable NMDAR currents at CA1 synapses of PO and P2 pups. In the brainstem trigeminal complex of the mutant mice, the connectivity and somatotopy of the primary afferent vibrissal fibres were largely preserved, but the terminal arbours were distributed diffusely and no distinct clustering was observed (Kutsuwada et al., 1996). This observation confirmed the finding made with NR1 -/- mice that NMDARs are necessary for the correct patterning of somatosensory connections.
NR1 knockout rescue

To support this result and formally exclude the possibility that a retardation of brain development could explain the absence of barrelettes, Iwasato et al. attempted to rescue NR1-/- mice by ectopic expression of a Grin1 transgene, encoding the NR1 subunit. The comparison of three transgenic mouse lines that differed by the amount of Grin1 transgene expression revealed that the correct formation of somatosensory maps was correlated with the amount of Grin1 expression and NMDAR activity (Iwasato et al., 1997).

Cortex specific NR1 knockout

Finally, Iwasato et al. made a knockin of a Cre recombinase gene downstream the promoter of the Emx1 homeobox gene to ablate the Grin1 gene only in cortical excitatory neurons. Thus, it was demonstrated that NMDARs in the cortex are necessary for the precise patterning of barrel boundaries (Iwasato et al., 2000).

NR1-N598R knockin

The replacement of a single amino acid (N598R) in the pore region of the NR1 subunit (NR1R) is sufficient to ablate the Mg²⁺ voltage-dependent blockade of NMDARs and prevent Ca²⁺ flux through the channel (Burnashev et al., 1992). This suppresses the coincidence detection property of NMDAR and its ability to activate intracellular signalling cascades, respectively. In mice, this mutation is dominant and lethal: both NR1R/+ mice and NR1R-/- mice became cyanotic shortly after birth and died within a few hours (Rudhard et al., 2003). Interestingly, in NR1R-/- mice, the projections of primary afferents from the whiskers to the brainstem trigeminal complex (BSTC) were normal, but the barrelettes were absent from the BSTC (Rudhard et al., 2003). Therefore, it is not the mere electrical activity of NMDARs that is necessary for the detailed patterning of neuronal networks, but rather its ability to detect coincident neuronal activity and to trigger intracellular signalling cascades involved in synaptic plasticity.
2) NMDARs are involved in sprouting and spine formation

**NR3A knockout**

The expression of the NR3 subunit of the NMDAR was investigated in the mouse at the mRNA level (Sucher et al., 1995), and in the rat at the mRNA (Ciabarra et al., 1995) and protein levels (Ali Hallaq et al., 2002; Wong et al., 2002). These studies have shown that the expression of the NR3A subunit is developmentally regulated. The expression level increases from postnatal day (P) 0 to 8, peaks at P8, and decreases gradually between P12 and P20, then stabilises in the adult brain at a level that is half that at birth. NR3A expression occurs in almost all brain areas, but is stronger in the cortex, striatum, hippocampus and amygdala and weaker in the cerebellum. Homozygous mice with a deletion of both Grin3A alleles (NR3A -/- mice) were obtained in Mendelian ratio, were viable and fertile, and grew into adulthood without apparent behavioural abnormalities (Das et al., 1998). The electrophysiological properties of NR3A containing NMDARs were investigated in transfected cells and in cortical neurons from NR3A -/- mice (Das et al., 1998; Sasaki et al., 2002; Sucher et al., 1995). NR3A containing NMDARs have a reduced channel conductance, a shorter open time, are less permeable to Ca2+, and are less sensitive to Mg2+ voltage dependent blockade. In NR3A -/- mice, the absence of the NR3A subunit augmented the NMDAR current density 2.8 times compared to wild type, but this did not generate gross structural abnormalities in the cerebral cortex and barrel field. However, in NR3A -/- mice, the number of dendritic spines was strikingly increased in cortical neurons of layers IV and V, and the spine morphology was modified too: spine heads were larger and spine necks were longer. The increase in spine density was observed at P19 and in 3-month-old mutant mice, but it was larger at P19 than in adulthood (Das et al., 1998). The expression of NR3A subunit is greatly reduced in cortical neurons of adult wild type mice compared to neonates, what suggests that the morphological changes in NR3A -/- mice were inversely correlated with the intensity of NR3A expression in wild-type mice.

As summarised in table 1, the plain and cortex specific deletion of the Grin1 gene, its rescue by Grin1 transgene expression, and the plain deletion of the Grin2B gene demonstrated that NMDAR activity is necessary for correct brain patterning. This finding was refined with NR1\(^R^+\) and NR1\(^R^-\) mice, showing that rather than the electrical
activity of NMDARs, it is their ability to detect coincident neuronal activity and trigger intracellular signalling cascades that is critical for precise somatosensory map formation. In addition, it was observed with NR3A -/- mice that increased NMDAR channel opening and Ca\(^{2+}\) flux was associated with increased spine size and spine density. This finding provides a mechanistic explanation for the role of NMDARs in the establishment of correct neuronal patterning, and suggests that NMDARs may be critical for memory formation.

### Table 1 Summary of the genetic investigations of NMDAR role in somatosensory map formation

<table>
<thead>
<tr>
<th>GENES</th>
<th>REF.</th>
<th>LINES</th>
<th>CHARACTERISATION</th>
<th>PHENOTYPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR1 KO</td>
<td>Li et al. 1994</td>
<td>NR1 -/-</td>
<td>No NR1 expression</td>
<td>Apnea, cyanosis and death 10-20h after birth</td>
</tr>
<tr>
<td></td>
<td>Forrest et al. 1994</td>
<td>NR1 -/-</td>
<td>No NR1 expression</td>
<td>No feeding, cyanosis and death 8-15h after birth</td>
</tr>
<tr>
<td></td>
<td>Kutsumada et al. 1996</td>
<td>NR2B -/-</td>
<td>No NR2B expression</td>
<td>Survival possible with hand-feeding</td>
</tr>
<tr>
<td></td>
<td>Kutsuwada et al. 1996</td>
<td>NR2B KO</td>
<td>No NR2B expression</td>
<td>Absence of barrelettes in BSTC</td>
</tr>
<tr>
<td></td>
<td>Kawasato et al. 1997</td>
<td>NR1 -/- LTg -/</td>
<td>Very low NR1 expression</td>
<td>Neonatal death</td>
</tr>
<tr>
<td></td>
<td>Kawasato et al. 1997</td>
<td>NR1 -/- LTg +/-</td>
<td>Low NR1 expression</td>
<td>Early death (&lt;24h)</td>
</tr>
<tr>
<td></td>
<td>Kawasato et al. 1997</td>
<td>NR1 -/- HTg +/-</td>
<td>Normal NR1 expression similar to WT</td>
<td>Wasting and spastic with ageing</td>
</tr>
<tr>
<td></td>
<td>Kawasato et al. 1997</td>
<td>EmxCre / fNR1</td>
<td>Normal NR1 activity in Cx</td>
<td>Normal body weight at P0, 70% of normal at P7</td>
</tr>
<tr>
<td></td>
<td>Rudhard et al. 2003</td>
<td>NR1 RI/- NS98R</td>
<td>All NMDARs are Mg2+ insensitive and Ca2+ impermeable</td>
<td>Death within 1h after birth</td>
</tr>
<tr>
<td></td>
<td>Miyamoto et al. 2002</td>
<td>NR2D KO</td>
<td>Reduced NMDAR density (25% less)</td>
<td>Normal whisker-related patterns in BSTC, Th and SI</td>
</tr>
<tr>
<td></td>
<td>Das et al. 1998</td>
<td>NR3A KO</td>
<td>2x increased NMDAR currents</td>
<td>Normal growth</td>
</tr>
</tbody>
</table>

BSTC, brain stem trigeminal complex; Cx, cortex; fNR1, NR1 allele flanked by loxP sites; Hi, hippocampus; P, post-natal day; SI, primary somatosensory cortex; Th, thalamus; WT, wild-type
B NMDAR ACTIVITY AND MEMORY

Hebb’s postulate: “When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A’s efficiency, as one of the cells firing B, is increased.” D.O. Hebb, The Organization of Behavior, 1949

Since D.O. Hebb (1904-1985) and his famous postulate, an ever-increasing amount of observations sustained the ideas that synaptic plasticity is the physical substrate of memory in the brain and that coincident presynaptic activity and postsynaptic depolarisation are necessary to strengthen synaptic connection. Due to the voltage-dependent Mg\(^2+\) blockade, NMDARs have the coincidence detection ability necessary to account for the Hebbian property of synaptic plasticity. This suggests a critical role for NMDARs in learning and memory processes. In this section, we summarise the studies that used genetic approaches to investigate in vivo the role of NMDARs for memory formation and storage.

1) NMDARs are necessary for heteroassociative memory formation

NR2A knockout

In contrast to the lethality and the profound effect on brain patterning of Grin1 or Grin2B gene disruption, mice with an homozygous deletion of the Grin2A gene (NR2A -/- mice) developed and mated normally and no anatomical changes were observed in their brains (Kadotani et al., 1996; Sakimura et al., 1995). NR2A -/- mice had a 50% reduction of NMDAR mediated currents in CA1 pyramidal cells (Sakimura et al., 1995), and granular cells of the cerebellum (Kadotani et al., 1996). NR2A -/- mice displayed reduced long-term potentiation (LTP) after tetanic stimulation, in agreement with the fact that LTP is an experimental form of synaptic plasticity that is NMDAR dependent in the hippocampus (Bliss and Collingridge, 1993; Sakimura et al., 1995). In addition, NR2A -/- mice had a reduced ability to learn with precision the platform position in the Morris water maze, a task challenging spatial memory that is also hippocampus and NMDAR dependent (Morris et al., 1986, 1989; Morris et al., 1982; Sakimura et al., 1995).
In a follow-up study, Kiyama et al. suggested that the reduction of LTP magnitude could be explained by a decrease in the number of active NMDAR channels. To demonstrate this hypothesis, it was shown that stronger stimulation, i.e. the repetition of the LTP eliciting stimulus or an increase of its intensity, could overcome the deficit of synaptic potentiation (Kiyama et al., 1998). Thus, the ability to display a maximum LTP was not impaired in NR2A -/- mice, but the response to the stimulation was decreased. A similar shift of the stimulus-response curve was also observed at the behavioural level. NR2A -/- mice tested on the fear-conditioning paradigm with a classical protocol did not show any deficit of memory. A deficiency in contextual learning became evident only when the conditioned stimulus was weakened by shortening the exposure time to the context, namely, the time interval between the placement in the conditioning chamber and the footshock (Kiyama et al., 1998). Thus, the deprivation of NR2A subunit caused a 50 % reduction of NMDAR currents, and raised the stimulus threshold necessary to induce LTP and contextual learning, but did not affect neither the maximum level of potentiation nor the maximum context-induced freezing.

**NR2B overexpression in forebrain neurons**

To continue the investigation of the relationship between NMDAR activity, LTP, and memory, Tang et al. generated two transgenic mouse lines in which the expression of NR2B subunit was under control of the forebrain neuron specific CaMKIIα promoter (Tang et al., 1999). In transgenic NR2B mice, the amount of NR2B protein was doubled in the cortex and hippocampus, and was unchanged in the brainstem and cerebellum. The brain anatomy of transgenic mice was normal, as was the spine morphology. In *vitro*, cultured cortical neurons from NR2B transgenic mice displayed NMDAR currents with longer decay time than that of wild-type mice (Tang et al., 1999), in agreement with the observation that NR2B-containing NMDARs have a slower decay constant than NR2A-containing NMDARs (Monyer et al., 1994). Field-recordings in the CA1 of NR2B transgenic mice demonstrated a profound modification of the synaptic plasticity induced by stimulations at different frequencies. Stimulation at 10 Hz for 90 s and stimulation at 100 Hz for 1 s strongly potentiated the CA3-CA1 synapses in NR2B transgenic mice, while little potentiation was observed in wild-type mice. At the behavioural level, NR2B transgenic mice displayed improved learning on the Morris water maze and the fear extinction paradigm, and better retention on the novel object
recognition task and the contextual and cued fear-conditioning paradigms (Tang et al., 1999).

In NR2A -/- mice and NR2B transgenic mice, the intensity of NMDAR currents was altered due to a change in the number of NMDARs and a change in their channel opening properties. Both the stimulation intensity necessary to trigger LTP and the amount of training necessary for correct learning of a behavioural task were directly correlated to the amount of NMDAR activity. In NR2A -/- mice, which have a 50% decrease of NMDAR currents, the stimulation thresholds necessary to induce LTP and contextual learning were raised compared to controls. On the contrary, in NR2B transgenic mice, a weaker stimulus than in controls was sufficient to induce LTP and the performances in learning and memory tests were better than control mice. These results suggest a direct, quantitative relationship between NMDAR activity, synaptic plasticity and memory.

CA1-restricted NR1 knockout

The application of Cre-Lox gene knockout technology in the mouse brain has also aided the understanding of NMDA receptor function. The CaMKIIα promoter was used to drive expression of the Cre recombinase specifically in forebrain neurons. Because transgene integration site has a strong influence on transgene expression pattern, one mouse line expressed the Cre recombinase only in CA1 region of the hippocampus (Figure 5) (Tsien et al., 1996a). This mouse line was used to ablate a floxed Grin1 gene, resulting in a CA1-restricted NR1 knockout (CA1-NR1-KO). This localised depletion of NMDAR did not disturb the development of the brain and did not affect mouse health (Tsien et al., 1996b). However, the CA1-NR1-KO mice showed a significant deficit in spatial learning, as they were unable to develop a navigation strategy in the Morris water maze. This behavioural impairment was correlated with the absence of LTP in CA1 area, suggesting that NMDARs are necessary for CA1 pyramidal neurons to establish long lasting changes in synaptic strength and encode new spatial memories (Tsien et al., 1996b). This learning deficit was not restricted to encoding spatial information. It has been shown that rats with hippocampal lesions or treated with NMDAR antagonist are deficient in learning tasks that include delays of seconds to minutes between relevant events (Chiba et al., 1994; Jackson et al., 1998; Lyford et al., 1993; McEchron et al., 1998). Tested on the trace fear-conditioning paradigm, i.e. with a 30 s gap between
conditioned and unconditioned stimuli (CS and US, respectively), CA1-NR1-KO mice were slower than controls in acquiring the conditioning, but eventually reached the same conditioning level. The retention was severely impaired, as CA1-NR1-KO mice did not show any CS-induced freezing during the memory test 24 h after conditioning (Huerta et al., 2000). However, on the delay fear-conditioning paradigm, i.e. with overlapping CS and US, CA1-NR1-KO mice displayed normal conditioning, and memory for the CS was intact after 24 h retention (Huerta et al., 2000).

In vivo multi-electrode recording of place cells was also performed on CA1-NR1-KO mice. Despite the absence of NMDARs and the deficit in spatial learning, place cells were still present in the CA1 area (McHugh et al., 1996). However, the spatial specificity of place fields was altered, disrupting the correlated firing of CA1 cells. As CA1 region receives convergent inputs from several sources, which include the CA3 region of the hippocampus, entorhinal cortex, and subcortical modulatory areas (Figure 5), these observations suggested that NMDAR mediated plasticity may be required for heteroassociative memory function between these inputs (McHugh et al., 1996).

**Figure 5:** Basic anatomy of the hippocampus. The hippocampus forms a principally unidirectional network. Main inputs originate from the entorhinal cortex and form connections with granular neurons of the dentate gyrus (DG), and CA1 and CA3 pyramidal neurons via the perforant path (pp). In addition, CA3 neurons receive inputs from the DG via the mossy fibers (mf) and from neighbouring CA3 neurons (loop arrow). They send axons to CA1 pyramidal cells via the Schaffer collateral pathway (sc) as well as to CA1 cells in the contralateral hippocampus via the associational commissural pathway (ac). The main hippocampal output consists in the CA1 neurons sending axons to the subiculum (Sb), which in turn projects back to the entorhinal cortex.
CA3-restricted NR1 knockout

In the CA3 area, pyramidal cells project to CA1 via Schäffer collaterals and receive excitatory inputs from three sources: the mossy fibres of the dentate gyrus granule cells, the perforant path axons of the stellate cells in the superficial layers of the entorhinal cortex, and the recurrent collaterals of the CA3 pyramidal cells themselves, the latter of which are the most numerous type of input to the CA3 pyramidal cells (Figure 5) (Ishizuka et al., 1990). The prominence of these recurrent collaterals has led to suggestions that CA3 might serve as an associative memory network.

To test NMDAR function in this network, Nakazawa et al. generated a mouse line in which NR1 subunit was ablated specifically in the CA3 area. A large, 120 kbp long fragment of the 5’ untranslated region of the mouse kainate receptor subunit 1 (KA1) was used to drive expression of the Cre recombinase. One mouse line, in which Cre recombinase expression was restricted to CA3 pyramidal cells, was used to ablate the floxed Grin1 gene. As shown by in situ hybridisation and immunostaining, Grin1 deletion was restricted to CA3 pyramidal neurons but it was progressive and slow, as 18 weeks were necessary for the recombination to occur in almost all neurons (Nakazawa et al., 2002). Due to the restricted and late NR1 depletion, the CA3-NR1-KO cells in the CA3 area had normal cytoarchitecture, fibre projections, and dendritic structures. NMDAR mediated currents were abolished in the commissural/associational (C/A) synapses but were normal at the medial perforant path (MPP)-dentate gyrus (DG) synapses and at the Schäffer collateral (SC)-CA1 synapses. Thus, LTP was abolished at the C/A-CA3 synapses, but was normal at the MPP-DG and the SC-CA1 synapses (Nakazawa et al., 2002).

At the behavioural level, CA3-NR1-KO mice displayed normal learning ability on the Morris water maze, and normal memory for the platform position during a probe trial performed with all the visual cues used for training. However, when three out of the four visual cues had been removed for the probe trial, control mice maintained the ability to orientate themselves and exhibited nominal performance levels, while CA3-NR1-KO mice failed to precisely locate the phantom platform position (Nakazawa et al., 2002). Arguing that CA1 activity is more likely to reflect behavioural performance than CA3 activity, as the CA1 is the final output region of the hippocampus, Nakazawa et al. recorded the electrical activity of the CA1 in vivo. They found that partial cue removal did not affect place cell activity in control mice compared to the no-cue removal condition, whereas the mutant cells showed significant reductions in burst spike
frequency, place field size and integrated firing rate (Nakazawa et al., 2002). Memory recall almost always occurs from limited cues in real-life situations. Theoretical analyses proposed that a recurrent network with modifiable synaptic strength, such as that in hippocampal CA3 area, could provide the pattern completion ability necessary to retrieve the full-memory from limited cues (Hasselmo et al., 1995; Marr, 1971). The impairment exhibited by CA3-NR1-KO mice in recalling the spatial memory after partial cue removal provided a direct demonstration of a role for the CA3 region and CA3 NMDARs in pattern completion at the behavioural level (Nakazawa et al., 2002).

It was also proposed that a recurrent network with modifiable synaptic strength could support rapid acquisition of memories of one-time experiences (Marr, 1971; McClelland et al., 1995). Thus, Nakazawa et al. tested the CA3-NR1-KO mice in a delayed matching-to-place (DMP) version of the Morris water maze. In this test, the animal’s ability to acquire a novel location of the hidden platform with just one visiting trial is tested by its performance in the second trial conducted a few minutes later (Steele and Morris, 1999). CA3-NR1-KO mice were impaired in rapidly acquiring the memory of novel hidden platform locations, but were normal when tested with previously experienced platform locations. In addition, compared to controls, CA1 place cells in CA3-NR1-KO mice had place field size that were significantly larger in novel environments, but normal in familiar ones (Nakazawa et al., 2003). It suggested that NMDARs in the CA3 play a crucial role in rapid hippocampal encoding of novel information for fast learning of one-time experience.

To summarise, in both CA1- and CA3-NR1-KO mice, deletion of the NR1 subunit prevented the formation of functional NMDARs. Thus, NMDAR currents were absent in these structures and NMDAR-dependent processes like LTP were altered. CA1-NR1-KO mice could not learn the Morris water maze task, and displayed slower learning and impaired retention of trace fear-conditioning but had normal learning and retention of delay fear-conditioning. Place cells were detected in the CA1 but had reduced spatial specificity, disrupting correlated firing of CA1 cells. In the Morris water maze, CA3-NR1-KO mice could not retrieve the platform position upon partial cued condition but displayed normal abilities upon full cued condition. In addition, CA3-NR1-KO mice learned more slowly than controls new platform positions in the delayed matching-to-place paradigm. Finally, CA1 place cells of CA3-NR1-KO mice displayed reduced spatial specificity in a new environment but not in a familiar one.
Together, data acquired from the analysis of mice with highly specific spatial deletion of NR1 subunits demonstrate that the behavioural consequences are strictly correlated with the processing abilities of the neuronal circuitry in which the NMDAR blockade occurred.

2) NMDARs are necessary for memory storage

The function of NMDARs in synaptic plasticity and memory formation has been intensively investigated with multiple pharmacological and genetic approaches, both *in vitro* and *in vivo*. However, dissociating the mechanisms involved in learning from the ones involved in long-term persistence of memory is a technically challenging question. Hereafter, we present one elegant genetic study, which investigated the role of NMDARs in long-term memory storage.

*Inducible and reversible forebrain specific NR1 knockout*

To study the role of NMDARs in long-term memory storage, an interesting genetic combination was implemented that resulted in an inducible, reversible and cell-specific ablation of the NR1 subunit (Cui et al., 2004). A line of CaMKIIα-Cre transgenic mice was used to recombine the floxed Grin1 gene and stop NR1 expression in forebrain neurons. In the meantime, the endogenous NR1 subunit was replaced by an NR1-GFP fusion protein, whose expression was triggered by the tetracycline-controlled transactivator (tTA) system. Incorporation of doxycycline (dox), a more potent analogue of tetracycline, in food pellets, shut down expression of NR1-GFP, resulting in the complete absence of any NR1 subunit specifically in the cortex, striatum and hippocampus within 5 days. The expression of NR1-GFP could be restored within 2 months even after prolonged dox treatment (Cui et al., 2004). These mutant mice were trained on the fear-conditioning paradigm and the effect of transient absence of NR1 subunit on the stability of the formed memory was tested. The short transient absence of NR1 during the retention phase (dox administration during 7 days), did not affect the strength of contextual and cued fear memory, while a long (dox for 30 days) transient absence of NR1 severely disrupted both types of memory (Cui et al., 2004). A conceptual model to explain long-term stability of memory suggests that the substrate of memory is the potentiation of synaptic connections and that strongly connected neurons need to reactivate together regularly to remain strongly connected. Cui *et al.* proposed
the NMDAR reactivation to be the key synaptic reinforcement mechanism for dynamically and stably preserving long-term stability of the brain. Interestingly, this supports the hypothesis that similar mechanisms are used for memory formation and memory stability.

