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Research Report

Blockade of NMDA receptors pre-training, but not post-training, impairs object displacement learning in the rat

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ABSTRACT

Several forms of hippocampal-dependent learning rely upon activation of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor. Here we have investigated the effects of administration of the NMDA receptor antagonist (±)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP) on the performance of rats in an object displacement task and the possible role of extracellular signal-regulated kinase (ERK) in this form of learning. The data show that rats injected intraperitoneally with CPP (10 mg/kg) before, but not after, training in the object displacement task displayed impairments in spatial learning when compared with saline-injected controls. The NMDAR may thus be involved in the acquisition, but not the consolidation, of this type of memory. In addition, a significant positive correlation was observed between learning and the expression of activated ERK in the dentate gyrus. No such correlation was apparent in the rest of the hippocampal formation. This study implicates the NMDARs in the acquisition phase of spatial learning and provides evidence for a role for ERK in spatial learning in the dentate gyrus of the rat.

1. Introduction

The N-methyl-D-aspartate (NMDA) subtype of glutamate receptor has long been implicated in mechanisms of synaptic plasticity such as long-term potentiation (LTP; Collingridge et al., 1983), spatial (Morris et al., 1986) and non-spatial forms of learning (Miserendino et al., 1990) in the hippocampus and other brain regions. While genetically-modified mouse strains lacking NMDAR subunits are a useful experimental tool (Tsien et al., 1996; Rondi-Reig et al., 2001; Weitlauf et al., 2005), a number of pharmacological inhibitors have also been used to investigate the role of the NMDA receptor in plasticity. Compounds such as 2-amino-5-phosphopentanoate (AP5; Morris et al., 1986), (+)-5-methyl-10,11-dihydro-5H-dibenzo-[*a*,*d*] cyclo-hepten-5,10-imine-maleate (MK-801; Butelman, 1986) and (6)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP; Ungerer et al., 1991), which we have chosen to use in this study due to its potent, selective and competitive properties (Lehmann et al., 1987), have all been used to demonstrate the importance of the NMDA receptor in hippocampal function.

Distinctions can be made between acquisition and consolidation of information, both theoretically and experimentally. The requirement of the NMDA receptor for each of these phases of information processing remains controversial. Early

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experimental evidence indicated that blockade of NMDA receptors with AP5 (Morris et al., 1986) or MK-801 (McLamb et al., 1990) impaired acquisition of hippocampal-dependent tasks. Subsequent studies, however, have provided evidence that while NMDA receptors may be involved in consolidation of memory, their activation may not be required for learning, e.g. McDonald et al. (2005) have used CPP to demonstrate a role for the hippocampal NMDA receptor in consolidation of spatial memory, but not initial memory encoding, while Kentros et al. (1998) reported that CPP compromised the long-term stability of new place fields but had no effect on formation or short-term stability of firing fields. Furthermore, MK-801 has recently been shown to impair recognition memory when injected either pre- or post-training (de Lima et al., 2005). The variations in the tasks and in the pharmacological properties of the NMDA antagonists used, as well as the intensity and duration of training the animals have undergone, may all contribute to the uncertainty regarding the precise nature of the role of NMDA receptors in the processing of information in the hippocampus, a role that remains to be clarified.

Activation of the NMDA receptor triggers a number of intracellular signaling cascades, including activation of the mitogen-activated protein kinase (MAPK) pathways. The MAPKs, particularly the serine/threonine kinase extracellular signal-regulated kinase (ERK), are crucial cellular mediators of plastic change in the hippocampus (Kelly et al., 2003; Blum et al., 1999). Hippocampal NMDA receptor activation has been shown to robustly induce ERK activation (Kurino et al 1995; Ivanov et al., 2006); the NMDA-associated ERK activation is thought to be critically involved in the expression of several forms of synaptic plasticity (Goldin and Segal, 2003; Adams and Sweatt, 2002). Here, we use the NMDA receptor antagonist CPP to examine, for the first time, the role of NMDAR activation and subsequent ERK phosphorylation in the performance of the object displacement task, a form of spatial learning, in the rat.

