Regulation by Metabotropic Glutamate Receptor 5 of LTP in the Dentate Gyrus of Freely Moving Rats: Relevance for Learning and Memory Formation

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Group I metabotropic glutamate (mGlu) receptors play a critical role in the regulation of hippocampal long-term potentiation (LTP) *in vivo*. Little is known, however, about the contribution of the individual subtypes mGlu1 and mGlu5 to learning processes and LTP. We investigated the involvement of mGlu5 in hippocampal LTP and spatial learning using the selective antagonist 2-methyl-6-(phenylethynyl)pyridine (MPEP). Rats were chronically implanted with recording and stimulating electrodes to enable measurement of evoked potentials from the medial perforant path — dentate gyrus granule cell synapses. An injection cannula was inserted into the ipsilateral cerebral ventricle to enable drug application. Experiments were begun 10 days subsequent to the implantation procedure. Robust LTP which lasted for over 25 h was generated using 200 Hz tetanization. MPEP, applied in concentrations which did not affect basal synaptic transmission, dose-dependently impaired the induction and expression of LTP. Application of MPEP 5 min after tetanization inhibited late LTP (>24 h). The effects of daily MPEP application on performance in an eight-arm radial maze were evaluated. MPEP-treated rats showed deficits in reference and working memory performance compared to vehicle-treated controls. Rearing, grooming and locomotor activity were unaffected in MPEP-treated animals. These data highlight the importance of mGlu5 for both LTP and spatial learning and emphasize the significance of these receptors for information storage on both synaptic and behavioural levels.

Keywords: hippocampus, *in vivo*, long-term potentiation, MPEP, synaptic plasticity, Wister

Introduction

Group I metabotropic glutamate (mGlu) receptors appear to play an important role in both hippocampal synaptic plasticity *in vivo* (Manahan-Vaughan and Reymann, 1996; Manahan-Vaughan, 1997; Manahan-Vaughan *et al.*, 1996, 1998, 1999a) and hippocampus-based learning (Balschun *et al.*, 1999; Balschun and Wetzel, 2002). Thus, pharmacological antagonism of these receptors results in a significant inhibition of both long-term potentiation (LTP) and long-term depression, as well as marked impairments in spatial learning.

Group I metabotropic glutamate receptors comprise mGlu1 and mGlu5, both of which are coupled to phospholipase C via Gq and mediate phosphoinositide hydrolysis. Despite their coupling to the same effector protein, these receptors appear to exhibit quite distinct functions. Selective activation of mGlu1 has been shown to result in an increase in intracellular calcium concentrations, a direct depolarization of CA1 pyramidal neurons, an increased frequency of spontaneous inhibitory postsynaptic potentials, as well as an inhibition of synaptic transmission in the hippocampal CA1 region (Mannaioni *et al.*, 2001). Activation of mGlu5, on the other hand, results in suppression of the calcium-activated potassium current (I_{AHP}) and a potentiation of NMDA receptor currents (Jia *et al.*, 1998; Attucci *et al.*, 2001; Mannaioni *et al.*, 2001).

The precise role of the group I mGlu receptor subtypes mGlu1 and mGlu5 in synaptic plasticity and behavioral learning remains unclear. A subtype specific involvement of mGlu1 and mGlu5 in synaptic plasticity has been suggested by studies in transgenic mice. Thus, in one study, mice lacking mGlu1 were demonstrated to show normal hippocampal LTD but impaired LTP (Aiba *et al.*, 1994), whereas in another study hippocampal LTP was found to be normal (Conquet *et al.*, 1994). In mice lacking mGlu5 hippocampal LTP expression was abnormal and deficits in both spatial learning and fear conditioning were reported (Lu *et al.*, 1997; Jia *et al.*, 1998). Interpretation of transgenic data is complicated, however, by possible developmental adaptations which may occur within the animal to compensate for the absent receptor. Pharmacological analysis of the contribution of the individual mGlu receptor subtypes to plasticity and learning has also been hindered by the lack of availability of selective ligands. The recent advent of highly specific antagonists for mGlu receptors offers new opportunities to clarify the contribution of these receptors to these processes.

In recent work, we have found that induction of LTP is coupled with an increase in the expression of mGlu5 but not mGlu1 receptor protein in the rat hippocampus (Manahan-Vaughan *et al.*, 1999b). An induction of mGlu5 receptor expression following certain learning behavior has been reported (Riedel *et al.*, 2000) and it has been demonstrated that dendritic protein synthesis is stimulated by activation of mGlu5 (Huber *et al.*, 2000, 2001), consistent with an important role for this receptor in information storage. This study therefore set out to address the involvement of mGlu5 in synaptic plasticity and spatial learning using the novel highly selective antagonist for mGlu5: methyl-6-(phenylethynyl) pyridine (MPEP).

Materials and Methods

Electrode Implantation

Male Wistar rats (7–8 weeks old) underwent electrode implantation into the dentate gyrus as described previously (Manahan-Vaughan *et al.*, 1998; Kulla and Manahan-Vaughan, 2000). Briefly, under sodium pentobarbitone anaesthesia (Nembutal, 40 mg/kg, i.p.; Serva, Germany), animals underwent implantation of a monopolar recording and a bipolar stimulating electrode (made from 0.1 mm diameter teflon coated stainless steel wire). A drill hole was made (1.0 mm diameter) for the recording electrode (3.1 mm posterior to bregma, 1.9 mm lateral to the midline) and a second drill hole (1.5 mm diameter, 6.9 mm posterior to bregma and 4.1 mm lateral to the midline) for the stimulating electrode. The dura was pierced through both holes and the recording and stimulating electrodes lowered into the

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dentate gyrus granule cell layer and the medial perforant path, respectively. Recordings of evoked field potentials via the implanted electrodes were taken throughout surgery. A cannula was also implanted into the ipsilateral cerebral ventricle, through which drug application could subsequently be made. Once verification of the location of the electrodes was complete, the entire assembly was sealed and fixed to the skull with dental acrylic (Paladur; Heraeus Kulzer GmbH, Germany). The animals were allowed 7–10 days to recover from surgery before experiments were commenced. Experiments were carried out using 9–13-week-old rats. Data were recorded and stored via a PC. Animals were connected via a flexible cable to the amplifier and stimulator. Throughout experiments the animals could thus move freely. Experiments were consistently conducted at the same time of day (commencing 8.00 a.m.). Baseline experiments to confirm stability of evoked responses were routinely carried out (at least 24 h) before LTP experiments were conducted. Where possible, the animals served as their own controls. Thus, basal synaptic transmission (in the absence of injection) was monitored over a 24 h period in all animals to confirm stability of evoked responses. Subsequently, a control experiment (e.g. potentiation or basal synaptic transmission) was carried out in the presence of vehicle injection and ~1 week later the same experiment was carried out in the same animal in the presence of a drug injection.

