Clozapine reverses schizophrenia-related behaviours in the metabotropic glutamate receptor 5 knockout mouse: association with N-methyl-D-aspartic acid receptor up-regulation

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Abstract

Abnormalities in glutamatergic signalling are proposed in schizophrenia in light of the schizophreniform psychosis elicited by NMDA antagonists. The metabotropic glutamate receptor 5 (mGluR5) interacts closely with the NMDA receptor and is implicated in several behavioural endophenotypes of schizophrenia. We have demonstrated that mice lacking mGluR5 have increased sensitivity to the hyperlocomotive effects of the NMDA antagonist MK-801. Mice lacking mGluR5 also show abnormal locomotor patterns, reduced prepulse inhibition (PPI), and deficits on performance of a short-term spatial memory task on the Y-maze. Chronic administration of the antipsychotic drug clozapine ameliorated the locomotor disruption and reversed the PPI deficit, but did not improve Y-maze performance. Chronic clozapine increased NMDA receptor binding ([³H]MK-801) but did not alter dopamine D₂ ([³H]YM-09151), 5-HT₂A ([³H]ketanserin), or muscarinic M₁/M₄ receptor ([³H]pirenzepine), binding in these mice. These results demonstrate behavioural abnormalities that are relevant to schizophrenia in the mGluR5 knockout mouse and a reversal of behaviours with clozapine treatment. These results highlight both the interactions between mGluR5 and NMDA receptors in the determination of schizophreniform behaviours and the potential for the effects of clozapine to be mediated by NMDA receptor regulation.

Key words: Antipsychotic, behaviour, mGluR5, NMDA.

Introduction

Disturbances in glutamatergic signalling are now central to many models of schizophrenia aetiology (Coyle, 2006; Konradi and Heckers, 2003; Krystal et al., 2003; Moghaddam, 2003; Tamminga, 1998). This stems from our increasing understanding of the role of glutamate in many of the key pathways implicated in psychiatric neuropathology, including the corticocortical, thalamocortical and corticofugal tracts (Javitt, 2004). Glutamatergic systems interact closely with the monoamine networks known to be disturbed in the CNS of subjects with schizophrenia, and are essential for the regulation of both the motor and higher brain functions disrupted in the disease (Millan, 2005). Both genetic linkage and post-mortem studies have provided further support for a dysfunctional glutamatergic system in schizophrenia (Goldman-Rakic and Selemon, 1997; Harrison, 1999; Lewis and Anderson, 1995; McCullumsmith and Meador-Woodruff, 2002; Weinberger et al., 2001). However, the most compelling evidence is the schizophrenia-like syndrome evoked by phencyclidine (PCP) and ketamine, which act to antagonize the major ionotropic glutamate receptor, the N-methyl-D-aspartic acid (NMDA) receptor (Adler et al., 1999; Krystal et al., 1994).

The widely expressed ionotropic glutamate receptors, comprising NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate...
subtypes, are key to the glutamatergic system. Metabotropic glutamate receptors (mGluR) are also widely distributed and mediate glutamatergic responses through G-protein-dependent mechanisms (Kew and Kemp, 2005). The metabotropic glutamate receptor type 5 (mGluR5) is coupled to downstream signalling pathways via Gq and phospholipase C-β1, and has been implicated in several behavioural endophenotypes relevant to psychiatric illness (Arguello and Gogos, 2006; Lipska and Weinberger, 2000; Seong et al., 2002; van den Buuse et al., 2005a). Pharmacological manipulations of mGluR5 activity have demonstrated a role for this receptor in the regulation of sensorimotor gating, locomotor behaviour, anxiety and cognitive processes (Ballard et al., 2005; Balschun et al., 2006; Barker et al., 2006; Bordi et al., 1996; Campbell et al., 2004; Pietraszek et al., 2005b; Rodrigues et al., 2002). These behavioural processes are reminiscent of schizophrenic endophenotypes. Significantly, mGluR5 knockout (KO) mice also show altered sensorimotor gating (prepulse inhibition; PPI), altered locomotor activity, and differential sensitivity to drugs of abuse (Brody et al., 2004b; Chiamulera et al., 2001; Lu et al., 1997). Ablation of mGluR5 or the use of mGluR5 antagonists induces reductions in long-term potentiation (LTP) (Balschun and Wetzel, 2002; Fendt and Schmid, 2002; Jia et al., 1998; Lu et al., 1997) which could potentially underpin some of the cognitive disruption observed in models of mGluR5 hypofunction.