2. Results

2.1. *CPP blocks object displacement learning when injected pre-, but not post-training*

Here we have used the antagonist CPP to examine the role of the NMDAR in acquisition and consolidation of spatial memory in the rat. As shown in Fig. 1a, rats injected with saline either 30 min before or immediately after training demonstrate significantly increased exploration of a moved object when expressed as a percentage of total exploration after a 24 h time delay (Fig. 1a; $p<0.001$ in each group; mean % exploration of the moved object was 22.96±2.04 and 55.45±4.19 for saline preinjected and 23.25±2.04 and 62.13±2.03 for saline postinjected, on day 1 and day 2 respectively). Blockade of the NMDAR with CPP results in blockade of learning only if injection occurs prior to, but not after training ($p<0.001$; Fig. 1a; 19.38±4.67 and 22.80±1.64 for CPP preinjected and 15.43±7.09 and 58.53±3.43 for CPP postinjected), suggesting that activation of the NMDAR is necessary for acquisition, but not consolidation, of spatial learning. Total exploration time during either the training or the testing phase did not differ between saline- and CPP-injected rats (Fig. 1b). Exploration was decreased in the testing phase compared with the training phase in all groups, but this decrease did not reach statistical significance.

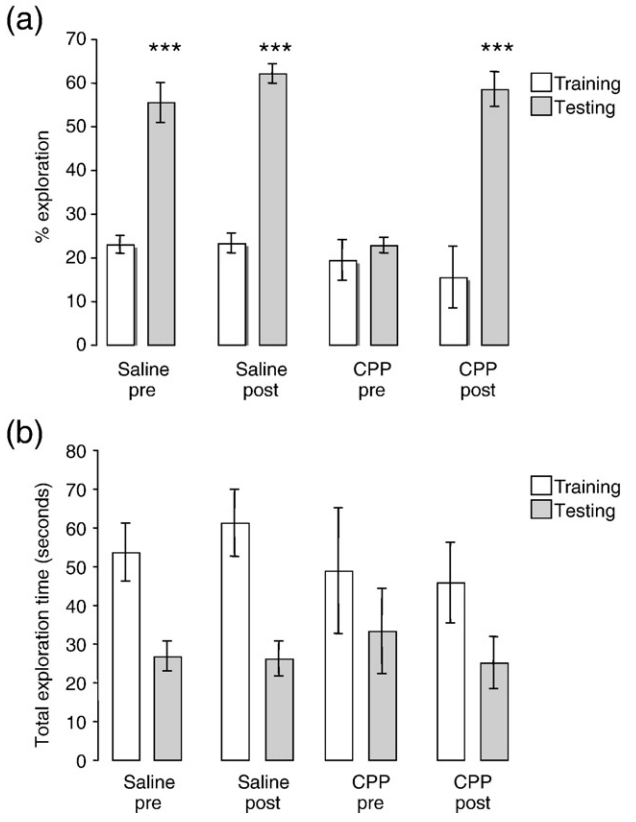

Fig. 1 – (a) Effect of CPP on exploration of the moved object as a percentage of total exploration of objects. Data expressed as mean % exploration ± SEM ($n=6$). One-way ANOVA revealed a significant increase in exploration of the moved object on day 2 in the saline preinjected, saline postinjected and CPP postinjected groups ($p<0.01$), but not the CPP preinjected group. (b) Effect of CPP on total exploration of all objects. Data expressed as mean seconds ± SEM ($n=6$). One-way ANOVA revealed no significant effect of CPP or timing of injection on exploratory behaviour.**

2.2. *ERK activation in the dentate gyrus is associated with object displacement and is blocked by CPP*

Numerous studies have provided evidence for a role for the mitogen-activated protein kinase extracellular signal-regulated kinase (ERK) in spatial learning, hence we analysed activation and expression of ERK in the dentate gyrus and hippocampus of these rats by SDS-PAGE and Western immunoblotting. This analysis revealed no significant difference in ERK activation between naive and control groups in samples of either dentate gyrus or hippocampus. Mean ERK phosphorylation expressed as % of total ERK expression was 14.27±4.077 and 13.83±3.44 in naive and control groups respectively. All saline- or CPP-treated groups showed increased ERK phosphorylation in the dentate gyrus when compared with naive and control groups (Fig. 2; $p<0.001$; ANOVA). In parallel with learning ability, activation of ERK, as measured by expressing phosphoERK as % of total ERK, was identical in the dentate gyrus of both saline-treated

