The effect of the mGlu5 negative allosteric modulator MTEP and NMDA receptor partial agonist D-cycloserine on Pavlovian conditioned fear

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Abstract

The metabotropic glutamate receptor 5 (mGlu5) and N-methyl-D-aspartate (NMDA) receptor are critical for processes underlying synaptic plasticity, such as long-term potentiation. mGlu5 signaling increases neuronal excitability and potentiates NMDA receptor currents in the amygdala and the hippocampus. The present study examined the involvement of mGlu5 in the acquisition and consolidation of conditioned fear to a tone and context in mice, and explored the functional relationship between mGlu5 and NMDA receptors in this regard. Experiment 1 showed that systemic administration of the mGlu5 negative allosteric modulator 3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine (MTEP) prior to conditioning significantly attenuated cue-elicited freezing during fear conditioning, which suggests that mGlu5 is necessary for the formation of a tone–shock association. This effect was dose-related (Experiment 2) and not due to any effects of MTEP on shock sensitivity or state-dependency (Experiment 3). Post-conditioning injection of MTEP had no effects (Experiment 4). Although post-conditioning injection of the NMDA receptor partial agonist D-cycloserine (DCS) alone facilitated consolidation of conditioned fear (Experiment 6), it was not able to rescue the acquisition deficit caused by MTEP (Experiment 5). Taken together, these findings indicate a crucial role for mGlu5 signaling in acquisition and NMDA receptor signaling in consolidation of conditioned fear.

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Introduction

Pavlovian fear conditioning is used as a translational model to study acquisition of fears involved in some anxiety disorders, such as post-traumatic stress disorder (Shin and Liberonz, 2009). It typically involves the pairing of a neutral cue (conditioned stimulus; CS) with an intrinsically aversive experience (unconditioned stimulus; US). After repeated pairings of the CS (such as a tone) and the US (typically a footshock), the animal expresses fear to the CS alone, referred to as a conditioned response (CR). Such learning requires long-term potentiation (LTP) (Clugnet and LeDoux, 1990; Kim et al., 1991; Maren, 1999; Sigurdsson et al., 2007; Sah et al., 2008), the most studied cellular mechanism for neural plasticity (Bliss and Lomo, 1973).

Induction of LTP in most cases requires activation of the metabotropic glutamate receptor 5 (mGlu5) and/or N-methyl-D-aspartate (NMDA) receptors (Collingridge et al., 1983; Anwyl, 1999). mGlu5 are located predominantly on post-synaptic dendritic elements where they are coupled to NMDA receptor potentiation during LTP (Jia et al., 1998). These two receptors share the common endogenous ligand L-glutamate (Mayer and Westbrook, 1987), and interact via scaffolding proteins such as Homer and Shank (Ango et al., 2002). mGlu5 increase neuronal excitability and potentiate NMDA currents in brain regions implicated in fear, such as the amygdala (Niswender and Conn, 2010) and the hippocampus (Fitzjohn et al., 1996). NMDA receptors in turn can enhance mGlu5 signaling (Alagarsamy et al., 1999). Thus, there appears to be reciprocal regulation between these receptors, although the relationship is as yet unclear. Because LTP involved in fear learning is NMDA-dependent (Sah et al., 2008), this suggests a role for mGlu5 in fear-related learning.

The initial demonstration showing involvement of mGlu5 in conditioned fear reported that mutant mice devoid of mGlu5 exhibited impaired fear conditioning to a context but not to a discrete tone, which suggests that mGlu5 are important for hippocampus-dependent aversive learning (Lu et al., 1997). In contrast,
Schulz et al. (2001) observed that an i.p. injection of the mGlu5 negative allosteric modulator 2-methyl-6-(phenylethynyl)-pyridine (MPEP) prior to light CS-shock pairings significantly attenuated potentiated startle to the light CS when tested drug-free the next day, which on the surface appears contrary to the findings using mGlu5 knockout (KO) mice. Additionally, although systemic injection of MPEP provides greater clinical relevance compared to gene modulation, startle was not measured during conditioning in the study by Schulz et al. (2001), hence it is not known whether MPEP attenuated the acquisition or consolidation of light CS-US association. Identifying the stage at which mGlu5 signaling is critical in fear learning would aid in determining the potential of mGlu5 agents to affect fear memories. Further, MPEP was not used to assess context–shock learning. Importantly, MPEP has a number of off-target effects, such as antagonism of NMDA receptors (O’Leary et al., 2000) and positive allosteric modulation of the mGlu4 receptor (Mathiesen et al., 2003).