The glutamatergic system is noteworthy for the extent of the interactions possible between the various receptor types. The mGluR5 subtype displays an expression pattern mirroring that of the NMDA receptor (NMDAR), which is indicative of the functional and spatial interactions between the two receptor types (Alagarsamy et al., 2002; Awad et al., 2000). The mGluR5 receptor is located perisynaptically, on the borders of the post-synaptic density (PSD), and is linked to the NMDAR via homer, shank and PSD-95, members of this multimeric protein complex (Ehlers, 2002; Tu et al., 1999; Yang et al., 2004); such linkages allow for reciprocal potentiation of function and synergistic activity (Awad et al., 2000; Bleakman et al., 1992; Mao and Wang, 2002). Indeed, many of the outcomes of mGluR5 blockade or ablation may stem from loss of the cooperative effects of mGluR5 and NMDAR. The disturbances in hippocampal LTP observed in mGluR5 KO mice are restricted to NMDAR-dependent pathways (Jia et al., 1998; Lu et al., 1997). The mGluR5 antagonists MPEP and MTEP augment the effects of the NMDAR antagonists PCP and 5-methyl-10,11-dihydro-5H-dibenzo cyclohepten-5,10-imine hydrogen maleate (MK-801, or dizocilpine) (Campbell et al., 2004; Henry et al., 2002; Homayoun et al., 2004; Kinney et al., 2003; Pietraszek et al., 2005a; Pietraszek et al., 2004). mGluR5 has been highlighted as a potential target for treatment of glutamatergic dysfunction in schizophrenia (Large, 2007), and administration of a novel, specific mGluR5 allosteric potentiator reversed amphetamine-mediated hyperactivity and sensorimotor gating disruptions in rodent models (Kinney et al., 2005).

Despite the fact that clozapine does not directly target glutamatergic receptors, the ability to modulate the glutamatergic system has been proposed as the basis of clozapine’s atypical profile (Heresco-Levy, 2003; Large, 2007). Clozapine is the archetypal atypical antipsychotic and shows marked polypharmacology, with affinities for D1, D2, D3, D4, 5-HT2A, 5-HT2C, muscarinic acetylcholine, adrenergic and histamatergic receptors. Through this complex pharmacology, clozapine has been shown to alter serum glutamate concentrations (Evins et al., 1997) and up-regulate NMDAR binding (Giardino et al., 1997; Ossowska et al., 1999; Pilowsky et al., 2006). The drug may also facilitate NMDAR activity indirectly through inhibition of the glycine transporter, up-regulating glycine binding to its positive modulatory site on the NMDAR (Javitt, 2004). Behaviourally, chronic administration of clozapine restores NMDAR function after PCP treatment (Ninan et al., 2003) and reverses the PPI deficits induced by ketamine and MK-801, NMDAR antagonists (Abdul-Monim et al., 2006; Bubenikova et al., 2005; Levin et al., 2005).

Given the interrelationship between NMDA and mGluR5 receptors and the sensitivity of people with schizophrenia to psychotomimetics, we wished to assess the response of mGluR5 KO mice to NMDAR antagonists. Thus, mGluR5 KO mice and wild-type (WT) littermate controls were tested for sensitivity to the locomotor stimulant effects of the NMDAR antagonist MK-801. In light of the accumulating data indicating a modifying effect of clozapine on the glutamatergic system, we also assessed the response of mGluR5 KO mice to clozapine. An earlier study showed that acute administration of clozapine was not effective in reversing the PPI deficits of the mGluR5 KO mouse (Brody et al., 2004a). However, clozapine treatment in the clinical setting is chronic, often with a significant delay between the commencement of treatment and a reduction of symptoms, thus, a longer-term experimental paradigm is more representative of the clinical setting. We therefore examined the efficacy of chronic clozapine to ameliorate the behavioural abnormalities in the mGluR5 KO mouse.
In addition, since we anticipate that the effects of long-term administration include alterations in gene transcription patterns, the expression of key neurotransmitter receptors was assessed using radioligand binding and autoradiography.

Methods

Animals

The mGluR5 KO line (Grm5tm1Rod) was obtained from Jackson Laboratories (Bar Harbor, Maine, USA) (originally generated as described in Jia et al., 1998; Lu et al., 1997). All animals were on a C57/Bl6 background (backcrossed for >10 generations), and KO animals were bred from mice heterozygous for the null mutation. WT mice were littermates of KO animals, with the genotypes determined by PCR. Mice were housed in groups of five, with food and water available ad libitum. The animal holding area was maintained on a 12-h light–dark cycle (lights on 07:00 hours). Testing was performed blind to genotype, and all procedures were approved by the institutional Animal Ethics Committee.

Two separate cohorts were used, one for the MK-801 sensitivity testing, the second for clozapine administration and subsequent behavioural testing. This was done in order to avoid the confounds of multiple drug administration.

