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Divergent cellular pathways of hippocampal memory consolidation and reconsolidation

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Abstract

The reconsolidation of memories after their retrieval involves cellular mechanisms that recapitulate much of the initial consolidation process. However, we have previously demonstrated that there are independent cellular mechanisms of consolidation and reconsolidation in the dorsal hippocampus for contextual fear memories. Expression of BDNF was required for consolidation, while Zif268 expression was necessary for reconsolidation. Given the dichotomy between the obvious mechanistic similarity and notable dissociations between consolidation and reconsolidation, we sought to determine whether the separation at the level of gene expression reflected either parallel and independent upstream signalling pathways, or common upstream mechanisms that diverge by the level of transcriptional activation. Here we show that while consolidation and reconsolidation are commonly dependent upon NMDA receptor activation in the dorsal hippocampus there is a double dissociation between the effects of the MEK inhibitor U0126 and the IKK inhibitor sulfasalazine. Moreover, rescue experiments and western blot analyses show that there are functional NMDA receptor–ERK1–BDNF and NMDA receptor–IKK α –Zif268 pathways for consolidation and reconsolidation, respectively. Therefore, there are divergent pathways of hippocampal memory consolidation and reconsolidation, involving commonality at the cell surface, but separable downstream kinase cascades and transcriptional regulation.

Keywords

NMDA receptor; ERK; IKK; contextual fear conditioning; BDNF

Introduction

The reactivation of a memory through stimulus re-exposure induces a phase of plasticity that is known as memory reconsolidation (Nader and Hardt, 2009). Reconsolidation may serve to update memories (Lee, 2009) and is increasingly recognised as a critical process in long-term memory. Much of the recent research on reconsolidation has sought to determine its cellular mechanisms, and it has largely been found that they are similar to those of initial consolidation (Alberini, 2005; Dudai and Eisenberg, 2004; Tronson and Taylor, 2007). However, there are notable exceptions. While some have found that reconsolidation engages only a subset of those genes induced during consolidation (von Herten and Giese, 2005),

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Author Contributions

JL designed the study, analysed data and wrote the paper. RH collected and analysed data.

Competing Financial Interests

We have no competing financial interests to disclose.

microarray analysis has revealed that there appears to be both a quantitative and qualitative distinction between the cellular processes of consolidation and reconsolidation (Barnes et al., 2010).

Functional pharmacological and gene knockdown studies have demonstrated further that the consolidation and reconsolidation of a given memory are not identically dependent upon the same mechanisms. For example, tone fear memory reconsolidation, but not consolidation, is impaired by the infusion of the beta-adrenergic antagonist propranolol into the amygdala (Debiec and LeDoux, 2004). However, it is only the presence of a double dissociation between consolidation and reconsolidation that argues for a true independence of mechanisms that cannot be reduced to quantitative factors (Nader and Hardt, 2009). In an inhibitory avoidance procedure, doubly dissociable anatomical mechanisms have been identified, with the expression of the transcription factor *C/EBP β* being required in the dorsal hippocampus for consolidation and in the amygdala for reconsolidation (Milekic et al., 2007; Taubenfeld et al., 2001). We have previously demonstrated that the translational mechanisms of consolidation and reconsolidation within the dorsal hippocampus for contextual fear memory consolidation and reconsolidation are also doubly dissociable (Lee et al., 2004). Expression of the neurotrophic factor BDNF is required for initial memory consolidation only, whereas that of the transcription factor *Zif268* is selectively necessary for reconsolidation. This distinction applies whether reconsolidation is induced through standard non-reinforced context re-exposure (Lee et al., 2004), a second learning trial (Lee, 2008) or through a qualitative updating of the hippocampal memory (Lee, 2010).

An important question is to what extent does a singular cellular double dissociation reflect independent mechanisms of consolidation and reconsolidation? Given that the majority of cellular mechanisms that have been implicated in consolidation also appear to play an important role in reconsolidation, is the difference between BDNF and *Zif268* in the hippocampus meaningful beyond being a useful tool for understanding the reconsolidation process (Lee, 2008; Lee, 2010)? Therefore, we studied the upstream mechanisms leading to the expression of BDNF and *Zif268*. The primary cell surface receptor implicated in synaptic plasticity and memory is the NMDA receptor (Riedel et al., 2003). There are several reports that infusion of the NMDA receptor antagonist D-AP5 into the dorsal hippocampus impairs the acquisition/consolidation of neutral contextual (Matus-Amat et al., 2007) and context fear (Sanders and Fanselow, 2003; Schenberg and Oliveira, 2008) memories. However, while there has been no report of the effect of intra-hippocampal NMDA receptor antagonism on the reconsolidation of contextual fear memories, intra-hippocampal D-AP5 did impair the reconsolidation of spatial memories as assessed in a water maze (Kim et al., 2011). Therefore, given that systemic NMDA receptor antagonism impairs contextual fear memory reconsolidation (Suzuki et al., 2004), a primary locus of action is likely to be the dorsal hippocampus.

Downstream of the NMDA receptor, two major kinase cascades regulate the expression of plasticity-associated genes. These are the MEK-ERK cascade and the $\text{I}\kappa\text{B}$ kinase (IKK)-NF- κB pathway. Selective inhibitors of these pathways have been used previously in studies of memory consolidation and reconsolidation. The MEK inhibitor U0126 impairs both memory consolidation (Schafe et al., 2000; Trifilieff et al., 2006) and reconsolidation (Duvarci et al., 2005; Kelly et al., 2003). However, again there has been no demonstration that hippocampal inhibition of the MEK-ERK cascade impairs contextual fear memory reconsolidation. Indeed there is some evidence to suggest that intra-hippocampal U0126 does not impair contextual fear memory reconsolidation (Chen et al., 2005; Fischer et al., 2007). In contrast, inhibition of $\text{I}\kappa\text{B}$ kinase (IKK) in the dorsal hippocampus disrupts the reconsolidation of inhibitory avoidance memories (Boccia et al., 2007), and intracerebroventricular infusions of the IKK inhibitor sulfasalazine impairs contextual fear memory reconsolidation (Lubin and

Sweatt, 2007). Therefore, we tested the functional involvement of hippocampal NMDA receptors, MEK activity and IKK activity on contextual fear memory consolidation and reconsolidation, as well as their functional links with the established dissociable translational mechanisms of consolidation and reconsolidation (BDNF and Zif268, respectively).