Therefore, the present study investigated whether mGlu5 was necessary in the acquisition or the consolidation of Pavlovian conditioned fear, observing CS-elicited freezing during conditioning and tests in C57BL/6J mice. We used systemic injection of 3-[(2-methyl-1,3-thiazol-4-yl)ethynyl] pyridine (MTEP), which is a mGlu5 negative allosteric modulator with ten-fold greater selectivity than MPEP (Anderson et al., 2002; Lea and Faden, 2006). To also examine whether NMDA signaling can have a down-stream effect on mGlu5 signaling, we increased NMDA signaling using systemic injection of the NMDA receptor partial agonist D-cycloserine (DCS) post-conditioning to see if it could rescue any detrimental effects of pre-conditioning injection MTEP on fear learning.

Methods

Subjects

Experimentally naive male C57Bl/6J mice (Animal Resource Centre, Australia) 8–9 wk of age at arrival were housed in groups of 4–6 in individually ventilated cages in a temperature- and humidity-controlled room under a 12 h light/dark cycle (lights on at 06:30 hours) at the Florey Behaviour Core Facility. Food and water was available ad libitum. Mice were allowed a minimum of 5 d acclimation period prior to experimentation. All procedures were approved by the local Animal Care and Ethics Committee and followed the guidelines of Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHMRC 2004).

Drugs

MTEP hydrochloride (Ascent, UK) was dissolved in 3% (wt/vol) dimethyl sulfoxide (Sigma-Aldrich, Australia) and sterile physiological saline. D-Cycloserine (C6880-1G, Sigma, USA) was dissolved in sterile physiological saline. All mice were injected i.p. at a volume of 10 ml/kg.

Apparatus

Two types of experimental chambers (Med Associates, USA), equipped with a Med Associates VideoFreeze system, were used to provide different contexts. Both types were rectangular (31.8 cm × 25.4 cm × 26.7 cm) with stainless-steel grid floors with 1/8 in rods (36 rods 3.2 on 7.9 mm centres). A constant-current shock generator could deliver electric shock to the floor of the chambers as required. A programmable tone generator, speaker and sound calibration package was used to deliver auditory cues. One type of chamber contained a white house light, curved striped walls and a tray lined with paper towels placed 10 cm beneath the grid floor. The other type had no house light, and contained plain white walls and a tray lined with bedding beneath the grid floor. Individual chambers were enclosed in sound-attenuating boxes. The experimental room in which the chambers were located was brightly lit with overhead lights. Animals were transported to the chambers in squads of four in carrier cages. Animals were assigned to the chambers so that groups were counterbalanced.

Procedures

The software and hardware used was purchased from Med Associates, Inc (St Albans, USA). The conditioned stimulus (CS) was a tone (volume: 80 dB; frequency: 5000 Hz). The unconditioned stimulus (US) was a 0.6 mA footshock. All sessions were video recorded.

Conditioning

Mice were placed in an experimental chamber for 790 s in total. They were allowed a 2 min period which was used as a baseline freezing measurement and then received six tone-footshock pairings. One pairing consisted of a 10 s tone that co-terminated with 1 s shock. Inter-trial intervals (ITI) ranged from 85 to 135 s, with an average of 110 s. Unpaired groups were placed in an experimental chamber for 791 s in total. They were allowed a 2 min baseline period and then received six tone (10 s) trials and then six footshock (1 s) trials. ITIs ranged from 30 to 80 s with an average of 55 s. For all mice, freezing was calculated from the first 9 s of each CS presentation to avoid confounding effects of the shock presentation.

Context test

Mice were exposed for 180 s to the chamber in which they were conditioned. Percentage freezing reported is based on all 180 s of test.

CS test

CS tests occurred in a chamber that was different to the conditioning context, and they commenced 1 h after
each context test. Mice were allowed a 2 min period during which baseline freezing was measured. Then they received 45 presentations of a 10 s tone in the absence of the shock with ITI of 10 s. Percentage freezing reported is based on 10 s of tone blocked into average freezing of five tones.

**Shock sensitivity testing**

Mice were allowed 20 s period after which they received up to 30 shocks increasing in intensity from 0.15 to 0.30 mA. The ITIs were 8 s and the intensity of the shock was increased by 0.01 mA every second shock. Three behavioural responses (flinching, shuffling and vocalizations) were measured by two independent scorers blind to the drug condition (Graham and Richardson, 2009). Specifically, *flinching* was defined as a sudden, brief, muscle contraction, not involving any locomotion, while *shuffling* was a more pronounced movement involving locomotion. *Vocalization* was defined as any audible vocal sound. Thresholds were defined as the lowest shock intensity at which the behaviour was exhibited, and the test terminated when all three behaviours were observed.

