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The IκB Kinase Regulates Chromatin Structure during Reconsolidation of Conditioned Fear Memories

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SUMMARY

Previously formed memories are susceptible to disruption immediately after recall due to a necessity to be reconsolidated after retrieval. Protein translation mechanisms have been widely implicated as being necessary for memory reconsolidation, but gene transcription mechanisms have been much less extensively studied in this context. We found that retrieval of contextual conditioned fear memories activates the NF-κB pathway to regulate histone H3 phosphorylation and acetylation at specific gene promoters in hippocampus, specifically via IKKα and not the NF-κB DNA-binding complex. Behaviorally, we found that inhibition of IKKα regulation of either chromatin structure or NF-κB DNA-binding complex activity leads to impairments in fear memory reconsolidation, and that elevating histone acetylation rescues this memory deficit in the face of IKK blockade. These data provide insights into IKK-regulated transcriptional mechanisms in hippocampus that are necessary for memory reconsolidation.

INTRODUCTION

Formation of long-term memory involves activation of multiple transcription pathways that affect a highly coordinated pattern of gene transcription that is necessary for memory stabilization. The transcription factor nuclear-factor kappa B (NF-κB) has been implicated in the induction of synaptic plasticity and initial formation of long-term memory (Dash et al., 2005; Freudenthal et al., 2005; Levenson et al., 2004a; Liou and Hsia, 2003; Meffert et al., 2003; Yeh et al., 2002, 2004). In addition, recent investigations into the role of NF-κB signaling in memory formation have identified involvement of this pathway in the process of long-term memory reconsolidation in the crab *Chasmagnathus* (Merlo et al., 2005). These findings suggest that specific mechanisms exist for activation of the NF-κB transcriptional pathway during various stages of memory formation. However, the regulatory mechanism and molecular targets through which the NF-κB pathway mediates transcriptional regulation to stabilize long-term memory have not been experimentally investigated.

Memories, when retrieved or recalled, can become labile and susceptible to disruption, which implies the necessity for a process that restabilizes previously formed memories. This process is commonly referred to as memory reconsolidation (Nader et al., 2000; Sara, 2000). For example, in a rodent contextual fear conditioning paradigm, a novel context (training chamber) is paired with a footshock, and after this training event, a long-term memory for this association is formed. After memory formation, reexposing the animal to the training chamber triggers memory retrieval and subsequent reconsolidation of the associative memory. Reestablishment of the contextual conditioned fear (CCF) memory is subject to disruption through inhibition of protein synthesis, or when signaling cascades such as the extracellular signal-regulated kinase-mitogen-activated protein kinase (ERK/MAPK) are inhibited (Duvarci and Nader, 2004; Duvarci et al., 2005; Suzuki et al., 2004). The goal of the present study was to investigate the involvement of the NF-κB signaling cascade, and molecular targets of this pathway, during reconsolidation in a mammalian long-term memory paradigm, contextual fear conditioning.

The NF-κB/Rel transcription factors are highly regulated and require modification of inhibitor kappa B (IκB) proteins for activation. In most cells, the binding of IκB to NF-κB causes cytoplasmic retention of the complex, blocking its capacity for transcriptional regulation. IκB proteins are marked for proteolytic degradation when they are phosphorylated by the IκB kinase (IKK) complex. The IKK complex consists of two kinase catalytic subunits, IKKα and IKKβ, and a regulatory subunit IKKγ (DiDonato et al., 1997; Zandi et al., 1998). Once released from IκB proteins by the action of the IKK complex, NF-κB translocates to the nucleus and binds to the promoter region of target genes by recognizing the κB consensus sequence within DNA (reviewed in Albensi and Mattson, 2000).

Several mechanisms have been described for NF-κB transcriptional regulation in addition to the binding of the NF-κB DNA-binding complex to κB regulatory elements in DNA. For example, signaling components of the NF-κB