To summarise, the NR2A -/- and NR2B transgenic mouse studies illustrated on one hand how the intrinsic properties of NMDAR activity influence behaviour. The number of NMDARs was decreased in NR2A -/- mice, whereas the opening time constant of NMDARs was increased in NR2B transgenic mice. Most probably acting on the size of the Ca\(^{2+}\) flux following NMDAR activation, these modifications influenced in an opposing fashion the learning ability of the mutated mice. On the other hand, the CA1 and CA3 specific NR1 knockout studies illustrated how the extrinsic properties of NMDARs influence their biological function. The specific behavioural abilities altered in CA1- and CA3-NR1-KO mice were dependent on the information processing steps disrupted by NR1 depletion. Finally, memory stability was dependent on NMDAR activity. These observations suggest that any change in NMDAR activity would affect cognition. The localisation of the NMDAR dysfunction would determine which cognitive process is affected, and the type of NMDAR alteration would determine how this cognitive process is altered.
C- NMDAR HYPOFUNCTION IN MICE AND SCHIZOTYPAL BEHAVIOURS

As presented previously, glutamatergic hypofunction is one of the main hypotheses underlying the pathophysiology of schizophrenia. This conceptual framework originates from the similarities between the psychotic state induced by NMDAR antagonist drugs and the one displayed by patients with schizophrenia. As the acute pharmacological approach may not be sufficient to model a complex syndrome like schizophrenia, multiple studies investigated the influence of genetic alteration of NMDAR activity on behaviour. The present section aims at reviewing these studies.

1) NMDAR activity influences maternal behaviour

The asparagine residue in position 598 (N598) of NR1 subunit is a critical determinant for the key properties of NMDARs. It contributes to the narrow constriction of the channel pore (Wollmuth et al., 1996), and is necessary for high Ca$^{2+}$ permeability and voltage dependent Mg$^{2+}$ block (Burnashev et al., 1992). The exchange of asparagine 598 with glutamine (N598Q) in the NR1 subunit (NR1Q) dramatically alters NMDAR properties in vitro and in vivo. Recordings from coronal brain sections of P0 mice homozygous for the N598Q mutation (NR1Q/Q) revealed that mutated NMDARs displayed normal current amplitude, four fold reduction in Ca$^{2+}$ permeability, incomplete block by Mg$^{2+}$ at resting potential and partial Ca$^{2+}$ blockade at depolarising potentials (Single et al., 2000). In other words, after glutamate stimulation at depolarising potentials, the NMDAR-mediated influx of Ca$^{2+}$ and Na$^{2+}$ is reduced in this mutant and at resting potential Ca$^{2+}$ and Na$^{2+}$ ions are able to flow through the channel that is normally blocked by Mg$^{2+}$ in wild-type mice.

Mutant mice homozygous for the N598Q mutation (NR1Q/Q) die within an hour after birth due to severe respiratory distress. Assuming that all NMDARs are tetramers made of two NR1 and two NR2 subunits that assemble randomly (Behe et al., 1995), heterozygous mice for the N598Q mutation (NR1+$\ast$Q) have an heterogeneous population of NMDARs: 50 % of them would contain either two NR1$^{+}$ or two NR1Q subunits, and 50 % a combination of NR1$^{+}$ and NR1Q. NR1+$\ast$Q mice displayed normal NMDAR
current amplitude, normal voltage dependence and normal Mg$^{2+}$ block at resting potential, but there was a small shift in Ca$^{2+}$ reversal potential reflecting the 25% of NMDARs that contained two NR1Q subunits. This was sufficient to alter dramatically the maternal behaviour of NR1rQ mice. Pregnant NR1rQ females were hyperactive before delivery, could not build a nest, did not take care of the pups and displayed aggressive behaviours towards them. The litters were underfed and died within two days. The maternal behaviour of NR1rQ females did not improve after repeated breedings (Single et al., 2000).

2) NMDAR hypofunction

_Hypomorphic NR1 allele_

In vivo, the integration of a neomycin resistance gene into the intron 20 of the Grin1 gene had a strong negative influence on the expression level of the NR1 subunit. Mice homozygous for the neomycin insertion (NR1Neo/-) exhibited NR1 mRNA and protein levels reduced to 8% and 7% of the normal ones, respectively (Mohn et al., 1999). Surprisingly, this very low level of NR1 subunit was not lethal. NR1Neo/- mice were slightly smaller and lighter than control littermates but their weight was still in the normal range. Radioligand binding experiments of $^3$H-MK801 to membrane homogenates from the prefrontal cortex showed that in NR1Neo/- mice, the NMDAR binding capacity was reduced to 10% of wild-type levels (Mohn et al., 1999). Using similar ligand binding technique on brain sections, it was shown that the drastic reduction of NMDAR density occurred evenly in all brain structures, while no compensatory changes in non-NMDA ionotropic glutamate receptors were observed (Duncan et al., 2002; Duncan et al., 2004). In NR1Neo/- mice, the regional brain metabolic activity was reduced in some primary sensory and motor cortical areas, which exhibited altered laminar pattern of $^{14}$C-2deoxy-glucose uptake. The anatomically selective alterations in brain metabolism suggested a dissociation between thalamic inputs and cortical outputs (Duncan et al., 2002). However, the dopamine metabolism in the striatum of NR1Neo/- mice was normal, as was the extracellular dopamine levels in the striatum of freely moving mice (Mohn et al., 1999).

At the behavioural level, NR1Neo/- mice displayed an increase in locomotor activity and stereotypy during habituation to a new environment, which was very similar to that observed when control mice were administered the non-competitive NMDAR
antagonists phencyclidine (PCP) and MK-801. However, NR1Neo-/- mice were themselves resistant to such a PCP and MK-801 challenge, what was in agreement with their very low level of NMDAR (Duncan et al., 2002; Mohn et al., 1999). Accordingly, the regional brain metabolism of NR1Neo-/- mice was not altered by MK-801 challenge (Duncan et al., 2002). NR1Neo-/- mice displayed a major reduction in social contacts: they did not build a nest and avoided contacts with cage mates, they conducted reduced investigation and displayed no aggressive behaviour towards the intruder in the resident-intruder test, and the males lacked normal sexual behaviour. These behavioural alterations were normalised upon acute administration of typical and atypical antipsychotic drugs haloperidol and clozapine, respectively (Duncan et al., 2004; Mohn et al., 1999).

Considering the range of behavioural deficits observed in the NR1Neo-/- mice, and reversal by antipsychotic treatment, Mohn et al. proposed these mice to be a genetic model presenting some schizophrenia-like behaviours.

**NR2A knockout**

The first analysis of NR2A -/- mice revealed a 50 % reduction of NMDAR mediated currents in both CA1 pyramidal neurons (Sakimura et al., 1995) and granular cells of the cerebellum (Kadotani et al., 1996). A second study demonstrated that it was the stimulation threshold to induce LTP and not the maximum potentiation level that was altered in NR2A -/- mice (Kiyama et al., 1998). In a third study, ³H-MK801 binding assay and Ca²⁺ uptake assay in synaptosome preparation confirmed that NR2A -/- mice had a reduced amount of functional NMDARs (Miyamoto et al., 2001). Finally, quantitative autoradiography of ³H-MK801 binding demonstrated that the strong reduction of NMDAR density in NR2A -/- mice occurred in all brain structures expressing NR2A in wild-type mice (Miyamoto et al., 2004). These multiple assays permitted to conclude that in NR2A -/- mice, both the density and the activity of NMDARs are halved in the olfactory bulbs, frontal and parietal cortex, hippocampus, striatum and thalamus.

NR2A -/- mice exhibited increased NMDA-induced dopamine release in the striatum compared to wild-type mice, which was not augmented by the simultaneous blockade of GABA receptors. NMDA-induced serotonin release in the cortex was slightly increased, and NMDA-induced GABA release in the striatum was markedly reduced (Miyamoto et al., 2001). This indicated that the reduction of NMDAR activity
in NR2A -/- mice induced a deficit of GABAergic tone, and thus, an increase of dopaminergic and serotonergic neuronal activities. Previous pharmacological experiments demonstrated that locomotor activity is mainly regulated by the dopaminergic system, and that activation of this system induces hyperlocomotion (Gainetdinov et al., 1999; Giros et al., 1996). Accordingly, when placed in a novel environment, NR2A -/- mice displayed more novelty-induced hyperactivity than control mice. Consistently with their ability to antagonise the dopamine D2 receptor, haloperidol and risperidone were effective in attenuating hyperlocomotion of NR2A -/- mice at doses that did not affect locomotor activity in wild-type mice (Miyamoto et al., 2001). Furthermore, NR2A -/- mice were less sensitive than control littermates to the stimulating effects of a low dose (1 and 3 mg/kg) of the NMDAR antagonist phencyclidine (PCP), but this reduced sensitivity disappeared with administration of a higher dose (10mg/kg) of PCP (Miyamoto et al., 2004). As PCP is an open-channel blocker, this observation was consistent with the reduced density and activity of NMDARs in NR2A -/- mice. Tested on the Morris water maze, NR2A -/- mice were slower in learning the platform position although were able to finally complete the task. However, the precision of the memory for the platform position was significantly altered (Sakimura et al., 1995). Tested on the fear-conditioning paradigm, NR2A -/- mice displayed impaired contextual learning only when the duration of the exposure to the context was short, but not when it was long (Kiyama et al., 1998). Tested on the water-finding task, NR2A -/- mice had some deficits in latent learning, which is depending on selective attention during the training phase (Miyamoto et al., 2001).

To summarise, NR2A -/- mice possess hypofunction of the glutamatergic and GABAergic systems, and hyperactivity of the dopaminergic and serotonergic systems. NR2A -/- mice also displayed novelty-induced hyperactivity, which was reversed by antipsychotic drugs. Finally, they had some cognitive impairment, as revealed by deficits of spatial memory, contextual learning and latent learning. These mice are a very interesting model presenting good construct, face and predictive validity regarding the hypothesis of glutamatergic hypofunction in schizophrenia.

However, genetic animal models have intrinsic limitations in their ability to mimic complex environmental and polygenic brain disorders like schizophrenia. NR2A -/- mice were tested on the prepulse inhibition (PPI) paradigm, a test that assesses a sensorimotor gating deficiency commonly found in schizophrenic patients. All non-
competitive NMDAR antagonists like the psychotropic drugs PCP, MK-801 and ketamines induce strong deficit of PPI in humans and in animals. In an interesting study, Spooren et al. demonstrated that NR2A -/- mice, which have a 50 % reduction in NMDAR activity, had no deficit in PPI. Sensorimotor gating in NR2A -/- mice was disrupted only after acute administration of the selective NR2B antagonist Ro-63-1908, whereas Ro-63-1908 by itself was not able to disrupt PPI in wild-type control mice (Spooren et al., 2004). This demonstrated that the simultaneous inhibition of NR2A- and NR2B- containing NMDARs was necessary to disrupt PPI in mice. Considering that the vast majority of NMDARs in forebrain regions contain either of the NR2A or NR2B subunits (Kew et al., 1998; Monyer et al., 1994; Wenzel et al., 1997), and that glutamate signalling through either one of these NMDAR subtypes is sufficient for normal sensorimotor gating, Spooren et al. proposed that the critical factor for PPI disruption is the absolute degree of NMDAR inhibition rather than the receptor subtype (Spooren et al., 2004). Therefore, by selectively targeting NR2A, one can only ever achieve a maximal block of approximately 50 % of forebrain NMDARs, what is not sufficient to mimic the PPI deficit observed in schizophrenic patients.

**GlyB site point mutations**

Glycine is an obligatory co-agonist of glutamate for NMDAR activity. The glycine concentration in the cerebrospinal fluid lies in the micromolar range (Kemp and Leeson, 1993; McGale et al., 1977; Westergren et al., 1994), which should saturate the NMDAR glycine-binding site (GlyB), as its affinity for glycine varies between 100 and 300 nM depending on the NR2 subunit (Ikeda et al., 1992; Johnson and Ascher, 1992; Kutsuwada et al., 1992; Mayer et al., 1989). However, it was demonstrated using NFPS, a specific inhibitor of glycine transporter 1 (GLYT1), that synaptic glycine concentration is tightly regulated and does not saturate NMDARs (Bergeron et al., 1998). In addition, the existence of three populations of NMDARs differing by their affinity for glycine has been described in rat cortical neurons, and a time course analysis suggested that NR2B containing NMDARs have a much higher affinity for glycine than NR2A containing NMDARs (Kew et al., 1998). To investigate the modulatory function of synaptic glycine concentration in vivo, Kew et al. employed site-directed mutagenesis and homologous recombination to generate two mouse lines with point mutations in the GlyB site.
NR1-K483Q line

In one mouse line, the basic residue lysine in position 483 was replaced by the neutral residue glutamine (line NR1-K483Q). In vivo, this mutation resulted in a 85-fold decrease of GlyB site affinity, but the receptor glutamate affinity was not affected. Homozygous NR1-K483Q mice were obtained in Mendelian ratio, but were unable to feed and died within two days after birth (Kew et al., 2000). NR1-K483Q neonates did not exhibit respiratory distress as observed in NR1 -/- mice (Forrest et al., 1994; Li et al., 1994), but rather resembled the NR2B -/- mice, which lack the suckling response and do not feed (Kutsuwada et al., 1996).

NR1-D481N line

In a second mouse line, the acidic residue aspartate in position 481 was replaced by the neutral residue asparagine (line NR1-D481N). In vivo, this mutation resulted in a global fivefold reduction of GlyB site affinity, although the affinity for glutamate was not affected. Homozygous NR1-D481N mice were obtained in Mendelian ratio, were viable and fertile and had normal brain morphology (Kew et al., 2000). In NR1-D481N mice, the GlyB site of mutated NMDARs was less occupied by glycine, and theta burst-induced LTP in CA1 pyramidal neurons was markedly reduced compared to control mice.

At the behavioural level, NR1-D481N mice displayed normal horizontal and vertical activity in a new environment over an 8h period, but spent more time in the centre of the arena. In the light-dark test, there was a trend toward spending more time in the lit area (Kew et al., 2000). These two results indicated a reduced anxiety in NR1-D481N mice, what was in agreement with the studies reporting anxiolytic activity of GlyB site antagonists (Kotlinska and Liljequist, 1998; Matheus et al., 1994). In the Morris water maze, NR1-D481N mice learned the platform position more slowly than control mice, but a longer training compensated this deficit. Finally, in the prepulse inhibition (PPI) test, NR1-D481N mice had a normal level of PPI, but the startling threshold was decreased and the startling amplitude was increased by almost 70 % (Kew et al., 2000).
NR1-\textit{D481N} mice and NR2A -/- mice share some interesting analogies. Both mouse lines displayed a reduction in NMDAR activity, which was correlated with a reduction of LTP. In the Morris water maze, both NR1-\textit{D481N} mice and NR2A -/- mice learned more slowly than wild-type mice the platform position, but could eventually reach similar performance level. Finally, both NR1-\textit{D481N} mice and NR2A -/- mice had normal PPI level, in contrast with the reduction of PPI induced by pharmacological inhibition of NMDAR activity.

\textbf{NR1-\textit{D481N/K483Q} line}

To obtain a mouse model that would be viable but would show a stronger phenotype than the NR1-\textit{D481N} mice, heterozygous mutant mice bearing both point mutations of the NR1 glycine-binding site were generated (line NR1-\textit{D481N/K483Q}) (Ballard et al., 2002). In NR1-\textit{D481N/K483Q} mice, the NMDARs displayed a biphasic affinity for glycine that was in agreement with data from homozygous NR1-K483Q and NR1-\textit{D481N} mice. The amplitudes of the high and low affinity components were of 16 and 84 \%, respectively. The normal affinity for glutamate was preserved. Using the greased gap cortical wedge technique, Ballard et al. demonstrated that the GlyB site was close to saturation in control mice, whereas in NR1-\textit{D481N/K483Q} mice, a large population of mutated NMDARs did not bind glycine. Accordingly, both NMDAR currents and theta burst induced LTP in CA1 pyramidal neurons were severely reduced (more than 50 \%) in NR1-\textit{D481N/K483Q} mice, and were both normalised by the addition of 100 \(\mu\)M D-serine. Finally, the levels of dopamine and serotonin were elevated in the striatum, but not frontal cortex, of mutants relative to wild-type mice.

At the behavioural level, NR1-\textit{D481N/K483Q} mice survived but were 30 to 40 \% lighter than control mice and had severe self-inflicted wounds. They were hyper-reactive, had impaired grip strength and motor performance on a rotarod, and nest building was also severely impaired. NR1-\textit{D481N/K483Q} mice displayed non-habituating hyperactivity that persisted across repeated testing when they were tested once for 24h or placed repeatedly for 1h in a new environment. Here again, NR1-\textit{D481N/K483Q} mice had a normal level of PPI, but the startling threshold was decreased and the startling amplitude almost tripled. In the Morris water maze, the erratic behaviour of the mutant mice was so strong that they could not even learn to find the visible platform.
The effects of acute injection of pharmacological compounds were assessed on the hyperactivity and the stereotypy of the mutant mice. NR1-D481N/K483Q mice were not sensitive to the stimulating effects of the NMDAR antagonist MK801, whereas they were still sensitive to amphetamine. The typical antipsychotic haloperidol and the atypical antipsychotic clozapine had only a weak effect on mutant mice behaviour: at doses that induced a strong sedation in wild-type mice, the decrease of locomotor activity was not sufficient to be statistically significant, whereas the reduction of the stereotypy count was statistically significant. The selective 5-HT2A receptor antagonist M100907 and the benzodiazepine α1 subunit-selective agonist zolpidem had both no effect on the motor activity and the stereotypy of mutant mice.

**GLYT1 heterozygous knockout mice**

A different approach to analyse the modulatory effect of glycine on NMDAR activity and its consequences on behaviour was to generate mice knockout for the GLYT1 transporter. Homozygous deletion of Glyt1 gene is lethal at birth (Gabernet et al., 2005; Gomeza et al., 2003a; Tsai et al., 2004b), due to excessive glycineergic neurotransmission that caused hyperinhibition of the respiratory network and respiratory deficiency (Gomeza et al., 2003a). This observation demonstrated that during early postnatal life, GLYT1 activity is necessary to modulate glycine receptor activity in caudal regions of the central nervous system. Mice heterozygous for the Glyt1 deletion (Glyt1 +/- mice) could develop and breed normally, but glycine uptake in forebrain was reduced by 50% (Gomeza et al., 2003a; Tsai et al., 2004b). Interestingly, this decrease of glycine uptake resulted in an augmentation of NMDAR activity at CA1 pyramidal cells. Two different studies reported increased NMDA/AMPA ratio in CA1 pyramidal neurons from Glyt1 +/- mice compared to controls. 70% augmentation was measured in 129/SvEvTac adult Glyt1 +/- mice (12-13 week old) (Tsai et al., 2004b), and 36% increase was measured in C57Bl/6 young mice (17-24 day old) (Gabernet et al., 2005). In addition, the amplitude of AMPA mEPSCs was not modified, indicating that NMDAR activity was augmented in Glyt1 +/− mice (Gabernet et al., 2005). This increased activity could be explained by a saturation of the GlyB site, as application of 10 μM glycine or D-serine potentiated NMDARs response in wild-type neurons but not in Glyt1 +/- neurons (Tsai et al., 2004b).

At the behavioural level, Glyt1 +/- mice learned the platform position in the Morris water maze at the same speed than control mice. However, performances during probe trial indicated that Glyt1 +/- mice remembered better the platform position (Tsai
et al., 2004b). Spontaneous activity and habituation to a new environment, and level of prepulse inhibition were similar in Glyt1 +/- and control mice. However, Glyt1 +/- mice were more sensitive to a PPI disruption by MK801 than control mice, and more resistant to PPI disruption by amphetamine administration, but equally sensitive to the effect of these treatments on activity level (Tsai et al., 2004b).

These changes in behaviour were consistent with previously published data. First, mice overexpressing NR2B in forebrain neurons, thus with increased NMDAR activity, displayed similar improved memory for the platform position (Tang et al., 1999). Second, the increased sensitivity of Glyt1 +/- mice to MK801-induced PPI disruption was consistent with the fact that MK801 is an open channel blocker. Given that Glyt1 +/- mice displayed increased NMDAR activity, this would augment the frequency at which MK801 can block NMDARs. Third, the observation that Glyt1 +/- mice had increased glutamatergic tone and reduced sensitivity to amphetamine fits with the opposite observation that decreased NMDAR activity increased sensitivity to monoaminergic psychotropic drugs (Geyer et al., 2001).