**Data analysis**

To assess freezing we used automated near-infrared video tracking equipment and computer software (Video Freeze, Med Associates). Manual scoring of experiments 1 and 2 was first used to determine a motion threshold for use with the Med Associates software. We used freezing, as the species-specific fear defense response, as the dependent variable (Blanchard and Blanchard, 1969), and it was defined as the absence of all movement other than that required for respiration. Freezing was scored with a time-sampling procedure in which each mouse was observed every 2 s and scored as either freezing or moving, and a percentage score was calculated. Two observers who were unaware of the subject’s group designation participated in manual scoring. There was a high degree of agreement between the two observers (r>0.9), and both observers scores correlated with the Video Freeze software when a motion threshold of 50 was used (r>0.9). Hence, all data reported in the present study used automated scoring by Video Freeze software with the motion threshold of 50.

Freezing data was statistically analysed using between-subjects analysis of variances (ANOVA), repeated-measures ANOVAs and independent samples t-tests where appropriate. For experiments involving more than two groups per between-subjects factor, post-hoc tests (Tukey’s HSD) were performed following significant overall ANOVA. The level of significance used for all analyses was p<0.05. In Experiment 6, data from one mouse from the saline group was excluded from the entire experiment, because its CS-elicited freezing during test was 3 S.D. different to the mean. No other mice were excluded from the study.

**Results**

**Experiment 1: mGlu5 is important for the acquisition of conditioned fear**

To examine the role of mGlu5 on conditioned fear to a tone CS and the context, C57Bl/6j mice were injected with MTEP (30 mg/kg) or vehicle 30 min prior to conditioning. The next day, fear memory to the context was first tested (by placing the mice in the conditioning context). One hour following the context test, memory to the tone CS was tested in a different context to the conditioning context. The context and cue tests were then repeated the next day in a similar manner (Fig. 1a).

Baseline levels of freezing at all phases of the experiments are reported in Table 1. There were no significant differences in baseline levels of freezing in any experiment. A repeated-measures analysis of variance (ANOVA) of the data during conditioning showed a main effect of Conditioning Trial (F\(_{5,170}=31.626, p<0.001\)) and Drug (F\(_{1,34}=10.125, p<0.05\)) as well as a Block 
\(F_{8,272}=3.100, p<0.05\)) a three-way Conditioning Trial–Drug–Pairing Condition interaction (F\(_{5,170}=6.094, p<0.001\)) (Fig. 1b). These results indicate that unpaired groups did not exhibit any increase in fear to the cue compared to the paired groups. Importantly, MTEP significantly attenuated the acquisition of fear conditioning to the cue in the paired groups, but had no effects in unpaired groups, which indicates that mGlu5 is important for the acquisition of conditioned fear to a tone cue.

All CS tests were analysed in blocks of five CSs. Repeated-measures ANOVA on the first day of cue testing revealed a main effect of Block (F\(_{8,272}=9.791, p<0.001\), Drug (F\(_{1,34}=24.045, p<0.001\)) and Pairing Condition (F\(_{1,34}=19.739\)) as well as a Block–Pairing Condition interaction (F\(_{8,272}=10.045, p<0.001\), a Block–Drug interaction (F\(_{8,272}=2.703, p<0.05\)), and a Pairing Condition–Drug interaction (F\(_{1,34}=9.388, p<0.05\)) but no other effects (biggest F = 1.752) (Fig. 1c). Similar analyses on the second day of cue testing revealed effects of Block (F\(_{8,272}=3.873, p<0.001\), Drug (F\(_{1,34}=7.762, p<0.05\)), and interactions between Block–Pairing Condition (F\(_{8,272}=3.100, p<0.05\)), and Block–Drug (F\(_{8,272}=2.196, p<0.05\)) but no other effects (biggest F=2.603) (Fig. 1d). As before, unpaired groups exhibited no fear to the cue compared to the paired groups. In the paired groups, the levels of freezing of the MTEP groups were significantly less than the vehicle group, and the effects were maintained on both days of testing. This indicates that MTEP significantly impaired fear acquisition to the tone CS, and the subsequent fear memory is reduced.

A two-way ANOVA of the data obtained from the context tests revealed no significant effects of pairing condition, but a main effect of Drug on the first day of
testing \(F_{1,37} = 22.843, p<0.001\) (Fig. 1e), which was maintained on the second day of testing \(F_{1,37} = 6.838, p<0.05\) (Fig. 1f). The absence of difference between the paired and unpaired group was expected as their contextual fear should be unaffected by the tone–shock contingency. The results indicate that MTEP attenuated contextual fear learning, and the effects were maintained on both days of testing.