Materials and Methods

Subjects

The subjects were 228 experimentally-naïve adult male Lister Hooded rats, weighing 275-300g at the start of the experiments. They were housed in groups of 4, in a holding room maintained at 21 °C on a normal light cycle (12 hr light: 12 hr dark; lights on at 0700). All rats were given free access to food and water. All procedures were conducted in accordance with the United Kingdom 1986 Animals (Scientific Procedures) Act (PPL 40/3205).

Surgical procedures

212 rats were implanted with bilateral chronic indwelling cannulae targeting the dorsal hippocampus. Under isoflurane anaesthesia, 11-mm 24G stainless steel cannulae (Coopers Needleworks, UK) were implanted dorsal to the target area (AP -3.5 mm, ML $\pm$ 2.0 mm, DV -1.5 mm; from bregma/skull surface). The rats were administered opiate analgesia perioperatively, and were given at least 6 days to recover before behavioral training began.

Drug infusions

Rats were subjected to two habituation PBS infusion procedures in order to minimise non-specific effects of the experimental infusion. All infusions were conducted using 28G injectors, custom-made to extend 2 mm beyond the indwelling cannulae, connected to a 5- μ l Hamilton syringe by polyethylene tubing. An infusion pump (Harvard Apparatus, UK) was used to deliver the infusion, and all infusions were carried out using a volume of 1 μ l and at a rate of 0.5 μ l/min. The drugs infused were D-2-Amino-5-phosphovaleric acid (D-AP5; 5 μ g/side; in PBS vehicle; Ascent Scientific, UK), U0126 (2 & 4 μ g/side; in 2% DMSO in PBS; Ascent Scientific, UK), sulfasalazine (Sulf; 2 μ g/side; in saline with 10 mM HEPES (pH 7.6) plus 20% DMSO; Sigma, UK) and human recombinant BDNF protein (h-BDNF; 1 μ g/side; in PBS vehicle; Caltag MedSystems Ltd, UK). D-AP5 was infused 15 min before the behavioral session. U0126 was infused 30 min before the session. Sulfasalazine was infused immediately after the session. h-BDNF was co-infused with D-AP5 or U0126 15 or 30 min prior to the session, respectively. The injector was left in place for 1 min after the end of the infusion in order to allow for diffusion away from the injector tip.

Behavioral Apparatus

The rats were trained and tested in 4 operant chambers (MedAssociates, Vermont) as previously described (Lee, 2010). Among other, unused modules, there was a houselight mounted at the top of one of the walls and the grid floor was connected to a shock generator. Infra-red video cameras (Viewpoint Life Sciences, France) were mounted above the chambers in order to record behavior and automatically score freezing behavior.

Behavioral Procedures

For fear conditioning, the rats were placed into the operant chambers for 3 min with the houselight on, and after 2 min a single footshock (0.5 mA, 2 s) was delivered. For the consolidation experiments, drugs were infused into the dorsal hippocampus before or after the conditioning session. Short-term memory (STM) and long-term memory (LTM) were

then tested 3 and 24 hr later by returning the rats to the operant chambers for 2 min with the houselight on. A second long-term memory test (LTM2) was conducted 8 days after conditioning. For the reconsolidation experiments, the fear memory was reactivated through a 2-min LTM test on the day after conditioning, and infusions were carried out before or after this session. Post-reactivation STM (PR-SRM) and LTM (PR-LTM & PR-LTM2) tests were subsequently carried out at the equivalent timepoints after reactivation to those after conditioning for the consolidation experiments. All behavior was video-recorded and automatically scored for freezing behavior using Videotrack software (Viewpoint Life Sciences, France).

Western Blot analysis

For the analysis of protein levels, rats were killed by rising concentration of CO₂, either 15 min (for kinase activity) or 2 hr (for protein quantification) after conditioning or memory reactivation. The dorsal hippocampus was immediately dissected out on wet ice and homogenised in lysis buffer before being stored at -20°C. Loaded samples were separated by electrophoresis on 7.5% (pIKK α and Zif268 analyses) or 10% (pERK1 analyses) Mini-PROTEAN® TGX™ Precast gels (Biorad, UK) at 100 V for 60-75 min. Separated proteins were blotted onto PVDF membrane at 100 mA for 1 hr under ice-cold conditions. The membrane was subsequently blocked for 1 hr in either 5% non-fat milk or 5% BSA. Incubation with primary antibody (Zif268: Santa cruz biotechnology [sc-110], 1/400 in TBST; phospho-ERK1/2: Cell Signalling [#4370], 1/2000 in 5% BSA; ERK1: Cell Signalling [#4372], 1/2000 in 5% BSA; phospho-IKK α : Santa cruz biotechnology [sc-101706], 1/400 in 5% BSA; IKK α : Santa cruz biotechnology [sc-7606], 1/1000 in 5% BSA; phospho-IKK β : Abcam [ab59195], 1/1000 in 5% BSA; IKK β : Cell Signalling [#2370], 1/1000 in 5% BSA; β -actin: Abcam [ab6276], 1/50000 in TBST or 5% BSA) took place overnight at room temperature. Secondary antibody was anti-rabbit HRP-linked antibody (Cell Signalling [#7074], 1/2000) for Cell Signalling primary antibodies. Otherwise anti-rabbit and anti-mouse HRP-linked antibodies (Sigma [A4416 & A6154], 1/10000 in TBST) were used as appropriate. The ECL reaction (Amersham Life Sciences) was developed for 1 min, and images captured using a cooled-CCD camera (G:Box Chemi XL, Syngene). The images were quantified using ImageJ software. The area and mean optical density of the target bands were used to calculate an amount of target protein. Levels of phospho-protein were normalised against total protein levels to give a proportion of protein phosphorylated.