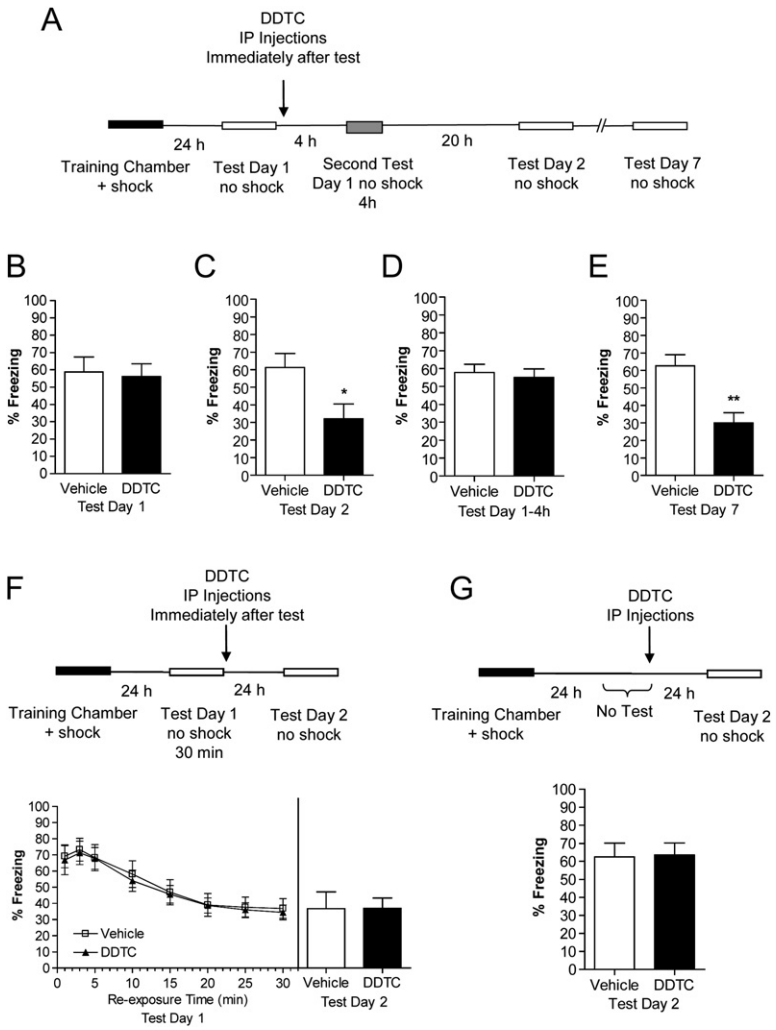

Figure 1. Effect of Inhibition of the NF-κB Signaling Pathway on Contextual Fear Conditioning after Context Reexposure

(A) Diagram outlines the experimental design used with data presented below in (B)–(E) (vehicle, n = 10; DDTC, n = 9).

(B) Freezing behavior on Test Day 1.

(C) Freezing behavior during reexposure on Test Day 2.

(D) Short-term memory test, assessed 4 hr after reexposure to chamber.

(E) Freezing behavior on Test Day 7.

(F) Effect of context reexposure for 30 min (Test Day 1) on freezing behavior assessed on Test Day 2.

(G) Injection of DDTC at the same time interval experienced by the test animals in (B), but with the first test given 48 hr later.

Student's t test; *$p < 0.05$, **$p < 0.01$ compared with vehicle. Error bars are SEM.

pathway have been shown to be involved in the regulation of gene expression through modification of histone phosphorylation and acetylation in concert with histone deacetylases (HDAC) in nonneuronal cells (Ashburner et al., 2001; Ito et al., 2001; Kumar et al., 2005; Viatour et al., 2003; Yamamoto et al., 2003). The IκB protein isoform IκBα has been shown to regulate transcription independent of NF-κB DNA binding activity through interaction with HDAC1 and HDAC3 (Viatour et al., 2003). Moreover, the IKKα subunit has been shown to function independently of the IKK complex to regulate cytokine-induced gene expression through regulation of histone H3 phosphorylation in vitro (Anest et al., 2003; Park et al., 2006; Yamamoto et al., 2003). Overall, these studies identify new roles for signaling components of the NF-κB pathway, such as IκBα and IKKα, in regulating chromatin structure and controlling gene expression independent of NF-κB binding to regulatory elements in DNA.

These unique regulatory mechanisms have heretofore not been investigated in the nervous system. However, a number of studies suggest that altered chromatin structure allows robust and lasting changes in gene expression, particularly in the nervous system (Alarcon et al., 2004; Battaglioli et al., 2002; Colvis et al., 2005; Huang et al., 2002; Korzus et al., 2004; Levenson et al., 2004b; Wood et al., 2006). In this study, we investigated whether activation of the IKK/NF-κB pathway and regulation of chromatin structure in hippocampus are important in memory reconsolidation in the mammalian CNS.

RESULTS

Inhibition of NF-κB Signaling after Memory Recall Impairs Memory Reconsolidation

To assess the effect of inhibiting NF-κB signaling on memory recall, we used a well-known NF-κB inhibitor, diethyldithiocarbamate (DDTC) (Hayakawa et al., 2003; Miyajima et al., 2003; Morais et al., 2006; Schreck et al., 1992). Figure 1A depicts the timing of DDTC administration and the time points at which we determined its effects on freezing behavior after recall of CCF memories. Immediately following reexposure to the training chamber,

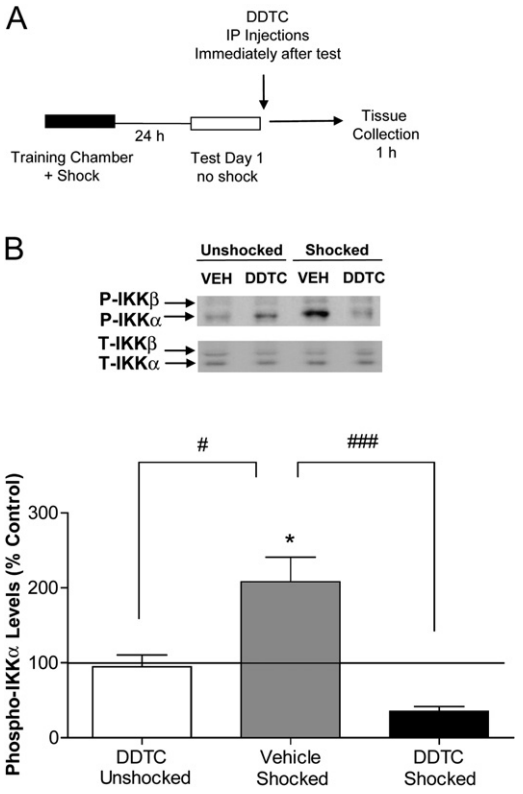

Figure 2. Activation of IKKα in Hippocampus after Context Reexposure

(A) Experimental design used with data presented below.