Only animals which expressed robust LTP (in the presence of vehicle) were used for MPEP experiments. Input–output curves were compared 1 week after this vehicle LTP experiment to ensure that evoked potentials had returned to basal levels. In some cases, MPEP/LTP experiments were conducted first and 1 week later vehicle/LTP experiments were conducted to exclude the possibility that metaplastic changes (arising as a consequence of the initial LTP/vehicle experiment) could influence the outcome of the MPEP/LTP experiments. The order of the vehicle and MPEP experiments was not found to play any role in the outcome of the experiments.

Measurement of Evoked Potentials

Responses were evoked by stimulating at low frequency (0.025 Hz, 0.2 ms stimulus duration, 16 000 Hz sample rate). For each time-point, five evoked responses were averaged. Both field excitatory postsynaptic potential (fEPSP) slope and population spike (PS) amplitude were monitored. The amplitude of PS was measured from the peak of the first positive deflection of the evoked potential to the peak of the following negative potential. fEPSP slope was measured as the maximal slope through the five steepest points obtained on the first positive deflection of the potential. By means of input–output curve determination the maximum PS amplitude was found for each individual animal and all potentials employed as baseline criteria were evoked at a stimulus intensity which produced 40 % of this maximum.

LTP was induced by a high frequency tetanus (HFT) of 200 Hz (10 bursts of 15 stimuli, 0.2 ms stimulus duration, 10 s interburst interval). The stimulus amplitude for this protocol was the same as that used for recordings.

The cortical electroencephalogram (EEG) was monitored throughout the course of each experiment; however, no alteration in EEG was seen as a result of HFT or drug application.

To examine the possible modulation by mGlu5 of presynaptic glutamate release, responses to paired-pulse stimulation were monitored. Pairs of stimuli were delivered at interpulse intervals (IPIs) of 20, 25, 40, 50, 100, 300, 500 and 1000 ms. Responses to paired-pulse stimulation were recorded 30 min after vehicle or MPEP (1.8 μg) injections. To determine whether MPEP treatment affected the intrinsic synaptic excitability, input–output curves were obtained 30 min following MPEP (1.8 μg) or vehicle treatment.

Compounds and Drug Treatment

The metabotropic glutamate receptor antagonist MPEP was obtained from Tocris Cookson, Bristol, UK Ltd. For injection, MPEP was dissolved in 0.9% NaCl. MPEP or vehicle were injected in a 5 μl volume over a 6 min period via a Hamilton syringe. The Hamilton syringe was connected by means of a flexible polyurethane tube (~30 cm) to an injection cannula which was inserted into the permanently implanted cannula. Antagonist or vehicle injection was carried out 30 min prior to tetanization to enable diffusion from the lateral cerebral ventricle to the hippocampus to occur (Manahan-Vaughan *et al.*, 1998).

Throughout the experiments, injections were administered following measurement of the baseline for 30 min. In LTP experiments, a tetanus was applied 30 min following injection, with measurements then taken at t = 5, 10, 15 and then 15 min intervals up to 4 h, with additional measurements taken after 24 and 25h.

Alternatively, injection was conducted 5 min after HFT in order to ascertain the effects of MPEP or vehicle application on LTP maintenance. In this case basal synaptic transmission was followed for 60 min prior to HFT and measurements were then taken at t = 5, 10, 15 and then 15 min intervals up to 4 h, with additional measurements taken after 24 and 25 h.

Data Analysis

The baseline fEPSP or PS data were obtained by averaging the response to stimulating the perforant path, to obtain five sweeps at 0.025 Hz, every 5 or 15 min as described above. The data were then expressed as mean % pre-injection baseline reading ± standard error of the mean (SEM). Statistical significance was estimated using analysis of variance (ANOVA) with repeated measures and by Student's *t*-tests. The probability level interpreted as statistically significant was $P < 0.05$.

Behavioural Experiments

The Radial Maze

The radial maze consisted of a central octagonal platform (26 cm in diameter) from which eight arms (67 cm long, 20 cm deep, 10 cm wide) radiated (Fig. 4*A*). The floor of the maze was made of dark grey polyvinylchloride, whereas the walls were made of transparant Plexiglas. The maze was elevated 80 cm above floor-level. The end of each arm, possessed a small circular indentation (1 cm deep, 3 cm diameter) ~3.5 cm from the tip. In the centre of this indentation was a 3 mm deep 'food cup' (3 mm diameter), in which a 45 mg food pellet could be placed. The indentation served to prevent visibility of the food pellet from the centre of the maze. The sides but not the ends of the arms were walled.

The experimental room was brightly lit and had white walls which were decorated with conspicuous extra-maze cues. For example, on one of the white walls was placed a black cross (30 × 20 cm with the arms of the cross having a diameter of 10 cm); on another wall were two large black rectangles (50 × 70 cm), 10 cm apart. The cues remained constant throughout the study. The maze was placed centrally in the room.

Experimental Procedure

Male Wistar rats (9–13 weeks old) which had undergone implantation of an injection cannula, were used for the behavioural study.

For 2 days before commencement of radial maze training animals were habituated to the maze. Food pellets were placed at the end of each radial maze arm. Animals were taken individually from their home cages and placed in the centre of the maze for 15 min. Rats had access to all arms and could eat the pellets *ad libitum*. During these habituation days home cage food access was reduced so that animal weight decreased by 10–15% of pre-habituation levels.

On training days four arms were baited with a single food pellet (Dustless Precision Pellet; Bioserv, New Jersey). For each animal a different constellation of baited arms was randomly chosen. This constellation remained constant throughout the 10 days of training. The trial commenced with placement of the animal on the central platform. A trial was deemed finished as soon as the food pellets had been found or when 15 min had elapsed: whichever occurred first. Once retrieved by the animals, the food pellets were not replaced. The number of arm entries was recorded until the trial was finished. The exact position of the entered arms was noted, together with the time spent in the maze, the frequency of freezing and number of rearings. At the end of each trial the number of fecal boli was counted and the maze was cleaned. In order to avoid the use of intramaze cues (odor trails etc.) the maze was rotated by 45° after each training day.

Thirty minutes prior to the commencement of each trial, drug or vehicle injection was applied in a volume of 5 μl via the lateral

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cerebral ventricle with exactly the same procedure as for electrophysiological experiments.

Performance Scoring

Entry into an unbaited arm or entry into a baited arm without removing the food pellet was scored as a reference memory error. This reflects memory of information which remains constant across trials (Eichenbaum, 2002) and is a measure of long-term memory. Reentry into a baited arm from which the food pellet had already been retrieved was scored as a working memory error. This mirrors the ability of the animal to retain information for the duration of the trial, i.e. to temporarily hold information online and is a measure of short-term memory (Eichenbaum, 2002). Reentry into an unbaited arm was scored as a 'double' working and reference memory error: it comprises a reference memory error as an entry into an unbaited arm occurred but also comprises a working memory error as the animal has forgotten that it recently entered this arm.