Clozapine administration

Clozapine (Sigma-Aldrich Pty Ltd, Castle Hill, NSW, Australia) was made up in a concentrated stock solution every 4 d, and diluted to an appropriate dilution for injection each day. The initial dilution was made in 0.1 m HCl, with the solution adjusted to around pH 7, subsequent dilutions were in 0.9% saline. Clozapine was administered at 5 mg/kg, in an injection volume of 100 μl/20 g mouse. A relatively low dose was chosen as mice are sensitive to the sedative effects of this drug. However, this dose has previously been shown to reverse the effects of PCP treatment in mice (Hashimoto et al., 2005). Pilot studies demonstrated that the sedative effects did not increase with chronic treatment and could be avoided completely by performing behavioural tests at least 20 h after the daily dose was last administered.

KO and WT mice were divided into clozapine- and saline-treated groups, balanced as closely as possible by gender. The final numbers in each group were: KO/clozapine (n=7), KO/saline (n=9), WT/clozapine (n=7), WT/saline (n=8). Intraperitoneal injections of saline or clozapine were administered daily by the same experimenter. Injections were commenced when the animals were aged 8 wk, and continued for 4 wk prior to the commencement of behavioural testing. During the behavioural testing period (12–16 wk), the injections were administered immediately after each test. Hence the mice received a total of 8 wk daily injections.

Behavioural testing

For the MK-801 sensitivity testing, locomotor behaviour was assessed following an acute dose of MK-801 (Sigma-Aldrich). As a pilot study demonstrated a strong habituation response over sequential exposures to the novel environment, each mouse was used once only, and received either 0.15 mg/kg, 0.25 mg/kg MK-801, saline vehicle, or no intervention (injection control). Mice were placed into photo-beam activity arenas (E63-10, TruScan Coulbourn Instruments, Allentown, PA, USA), for 30 min before those in the saline or MK-801 groups were given an injection. Activity was then monitored for a further 60 min, and the resulting measurements were quantified using the TruScan 2.0 software (Coulbourn Instruments). For the clozapine administration cohort, a minimum of 1 wk was allowed between each behavioural testing series. The mice were placed into the dim, sound-attenuated room containing the testing apparatus for at least 1 h prior to the 15-min session. The exploratory activity of the mice (in horizontal and vertical planes) was assessed using photo-beam activity arenas (as above).

The Y-maze was performed as previously described (Sarnyai et al., 2000). Briefly, a Y-shaped maze was placed beneath a CCD camera coupled to the Noldus Ethovision recording system (Wageningen, The Netherlands). The test comprises two sections, separated by a 1-h interval. In the first session, one of the arms is blocked off, in a pseudo-random pattern, and the mouse is introduced to one arm (the home arm). The mouse is then given 10 min to explore the home arm and the second arm (designated the familiar arm). After the interval period, during which the mouse is removed from the maze, the mouse is reintroduced to the maze, with all three arms open. The animal is then given 5 min to freely explore. The duration of time spent in each arm is then tracked, along with the control measures of total distance moved and velocity.

The acoustic startle response and the PPI were assessed using automated SR Lab startle chambers (San Diego Instruments, San Diego, CA, USA). In each sound-attenuated chamber a mouse is placed into a Plexiglas cylinder attached to a piezoelectric sensor.
The startle response to the acoustic startle stimulus (pulse) is measured by the SR laboratory startle equipment. Throughout the testing session mice were exposed to a 70 dB background white noise, and this was present during a 5-min habituation period at the start of each session. Each session consisted of 80 trials where the first and last ten trials consisted of ‘pulse only’ startle-inducing stimuli of 115 dB lasting 40 ms. The central 60 pulses were a pseudo-randomized program of 20 PI15 trials, 10 with no stimulus, to measure baseline movement, and 3 × 10115-dB pulses preceded by a prepulse of 4, 8 or 16 dB above the baseline ‘white noise’ to measure the animals’ capacity for sensorimotor gating. In these instances the prepulse preceded the pulse by 100 ms and lasted for 20 ms. The PPI was expressed as a percentage inhibition of the pulse-alone startle response. This test was conducted last as it is potentially the most anxiogenic.

Tissue collection and processing

To avoid confounds arising from the administration of MK-801, tissue was only collected from the animals in the chronic clozapine study. Twenty-four hours after the final injection, animals were euthanized by cervical dislocation and the whole brain removed. Brains were immersed in Tissue Tek O.C.T. (Sakura Finetek, Japan), rapidly frozen and stored at −80 °C. Serial parasagittal sections were cut at 20 μm and mounted on gelatin/chrome alum-coated slides. Two regions were selected for analysis, medial (bounding 1.32 mm lateral to the midline) and lateral (bounding 3 mm lateral to the midline), in order to assess dorsal and ventral hippocampus, nucleus accumbens and frontal/motor and somatosensory cortex.