Moreover, levels of total protein were normalised against actin levels in order to assess any group differences in the absolute levels of both total protein.

Histological procedures

For the analysis of cannula placements, rats were killed by rising concentration of CO₂, their brains extracted and fixed in 4% paraformaldehyde for 7 days. The brains were then cut into 50 μ m coronal sections and stained for nissl substance using cresyl violet. All rats that completed the full testing schedule had cannulae located successfully in the dorsal hippocampus bilaterally.

Statistical analysis

The conditioned freezing data were analysed using mixed factorial ANOVA. The data were checked for normality, using the Shapiro-Wilk test, and for sphericity. The Greenhouse-Geisser correction was employed when the assumption of sphericity was violated. As the anticipated effects of consolidation and reconsolidation impairments involve a selective impairment of long-term memory, with intact short-term memory, planned one-way ANOVAs were conducted on the effect of drug treatment at each test. Western blot analyses

were also conducted using one-way ANOVA. A significance level of $p < 0.05$ was used for all analyses.

Results

Of the total of 228 rats that were used in the study, 11 were excluded from the final analysis. 4 did not complete the experiment due to ill health, and 7 were excluded due to equipment malfunction leading to a loss of data. All remaining rats had their cannulae correctly located within the dorsal hippocampus (Fig. 1).

Memory consolidation

The acquisition/consolidation of contextual fear conditioning was impaired by the infusion of D-AP5 into the dorsal hippocampus 15 min before conditioning (Fig. 2A). Mixed ANOVA revealed a main effect of D-AP5 ($F(1,9)=11.478$, $p=0.008$), with no D-AP5 $\times$ test interaction ($F(2,18)=2.468$, $p=0.113$). While planned comparisons at each test ($p < 0.05$) revealed a significant difference between the D-AP5 and PBS groups only at the LTM and LTM2 tests, the overall pattern of results suggests that NMDA receptor antagonism impaired both the acquisition and subsequent consolidation of contextual fear conditioning.

Infusion of the MEK inhibitor U0126 into the dorsal hippocampus 30 min before conditioning resulted in an impairment of memory consolidation, with intact fear memory acquisition (Fig. 2B; U0126 $\times$ test interaction: $F(2,20)=3.927$, $p=0.036$; main effect of U0126: $F(1,10)=7.776$, $p=0.019$). Planned comparisons ($p < 0.05$) confirmed that U0126-infused rats were impaired only at the LTM and LTM2 tests. In contrast, infusion of sulfasalazine into the dorsal hippocampus immediately after conditioning had no effect upon the acquisition and consolidation of contextual fear memories (Fig. 2C; sulfasalazine $\times$ test interaction: $F(1,9)=0.038$, $p=0.850$; main effect of sulfasalazine: $F(1,9)=0.002$, $p=0.968$). Planned comparisons ($p < 0.05$) confirmed that there were no effects of sulfasalazine at either the STM or LTM tests. Therefore, the consolidation of contextual fear memories is dependent upon MEK, but not IKK in the dorsal hippocampus.

To confirm the selective involvement of the MEK-ERK cascade in memory consolidation, we analysed the conditioning-induced activation of phospho-ERK1 and phospho-IKK α in the dorsal hippocampus 15 min after training. Western blots revealed a significant induction of phospho-ERK1, but not phospho-IKK α . There was no conditioning-induced regulation of IKK α phosphorylation (Fig. 3A; $F(1,6)=0.247$, $p=0.637$), nor total IKK α protein (Fig. 3B; $F(1,6)=1.133$, $p=0.328$). There was also no change in the phosphorylation of IKK β (see Supplementary Fig 1). In contrast, the levels of phospho-ERK1 were greater in the conditioned group than in a group that was previously habituated to the context (Fig. 3C; $F(1,6)=6.838$, $p=0.040$), with no difference in the total amount of ERK1 protein (Fig. 3D; $F(1,6)=1.340$, $p=0.291$). Moreover, the phosphorylation of ERK1 was dependent upon NMDA receptor activation. Infusion of D-AP5 into the dorsal hippocampus prior to conditioning attenuated the phosphorylation of ERK1. D-AP5 reduced the proportion of ERK1 that was phosphorylated compared to PBS control (Fig. 3E; $F(1,6)=21.726$, $p=0.003$), but had no effect upon the amount of total ERK1 protein (Fig. 3F; $F(1,6)=1.643$, $p=0.247$). Therefore, contextual fear memory consolidation is associated with, and dependent upon an NMDA receptor–ERK1 signalling pathway.