Animal activity (locomotion) was determined by a simple calculation based on the amount of time spent in the maze and the number of arms crossed: (number of arms entered × 160)/(time spent in maze (s)), where 160 equals the length of the maze from arm tip to opposite arm tip.

Rearing, grooming and fecal boli were quantitatively assessed. Thus, number of rears, grooms and fecal boli were counted and compared between drug and vehicle-treated groups.

Data Analysis

The Mann–Whitney *U*-test was used to assess between group differences for individual trials. ANOVA with repeated measures was used to assess behavior across days of training. The Wilcoxon matched pairs signed rank test was used to evaluate within-group differences. The probability level interpreted as statistically significant was $P < 0.05$.

Results

Antagonism of mGlu5 Has No Effect on Basal Synaptic Transmission

When MPEP was injected into the lateral cerebral ventricle as 1.8 µg in a 5 µl injection volume, no effect on synaptic transmission was seen over the 25 h monitoring period [n = 11, compared to controls n = 8; Fig. 1*A–C*; confirmed by ANOVA: between-factor, $F(1,31) = 0.53$, $P < 0.985$ for PS; $F(1,31) = 0.77$, $P < 0.8122$ for fEPSP]. Similarly, no effect was seen when 3.6 µg MPEP was injected [n = 4; Fig. 1*A*; confirmed by ANOVA: between-factor, $F(1,31) = 0.69$ $P < 0.8902$ for PS; $F(1,31) = 0.47$, $P < 0.9937$ for fEPSP].

Analysis of input–output curves obtained 30 min following vehicle (n = 6) or MPEP (3.6 µg, n = 6) injection revealed no differences in the responsiveness to stimulation intensities varying from 100 through 900 µA (Fig 1*D*).

To confirm that MPEP did not produce changes in basal transmission, responses to paired pulse stimulation were examined. Pairs of stimuli were delivered at interpulse intervals (IPIs) of 20, 25, 40, 50, 100, 300, 500 and 1000 ms. Responses to paired-pulse stimulation were recorded 30 min after vehicle (n = 6) and MPEP (3.6 µg, n = 8) injections. No significant difference in paired pulse responsiveness was seen (Fig 1*E*).

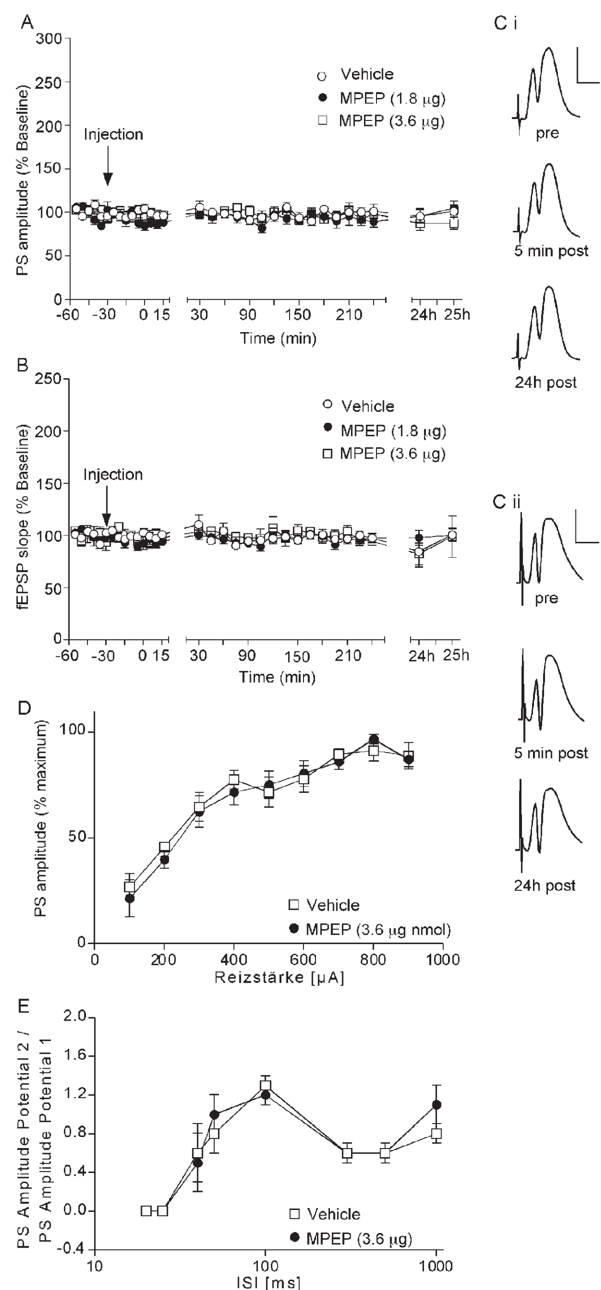

Figure 1. Antagonism of mGlu5 has no effect on basal synaptic transmission. (*A*, *B*) Test-pulse stimulation when given in the presence of the mGlu5 receptor antagonist MPEP (1.8 µg, $n = 11$ or 3.6 µg, $n = 4$) does not affect basal PS amplitude (*A*) or fEPSP slope (*B*) compared to vehicle-injected controls ($n = 8$). (*C*) Original analog traces showing the field potentials evoked from the dentate gyrus pre injection, 5 min and 24 h following injection of (i) vehicle or (ii) 3.6 µg MPEP. Vertical scale-bar corresponds to 5 mV, horizontal scale-bar to 4 ms. (*D*) Input–output curves obtained 30 min following application of vehicle ($n = 6$) or MPEP (3.6 µg, $n = 6$) showed no significant difference in the profile of responses. Paired pulse stimulation given 30 min following application of vehicle ($n = 6$) or MPEP (3.6 µg, $n = 8$) revealed no differences in the responses obtained.