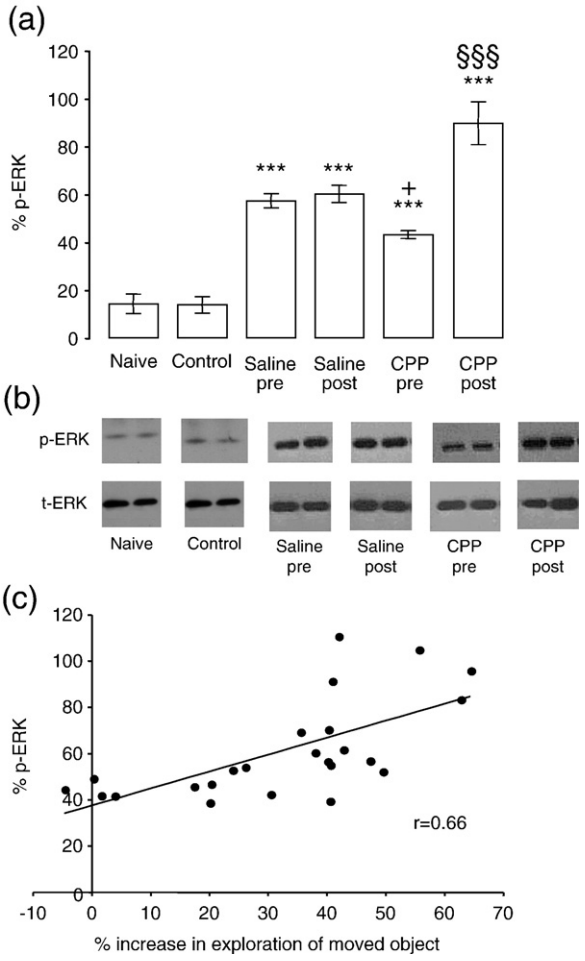

Fig. 2 – Effect of CPP on activation of ERK in the dentate gyrus. (a) Histograms represent mean % phosphoERK ± SEM and are the mean of at least 2 separate experiments. One-way ANOVA revealed significant increases in ERK activation in all groups when compared with the naive and control groups (*$p<0.001$). In parallel with behaviour, there was no significant difference in ERK activation between saline-treated groups, but a significant decrease in ERK activation in rats injected with CPP pre-training was observed when compared with both saline-treated groups (*$p<0.01$). Furthermore, CPP postinjection resulted in significantly increased ERK activation compared with all other treated groups (§§§$p<0.001$). (b) Sample representative immunoblots are shown for p42 isoform of phosphoERK and total ERK. (c) Linear regression analysis revealed a significant correlation between learning, as expressed by increased exploration of the moved object on day 2, and ERK activation as measured by expression of phosphoERK as a % of total ERK (**$p<0.01$; $n=24$).**

groups (Fig. 2). However, ERK activation in the CPP preinjected group was significantly reduced when compared with both saline-treated groups ($p<0.05$; ANOVA; mean %ERK activation ± SEM was 57.33 ± 2.97, 60.21 ± 3.57, 43.25 ± 1.64 and 89.71 ± 8.96 in saline preinjected, saline postinjected, CPP preinjected and CPP postinjected groups respectively). Injection of CPP post-training resulted in a significantly increased activation of ERK when compared with all other groups ($p<0.001$; ANOVA). To further probe the role of ERK phosphorylation in this form of spatial learning, regression analysis was performed to assess whether there was a correlation between learning, as evidenced by increased exploration of the moved object, and %ERK activation in all groups of rats. This analysis revealed a statistically significant positive correlation between exploration of the moved object and ERK activation in the dentate gyrus ($p<0.001$, Fig. 2c).

2.3. *Object displacement learning is not associated with ERK activation in the hippocampus*

In contrast to the results observed in the dentate gyrus, no significant differences in ERK activation were observed in samples of hippocampus prepared from any of the groups, with the