[^3H] radioligand binding and autoradiography

For all ligands, sections were processed in a similar manner: slides were allowed to equilibrate to room temperature for 1 h prior to preincubation in the appropriate assay buffer. Slides were dried, then incubated for 1 h in the appropriate ligand, with or without the displacing agent for non-specific binding. After 3 × 10-min washes in ice-cold buffer and a brief dip in distilled water, the slides were dried thoroughly and placed in paraformaldehyde fumes overnight. Slides are then apposed to Fuji BAS TR2025 tritium-sensitive phosphor imaging plates (Fuji Photo Film Co., Tokyo, Japan). Following an appropriate length exposure, plates were read using the Fujifilm BAS-5000 bioimaging analyser (Fuji Photo Film Co.). A standard curve was generated using [^3H] microscales (Amersham Pharmacia Biotech AB, Sweden), and this was used to convert the optical density of the image to femtomoles per milligram estimated tissue equivalent (fmol/mg ETE). The results were expressed as total binding minus non-specific binding.

For [^3H]ketanserin binding, the buffer was 170 mM Tris–HCl (pH 7.7), and the ligand was used at a concentration of 10 nM. The displacing agent was spiperone, at 10 μM (Dean et al., 2003). The [^3H]pirenzepine assay was conducted in 10 mM KH2PO4, 10 mM Na2HPO4 buffer (pH 7.4), with the ligand at 15 nM, and the displacer, QNX, at 1 μM (Dean et al., 1996). [^3H]MK-801 binding was conducted in 50 mM Tris–acetate buffer, with 100 μM glutamate, 50 μM glycine, 50 μM spermidine (pH 7.4). The ligand was used at a concentration of 10 nM, with unlabelled MK-801 as a displacer at 100 μM (Scarr et al., 2005). [^3H]YM-09151 binding was conducted at 1 nm in 50 mM Tris–HCl, 120 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 0.1 μM pindolol, 0.5 μM DTG (pH 7.4). The displacer was sulpiride at 10 μM (van den Buuse et al., 2005b).

Statistical analysis

Results of each test were assessed for normal distribution. Multiple factor ANOVA tests (with repeated-measures testing for PPI, autoradiography and MK-801 locomotor testing), were employed to assess group variations across the data, with subsequent post-hoc Bonferroni analyses performed (a conservative test encompassing correction for multiple comparisons). For the Y-maze, one-way ANOVA were performed by sex, with subsequent post-hoc Bonferroni analyses performed (a conservative test encompassing correction for multiple comparisons). Variation with sex was tested within each group dataset (as a factor in the ANOVA) before combining groups for further analysis; in no case was a significant effect observed. However, the limited availability of animals of each sex does not give this study statistical power to identify subtle or complex sex-related variation.

Results

MK-801-induced hyperactivity

In the 20-min habituation phase prior to drug administration, the KO mice showed hyperactivity relative to the WT animals (Figure 1; two-way ANOVA, genotype: F = 18.35, p = 0.0002). KO and WT animals demonstrated a similar decline in activity with habituation to the arena (time: F = 11.8, p < 0.0001; genotype × time interaction: F = 2.3, p = 0.090).
Analysis of activity after drug or saline injection showed that there was an overall significant effect of both drug administration and genotype (three-way ANOVA, drug: $F = 6.30, p = 0.0055$; genotype: $F = 14.38, p = 0.0007$). Activity levels varied over time, and the change in activity over time was influenced by drug administration (time: $F = 11.89, p = 0.0001$; time x drug interaction: $F = 4.31, p < 0.0001$). The interaction between drug administration and response over time was further modified by genotype (drug x time x genotype interaction: $F = 2.31, p = 0.0147$). In order to further dissect out the drug response, each genotype was considered separately for subsequent analyses.

The WT mice showed a dose-dependent hyperlocomotive response to MK-801 (Figure 1a; two-way ANOVA, drug: $F = 19.13, p < 0.0001$; time: $F = 2.147, p = 0.019$), with post-hoc tests demonstrating a significant effect of 0.25 mg/kg MK-801 but not 0.15 mg/kg MK-801 (Bonferroni tests, saline vs. 0.25 mg/kg, $p < 0.05$ at 60, 70, 80 min). A non-injected control group showed no variation in distance travelled compared to the saline-injected animals (data not shown, two-way ANOVA, $F = 1.374, p = 0.244$).