We next demonstrated that there is a functional link between both NMDA receptors and BDNF and between MEK-ERK1 and BDNF. We made use of a human recombinant BDNF protein (hBDNF) that has previously been infused into the dorsal hippocampus to rescue BDNF knockdown deficits (Lee et al., 2004). Here, infusion of hBDNF rescued the impairments in memory consolidation induced by both D-AP5 and U0126 (Fig. 4). hBDNF

and D-AP5/U0126 were infused either individually, or in conjunction, enabling analysis of the independent effects of each infusion. For D-AP5, there was a significant D-AP5 $\times$ BDNF interaction ($F(1,22)=6.471$, $p=0.019$), with no effects of D-AP5 ($F(1,22)=1.310$, $p=0.265$) or BDNF ($F(1,22)=2.684$, $p=0.116$) alone. The effect of BDNF to rescue the D-AP5-induced deficit was apparent across each of the tests, as evidenced by the lack of an D-AP5 $\times$ BDNF $\times$ test interaction ($F(2,44)=1.750$, $p=0.186$). Planned comparisons revealed that when the groups not infused with hBDNF were analysed separately, the pattern of results was almost identical to the previous experiment (Fig. 2). D-AP5 appeared to impair freezing at each test, and planned comparisons revealed this time an impairment also at the STM test. In order to confirm that hBDNF rescued the D-AP5-induced deficit, the BDNF-infused groups were analysed separately. There was both an effect of BDNF ($F(1,14)=9.800$, $p=0.007$) and a BDNF $\times$ test interaction ($F(2,28)=4.264$, $p=0.024$). Planned comparisons ($p<0.05$) revealed that BDNF infusion increased conditioned freezing at the LTM and LTM2 tests, but not the STM test. Finally, there was no evidence for a potentiative effect of hBDNF in isolation. hBDNF did not increase conditioned freezing compared to PBS-infused controls (BDNF: $F(1,8)=0.463$, $p=0.516$; BDNF $\times$ test: $F(2,16)=0.155$, $p=0.858$).

For U0126, while there were no U0126 $\times$ BDNF ($F(1,27)=0.356$, $p=0.556$) or U0126 $\times$ BDNF $\times$ test ($F(2,54)=1.888$, $p=0.161$) interactions, there were significant main effects of U0126 ($F(1,27)=9.633$, $p=0.004$) and BDNF ($F(1,27)=10.331$, $p=0.003$) alone. Planned comparisons revealed that when the groups not infused with hBDNF were analysed separately, the pattern of results was identical to the previous experiment (Fig. 2). In order to confirm that hBDNF rescued the U0126-induced deficit, the BDNF-infused groups were analysed separately. There was an effect of BDNF ($F(1,15)=14.612$, $p=0.002$), but no BDNF $\times$ test interaction ($F(2,30)=2.585$, $p=0.092$). Planned comparisons ($p<0.05$) revealed that BDNF infusion increased conditioned freezing at the LTM and LTM2 tests, but not the STM test. Finally, there was again no evidence for a potentiative effect of hBDNF in isolation. hBDNF did not increase conditioned freezing compared to vehicle-infused controls (BDNF: $F(1,12)=2.001$, $p=0.183$; BDNF $\times$ test: $F(2,24)=0.238$, $p=0.790$). Therefore, the patterns of behavioral and molecular data indicate that the consolidation of contextual fear conditioning depends upon a dorsal hippocampal D-AP5–ERK1–BDNF pathway. Moreover, there is no evidence for a functional involvement of IKK α in contextual fear memory consolidation.

Memory reconsolidation

The retrieval and reconsolidation of a contextual fear memory was impaired by the infusion of D-AP5 into the dorsal hippocampus 15 min before memory retrieval/reactivation (Fig. 5A–B). Mixed ANOVA revealed a D-AP5 $\times$ reactivation interaction ($F(1,22)=6.676$, $p=0.017$), with no D-AP5 $\times$ reactivation $\times$ test interaction ($F(2,44)=1.945$, $p=0.155$). While D-AP5 had no effect upon subsequent freezing when infused in the absence of memory reactivation (D-AP5: $F(1,12)=0.180$, $p=0.679$; D-AP5 $\times$ test: $F(2,24)=0.373$, $p=0.692$), it significantly impaired freezing when infused prior to memory reactivation (D-AP5: $F(1,10)=15.446$, $p=0.003$; D-AP5 $\times$ test: $F(2,20)=3.429$, $p=0.052$). Planned comparisons ($p<0.05$) confirmed that D-AP5-infused rats were impaired at each of the post-reactivation tests. However, they also revealed that the infusion of D-AP5 acutely impaired contextual fear memory retrieval at the reactivation session ($F(1,10)=51.170$, $p<0.001$). Therefore, the overall pattern of results suggests that NMDA receptor antagonism impaired both the retrieval and subsequent reconsolidation of contextual fear conditioning.

Infusion of the IKK inhibitor sulfasalazine into the dorsal hippocampus immediately after memory reactivation resulted in an impairment of memory reconsolidation, with intact fear memory retrieval and expression (Fig. 5C–D; sulfasalazine $\times$ reactivation $\times$ test interaction: $F(2,44)=3.402$, $p=0.046$; sulfasalazine $\times$ reactivation: $F(1,22)=1.281$, $p=0.270$). While sulfasalazine had no effect upon subsequent freezing when infused in the absence of

memory reactivation (sulfasalazine: $F(1,10)=0.001$, $p=0.977$; sulfasalazine $\times$ test: $F(2,20)=0.083$, $p=0.920$), it significantly impaired freezing when infused after memory reactivation (sulfasalazine $\times$ test: $F(2,24)=4.593$, $p=0.020$; sulfasalazine: $F(1,12)=3.429$, $p=0.052$). Planned comparisons ($p<0.05$) confirmed that sulfasalazine-infused rats were impaired only at the PR-LTM and PR-LTM2 tests. Moreover, there was no difference between the sulfasalazine- and vehicle-infused groups at memory reactivation prior to infusion $F(1,12)=1.374$, $p=0.264$. In contrast, infusion of U0126 into the dorsal hippocampus 30 min before memory reactivation had no effect upon the retrieval and reconsolidation of contextual fear memories (Fig. 5E and Supplementary Fig. 2). Analysis of the post-reactivation tests revealed no effect of U0126 on contextual freezing (U0126 $\times$ test: $F(2,26)=0.306$, $p=0.739$; main effect of U0126: $F(1,13)=0.301$, $p=0.593$). Planned comparisons ($p<0.05$) confirmed that there were no effects of U0126 at either the PR-STM or PR-LTM tests. Moreover, there was no effect of U0126 acutely on memory retrieval and expression at the reactivation session ($F(1,13)=0.028$, $p=0.870$). Therefore, the reconsolidation of contextual fear memories is dependent upon IKK, but not MEK in the dorsal hippocampus.