Table 2: Summary of the genetic investigations of the role of NMDARs in some schizophrenia-like behaviours
5-HT, serotonin; CS, conditioning stimulus; Cx, cortex; DA, dopamine; FCx, frontal cortex; fEPSP, field excitatory post-synaptic potential; Hi, hippocampus; ICV, intra-cerebro-ventricular injection; I-O input-output relationship; LTP, long-term potentiation; MAP, metamphetamine; MOR, morphine; MWM, Morris water maze; NE, norepinephrine; PCP, phencyclidine; PPI, prepulse
## Part 1: Introduction

<table>
<thead>
<tr>
<th>GENES</th>
<th>REF.</th>
<th>MOLECULAR CHARACTERISATION</th>
<th>BEHAVIOUR</th>
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<tbody>
<tr>
<td>NR2A-/-</td>
<td>Sakamura et al. 1995</td>
<td>Normal fEPSP I-O in CA1</td>
<td>Developed and mated normally</td>
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<td>Normal paired-pulsed facilitation</td>
<td><em>“Jumpy”</em> mice</td>
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<td>Reduced NMDA/AMPA ratio (-50%)</td>
<td>Slower learning on MWM and less precise retention of platform position</td>
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<td>Reduced LTP (126% instead of 187%)</td>
<td>Seemed to have enhanced startle response</td>
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<td></td>
<td>Kiyama et al. 1998</td>
<td>Stronger stimulation restored normal LTP</td>
<td>Impaired contextual learning under weak CS</td>
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<td></td>
<td>Miyamoto et al. 2001</td>
<td>Reduced NMDAR currents at mossy fibres-granular cells synapses</td>
<td>Increased activity in new environment (during 1st h)</td>
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<td>Hyperactivity reversed by haloperidol and risperidone</td>
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<td>Reduced latent learning in the water finding task</td>
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<td>Miyamoto et al. 2004</td>
<td>Reduced NMDAR currents at mossy fibres-granular cells synapses</td>
<td>Less sensitive to PCP-induced hyperlocomotion</td>
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<td>Reduced LTP</td>
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<td>Increased DA and 5HT metabolism in FCx and St</td>
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<td>Decreased NE metabolism in Hi</td>
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<td>Increased NMDA-induced DArelease in St</td>
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<td>Decreased NMDA-induced GABA release in St</td>
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<tr>
<td>NR2A-/-</td>
<td>Kadotani et al. 1996</td>
<td>Reduced NMDAR currents at mossy fibres-granular cells synapses</td>
<td>Normal locomotor activity in open-field (3min test)</td>
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<td>Normal grip strength and righting reflex</td>
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<tr>
<td>NR1-K483Q</td>
<td>Kew et al. 2000</td>
<td>85x reduction in glycine affinity</td>
<td>Death within 48th after birth - no feeding</td>
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<td>More lighter at birth</td>
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<td>NR1-D481N</td>
<td>Kew et al. 2000</td>
<td>5x reduction in glycine affinity</td>
<td>More time spent in the cage centre (8th test)</td>
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<td>Reduced LTP</td>
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<td>Greater increase of NMDAR currents by D-serine</td>
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<td>Increased DA metabolism in St but normal in Cx</td>
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<td>Increased 5HT in St but normal in Cx</td>
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<tr>
<td>NR1-/-</td>
<td>Ballard et al. 2002</td>
<td>84% of low affinity for glycine (95x reduction)</td>
<td>Reduced weight and grip strength</td>
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<td>16% of high affinity (5x reduction)</td>
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<td>Almost no LTP</td>
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<td>Greater increase of NMDAR currents by D-serine</td>
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<td>Increased DA metabolism in St but normal in Cx</td>
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<td>Increased 5HT in St but normal in Cx</td>
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<td>Hyperactivity NOT decreased by clozapine</td>
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<td>Stereotopy decreased by haloperidol</td>
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<td>NR1-/-</td>
<td>Mohn et al. 1999</td>
<td>10% of normal NMDAR amount</td>
<td>Reduced weight</td>
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<td>Normal DA levels in St (?)</td>
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<td>Gly1 +/-</td>
<td>Tse et al. 2004</td>
<td>NMDAR currents not potentiated by glycine or D-serine</td>
<td>Normal learning on MWM</td>
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D DISCUSSION

The multiple mouse lines presented above exhibit an almost continuous range of NMDAR alteration, from the complete abolition to an increase in activity level, and form a very interesting ensemble to understand the relationship between NMDAR activity and behaviour. Altogether, consistent behavioural alterations were reported, suggesting a quantitative correlation between NMDAR activity and certain behavioural abilities.

1) NMDAR activity is necessary for survival

Complete abolition of NMDAR activity was observed in NR1 and NR2B knockout mice, and in NR1-K483Q mice. These mutations caused no embryonic lethality but prevented survival ex-utero: mutant mice died within a few hours after birth due to respiratory distress or feeding problems (Forrest et al., 1994; Kew et al., 2000; Kutsuwada et al., 1996; Li et al., 1994). In addition, NR1K- mice, which NMDARs are Ca\(^{2+}\) impermeable and insensitive to Mg\(^{2+}\) blockade, and NR1 Q/Q mice, which NMDARs are Ca\(^{2+}\) permeable at resting potential, displayed a similar rapid death after birth (Rudhard et al., 2003; Single et al., 2000). Interestingly, NR1Neo-/- mice, which had only 7% of normal NR1 protein amount, could develop and grow, suggesting that a very low level of NMDAR activity was sufficient for survival (Mohn et al., 1999).

2) NMDAR activity influences locomotor activity

The amount of locomotor activity seems directly correlated with NMDAR activity. In a familiar environment, mice with a mild reduction of NMDAR activity (NR2A -/-, NR1-D481N) displayed normal locomotor activity, while mice with a strong reduction of NMDAR activity (NR1-D481N/K483Q, NR1Neo-/-) displayed permanent locomotor hyperactivity (Ballard et al., 2002; Kew et al., 2000; Miyamoto et al., 2001; Mohn et al., 1999). However, mice with a mild reduction of NMDAR activity displayed more novelty-induced hyperactivity than control mice. For example, NR2A -/- mice displayed novelty-induced hyperactivity during the first hour of the test but not during the second hour (Miyamoto et al., 2001). This suggests that the effect of mild reduction of NMDAR activity on locomotion is visible only in a slightly stressful situation.
3) Drug sensitivity depends on NMDAR activity

In good agreement with their biochemical definition, sensitivity to NMDAR antagonists was directly correlated with the level of NMDAR activity. This is illustrated by NR2A -/- mice, which displayed less PCP-induced hyperactivity, less sensitisation to PCP and no PCP-induced place preference compared to control mice (Miyamoto et al., 2004). In addition, NR1-D481N/K483Q and NR1Neo-/- were no longer sensitive to PCP and MK801 administration (Ballard et al., 2002; Mohn et al., 1999). Conversely, Glyt1 +/- mice were more sensitive to MK801-induced PPI disruption (Tsai et al., 2004b).

In contrast, differences in the sensitivity to antipsychotic drugs were observed. Typical and atypical antipsychotics could reverse hyperactivity of NR2A -/- and NR1Neo-/- mice, but were almost without effect on the hyperlocomotion and stereotypy displayed by NR1-D481N/K483Q mice.

4) Learning ability and LTP are correlated with NMDAR activity

A positive correlation between NMDAR activity and learning ability was demonstrated by improved learning performances upon augmentation of NMDAR levels and decreased learning ability upon reduction of NMDAR level. In the Morris water maze, NR2A -/- and NR1-D481N mice needed more training than control mice to learn the platform position and searched less precisely the platform during probe trials (Kew et al., 2000; Sakimura et al., 1995). Conversely, Glyt1 +/- mice and NR2B overexpressing mice displayed improved learning ability (Tang et al., 1999; Tsai et al., 2004b). Interestingly, LTP amplitude was correlated with NMDAR activity in a similar way. NR2A -/- and NR1-D481N mice had reduced LTP, whereas LTP could be triggered more easily in NR2B overexpressing mice than in controls.

5) Startle response but not PPI is correlated with NMDAR activity

Contrasting with the well-established PCP-induced disruption of PPI, genetic decrease of NMDAR activity altered the startling threshold but not the PPI levels. Pharmacological inhibition of NMDAR activity with non-competitive antagonists such as PCP, MK801 and ketamines, is well known to decrease the level of PPI in mammalian species (including human-beings), without consistent effect on the startle response (Geyer et al., 2001). However, mice with genetic disruption of NMDAR
activity, namely NR2 -/-, NR1-D481N and NR1-D481N/K483Q mice, displayed increased amplitude of the startle response and decreased startling threshold, but normal PPI levels (Ballard et al., 2002; Kew et al., 2000; Spooren et al., 2004).

Spooren et al. suggested that the 50 % reduction of NMDAR activity in NR2 -/- mice was not sufficient to disrupt PPI. Indeed, NR2 -/- mice displayed reduced PPI only after acute administration of the selective NR2B antagonist Ro-63-1908, which by itself was not able to disrupt PPI in wild-type mice. This suggested that a larger reduction of NMDAR activity than the one obtained by NR2A deletion or NR2B inhibition was necessary to disrupt PPI (Spooren et al., 2004). However, the normal PPI levels displayed by NR1-D481N/K483Q mice, which NMDAR currents are almost abolished, challenge this hypothesis. We propose and discuss at the end of this dissertation that the duration rather than the amplitude of NMDAR inhibition may be a critical factor explaining the opposite effects observed upon long-lasting genetic versus acute pharmacological inhibition of NMDAR activity.

6) NMDAR hypofunction and disorganised behaviours

With regard to the hypothesis of NMDAR hypofunction in schizophrenia, it is of interest to consider not only the classical behavioural tests presented so far, but also the “unclassifiable” observations that were reported in the original papers. Many of them support the idea that strong NMDAR hypofunction induces a “disorganised” behaviour that may resemble a psychotic state. Thus, NR1Neo-/-, NR1-D481N/K483Q and NR1+/Q mice displayed no nest-building activity (Ballard et al., 2002; Mohn et al., 1999; Single et al., 2000). NR1+/Q mice did not have proper maternal behaviour, let the litters underfed and even displayed aggressive behaviours toward them, what eventually led the pups to death (Single et al., 2000). NR1-D481N/K483Q and NR2 -/- mice displayed a “jumpy” behaviour, occasionally jumping of the behavioural test, what might indicate hyper-reactivity to situations (Ballard et al., 2002; Sakimura et al., 1995). Finally, NR1Neo-/- mice displayed reduced interactions in the resident-intruder test and lacked normal sexual behaviour (Mohn et al., 1999). It would be of major interest to test whether, alike the social and sexual behaviour of NR1Neo-/- mice, these “disorganised” behaviours could be ameliorated by antipsychotic administration.

To summarise, the investigation of NMDAR function with a genetic approach revealed that the absence of NMDAR activity is lethal at birth, and that NMDAR
activity is required for correct somatosensory map formation, putatively via the same mechanisms than the ones involved in sprouting and spine formation. In addition, NMDAR activity is necessary for the synaptic plasticity mechanisms that sustain memory formation and maintenance. Finally, genetic reduction of NMDAR activity influenced mouse behaviour in a comparable but not identical manner as NMDAR antagonists. Hyperactivity, stereotypical behaviours and learning deficit resembled closely the ones observed upon acute administration of PCP, MK801 or ketamines. However, threshold and amplitude of the startle response and PPI levels were altered differently upon acute versus constitutive reduction of NMDAR activity. Collectively, these results suggest that NMDAR hypofunction could sustain at least some of the symptoms observed in patients with schizophrenia.

Investigating the consequences of genetic inhibition of NMDAR activity revealed the critical role that NMDARs have in multiple processes sustaining balanced brain functioning. It suggests that the endogenous processes that regulate NMDAR activity have themselves a major significance for cognitive functions and behaviour. One of such regulatory mechanism is mediated by the synaptic concentration of glycine. The major impact of glycine on NMDAR activity and behaviour has been demonstrated in vivo by genetic alteration of the binding affinity. However, little is known about the mechanisms controlling glycine concentration in vivo. It has been proposed that synaptic glycine concentration is set to sub-saturating level by an active process mediated by GLYT1. The aim of this thesis was thus to investigate how the activity of GLYT1 would affect synaptic glycine concentration, NMDAR activity and behaviour.

We elaborated a strategy set on a dual genetic approach to modulate GLYT1 activity in vivo. The first approach consisted in overexpressing GLYT1 specifically in forebrain neurons with a ligand-controlled gene expression system. We proposed that GLYT1 overexpression would increase glycine uptake, reduce synaptic glycine concentration and decrease NMDAR activity. Conversely, the second approach took advantage of heterozygous deletion of Glyt1 gene to reduce GLYT1 expression and glycine uptake, and increase glycine concentration and NMDAR activity. Finally, to improve control of gene expression in vivo, a mouse line expressing the rtTA2s-M2 factor in the brain was generated. This new mouse line allows efficient, rapid and reversible induction of gene expression specifically in forebrain neurons.
Part 2: Generation and characterisation of Glyt1 overexpressing mice
I- INTRODUCTION

Glutamate is the most important neurotransmitter in the brain where it mediates up to 60% of all synaptic neurotransmissions. Among the different types of glutamate receptors, the NMDARs are present in almost all glutamatergic synapses and have very specific properties conferring them a critical role in multiple brain processes. The most significant of their specific properties are the permeability to Ca2+, voltage-dependent Mg2+ blockade and activity regulation by glycine, a necessary coagonist. NMDARs are able to detect coincident neuronal activity and are strongly connected via their Ca2+ permeability to intracellular signalling. Thus, NMDARs play a major role in synaptic plasticity, supporting learning and memory and most cognitive processes, what suggested that NMDAR malfunction could underlie the pathophysiology of brain disorders. In this respect, the hypothesis of glutamatergic hypofunction has gained much favour as causative agent in schizophrenia. It was recently discovered that regulation of NMDAR activity could occur in vivo, by modulation of synaptic glycine concentration mediated by glycine transporters. To better understand this specific mechanism and its implication on cognitive processes and behaviour, we proposed to generate transgenic mice overexpressing the glycine transporter 1 (GLYT1), with a system allowing temporal and spatial control. This genetic manipulation was expected to reduce synaptic glycine concentration leading to a reduction in NMDAR activation. Glutamatergic hypoactivity in forebrain regions was predicted to mimic some of the mechanisms underlying the aetiology of schizophrenia.

To evaluate the impact of glycine-mediated modulation of NMDAR activity on behaviour without confounding effects on autonomic systems and locomotion, we restricted the genetic manipulation to forebrain structures. In addition, we used a ligand-controlled gene expression system to prove convincingly that the observed phenotype in adult mice is not due to developmental adaptation. The most effective tool to temporally and spatially regulate gene expression in vivo is the tetracycline-controlled transactivator system. It is based on regulatory elements of the Tn10 tetracycline resistance operon of Escherichia coli, in which the tetracycline repressor (tetR) negatively regulates transcription of resistance genes (Hillen and Berens, 1994). These regulatory elements were modified to generate a synthetic system allowing constitutive gene expression that is suppressed in presence of the antibiotic tetracycline. The tetR
was fused to the C-terminal domain of the transcription activator VP16 from Herpes Simplex Virus, known to be essential for transcription of immediate early viral genes (Triezenberg et al., 1988). It resulted in a hybrid tetracycline controlled transactivator (tTA) that, in absence of tetracycline, binds the tetracycline operator (tetO) and activates a minimal promoter (Gossen and Bujard, 1992). Activation of this promoter is prevented in presence of low concentrations of tetracycline or its analogue doxycycline (dox) (Figure 6). The tTA system was also named tet-Off, as it is constitutively active but switched off by administration of tetracycline.

The use of this technology involves two independent constructs, to have both the spatial specificity and the inducibility of transgene expression. One construct consists of a specific promoter upstream the tTA coding sequence and allows tTA expression in a desired cell type. The second construct is made of the gene of interest under control of the tetO promoter, which activation requires the tTA factor and is repressed by dox. To limit transgene expression to forebrain structures, we took advantage of a transgenic mouse line in which the CaMKIIα promoter drives the expression of the tTA factor (CaMKIIα-tTA). This mouse line was reported to induce transgene expression throughout the neocortex, hippocampus, amygdala and striatum (Mayford et al., 1996a). The second genetic construct consisted of the Glyt1a cDNA under the control of tetO promoter (tetO-Glyt1a). These two transgenic mouse lines were crossed to obtain double-transgenic mutant mice, which overexpressed GLYT1 specifically in forebrain neurons. In relation with the hypothesis of glutamatergic hypofunction in schizophrenia, these mutant mice were evaluated on a series of behavioural tests assessing exploratory activity, anxiety, learning and memory and sensorimotor gating abilities.

![Figure 6 Principle of gene expression regulation with the tTA system](image)

Expression of the tetracycline-controlled transactivator (tTA) is driven by a specific promoter, which restricts expression to the targeted organ(s) or cell type(s). tTA binds constitutively the tetracycline operator (tetO), activates the promoter and triggers the expression of target gene (ON state). In the presence of doxycycline, an analogue of tetracycline, tTA binding to tetO sequences is prevented, and the expression of target gene is silenced (OFF state).
II- MATERIAL AND METHODS

1) Cloning

Glyt1a cDNA was generously contributed by Dr. A. Huber and Dr. D. Boison. It was excised from the pBK-Glyt1a plasmid by digestion with EcoR1 and SacII, and blunted with the Klenow fragment of Taq DNA polymerase. Glyt1a cDNA fragment was separated from vector backbone by agarose gel electrophoresis and purified. The pMM400 plasmid, originally made by Dr. M. Mayford, contains the tetO promoter upstream of 5’ and 3’ synthetic regulatory sequences including intronic sequences that increase gene expression in transgenic mice (Choi et al., 1991). The pMM400 plasmid was open by digestion with EcoRV, dephosphorylated with calf intestinal phosphatase to prevent self-ligation, and purified. The blunt Glyt1a cDNA fragment was ligated into the EcoRV site of the pMM400 plasmid. The tetO-Glyt1 gene was excised by digestion with SfiI, separated from vector backbone by agarose gel electrophoresis and purified by electroelution (Elutrap, Schleicher and Schuell, GE).

2) Housing conditions

Mice were kept in a room with controlled temperature and humidity level, under inverted 12-hour light/dark cycle, lights on at 21h30. All mice were allowed access to food and water ad libitum. All experiments were conducted approximately at the same time each day, between 10h and 18h. Facility, experimenters and behavioural experiments were accredited by the Swiss veterinary office.

3) Genotyping PCR

Mice were identified by ear clipping, and genotyped by PCR. The HotSHOT method was used for rapid extraction of genomic DNA from tail biopsies (Truett et al., 2000). CaMKIIα-tTA and tetO-Glyt1 transgenes were detected simultaneously in a multiplex PCR, containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 10 mM of each primer, 10 mM dNPTs, 2.5 mM MgCl2, 0.001% gelatine, 0.5 u Taq polymerase and 1 μL of DNA sample, in a final reaction volume of 21 μL. Primers F1 5’-TGC CTT TCT...
CTC CAC AGG TGT CC and tet23 5'- GCG GAC CCA CTT TCA CAT TTA AG amplified a 200 bp fragment of the CaMKIIα-tTA transgene. Primers Fl and Glyt1α-2R 5’-AGG TGT GTT CCA GGG GTT GTT G amplified a 114 bp fragment of the tetO-Glyt1 transgene.

4) Southern blot

Genomic DNA was purified from mouse-tail biopsies by phenol-chloroform extraction followed by ethanol precipitation, dried, and dissolved in TE buffer (10 mM Tris-HCl pH8.3, EDTA 1mM). 20 μg of genomic DNA were digested with BamH1, and fractionated by 0.8% agarose gel electrophoresis. DNA was transferred onto Hybond N1 membrane (Amersham, Zurich, Switzerland) with a vacuum system. The membrane was hybridised with a 32P radiolabeled probe, and exposed to X-ray film with an intensifying screen at -80°C for 1 to 6 days.

5) Rotarod

A revolving rotarod apparatus (Ugo Basile, Varese, Italy) was used to measure the motor coordination of mice. Mice were placed on the rotating rod, which speed accelerated constantly from 4 to 40 rpm during the 5 min trial, and the latency to fall was recorded. Two measurements were done on two consecutive days. The mean latency to fall was calculated and group means were compared using unpaired t-test with a significance level of 0.05.

6) Open field

Exploratory locomotor activity was recorded in a square open field measuring 70x70x30 cm. The 10 min test was realised under dim-light condition (17 lux). An overhead CCD camera tracked the movement of the mice and sent the feed to a video-recorder and a computerised tracking system (Ethovision, Noldus, NE), which allowed analysis of the total distance moved, the speed of displacement and the time spent in each zone of the open field. Rearings were counted manually as the software did not count them with a sufficient reliability. Data were analysed using unpaired t-test. An effect was considered statistically significant when p<0.05.
7) Elevated plus maze

The elevated plus-maze consisted of two open (30x5x0.5 cm) and two closed (30x5x20 cm) arms emanating from a common central platform (5x5 cm) to form a plus shape. The entire apparatus was elevated 70 cm above the floor. The test was realised under dim red light condition (1 lux), and started by placing a mouse on the central platform of the maze facing an open arm. During the 5 min duration of the test, mouse behaviours were viewed with a top camera, and recorded on tape. The Ethovision (Noldus, NE) videotracking system was used to measure the total distance moved and the time spent in each arm of the maze. Entries and rearings were counted manually. Data were analysed using unpaired t-test. An effect was considered statistically significant when p<0.05.

8) NOR

Before training, mice were habituated to the arena (63x51x25 cm) and familiarised with the presence of objects in the arena. This habituation was done on two consecutive days with two sessions of 10 min duration each day. Mice were trained with 3 objects for 5 sessions, 5 min per session, at 5 min intertrial interval. Relocation of one of the three objects occurred between the third and the fourth training sessions. Mice readily noted such a spatial change in the position of one object. After 5 training sessions, a new object substituted a familiar one, and the mouse ability to discriminate the new object was tested after 5 min, 3 h or 24 h of retention. The exploration duration for each object was recorded and used to calculate the discrimination ratio (DR), which is the quotient of the time spent exploring the new object over the sum of the new object exploration and the mean exploration of the two familiar objects.

\[ DR = \frac{\text{new}}{\text{new} + \left(\frac{\text{familiar1} + \text{familiar2}}{2}\right)} \]

Thus, equal exploration of the three objects gives a DR=0.5, and preferential exploration of the new object gives a DR value between 0.5 and 1. To measure precisely the duration of object exploration, a videotracking system (Video-Track, ViewPoint,
FR) was used to detect the position of the mouse head only, and not the position of the whole body. In addition, a speed criterion was applied to exclude the fast locomotion from the slow exploration. The same group of mice was used for the three retention durations. Mice were trained with a new set of object each time and received a rest period of two weeks between trainings. Retention durations were distributed evenly over each training sessions to avoid potential confounding effects. Data were analysed using repeated-measures ANOVA with retention time as within-group factor. An effect was considered statistically significant when \( p < 0.05 \).