The mGluR5 KO mice also showed a significant response to MK-801 (Figure 1b; two-way ANOVA, treatment: $F = 43.90, p < 0.0001$; time: $F = 2.391, p = 0.0091$). However, in the KO mice this was not dose-dependent, with both low and high doses inducing marked hyperactivity (Bonferroni tests, saline vs. 0.15 mg/kg, $p < 0.01$ at 50 min, 60 min, $p < 0.05$ at 55 min, 75 min. Saline vs. 0.25 mg/kg, $p < 0.01$ at 75 min, 90 min, $p < 0.05$ at 65 min, 85 min). The effect of MK-801 was extended in the KO mice, with both 0.15 mg/kg and 0.25 mg/kg inducing a prolonged hyperactive response, and the higher dose eliciting a significant effect even at the 90-min time-point. The flattened curves may indicate a ceiling effect, as high doses of MK-801 induce stereotypy and/or ataxia that do not count towards the distance travelled. This does not represent a wholesale change in the response to MK-801, but a moderate increase in sensitivity to the drug. As in the WT animals, an injection of saline did not have a significant effect on the movements tracked (data not shown, two-way ANOVA, $F = 0.993, p = 0.321$).

**Locomotor behaviour**

The mGluR5 KO mice receiving saline displayed significant hyperactivity in comparison to the WT mice (Figure 2). A two-way ANOVA showed a significant effect of genotype ($F = 7.231, p = 0.012$), but no overall effect of clozapine ($F = 0.596, p = 0.447$). The interactive effect fell just short of significance ($F = 3.961, p = 0.056$). Post-hoc Bonferroni analyses demonstrated increased distance travelled in the KO mice receiving saline compared to the saline-treated WT mice ($p < 0.01$). Clozapine treatment did not alter the distance
travelled by WT mice (WT saline vs. WT clozapine, \( p > 0.05 \)). Clozapine treatment ameliorated the hyperactivity observed in the KO animals; the distance travelled by KO mice treated with clozapine was not statistically different to that of WT mice treated with either saline or clozapine (KO clozapine vs. WT saline and WT clozapine, \( p > 0.05 \)). However, the reduction in activity in the KO mice treated with clozapine did not achieve statistical significance (KO saline vs. KO clozapine, \( p > 0.05 \)), indicating that the effect size was small.

**Acoustic startle and PPI**

Despite a slightly reduced average startle response in the mGluR5 KO mice compared to WT mice, the variation between groups did not achieve statistical significance [Figure 3; two-way ANOVA: genotype \( F = 2.604, p = 0.118 \), drug \( F = 0.004, p = 0.948 \)]. All groups showed significant inhibition of the startle response when presented with a pre-pulse, which increased with prepulse intensity (three-way ANOVA, prepulse level: \( F = 58.55, p < 0.001 \)). There was a significant effect of clozapine treatment \( (F = 4.282, p = 0.049) \), but not of genotype \( (F = 2.51, p = 0.126) \) but no interaction between genotype and drug treatment \( (F = 2.72, p = 0.112) \), or genotype, drug and prepulse intensity \( (F = 1.012, p = 0.379) \). However, there was a significant interaction between prepulse intensity and genotype \( (F = 4.234, p = 0.02) \), indicating that there was variation between genotypes at some prepulse levels.

Subsequent analyses were performed within each prepulse (PP) level, to further assess the effect of clozapine administration and genotype. At PP4, there was a significant overall effect of clozapine (two-way ANOVA: \( F = 4.346, p = 0.047 \)), but not of genotype \( (F = 0.066, p = 0.799) \), nor a significant interactive effect \( (F = 2.655, p = 0.115) \). Post-hoc Bonferroni analysis showed significantly greater PPI in KO mice treated with clozapine relative to KO mice treated with saline \( (p < 0.05) \). Similarly, at PP8 there was a significant effect of clozapine (two-way ANOVA: \( F = 4.328, p = 0.047 \)), but not of genotype \( (F = 0.178, p = 0.676) \). There was a non-significant trend to an interactive effect \( (F = 4.051, p = 0.054) \). Post-hoc tests also showed increased PPI in clozapine-treated relative to saline-treated KO mice. At PP16, however, the effect of clozapine was no longer significant \( (F = 0.596, p = 0.447) \), whilst there was a significant effect of genotype \( (F = 7.273, p = 0.012) \), and no interactive effect \( (F = 1.080, p = 0.308) \). Post-hoc analysis showed this to be due to reduced PPI in the saline-treated KO mice relative to saline-treated WT mice.