**Experiment 2: reducing mGlu5 signaling attenuates conditioned fear acquisition in a dose related manner**

To investigate whether the effects of MTEP on the acquisition of fear conditioning were dose related, we gave 3, 10 or 30 mg/kg of MTEP 30 min prior to conditioning. A repeated-measures ANOVA of the conditioning data showed a main effect of Conditioning Trial \(F_{5,180} = 62.658, p<0.001\) and Dose \(F_{3,36} = 8.751, p<0.001\) as well as an interaction between the two \(F_{15,180} = 2.443, p<0.05\). Post-hoc Tukey’s HSD tests revealed this was due to significant differences between the vehicle vs. 10 and 30 mg/kg MTEP \(p<0.05\), as well as a significant difference between the doses of 3 mg/kg MTEP and 30 mg/kg MTEP \(p<0.05\). These results suggest that doses of 10 and 30 mg/kg, but not 3 mg/kg of MTEP, attenuate the acquisition of auditory fear in mice (Fig. 2a).

Consistent with experiment 1, impaired acquisition of conditioned fear led to impaired fear memory the next day (Fig. 2b). On the first day of cue testing, repeated-measures ANOVA revealed a main effect of Block \(F_{8,288} = 20.883, p<0.001\) and Dose \(F_{3,36} = 5.739, p<0.05\). Tukey’s HSD tests showed significant differences between the vehicle vs. 10 and 30 mg/kg MTEP \(p<0.05\), as well as a difference between the doses of 3 and 30 mg/kg MTEP \(p<0.05\) but no other significant differences. On the second day, there was a main effect of Block \(F_{8,288} = 19.234, p<0.001\) and a Block–Dose interaction \(F_{24,288} = 1.688, p<0.05\) but no other effects (Fig. 2c).

A one-way ANOVA of the first context test revealed an overall significant effect of Dose \(F_{3,288} = 10.586, p<0.001\) and subsequent Tukey’s HSD tests revealed significant differences between vehicle vs. 10 and 30 mg/kg MTEP \(p<0.05\), as well as a significant difference between the doses of 3 and 30 mg/kg MTEP \(p<0.05\) (Fig. 2d). For the second context test there was a main effect of Dose \(F_{3,288} = 3.545, p<0.05\). Tukey’s HSD tests showed a significant difference between the vehicle and 30 mg/kg MTEP \(p<0.05\), but no other differences (Fig. 2e).

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**Fig. 1.** (a) General experimental design used in the present study. The mean (±S.E.M) levels of CS-elicited freezing for experiment 1 during conditioning (b), the first cue test (c) and the second cue test (d). The mean (±S.E.M) levels of context-elicited freezing during the first (e) and second (f) day of testing. Pre-conditioning injection of MTEP impaired the acquisition of auditory and contextual fear in mice (n=10 per group).
These results indicate that pre-conditioning administration of MTEP significantly attenuated the acquisition of contextual fear at doses of 10 and 30 mg/kg.

**Experiments 3a & b: MTEP does not affect shock sensitivity, and the effect of MTEP on memory is not due to state-dependent learning**

To determine whether the observed effect of MTEP on conditioned fear was due to any potential changes in shock sensitivity, mice were injected with the highest effective dose of MTEP (30 mg/kg) vs. vehicle 30 min prior to testing in experiment 3a. They were then placed in a chamber where they received up to 30 shocks increasing in intensity from 0.15 to 0.30 by 0.01 mA every second shock (Graham and Richardson, 2009). Flinching, shuffling and vocalizations to the shock were measured, and the shock intensity at each behaviour was first exhibited was recorded. There was a main effect of the behavioural measure \( F(2,12) = 27.8, p < 0.001 \) indicative of differences in the mean shock intensity for mice to first display flinching, shuffling and vocalization. There was no effect of Drug and no other interactions (biggest \( F = 1.4 \) ) (Fig. 2f).

In experiment 3b, we tested whether the reduced context- and CS-elicited freezing observed during tests were due to MTEP providing a particular internal context during fear acquisition that was substantially different from the drug-free tests. Such a change in internal context may interfere with the retrieval of the context- and the CS-related memories. Mice were first injected with vehicle or MTEP (10 mg/kg) before conditioning as described in experiment 1. Mice were then injected with vehicle or MTEP (10 mg/kg) 30 min before the context or the CS test. One type of test was conducted each day to keep the injection–test interval constant. The test order was counterbalanced. Repeated-measures ANOVA of the conditioning day revealed a main effect of Conditioning Trial \( (F_{5,135} = 53.423, p < 0.0001) \), and Group–Conditioning Trial interaction \( (F_{10,135} = 4.065, p < 0.001) \), replicating the original MTEP effect (Fig. 3a). Similar analyses of cue testing revealed a main effect of Block \( (F_{8,216} = 19.727, p < 0.001) \), Groups \( (F_{2,27} = 4.433, p < 0.05) \), and a Block–Group interaction \( (F_{16,216} = 2.703, p < 0.05) \) (Fig. 3b). Post-hoc Tukey’s HSD tests revealed significant differences between the vehicle–vehicle vs. MTEP–vehicle and MTEP–MTEP (Ps<0.05). A one-way ANOVA of the context test data revealed an overall significant effect of Groups \( (F_{2,27} = 7.439, p < 0.005) \), and Tukey’s HSD tests revealed significant differences between the vehicle–vehicle vs. MTEP–vehicle and MTEP–MTEP (Ps<0.05) (Fig. 3c). These results show that context- and CS-associated fear acquisition deficit caused by MTEP is not reversed by pre-test injection of MTEP.