9) **Morris water maze**

The circular pool was 160 cm in diameter and 60 cm deep. It was filled with water at 22°-24°C supplemented with a non-toxic white paint that rendered the water opaque. An overhead CCD camera tracked the movement of the mice and sent the feed to a video-recorder and a computerised tracking system (Video-Track, ViewPoint, FR). The escape latency (platform search time), path length (the distance travelled to reach the platform) and swimming velocity were measured for each trial. Mice were trained to find a square platform (10 x 10 cm) hidden 1.5 cm below the surface of water and placed at the centre of a quadrant. The training was carried out with 3 trials per day for 9 days. At each trial, the mice were released from three pseudo-randomly assigned start locations and allowed to swim for 90 s. If a mouse did not find a platform within 90 s, it was manually guided to the platform and allowed to rest on the platform for 15 s. Performance during the learning phase was assessed by the latency to reach the platform. To evaluate the stability of the formed memory, four probe trials were performed respectively 1, 14, 29 and 42 days after the end of training. In probe trials, the mice were released at one of the three starting position, facing the wall of the pool, and were allowed to swim for 60 s in the absence of the platform. The percentage of time spent in the platform quadrant and the number of crossings over the phantom platform position were used as indicators of the memory strength. Data were analysed using repeated-measures ANOVA with training days or retention time as within-group factor. An effect was considered statistically significant when \( p < 0.05 \).

10) **PPI**

Two SR-Lab (San Diego Instruments, San Diego, CA) acoustic startle chambers were used in the present studies. Each system contains a Plexiglas cylinder mounted on
a platform with a piezoelectric accelerometer unit attached below the Plexiglas cylinder. The piezoelectric unit transduces vibrations into signals that are rectified and stored by a computer interface. The Plexiglas cylinder and platform are located in a sound-attenuated chamber with a loudspeaker, a light, and a ventilation system. SR-Lab software controlled the delivery of the stimuli to the animals and recorded the response. Before the first session of any day, the chambers were calibrated for both movement and sound to ensure across chambers reliability of the measurements. The background sound level was set-up at 68 dB. The startle-eliciting stimulus, also called pulse, was a white noise burst lasting 30 ms at 120 dB. The prepulses were white noise bursts of 20 ms duration at 72, 76, 80 and 84 dB. The peak amplitude of the startle response was measured as the highest value during a 65 ms time window beginning at the onset of the pulse. The three types of trials consisted in presentation of the pulse alone to measure the amplitude of the startle response, presentation of the prepulses alone to verify the absence of motor response, and presentation of the prepulse plus pulse with 100 ms onset interval to measure the prepulse-induced inhibition of the startle response. The test session consisted of a 5 min acclimation period, a starting series of five pulse presentations, 12 presentations of each trial in a pseudo-random order with an inter-trial interval random between 10 and 20 s, and a final series of five pulse presentation. The percentage of prepulse inhibition (PPI) was calculated as follow:

$$PPI = \left(1 - \frac{PP_{xx}}{P_{120}}\right) \times 100$$

$PP_{xx}$ is the mean startle intensity during prepulse plus pulse trials with 4 different prepulse intensities, and $P_{120}$ is the mean startle intensity upon pulse presentation. Thus, high PPI value indicates strong inhibition of the startle response during the prepulse plus pulse trials compared to presentation of the pulse alone. Conversely, low PPI value indicates poor prepulse-induced inhibition of the startle response, i.e. the startle response was similar with and without the prepulse. Data were analysed using repeated-measures ANOVA with the prepulse intensity as the within-group factor, followed when appropriate by post hoc analysis using the Student’s test. An effect was considered statistically significant when $p<0.05$. 
11) Gly uptake assay

Mouse brain samples were homogenised in 1ml of ice-cold isolation medium (0.33 M sucrose, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM HEPES-Tris pH 7.4) using a Dounce-type glass homogenizer. The homogenate was centrifuged (1000 g, 5 min) at 4°C, the pellet was saved for Western-blot, and the supernatant centrifuged at 17,000 g for 10 min. The resulting high-speed pellet (P2) was washed once and resuspended in modified Krebs-Henseleit medium (125 mM NaCl, 5 mM KCl, 2.7 mM CaCl2, 1.3 mM MgSO4, 10 mM glucose, 25 mM HEPES-Tris pH 7.4). Protein concentrations were determined using a protein assay system (Bio-Rad, München, Germany). 20 μL aliquots of the membrane suspension (equivalent to 30–50 μg of protein) were preincubated for 2 min at 37°C. Uptake was initiated by addition of 80 μL of a modified Krebs-Henseleit solution kept at 37°C containing 3H-glycine (2 μM) (1.52 GBq/ mol, Amersham, UK). After a 1 min incubation with gentle agitation, uptake was terminated by diluting the incubation mixture with 3 ml of modified Krebs-Henseleit medium kept at room temperature followed by rapid filtration through a moistened filter (SM 11106, 0.45 μm pore size) (Sartorius, Göttingen, Germany). Filters were rinsed twice with 3 ml of modified Krebs-Henseleit medium. All dilution, filtration, and washing procedures were performed within 20 s. Filters were dried and placed in microvials, and their radioactivity measured by scintillation spectrometry.

12) Western-blot analysis

The amount of GLYT1 protein was quantified by western-blot in the crude synaptosomal and cell-body fractions prepared for the glycine uptake assay. Protein concentration in the samples was determined with the Bio-Rad Bradford assay. For SDS-PAGE, samples containing 5 μg of protein were supplemented with one third of 4x gel loading buffer (200 mM Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 0.4% (w/v) Bromophenol blue, and 10% β-mercaptoethanol). SDS-PAGE on 10% gels was followed by electrotransfer onto polyvinylidene difluoride membrane (Hybond-P; Amersham Biosciences). Immunodetection was performed with primary antibodies used at the appropriate dilution: rabbit anti-GLYT1, 1:1000 (kind gift from Dr. D. Boison), mouse anti-β-actin, 1:1000 (Sigma). HRP-coupled anti-rabbit and anti-mouse secondary antibodies (Sigma) were used at the dilution 1:5000. Specific immunoreactivity was detected using enhanced chemiluminescence (ECL Plus; Amersham Biosciences).
III- RESULTS

1) Generation of tetO-Glyt1a transgenic mice

Mouse Glyt1a cDNA was generously contributed by Dr. A. Huber and Dr. D. Boison. It was flanked with synthetic regulatory sequences improving in vivo transcription and translation levels, as well as mRNA stability (Choi et al., 1991). Then, the construct was inserted downstream of the tetO promoter to generate the tetO-Glyt1a transgene (Figure 7a). A service laboratory at the university conducted the pronuclear injection of the DNA construct into fertilised oocytes to create pseudo-founders. Both male and female mice used as gamete donors for transgenesis were of a mixed C57BL/6J and DBA2 F1 background. Tail DNA samples from the pseudo-founders were analysed by PCR and Southern-blot (Figure 7b). Stable genomic integration of the tetO-Glyt1a transgene occurred in 3 out of 30 pseudo-founders (Figure 7c, d). These 3 founders were backcrossed to C57BL/6J mice and gave rise to 3 independent tetO-Glyt1a lines, numbered 876, 877, and 878.

Figure 7: Generation of the tetO-Glyt1a transgenic mouse lines
a) Cloning strategy: Glyt1a cDNA was excised with EcoR1 and SacI from the pBK-Glyt1a plasmid obtained from Dr. D. Boison, and blunted. This fragment was inserted into the EcoRV site of the pMM400 plasmid, which contained the 5' and 3' untranslated regions (UTR) and the tetO promoter. The tetO-Glyt1a transgene was excised with Not1, purified by electroelution, and given for pronuclear injection.
b) Positions of the F1 and Glyt1a-802R primers (arrowheads) used for genotyping by PCR. The thick bar shows the DNA probe used for Southern-blotting.
c) Determination of transgene integration by PCR. Only numbers of the transgenic founders are shown (6, 15, 20), plus the negative (-) and positive (+) controls. The fragment amplified with the F1 and Glyt1a-802R primers was 538 bp long.
d) Determination of transgene integration by Southern-blot. The first lane shows the DNA size marker. Lanes a and b contains 10 pg and 1 pg of pMM400-Glyt1 plasmid, respectively. Only numbers of the transgenic founders are shown (6, 15, 20). Partial degradation of the genomic DNA samples occurred, as smears and not bands were visible.
Part 2: Glyt1 overexpression

a 5'UTR, 324bp

7x tetO

Not1

EcoRV/blunt EcoRI

Glyt1a cDNA, 2340 bp

blunt SaclI/EcoRV

3'UTR, 1119bp

pMM400, 5636 bp

pMM400-Glyt1a 7976 bp

b

tetO

Glyt1a

Glyt1-802R

A+

538 bp

C

15 6 20

- +

D

bp

a b 6 15 20

10000 -

2000 -

1000 -

600 -

400 -
2) Characterisation of Glyt1a transgene expression

CaMKIIα-tTA and tetO-Glyt1a transgenic mice were crossed with each other to generate double-transgenic mice. Expression of Glyt1a transgene in double transgenic mice was determined by RT-PCR. Glyt1a transgene mRNA was detected in samples from line 878, but not from lines 876 and 877 (data not shown). In line 878, Glyt1a transgene mRNA was detected in cortical and hippocampal samples but not in cerebellar samples (Figure 8), what suggested that Glyt1a transgene expression was restricted to forebrain structures. This observation was in agreement with the endogenous CaMKIIα expression pattern (Bürgin et al., 1990; Herms et al., 1993). Thus, we selected the line 878 for characterisation of the phenotype at the behavioural level. For simplicity reasons, the double transgenic mice in which the CaMKIIα-tTA transgene drives the expression of the tetO-Glyt1a gene are named tTA-Glyt1 mice or mutant mice.

Figure 8: Expression of Glyt1a transgene mRNA in tTA-Glyt1 mice
a) Primers (arrowheads) used for RT-PCR spanned an intron-containing region. Thus, the fragment amplified from genomic DNA (791 bp) was longer than the one amplified from mRNA (561 bp).
b) RT-PCR showing expression of the Glyt1a transgene mRNA. Lanes contain DNA ladder (L), negative control (water) (-), positive control (genomic DNA) (+), and hippocampal (Hi) and cortical (Cx) cDNA samples from tTA-Glyt1 mice, line 878.
3) Behavioural characterisation of tTA-Glyt1 mice

a- Health, motor coordination and balance assessment

First, we assessed general health status and motor activities, which could interfere with further behavioural testing. tTA-Glyt1 mice showed normal body weight (Figure 9a, b), normal grooming and normal activity in the home cage, and their reaction to handling was similar to control littermates. In addition, gross neurological function was assessed by looking at stereotyped motor behaviours. Adult tTA-Glyt1 mice, males and females, displayed no obvious abnormalities when tested for bar gripping, limb extension, object sniffing and rearing. Motor coordination and balance were evaluated with the accelerating rotarod. Each mouse was placed on a rotating rod, whose rotation speed was increased from 4 to 40 revolutions per minute over a 5-min period, and the latency to fall was recorded. Adult tTA-Glyt1 mice, males and females, showed identical latency to fall as their control littermates (Figure 9c, d).

Thus, we concluded that tTA-Glyt1 mice were healthy, had normal gross neurological function and normal motor coordination and balance. To avoid the influence of the oestrous cycle on behaviour, the following tests were conducted with male mice only.
Figure 9: tTA-Glytl adult mice have normal weight and normal motor coordination.

a-b) Weight: Female and male adult tTA-Glytl mice had the same weight as their control littermates. Bars represent the mean weight ± SD a) females: controls n= 20, tTA-GlyT1 n=10, b) males: controls n=15, tTA-Glytl n=9.

c-d) Motor coordination: Performances on the accelerating rotarod were identical between control and tTA-Glytl mice. The latency to fall was measured on two consecutive trials. Bars represent the mean ± SD c) females: controls n= 12, tTA-Glytl n=6, d) males: controls n=13, tTA-Glytl n=9.
b- Motor activity and anxiety

tTA-Glyt1 mice were tested for general motor activity and anxiety in the open field and elevated plus maze.

In the open field, control and tTA-Glyt1 mice had the same level of horizontal motor activity, indicated by the total distance (d) moved (25 controls, d=6525 cm ±1279; 20 tTA-Glyt1, d=6971 cm ±1782; t(43)=-1; p=0.3) (Figure 10a). The vertical activity, indicated by the number of rearings (r), was also identical between control and tTA-Glyt1 mice (controls, r=107 ±30; tTA-Glyt1 r=102 ±23; t(43)=0.7; p=0.5) (Figure 10b). In addition, controls and tTA-Glyt1 mice spent the same amount of time (t) in the centre of the open field (expressed in percentage of the 10 min trial duration; controls, t=9.6 % ±4.4; tTA-Glyt1 t=8.2 % ±3.2; t(43)=1.2; p=0.2), which indicated equivalent anxiety levels (Figure 10c).

In the elevated plus maze, the total distance moved (d), the total number of entries (e) in closed and open arms (Figure 10d), and the number of rearings (r) were similar between controls and tTA-Glyt1 mice (22 controls, d=1314 cm ±216, e=15.5 ±4.4, r=18.0 ±5.4; 18 tTA-Glyt1, d=1319 cm ±206, e=14.6 ±3.9, r=17.1 ±4.9; all p≥0.5). In addition, the number of entries in the open arm (oe) (Figure 10e) and the time spent in open arms (t) (Figure 10f) were equivalent between controls and tTA-Glyt1 mice. The number of open arm entries is expressed in percentage of the total number of arm entries, and the duration in open arms is expressed in percentage of the 5 min test duration (22 controls oe=32 % ±14, t=29 % ±14; 18 tTA-Glyt1 oe=37 % ±10, t=30 % ±12; all p≥0.2).

These results indicate that the general motor activity and anxiety levels are similar between tTA-Glyt1 and control mice.
**III- Results**

Open field

a

![Graph showing distance moved](image)

b

![Graph showing total rearings](image)

c

![Graph showing time in centre](image)

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Elevated plus maze

d

![Graph showing total number of entries](image)

e

![Graph showing percent number of entries](image)

f

![Graph showing time in open arms](image)

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**Figure 10:** tTA-Glyt1 mice display normal levels of activity and of anxiety. 

a-c) Open field. (a) Total distance moved, (b) total number of rearings, and (c) time spent in the centre of the open field expressed as percentage of test duration (10 min.). Bars represent the mean ± SD for controls (white) n=25 and tTA-Glyt1 mice (black) n=20.


d-f) Elevated plus maze. (d) Total number of entries, (e) percent number of entries to open arms and (f) time spent in open arms as percentage of trial duration (5 min.). Bars represent the mean ± SD for controls (white) n=22 and tTA-Glyt1 mice (black) n=18.
c- Learning and memory

NMDAR activity is critical for many forms of synaptic plasticity, including long-term potentiation, long-term depression and depotentiation in the hippocampus. Accordingly, numerous studies revealed the importance of NMDAR for the formation of new memories and the stable storage of existing memory traces. Thus, we assessed the learning ability and the short- and long-term memory of the tTA-Glyt1 mice with two different behavioural paradigms, the Morris water maze and novel object recognition.

i- Morris water maze

Both controls and tTA-Glyt1 mice learned equally well to reach the platform position. The 9 days of training reduced significantly the latency to reach the platform (days, F(8, 200)=13.9 p<0.0001), and there was no difference between control and tTA-Glyt1 mice (genotype, F(1, 25)=2.6, p=0.12). In addition, there was no significant interaction between days and genotype, indicating similar learning efficiency between control and tTA-Glyt1 mice (days*genotype, F(8, 200)=0.40, p=0.9) (Figure 1 la).

To evaluate the stability of the formed memory, four probe trials were performed respectively 1, 14, 29 and 42 days after the end of training. In probe trials, mice were allowed to swim for 60 s in the absence of the platform. The percentage of time spent in the platform quadrant and the number of crossings over the phantom platform position were used as indicators of the memory strength. On the first day of probe trial, control and tTA-Glyt1 mice swam in the correct quadrant 46 % of the time, which was statistically different from the average time spent in each of the other quadrant (18 %) (quadrant, F(1,25)=91.6, p<0.0001). This confirmed that both control and tTA-Glyt1 mice could learn and remember the platform position. Furthermore, there was no difference between genotypes for the time (t) spent in the correct quadrant (16 controls t=43.5 % ±12.2, 11 tTA-Glyt1 t=49.7 % ±10.3; t(25)=1.37, p=0.18), the number of crossings (c) of the platform position (controls c=4.1 ±2.1, tTA-Glyt1 c=3.6 ±1.6, t(25)=0.58, p=0.57), and the latency (l) to swim over the platform position (controls l=11.1 s ±6.5, tTA-Glyt1 l=13.2 s ±11.1, t(25)=0.61, p=0.54).
Additional probe trials were done 14 days, 29 days and 42 days after the end of the learning period to assess memory decay. There was a significant decrease of the preference for the platform quadrant with time (retention time, \( F(3,75)=10.4, p<0.0001 \)), that was identical between controls and tTA-Glyt1 mice (genotype, \( F(1,25)=0.20, p=0.9 \)) (Figure 11b). Memory decay was also indicated by the decrease in the number of crossings of the platform position, and the increase in latency to swim over the platform position, for both controls and mutants (data not shown).

Thus, on the Morris water maze, tTA-Glyt1 mice have normal spatial learning ability and long-term memory. However, because other forms of memory and short-term memory may use different pathways and have a different sensitivity to GLYT1 overexpression, mice were tested on the novel object recognition task.

![Figure 11: tTA-Glyt1 mice display normal spatial learning and long-term memory in the Morris water maze.](image)

a) Learning curve. Mice were trained with 3 trials per day over 9 consecutive days. The mean daily latency to reach the platform (in s) is plotted over the training duration (in days). Controls \( n=16 \), tTA-Glyt1 \( n=11 \), Mean ± SEM.

b) Retention curve. The time spent in the platform quadrant is plotted over the retention time (in days), as a percentage of the total probe trial duration (60 s). The chance level, 25 % of the time in each quadrant, is used as baseline. Controls \( n=16 \), tTA-Glyt1 \( n=11 \), Mean ± SEM.
Part 2: Glyt1 overexpression

ii- Novel object recognition

The novel object recognition (NOR) task takes advantage of the novelty seeking behaviour of rodents to assess their ability to distinguish familiar and novel objects. This is typically observed as preferential exploration of the novel object over familiar objects. Because the ability to discriminate novel and familiar objects require intact memory, the NOR task allows assessment of the learning and memory functions. The duration of the training sessions (or trials) and the duration of the intertrial intervals (ITI) have a major influence on the strength of the formed memory (Genoux et al., 2002). Thus, mice were trained for 5 training sessions, 5 min per session at 5 min intertrial intervals, to avoid floor and ceiling effects.

Their ability to discriminate the novel object was evaluated after 5 min, 3 h or 24 h of retention. At every time point, control and tTA-Glyt1 mice recognised the novel object, as evidenced by a discrimination ratio value statistically different from 0.5, the value indicating equal exploration of the three objects (for 5 min, 3 h and 24 h, p<0.05), and the performance level was not influenced by the retention duration (retention, F(2, 22)=2.15, p=0.13). Overall, tTA-Glyt1 mice exhibited a tendency to discriminate more the new object than control mice (genotype, F(1,22)=3.61, p=0.07) (Figure 12b).

In addition, the training protocol included relocation of one object between the third and fourth training sessions. Assessment of exploratory behaviour during the fourth training session revealed that control and tTA-Glyt1a mice displayed a similar preference for the moved object, as indicated by a discrimination ratio value (DR) significantly larger than 0.5 (16 controls, DR=0.57 ±0.08, t(15)=3.48, p<0.01; 14 tTA-Glyt1a DR=0.58 ±0.07, t(13)=4.39, p<0.001), and by the absence of mean difference (Δ=-0.1, t(28)=0.45, p=0.65) (Figure 12a).

Thus, the ability of control and tTA-Glyt1 mice to recognise the novel object and the relocated object were similar, indicating that the challenged memory processes were not altered by GLYT1 overexpression. More generally, performances in the Morris water maze and the novel object recognition task suggest that tTA-Glyt1 mice have normal learning and memory abilities.
Figure 12: tTA-Glyt1 mice have normal short- and long-term memory for objects.

a) Experimental design: Mice were trained with 3 objects inside the arena for 5 times 5 min (grey boxes), with 5 min intertrial interval (ITI) (white boxes). One object was relocated between the 3rd and 4th training sessions (arrows), object exploration was quantified (black boxes) during the 4th training session, and training continued. After 5 min, 3 h or 24 h of retention and replacement of one object by a novel one, mice were placed back in the arena and exploratory behaviour was quantified.

b) Object relocation: Mice displayed preferential exploration of the moved object during the 4th training session. Bars represent the mean discrimination ratio ± SEM of triplicate measurements (once per retention time). Baseline at 0.5 represents the discrimination ratio value for equal exploration of the objects.

c) Object substitution: Mice displayed preferential exploration of the novel object after 5 min, 3 h and 24 h of retention. Each point represents the mean ±SEM for 17 controls (open circles) and 13 tTA-Glyt1 mice (black squares)
d- Sensorimotor gating ability

Pharmacological blockade of NMDAR activity is known to impair sensorimotor gating abilities, in a way that resembles the deficit displayed by patients with schizophrenia. Thus, to investigate the impact of GLYT1 overexpression on NMDAR activity and behaviour, we tested tTA-Glyt1 mice in the prepulse inhibition (PPI) test, a classical paradigm challenging sensorimotor gating abilities. The PPI test measures the ability of a low intensity auditory stimulus to decrease the motor response to a startling stimulus (ST), which was in our set-up a loud noise burst.

The mean amplitude of the startle response was identical between control and tTA-Glyt1 mice (25 controls and 20 mutants, 32 ST presentations, t(1438)=−1.36, p=0.18) (Figure 13a). In addition, no habituation to the startling stimulus was observed, as indicated by the stability of the startle response across the whole session duration (ST presentations, F(1, 31)=0.87, p=0.66) (Figure 13b).