(B) Western blot densities for phosphorylated IKKα (P-IKKα) normalized to total IKKα (T-IKKα) levels from area CA1 (vehicle-unshocked, n = 3; DDTC-unshocked, n = 3; vehicle-shocked, n = 6; DDTC-shocked, n = 6). At the 1 hr time point assessed, there were no changes in P-IKKα levels in area CA3 or dentate gyrus after context reexposure (data not shown). Two-way ANOVA with post hoc test; $^{*}p < 0.05$, $^{**}p < 0.01$ compared with unshocked-DDTC, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with vehicle-shocked group. Error bars are SEM; solid lines represent normalized vehicle control levels.

animals were injected with vehicle or DDTC (Test Day 1). Because their injections were given after context reexposure, both vehicle and DDTC-treated animals displayed normal freezing behavior on Test Day 1 (Figure 1B). On Test Day 2, DDTC-treated animals exhibited a significant decrease in freezing behavior compared with vehicle-treated animals ($t_{(14)} = 2.50$, $p < 0.05$; Figure 1C). We also performed a short-term memory test 4 hr after context reexposure and found that vehicle and DDTC-treated animals displayed similar freezing behavior ($t_{(10)} = 0.41$, $p > 0.05$; Figure 1D). These results indicate that the NF-κB pathway participates in retrieval-induced reconsolidation of long-term CCF memories.

In order to ensure that the drug-induced interference of CCF memories did not fade at longer drug-retest time intervals, we also delayed retest for 7 days (Lattal and Abel, 2004). For these experiments, vehicle or DDTC was administered immediately after reexposure (Test Day 1) and the animals were given a second test (Test Day 7). Freezing was significantly reduced in the DDTC-treated animals on Test Day 7 ($t_{(14)} = 3.80$, $p < 0.05$; Figure 1E).

A possible effect of inhibiting NF-κB signaling activity after memory recall could be an enhancement of memory extinction instead of a blockade of memory reconsolidation. For assessment of this possible effect, we used a longer reexposure trial (30 min) on Test Day 1 to initiate memory extinction. At the end of the 30 min session, freezing behavior decreased in both vehicle and DDTC-treated animals (Vehicle: $F_{(1,8)} = 14.90$, $p < 0.001$; DDTC: $F_{(1,8)} = 10.41$, $p < 0.001$; Figure 1F). DDTC was then administered to animals immediately after the 30 min reexposure trial and animals were given a second test (Test Day 2). Reassuringly, both vehicle and DDTC-treated animals displayed similar freezing behavior (Figure 1F). These results suggest that inhibition of NF-κB signaling activity after memory retrieval does not result in an enhancement of memory extinction.

Another possible effect of inhibiting NF-κB signaling activity with DDTC is that DDTC may have induced amnesia without reactivation of memory. To control for this possible effect, DDTC was administered at the same time interval the test animals experienced on Test Day 1 without reexposure to the training chamber. On Test Day 2, we found that freezing behavior persisted in both vehicle and DDTC-treated animals (Figure 1G), indicating that the behavioral effects of DDTC are specifically related to inhibition of reconsolidation of CCF memories.

NF-κB Signaling Activity after Memory Recall Is Specific to Context Reexposure

The hypothesis of an involvement of the NF-κB pathway in memory reconsolidation predicts that NF-κB signaling activity should increase when memory recall is triggered. Therefore, we confirmed NF-κB signaling activity by evaluating two molecular markers of the pathway: IKK activation and NF-κB DNA-binding complex activity. Evaluation of IKK activity indicates whether recall triggers activation of the NF-κB signaling pathway at the IKK level of the cascade. An assessment of NF-κB DNA-binding complex activity indicates whether recall triggers active binding of NF-κB to κB regulatory elements in DNA.

To evaluate IKK activity after retrieval, protein extracts were prepared from area CA1 of hippocampus 1 hr after context reexposure, and IKK phosphorylation was assessed by western blotting with a phosphoselective antibody (Figure 2A; Test Day 1). Western blotting analysis revealed significant increases in IKKα phosphorylation at Ser-180 after reexposure ($F_{(3,14)} = 12.15$, $p < 0.05$; Figure 2B) with no change in IKKβ phosphorylation at Ser-181. These results suggest that memory recall triggers IKKα, but not IKKβ, activation in hippocampus after reexposure to the training chamber.

As a control, we confirmed that DDTC administered immediately after context reexposure blocked IKKα activation in hippocampus. Similar to inhibition of CCF memories, recall-mediated increases in phosphorylated