To confirm the selective involvement of IKK α in memory reconsolidation, we analysed the reactivation-induced activation of phospho-ERK1 and phospho-IKK α in the dorsal hippocampus. Western blots revealed a significant induction of phospho- IKK α , but not phospho- ERK1. There was no conditioning-induced regulation of ERK1 phosphorylation (Fig. 6A; $F(1,6)=2.662$, $p=0.154$), nor total ERK1 protein (Fig. 6B; $F(1,6)=0.068$, $p=0.803$). In contrast, the proportion of IKK α that was phosphorylated was greater in the reactivated group than in the non-reactivation control group (Fig. 6C; $F(1,6)=7.944$, $p=0.030$), whereas the amount of total IKK α protein was unaffected (Fig. 6D; $F(1,6)=3.452$, $p=0.113$). Moreover, the phosphorylation of IKK α was dependent upon NMDA receptor activation. Infusion of D-AP5 into the dorsal hippocampus prior to memory reactivation attenuated the phosphorylation of IKK α . D-AP5 reduced the proportion of IKK α that was phosphorylated compared to PBS control (Fig. 6E; $F(1,6)=7.951$, $p=0.030$), but had no effect upon the amount of total IKK α protein (Fig. 6F; $F(1,6)=0.159$, $p=0.704$). Therefore, contextual fear memory reconsolidation is associated with, and dependent upon an NMDA receptor– IKK α signalling pathway.

Not only was the activation of NMDA receptors linked to IKK α phosphorylation, but the cellular pathway extended to the expression of Zif268. As well as impairing the phosphorylation of IKK α , infusion of D-AP5 into the dorsal hippocampus prior to memory reactivation also impaired the expression of Zif268 protein 2 hr later (Fig. 6G; $F(1,6)=43.237$, $p=0.001$). Moreover, infusion of sulfasalazine immediately after memory reactivation similarly impaired the expression of Zif268 (Fig. 6H; $F(1,6)=19.475$, $p=0.005$). Therefore, the patterns of behavioral and molecular data indicate that the reconsolidation of contextual fear conditioning depends upon a dorsal hippocampal NMDA receptor–IKK α –Zif268 pathway. Moreover, there is no evidence for a functional involvement of MEK–ERK1 in contextual fear memory reconsolidation.

Discussion

The present results demonstrate that intra-dorsal hippocampal infusions of D-AP5 impair the acquisition/consolidation and retrieval/reconsolidation of contextual fear memories. In contrast, infusions of U0126 into the dorsal hippocampus disrupted contextual fear memory consolidation, but not reconsolidation. The reverse pattern of results was observed with infusions of sulfasalazine, demonstrating a selective impairment of reconsolidation. An NMDA receptor–ERK1–BDNF neurobiological pathway of contextual fear memory consolidation was substantiated by both the attenuation of the conditioning-induced

phosphorylation of ERK1 by D-AP5, and by the rescue of the D-AP5- and U0126-induced deficits by concomitant infusions of recombinant BDNF protein. Moreover, an NMDA receptor-IKK α -Zif268 pathway of contextual fear memory reconsolidation was supported by the attenuation of the reactivation-induced phosphorylation of IKK α by D-AP5, and of the reactivation-induced upregulation of Zif268 by both D-AP5 and sulfasalazine. Finally, western blot analysis revealed that ERK1 and IKK α were selectively phosphorylated by conditioning and memory reactivation, respectively. These patterns of results strongly suggest that there are divergent cellular pathways of hippocampal contextual fear memory consolidation and reconsolidation.

Mechanisms of contextual fear memory consolidation

Intra-dorsal hippocampal infusions of D-AP5 have previously been demonstrated to disrupt the acquisition of contextual fear memories. As in the present study, D-AP5 was infused immediately prior to conditioning, resulting in a reduction in subsequent contextual freezing 24 hours later (Sanders and Fanselow, 2003; Schenberg and Oliveira, 2008; Young et al., 1994). These results are consistent with those using MK-801, rather than D-AP5, infused into the dorsal hippocampus prior to conditioning (Bast et al., 2003). The use of pre-training infusions of D-AP5 in the present study does not allow a conclusive demonstration of the involvement of NMDA receptors in consolidation rather than acquisition. Indeed, we also observed an impairment in contextual freezing at the 3-hr short-term memory test. This may appear to contradict the preserved post-shock freezing observed previously (Sanders and Fanselow, 2003; Young et al., 1994). However, in the present study, post-shock freezing was similarly unimpaired (PBS: 22.1 ± 5.7 ; D-AP5: 18.0 ± 1.6 ; $F(1,9) = 0.478$, $p=0.507$). While post-shock freezing seems to be a genuine measure of immediate associative memory (Fanselow, 1980; Wood and Anagnostaras, 2011), it may be dissociable from the 3-hr short-term memory, especially as it is recognised that there are intermediate forms of memory (Dudai, 2004). Therefore, while immediate memory expression may be independent of NMDA receptor activation, intermediate as well as long-term memories are NMDA receptor-dependent. It may be argued that the use of post-conditioning infusions of D-AP5 would have isolated consolidation mechanisms, and rendered the issue of post-shock freezing irrelevant. However, a comprehensive analysis of the effects of intra-dorsal hippocampal infusions revealed that pre-conditioning, but not post-conditioning, infusions were effective in disrupting long-term contextual freezing (Schenberg and Oliveira, 2008). This occurred regardless of whether contextual fear conditioning was conducted in a background manner (alongside the conditioning of a discrete cue) or in a foreground manner as in the present study. Therefore, it appears likely that NMDA receptor activation during and up to only a very limited period following conditioning is necessary and sufficient to support the consolidation of long-term contextual fear memories.