**Experiment 4: mGlu5 is not necessary for the consolidation of conditioned fear**

To investigate whether mGlu5 are involved in the consolidation of fear memory, mice were injected with 30 mg/kg MTEP or vehicle immediately after conditioning.

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**Table 1. Mean (±S.E.M) % freezing at baseline for all phases of the experiments**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Group</th>
<th>Conditioning</th>
<th>1st cue test</th>
<th>2nd cue test</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Paired–MTEP</td>
<td>0.3±0.2</td>
<td>1.2±0.4</td>
<td>2.3±0.6</td>
</tr>
<tr>
<td></td>
<td>Paired–vehicle</td>
<td>0.5±0.3</td>
<td>5.5±2.4</td>
<td>6.8±2.2</td>
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<tr>
<td></td>
<td>Unpaired–MTEP</td>
<td>0.4±0.3</td>
<td>6.5±4.0</td>
<td>8.2±4.1</td>
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<tr>
<td></td>
<td>Unpaired–vehicle</td>
<td>0.3±0.2</td>
<td>18.7±7.9</td>
<td>8.1±3.3</td>
</tr>
<tr>
<td>2</td>
<td>Vehicle</td>
<td>1.0±0.4</td>
<td>20.1±7.1</td>
<td>22.3±8.9</td>
</tr>
<tr>
<td></td>
<td>3 mg/kg MTEP</td>
<td>0.1±0.1</td>
<td>17.8±7.3</td>
<td>17.3±5.9</td>
</tr>
<tr>
<td></td>
<td>10 mg/kg MTEP</td>
<td>0.7±0.4</td>
<td>7.8±3.5</td>
<td>15.7±5.0</td>
</tr>
<tr>
<td></td>
<td>30 mg/kg MTEP</td>
<td>0.3±0.4</td>
<td>3.8±1.7</td>
<td>10.6±3.3</td>
</tr>
<tr>
<td>3b</td>
<td>Vehicle–vehicle</td>
<td>0.2±0.2</td>
<td>3.2±1.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MTEP–vehicle</td>
<td>0.2±0.2</td>
<td>4.2±1.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MTEP–MTEP</td>
<td>0.2±0.2</td>
<td>7.0±2.3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Paired–MTEP</td>
<td>0.1±0.1</td>
<td>11.8±7.8</td>
<td>13.4±6.5</td>
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<tr>
<td></td>
<td>Paired–vehicle</td>
<td>0.5±0.4</td>
<td>9.1±4.8</td>
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<td>Unpaired–MTEP</td>
<td>0.0±0.0</td>
<td>12.9±6.4</td>
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<tr>
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<td>Unpaired–vehicle</td>
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<td>15.2±6.6</td>
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<td>5</td>
<td>Vehicle–vehicle</td>
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<td>10 mg/kg MTEP–vehicle</td>
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<td>1.9±0.4</td>
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</tr>
<tr>
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<td>10 mg/kg MTEP–15 mg/kg DCS</td>
<td>0.4±0.1</td>
<td>4.2±1.5</td>
<td>15.1±3.5</td>
</tr>
<tr>
<td></td>
<td>10 mg/kg MTEP–30 mg/kg DCS</td>
<td>1.9±0.5</td>
<td>4.6±0.8</td>
<td>23.3±4.1</td>
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<tr>
<td>6</td>
<td>Vehicle</td>
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<td>15.3±6.2</td>
<td>12.0±6.0</td>
</tr>
<tr>
<td></td>
<td>15 mg/kg DCS</td>
<td>0.2±0.2</td>
<td>38.9±16.0</td>
<td>26.4±9.1</td>
</tr>
</tbody>
</table>
A repeated-measures ANOVA of the data during conditioning showed a main effect of Conditioning Trial ($F_{5,190}=24.650$, $p<0.001$), and Pairing Condition ($F_{1,38}=83.046$, $p<0.001$) and a Conditioning Trial–Pairing interaction ($F_{5,190}=22.709$, $p<0.001$) (Fig. 4a), showing that unpaired groups failed to acquire CS-elicited freezing during conditioning compared to paired groups.