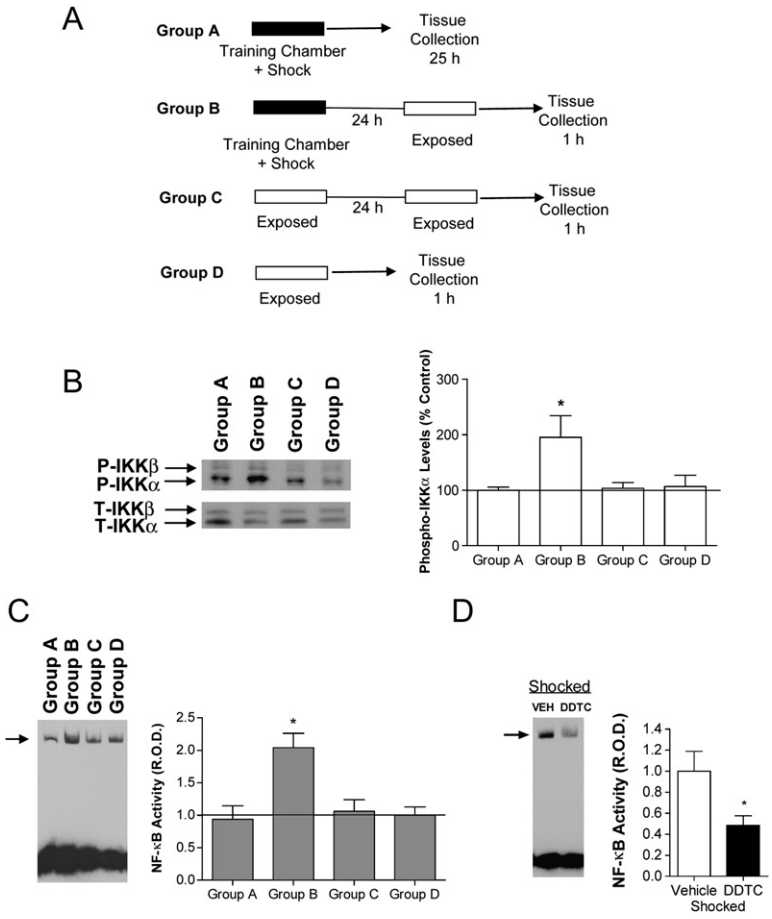

Figure 3. Activation of the NF-κB Signaling Pathway Is Regulated after Reexposure to the Context

(A) Outline of the experimental procedure performed.

(B) Representative western blots and densitometry analysis for phosphorylated IKKα (P-IKKα) normalized to total IKKα (T-IKKα) levels are shown (Group A, n = 9; Group B, n = 9; Group C, n = 6; Group D, n = 8). Student's t test; *p < 0.05 compared with naive group.

(C) Nuclear extracts were prepared from area CA1 from all groups in parallel. NF-κB DNA binding activity was measured using EMSA (Group A, n = 5; Group B, n = 10; Group C, n = 8, Group D, n = 5). Student's t test; *p < 0.05 compared with naive group.

(D) Nuclear extracts were prepared from area CA1 1 hr after reexposure (vehicle-shocked, n = 6; DDTC-shocked, n = 6). The specific band is indicated with an arrow. The relative optical density (R.O.D) values of the specific NF-κB-shifted-band normalized to vehicle group (VEH) are shown. Student's t test; *p < 0.05 compared with vehicle-shocked group.

Error bars are SEM; solid lines represent normalized naive control levels.

IKKα were significantly inhibited with DDTC treatment ($F_{(3,14)} = 12.55$, $p < 0.001$; Figure 2B).

It is possible that the upregulation in IKKα activity in hippocampus was not specific to memory recall, but rather was simply due to context exposure or to a persistent effect from the prior day's training. Thus, we evaluated IKKα activity in the following four groups of animals: (1) Group A, animals trained but not reexposed to the training chamber; (2) Group B, animals trained and reexposed to the training chamber (as described in the previous section); (3) Group C, untrained animals that were exposed to the training chamber and 24 hr later reexposed without being shocked; and (4) Group D, untrained animals that were exposed to the training chamber once without being shocked. All groups were compared to naive animals that were not exposed to the training chamber (Figure 3A).

Western blotting analysis revealed no change in IKKα phosphorylation 25 hr after training with no context reexposure (Figure 3B, Group A). Animals that were trained and reexposed to the training chamber (24 hr later) showed a significant increase in IKKα phosphorylation 1 hr after context reexposure ($t_{(16)} = 2.41$, $p < 0.05$; Figure 3B, Group B), as we described above. Reexposure of untrained animals to the training chamber resulted in no change in IKKα phosphorylation ($t_{(7)} = 0.13$, $p < 0.05$; Figure 3B, Group C). One hour after a seven-minute exposure to the training chamber, which was similar to the duration of the training session, we found no significant change in IKKα phosphorylation in Group D (Figure 3B). Together these results indicate that reexposure to the training chamber, in previously trained animals only, selectively triggered an increase in IKKα activity in hippocampus.

Using an electrophoretic mobility shift assay (EMSA), we assessed NF-κB DNA-binding complex activity in hippocampal nuclear extracts after context reexposure. This assay is a technique for measurement of DNA binding activity of the NF-κB DNA-binding complex. NF-κB DNA binding activity in this in vitro assay system reflects nuclear translocation and activation of the NF-κB transcription factor. In nuclear extracts from area CA1, only one shifted band was detected with this assay (see Figures 3C and 3D). This band corresponds to NF-κB-subunit-containing protein complexes that specifically bind to the κB consensus sequence, as we previously demonstrated using competition assays (Lubin et al., 2005).

One hour after context reexposure, a significant increase in NF-κB DNA binding activity was observed in area CA1 of hippocampus ($t_{(12)} = 2.91$, $p < 0.05$; Figure 3C). Similar to IKKα activity, NF-κB DNA-binding activity after context reexposure was significantly attenuated following

treatment of animals with DDTC ($t_{(10)}$ = 2.45, $p < 0.05$; Figure 3D). These results demonstrate that the NF-κB DNA-binding complex is activated in hippocampus after recall of CCF memories, and that DDTC blocks this effect. Overall, then, we observed hippocampal NF-κB pathway activation after memory recall using two independent biochemical assay procedures.

Like IKKα, NF-κB DNA binding activity in area CA1 of hippocampus showed no change 25 hr after training with no context reexposure (Figure 3C, Group A). In animals that were trained and reexposed to the same training chamber, we found a significant increase in NF-κB DNA binding activity ($t_{(16)}$ = 3.86, $p < 0.01$; Figure 3C, Group B), as described above. Animals that were exposed to the training chamber twice showed no change in NF-κB DNA binding activity (Figure 3C, Group C). At 1 hr after a single exposure to the training chamber, no significant change in NF-κB DNA binding activity was detected in area CA1 of hippocampus (Figure 3C, Group D). These results indicate that activation of the NF-κB DNA-binding complex in hippocampus occurred selectively after reexposure of trained animals to the context.