**Y-maze**

WT mice showed a significant preference for the novel arm, irrespective of saline or clozapine treatment [Figure 4; one-way ANOVA: WT saline \( F = 7.08, p = 0.006 \), WT clozapine \( F = 12.54, p = 0.0003 \)]. The KO mice spent an equal amount of time in each arm, for both saline- and clozapine-treated mice [one-way ANOVA: KO saline \( F = 0.23, p = 0.798 \), KO clozapine \( F = 0.148, p = 0.863 \)], which indicated a failure to accurately recognize the novel arm. The control measures of distance travelled and velocity of movement did not differ between groups, demonstrating that the KO mice did not show hyperactivity in this test [two-way ANOVA, distance: genotype \( F = 0.0004, p = 0.985 \), drug \( F = 0.842, p = 0.367 \); velocity: genotype \( F = 0.00001, p = 0.997 \), drug \( F = 0.882, p = 0.356 \)].

**[3H]MK-801 binding**

[3H]MK-801 binding was homogenous across the cortex hence an integrated measure was taken. Within the
In both medial and lateral sections, a distinct layering pattern was visible throughout the cortex. Three measurements were taken through the cortical layers of both sections. CA1, CA3 and dentate gyrus were distinct in both dorsal and ventral hippocampus. Strong binding was observed in the striatum, and the nucleus accumbens core and shell could be distinguished in the medial sections. Three-way ANOVA showed a significant effect of region (Figure 7; $F = 37.21, p < 0.001$), but no effect of genotype ($F = 0.557, p = 0.466$), or drug ($F = 0.091, p = 0.766$).

${}^{[3]H}$Pirenzepine binding

In both medial and lateral sections, a distinct layering pattern was visible throughout the cortex. Three measurements were taken through the cortical layers of both sections. CA1, CA3 and dentate gyrus were distinct in both dorsal and ventral hippocampus. Strong binding was observed in the striatum, and the nucleus accumbens core and shell could be distinguished in the medial sections. Three-way ANOVA showed a significant effect of region (Figure 7; $F = 37.21, p < 0.001$), but no effect of genotype ($F = 0.557, p = 0.466$), or drug ($F = 0.091, p = 0.766$).

${}^{[3]H}$YM-09151 binding

Strong binding was observed in the striatum in both lateral and medial sections, and the nucleus accumbens core and shell. Three-way ANOVA showed no significant effect of region (Figure 8; $F = 0.368,$

Figure 4. Y-maze. (a) Duration in maze arms (mean ± S.E.M.) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (b) Velocity of movement (mean ± S.E.M.). (c) distance moved (mean ± S.E.M.). WT, Wild type; KO, knockout.
Discussion

In light of the literature suggesting spatial and functional interactions between mGluR5 and the NMDAR, we assessed MK-801-induced locomotor hyperactivity in mGluR5 KO mice and demonstrated hypersensitivity to this NMDAR antagonist in KO animals. In addition, we found that mice deficient in mGluR5 receptor show abnormalities in locomotor activity and reduced short-term spatial memory and sensorimotor gating: behaviours relevant to schizophrenia endophenotypes. Chronic administration of clozapine ameliorated the locomotor and sensorimotor deficits, but was unable to improve the cognitive impairment. Treatment with clozapine was
accompanied by an up-regulation of hippocampal and cortical NMDAR binding, suggesting that altered NMDAR activity may play a role in generating the phenotype of the mGluR5 KO, and that increases in the NMDAR reverse the effect of mGluR5 ablation. Together these findings highlight the functional interactions between mGluR5 and NMDAR, and the potential for rectifying schizophrenia-related behavioural symptoms through this pathway.

mGluR5-mediated behavioural abnormalities modified by clozapine

In the tests of locomotor activity, mGluR5 KO mice showed a hyperactive response to the novel environment of the locomotor testing arena. Such a behavioural phenotype cannot be compared directly with human symptoms, but aspects of this behaviour are relevant to the psychomotor agitation observed in schizophrenia (Sachs, 2006). Clozapine ameliorated this abnormal behaviour, which may be related to the ability of atypical antipsychotics to reverse acute agitation in the clinical setting (Caine, 2006). This reduction in activity is unlikely to be attributable to the acute sedative effects of clozapine, as the WT animals treated with saline and clozapine did not differ in activity, and at least 20 h had elapsed between the previous injection and the testing session [the half-life of clozapine is 1-2 h in mice (Baldessarini et al., 1993)].

The mGluR5 KO mice show reduced sensorimotor gating ability, as measured by PPI. This finding replicates that of other groups (Brody et al., 2004b; Kinney et al., 2003), who observed significant disruption in PPI in mice deficient in the mGluR5 receptor. Similarly, in a comparative microarray study of C57B/6 mice grouped into those with high and low PPI, down-regulation of mGluR5 was associated with lower PPI (Grottick et al., 2005). Chronic clozapine administration restored PPI to levels approximating those of WT animals at the two lower prepulse intensities. This effect is reminiscent of clozapine’s ability to reverse PCP-, ketamine- and MK-801-induced PPI deficits.