Repeated-measures ANOVA on the first day of cue testing revealed effects of Block ($F_{8,288}=8.603$, $p<0.001$), Pairing Condition ($F_{1,36}=18.656$, $p<0.001$) and a Block–Pairing interaction ($F_{8,288}=5.732$, $p<0.001$), but no effect of Drug or any interactions (biggest $F=1.873$) (Fig. 4b). The results were the same for the second day of testing, with significant effects of Block ($F_{8,288}=6.814$, $p<0.001$),
and a Block–Pairing Condition interaction ($F_{8,288} = 5.861$, $p < 0.001$), but no other effects or interactions (biggest $F = 2.432$) (Fig. 4c). A two-way ANOVA of context tests revealed no effects for both the first and the second (biggest $F = 1.014$) day of testing (Fig. 4d, e). These results indicate that post-conditioning injection of MTEP had no effects on cue or context fear memory, which suggest mGlu5 signaling is not critical for the consolidation of conditioned fear.

**Experiment 5: increasing NMDA signaling post-conditioning does not rescue the deficit in fear learning caused by pre-conditioning injection of MTEP**

mGlu5 and NMDA receptors share the common endogenous ligand L-glutamate (Mayer and Westbrook, 1987), and interact via scaffolding proteins such as Homer and Shank via protein kinase C (PKC) (Ango et al., 2002). Agonists of mGlu5 increase NMDA receptor-mediated currents in brain regions implicated in learned fear such as the amygdala (Niswender and Conn, 2010), and the hippocampus (Fitzjohn et al., 1996; Doherty et al., 1997). Conversely, MPEP potentiates the effects of NMDA receptor antagonists (Homayoun and Moghaddam, 2006), whilst the mGlu5 positive allosteric modulator 3-cyano-N-(1,3 diphenyl-1H-hyrazol-5-yl)benzamide (CDPPB) can reverse the effect of the NMDA antagonist MK801 on cortical neurons (Lecourtier et al., 2007). The latter finding especially suggests that mGlu5 signaling is downstream from NMDA signaling, and manipulation of the mGlu5 may ‘trump’ the effects of NMDA. This notion is consistent with a recent finding that showed systemic administration of CDPPB reversed the attenuated conditioned aversion learning caused by systemic injection of the NMDA antagonist MK801 (Fowler et al., 2011).

However, NMDA receptors in turn can affect mGlu5 signaling. For example, low doses of NMDA potentiate mGlu5 function, whilst higher doses result in a decrease in mGlu5 function (Challiss et al., 1994; Alagarsamy et al., 1999), suggesting a reciprocal regulation between NMDA receptors and mGlu5. Moreover, in Fowler et al. (2011), CDPPB injection occurred immediately after MK801 injection, therefore the direction of effect is unclear. Therefore, we tested for the first time whether increasing NMDA signaling after conditioning can reverse the impairment of fear learning caused by MTEP. Mice were injected with vehicle or MTEP (10 mg/kg) 30 min prior to conditioning. All MTEP groups were then injected with vehicle or DCS (15 or 30 mg/kg) immediately after conditioning. Repeated-measures ANOVA of the conditioning data revealed a main effect of Conditioning Trial ($F_{5,110} = 51.181$, $p < 0.001$) and Group
Vehicle and all three MTEP groups (Ps < 0.05) (Fig. 5). The repeated-measures ANOVA of the second cue test showed a main effect of Block (F\(_{1,110} = 2.798, p < 0.05\)). Tukey’s HSD tests revealed significant differences between the vehicle and all other MTEP groups (Ps < 0.05) (Fig. 5a).

Repetitive-measures ANOVA of the data from the first cue test revealed a main effect of Block (F\(_{8,176} = 11.262, p < 0.001\)), and Group (F\(_{3,22} = 5.281, p < 0.05\)), but no other effects (biggest F = 1.071). Tukey’s HSD test showed significant differences between the vehicle and all three MTEP groups (Ps < 0.05) (Fig. 5b). Repeated-measures ANOVA of the second cue test showed a main effect of Block (F\(_{8,176} = 12.464, p < 0.001\)), and a Group–Block interaction (F\(_{24,176} = 1.910, p < 0.05\)), but no other significant effects (biggest F = 2.393) (Fig. 5c).

An overall one-way ANOVA of the context tests revealed no significant effects on either day (1st test: F\(_{3,22} = 2.701, p = 0.070\); 2nd test: F\(_{3,22} = 2.275, p = 0.108\)). However, due to an observed trend towards significance, the MTEP groups were collapsed and an independent samples t-test was performed. This revealed a significant effect of pre-conditioning MTEP compared to vehicle (1st test: t\(_{24} = 2.933, p = 0.07\); 2nd test: t\(_{24} = 2.082, p = 0.048\)). These results indicate that impairments in acquisition of conditioned fear to a cue or context caused by MTEP cannot be reversed by post-conditioning injection of DCS (Fig. 5d, e).