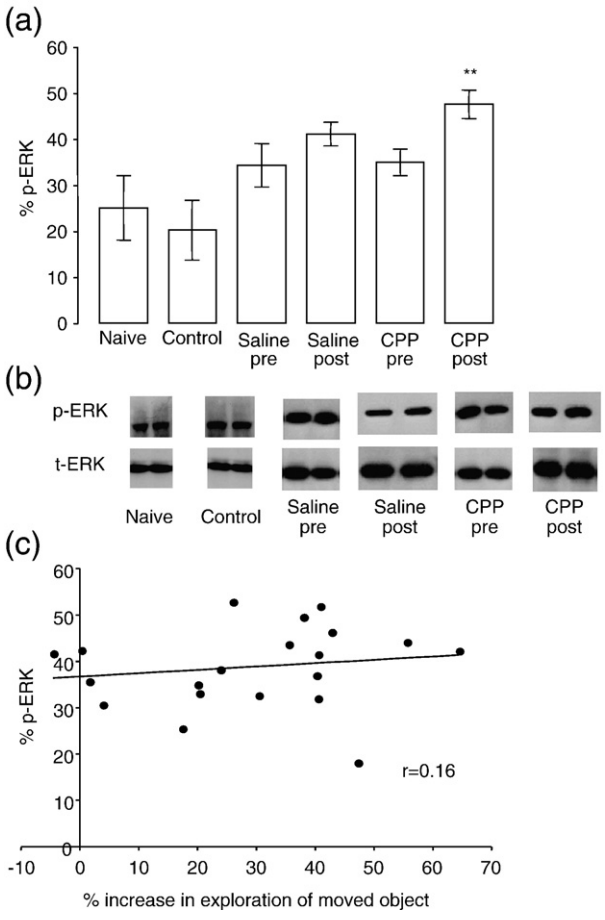

Fig. 3 – Effect of CPP on activation of ERK in hippocampus. (a) Histograms represent mean % phosphoERK ± SEM and are the mean of at least 2 separate experiments. One-way ANOVA revealed a significant increase in ERK activation between the CPP postinjected group compared with the naive and control groups ($p<0.01$). (b) Sample representative immunoblots are shown for p42 isoform of phosphoERK and total ERK. (c) Linear regression analysis revealed no correlation between learning, as expressed by increased exploration of the moved object on day 2, and ERK activation as measured by expression of phosphoERK as a % of total ERK ($p=0.5123$; $n=24$).**

exception of the CPP postinjection group; ERK activation was significantly increased in this group when compared with either naive or control groups (Fig. 3; $p<0.01$; ANOVA). Mean %ERK activation±SEM was 25.00±7.00, 20.22±6.2, 34.29±4.7, 41.09±2.56, 41.09±2.56, 34.94±2.89 and 47.56±3.09 in naïve, control, saline preinjected, saline postinjected, CPP preinjected and CPP postinjected groups respectively. Regression analysis demonstrated no correlation between learning and ERK activation in the hippocampus ($p=0.5123$; Fig. 3c).

3. Discussion

The objectives of these experiments were to investigate the role of the NMDA subtype of glutamate receptor in the acquisition and consolidation phases of spatial learning in the hippocampal formation using a potent, selective and competitive inhibitor, and to assess whether this NMDAR-dependent learning was reliant upon the MAPK intracellular signaling cascade. Our results reveal, for the first time, that injection of CPP pre-, but not post-, training, inhibits performance in the object displacement task, suggesting a role for the NMDA receptor in the acquisition, but not consolidation, of this form of learning. Furthermore, this NMDAR-dependent learning appears to be associated with ERK activation in the dentate gyrus, but not the hippocampus.

CPP was the NMDAR antagonist of choice in this study as it is a potent, selective, competitive drug that binds reversibly to the receptor (Porter et al., 1992). It has been suggested that the effects of NMDAR antagonists on sensory and/or motor function may contribute to their impairment of learning behaviours (Bannerman et al., 2006). Previous reports have found that, at high doses, CPP may produce ataxia and increased locomotor activity, along with its inhibitory effects on learning and memory (Jerram et al., 1996). However, we observed no motor impairments in our CPP-treated groups, and no significant differences in exploratory behaviour when compared with saline-treated controls (Fig. 1b), suggesting that the effects of the compound in the present study are likely to be largely mnemonic.