Both control and tTA-Glyt1 mice displayed 30 to 70 % reduction of the startle response when a prepulse preceded presentation of the startling stimulus. The amount of PPI was correlated with prepulse intensity (prepulse intensity, F(3, 43)=106.6, p<0.0001), but not with GLYT1 overexpression (genotype, F(1, 43)=0.7, p=0.80) (Figure 13c), and there was no interaction between prepulse intensity and genotype (F(3, 129)=73.1, p=0.67) (Figure 13d). To exclude the possibility that motor responses interfere with PPI, we verified that the prepulses presented alone did not trigger measurable motor responses (data not shown).

Thus, adult male control and tTA-Glyt1 mice displayed similar startle response and similar PPI levels, indicating that the sensorimotor gating ability was not influenced by GLYT1 overexpression.
Figure 13: tTA-GlyT1 mice display normal startle response and PPI levels

(a, b) Startle response. (a) Mean amplitude of the startle response and (b) stability of the startle response upon repeated presentations of the startling stimulus (ST). (a) Bars represent the mean ±SEM response to 32 presentations of the startling stimulus alone. (b) Means ±SEM response to each ST presentation for 25 control mice (open circles) and 20 tTA-GlyT1 mice (black squares).

c, d) Prepulse inhibition (PPI). (c) Mean PPI and (d) effect of prepulse intensity on PPI levels. Prepulses (PP) of 20 ms duration and of 72 dB, 76 dB, 80 dB and 84 dB intensity (PP72, PP76, PP80, PP84) preceded the onset of the startling stimulus (120dB, 30 ms) by 100ms. Bars represent the mean ±SEM response to 12 presentations of each prepulse plus pulse combination in a pseudo-random order.
Part 2: Glyt1 overexpression

e- Conclusion for behavioural analysis

tTA-GlyT1 mice were tested for gross neurological deficits, motor coordination and balance abilities on the rotarod, locomotor activity and anxiety on the open field and elevated plus maze, learning and memory on the Morris water maze and novel object recognition test, and sensorimotor gating ability on the prepulse inhibition paradigm. In all of these tests, the behavioural responses were similar between tTA-Glyt1 mice and control littermates. It became, therefore, critical to evaluate the effect of Glyt1 transgene expression in forebrain neurons at the biochemical level.

4) Biochemical characterisation of tTA-Glyt1 mice

To investigate the effect of Glyt1 transgene expression at the biochemical levels, glycine uptake in forebrain synaptosomes and the amount of GLYT1 protein in diverse cellular fractions were measured.

a- Glycine uptake activity

To detect the changes in glycine uptake activity induced by Glyt1 transgene expression, synaptosomes from a 2 mm thick forebrain coronal section were prepared. Because there is a low abundance of endogenous GLYT1 in the forebrain (Figure 14a), the use of forebrain ensured a high probability of detecting glycine uptake changes due to the Glyt1 transgene expression.

Crude synaptosomes were prepared by a two-step process. Each forebrain sample was homogenised in a high-density physiological buffer, and spun at low speed to remove cell nuclei and debris. Then, supernatant was collected and spun at high speed to precipitate the membrane fraction enriched in synaptosomes that was used for the assay. After resuspension, aliquots of the crude synaptosome fraction were incubated with 3H-glycine at 37°C for 1 min to measure glycine incorporation, and GLYT1-specific glycine uptake was calculated by subtracting glycine uptake in the presence of sarcosine from the total glycine uptake.

All measurements were recorded in triplicates using 5 controls and 5 tTA-Glyt1 adult mice. No difference was observed between controls and tTA-Glyt1 mice, for total
Ill-Results

glycine uptake (controls: 28.5 ±4.1 pmol/min/µg, tTA-Glytl: 28.0 ±4.0 pmol/min/µg, t(8)=0.21, p=0.8), and sarcosine-inhibited glycine uptake (controls: 17.7 ±1.0 pmol/min/µg, tTA-Glytl: 16.8 ±1.7 pmol/min/µg, t(8)=1.08, p=0.31) (Figure 14b). Consequently, there was no genotype difference in GLYT1 specific uptake (controls: 10.8 ±3.2 pmol/min/µg, tTA-Glytl: 11.2 ±3.3 pmol/min/µg, t(8)=-0.20, p=0.8) (Figure 14c).

Therefore, expression of Glytl transgene mRNA in forebrain neurons did not increase glycine uptake in synaptosomes.

**Figure 14:** GLYT1 mediated glycine uptake is not increased in tTA-Glytl mice

a) Endogenous GLYT1 expression pattern in adult rat brain revealed by immunostaining (adapted from F. Jursky and N. Nelson, 1996). A 2mm thick slice of the forebrain, at the position indicated by the vertical bars, was used to prepare the crude synaptosome fraction used for the glycine uptake assay. It corresponds to an area of low endogenous GLYT1 expression to maximise the probability of detecting transgenic GLYT1 activity.

b) Total glycine uptake was measured in the absence (-) and in the presence (+) of sarcosine, a specific GLYT1 inhibitor. Bars represent the mean ±SEM of triplicate measurements for 5 controls (white bars) and 5 tTA-Glytl mice (black bars).

c) GLYT1 specific 3H-glycine uptake was calculated as the difference between total and sarcosine-inhibited glycine uptake.
b- GLYT1 protein detection

To delineate the cause of the lack of increased glycine transport activity in tTA-Glytl mice, the amount of GLYT1 protein was semi-quantified by Western-blot. Both the membrane and cell-body fractions prepared precedently were used for quantification purpose, to verify GLYT1 export to the membranes. Rabbit polyclonal antibody against GLYT1 was a kind gift from Dr. D. Boison (Gabernet et al., 2005). The same amount of total protein was loaded for each samples in 10 % SDS-PAGE, transferred to a membrane, and the signal intensity for GLYT1 was semi-quantified by chemiluminescence. In addition, to correct for possible variations in sample loading and uneven transfer, β-actin was also detected and quantified.

In the crude synaptosomal fraction prepared for the glycine uptake assay, the GLYT1/β-actin ratios (r) were the same between control and tTA-Glytl mice (5 controls, r=6.3 ±0.8; 5 tTA-Glytl mice, r=6.8 ±0.9, t(8)=−0.9, p=0.39) (Figure 15 a, b). A similar result was obtained with samples containing the cell-body fraction (5 controls, r=6.8 ±0.6; 5 tTA-Glytl mice, r=6.8 ±2.3, t(8)=−0.02, p=0.98) (Figure 15 c, d).

Thus, expression of Glytl transgene mRNA was not associated with a detectable increase in GLYT1 protein amount.

c- Conclusion for biochemical analysis

Despite expression of Glytl transgene mRNA, no increase in GLYT1 protein amount were detected by Western-blot in both the cell-body and crude synaptosome fractions of tTA-Glytl mice compared to control mice. This observation sustains the similar glycine uptake activity measured in tTA-Glytl and control mice.
Figure 15 GLYT1 protein amount is not increased in tTA-GlyT1 mice.
Semi-quantification of GLYT1 protein levels in crude synaptosome fractions (a, b) and in cell-body fractions (c, d), using Western-blot. Pictures of representative Western-blot for GLYT1 and β-actin (a, c), and semi-quantification of the signal intensity for GLYT1, normalised to β-actin. (b, d). Bars represent the means ± SEM for 5 controls (C) and 5 tTA-GlyT1 (T) mice. Both Western-blot and quantifications were done in duplicates.
IV DISCUSSION

To investigate glycine-mediated modulation of NMDARs, we generated a transgenic mouse line to overexpress GLYT1 in forebrain neurons. The proposed hypothesis was that increased GLYT1 activity would decrease both synaptic glycine concentration and NMDAR activation, and influence behaviour. tTA-Glyt1 mice were generated, and the expression of Glyt1 transgene mRNA was detected by RT-PCR in the cortex and hippocampus but not in the cerebellum. This expression profile was consistent with the endogenous expression pattern of CaMKIIα promoter, which was used to express tTA factor in forebrain neurons. Because we hypothesised a reduction of NMDAR activation, we tested tTA-Glyt1 mice on a series of behavioural tests designed to assess some of the symptoms frequently found in schizophrenic patients (activity, anxiety, sensorimotor gating), plus learning and memory. Motor activity and anxiety-related behaviours were tested on the rotarod, open-field and elevated plus-maze. Sensorimotor gating ability was evaluated on the prepulse inhibition paradigm. Two forms of learning and memory were assessed in the Morris water maze and the new object recognition task. tTA-Glyt1 mice behaved identically to their control littermates on all these tests. To understand this absence of phenotype, we undertook a detailed biochemical investigation. It revealed that synaptic glycine transport was not augmented in tTA-Glyt1 mice, and that the amount of GLYT1 protein was not increased in cell-body and synaptosomal fractions. The fact that neither the amount of GLYT1 protein nor GLYT1 activity were increased in the tTA-Glyt1 mice explained the normal behaviour of these mice. Thus, only one question remained open, regarding the discrepancy between transcription and translation of Glyt1 transgene.

We propose to discuss three different aspects of this issue. First, we will analyse the ability of RT-PCR and Western-blot to provide sensitive and quantitative information. Second, we will re-evaluate the decision to overexpress glial GLYT1 protein in neurons, instead of neuronal GLYT2. Finally, we will discuss a technical improvement that could ameliorate the outcome of other similar projects, and we will conclude with a proposal for a different approach to investigate the impact of GLYT1 activity on behaviour.
1) Discrepancy between transcription and translation of Glyt1 transgene

Expression of Glyt1 transgene mRNA was detected by RT-PCR in the cortex and hippocampus of tTA-Glyt1 mice, but neither the amount of GLYT1 protein nor GLYT1 activity were augmented. It suggests that Glyt1 transgene mRNA was not translated, supposedly because of expressing a glial protein in neurons. However, the methods used do not allow precise quantification neither of the mRNA nor of the protein amounts. Thus, the possibility of weak Glyt1 transgene transcription but normal mRNA translation cannot be excluded.

a- Absence of quantitative information about Glyt1 transgene mRNA

RT-PCR is a highly sensitive method but is very limited in terms of quantification because of the amplification step by PCR. With optimised conditions, very low initial amounts of target DNA can be successfully amplified, but a saturated non-exponential amplification rate is usually attained much before the end of the amplification phase, compensating the initial differences in starting material. In addition, there is basically no limitation in the sensitivity of this method, which is now frequently used to assess gene expression in single cells.

RT-PCR was used to screen the different tetO-Glyt1 lines for transgene expression, and to verify expression specificity for forebrain structures. RT-PCR was also used to verify the absence of expression in the control mice that had Glyt1 transgene but not CaMKIIα-tTA transgene. Finally, RT-PCR was used to demonstrate the silencing of expression upon doxycycline administration (data not shown). Thus, a robust and efficient RT-PCR was necessary in order to trust also the negative results. Due to the aforementioned limitations, these experiments did not provide any information about the amount of Glyt1 transgene mRNA. Therefore, the possibility that transgene transcription rate was very low cannot be excluded. The use of different methods, like Northern-blot analysis, RNase protection assay or “TaqMan” assay would have been necessary to obtain a quantitative information and verify the strength of transgene transcription.
Part 2: Glyt1 overexpression

b- Limits of western-blot quantification

In addition to demonstrating the expression of Glyt1 transgene by RT-PCR, Western-blot was used to semi-quantify the amount of GLYT1 protein. The mouse Glyt1 cDNA was used to generate Glyt1 transgene, but no tag was fused to the protein because it is very difficult to add a functional tag to a membrane protein without disrupting its activity. Thus, it was not possible to have an antibody specific for transgenic GLYT1 protein. Furthermore, endogenous GLYT1 is expressed at different levels in almost all brain structures including the hippocampus and the cortex. The absence of specific antibody and the basal expression of endogenous GLYT1 in the forebrain prevented the detection of transgenic GLYT1 alone, leaving only the possibility to compare signal intensities between controls and tTA-Glyt1 mice. However, Western-blot entails two signal amplification steps, which generate some variability and prevents precise quantification. Therefore, despite our results showing normal GLYT1 protein amount in the forebrain of tTA-Glyt1 mice, it cannot be excluded that there was a weak overexpression that could not be demonstrated with Western-blot.

Detection of Glyt1 transgene mRNA by RT-PCR and normal GLYT1 protein amount in tTA-Glyt1 mice suggested that no translation of Glyt1 transgene mRNA occurred. However, due to technical limitations, we could not exclude the possibility of a weak transcription of the transgene with normal translation of the mRNA, which would result in an increase of GLYT1 protein too low to be detectable using the available technique in our laboratory.

2) GLYT1 expression in forebrain neurons

The discrepancy between transcription and translation of Glyt1 transgene may be due to a neuron specific process regulating endogenous GLYT1 expression. We review the arguments sustaining this hypothesis.

GLYT1 and GLYT2 differ in their expression pattern. In adult mice and rats, in situ hybridisation and immunohistochemical studies revealed that strong GLYT2
IV- Discussion

expression occurred exclusively in the spinal cord and hindbrain (Borowsky et al., 1993; Guastella et al., 1992; Jursky and Nelson, 1995, 1996; Luque et al., 1995; Smith et al., 1992; Zafra et al., 1995a; Zafra et al., 1995b). Weaker expression was observed in the cerebellum, and there was no GLYT2 expression in the whole forebrain. Morphological evidences indicated that GLYT2 was expressed in neurons but not in glial cells. Thus, GLYT2 expression pattern was similar to the one of glycine receptor, suggesting that GLYT2 was involved in the termination of glycine inhibitory neurotransmission. This proposal was strengthen by observations at the microstructural level, which detected GLYT2 in processes with varicosities associated with glycine receptor (Jursky and Nelson, 1996; Spike et al., 1997). In addition, double-immunostaining experiments have shown a perfect co-localisation of GLYT2 and glycine immunoreactivities, indicating that GLYT2 is a reliable marker for glycinergic neurons (Poyatos et al, 1997). Finally, recent evidences obtained in knock-out mice confirmed the involvement of GLYT2 in glycinergic inhibitory neurotransmission (Gomeza et al., 2003b).

GLYT1 was also expressed at high level in the spinal cord and most hindbrain and midbrain structures, fibre tracts and olfactory bulbs (Adams et al., 1995; Borowsky et al., 1993; Guastella et al., 1992; Jursky and Nelson, 1996; Smith et al., 1992; Zafra et al., 1995a; Zafra et al., 1995b). This strong expression in caudal brain areas suggested that GLYT1 was also involved in the termination of glycine inhibitory neurotransmission. This function was demonstrated with the Glyt1 knock-out mice, in which the absence of GLYT1 activity caused lethal inhibition of the neuronal activity commanding inspiration (Gomeza et al., 2003a). In addition, GLYT1 expression was detected in the cerebral cortex and the hippocampus, areas that are devoid of glycinergic neurotransmission. A second function was proposed for GLYT1, which would act as a regulator of glycine concentration at synapses containing NMDAR (Guastella et al., 1992). This function was indeed demonstrated in vitro (Berger et al., 1998; Bergeron et al., 1998; Lim et al., 2004) and in vivo (Kinney et al., 2003), using GLYT1 inhibitors whose administration resulted in a potentiation of NMDAR activity.

Because GLYT1 but not GLYT2 is expressed in forebrain areas, and GLYT1 activity modulates NMDAR activity in vivo, we decided to overexpress GLYT1 in forebrain to study how the regulation of NMDAR activity impacts behaviour. However, the CaMKIIα-tTA system drives the expression of Glyt1 transgene in neurons only, whereas immunostaining experiments described GLYT1 expression in glial cells only. It suggests that cell-type specific regulatory processes could prevent translation of Glyt1 transgene mRNA in neurons, which could explain why the mRNA but not the protein
Glyt1 overexpression was detected in tTA-Glyt1 mice. This hypothesis is supported by the literature. In situ hybridization experiments supported a moderate to high expression of Glyt1 mRNA in neurons of the cortex, hippocampus, thalamus, hypothalamus and olfactory bulbs, indicating the existence of a neuronal form of GLYT1 (Borowsky et al., 1993; Smith et al., 1992; Zafira et al., 1995b). However, immunohistostaining experiments failed to detect this form of the protein. Antibodies against GLYT1 were not able to detect GLYT1 expression in neurons, except in the amacrine cells of the retina (Adams et al., 1995; Jursky and Nelson, 1996; Zafira et al., 1995a). This discrepancy between in situ hybridization and immunostainings about GLYT1 expression in forebrain neurons sustains the hypothesis that some cell-type specific regulatory processes prevent translation of the Glyt1 transgene mRNA in neurons.

3) Alternatives

Considering that endogenous GLYT1 expression occurs in forebrain neurons, another hypothesis was formulated to explain why transcription of Glyt1 transgene did not lead to an increase of GLYT1 protein. We propose the existence of a feedback mechanism, which would control the total amount of GLYT1 protein.

The tTA system used to drive transgene expression in forebrain neurons is constitutively active and repressed by doxycycline (Gossen and Bujard, 1992). In addition, prenatal transgene expression was already observed in some transgenic mice using the CaMKIIα promoter (Krestel et al., 2001). Thus, constitutive and early expression of Glyt1 transgene in tTA-Glyt1 mice could have triggered compensatory mechanisms during development. For example, methylation of foreign DNA sequences is a well-described process, which evolved to protect organisms against viral infections. However, this process is not originally the cause of a lack of Glyt1 transgene expression, because the mRNA was amplified by RT-PCR. Thus, a more likely scenario is that permanent expression of Glyt1 transgene could have been compensated by a down-regulation of endogenous GLYT1 expression, or by an accelerated turnover of the protein pool. To limit the chance of such compensation to occur, the use of an inducible expression system would be advantageous.
The rtTA system is a mutant form of the tTA factor, which is constitutively silent but activated by doxycycline. A transgenic mouse line expressing the rtTA factor under control of CaMKIIα promoter is available in our laboratory (Genoux et al., 2002; Malleret et al., 2001; Mansuy et al., 1998). Its use would allow quick induction of transgene expression in adulthood, which would presumably lower the risks of compensatory mechanisms. However, the rtTA system has some drawbacks also. Rapid degradation and alternative splicing of rtTA mRNA were reported, what resulted in a low expression level of the rtTA factor. In addition, a high amount of doxycycline is required to permit transgene expression, and its administration on a daily basis is both time-consuming and expensive. Finally, the expression level obtained with the rtTA factor is low (Giménez et al., 2004; Robertson et al., 2002; Wells et al., 1999). To ameliorate the properties and the usefulness of the rtTA system, an improved version was generated, the rtTA2S-M2, which has a coding sequence adapted to mammalian codon-usage, more stable mRNA sequence, and much higher affinity for doxycycline in vitro (Urlinger et al., 2000). Due to the limitations of the rtTA system, we decided to use the tTA factor to drive Glyt1 transgene expression. However, to benefit from the improved properties of the rtTA2S-M2 system, we generated a transgenic mouse line that expresses the rtTA2S-M2 in forebrain neurons. The generation of this mouse line and its characterization are reported in the third chapter.

Considering the problems encountered with this transgene expression approach, we devised an alternative strategy to investigate the influence of synaptic glycine concentration on NMDAR activity and behaviour. Gomeza et al. generated a knock-out allele of Glyt1 gene, and reported that heterozygous Glyt1 knock-out mice (Glyt1 +/-) developed normally but displayed a gene dosage effect on glycine transport (Gomeza et al., 2003a). This reduction of glycine transport suggested that synaptic glycine concentration and NMDAR activity would be increased in Glyt1 +/- mice. We proposed that these alterations would influence mouse behaviour, and started a collaboration to investigate this point. We report the behavioural characterisation of these Glyt1 +/- mice in the next chapter.
Part 3: Behavioural characterisation of mice with heterozygous deletion of Glyt1 gene
I- INTRODUCTION

Considering the difficulties encountered with the Glyt1 transgene expression strategy, we devised a new approach to investigate the impact of GLYT1-mediated modulation of NMDAR activation on behaviour. This new strategy is conceptually symmetrical to the overexpression approach, and consists in a genetic decrease of GLYT1 expression in heterozygous knock-out mice for Glyt1 gene.

Using a homologous recombination approach, Gomez et al. generated homozygous mice for Glyt1 gene disruption (Glyt1 +/- mice), which developed normally in utero but died within a few hours after birth (Gomez et al., 2003a). Glyt1 gene disruption prevented GLYT1 protein synthesis and abolished GLYT1 mediated glycine transport (Figure 16 b). Newborn Glyt1 +/- mice could not move nor feed and assumed an abnormal body posture (Figure 16 a). Mutant pups died of suffocation, as indicated by the dramatic decrease in respiratory rate (Figure 16 c). However, anatomical development of Glyt1 +/- mice was normal, as were the expression level and localisation of major synaptic proteins. Electrical activity of the pre-Bötzinger complex was recorded in transversal slices of the caudal medulla to observe in vitro respiratory activity. A dramatic reduction of burst frequency, which could be reversed by strychnine application, was observed in Glyt1 +/- mice compared to wild-type mice. In addition, patch-clamp recordings from hypoglossal motoneurons revealed that glycine receptor mediated inhibitory currents were more frequent and had a longer decay time constant. These results suggested that the suppression of respiratory activity seen in Glyt1 +/- mice was due to increased accumulation of extracellular glycine, leading to sustained activation of inhibitory glycine receptors (Gomez et al., 2003a). In addition, these results demonstrated the critical role of GLYT1 activity for the termination of glycineergic neurotransmission.

Glyt1 gene disruption is not a dominant mutation. Mice heterozygous for Glyt1 gene deletion (Glyt1 +/- mice) were viable (Figure 16 a), but displayed a gene-dosage effect on GLYT1 expression level causing a 50 % reduction of GLYT1-mediated glycine uptake in the frontal brain, brain stem and spinal cord (Figure 16 b) (Gomez et al., 2003a). Despite this reduction of glycine transport, the respiratory frequency was not altered in Glyt1 +/- mice compared to wild-type mice (Figure 16 c). Interestingly,
Glyt1 +/- mice developed normally, were able to mate and displayed no overt behavioural phenotype (Gomeza et al., 2003a). It was shown that pharmacological inhibition of GLYT1 activity increased NMDAR activity in vivo (Chen et al., 2003; Lim et al., 2004) and had an “antipsychotic-like” effect on behaviour (Kinney et al., 2003). Thus, Glyt1 +/- mice were an elegant model to test the impact of genetic long-term reduction of GLYT1 activity on behaviour. A group of young adult male Glyt1 +/- mice and their wild-type littermates was obtained by collaboration. Because modulation of NMDAR activity has been shown to influence activity and anxiety levels and sensorimotor gating ability, the behaviour of Glyt1 +/- mice was assessed in the open field, elevated plus maze, home cage and prepulse inhibition tests.