This result diverges somewhat from the findings of Brody et al. (2004a), who found that the PPI deficit of mGluR5 KO mice was not reversed by acute administration of 3 mg/kg clozapine. This discrepancy may stem from the requirement of long-term treatment to effect changes in receptor expression. As the use of antipsychotic drugs characteristically spans years or decades in the clinical setting, chronic administration may model more accurately the pharmacological status of humans. In addition, the propensity of acutely administered clozapine to elicit sedation and hence reduced startle amplitude may inhibit accurate quantification of effects of small magnitude.

The inability of mGluR5 KO mice to recognize the novel arm in the Y-maze test is indicative of a deficit in short-term memory. Elsewhere, mGluR5 KO mice have shown similar deficits in spatial learning and recall in a longer-term paradigm, the Morris water maze, and contextual fear conditioning (Lu et al., 1997). This
may be correlated to the disturbances in hippocampal synaptic plasticity observed in this KO line (Jia et al., 2001; Lu et al., 1997).

The memory deficit in mGluR5 KO mice was not reversed by chronic administration of clozapine. Clozapine is generally ascribed with improved efficacy with regards to cognitive symptoms than typical antipsychotics, although the effect size may be small (Hori et al., 2006; Keefe et al., 1999; Sharma et al., 2003). Clearly the translation of these results to a preclinical model is problematic, but it is of note in the context of the large number of differing approaches to the assessment of cognition in animal models. Clozapine has been shown to be effective in some paradigms, but both the specific memory tests employed and the mechanism of the underlying impairment varies. For example, clozapine reversed a deficit in a test of delayed spatial alternation that was elicited by a hippocampal lesion (Bardgett et al., 2006). In a separate hippocampal lesion model, clozapine did not ameliorate, but potentiated, deficits in the radial arm maze (Levin and Christopher, 2006). The ability of
Clozapine to reverse hyperactivity and sensorimotor gating deficits but not the spatial memory deficit in mGluR5 KO mice is consistent with the clinical findings of a greater impact on positive symptoms than in the cognitive domain.

Clozapine exhibits affinities for several key neurotransmitter receptors. However, in contrast to the up-regulation of NMDAR binding, we did not observe any alteration in the expression of $D_2$, $5-HT_{2A}$, or $M_1$/$M_4$ receptor subtypes in response to chronic clozapine administration. This highlights the potential role of the NMDAR in the behavioural effects of clozapine.

The role of the NMDAR in mGluR5-mediated behaviours

We observed increased sensitivity to the effects of the NMDA antagonist MK-801 in mGluR5 KO animals. In the context of the close spatial and functional associations between the NMDA and mGluR5 receptors, this finding is indicative of reduced glutamatergic function in the absence of mGluR5. Clozapine treatment up-regulates the number of open NMDAR ion channels (as measured by $[^3H]$MK-801 binding), which may reverse a deficit in NMDAR-mediated signalling in the mGluR5 KO mouse. Notably, although there was a trend towards reduced NMDAR binding in untreated KO compared to WT animals, this did not achieve statistical significance. Taken together, these results suggest that expression patterns of NMDAR are close to normal in mice lacking mGluR5, but that the signalling through these receptors may be impaired or dysregulated. Thus the antagonist activity of MK-801 is potentiated in a system where the lack of mGluR5-mediated facilitation of NMDAR currents elicits reduced glutamatergic activity. These findings are in support of several earlier studies which have demonstrated a role for the NMDAR in mGluR5-mediated function and behaviours (Campbell et al., 2004; Gravius et al., 2005; Koros et al., 2007). In WT animals, stimulation of mGluR5 receptors induces a potentiation of NMDAR currents (Pisani et al., 2001), whilst mGluR5 antagonists impair these currents (Rodrigues et al., 2002). This effect was absent in mGluR5 KO mice, indicating that these animals lack a key mechanism for amplification of NMDAR signalling. Such synergism is also observed with co-administration of mGluR5 antagonists and MK-801, where blockade of both mGluR5 and NMDA elicits greater effects on dopamine release and cognition (Homayoun et al., 2004), and on PPI and locomotor behaviour (Pietraszek et al., 2005a).

The finding of clozapine-mediated reversal of mGluR5 behavioural deficits in association with up-regulation of hippocampal and cortical NMDAR expression is novel. Disruption of NMDAR function can elicit behavioural abnormalities akin to those observed in the mGluR5 KO animal. NMDAR antagonists induce PPI disruption, locomotor hyperactivity, and cognitive disruption (reviewed in Large, 2007), behaviours observed here in the mGluR5 KO mice. The NMDAR hypomorphs also failed to respond to doses of clozapine that robustly increase PPI in WT mice, suggesting that clozapine’s effects on PPI require functional NMDAR (Duncan et al., 2006).