The hippocampal formation is believed to play a key role in spatial representation (Nadel and Hardt, 2004). The object displacement task is a form of intrinsically-motivated spatial learning that is suggested to be hippocampal-dependent (Poucet, 1989; Anderson and O'Mara, 2004; Zhu et al., 1995a,b). Reliant as it is on the natural propensity of rodents to explore their environment, performance is independent of external motivators inherent in other classic spatial learning tasks such as the water maze, and as such it may be argued that it is less stressful for the animals when compared with such tasks. Zhu et al. (1995a,b) used the expression of the immediate early gene c-*fos* to analyse the activation of the hippocampal formation and perirhinal cortex following exposure to novel objects in either a familiar or a novel environment: they showed that whereas perirhinal neurons are activated by novel rather than familiar objects, hippocampal formation neurons are preferentially activated by novel rather than familiar environments (see also Anderson and O'Mara, 2004). The spatial object exploration task used here is based on that of Poucet (1989), in which rats freely explored a group of objects in an arena; initially, normal rats displayed much exploratory behaviour, but this decreased over time. At this time, one of the objects was moved to a new location in the arena, which led to the resumption of exploratory behaviour. Few studies have investigated the requirement for NMDAR activation in this type of learning. Mele and colleagues have suggested a role for the NMDAR in the nucleus accumbens, a region that receives glutamatergic projections from the hippocampus, in the detection of spatial novelty by mice (Usiello et al., 1998; Sargolini et al., 1999; Mandillo et al., 2003); their data indicate a role for the receptor in both acquisition and consolidation of the motivated components of learning in this task.

Other forms of spatial learning have been extensively used to analyse the role of NMDAR in hippocampal plasticity, the most commonly-used of which has been the water maze task. Morris et al. (1986) found that injection of AP5 prior to training impaired spatial learning in the water maze, while post-training injection had no effect, thus supporting the idea that NMDARs are needed for memory acquisition but not necessarily consolidation or retrieval. Similarly, Heale and Harley (1990) found that intracerebroventricular administration of either AP5 or MK-801 blocked acquisition of spatial learning in the water maze, but had no effect on retention when administered post-training. The data presented here are in broad agreement with these previous findings in the literature i.e. that NMDA receptors play a role in acquisition but not consolidation. However, based on the current data, we cannot rule out the possibility that pre-training injections of CPP also affected the early phase of consolidation. It is possible that this early period of consolidation was not affected by post-training injections because of the delay required for the drug to reach the brain after systemic administration. Further experiments using an intracerebroventricular route of drug administration are required to allow confirmation of our present conclusions. It should be noted that some other researchers have failed to observe impairments in spatial learning following NMDAR blockade pre-training. A recent paper by McDonald et al. (2005) reports that CPP had no effect on acquisition of spatial learning in the water maze. However, these authors used a three-phase variant of this task in which animals received training prior to drug administration, in contrast to the current study, in which the rats were naive to the experimental apparatus. This may account for the disparity in findings.

Elucidation of the cell signaling pathways that may be activated downstream of the NMDAR during spatial learning is of considerable importance in furthering our understanding of the cellular mechanisms underlying learning and memory; here we have focused on the potential role of the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK). A role for ERK in various forms of hippocampal-dependent learning has been well-established (Atkins et al., 1998; Berman et al., 1998; Gooney et al., 2002; Kelly et al., 2003). Studies by Selcher et al. (1999) and Blum et al. (1999) have demonstrated a specific role for ERK in spatial learning. Various receptors and protein kinases, including the NMDAR, can activate the ERK cascade, leading to downstream events such as protein synthesis, changes in gene expression, dendritic spine stabilisation, ion channel modulation and increase of receptor numbers (Sweatt, 2004), all of which may be vital for the expression of various forms of synaptic plasticity.

Here we have shown that performance of the object displacement task is associated with an increase in activation of