Figure 16 Glyt1 knockout mice
a) Compared to wild-type (+/+), heterozygous (+/-) and homozygous mutant animals (-/-) did not move spontaneously, had no milk in their belly and assumed an abnormal body posture with dropping forelimbs.

b) 3H-glycine uptake in different CNS regions of newborn mice. Data are given as means ± SD (n=3-4 for each genotype). Asterisk indicates significantly different from wild-type mice, p<0.001 (Student’s t test).

c) Mean respiratory frequency, measured by whole-body plethysmographic recordings. Data are presented as means ± SD (n=18-38 for each genotype). Asterisk indicates significantly different from wild-type mice, p<0.001 (Student’s t test).

Adapted from Gomeza et al., 2003
II MATERIAL AND METHODS

For description of mouse maintenance conditions, and open field, elevated plus maze and prepulse inhibition tests, please refer to the corresponding sections in previous chapter (p 59).

Home cage activity

To measure home cage activity, mice were isolated in clean cages and transported to the test room, in which temperature, humidity and light-dark cycle conditions were maintained similar to the mouse facility. After a 24 h habituation period and during 24 h, an overhead CCD camera tracked the movement of the mice through the cage’s lid and sent the feed to a computerised video-tracking system (Ethovision, Noldus, NE), which calculated the distance moved twice per second. During dark phase, a very dim red light (<1 lux) was used to permit mouse tracking. No entries in the test room were allowed during habituation and testing periods. The total distance travelled in 24 h was analysed with unpaired t test. To observe the variations of activity levels during 24 h, the distance travelled was calculated per bin of 10 min and was analysed with an ANOVA conducted with genotype (mutants vs. controls) as between-subject factor and time (bin of 10 min) as repeated factor.
III RESULTS

Given that in vivo pharmacological inhibition of GLYT1 potentiated NMDAR activity and modified sensorimotor gating ability (Chen et al., 2003; Kinney et al., 2003; Lim et al., 2004), we suggested that the genetic decrease of GLYT1 activity in Glyt1 +/- mice would result in an increased synaptic glycine concentration that would augment NMDAR activity and influence behaviour. The level of NMDAR activity has been shown to influence locomotor activity, anxiety levels and sensorimotor gating ability, thus, the behaviour of Glyt1 +/- mice was assessed in the open field, elevated plus maze, home cage and prepulse inhibition tests.

1) Motor activity and anxiety

Glyt1 +/- mice were tested for motor activity and anxiety in the open field and elevated plus maze.

In the open field, the horizontal locomotor activity displayed by Glyt1 +/- mice was significantly increased compared to their control littermates, but the vertical activity and time in centre were similar to controls. During the 10 min test duration, the total distance (d) travelled by Glyt1 +/- mice was 17 % longer than controls (20 controls, d=5306 cm ±1159; 17 Glyt1 +/- mice, d=6224 cm ±1560; t(35)=-2.05, p<0.05) (Figure 17 a). However, the number of rearings (r) was identical in both groups (19 controls, r=76 ±18; 15 Glyt1 +/- mice, r=80 ±22; t(32)=-0.57, p=0.6) (Figure 17 b). Controls and Glyt1 +/- mice stayed for the same duration (t) in the centre of the open-field (expressed in percentage of test duration) (20 controls, t=5.4 % ±2.3; 17 Glyt1 +/- mice, t=6.2 % ±2.6; t(35)=-1.05, p=0.3) (Figure 17 c).

In the elevated plus maze, Glyt1 +/- mice displayed a trend toward reduced horizontal locomotor activity compared to controls, but vertical activity, number of entries and duration in open arms did not differ from controls. The total distance (d) travelled by Glyt1 +/- mice was 12 % less than controls (21 controls, d=1054 cm ±184; 15 Glyt1 +/- mice, d=932 cm ±172; t(34)=2.01, p=0.05) (Figure 17 d). Accordingly, Glyt1 +/- mice did less entries (n) in closed and open arms (21 controls, n=12.1 ±4.4; 15 Glyt1 +/- mice, n=8.3 ±4.8; t(34)=2.04, p=0.05). However, the number of rearings (r) was similar for both groups (21 controls, r=13.4 ±4.5; 15 Glyt1 +/- mice, r=12.4 ±4.9;
Finally, the percentage of entries (p) and time (t) in open arms (t) were similar between controls and Glyt1 +/- mice (21 controls, \(p=19.0\% \pm 13.5\) and \(t=9.1\% \pm 7.4\); 15 Glyt1 +/- mice, \(p=18.5\% \pm 15.8\) and \(t=8.6 \pm 12.5\)) (Figure 17 e, f).

Surprisingly, Glyt1 +/- mice did not display consistent levels of horizontal activity in the open field and elevated plus maze. A small increase (17\%) of horizontal activity was observed in the open field compared to controls, whereas a small decrease (12\%) was observed in the elevated plus maze. However, vertical activity, indicated by the number of rearings, was similar between controls and Glyt1 +/- mice on both tests, suggesting that the changes in activity level were limited. Anxiety level, indicated by the time spent in the centre of the open field and the number of entries in the open arms, was similar between controls and Glyt1 +/- mice in both tests.

**Figure 17:** Glyt1 +/- mice display normal level of anxiety

(a-c) Open field test. (a) Total distance moved, (b) number of rearings, and (c) time spent in the centre of the arena expressed as percentage of test duration (10 min). Bars represent the means \(\pm\) SD for controls (white, +/+ \(n=20\) and Glyt1 +/- mice (black, +/- \(n=17\)).

(d-f) Elevated plus maze test. (d) Total number of entries, (e) percentage of entries done in the open arms, and (f) time spent in the open arms as percentage of trial duration (5 min). Bars represent the means \(\pm\) SD for controls (white, +/+ \(n=21\) and GlyT1 +/- mice (black, +/- \(n=15\)).
2) Motor activity and circadian cycle

In both the open field and elevated plus maze tests, locomotor activity differed between control and Glyt1 +/- mice, but the variations were not consistent. To complement these observations with recordings in a non-stressful situation over a longer time-period, locomotor activity was assessed in the home-cage for 24 h. Each mouse was isolated in a cage and given 24 h to habituate to the new environment before the activity was recorded. For analysis, the total distance travelled per 10 min was calculated and plotted over the 24 h recording.

In this non-stressful situation, the total distance (d) moved in 24 h was similar between controls and Glyt1 +/- mice (16 controls, d=280 m ±126; 13 Glyt1 +/- mice, d=314 m ±117, t(27)=-0.8, p=0.5). The effect of circadian cycle was clearly recognisable, with a low level of activity during light phase (21:30 - 9:30) and a high level of activity during dark phase (9:30 - 21:30), and influenced activity level significantly (time, F(1,128)=8.8, p<0.0001). Glyt1 +/- mice displayed a circadian cycle identical to the one of control mice (time*genotype interaction, p=0.6) (Figure 18). Sleep phases could be visually recognised and no difference between controls and Glyt1 +/- mice was visible.

Thus, in the open field, elevated plus maze and home-cage, Glyt1 +/- mice displayed increased, decreased and similar locomotor activity levels compared to control mice, respectively.

Figure 18: Home cage activity
Horizontal locomotor activity in the home-cage over a 24 h period. For this test, mice were isolated and given 24 h to habituate to the new environment. Activity was recorded with an overhead camera. Light shifts were automatic. A photographic bulb was used to produce a dim red light (<1 lux) for recording during the dark phase. The total distance moved was plotted per bin of 10 min in order to smoothen the activity curve while keeping a certain level of precision. Each point represents the mean ±SEM for 16 controls (+/+, open circles) or 13 Glyt1 +/- (black squares) adult male mice.
3) Sensorimotor gating ability

The prepulse inhibition paradigm was used to assess the influence of heterozygous Glyt1 deletion on sensorimotor gating ability.

The startling stimulus (ST), a loud noise burst, was presented 22 times randomly during the PPI testing session to measure the amplitude of the startle response. The mean amplitude of the startle response (s, in arbitrary units) was identical between control and Glyt1 +/- mice (19 controls $s=602 \pm 240$, and 14 GlyT1 +/- mice $s=609 \pm 283$, 22 ST presentations, $F(1, 20)= 0.01$, $p=0.9$), and no habituation to the startling stimulus was observed (ST presentations, $F(1, 20)=1.14$, $p=0.3$) (Figure 19 b).

Based on published data, four different prepulse intensities were used, i.e. 72, 76, 80 and 84 dB, that were respectively 4, 8, 12 and 16 dB above the background noise (Geyer, 1999). It was noticed that the prepulse at 84 dB could trigger a motor response (data not shown). To avoid any bias in the results, all measurements done with this prepulse intensity were excluded from the analysis. The other prepulse intensities did not trigger any motor response.

Depending on prepulse intensity, control mice displayed from 26 % to 53 % PPI, and Glyt1 +/- mice displayed from 12 % to 41 % PPI, which was significantly less than controls (genotype, $F(1, 31)=4.4$, $p<0.05$). There was no interaction between prepulse intensity and genotype (prepulses*genotype, $F(2, 31)=0.5$, $p=0.6$).
The measurement of startle response intensity is depending on the weight of the animal. Mice were weighted immediately before testing, and control and Glyt1 +/- mice displayed similar weight (18 controls w=35.0 ±4.3 g, 13 Glyt1 +/- mice w=36.8 ±4.2 g, t(29)=-1.17, p=0.2). Therefore, we can exclude the possibility that PPI levels were biased by a weight difference between control and Glyt1 +/- mice.

Thus, in the prepulse inhibition test, control and Glyt1 +/- mice displayed similar startle response, but the PPI levels of Glyt1 +/- mice were on average 38 % lower than control’s one, indicating a reduction of sensorimotor gating ability in Glyt1 +/- mice.

**Figure 19**: Glyt1 +/- mice have a deficit of prepulse inhibition

a, b) Startle response. (a) Mean startle amplitude and (b) stability of the startle response upon repeated presentations of the startling stimulus (ST). (a) Bars represent the mean ± SEM intensity of the startle response to 22 presentations of the startling stimulus, for 19 controls (+/+)) and 14 Glyt1 +/- mice. (b) Means ± SEM responses to each of the 22 ST presentations.

b, d) Prepulse inhibition (PPI). (c) Mean PPI and (d) effect of the prepulse intensity on PPI levels. Prepulses (PP) of 20 ms duration, at an intensity of 72 dB, 76 dB and 80 dB (PP72, PP76, PP80) preceded the onset of the startling stimulus (120dB, 30 ms) by 100ms. Bars represent the means ± SEM of 12 presentations of each prepulse intensity.
IV DISCUSSION

We took advantage of the knock-out allele of Glyt1 gene that was generated by Gomeza et al. to investigate how permanent reduction of glycine transport activity influenced the behaviour of adult male mice. Because GLYT1 activity modulates NMDAR activity *in vivo* (Chen et al., 2003; Kinney et al., 2003; Lim et al., 2004), Glyt1 +/- mice were tested for locomotor activity, anxiety levels and sensorimotor gating ability. The locomotor activity displayed by Glyt1 +/- mice was significantly increased in the open field compared to wild-type mice, but significantly decreased in the elevated plus maze, and normal in the home cage. The anxiety level, evaluated in the open field and elevated plus maze, did not differ between control and Glyt1 +/- mice. Finally, Glyt1 +/- mice displayed on average a significant 38 % decrease of PPI.

1) Locomotor activity level

The horizontal distance travelled by Glyt1 +/- mice was significantly increased by 17 % compared to control mice in the open field, but significantly decreased by 12 % in the elevated plus maze, and similar to controls in the home cage. In addition, the vertical activity, indicated by the number of rearings, was similar between control and Glyt1 +/- mice in both the open field and the elevated plus maze.

The difference between the activity levels observed in the home cage on one hand, and in the open field and elevated plus maze on the other hand, are most probably due to differences in stress level and test duration, and are not surprising. Because mice are forced to enter the open field and elevated plus maze and cannot escape from the arenas, these tests are highly stressful. In addition, test durations are short, 10 min in open field and 5 min in elevated plus maze. Thus, both tests quantify novelty-induced exploratory behaviour in stressful conditions. Contrarily, in the home cage assessment, mice were given 24 h to habituate to isolation and new environment, and no mouse handling occurred before locomotor activity was quantified during a 24 h period. Thus, conditions in the home cage were not novel and not stressful compared to the open field and elevated plus maze. Finally, reports of novelty-induced locomotor activity
differences are usual. For example, in an open field test of 2 h duration, NR2A knock¬
out mice displayed increased locomotor activity compared to controls during the first
hour but not during the second hour of testing, indicating that hyperactivity was novelty-
dependent (Miyamoto et al., 2001).

It is, however, not usual to observe opposite locomotor activity levels in the open
field and elevated plus maze. Due to its elevation and narrowness, the elevated plus maze is more stressful than the open field, and it could be argued that the stress response
was altered in Glyt1 +/- mice, leading to a decrease of locomotor activity. However, the
similar levels of anxiety related behaviours between control and Glyt1 +/- mice on both
tests do not support this hypothesis. In addition, although the differences of locomotor
activity between control and Glyt1 +/- mice were statistically significant, their
amplitudes were low, as it was only 17 % increase in the open field and 12 % decrease
in the elevated plus maze. In addition, the vertical exploratory activity indicated by the
number of rearings was similar between control and Glyt1 +/- mice in both tests. It
suggests that the observed differences may have occurred by chance. Replication of the
experiments would be necessary to determine if these activity patterns were false
positive observations or if they were a complex but real endophenotype.

Finally, behavioural analysis of similar heterozygous knockout mice for Glyt1
gene was recently reported. Total activity, including locomotion, rearings, and repetitive
behaviours, was assessed in an open field set-up, and was quantified during 3 h after 1 h
of habituation to the arena. In these conditions, no differences of activity level were
reported between control and Glyt1 +/- mice (Tsai et al., 2004b).

2) Anxiety

In the open field and elevated plus maze, the time spent in the centre of the arena
and in the open arms, respectively, did not differ between control and Glyt1 +/- mice,
indicating that decreased glycine transport activity did not influence anxiety level. This
observation is consistent with a recent published report about GLYT1 inhibition and
anxiety in rodents. Harsing et al. tested the effect of two different specific GLYT1
inhibitors, namely N[3-(40-fluorophenyl)-3-(40-phenylphenoxy)-propyl]-sarcosine
(NFPS) and R,S-(+-)N-methyl-N-[(4-trifluoromethyl)-phenoxy]-3-phenyl-
propylglycine (Org 24461), on three different tests related to anxiety-like behaviours.
They found that NFPS and Org 24461 did not exhibit anxiolytic effects in the light-dark
test in mice, in the meta-chlorophenylpiperazine (mCPP)-induced anxiety test and in the Vogel conflict drinking test in rats (Harsing et al., 2003). Thus, pharmacological and genetic reduction of GLYT1 activity did not alter anxiety-related behaviours in rodents.

3) Circadian cycle

Levels of locomotor activity in the home cage were strongly correlated with the light cycle. Activity was much more intense and less interrupted during the dark phase than during the light phase. The synchronisation of the active phases was very precise, and the activity profiles were identical between control and Glyt1 +/- mice. This suggested that the circadian cycle was not affected by the reduction of glycine transport activity. Consistently with this observation, NR1-D481N/K483Q mice, which have point mutations in the GlyB site of NMDAR that decreased glycine affinity 72 times on average, displayed normal circadian changes in activity levels (Ballard et al., 2002).

4) Sensorimotor gating

Glyt1 +/- mice displayed normal amplitude of the startle response, but the prepulse inhibition was reduced by 38% on average. This observation is not consistent with two published studies investigating the effect of pharmacological or genetic inhibition of GLYT1 on PPI.

In the first study, Kinney et al. tested the effect of intra-peritoneal injection of (+)-NFPS on PPI levels. NFPS administration at 1 mg/kg and 10 mg/kg increased significantly the mean PPI level in a dose dependent manner but did not modify the amplitude of the startle response (Kinney et al., 2003). Thus, acute pharmacological GLYT1 inhibition augmented PPI levels, whereas constitutive reduction of GLYT1 activity decreased PPI levels. However, the duration of reduced GLYT1 activity and the different mouse strains used for these experiments could account for these contradictory observations. The genetic reduction of GLYT1 activity in Glyt1 +/- mice is constitutive, and thus, occurred at all developmental stages, what could have favoured compensatory mechanisms decreasing PPI levels. In addition, adult male mice from the DBA/2J strain displayed PPI levels that were significantly lower than the ones displayed by mice of the 129S6/SvEv and C57BL/6 strains, indicating that genetic background has a significant
Part 3: Reduced GLYT1 expression

influence on PPI levels (Kinney et al., 2003; Olivier et al., 2001). Thus, it is noteworthy that DBA/2J mice were selected to the effect of NFPS on behaviour, because the low basal PPI level would augment sensitivity to PPI enhancement.

In the second study, the behaviour of another line of Glyt1 +/- mice was evaluated on the PPI test (Tsai et al., 2004b). Interestingly, their Glyt1 +/- mice displayed also a 50 % reduction of GLYT1 mediated glycine transport, indicating that the two Glyt1 +/- mouse lines were equivalent at the molecular level. However, their Glyt1 +/- mice displayed normal PPI levels compared to controls (Tsai et al., 2004b). Thus, in two independent Glyt1 +/- mouse lines, a similar genetic mutation causing the same 50 % reduction in GLYT1 activity had either no effect or decreased significantly PPI levels. The two studies differed on a couple of points that could help to explain this discrepancy.

First, the Glyt1 +/- mice used in the present study were generated with 129/OLA mouse embryonic stem cells, and injected into C57BL/6 blastocysts. Then, the chimeras were backcrossed for at least two generations with C57BL/6 mice (Gomeza et al., 2003a), whereas the Glyt1 +/- mice made by Tsai et al. were backcrossed at least nine generations to the 129/SvEvTac background (Tsai et al., 2004b). Paylor and Crawley analysed systematically the influence of the genetic background on PPI level. Of the 12 different inbred strains that were tested, the 129/SvEvTac mice had the highest PPI level, ranging from 60 to 85 %, while the C57BL/6J mice had the lowest PPI level, ranging from 20 to 40 % (Paylor and Crawley, 1997). This suggests that the process involved in the plasticity of the startle response would be more robust in 129/SvEvTac mice, which would therefore resist to the reduced GLYT1 activity. Contrarily, C57BL/6J mice would have a “fragile” sensorimotor gating system that would be sensitive to the decreased GLYT1 activity.

Secondly, Tsai et al. tested in parallel the basal PPI level and its sensitivity to amphetamine and MK-801 treatments. A Latin-square design was used to allow a within-subjects analysis. The consequence is that each mouse was tested repeatedly, with only one week between two consecutive tests (Tsai et al., 2004b). On the contrary, all of our mice were tested only once on the PPI test, as we did not incorporate drug treatments in the experimental design. With rats, multiple PPI assessments were reported to be beneficial (Bakshi et al., 1998) and detrimental (Weiss and Feldon, 2001) to the reliable characterisation of a phenotype. The mice tested by Tsai et al. received a different treatment for every test sessions, but the treatments were distributed to avoid a confounding effect with the test session itself. Thus, they could not benefit from a
potential increase in power due to repeated measurements, but could suffer from an increase in variance due to testing the same condition on different days. In addition, habituation to the test procedure could influence the expression of the phenotype. Therefore, the experimental design used by Tsai et al., which involved repeated testing of the same mice, could have increased the variance and decreased the penetrance of the phenotype.

Finally, because mice are nocturnal animals, our mice were kept under inverted light cycle with lights on at 21:30, and therefore, were tested during the active phase. Contrarily, mice used by Tsai et al. were on normal light cycle with lights on at 6:00 AM, and therefore, were tested during the light phase (Tsai et al., 2004b). As clearly illustrated by the graph showing the home-cage activity over a 24 h period (Figure 18), mice were much more active during the dark phase, and were inactive, supposedly asleep, during most of the light phase. This difference could influence significantly the outcome of behavioural testing.

GLYT1 function in vivo serves the regulation of two antagonistic neurotransmitter systems. In the spinal cord, brain stem and cerebellum, GLYT1 activity is necessary for the termination of inhibitory glycinergic neurotransmission, whereas GLYT1 modulates activation of excitatory NMDARs in forebrain structures. Thus, heterozygous Glyt1 gene deletion could impact both neurotransmitter systems, and careful electrophysiological analysis of both of them would be required to infer the molecular mechanisms sustaining the observed behavioural phenotype.
Part 4: Inducible and neuron-specific gene expression in the adult mouse brain with the rtTA2S-M2 system

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Technology report submitted to Genesis
ABSTRACT

To exploit the latest improvement of the tetracycline-controlled transactivator-based system and achieve doxycycline-dependent gene expression in the mouse brain, we combined the rtTA2S-M2 factor with the CaMKIIα promoter in transgenic mice. Several independent lines of mutant mice were obtained in which restricted gene expression can be reversibly induced in forebrain neurons. The optimal dose of doxycycline, time course of gene expression and expression profile in adult and newborn mice have been determined with a lacZ reporter gene. In adult mice, doxycycline-enriched diet (6 mg/g) could induce within 6 days strong expression of the reporter gene specifically in the cortex, striatum, hippocampus, amygdala and olfactory bulbs, whereas no expression occurred in the midbrain and hindbrain areas. The consistency of the gene expression pattern was confirmed using a second reporter line. Finally, gene expression could be activated in the developing brain (P0) in similar structures. The CaMKIIα promoter–rtTA2 transgenic mouse lines thus provide a useful tool to inducibly and reversibly overexpress genes specifically in the developing and adult forebrain.
Cellular functions recruit multiple proteins which activity and role may vary depending on cell type, compartment or developmental stage. Genetic manipulations such as gene knock-out, or transgenic expression of dominant-negative, inhibitory or activatory mutant proteins have been extensively used to study the functions and biological significance of many proteins. To gain in specificity, such manipulations need to be spatially- and temporally-regulated such that the activity of the protein of interest is altered in a selected cell type over a precise time window. Spatial control is generally achieved with cell-specific promoters while temporal control can be obtained with ligand-dependent expression systems. The tetracycline-controlled transactivator (tTA) systems, developed by the laboratory of H. Bujard (Gossen and Bujard, 1992) have been widely used for inducible gene expression in vitro and in vivo. The tTA, also called Tet-Off, is a fusion between the tetracycline repressor from the Tn10 tetracycline resistance operon of E. coli and the C-terminal domain of the transcription factor from Herpes Simplex Virus (HSV) VP16. The resulting hybrid transcriptional activator is able to activate cognate sequences made of a minimal promoter placed downstream from seven consecutive repeats of the tetracycline operator (tetO). It is constitutively active but its activity can be inhibited by low concentrations of tetracycline or analogues such as doxycycline (dox). The reverse tetracycline-controlled transactivator (rtTA, or Tet-On) by contrast has opposite properties: it is constitutively silent and needs dox to bind to tetO sequences and induce transcription (Gossen et al., 1995; Orth et al., 2000). The rtTA factor was developed from the tTA factor by random mutagenesis followed by screening for drug-dependent transcriptional activation in yeast.