Antagonism of mGlu5 Dose-dependently Impairs Long-term Potentiation

High frequency tetanization (200 Hz; HFT = 10 bursts of 15 stimuli, 0.2 ms stimulus duration) resulted in robust long-term potentiation in the dentate gyrus *in vivo* (Fig. 2). MPEP (1.8 µg, n = 7) when applied 30 min prior to HFT did not significantly alter the initial magnitude of potentiation (5 min post-HFT) when compared to vehicle-injected animals (n = 7). Approximately 2.5 h after tetanization the fEPSP slope and the PS amplitude had significantly decreased compared to LTP levels in vehicle-injected animals. For PS amplitude the response was significantly different from controls from 2.5 h post-HFT (P <

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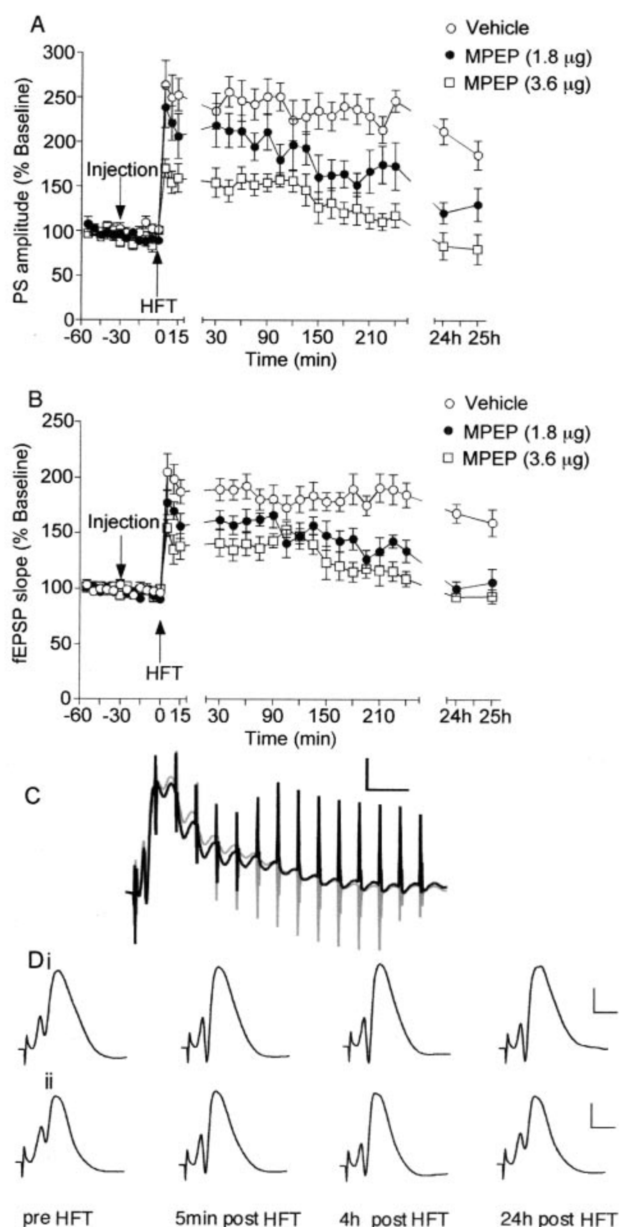

Figure 2. Antagonism of mGlu5 dose-dependently impairs long-term potentiation. (*A*, *B*) 200 Hz HFT in the presence of vehicle ($n = 7$) results in a robust long-term potentiation of both PS (*A*) and fEPSP (*B*). Administration of the mGlu5 antagonist MPEP (1.8 μg, $n = 7$) or (3.6 μg, $n = 7$) dose-dependently inhibits LTP compared to vehicle injected controls. Effects become evident ~2.5 h post-HFT in 1.8 μg-treated animals. Animals treated with 3.6 μg MPEP show a significant reduction in LTP immediately after HFT. (*C*) Application of 1.8 μg MPEP 30 min prior to the tetanus had no effect on the fEPSP. Original analog traces showing the fEPSP response during 200 Hz tetanization (grey trace, MPEP; black trace, vehicle). Vertical scale-bar corresponds to 5 mV, horizontal scale bar 5 ms for MPEP; vertical scale-bar corresponds to 6.1 mV, horizontal scale-bar 5 ms for vehicle-treated animals. (*D*) Original analog traces showing the field potentials evoked from the dentate gyrus pre-HFT, 5 min, 4 h and 24 h following HFT in the presence of (i) vehicle or (ii) MPEP (1.8 μg). Vertical scale-bar corresponds to 5 mV, horizontal scale-bar to 4 ms.

0.05). A similar decrease was also seen in fEPSP, with a significant difference from controls appearing at 165 min post-HFT ($P < 0.05$; Fig. 2). A complete return of evoked potentials to pre-HFT levels did not occur in the initial 4 h monitoring period, however. Four hours post-HFT a significantly ($P < 0.01$) higher magnitude of PS amplitude and fEPSP slope was detected compared to animals which received MPEP in the absence of HFT (baseline controls; Fig. 1).

Twenty four hours post-HFT, LTP was still evident in control animals, but was significantly inhibited in MPEP treated animals (Fig. 2). Thus, both PS amplitude and fEPSP slope had returned to basal levels when compared to animals which received MPEP in the absence of HFT (Fig. 2). MPEP in the 1.8 μg concentration had no effect on the fEPSP properties during the tetanus ($n = 6$, Fig 2*C*). Although the higher concentration of 3.6 μg show tendencies in some cases towards an inhibition of the fEPSP during the tetanus, no consistent responses were obtained. This suggests that the higher concentration of MPEP may have been at the threshold for impairment of NMDA currents (Mannaioni *et al.*, 2001; Lee *et al.*, 2002), but not suprathreshold enough to generate consistent effects.

Raising the concentration of MPEP to 3.6 μg ($n = 7$) resulted in a more potent inhibition of LTP. Thus, 5 min after MPEP application a significant reduction in LTP induction was seen. Four hours after HFT, evoked potentials had returned to basal levels compared to baseline controls (Fig. 2). Furthermore, the PS amplitude immediately following application of HFT was statistically lower than in 1.8 μg MPEP-injected controls ($P <$ 0.05; $t = 5$ min post-HFT; Fig. 2).

ANOVA confirmed the statistical significance between the control and 1.8 μg MPEP-treated LTP groups, as well as between the two groups which received different concentrations of MPEP. Comparing the PS values of the control and 1.8 μg MPEP groups, the statistical results for the between-factor analysis was $F(1,31) = 72.29$, $P < 0.0001$. For fEPSP values the between-factor analysis yielded $F(1,31) = 119.09$, $P <$ 0.0001. Comparing the 1.8 μg MPEP group with the 3.6 μg MPEP group results in the between-factor analysis yielded $F(1,31) = 65.29$, $P < 0.0001$ for the PS and $F(1,31) = 27.85$, $P <$ 0.0001 for the fEPSP values.

Application of the mGlu5 Antagonist MPEP 5 min after High-frequency Tetanization Results in a Significant Impairment of Late LTP

The effects of MPEP in the lower dose (1.8 μg) on LTP maintenance could have occurred through modulation of LTP induction by HFT. To examine this possibility, MPEP was applied 5 min after HFT. In this case, a significant impairment of LTP with regard to both PS and fEPSP slope was seen which became evident 24 h after MPEP was applied [Fig 3; $P < 0.05$; confirmed by ANOVA, between factor: $F(1,29) = 4.00$, $P <$ 0.0001 for PS; $F(1,29) = 19.12$, $P < 0.0001$ for fEPSP slope]. These data support a modulation by MPEP of LTP maintenance corresponding to an inhibition of late LTP.