**Experiment 6: DCS facilitates conditioned fear to a tone but not to the context**

To validate that increasing NMDA signaling by DCS can in fact facilitate conditioned fear regardless of MTEP treatment, we systemically injected mice with DCS (15 mg/kg) or vehicle immediately following fear conditioning. During conditioning, there was a main effect of Conditioning Trial (F\(_{5,35} = 16.900, p < 0.001\)), but no other effects (biggest F = 1.371), indicating that there were no pre-existing differences in acquisition of conditioned fear before the DCS or vehicle injection (Fig. 5a).

Interestingly, repeated-measures ANOVA of the first cue test showed a main effect of Block (F\(_{8,88} = 5.660, p < 0.001\)) and Drug (F\(_{1,11} = 6.262, p < 0.05\)) but no interaction between the two (F = 1.063). These results suggest that post-conditioning injection of DCS significantly enhanced consolidation of conditioned fear to a tone in mice (Fig. 6b). On the second day of testing, repeated-measures ANOVA revealed a main effect of Block (F\(_{8,88} = 9.626, p < 0.001\)), Drug (F\(_{1,11} = 7.697, p < 0.05\)), as well as a Block–Drug interaction (F\(_{8,88} = 3.345, p < 0.05\)), indicating that the enhanced conditioned fear to a tone was maintained on the second day of testing (Fig. 6c). An independent samples t-test of the context tests revealed no significant differences on either day (1st Context...
Test: $t_{11} = 0.1, p = 0.9; 2\text{nd Context Test: } t_{11} = 1.5, p = 0.2$. It appears that DCS may have a selective effect in enhancing consolidation of conditioned fear to a tone but not to a context (Fig. 6d, e).

Discussion

Here we demonstrate for the first time that systemic injection of MTEP significantly attenuates acquisition of conditioned fear as measured during conditioning. The effect of MTEP was not due to any potential effects on shock sensitivity or changes in the internal context. The lack of effect of MTEP on shock sensitivity is consistent with numerous previous findings that show mGlu5 antagonism affects chronic, but not acute, pain (Kozela et al., 2003; Sevostianova and Danysz, 2006; Zhou et al., 2013). Importantly, MTEP had no effects on baseline levels of freezing in any experiment. This is consistent with a previous study that showed MTEP does not affect locomotor activity in the same strain of mice when injected at 10 mg/kg (Cowen et al., 2007). Moreover, the latter study (Cowen et al., 2007) showed that 20 mg/kg MTEP also did not affect locomotor activity, while 40 mg/kg MTEP did cause hypolocomotion. Therefore, it is hypothetically possible that our highest dose of MTEP in the current study (30 mg/kg) may conceivably cause decreases in locomotor activity. However, we feel this is most unlikely since such an outcome would lead to increased (not decreased as we observed) freezing.

We also observed memory deficits during drug-free tests, which further highlights that the present results are not due to MTEP affecting freezing behaviour. We observed no differences in the orienting responses of the mice to the tone CS, or freezing to the first CS during conditioning, highlighting that MTEP did not affect processing of the CS.

Nevertheless, it may be the case that reduction in mGlu5 signaling affected the formation of contextual representation, rather than the context–shock association. Interestingly, systemic or central injection of the mGlu5 negative allosteric modulator MPEP has no effects on rat spatial learning ability in the Morris Water Maze (Ballard et al., 2005; Car et al., 2007), which suggest that pharmacological reduction of mGlu5 signaling may not affect configurational learning to associate different neutral stimuli in the environment. Collectively, the present results suggest that learning to associate stimuli with aversive events requires mGlu5 signaling.

Our findings of a crucial role for mGlu5 in conditioned fear learning processes are consistent with the previous literature (Maciejak et al., 2003). For example, Rodrigues and colleagues gave bilateral intra-amygdala infusions of MPEP prior to fear conditioning in rats, and demonstrated an impairment of short-term and long-term expression of fear compared to vehicle (Rodrigues et al., 2002). Our results provide strong evidence that the mechanism behind these findings is likely due to the failures in the actual acquisition of stimuli–shock association during...
fear conditioning (Experiments 1, 2 and 5). We also observed that once the stimuli–shock association was strongly established, the consolidation of that learning appears mGlu5 independent because post-conditioning MTEP injection had no effects (Experiment 4). Consistent with our results, post-training infusions of MPEP into the amygdala were found to have no effects when rats were subsequently tested for conditioned fear (Rodrigues et al., 2002).