Similar to D-AP5, several studies have previously demonstrated that the infusion of U0126 into the dorsal hippocampus impairs the consolidation of contextual fear memories. In particular, Trifilieff et al. (2006) observed in mice that U0126 infusion prior to conditioning impaired subsequent contextual freezing at 24 hr, but not at a 1-hr short-term memory test. Other than the fact that the present study was carried out in rats, rather than mice, a further notable difference is that Trifilieff et al. used a procedure in which a tone was either paired or unpaired with footshock. The effect of U0126 was observed only in the unpaired condition, which has similarity to the foreground conditioning procedure used in the present study. Moreover, the failure of U0126 to impair contextual fear memory consolidation in a background conditioning setting has been independently demonstrated (Ahi et al., 2004). Therefore, it is evident that the vulnerability of newly-formed contextual fear memories to MEK inhibition in the dorsal hippocampus is acutely dependent upon behavioral conditions.

The rescue of the D-AP5- and U0126-induced deficits by co-infusion of recombinant BDNF protein demonstrates that BDNF is a critical effector protein of upstream NMDA receptor and MEK-ERK signalling. We have previously demonstrated that recombinant BDNF protein rescues the contextual fear memory consolidation deficit induced by antisense oligodeoxynucleotide-mediated BDNF knockdown (Lee et al., 2004). Moreover, infusion of recombinant BDNF into the dorsal hippocampus also rescued the deficit in inhibitory avoidance conditioning induced by dopamine D1 receptor antagonism (Rossato et al., 2009). We have used the same strategy to link NMDA receptors and MEK/ERK to BDNF in the current contextual fear conditioning setting, supporting previous evidence that the expression of BDNF is regulated by both NMDA receptor activity (Hardingham et al., 2002) and intracellular signalling cascades including ERK1/2 (West et al., 2001). One notable difference, however, is that we have no evidence both in the current study and previously (Lee et al., 2004) that BDNF enhanced contextual fear conditioning by itself. This is in contrast to the observation that both short-term and long-term inhibitory avoidance memories were increased by pre-training BDNF infusions (Alonso et al., 2002a; Alonso et al., 2002b). This potentiative effect of recombinant BDNF protein was associated with an elevation of ERK1/2 phosphorylation (Alonso et al., 2002a; Alonso et al., 2002b), suggesting that ERK1/2 acts downstream of BDNF signalling. This is equally possible in our contextual fear memory consolidation setting, although the effect of BDNF to rescue U0126-induced deficits in the absence of the capacity to enhance memory in isolation suggests strongly that the MEK/ERK cascade also acts upstream of BDNF expression. The further demonstration that the conditioning-induced phosphorylation of ERK1 is attenuated by D-AP5 indicates that ERK1 phosphorylation is a primary mechanism by which NMDA receptor activation leads to BDNF upregulation. This does not, however, preclude the involvement of other kinase mechanisms, including ERK2, which would be similarly disrupted by U0126. Moreover, the observation that ERK1 phosphorylation, a demonstrated mechanism of memory consolidation, is dependent upon NMDA receptor activation indicates that the impairment of memory acquisition by D-AP5 results in a failure to engage the normal consolidation process, leading to long-term amnesia.

Mechanisms of contextual fear memory reconsolidation

The dependence of contextual fear memory reconsolidation on NMDA receptor activity has not previously been studied extensively. To our knowledge, the only studies to have investigated the effects of NMDA receptor antagonists upon the reconsolidation of a contextual fear memory have used systemic routes of administration (Charlier and Tirelli, 2011; Suzuki et al., 2004). The injection of CPP (Suzuki et al., 2004) or MK-801 (Charlier and Tirelli, 2011) in mice successfully disrupted contextual fear memory reconsolidation. However, the neuroanatomical locus of action of these systemic injections was not investigated. Given that systemic injections of MK-801 also impair the reconsolidation of hippocampal-independent discrete fear memories (Lee et al., 2006), it is likely that the effect of systemic NMDA receptor antagonism on contextual fear memory reconsolidation is mediated, at least in part, by its actions in extra-hippocampal areas. However, this may not include the amygdala, as D-AP5 infusions into the basolateral amygdala appear to prevent the initial destabilisation of memories rather than their reconsolidation (Ben Mamou et al., 2006). Therefore, while not unexpected, the present demonstration that infusions of D-AP5 directly into the dorsal hippocampus impair the reconsolidation of contextual fear memories has not previously been observed. The interpretation is again complicated by the use of pre-reactivation infusions. There is strong evidence that the timecourse of contextual fear memory reconsolidation is shorter than that for its initial consolidation (Debiec et al., 2002), which along with the aforementioned failure of post-conditioning D-AP5 infusions to impair consolidation (Schenberg and Oliveira, 2008) strongly predicts that post-reactivation D-AP5 infusions would have been without amnesic effect. However, in the present study, the

infusions of D-AP5 disrupted contextual freezing not only at the post-reactivation long-term memory tests, but also more acutely at the both the 3-hour post-reactivation short-term memory test and during the reactivation session itself. The acute effect at the reactivation session is somewhat unexpected, as previous studies have observed that pre-test infusions of D-AP5 do not impair the expression of contextual fear memories (Kim et al., 1991; Matus-Amat et al., 2007). One potential difference that may account for this discrepancy is the intensity of the conditioning. We used a mild conditioning procedure involving a single 0.5-mA footshock, whereas the previous studies employed either one (Matus-Amat et al., 2007) or three (Kim et al., 1991) 1-mA shocks. Therefore, it is possible that the retrieval of weak, but not strong contextual fear memories requires NMDA receptor activity. Regardless of the acute retrieval effect, it is not the case that the infusion of D-AP5 into the dorsal hippocampus resulted directly in a long-lasting (up to 7 days) impairment in fear memory retrieval, as the deficit in contextual freezing was not observed at any of the tests when D-AP5 was infused in the absence of memory reactivation. Moreover, the acute inhibition of memory retrieval by D-AP5 is not inconsistent with the successful reactivation of the memory, as memory reactivation has previously been demonstrated to be independent of memory retrieval and expression (Ben Mamou et al., 2006). Therefore, the reactivation-dependence of the amnesic effect of D-AP5 is characteristic of reconsolidation impairments. However, amnesia is not normally also observed at the post-reactivation short-term memory test. In fact the temporal profile of amnesia induced by D-AP5 following memory reactivation is similar to that following initial acquisition. Perhaps then, the pattern of results suggests that NMDA receptor activation during memory reactivation is required for the subsequent maintenance of the memory in both the short and long term.