Antagonism of mGlu5 Significantly Impairs Reference Memory

Administration of MPEP (1.8 μg, $n = 7$) caused a significant impairment of reference memory performance compared to vehicle-treated controls ($n = 5$) which became evident from day 5 onwards (Fig. 4*B*).

Thus, on days 1–4 no significant effect was seen [Mann–Whitney U ($n_1 = 5$, $n_2 = 7$) for day 1 = 14; for day 2 = 11; for day 3 = 7 for day 4 = 12]. However, a significant effect was seen on day 5 [Mann–Whitney U ($n_1 = 5$, $n_2 = 7$) = 2.5 ($P <$ 0.05); day 6 = 3 ($P < 0.05$); day 7 = 3 ($P < 0.05$); day 8 = 2.5 ($P <$

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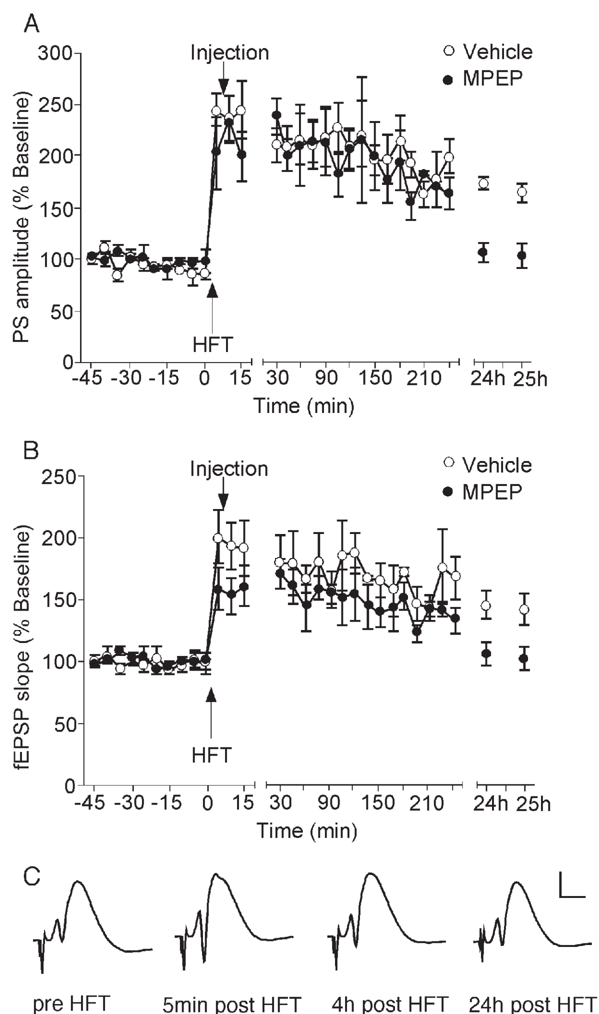

Figure 3. Application of the mGlu5 antagonist MPEP 5 min after HFT results in a significant impairment of late LTP. (*A*, *B*) Vehicle ($n = 5$) applied 5 min after 200 Hz HFT results in a robust long-term potentiation of both PS (*A*) and fEPSP (*B*). Administration of the mGlu5 antagonist MPEP (1.8 µg, $n = 4$) 5 min after HFT produces a significant inhibition of late LTP. Effects become evident ~24 h post-HFT. (*C*) Original analog traces showing the field potentials evoked from the dentate gyrus pre-HFT, 5 min, 4 h and 24 h following HFT where MPEP (1.8 µg) was given 5 min after HFT. Vertical scale-bar corresponds to 5 mV, horizontal scale-bar to 4 ms.

0.05); day 9 = 1 ($P < 0.05$) and day 10 = 1.5 ($P < 0.05$)]. ANOVA confirmed a significant effect between the MPEP- and vehicle-treated groups across trial days: between factor, $F(1,9) = 3.95$, $P < 0.0003$.

Antagonism of mGlu5 Significantly Impairs Working Memory

Application of MPEP (1.8 µg, $n = 7$) caused a significant impairment of working memory performance compared to vehicle-treated controls ($n = 5$) which became evident from day 5 onwards (Fig. 4*C*).

Thus, on days 1–4 no significant effect was seen [Mann–Whitney U ($n_1 = 5$, $n_2 = 7$) for day 1 = 9; for day 2 = 13.5; for day 3 = 7; for day 4 = 10.5]. However, a significant effect was seen on day 5 (Mann–Whitney U ($n_1 = 5$, $n_2 = 7$) = 6 ($P < 0.05$); day 6 = 2.5 ($P < 0.05$); day 7 = 2.5 ($P < 0.05$); day 8 = 5 ($P < 0.05$); day 9 = 4 ($P < 0.05$) and day 10 = 1 ($P < 0.05$). ANOVA confirmed a significant effect between the MPEP- and vehicle-treated groups across trial days: between factor, $F(1,9) = 2.45$, $P < 0.04$).

An analysis of 'double' working and reference memory scores (i.e. re-entry into an unbaited arm) showed that a significant effect impairment was caused by MPEP application (1.8 µg, $n = 7$) which became evident from day 7 onwards (Fig. 4*D*) compared to vehicle injected controls ($n = 5$).

On days 1–4 no significant effect was seen [Mann–Whitney U ($n_1 = 5$, $n_2 = 7$) for day 1 = 11; for day 2 = 11; for day 3 = 8 for day 4 = 9; for day 5 = 7.5; for day 6 = 8.5]. However, a significant effect was seen on day 7 [Mann–Whitney U ($n_1 = 5$, $n_2 = 7$) = 4.5 ($P < 0.05$); day 8 = 4.5 ($P < 0.05$); day 9 = 4 ($P < 0.05$) and day 10 = 3.5 ($P < 0.05$)]. ANOVA confirmed a significant effect between the MPEP- and vehicle-treated groups across trial days: between factor, $F(1,9) = 3.95$, $P < 0.0003$).

Antagonism of mGlu5 Has No Effect on Locomotion, Rearing, Grooming or Defecation

The significant effects of MPEP on learning performance could perhaps be explained by a negative influence of the drug on locomotion. We therefore closely assessed locomotory behaviour on each of the experimental days. No difference was found in the activity of the MPEP treated group in comparison to vehicle injected controls [Fig. 5*A*; ANOVA: between factor, $F(1,9) = 0.105$, $P < 0.409$]. Thus, the effects of MPEP on working and reference memory performance are likely to have been mediated by a direct effect on learning.