Overall, these results are consistent with the hypothesis that increases in both IKKα activity and NF-κB DNA-binding complex activity are part of the retrieval-induced memory reconsolidation process.

Regulation of Histone H3 Modifications by IKKα Signaling Activity

The binding of transcription factors to promoter regions within DNA can potentially trigger chromatin remodeling, thereby inducing a mechanism for regulating the transcription of the gene (for review see, Natoli et al., 2005). Thus, we assessed histone H3 posttranslational modifications in area CA1 of hippocampus 1 hr after context reexposure of trained animals (Figure 4A). We observed significant increases in hippocampal histone H3 phosphorylation and acetylation levels after context reexposure (P-H3: $F_{(3,25)} = 6.43$, $p < 0.05$; AcH3: $F_{(3,23)} = 8.14$, $p < 0.05$; Figures 4B and 4C). These data suggest that chromatin remodeling occurs in response to memory recall. To determine if these changes were dependent on NF-κB pathway activation, we assessed the effects of the IKK inhibitor DDTC on histone phosphorylation and acetylation. The increases in histone H3 phosphorylation and acetylation after reexposure were significantly attenuated with DDTC treatment (P-H3; $F_{(3,25)} = 6.43$, $p < 0.01$, AcH3; $F_{(3,23)} = 8.14$, $p < 0.01$; Figures 4B and 4C). In additional experiments we found no change in histone H4 acetylation at 1 hr after reexposure (Figure 4D). Based on these data we hypothesize that altered histone H3 phosphorylation and acetylation in hippocampus after recall of CCF memories is regulated by IKKα/NF-κB signaling activity.

Histone H3 Regulation after Recall Is Specific to Context Reexposure

To determine whether histone H3 regulation in hippocampus was specific to recall of the learned context-plus-shock association or whether it was induced by context exposure alone, we evaluated histone regulation in the four groups of animals previously described (Figure S1A in the Supplemental Data available with this article online). We found no significant change in histone H3 phosphorylation and acetylation 25 hr after training with no context reexposure (Figures S1A and S1B; Group A). Histone H3 phosphorylation and acetylation significantly increased 1 hr after context reexposure in previously trained animals (P-H3: $t_{(17)}$ = 3.90, $p < 0.01$; AcH3: $t_{(17)}$ = 3.72, $p < 0.01$; Figures S1A and S1B; Group B). Reexposure of untrained animals to the training chamber 24 hr later elicited no change in histone H3 phosphorylation and acetylation (P-H3: $t_{(17)}$ = 3.904, $p < 0.01$; AcH3: $t_{(17)}$ = 3.723, $p < 0.01$; Figures S1A and S1B; Group C). Exposure to the training chamber alone elicited no change in histone H3 phosphorylation and acetylation (Figures S1A and S1B; Group D). In all groups we observed no change in histone H4 acetylation (Figure S1C). Together these results suggest that histone H3 regulation in hippocampus was specific to reexposure to the context used for conditioned training. These findings implicate the regulation of chromatin structure as a mechanism contributing to memory reconsolidation.

IKK Activity Regulates Histone H3 Phosphorylation and Acetylation after Recall

Next, we examined the effects of directly inhibiting IKKα activity on recall-mediated histone H3 regulation. For these experiments we used sulfasalazine (SSZ), which is a direct pharmacologic inhibitor of IKK (Wahl et al., 1998; Weber et al., 2000). We first determined the effects of inhibiting IKKα activity on memory reconsolidation, as assessed behaviorally. Two different doses of SSZ, 5 mM and 10 mM in 5 μl of vehicle, were administered intracerebroventricularly (i.c.v.), and their effects were assessed on CCF memory reconsolidation (Figure 5A). On Test Day 1, no significant differences in freezing behavior were observed between the groups of animals used for subsequent vehicle and SSZ treatment (Figure 5B). Upon retesting, animals infused with the lower dose of SSZ immediately after context reexposure showed a trend toward a decrease, but no significant difference in, freezing behavior when compared to the vehicle-treated animals (Figure 5C). However, animals infused with the higher dose of SSZ showed significantly less freezing behavior compared with animals injected with vehicle ($t_{(9)}$ = 3.28, $p < 0.01$; Figure 5C). We found no effect of the higher dose of SSZ on short-term memory ($t_{(9)}$ = 0.61, $p > 0.05$; Figure 5D), which confirms the selective actions of SSZ for long-term memory after memory recall (24 hr). These results demonstrate that inhibition of IKKα activity with SSZ after recall impairs reconsolidation of CCF long-term memories.

As a control, we confirmed the efficacy of the higher dose of SSZ to inhibit IKKα signaling after recall. Western blotting analyses show that increases in IKKα