Of additional note is the observation that clozapine administration did not produce statistically significant effects on NMDAR expression in WT animals.
may suggest that mice lacking the mGluR5 receptor have increased sensitivity to the effects of clozapine, and is reflective of the slightly (but not significantly) reduced levels of NMDA binding in saline-treated KO mice relative to WT mice. Although the underlying mechanism is unclear, this is interesting in the context of the variable clinical response to clozapine and the potential that a subgroup within the schizophrenia population (with more predominant glutamatergic dysfunction) may respond better to clozapine.

**Mechanism of clozapine up-regulation of \[^{3}H\]MK-801 binding**

Our observation of up-regulated \[^{3}H\]MK-801 binding after clozapine treatment correlates with recent findings of increased NMDAR binding in vivo in schizophrenia patients treated with clozapine (Pilowsky et al., 2006). Increasing evidence points to effects of clozapine on NMDAR function, although the precise mechanism remains unclear. Clozapine treatment increases serum glutamate levels (Evins et al., 1997), facilitates NMDA-mediated neurotransmission (Arvanov et al., 1997), and antagonizes NMDA antagonist-mediated behaviours. Of note are the findings from several clinical trials indicating that agonists at the glycine site of the NMDAR improve the symptomatology of schizophrenia when given in conjunction with other antipsychotic drugs, but do not further improve the response to clozapine (reviewed in Millan, 2005; Shim et al., 2008).

The chronic administration of clozapine affords greater likelihood of changes in receptor transcription, translation and trafficking to the membrane than a single acute administration. However, it remains unclear whether the increased \[^{3}H\]MK-801 binding can be attributed to increased NMDAR expression, an alteration of receptor conformation or affinity for MK-801. It should be noted that the increase in \[^{3}H\]MK-801 binding in KO mice is unlikely to be a product of increased glycine occupancy resulting from the acute effects of clozapine on the glycine transporter as the reaction buffer contains sufficient glycine to maximally stimulate this co-agonist site. The increase in \[^{3}H\]MK-801 binding may also result from increases in any one of the three receptor subunits, as this compound exhibits equal affinity for the combination of NR1 + NR2a and NR1 + NR2b (Kendrick et al., 1996; Laurie and Seeburg, 1994).

**Limitations of the study**

One factor potentially inhibiting clear interpretation of the results of this study was the necessity of including both female and male animals. The mGluR5 KO line tends to display slightly reduced litter size and increased neonate mortality, which are potential contributors to behavioural variance that we cannot control. In order to obtain sufficient numbers in a single cohort, both sexes were needed. Each dataset was analysed for the effect of sex, with no significant effects observed; however, these results require replication in single-sex studies. However, it would be necessary to replicate our studies in larger cohorts if small or complex sex-related effects were to be explored. In addition, the availability of animals limited the study to a single dose of clozapine. Establishing dose responsivity would strengthen these findings.

**Conclusions and implications for antipsychotic treatment and schizophrenia**

The prominent role of mGluR5 in the regulation of NMDAR function suggests that aberrant regulation or expression of this receptor may contribute to the glutamatergic dysfunction in schizophrenia. However, thus far the evidence for a direct aetiological role for mGluR5 in schizophrenia is not conclusive. Ohnuma and colleagues found small increases in the mRNA for mGluR5 in a limited cohort of schizophrenia subjects \((n=5–6)\) in the frontal cortex but not hippocampus (Ohnuma et al., 1998, 2000). This change was restricted to layer III of Brodmann’s area 11. A study of mGluR5 protein expression showed no change in schizophrenia subjects in cortical or striatal areas (Gupta et al., 2005). However, this does not preclude there being a change in conformation, functionality, or linkage with the post-synaptic density proteins that mediate the interactions of mGluR5 with NMDAR. Intriguing data from a case-control study revealed an increase in one particular mGluR5 allele frequency in patients with schizophrenia compared to controls (Devon et al., 2001). Thus the possibility remains that this gene is an aetiological factor, at least in some segments of the schizophrenia syndrome; this remains to be investigated more fully. In addition, it is important to note in the context of schizophrenia as a developmental disorder, that there are parallel disruptions of somatosensory cortex development in mGluR5 KO animals and mice selectively lacking the NMDAR1 gene in excitatory cortical neurons (Hannan et al., 2001; Iwasato et al., 2000).

This series of experiments has afforded greater insight into one possible mechanism of action of clozapine, which was shown to restore some of the behavioural deficits elicited by ablation of mGluR5, an effect associated with up-regulation of NMDAR. This
further illustrates the functional interactions between NMDA and mGlu5 receptors and the potential relevance of this interaction to the pathophysiology and pharmacotherapy of schizophrenia-related behaviours.

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Statement of Interest

None.

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