Stress can impair the ability of an animal to learn or retrieve information. An anxiolytic or stress-enhancing effect of MPEP could thus alter learning performance. Defecation (i.e. number of fecal boli), number of rears and number of grooms were used as a general indication of the degree of stress and anxiety experienced by the animal. No significant difference in the number of fecal boli [ANOVA: between factor, $F(1,9) = 1.367$, $P < 0.2167$], rears [ANOVA: between factor, $F(1,9) = 1.131$, $P < 0.3496$], or grooms [ANOVA: between factor, $F(1,9) = = 1.289$, $P < 0.2523$] was noted between the MPEP- and vehicle-treated groups (Fig. 5*B*–*D*).

Discussion

The results of this study indicate that antagonism of mGlu5 receptors dose-dependently and differentially inhibits the induction and expression of LTP and causes a distinct impairment of working and reference memory of a spatial learning task. These data implicate mGlu5 as a critical link between synaptic plasticity and learning.

MGlu5 is predominantly postsynaptically localized (Shigemoto *et al.*, 1997; Takumi *et al.*, 1999) although presynaptic phospholipase C-coupled mGlu receptors exist (Sistiaga *et al.*, 1998; Manahan-Vaughan *et al.*, 1999a). MGlu5 typically occurs in membrane domains peripheral to postsynaptic specializations (Lujan *et al.*, 1996). In the rat brain, the hippocampus, and in particular the dentate gyrus is among the regions with the highest density of mGlu5 receptors. They are located in the distal dendritic compartments of the dentate gyrus molecular layer but also expressed on interneurons (Baude *et al.*, 1993; Lujan *et al.*, 1996; Blümcke *et al.*, 1996). Given the wide distribution of mGlu5 throughout the hippocampus it is thus quite possible that the CA1 region contributed to the effects on spatial memory seen in the present study. However, the greater density of mGlu5 receptors in dentate

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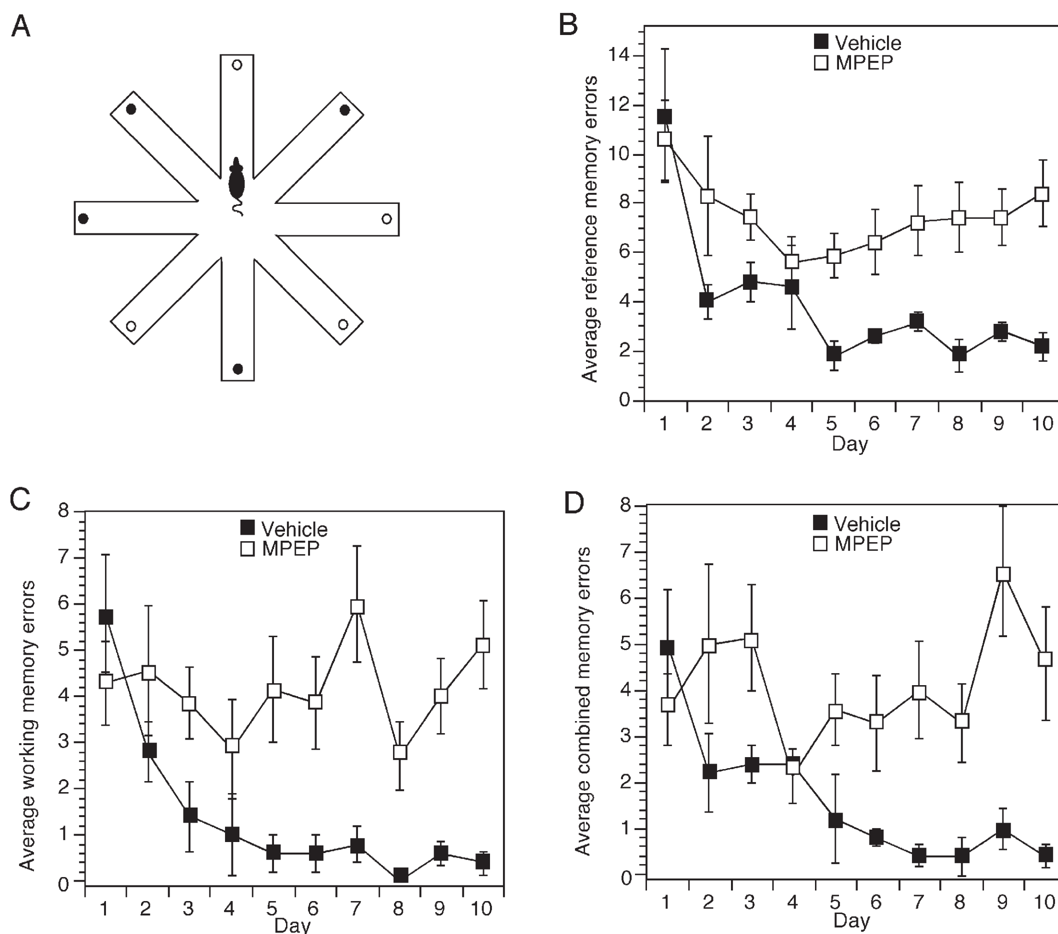

Figure 4. Antagonism of mGlu5 significantly impairs working and reference memory. (*A*) Animals were placed in an eight-arm radial maze with four baited arms. (*B–D*) Daily application of MPEP (1.8 μg, $n = 7$) resulted in a significant increase in performance errors with regard to reference (*B*), working (*C*) and double working and reference memory (*C*) compared to vehicle injected controls ($n = 5$).

gyrus would support a significant contribution of this region to the impairments in learning observed.

Activation of mGlu5 results in the stimulation of dendritic protein synthesis (Huber *et al.*, 2000), suppression of the calcium-activated potassium current (I_{AHP}) and potentiation of NMDA receptor currents (Jia *et al.*, 1998; Attucci *et al.*, 2001; Mannaioni *et al.*, 2001). Consistent with this latter observation, in the hippocampal slice preparation, antagonism of mGlu5 receptors results in an impairment of LTP induction due to inhibition of mGlu5 receptor-mediated NMDAR currents (Doherty *et al.*, 2000; Mannaioni *et al.*, 2001; Lee *et al.*, 2002). MPEP is a highly selective antagonist at mGlu5. This compound exhibits an IC_{50} of 36 nM at mGlu5 with no activity at any other mGlu receptor subtype (Gasparini *et al.*, 1999). We found that although basal synaptic transmission, paired pulse responses and input–output curves were unaffected, MPEP dose-dependently inhibited LTP and that the higher concentration of MPEP significantly inhibited the induction of LTP and was coupled with a rapid decline in potentiated responses. The latter effect is perhaps consistent with an inhibition of receptor-mediated NMDA receptor currents as reported by others *in vitro* (Mannaioni *et al.*, 2001; Lee *et al.*, 2002). However, analysis of the fEPSP during the tetanus in experiments where MPEP was applied at the higher concentration of 3.6 μg, did not reveal consistent inhibitory effects on the fEPSP. The higher concentration of MPEP may thus have been at the threshold for impairment of NMDA currents, but not suprathreshold enough to generate consistent effects. Effects on LTP induction were obtained with a concentration of MPEP (3.6 μg) which was far lower than that used to inhibit NMDA receptors *in vitro* (10 μM). This disparity may be derived from the very different experimental designs used. The *in vitro* experiments used a wash-in/wash-out approach to MPEP application. A wash-out is not practicable *in vivo*. The presence of the low concentration of MPEP in the hippocampus for a comparatively longer time period may explain why less drug was needed to elicit an inhibition of LTP induction than that used *in vitro*.