The observed effect of sulfasalazine on contextual fear memory reconsolidation is more characteristic of a standard reconsolidation impairment, and replicates previous findings in both contextual freezing (Lubin and Sweatt, 2007) and inhibitory avoidance (Boccia et al., 2007) settings. While sulfasalazine was infused directly into the dorsal hippocampus to impair inhibitory avoidance memory reconsolidation (Boccia et al., 2007), the study on contextual freezing used intracerebroventricular infusions (Lubin and Sweatt, 2007), the precise locus of action of which is difficult to conclude. However, in accordance with the study by Lubin & Sweatt (2007), we also observed a reactivation-induced phosphorylation of IKK α in the dorsal hippocampus. Moreover, the phosphorylation of IKK α was dependent upon upstream NMDA receptor activation, further supporting the conclusion that D-AP5 impairs contextual fear memory reconsolidation. It has previously been demonstrated *in vitro* that the activation of NF- κ B, which is ultimately regulated by the IKK complex (including IKK α ; Zandi et al., 1997), can be achieved through NMDA receptor activation (Meffert et al., 2003; Scholzke et al., 2003). Therefore, the present results show that the NMDA receptor-regulation of the IKK-NF- κ B cascade is also functionally relevant *in vivo*.

Both D-AP5 and sulfasalazine attenuated the reactivation-induced upregulation of Zif268 in the dorsal hippocampus. NMDA receptors have previously been linked functionally to the expression of Zif268 in the amygdala (Lee et al., 2009; Malkani and Rosen, 2001; Milton et al., 2008). Infusion of D-AP5 into the amygdala impaired both the acquisition/consolidation of contextual fear memories and the normal conditioning-induced upregulation of Zif268 mRNA levels (Malkani and Rosen, 2001). Similarly, intra-amygdala infusions of D-AP5 impaired the reconsolidation of a light-cocaine association and attenuated the reactivation-induced upregulation of Zif268 protein levels (Milton et al., 2008). The functional regulation of Zif268 expression by NMDA receptor activity was also potentiated by the NMDA receptor partial agonist D-cycloserine, which enhanced the reconsolidation of the light-cocaine memory (Lee et al., 2009). Moreover, it has previously been demonstrated both that Zif268 is a target of IKK/NF- κ B-mediated transcription (Carayol et al., 2006), and that IKK α regulates histone phosphorylation and acetylation in the promoter region of Zif268

(Lubin and Sweatt, 2007). Therefore, the impact of sulfasalazine to impair contextual fear memory reconsolidation is mediated, at least in part, by the failure to upregulate Zif268 expression. Similarly, the amnesic effect of D-AP5 results, again at least in part, from its attenuation of IKK α phosphorylation and Zif268 expression.

Dissociable mechanisms of consolidation and reconsolidation

Although there was a common amnesic effect of D-AP5 in both the consolidation and reconsolidation settings, we observed a double dissociation between the effects of U0126 and sulfasalazine on contextual fear memory consolidation and reconsolidation, respectively. This mirrors our previous observation that BDNF and Zif268 are independent mechanisms of consolidation and reconsolidation (Lee et al., 2004). Here, U0126 failed to impair memory reconsolidation, even at double the dose sufficient to impair consolidation. Moreover, sulfasalazine had no impact upon consolidation at the dose sufficient to disrupt reconsolidation. Given that reconsolidation is more vulnerable to disruption than consolidation (Anokhin et al., 2002; Mactutus et al., 1979), alternative doses of sulfasalazine are unlikely to have had any greater impact upon consolidation. The apparent independence of these mechanisms was further supported by the lack of significant phosphorylation of ERK1 following memory reactivation and of IKK α or IKK β after conditioning.

The failure of U0126 to impair contextual fear memory reconsolidation may appear surprising, especially given that ERK1/2 is a major regulator of IKK α and NF- κ B (Cruise et al., 2000). However, the regulation of IKK and NF- κ B by ERK1/2 was observed in a preparation that was not sensitive to NMDA receptor modulation (Cruise et al., 2000). Therefore, the mechanism by which NMDA receptor activation leads to IKK α phosphorylation may not involve ERK1/2. Moreover, two previous studies have failed to find any evidence that ERK1/2 inhibition impairs contextual fear memory reconsolidation (Chen et al., 2005; Fischer et al., 2007). Chen et al. (2005) observed that infusion of the MEK inhibitor PD98059 into the dorsal hippocampus had no impact upon the reconsolidation of a contextual fear memory. However, it did acutely impair memory retrieval, in contrast to the normal memory retrieval following U0126 in the present study. Similarly, Fischer et al. (2007) demonstrated that neither U0126 nor PD98059 affected reconsolidation when infused immediately after a retrieval trial. Instead, both studies (Chen et al., 2005; Fischer et al., 2007) observed a requirement for MEK-ERK in the extinction of contextual fear memories. Therefore MEK/ERK may be selectively required for contextual fear memory extinction, as is also the case for calcineurin (de la Fuente et al., 2011).