Both the tTA and rtTA factors have been used in the mouse to generate transgenic models with dox-controlled gene expression in various organs (Morozov et al., 2003; Yamamoto et al., 2001). However, their use has been hampered by several technical limitations. For instance with the tTA system, the kinetic of transgene induction in vivo is constrained by the time needed to clear the body from the inducer drug. It can be slow when transgene expression was suppressed for long periods of time, and can sometimes be blocked (personal observation). In that respect, the rtTA system is better because its kinetic is faster after dox treatment. Full transgene expression could for instance, be achieved in the adult mouse brain within 6 days (Mansuy et al., 1998). However, rtTA is
Part 4: Inducible and neuron-specific gene expression

generally less efficient than tTA and leads to lower levels of transgene expression. It also often has background residual activity in the absence of dox and requires high amount of dox for induction (Giménez et al., 2004; Urlinger et al., 2000).

In an attempt to improve the use of the rtTA system for in vitro and in vivo applications, Urlinger et al. recently developed improved versions of the factor with increased mRNA stability, optimized codon-use for mammals, lower background activity and higher affinity for dox (Urlinger et al., 2000). To take advantage of these new properties for use in the adult mouse brain, we expressed the rtTA2S-M2 factor (rtTA2 thereafter) under the control of the forebrain-specific CaMKIIα promoter. The dox-dependence and transcriptional activity of rtTA2 was evaluated using a tetO-LacZ reporter gene (Figure 20). Several independent lines of CaMKIIα promoter-rtTA2 were obtained and crossed with tetO-LacZ reporter mice. For each line, double mutant animals and control littermates were obtained and treated with dox to determine the pattern, dox-dependence and time course of expression.

Figure 20: Cell-specific and doxycycline-controlled expression system.
A first transgene carries the CaMKIIα promoter that drives expression of the transcription activator rtTA2S-M2 only in forebrain neurons. A second independent transgene carries seven tetracycline operator sequences (tetO) placed upstream from a minimal promoter and a lacZ reporter gene encoding β-galactosidase. Doxycycline (Dox) is an analogue of tetracycline that is more potent (Gossen et al., 1995) and more brain permeable (Andersson and Alestig, 1976). Dox is used to switch rtTA2S-M2 protein into an active conformation that binds specifically tetO sequences (Orth et al., 2000) and induces expression of the reporter gene (ON state). In absence of dox, rtTA2S-M2 cannot bind tetO sequences, the minimal promoter is not activated, and there is no lacZ expression (OFF state).
In two lines of rtTA2-expressing animals (894 and 898), forebrain-specific expression of the lacZ reporter gene was obtained after dox treatment. β-galactosidase activity was detected in neurons of the olfactory bulb, piriform cortex, most cortical areas, striatum, septum, hippocampus and amygdala (Figure 21). No staining was detected in midbrain, cerebellum or spinal cord. The pattern of expression was reproducible and was consistent with the endogenous pattern of CaMKIIα expression (Bürgin et al., 1990; Herms et al., 1993; Sola et al., 1999; Zou et al., 2002). All following experiments were done with the line 898 only. To test the dependence of rtTA2-controlled expression on dox, adult double mutant mice were treated with various concentrations of drug for a month. They either received 6 mg/g dox in the diet, or 3.5 mg/ml, 2 mg/ml or 0.05 mg/ml dox in the drinking water. Compared analyses showed that the level of lacZ reporter gene expression correlated with the amount of dox received without any remarkable change in the expression profile (Figure 22). The strongest expression was achieved when dox was incorporated at 6 mg/g in the food, while little or no expression was induced with low concentrations or in the absence (data not shown) of dox. These results demonstrate that, when placed under the control of the CaMKIIα promoter, rtTA2 has no background residual activity and allows tight and dose-dependent control of gene expression.

We next examined the time course of induction of the reporter lacZ expression by treating adult mutant mice with dox (6 mg/g food) for 2, 4, 6 or 8 days. We observed that β-galactosidase activity was induced already after 4 days of dox treatment, but the staining intensity and number of positive cells were increased after 6 or 8 days of dox administration (Figure 24). This most likely results from an increase in expression and an accumulation of β-galactosidase. The expression after 4 days of dox treatment occurred qualitatively in the same brain structures than after 8 days. The slow time course of induction (when compared to cell culture (Gossen et al., 1995) might be due to the slow diffusion and low penetrability of dox through the blood brain barrier, and the time necessary to accumulate enough dox to reach sufficient concentration (Andersson and Alestig, 1976; Riond and Riviere, 1988). Here again, the absence of β-galactosidase activity in the mutant mice treated with dox for 2 days demonstrates the tightness of the rtTA2 system in the brain.
Figure 21: β-galactosidase expression pattern in double transgenic mice fed with dox-supplemented food. Adult mice bearing both the CaMKIIαtTA2S-M2 and the tetO-LacZ transgenes were fed for 11 days with 6 mg dox per gram of wet food. β-galactosidase activity was observed exclusively in the following structures of the forebrain: amygdala nuclei (AMG), caudate putamen (CPU), cingulate cortex (CCX), frontal cortex (FCX), insular cortex (ICX), orbital cortex (OCX), somatosensory cortex (SCX), dentate gyrus (DG), cornu ammonis regions 1,2,3 (CA1,2,3), lateral septum (LS), anterior olfactory nuclei (ON), olfactory tubercles (OT), piriform cortex (PIR). No β-galactosidase activity was present in the midbrain structures, cerebellum (CB), pons and spinal cord (not shown).
Figure 22: The level of transgene expression is controlled by the dose of doxycycline. Double transgenic mice were treated with dox at a) 6 mg/g wet food b) 3.5 mg/mL c) 2 mg/mL d) 0.05 mg/mL drinking water for one month before analysis. The treatment duration was chosen to avoid any interference between the dose of dox and the induction speed. The doses 6 mg/g, 3.5 mg/mL and 2 mg/mL induced expression of the lacZ gene, while 0.05 mg/mL did not induce any expression.
Because inducible expression systems can be useful for studying developmental processes, we next examined whether the lacZ reporter gene could be induced during embryogenesis and early postnatal life in our rtTA2 expressing animals. To test the earliest possible time point of induction, dox was administered first during breeding then maintained through gestation, and lacZ gene expression was tested in newborn pups. A strong β-galactosidase staining was detected in the olfactory bulb, cortex, striatum and hippocampus at birth (Figure 23). Incidentally, there was ectopic staining in midbrain areas in newborns that however, was not observed when dox was administered only in adulthood. Such early gene expression was not expected as the CaMKIIα promoter has been reported to drive gene expression essentially postnatally, consistent with the late expression of endogenous CaMKIIα reported in rat and mouse (Burgin et al., 1990; Herms et al., 1993; Sola et al., 1999; Zou et al., 2002). However, prenatal expression has also been observed in transgenic mice carrying this promoter (Krestel et al., 2001), suggesting that the activity of this promoter is highly variable depending on the site of genomic integration and copy number.

In summary, we have generated new lines of transgenic mice allowing dox-dependent gene expression in forebrain neurons. In stably transfected cell lines expressing a luciferase reporter gene, the rtTA2s-M2 proved to be virtually silent in absence of dox, and needed 10 times less dox than the rtTA to be fully activated (Urlinger et al., 2000). Considering that the blood brain barrier limits drastically dox diffusion into the brain (Andersson and Alestig, 1976), we proposed that the increased sensitivity toward dox of the rtTA2s-M2 could have a positive influence on the induction speed and, possibly, on the maximum expression level too. Unfortunately in our transgenic lines, the induction of full rtTA2s-M2 activity in mouse forebrain neurons in vivo needed the same amount of time (6 days) and the same high dose of dox (6 mg/g of food) than the older rtTA factor (Mansuy et al., 1998). However, for technical reasons, no formal comparisons of the performances of these two systems could be undertaken in our laboratory. The discrepancy between the improved dox sensitivity in vitro and the absence of improvement of the induction speed in vivo suggests either that dox sensitivity is actually not a critical parameter in the brain, or that independent intracellular mechanisms could interfere with the induction of transgene expression.

However, the early inducibility, dose-dependence, forebrain and neuron-specificity, tightness of induction, and reproducibility of gene expression achieved with
our rtTA2 mice are valuable properties that when combined with an improved inducer drug, should be extremely useful for studies of brain functions. For instance, 4-epidoxycycline (4-ED), an hepatic metabolite of dox without antimicrobial activity and no side effect on intestinal flora, has recently been used to control gene expression in vitro in mice (Eger et al., 2004). Another exciting ongoing work is the development of photolabile caged doxycycline for cell-specific delivery and local gene expression (Cambridge et al., 2004), a system that should dramatically increase the spatial and temporal resolution of genetic manipulations.

Figure 23: Expression in newborn pups. Breeding pairs were fed with dox-supplemented food, and dox was maintained through gestation. β-galactosidase activity was revealed in the brain of one-day old pups. Like in adult mice, LacZ expression was detected in forebrain neurons. However, some additional ectopic expression in nuclei of the brain stem are also visible.
Figure 24: Time course of induction of transgene expression. Double transgenic mice were fed with dox-supplemented food (6mg/g) for 2, 4, 6 or 8 days before brain dissection and staining for β-galactosidase activity. 4 days of dox feeding were required to induce lacZ expression and β-galactosidase accumulation to a detectable level. No expression was observed after 2 days of dox treatment.
MATERIAL AND METHODS

Cloning and generation of CaMKIIα-rtTA2S-M2 transgenic mice:

rtTA2S-M2 coding sequence was flanked with hybrid regulatory sequences and fused to 8.5 kb promoter sequence of the αCaMKII gene. In details, rtTA2S-M2 synthetic gene was excised with EcoRI and BamHI from the pUHD-rtTA2-MT2 plasmid (Urlinger et al., 2000) and blunted. This fragment was inserted into the EcoRV site of the pNN265 plasmid, which carries 5’ and 3’ regulatory sequences with hybrid introns favouring in vivo transgene expression (Choi et al., 1991), plus a polyA signal from SV40. This construct was removed by NotI excision and inserted downstream CaMKIIα promoter at the NotI site of the pMM403 plasmid (Mayford et al., 1996a). The full construct was excised off the vector with SfiI, separated from the vector backbone on an agarose gel and purified by electro-elution followed by ethanol precipitation. Pronuclear injection into B6D2F1 eggs was realised in the service laboratory of the university, following a classical procedure (Hogan et al., 1994) (Rülicke T, 2000). Of the eight founder mice that were obtained and backcrossed to C57/B16J mice, seven of them transmitted the transgene to their litters. To observe rtTA2S-M2 activity, mice from the F1 generation were crossed with mice heterozygous for tetO-LacZ reporter gene (Mayford et al., 1996b), and animals carrying both transgenes were obtained in Mendelian ratio.

Mouse housing

Mice were housed under constant temperature, humidity and inverted light-dark cycle of 12 h (dark 9.30AM-9.30PM). They were cared for according to the guidelines established by the Federation of Swiss Cantonal Veterinary Officers.

Genotyping

Mice were marked by ear punching and tail biopsies were collected to determine the genotype by PCR. The HOTSHOT method was used for genomic DNA extraction (Truett et al., 2000). For rtTA2, primers F1 5’-TGCCCTTCTCTCCACAGGTTGC-3’ and rtTA2-260R 5’-GAGAGCACAGCGGAATGAC-3’ were used. For lacZ gene,
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primers **lacZ-s 5’-CCCATTACGGCTAATCCGCCG-3’** and **lacZ-as 5’-GCCTCCAGTACAGCGCGGCTG-3’** were used. The PCR reactions had 35 cycles at 94°C for 30 s, 62°C for 40 s, and 72°C for 1 min, in a buffer containing 2.5 mM MgCl2.

**Doxycycline administration**

Two ways of providing dox were used. One method was to soften food pellets in water and supplement it with 6mg dox per g of wet food (Westward Pharmaceutical). This food was prepared and given every day to the mice, at the beginning of the dark phase (morning). The second method was to sweeten drinking water with 4 mg/mL sugar, and add 3.5 mg/mL or 2 mg/mL or 50 μg/mL dox (Sigma). Bottles were protected from light to prevent light-induced degradation of dox. Bottles containing 3.5 mg/mL were replaced twice a week, whereas bottles with 2 mg and 0.05 mg were replaced on a weekly basis only.

**β-galactosidase staining**

16 μm thick fresh frozen brain sections were fixed for 10 min in ice-cold 100 mM phosphate buffer pH7.2 containing 0.2% glutaraldehyde, and rinsed 3 times. The sections were then incubated overnight at 37°C in a solution made of 5 mM potassium ferrocyanide, 5 mM potassium ferrocyanate, 2 mM MgCl2, 1 mM X-Gal, 0.01% sodium deoxycholate, 0.02% NP40 and 100 mM phosphate buffer pH7.2. Sections were counterstained with acidified hematoxylin, dehydrated, dried, mounted with Neo-mount (Merck) and photographed.
Part 5: General discussion
I- SUMMARY OF THE RESULTS

The aim of this thesis was (1) to investigate the impact of glycine-mediated modulation of NMDAR activity on behaviour and (2) to generate an animal model for testing the hypothesis of NMDAR hypofunction in schizophrenia. For this purpose, two mouse lines with genetic up- or down-regulation of GLYT1 activity were characterised behaviourally. In addition, a transgenic mouse line was generated, which expressed the new reverse tetracycline controlled transactivator (rtTA2) in the forebrain neurons.

1) tTA-Glyt1 mice

GLYT1 was overexpressed in forebrain neurons to decrease synaptic glycine concentration and thus, NMDAR activity. This project was interesting in two respects. First, it could provide information about the relationship between forebrain NMDAR activity and cognitive functions and behaviour. Second, because NMDAR hypofunction is thought to be one of the causes of schizophrenia, it could have been an interesting animal model to test new drug candidates for antipsychotic activity.

We generated transgenic mice using the tTA system to overexpress Glyt1 mRNA in forebrain neurons. The use of the tTA system gave the possibility to suppress gene expression by administration of dox. tTA-Glyt1 mice were evaluated on behavioural tests related to symptoms commonly observed in schizophrenic patients, including general motor activity, anxiety, learning and memory, and sensorimotor gating ability. tTA-Glyt1 mice displayed normal abilities on all these tests. A biochemical investigation revealed that although Glyt1 mRNA was upregulated, the amount of GLYT1 protein and the glycine transport activity were not changed. This result represents a discrepancy between transcriptional and translational regulation of the transgene, which may be the cause for the absence of behavioural alterations.

There are several possible explanations for such discrepancy. First, the translational silencing of the transgene may be due to the ectopic expression of GLYT1 in neuronal population rather than in glial cells. The review of previous publications suggests that a similar discrepancy may have been observed in vivo. In fact, expression
of GLYT1 protein was predicted in forebrain neurons by studies that used the in situ hybridisation technique, but this expression was never detected by immunohistostaining. Second, the lack of GLYT1 protein overexpression may be due to a feedback mechanism controlling the expression level of endogenous GLYT1 protein, where the endogenous GLYT1 may have been downregulated to compensate for the expression of Glyt1 transgene.

2) Glyt1 +/- mice

To determine the effect of reduced NMDAR activity on behaviour, heterozygous GLYT1 knockout was used. These heterozygous knockout mice displayed a 50% reduction in glycine transport activity. These mice displayed no sign of anxiety, but the locomotor activity was increased in the open field and decreased in the elevated plus maze. In the home cage during a 24 h period, Glyt1 +/- mice had a normal activity level and their circadian cycle was synchronised with the one of control mice. Finally, Glyt1 +/- mice displayed a 38% reduction of PPI on average, while the amplitude of the startle response was not modified.

Some of these results were in agreement with the literature. It was reported that another line of Glyt1 +/- mice, which had a similar 50% reduction of glycine transport activity, displayed normal locomotor activity level (Tsai et al., 2004b), and that pharmacological inhibition of GLYT1 with NFPS had no effect on anxiety level (Harsing et al., 2003). However, contradictory results were published regarding GLYT1 activity and PPI. The Glyt1 +/- mice generated by Tsai et al. displayed normal PPI levels, while inhibition of GLYT1 with NFPS increased PPI levels (Kinney et al., 2003). Differences in the genetic background of the mouse strains used for these experiments and differences of experimental designs and set-ups could explain the heterogeneity of these observations.

3) CaMKIIα-rtTA2 mice

The use of CaMKIIα promoter restricted the rtTA2 expression to forebrain neurons, thus controlling spatial and cell-type specificity of transgene expression. The temporal control of the transgene expression is mastered by dox administration. The
transgene expression pattern of this mouse line was characterised by crossing the CaMKIIα-rtTA2 transgenic mice with transgenic mice possessing tetO-LacZ reporter gene. The mouse expressing both CaMKIIα-rtTA2 and tetO-LacZ transgenes showed LacZ reporter expression only upon dox administration and restricted to forebrain neurons. The LacZ expression was detected after 4 d of dox feeding, but full expression was observed only after 6 d. The optimal dosage to induce gene expression was 6 mg dox per g of food. When dox was administered to breeding pairs, expression of the reporter gene was already detected in neonates. Thus, we generated a new mouse line permitting inducible gene expression in forebrain neurons, which is now being used in our laboratory and by collaborators to investigate protein function in vivo.

Regarding the aim of this thesis, which was to investigate the impact of glycine-mediated modulation of NMDAR activity on behaviour, we made two significant observations. The genetic reduction of glycine transport (1) did not affect anxiety levels and (2) impaired significantly the sensorimotor gating ability. The first observation was consistent with the effects of pharmacological inhibition of GLYT1 activity, whereas the second was not. We will discuss first the differences between genetic and pharmacological approaches to modulate protein activity. Genetic approaches alter molecular processes permanently and are more prone than pharmacological approaches to induce global modifications, for example at the connectivity level. Thus, genetic approaches are proposed to mimic better than pharmacological approaches the effect of a molecular dysfunction in human beings. Then, because we focused on glycine-mediated regulation of NMDAR activity and its putative participation to the aetiology of schizophrenia, we will review and discuss the clinical trials that tested the effect of glycine administration to patients as an adjunct therapy. Finally, we will discuss how the heterogeneous genetic contribution suggests synaptic dysfunction in schizophrenia.
II- GENETIC VERSUS PHARMACOLOGICAL ANIMAL MODELS

There are some discrepancies between pharmacological and genetic approaches in studying NMDAR hypofunction model in schizophrenia. We propose that the cause of this difference may be due to the duration of NMDAR impairment and that the genetic approach may be more appropriate to model human disorders, such as schizophrenia.

The NMDAR hypofunction model of schizophrenia emerged when it was noticed that PCP, a drug of addiction that caused behavioural alterations very similar to the psychotic state of schizophrenic patients, was a specific antagonist of NMDARs. Since then, this drug and similar non-competitive antagonists of NMDARs were used to induce schizophrenia-like behavioural symptoms in laboratory animals. Furthermore, these pharmacological animal models of schizophrenia were used to test the efficacy of new antipsychotic compounds. This approach had some limitations, such as the specificity of antagonists and difficulties in generating receptor subunit specific molecules. With the advancement in genetic technology, the biological function of neurotransmitter receptors could be investigated more directly. However, genetic technology is currently available for a limited number of laboratory animals, such as mice. Therefore, the NMDAR hypofunction hypothesis was tested in mice using genetic approaches as reviewed in the introduction.

The genetic animal models of NMDAR hypofunction reproduced some but not all of the behaviours observed with pharmacological models. For example, hyperactivity is a trait that was found consistently in pharmacological and genetic models, and was nicely correlated in strength with the reduction of NMDAR activity. In contrast, deficit of PPI was a robust effect of the pharmacological inhibition of NMDARs (Curzon and Decker, 1998; Geyer et al., 2001), but was not shown by any of the genetic models of NMDAR hypofunction. Another illustration of the differences between pharmacological and genetic animal models regards the effect of decreased GLYT1 activity on PPI levels. The use of the specific GLYT1 inhibitor NFPS increased PPI levels (Kinney et al., 2003). In rat in vivo, NFPS administration
increased NMDAR activity in hypoglossal motoneurons (Lim et al., 2004) and prefrontal cortex neurons (Chen et al., 2003), and increased LTP in the dentate gyrus (Kinney et al., 2003). If one considers the main effect of GLYT1 inhibition in vivo to be the increase of NMDAR activity, then, the NFPS-induced increase of PPI was an observation nicely symmetrical to the PCP-induced decrease of PPI (Curzon and Decker, 1998; Geyer et al., 2001). However, the 50% decrease of GLYT1 activity in Glyt1 +/- mice had either no effect on PPI levels (Tsai et al., 2004b), or caused a 38% reduction of PPI levels in our hands. Thus, pharmacological and genetic models do not provide systematically consistent observations.