Although the higher concentration of MPEP may have elicited an LTP impairment through inhibition of NMDA receptor currents, the lower concentration of MPEP impaired late LTP in an NMDA receptor-independent manner. Thus, the fEPSP response during the tetanus was unaffected by this concentration of MPEP. In addition, application of MPEP after the tetanus successfully impaired late LTP. Interestingly, the application of protein synthesis inhibitors *in vivo* also causes an impairment of late LTP (Frey *et al.*, 1988). Activation of mGlu5 receptors results in protein synthesis in conjunction with the induction of long-term depression *in vitro* (Huber *et al.*, 2000, 2001) and the induction of synaptic potentiation *in vivo* (Manahan-Vaughan and Braunewell, 1999). In addition,

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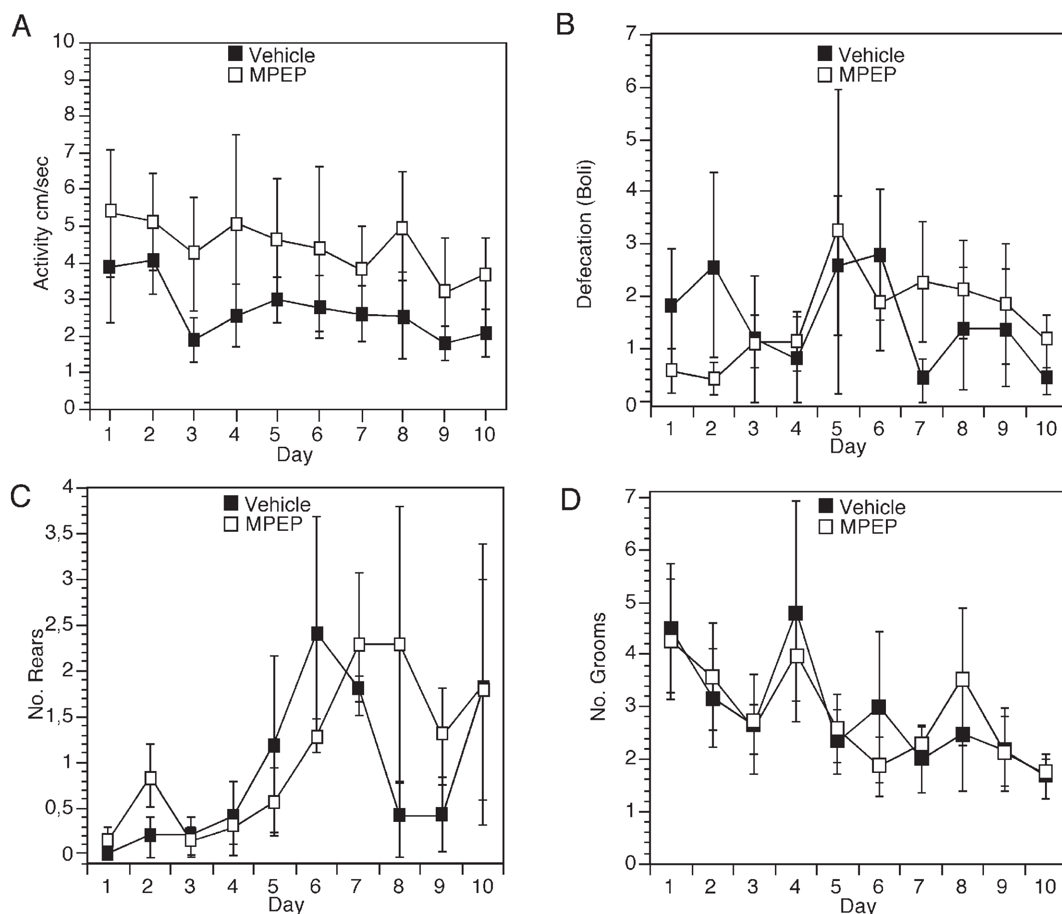

Figure 5. Antagonism of mGlu5 has no effect on locomotion, rearing, grooming or defecation. Daily application of MPEP (1.8 μg, $n = 7$) had no effect on locomotion, rearing, grooming or defecation compared to vehicle-injected controls ($n = 5$).

the induction of dentate gyrus LTP *in vivo* is associated with an increase in the expression of mGlu5 receptors (Manahan-Vaughan *et al.*, 1999b) and calcium sensor proteins (Manahan-Vaughan and Braunewell, 1999). In our study we saw an impairment of LTP by MPEP which became evident ~2.5 h after LTP induction. (This effect may not have been detectable in *in vitro* studies due to the short duration of the experiments of ~90 min post-tetanization). This impairment of late LTP may therefore be related to an inhibition of the synthesis of new proteins essential for the maintenance of LTP. This possibility is supported by the observation that application of MPEP after tetanization resulted in a significant inhibition of late LTP.

In our behavioral study, reference memory performance was determined by evaluating the number of times an animal entered an unbaited arm during a trial. By this means we could obtain information about the long-term storage of spatial information in the hippocampus. Working memory performance was determined by assessing the number of times during a trial that an animal returned to an arm from which the food pellet had been removed. Although working memory, by definition, reflects the ability of an animal to hold information 'online', it is also influenced by the performance of an animal within a given trial (Olton and Papas, 1979). Thus the working memory performance within a particular trial may differ, from a trial on a different day, depending on the arm first entered by the animal: the sequence of arm entries will also affect the working memory performance and the more the animal learns about the task the more effectively it can perform. From day to day the animal will learn to use its working memory more efficiently thus enabling an improvement in working memory performance towards the end of the trial series. This was indeed the case in the current study, as in control animals a progressive improvement in working memory was seen.

The behavioral study revealed that both working and reference memory were impaired by daily application of MPEP. These effects became evident ~5 days after drug treatment began. Thus the ability of the animals to hold information online (working memory) as well as their ability to retain information over prolonged periods (reference memory) were significantly impaired. One could speculate that the impairments in working memory might have been caused by a modulation of NMDA currents by mGlu5 antagonism whereas reference memory was impaired by an inhibition in protein synthesis caused by MPEP application. In support of this latter possibility, behavioral studies which examined the effects of protein synthesis inhibitors have found learning and memory deficits in different paradigms (Nakajima, 1969; Bourtchouladze *et al.*, 1998; Kogan *et al.*, 2000; Vianna *et al.*, 2001). The impairment of learning caused by the mGlu5 antagonist may therefore have been associated with an inhibition of mGlu5-stimulated protein synthesis and therefore a disturbance in information storage.