ERK in the dentate gyrus. There was no significant increase in ERK phosphorylation in the control group when compared with the naive group, indicating that exploration of the open field alone, in the absence of objects, does not increase ERK activation. However, all groups that were trained in the task, and hence explored objects, displayed increased ERK activation in the dentate gyrus following testing. Furthermore, regression analysis revealed a positive correlation between ERK activation in the dentate gyrus and performance of this task, as evidenced by increased exploration of the displaced object during the testing phase. The current experiments have focused on observation of behaviour across 2 days, with analysis of memory and, in parallel, ERK activation following testing. We have not assessed, in the present study, the phosphorylation of ERK immediately following training. However, we have previously reported an increase in phosphorylation of ERK in the dentate gyrus immediately following training in the object recognition task (Kelly et al., 2003, Hennigan et al., 2007). In this context, it is of interest that ERK phosphorylation is also associated with reconsolidation and retrieval (Kelly et al., 2003, Valjent et al., 2006). Interestingly, while the CPP preinjected group displayed increased ERK phosphorylation compared with controls, this phosphorylation was significantly decreased when compared with the saline-treated groups, paralleling the impairment in learning. We tentatively hypothesise that the more modest ERK response in this group following testing may indicate that the NMDAR-dependence of this form of learning is linked with NMDAR-stimulated ERK activation. In contrast, such inhibition of ERK phosphorylation was not observed in the CPP postinjected group; indeed, this group displayed more robust ERK phosphorylation than all other groups. This could indicate some enhancement of learning-associated intracellular signaling by post-learning injection of CPP, albeit that this was not paralleled by enhancement in learning itself. Such effects of other NMDAR antagonists have been reported; MK-801 administration after training enhanced spatial retention in aged rats (Norris and Foster, 1999), while both MK-801 and AP7 enhanced learning of passive avoidance tasks when injected post-training, but impaired learning when injected pre-training (Mondadori et al., 1989). ERK phosphorylation was upregulated in the hippocampus of the same group when compared with controls only. No change in ERK activation was observed in the hippocampus of any other group when compared with controls; this finding may be interpreted as indicating a more important role for the dentate gyrus than the other subfields of the hippocampal formation in this type of learning, however such a hypothesis requires further experimental investigation. In addition, we have restricted our analyses in this study to the hippocampal formation. We cannot discount the possibility that other brain regions, or indeed, other signaling pathways, may be of critical importance to this form of learning.

It has thus been shown that blockade of NMDARs by CPP pre-training, but not post-training, impaired learning of the object displacement task in rats and the associated phosphorylation of ERK in the dentate gyrus. In conclusion, our data demonstrate a pivotal role for NMDAR activation during the acquisition of the spatial version of the object displacement task.

4. Experimental procedures

4.1. *Subjects*

Adult (250–300 g) male Wistar rats (BioResources Unit, Trinity College Dublin) were housed 3 to a cage in a controlled environment (temperature: 20–22 °C; 12:12 h light/dark cycle), with access to food and water *ad libitum*. All experiments were carried out in accordance with local and national guidelines.

4.2. *Drugs*

Rats were injected intraperitoneally (i.p.; 200 μl) with the NMDAR antagonist, (±)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP; 10 mg/kg), or saline (0.9% NaCl (v/v) in water) vehicle. Rats in naive and control groups received no injections.

4.3. *Object displacement task*

The object displacement task exploits the natural tendency of rodents to explore their environment. The learning ability of rats can be demonstrated by their preferential exploration of an object which has been moved from its initial, familiar, position when compared with static objects. The experimental apparatus consisted of a black circular open field (diameter, 200 cm; height, 35 cm) placed in a dimly-lit room. Rats were extensively handled and habituated to the experimental apparatus in the absence of objects prior to commencement of experimental procedures. 4 objects, consisting of small pieces of laboratory equipment, were fixed to the floor of the open field in square formation at least 15 cm from the walls. Objects were cleaned thoroughly between trials to ensure the absence of olfactory cues. The criteria for exploration were based strictly on active exploration, where rats had to be touching the object at least with their noses. On day 1 (training), rats were placed in the open field containing 4 objects in a particular spatial arrangement. They were given 3×6 min periods in which to explore the objects with an inter-trial interval of 3 min. During the 3 min interval, rats were placed in a holding cage in the same room as the apparatus. 24 h later (testing), one of the objects was moved from its original position and rats were reintroduced into the open field for 3×6 min periods with an inter-trial interval of 3 min. Object exploration was recorded using two timers: one to record exploration of the object to be moved, the other to record total exploration of all 4 objects. The time (in seconds) spent exploring the moved object was expressed as a percentage of the total exploration time of all 4 objects for each trial. Mean exploration time was calculated for the training phase (trials 1 to 3) and the testing phase (trials 4 to 6) for each rat. To test whether activation of NMDARs was necessary for acquisition or consolidation of learning, the NMDAR antagonist CPP or vehicle control was injected at specific timepoints. Rats were randomly assigned to 6 groups ($n=6$ in each). The naive group were caged controls and did not explore the apparatus. Control rats explored the open field for the same time as the experimental groups but were not exposed to any objects. The saline pre-injected group received an intraperitoneal injection of saline 30 min prior to training, while the saline postinjected group were injected with saline immediately after training (within

1 min of removal from the arena). The remaining 2 groups were injected with CPP either 30 min prior to training (CPP pre-injected) or immediately after training (CPP postinjected).