The duration of the alteration is the most evident explanation for the discrepancy between pharmacological and genetic models. Pharmacological experiments are mostly done by acute drug administration shortly before behavioural testing, whereas gene-targeting experiments modify the cellular environment on a permanent basis. Such permanent modification is very likely to cause additional changes at the molecular, cellular, and neuronal network levels. An example of such an adaptation at the molecular level was the dramatic reduction of NR2B expression in NR1 -/- mice, whereas NR2A levels were unchanged (Forrest et al., 1994). Similarly, in NR1-D481N mice, increased mRNA levels for the NR1, NR2A and NR2C but not the NR2B subunits were detected in some specific brain structures (Kew et al., 2000). Changes at a more global scale than the molecular level are very likely to occur. This is even more critical in the particular case of NMDARs, as they are involved in many forms of synaptic plasticity. For example, NR2A -/- mice, which had a 50% reduction of NMDAR currents, displayed reduced LTP in CA1 region after tetanic stimulation (Sakimura et al., 1995), whereas NR3A -/- mice, which had increased NMDAR currents, displayed increased spine density and enlarged spine heads (Das et al., 1998). Finally, changes at the cellular level could alter structure and function of the whole network, and in the case of NMDARs, alteration of synaptic plasticity would prevent establishment of proper connections between neurons. This was demonstrated for short distance connections by the absence of whisker-related pattern in NMDAR deficient mice. Because of all the changes that can occur at the molecular, cellular and structural levels from the earliest developmental stage, observations based on genetic animal models could differ quite dramatically from the ones obtained with pharmacological approaches.
Due to multiple interdependent effects that occur in genetic models, these ones are more likely to simulate better what happen in human diseases than the acute injection of a drug. This should be especially true when the modelled disorder has genetic and developmental factors. Schizophrenia is a complex brain disorder that has environmental and genetic factors contributing to its aetiology. It was shown that the concordance rate for schizophrenia is 50% for monozygotic twins, whereas it is only 17% for dizygotic twins, 9% for siblings and only 1% for the general population, what suggests a strong genetic component to the disease (Gottesman, 1991). In addition, multiple changes in gene expression levels were reported in post-mortem cortex tissue from schizophrenic patients. Using gene-chip technology, decreased mRNA levels of multiple proteins involved in presynaptic activity (Mirmics et al., 2000) or in some specific metabolic pathways (Middleton et al., 2002) were described. Finally, despite schizophrenia onset occurs mainly during early adulthood (20-30 y), it was proposed that behavioural alterations are already visible during childhood and early adolescence. For example, delayed development of certain motor skills, such as posture control, standing and walking, is associated with a higher risk of developing schizophrenia (Fish et al., 1992; Marcus et al., 1993). Thus, genetic factors, involvement of multiple molecular pathways and interferences with development contribute to the complexity of schizophrenia. These multiple levels of interactions should be much better reproduced with a genetic animal model than with acute drug administration.

Because schizophrenia is a complex disorder with a strong genetic component, numerous alterations of gene expression levels and putative interferences with development already at very early stages, a genetic approach to generate animal models seems more appropriate than a pharmacological one. Indeed, genetic models reproduce also the interdependent relationship between molecular, cellular and network levels that cannot be mimicked by acute drug administration. Thus, genetic models test in a more realistic way the therapeutic efficacy of new compounds and should offer better chances to discover unsuspected molecular targets.
Clinical trials testing on patients with schizophrenia the effect of glycine administration as an adjunct to ongoing treatments started 15 years ago already. A meta-analysis of published data concluded that administration of glycine or D-serine ameliorated moderately negative symptoms but only marginally cognitive functions, and had no effect on positive symptoms, whereas D-cycloserine administration did not ameliorate the clinical status of patients. We propose that in reason of the low doses used in some of the trials incorporated in the meta-analysis, the full therapeutic potential of glycinergic therapy was underestimated. In addition, new data suggest that specific GLYT1 inhibition has a valuable therapeutic efficacy, similar to glycine or D-serine administration, and is more amenable than glycine to large-scale clinical use.

Historically, the first clinical trials testing the effect of glycine administration to patients with schizophrenia started more than 15 years ago. The discovery that glycine was able to potentiate NMDAR activity (Johnson and Ascher, 1987) suggested that glycine administration could have some therapeutic efficacy for the treatment of schizophrenia (Deutsch et al., 1989). Because glycine is the simplest amino acid and thus is devoid of toxicity, clinical trials could start almost immediately, and indicated the potential therapeutic usefulness of glycine administration (Costa et al., 1990; Rosse et al., 1989). Since then, numerous studies using more refined designs and larger groups investigated the effect of glycine, D-serine and D-cycloserine administration to patients with schizophrenia, always as adjunctive therapy to ongoing antipsychotic treatments. A recent meta-analysis of 18 different randomised, placebo-controlled, double-blind trials concluded that glycine and D-serine administration improved moderately but significantly the negative symptoms (Tuominen et al., 2005). The improvement of cognitive functions was mild and did not reach statistical significance, whereas no effects on positive symptoms were detected. D-cycloserine administration was less efficient than both glycine and D-serine on negative symptoms, and had no effect on cognitive and positive symptoms (Tuominen et al., 2005). The most probable explanation for this absence of effect is that, contrarily to glycine and D-serine which
are full agonists, D-cycloserine is a partial agonist on NMDARs in vitro (Hood et al., 1989) and in vivo (Emmett et al., 1991). It is noteworthy that the trial using the highest D-cycloserine dose showed significant worsening of positive and negative symptoms (van Berckel et al., 1999).

This meta-analysis revealed that the improvement of negative symptoms due to glycine or D-serine administration was only moderate and the improvement of cognitive functions did not reach statistical significance (Tuominen et al., 2005), but these results may not reflect the full potential of glycicnergic therapy. Due to the absence of proper dose-finding trials, i.e. randomised testing of multiple doses on parallel groups, the dose-response curve of glycicnergic drugs is undetermined and the doses used so far could have limited treatment efficacy. Indeed, the glycine trials reported to date suggest a dose-dependent glycine-induced treatment response. A 0.4g/kg/day glycine adjuvant regimen resulted in a significant 17 % mean reduction in negative symptoms (Javitt et al., 1994), whereas treatment with 0.8g/kg/day glycine, the highest dose used to date, resulted in an approximately 30 % mean improvement in negative symptoms (Heresco-Levy et al., 1999). However, the meta-analysis averaged the results from studies using low (3 studies) and high (4 studies) glycine doses together, what may have reduced the overall effectiveness of glycine administration. Indeed, a recent study tested the effect of high glycine dose (0.8g/kg/day) on patients treated with olanzapine and risperidone, two recent atypical antipsychotics, and demonstrated significant improvement of negative and positive symptoms and cognitive functions (Heresco-Levy et al., 2004). Thus, glycine adjunction to ongoing therapy seems a valuable approach to improve not only negative symptoms but also cognitive functions and, to a lesser extent, positive symptoms.

Finally, specific inhibition of GLYT1 has a similar therapeutic efficacy and is more amenable to clinical use. Because glycine is a small and neutral amino-acid, used by every cells in the body and transported by GLYT1 away from NMDARs, the administration of a massive dose of glycine is necessary to increase its concentration at NMDARs and obtain therapeutic efficacy. Indeed, the dose of 0.8g/kg/day glycine, which showed the best clinical efficacy, is not convenient for generalised clinical use because it corresponds to an intake of 50 to 70 g of glycine per day. In contrast, similar therapeutic efficacy was obtained with D-serine at a dose of 30 mg/kg/day only – around 2 g per day (Tsai et al., 1998; Tsai et al., 1999), most probably because it is not a
metabolite and not transported by GLYT1. Given that GLYT1 activity at glutamatergic synapses is limiting glycine concentration and thus NMDAR activity, the specific inhibition of GLYT1 should increase synaptic glycine concentration and potentiate NMDAR currents. This hypothesis has been validated by two independent studies showing that NFPS administration in vivo increased NMDAR activity in rodents (Chen et al., 2003; Kinney et al., 2003). In addition, the effect of administrating 2 g per day of sarcosine, a specific GLYT1 inhibitor, to patients with schizophrenia was investigated in a recent study. A significant amelioration of both positive and negative symptoms of 17 % and 14 %, respectively, and a 13 % improvement of cognitive abilities were reported (Tsai et al., 2004a). Thus, specific inhibition of GLYT1 activity is an alternative approach to potentiate NMDAR activity, which has a therapeutic efficacy comparable to administration of glycine or D-serine. In addition, the small doses necessary are preferable for clinical use.

Actual antipsychotics used for schizophrenia treatment are mostly dopamine and serotonin receptor antagonists, alleviate efficiently positive symptoms, but are relatively inefficient against negative symptoms and cognitive deficits. Considering the hypothesis of NMDAR hypofunction in schizophrenia, it has been proposed that the glycine modulatory site of NMDARs could be targeted to augment NMDAR activity. Glycine and D-serine, which are Gly-B site agonists, and sarcosine, a GLYT1 inhibitor, were tested in clinical trials and demonstrated efficient relief of negative symptoms and some attenuation of positive symptoms and cognitive deficits. Because large dose of glycine were necessary to obtain a therapeutic effect, its clinical use would not be convenient. Contrarily, only small doses of D-serine and sarcosine were necessary to observe clinical effect. D-serine is an endogenous molecule and sarcosine is a naturally occurring amino-acid, and no side-effects were reported during the course of the clinical trials, suggesting an absence of toxicity at the doses tested. Thus, after large scale and careful testing of the innocuousness of D-serine and sarcosine intake, their use as dietary supplement could improve significantly existing medication of schizophrenia.

However, the absence of effect of typical and atypical antipsychotics on negative symptoms and cognitive dysfunctions, and the moderate improvement brought by glycineric therapy indicates that other approaches are necessary to develop better drugs. A deeper understanding of the pathophysiology of schizophrenia is necessary to discover new drug targets, which could either replace existing treatments or complement them.
IV- HETEROGENOUS GENETIC CONTRIBUTION SUGGESTS SYNAPTIC DYSFUNCTION IN SCHIZOPHRENIA

In schizophrenia, a high level of heterogeneity is observed at the neuropathological and most of the observations have been subject to controversy. Despite numerous studies since A. Alzheimer (1864-1915) who was the first to investigate the neuropathology of schizophrenia, no consistent markers were found at the anatomical level.

Investigation of the genetic underlying schizophrenia revealed an even greater heterogeneity than the neuropathological observations. It is quite clear that the genetic contribution to schizophrenia follows a polygenic model, that is, multiple risk genes act additively or multiplicatively. In addition, each of these genes account for only a small increment in risk (e.g. no greater than a three-fold risk elevation in siblings (Risch, 1990)), which is likely to be modified by other genes, including protective ones. Replicating the studies showing gene association with schizophrenia in another population than the original one was often difficult and gave contradictory results, suggesting that different genes contribute to schizophrenia in different populations. However, in a recent review of the literature, P.J. Harrison and D.R. Weinberger (2004) considered four different lines of evidence, namely genome wide scans, association studies, biological plausibility and expression levels in patients, to determine the genes associated with schizophrenia with the highest level of reliability. Genes for which a role in the aetiology of schizophrenia was supported by published data in each of these four categories, were catechol-O-methyl transferase (COMT), dysbindin (DTNBP1), neuregulin 1 (NRG1), regulator of G-protein signalling 4 (RGS4), disrupted-in-schizophrenia (DISC1), metabotropic glutamate receptor-3 (mGluR3), G72, D-amino acid oxidase (DAAO), gamma catalytic subunit of calcineurin (PPP3CC), α7 nicotinic receptor (CHRNA7), proline dehydrogenase (PRODH2) and protein kinase B (Akt1) (Harrison and Weinberger, 2004). Interestingly, all these susceptibility genes impact upon the molecular biology of the synapse and the metabolism of neurotransmitters. The genetic influences on the synapse include effects on receptors (e.g. mGluR3, CHRNA7, G72), signal transduction (RGS4), and regulation of plasticity and synaptogenesis (NRG1, dysbindin, DISC1, calcineurin), and this for glutamatergic, dopaminergic and...
GABAergic synapses. Thus, the highly heterogeneous data set comprising genetic, biochemical and expression level data could be interpreted in a comprehensive way, suggesting that synaptic dysfunction is the core aetiological feature of schizophrenia (Harrison and Weinberger, 2004). According to this concept and the data supporting it, the molecular basis of schizophrenia is most unlikely to reside in any one neurotransmitter or receptor, and the disease should not prove reducible to a single molecular process or neurotransmitter signalling system.

Given that schizophrenia is fundamentally a disorder of abnormal information processing, the concept of synaptic dysfunction may sound vague and lacking proper explanatory power. However, the computational abilities of a neuronal network are strictly depending on synaptic properties. Thus, molecular dysfunction at the synapse would cause malfunction of cortical microcircuits, what would in turn affect the computational abilities of macrocircuits and disorganise brain activity. It illustrates the highly complex but fascinating continuum between genetic, molecular, synaptic and computational levels. The search for the mechanisms underlying a complex psychiatric disorder like schizophrenia is just one way toward understanding how the brain works, an important quest since the mind has been embodied.
Part 6: Reference list


59. Harrison PJ. 1999b. Neurochemical alterations in schizophrenia affecting the putative receptor targets of atypical antipsychotics. Focus on dopamine (D1, D3, D4) and 5-HT2a receptors. Br J Psychiatry Suppl:12-22.


Part 7: Appendices
DIAGNOSTIC CRITERIA FOR SCHIZOPHRENIA

The following specific diagnostic criteria are reproduced verbatim from the DSM-IV.

A. Characteristic symptoms:
Two (or more) of the following, each present for a significant portion of time during a 1-month period (or less if successfully treated):
• delusions
• hallucinations
• disorganized speech (e.g., frequent derailment or incoherence)
• grossly disorganized or catatonic behavior
• negative symptoms, i.e., affective flattening, alogia, or avolition

Note: Only one Criterion A symptom is required if delusions are bizarre or hallucinations consist of a voice keeping up a running commentary on the person’s behavior or thoughts, or two or more voices conversing with each other.

B. Social/occupational dysfunction:
For a significant portion of the time since the onset of the disturbance, one or more major areas of functioning such as work, interpersonal relations, or self-care are markedly below the level achieved prior to the onset (or when the onset is in childhood or adolescence, failure to achieve expected level of interpersonal, academic, or occupational achievement).

C. Duration:
Continuous signs of the disturbance persist for at least 6 months. This 6-month period must include at least 1 month of symptoms (or less if successfully treated) that meet Criterion A (i.e., active-phase symptoms) and may include periods of prodromal or residual symptoms. During these prodromal or residual periods, the signs of the disturbance may be manifested by only negative symptoms or two or more symptoms listed in Criterion A present in an attenuated form (e.g., odd beliefs, unusual perceptual experiences).

D. Schizoaffective and Mood Disorder exclusion:
Schizoaffective Disorder and Mood Disorder With Psychotic Features have been ruled out because either (1) no Major Depressive Episode, Manic Episode, or Mixed Episode have occurred concurrently with the active-phase symptoms; or (2) if mood episodes have occurred during active-phase symptoms, their total duration has been brief relative to the duration of the active and residual periods.

E. Substance/general medical condition exclusion:
The disturbance is not due to the direct physiological effects of a substance (e.g., a drug of abuse, a medication) or a general medical condition.

F. Relationship to a Pervasive Developmental Disorder:
If there is a history of Autistic Disorder or another Pervasive Developmental Disorder, the additional diagnosis of Schizophrenia is made only if prominent delusions or hallucinations are also present for at least a month (or less if successfully treated).

SUBTYPES

1. Paranoid Type
A type of Schizophrenia in which the following criteria are met:
Diagnostic criteria for schizophrenia

- Preoccupation with one or more delusions or frequent auditory hallucinations.
- None of the following is prominent: disorganized speech, disorganized or catatonic behavior, or flat or inappropriate affect.

2. Catatonic Type
A type of Schizophrenia in which the clinical picture is dominated by at least two of the following:
- motoric immobility as evidenced by catalepsy (including waxy flexibility) or stupor
- excessive motor activity (that is apparently purposeless and not influenced by external stimuli)
- extreme negativism (an apparently motiveless resistance to all instructions or maintenance of a rigid posture against attempts to be moved) or mutism
- peculiarities of voluntary movement as evidenced by posturing (voluntary assumption of inappropriate or bizarre postures),
- stereotyped movements, prominent mannerisms, or prominent grimacing
- echolalia or echopraxia

3. Disorganized Type
A type of Schizophrenia in which the following criteria are met:
- All of the following are prominent:
  - disorganized speech
  - disorganized behavior
  - flat or inappropriate affect
- The criteria are not met for Catatonic Type.

4. Undifferentiated Type
A type of Schizophrenia in which symptoms that meet Criterion A are present, but the criteria are not met for the Paranoid, Disorganized, or Catatonic Type.

5. Residual Type
A type of Schizophrenia in which the following criteria are met:
- Absence of prominent delusions, hallucinations, disorganized speech, and grossly disorganized or catatonic behavior.
- There is continuing evidence of the disturbance, as indicated by the presence of negative symptoms or two or more symptoms listed in Criterion A for Schizophrenia, present in an attenuated form (e.g., odd beliefs, unusual perceptual experiences).

Associated features
- Learning Problem
- Hypoactivity
- Psychosis
- Euphoric Mood
- Depressed Mood
- Somatic or Sexual Dysfunction
- Hyperactivity
- Guilt or Obsession
- Sexually Deviant Behavior
- Odd/Eccentric or Suspicious Personality
- Anxious or Fearful or Dependent Personality
- Dramatic or Erratic or Antisocial Personality
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EDUCATION

2000-2005 PhD thesis in Neuroscience, Swiss Federal Institute of Technology (ETHZ) and Zürich Neuroscience Centre (ZNZ), Zürich, CH
1997-2000 Master degree in Biotechnology at the European School of the Higher Rhine Universities (ESBS), Strasbourg, FR
1995-1997 DEUG (two-year degree) in Biology and Biochemistry at the University of Pau, FR

WORK EXPERIENCE

Dec. 2000 PhD student in the laboratory of Prof. Dr. Isabelle Mansuy, Swiss Federal Institute of Technology Zürich, CH
Jan 2005 Dual approach to study the impact of glycine-mediated modulation of glutamatergic transmission on behaviour

Techniques used:
- DNA: cloning, sequencing, PCR, southern-blot
- RNA: RT-PCR
- Protein: pseudo-quantitative western-blot
- Biochemistry: kinase activity assay, neurotransmitter (3H-Gly) uptake assay, LacZ staining
- Behaviour: activity: open-field, home-cage activity, rotarod
  - anxiety: elevated plus maze, free exploratory paradigm
  - depression: Porsolt test
  - learning and memory: novel object recognition, Morris water maze
  - sensorimotor gating: prepulse inhibition test

Experience in training and supervising students

IT responsible in the lab
- Familiar with computer maintenance, Macintosh and PC
- Easy handling of Mac OS X, Windows, Microsoft Office, Statview, EndNote, Photoshop

Teaching experience
- "Anatomical and Behavioural Insights into Brain Function"
- "Literature search in Biology"
- Practicals for Biology students at the ETH Zürich, CH, 2001 - 2004

Languages
- French native language
- English fluent written and spoken (language used at work since Jan. 2000)
- German fluently understood and good spoken (social use in Zürich for 4 years)
TRAIINEESHIP

Jan. 2000  Master-degree work in the laboratory of Dr. N.K. Spurr, Department of Biotechnology and Genetics, SmithKline Beecham Pharmaceuticals, Harlow, UK.

Determination of the GSK3-beta genomic structure and polymorphism detection by denaturing HPLC

June 1999  Undergraduate project in the laboratory of Dr. M. Labouesse on Molecular Embryology of Invertebrates, at the Institute for Genetic and Molecular and Cellular Biology (IGBMC), Strasbourg, FR

Transposition induction in sexual cell lines of the nematode Caenorhabditis elegans by in vivo injection of transposase mRNA.

July 1998  Trainee in the laboratory of endocrinology, MD PhD. O.E. Janssen, Central Hospital of Munich, DE.

Expression of the TSH hormone receptor in Xenopus oocytes.

PUBLICATIONS

Michalon A., Mansuy IM.
Genetic investigation of NMDA receptor function in vivo : A decade in review
Submitted

Michalon A., Koshibu K., Mansuy IM.
Inducible and neuron-specific gene expression in the mouse brain with the CaMKIIα-rtTA2S-M2 system
Submitted

Genoux D, Haditsch U, Knobloch M, Michalon A, Storm D, Mansuy IM.
Protein phosphatase 1 is a molecular constraint on learning and memory

PRESENTATIONS IN 2004

Centre of transgenesis expertise : Novel transgenic systems
Annual meeting of the National Centre of Competence in Research "Neural Plasticity and Repair", Constance, DE, 26-27.03.2004

Effects of synaptic glycine concentration acting via NMDA receptors on behaviour
Presentation at the Institute of Cell Biology, ETH Zürich, CH, 12.01.2004
POSTERS IN 2004

Modulation of synaptic glycine concentration in the mouse: a dual approach to study the impact on behaviour
Meeting of the Federation of European Neuroscience Societies (FENS), Lisbon, PT, 10-14.07.2004
Meeting of the Molecular and Cellular Cognition Society (MCCS), Lisbon, PT, 8-9.07.2004

A transgenic mouse model to test the hypothesis of glutamatergic hypofunction in schizophrenia
Meeting of the Swiss Society of Neuroscience (SSN) and the Swiss Society for Behavioural Neurobiology (SSBN), Lausanne, CH, 17.01.2004

GRANTS AWARDED

Travel grants from Julius Klaus Foundation and ETHZ to attend the meetings of the Federation of European Neuroscience Societies and the Molecular and Cellular Cognition Society, Lisbon, PT, 8-14.07.2004

Travel grant and registration waiver from the New York Academy of Science to attend the congress "Glutamate and Disorders of Cognition and Motivation", Yale, New Haven, CT, USA, 13-15.04.2003

Travel grant from the Swiss Society of Neuroscience to attend the meeting of the International Brain Research Organisation, Prague, CZ, 10-15.07.2003