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The correlation between the plasticity impairment and the delayed impairment of spatial learning seen in this study may perhaps be explained first of all by a task acquisition phase (which endures typically for 3–5 days in the radial maze), and secondly by the role of the hippocampus in learning as proposed by Lisman and Otmakhova (2001). According to the Lisman theory the dentate gyrus engages in (theta–gamma) phase coding of spatial information which is then stabilized in the CA3 region by sharp wave activity and then relayed onto to cortical storage regions by means of ripple activity in the CA1 region. If antagonism by MPEP of mGlu5 receptors and the associated disruption of LTP in the dentate gyrus interferes with the phase coding of a spatial map then a disruption of spatial memory as seen in our experiments would be expected. The delay in onset of the impairment may be caused by the fact that in the early days of the experiment the animal must first learn what the task is. Having acquired the 'rules of play' (task acquisition) the animal can then begin to store the spatial information required for locating the food pellets. The task acquisition usually takes 3–5 days in the radial maze, thus explaining why the differences in spatial learning first became apparent after 5 days.

The effects of MPEP on LTP appear to be tightly intertwined with the effects observed on learning in the present study, and furthermore suggest that the contribution of LTP to spatial learning is cumulative. Two days of acclimatization preceded the commencement of the learning trials. During this time the animals were given the opportunity to learn that the radial arms could contain food. The subsequent trials should have enabled precise learning of the pellet locations. Differences between working memory in the MPEP and control animals took 5 days to become apparent suggesting that the effects of MPEP were gradual. In other words, learning occurred because MPEP did not elicit a complete extinguishment of LTP; however, optimal learning did not occur because the trial repetitions were not accompanied by normal LTP. MPEP elicited an inhibition of LTP expression but no effect on LTP induction. STP (here meaning the phase of LTP which endured for ~90 min post-tetanus) was reduced by MPEP, however. If working memory is determined by short-term potentiation, then the impairment of STP by MPEP may correspond to the impairment of working memory seen. As STP was not fully blocked, but rather attenuated by MPEP, the effects on working memory took 5 days to appear, supporting that the contribution of LTP to spatial learning is cumulative. This possibility is further supported by the reference memory data, where effects also became first apparent after 5 days and may correlate with the impairment of late LTP by MPEP, seen in this study.

Interestingly, antagonism of mGlu5 results in an alteration of EEG activity, such that delta (slow wave) activity becomes increasingly evident (Binns and Salt, 2001). An increase in slow wave activity is an indication of a reduction in the state of arousal (Steriade *et al.*, 1993). Delta waves typically occur in deep sleep but are also believed to reflect decision-making processes (Basar *et al.*, 2001). Increased delta activity as a result of mGlu5 receptor antagonism may reflect an interference in normal cognitive processing and thus underlie the deficits in learning observed in the present study.

Unilateral application of MPEP was sufficient to impair learning performance in the present study. Earlier work conducted by this group followed ligand diffusion following injection into one lateral cerebral ventricle (Manahan-Vaughan *et al.*, 1998). Thirty minutes after injection a localized diffusion to the ipsilateral hippocampus was seen. One hour after injection, a gradual distribution to other brain regions via for example the third ventricle occurred. One therefore cannot exclude that repetitive daily application of MPEP would enable a brain distribution of the compound such that the hippocampus on the contralateral side would be affected.

Intraperitoneal MPEP injection inhibits locomotory activity (Spooren *et al.*, 2000). No significant influence on locomotion were found in the current study. The lack of effect can perhaps be explained by the relatively low concentrations of the compound used and its intracerebral administration route. Intraperitoneal MPEP reduces anxiety (Spooren *et al.*, 2000; Schulz *et al.*, 2001). However, no significant effects on grooming, rearing and defecation were seen in the current study suggesting that anxiolysis was not elicited by MPEP in the concentration used. In contrast, studies where anxiolytic effects were caused by MPEP involved concentrations which were considerably higher than those used in our study, and which were systemically administered. For example, Tatarczynska *et al.* (2001) used 1–30 mg/kg, i.p., Brodkin *et al.* (2002) used 3–30 mg/kg, i.p. These findings are consistent with the likelihood that the concentration of MPEP used was subthreshold for induction of these behavioral effects.

One must consider that MPEP treatment caused visual impairments which contributed to the deficits in learning performance seen. However, studies conducted by others suggest that antagonism of mGlu5 using MPEP does not alter visual transmission. For example Cirone *et al.* (2002) demonstrated that antagonism of mGlu5 by MPEP does not alter visual responses in the rat using a concentration of the compound (5 μM) which was far in excess of that used in our study. Studies into the role of mGlu1 and mGlu5 in synaptic transmission of thalamocortical neurons in the dorsal lateral geniculate nucleus also excluded a role for mGlu5 (Turner and Salt, 2000). Perhaps more convincingly, a behavioral study conducted to examine the role of mGlu5 in reversal of akinesia demonstrated that neither acute nor chronic (3 week) treatment with MPEP (1.5, 3 or 6 mg/kg, i.p.) had any effect on trained lever depression following a visual cue (Breysse *et al.*, 2002). These findings would argue against the likelihood that the deficits in spatial learning, which we observed, were derived from visual impairments caused by mGlu5 antagonism.

The preferential selectivity of MPEP at the mGlu5 isoforms mGlu5a and mGlu5b has not been determined although its efficacy at antagonizing human mGlu5a receptors has been shown (Gasparini *et al.*, 1999). It will be intriguing to ascertain whether these isoforms differentially mediate the contribution of the mGlu5 receptor to LTP and learning. An isoform specific regulation of LTP could account perhaps for the dose-dependent effects of the compound on LTP induction and late LTP. Thus, it has been demonstrated for example, that mGlu 5a inhibits whereas mGlu5b promotes the formation and development of neurites (Mion *et al.*, 2001).

In conclusion, the results of this study demonstrate that antagonism of mGlu5 receptors leads to differential effects on the expression of LTP: an inhibition of LTP induction, corresponding perhaps to an inhibition of NMDA receptor currents and an inhibition of late LTP correlating perhaps with an impairment of mGlu5-triggered protein synthesis. Antagonism of mGlu5 was also associated with marked deficits

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in working and reference memory in an eight-arm radial maze consistent with a role for this receptor in spatial memory processes. These findings emphasize the importance of this receptor in both synaptic plasticity and cognitive processes and indicate an important contribution of mGlu5 in the coordination of these phenomena.

Notes

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