Sulfasalazine has previously been demonstrated to impair the consolidation of inhibitory avoidance memories (Freudenthal et al., 2005). However, again, this was observed following intracerebroventricular infusions of sulfasalazine, and has not to our knowledge been replicated with infusions directly into the dorsal hippocampus. Nevertheless, NF- κ B activity was elevated in the dorsal hippocampus following inhibitory avoidance training (Freudenthal et al., 2005), strongly suggesting that the consolidation of inhibitory avoidance memories does engage dorsal hippocampal IKK phosphorylation. In contrast, there was no evidence in the present contextual fear conditioning study that conditioning increased IKK α phosphorylation. Why the consolidation of the memories mediating inhibitory avoidance and contextual freezing should be differentially sensitive to sulfasalazine remains unclear. However, differences between inhibitory avoidance and contextual freezing are not uncommon (Muravieva and Alberini, 2010; Takao et al., 2010; Wilensky et al., 2000).

In summary, the present pattern of results indicates that there are divergent neurobiological pathways of hippocampal contextual fear memory consolidation and reconsolidation. While there is commonality at the cell surface in terms of a common requirement for NMDA receptor activity, the pathways diverge at the level of intracellular signalling cascades and

subsequent gene expression. The molecular “switch” that controls the appropriate engagement of these differential pathways, as well as its ultimate regulation, remains to be determined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Schematic representation of the locations of injectors within the dorsal hippocampus. All injector tips across the behavioral experiments were located within the grey shaded area.

Fig. 2.

Intra-dorsal hippocampal infusions of D-AP5 and U0126, but not sulfasalazine, impair contextual fear conditioning. **A**, Pre-conditioning D-AP5 impaired contextual freezing at 3 hr (STM), 24 hr (LTM) and 8 days (LTM2) after conditioning. **B**, Pre-conditioning U0126 impaired contextual freezing at the LTM and LTM2 tests, but not the STM test. **C**, Post-conditioning Sulfasalazine had no impact upon contextual freezing at either the STM or LTM test. Data presented as mean + SEM.

Fig. 3.

Western blot analysis of conditioning-induced phosphorylation of ERK1 and IKKα in the dorsal hippocampus. Rats were either latently inhibited to the context prior to contextual fear conditioning (LI) or were subjected to fear conditioning alone (cond). Quantification of phospho-protein involved normalisation against the levels of total protein. Quantification of total protein involved normalisation against levels of actin. Representative bands for the group effects are presented. **A**, Contextual fear conditioning did not induce an increase in phospho-IKKα (pIKKα). **B**, Conditioning did not alter total IKKα (tIKKα) levels. **C**, Contextual fear conditioning resulted in an increase in phospho-ERK1 (pERK1). **D**, Conditioning did not alter total ERK1 (tERK1) levels. **E**, Infusion of D-AP5 into the dorsal

hippocampus prior to conditioning attenuated the phosphorylation of ERK1 relative to PBS control. **F**, D-AP5 infusion had no effect on total ERK1 levels. Data presented as mean + SEM.

Fig. 4.

Rescue of conditioning deficits with recombinant BDNF. **A**, D-AP5 and BDNF were infused into the dorsal hippocampus alone or in a mixed solution prior to contextual fear conditioning. While D-AP5 impaired contextual freezing at 3 hr (STM), 24 hr (LTM) and 8 days (LTM2) after conditioning compared to PBS control, co-infusion of BDNF rescued the deficits at each test. BDNF alone did not affect conditioning. **B**, U0126 and BDNF were infused into the dorsal hippocampus alone or in a mixed solution prior to contextual fear conditioning. While U0126 impaired contextual freezing selectively at the LTM and LTM2 tests compared to Vehicle control, co-infusion of BDNF rescued the deficits. BDNF alone did not affect conditioning. Data presented as mean $\pm$ SEM.

Fig. 5.

Intra-dorsal hippocampal infusions of D-AP5 and sulfasalazine, but not U0126, impair contextual fear memory reconsolidation. **A**, Pre-reactivation D-AP5 impaired contextual freezing at 3 hr (PR-STIM), 24 hr (PR-LTM) and 8 days (PR-LTM2) after memory reactivation (LTM). **B**, Infusion of D-AP5 in the absence of memory reactivation had no impact upon subsequent contextual freezing. **C**, Post-reactivation sulfasalazine impaired contextual freezing at the PR-LTM and PR-LTM2 tests, but not the PR-STIM test. **D**, Infusion of sulfasalazine in the absence of memory reactivation had no impact upon

subsequent contextual freezing. **E**, Pre-reactivation U0126 had no impact upon subsequent contextual freezing. Data presented as mean + SEM.

Fig. 6.

Western blot analysis of reactivation-induced phosphorylation of IKK α and ERK1 in the dorsal hippocampus. Rats were contextually fear conditioned and then either sacrificed without memory reactivation (NR) or subjected to memory reactivation (R). Quantification of phospho-protein involved normalisation against the levels of total protein. Quantification of total protein involved normalisation against levels of actin. Representative bands for the group effects are presented. **A**, Memory reactivation did not induce an increase in phospho-ERK1 (pERK1). **B**, Reactivation did not alter total ERK1 (tERK1) levels. **C**, Memory reactivation resulted in an increase in phospho-IKK α (pIKK α). **D**, Reactivation did not alter

total IKK α (tIKK α) levels. **E**, Infusion of D-AP5 into the dorsal hippocampus prior to memory reactivation attenuated the phosphorylation of IKK α . relative to PBS control. **F**, D-AP5 infusion had no effect on total IKK α levels. **G**, Infusion of D-AP5 into the dorsal hippocampus prior to memory reactivation attenuated the upregulation of Zif268 relative to PBS control. **H**, Infusion of Sulfasalazine into the dorsal hippocampus prior to memory reactivation attenuated the upregulation of Zif268 relative to PBS control. Data presented as mean + SEM.