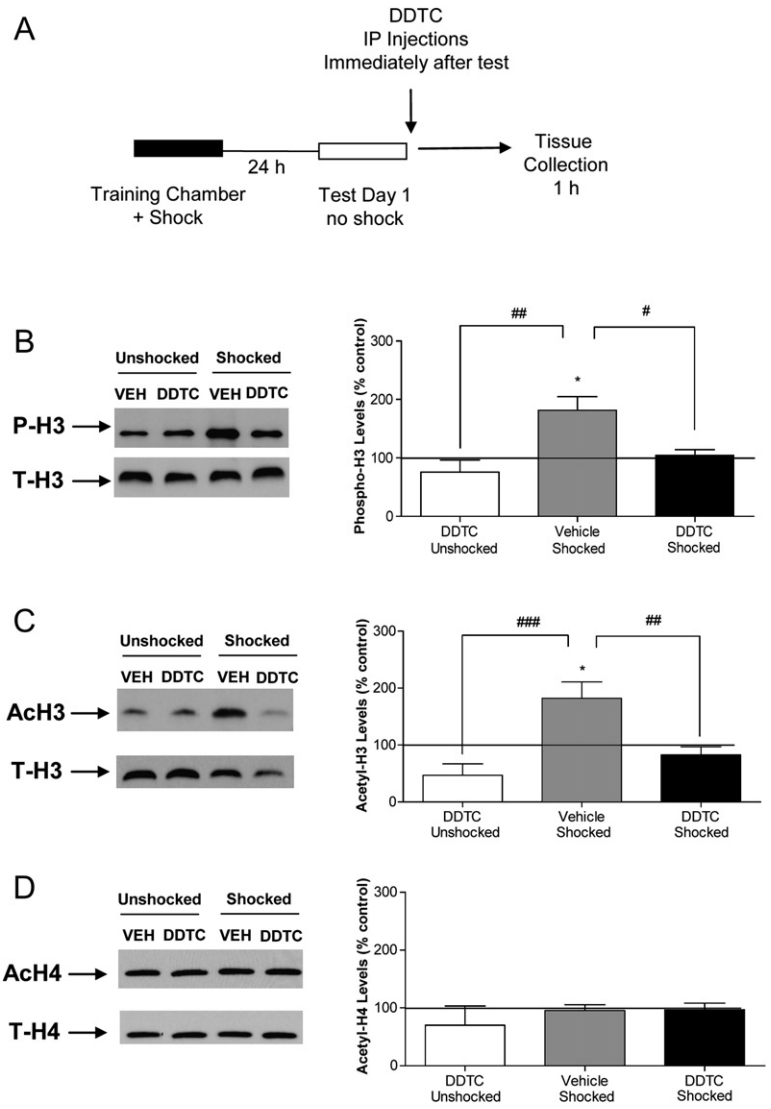

Figure 4. Regulation of Histone H3 Phosphorylation and Acetylation Is Associated with Activation of the NF-κB Signaling Pathway after Context Reexposure

(A) Histone extracts from area CA1 were prepared from animals 1 hr after DDTC or saline (vehicle) treatment.

(B) Phosphorylated histone H3 (P-H3) levels were normalized to total histone H3 (T-H3) protein levels from area CA1. There were no changes in histone H3 modifications in area CA3 or dentate gyrus at 1 hr after recall (data not shown) (vehicle-unshocked, n = 5; DDTC-unshocked, n = 6; vehicle-shocked, n = 9; DDTC-shocked, n = 9).

(C) Acetylated histone H3 (AcH3) levels were normalized to total histone H3 (T-H3) protein levels (vehicle-unshocked, n = 6; DDTC-unshocked, n = 6; vehicle-shocked, n = 6; DDTC-shocked, n = 9).

(D) Acetylated H4 (AcH4) levels normalized to total histone H4 (T-H4) levels (vehicle-unshocked, n = 4; DDTC-unshocked, n = 4; vehicle-shocked, n = 6; DDTC-shocked, n = 6).

Two-way ANOVA with post hoc test; $^{**}p < 0.01$, $^{***}p < 0.001$ compared with unshocked-DDTC, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with vehicle-shocked group. Error bars are SEM; solid lines represent normalized vehicle-unshocked control levels.

phosphorylation 1 hr after reexposure to the training chamber were significantly attenuated with SSZ treatment ($F_{(2,12)} = 12.13$, $p < 0.01$; Figure 5E). Recall-mediated increases in NF-κB DNA binding activity were also blocked with SSZ treatment ($t_{(6)} = 3.03$, $p < 0.05$; Figure 5F).

Having established a requirement for IKKα activity in memory reconsolidation, we next investigated the effect of inhibiting IKKα on histone H3 regulation in hippocampus after memory recall. We found that recall-mediated increases in histone H3 phosphorylation and acetylation were blocked with SSZ treatment (P-H3: $F_{(2,9)} = 9.32$, $p < 0.05$; AcH3: $F_{(2,8)} = 8.76$, $p < 0.05$; Figures 6A and 6B). These results suggest a mechanism for histone H3 regulation during memory reconsolidation; specifically, that IKKα regulates histone H3 phosphorylation and acetylation. Furthermore, these results confirm, using another inhibitor of the IKKα pathway, that this signaling cascade is involved in chromatin regulation in hippocampus after retrieval of CCF memories.

Effect of NF-κB Inhibition on Histone H3 Modifications after Memory Recall

Next, we considered that IKKα regulation of histone H3 in hippocampus after recall might have occurred as a result of two possible scenarios: (1) IKKα recruiting the NF-κB DNA-binding complex to mediate regulation of histone H3, or (2) IKKα directly mediating regulation of histone H3. We distinguished between these two scenarios by using a direct inhibitor of the NF-κB DNA-binding complex, SN50. The SN50 active peptide prevents the NF-κB transcriptional complex from interacting with its normal DNA binding sites and is known to be effective in neurons even when applied outside of the cell (Kubota et al., 2000; Lee and Rivier, 2005). We administered the SN50 active peptide i.c.v. to animals 2 hr prior to reexposure to the training chamber (Figure 7A).