4.4. *Preparation of tissue samples*

Rats were stunned and decapitated immediately after testing on day 2. Control and naive rats were killed at the same time. The hippocampal formation was dissected free and a subdissection yielded the dentate gyrus and the hippocampus (CA regions). The hippocampus and dentate gyrus were cross-chopped using a McIlwain tissue chopper (350×350 μm), then rinsed twice in ice-cold Krebs solution (composition in mM: NaCl, 136; KCl, 2.54; KH_2PO_4, 1.18; $MgSO_4{\cdot}7H_2O$, 1.18; $NaHCO_3$, 16; glucose, 10; $CaCl_2$, 2). Samples were then rinsed twice with 1 ml of ice-cold Krebs solution containing DMSO (10% (v/v)) before being stored at −20 °C in 1 ml Krebs solution containing DMSO (10% (v/v)) for later analysis.

4.5. *SDS-PAGE and Western immunoblotting*

Samples were thawed, washed twice with Krebs solution and homogenised in 350 μl of ice-cold Krebs solution. Samples were assayed for protein content according to the method of Bradford (1976) and diluted to a final volume of 100 μl to equalise for protein content. Samples were then diluted 1:2 in sample buffer (27.5% dH_2O, 12.5% Tris–HCl (pH6.8), 20% glycerol, 20% SDS (10%), 5% β-mercaptoethanol and 15% bromophenol blue (10x)) and boiled for 5 min. 5 μl of molecular marker (Sigma UK) and 10 μl aliquots of sample were loaded onto 10% SDS gels. Proteins were separated at 60 mA for 35–40 min. Proteins were then transferred onto nitrocellulose membrane (Amersham Biosciences, UK) at 225 mA for 80 min.

Membranes were blocked with agitation overnight at 4 °C in Tris-buffered saline-Tween 20 (TBS-T, 20 mM Tris–HCl, 150 mM NaCl, 0.05% Tween 20; pH 7.5) containing 5% bovine serum albumin (BSA). Membranes were washed four times (30 min) in TBS-T and incubated with agitation for 2 h at room temperature with 10 ml of anti-phosphoERK per blot (1:3000 dilution in TBS-T containing 2% BSA, Calbiochem UK). Membranes were again washed four times (30 min) with TBS-T, incubated for 1 h with agitation at room temperature with 10 ml of anti-mouse IgG per blot (1:1000 dilution in TBS-T containing 2% BSA, Sigma UK) and washed four times (30 min) with TBS-T. Chemiluminescent super-signal solution (Pierce, UK) was then added (2 ml per blot) and incubated for 5 min at room temperature. Blots were exposed to photographic film for approximately 30 s and the film was developed using a Fuji X-ray processor. Membranes were then stripped of antibody by incubating for 15 min at room temperature in reblot plus solution (Chemicon, UK), washed twice (15 min) and blocked overnight in TBS-T containing 5% BSA. To assess total ERK content, blots were washed four times (30 min) and incubated for 2 h at room temperature with agitation with 10 ml of anti-ERK per blot (1:700 dilution in TBS-T containing 2% BSA, Calbiochem UK). Membranes were again washed four times (30 min) with TBS-T, incubated for 1 h with agitation at room temperature with 10 ml of anti-mouse IgG per blot (1:1000 dilution in TBS-T containing 2% BSA, Sigma UK) and washed four times (30 min) with TBS-T. Chemiluminescent super-signal solution (Pierce, UK) was added (2 ml per blot) and incubated for 5 min at room temperature, blots were exposed to photographic film for approximately 30 s and the film was developed using a Fuji X-ray processor. Protein bands were quantitated by densitometric analysis using two software packages, Grab It (Grab It Annotating Grabber 2.04.7, Synotics; UVP Ltd., UK) and Gelworks (Gelworks ID, Version 2.51; UVP Ltd., UK) for photography and densitometry respectively. Densitometric analysis was performed blind. Gelworks provides a single value (in arbitrary units) representing the density of each blot and the values presented here are means of data generated from at least 2 separate experiments. Expression of phosphoERK2 is shown as a % of total ERK2.

4.6. *Statistical analysis*

A one-way analysis of variance was performed to determine whether there were significant differences between conditions. When this analysis indicated significance (at the 0.05 level), *post hoc* Student Newmann–Keuls test analysis was used to determine which conditions were significantly different from each other. In some cases, linear regression analysis was performed.

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