However, Lu et al. (1997) demonstrated that mGlu5 KO mice showed comparable acquisition but impaired consolidation of context fear conditioning to wild type mice. In that study, animals were exposed to a single tone co-terminating with a shock. Following the single shock, KO mice and wildtype littermates showed comparable freezing to the conditioning context for the rest of the session. When tested the next day, mGlu5 KO mice exhibited significantly less freezing to the conditioning context, whereas freezing to the discrete tone was unimpaired, hence the authors concluded that mGlu5 signaling is selectively important for consolidation of contextual fear. In that study, the deficits in LTP exhibited by the mGlu5 KO mice were only examined in the hippocampus and not in the amygdala. It is possible that by some compensatory mechanisms LTP in the amygdala was preserved in those mice, despite the importance of mGlu5 in some forms of amygdaloid LTP (Niswender and Conn, 2010). Additionally, the discrepancy between the present findings and Lu et al. (1997) may be because our acute pharmacological manipulation following conditioning did not produce a sufficiently sustained reduction in mGlu5 signaling compared to global mGlu5 KO mice, thus not affecting consolidation processes in the present study. Therefore, it is still possible that mGlu5 signaling modulates fear memory consolidation if manipulated more chronically. In any case, it is interesting that the effects resulting from the complete lack of mGlu5, are distinct from those resulting from acute pharmacological manipulation of these receptors. Conditional mutants, where anatomically discrete deletions of receptor are made in adult mice, will help to clarify how anatomically specific the role of mGlu5 is in acquisition and consolidation of conditioned fear.

Importantly, we observed significant facilitation of conditioned fear consolidation following the administration of DCS (Experiment 6). This is consistent with a role for NMDA signaling in conditioned fear consolidation (Miserendino et al., 1990), and it has been reported that DCS administration 30 min prior to a fear memory reactivation session can facilitate reconsolidation (Lee et al., 2006). However, to our knowledge, no one has yet demonstrated the ability of post-conditioning DCS to enhance the initial consolidation of fear, despite a number of demonstrations of DCS’s ability to facilitate the initial consolidation of extinction in animals as well as humans (Walker et al., 2002; Ressler et al., 2004). Our finding highlights that NMDA signaling is sufficient as well as necessary for conditioned fear consolidation.

We also observed that DCS was unable to rescue the deficit caused by pre-conditioning MTEP administration (Experiment 5). These results suggest that DCS can enhance fear learning only when the learning is optimal, or above a certain threshold. Hence, when pre-conditioning injection of MTEP impaired the formation of CS–US association in the present study, DCS was not able to alleviate the poor learning. Supporting this idea, it has been shown that DCS facilitated extinction to an olfactory cue in rats, but only when these rats had displayed substantial within-session extinction (Weber et al., 2007). In another study, rats that showed below-median levels of extinction acquisition did not show any effects of DCS, in contrast to those showing above median extinction acquisition (Bouton et al., 2008). These results indicate that optimal memory formation is first necessary for DCS to facilitate the consolidation of that memory, which explains some of the literature in which DCS had no effects on extinction and fear conditioning in humans (e.g. Guastella et al., 2007).

The modulating effect of DCS appears dependent on the level of activation of mGlu5. This suggests that manipulation of NMDA receptor signaling after mGlu5 manipulation has no effects, and NMDA signaling is not downstream of mGlu5, but rather that the effect of mGlu5 modulation is downstream of NMDA. This result strengthens the conclusions of Fowler et al. (2011) that changes in mGlu5 signaling following NMDA signaling via an injection of CDPPB immediately following the injection of the NMDA antagonist MK801 reverses the effects of MK801. During CS–US pairings in Pavlovian conditioning, mGlu5 signaling likely affects NMDA-potentiated PKC postsynaptically in the lateral amygdala where the information on CS and US converge (Rodrigues et al., 2002). In support of this idea, PKC inhibition can impair acquisition, but not consolidation of conditioned taste aversion (Sacchetti and Bielavska, 1998). In the present study, MTEP may have disrupted conditioning-induced NMDA-dependent LTP in the lateral amygdala via disruption of PKC (Malenka et al., 1989).

It is important to note that antagonism of mGlu5 also affects other types of learning, such as novel object recognition and inhibitory avoidance (Simonyi et al., 2007; Goh and Manahan-Vaughan, 2013). Future studies would also benefit from further exploring the interactions between the two receptors in fear extinction, because mGlu5 are also implicated in extinction of conditioned fear (Xu et al., 2009; Sepulveda-Orengo et al., 2013). Interestingly, mGlu5 are differentially regulated throughout development (Catania et al., 1994), which is significant considering that the nature of extinction appears to change across development (Kim and Richardson, 2008, 2010).

Our results also have important translational implications for future research seeking to identify potential
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Statement of Interest

None.

References


Kim JH, Richardson R (2008) The effect of temporary amygdala inactivation on extinction and re-extinction of fear in the developing rat: unlearning as a potential treatments for anxiety disorders, especially considering the increased receptor specificity and the reduced side effects that can be achieved by allosteric modulation of metabotropic glutamate systems (Niswender and Conn, 2010). Our findings suggest that although mGlu5 may be critical for moderating the emotional associations to the cues present during a fearul event, once a fear memory has been formed, mGlu5 modulation alone may not be effective in treating anxiety disorders.