As a positive control for the effectiveness of SN50, we examined nuclear extracts prepared from area CA1 1 hr after context reexposure using EMSA. As expected,

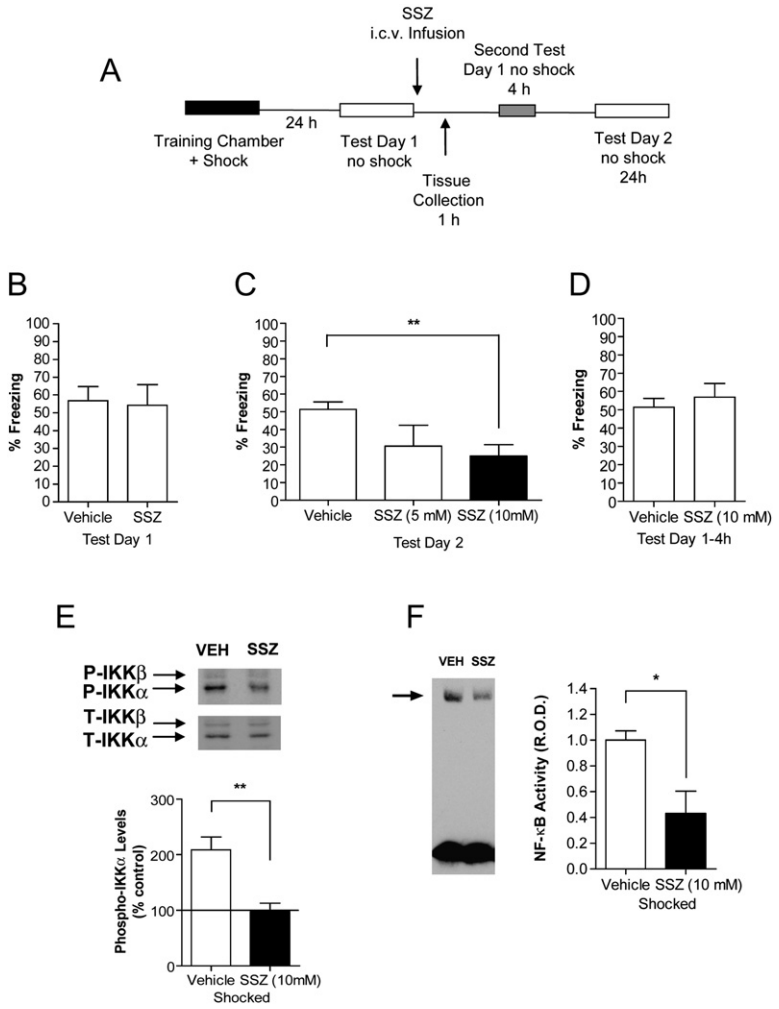

Figure 5. Inhibition of IKKα Affects CCF Memories after Reexposure

(A) The experimental design used with the data presented in (B)–(E).

(B) Freezing behavior of animals infused with either vehicle or SSZ immediately following reexposure on Test Day 1 (Vehicle, n = 6; SSZ, n = 10).

(C) Freezing behavior of animals during reexposure on Test Day 2 (Vehicle, n = 6; 5 mM SSZ, n = 5, 10 mM SSZ, n = 5).

(D) Assessment of freezing behavior 4 hr after reexposure (10 mM SSZ, n = 6).

(E) Western blots and graph summary of data for phosphorylated IKKα (P-IKKα) normalized to total IKKα (T-IKKα) levels (Vehicle, n = 5; 10 mM SSZ, n = 5).

(F) The binding activity for NF-κB measured 1 hr after 10 mM SSZ treatment. The specific band is indicated by an arrow. The specific NF-κB retarded band from the 10 mM SSZ treated group (n = 4) was normalized to the vehicle-treated group (n = 4). R.O.D., relative optical density.

Two-way ANOVA with post hoc test; *p < 0.05 compared with vehicle-shocked. Error bars are SEM; solid lines represent normalized vehicle control levels.

EMSA analysis showed a significant decrease in NF-κB DNA binding activity with SN50 treatment as compared with vehicle or SN50M treatment ($F_{(2,9)} = 10.58$, $p < 0.01$; Figure 7B).

We next determined whether direct inhibition of the NF-κB DNA-binding complex by SN50 could impair CCF memories after retrieval. On Test Day 1, all animals showed similar freezing behavior (Figure 7C), indicating that administration of SN50 2 hr prior to reexposure did not prevent the retrieval of CCF memories. On Test Day 2, animals infused with SN50 showed a significant decrease in freezing behavior when compared with vehicle- or SN50M-treated animals ($F_{(2,21)} = 4.19$, $p < 0.05$; Figure 7D). We also confirmed that the effect of SN50 was specific for long-term CCF memory by showing that SN50 did not affect short-term memory, assessed 4 hr later, relative to that of vehicle- or SN50M-treated animal controls ($F_{(2,13)} = 0.08$, $p > 0.05$; Figure 7E). These results demonstrate that disruption of CCF memories after recall requires activation of the NF-κB transcriptional complex. These experiments are also a third independent line of evidence indicating that the NF-κB transcriptional pathway is required for memory reconsolidation.

Once the amnestic effects of SN50 were determined, we next examined the effect of inhibiting NF-κB DNA binding activity on histone H3 regulation in area CA1 after memory recall. SN50 treatment had no significant effect on recall-induced increases in histone H3 phosphorylation and acetylation (P-H3: $F_{(3,12)} = 3.47$, $p < 0.05$; AcH3: $F_{(3,12)} = 3.45$, $p < 0.05$; Figures S2A and S2B). This indicates that NF-κB DNA binding is not involved in regulating histone H3 phosphorylation and acetylation after memory retrieval. However, it is possible that blocking NF-κB DNA binding might trigger alternative modifications of histones that are not normally seen. To assess this possibility we also measured histone H4 acetylation 1 hr after reexposure to the training chamber. No change in histone H4 acetylation was observed with SN50 treatment as compared to either vehicle or SN50M treatment (Figure S2C).

In summary, the results in this section suggest that the mechanism of regulation of histone H3 phosphorylation and acetylation during memory reconsolidation is not at

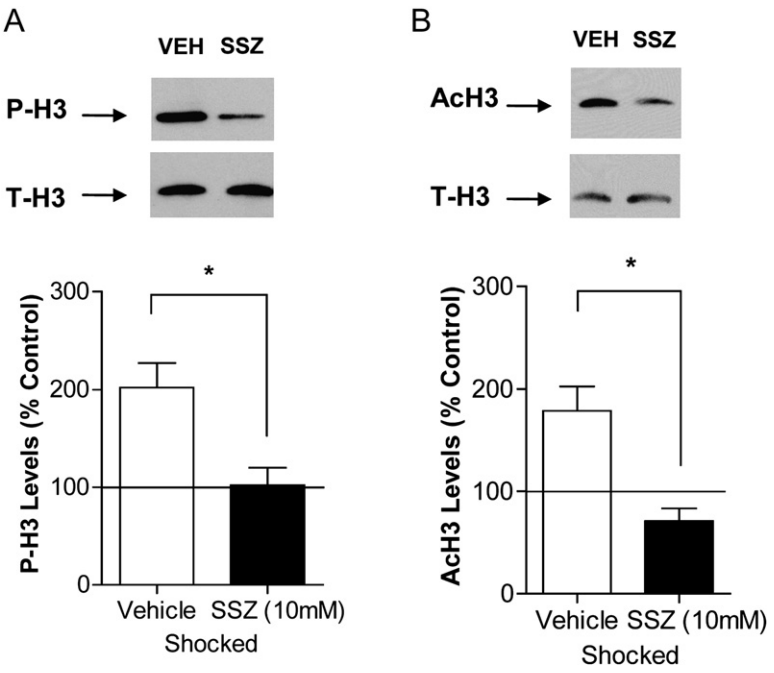

Figure 6. IKKα Contributes to Increases in Histone H3 Phosphorylation and Acetylation during Reconsolidation

(A) Western blot and quantitative analysis of phosphorylated histone H3 (P-H3) levels after reexposure to the training chamber (unshocked, n = 4; vehicle-shocked, n = 4; shocked-SSZ, n = 4).

(B) Western blot and quantitative analysis of acetylated histone H3 (AcH3) levels from area CA1 after 10 mM SSZ treatment (naive, n = 4; vehicle-shocked, n = 4; shocked-SSZ, n = 4). One-way ANOVA; *$p < 0.05$ compared with unshocked-vehicle. Error bars are SEM; solid lines represent normalized unshocked-vehicle control levels.

the level of the NF-κB DNA-binding complex, but rather at the level of upstream IKKα activity in the NF-κB signaling pathway. These surprising findings indicate an unexpected divergence of IKKα/NF-κB signaling upstream of the genome. Thus, IKKα appears to independently regulate chromatin structure in addition to triggering NF-κB DNA-binding complex activation.

IKK Signaling Activity Regulates Histone H3 Modifications at Gene Promoter Regions

In prior Affymetrix microarray studies, we identified 35 memory-associated genes expressed in area CA1 of hippocampus after fear conditioning (Levenson et al., 2004a). We found that seven of these genes contained NF-κB regulatory elements in their upstream regions. Specifically, we found NF-κB regulatory elements in the upstream regions of the immediate-early gene *Zif268* (Figure 8A; also known as *EGR-1*, *NGFI-A*, *Krox 24*, *TIS 8*, and *ZENK*) that is upregulated in hippocampus after memory reactivation (Bozon et al., 2003; Hall et al., 2001; Lee et al., 2004). This is consistent with recent studies that have identified *Zif268* as a bona fide NF-κB pathway gene target (Carayol et al., 2006). These findings suggested the intriguing possibility that IKKα activity might be directly involved in chromatin remodeling across the *Zif268* promoter to mediate memory reconsolidation. To this end, we investigated whether recall triggered changes in histone H3 phosphorylation and acetylation specifically at the level of the *Zif268* gene promoter and examined whether IKKα inhibition can alter histone modifications at this gene promoter.

Using a chromatin immunoprecipitation (ChIP) assay combined with quantitative real-time PCR, we found that recall triggered a significant increase in histone H3 phosphoacetylation and acetylation levels at the *Zif268* promoter that were significantly attenuated with DDTC treatment relative to naive controls (PH3/AcH3: $F_{(3,12)} = 18.49$, $p < 0.01$; AcH3: $F_{(3,11)} = 25.18$, $p < 0.001$; Figure 8B). There were no significant changes in histone H4 acetylation at the *Zif268* promoter after memory recall (Figure 8B). These findings indicate that IKKα regulates histone H3 phosphorylation and acetylation at the *Zif268* promoter in response to memory recall.

As a positive control for our ChIP assay, we also tested the effect of IKKα inhibition on histone modifications around the IκBα promoter, a well-defined gene target of the NF-κB signaling pathway (Baldwin, 1996). Reexposure of animals to the training chamber triggered a significant increase in histone H3 phosphorylation and acetylation at the IκBα promoter that was blocked with IKKα inhibition with DDTC compared with naive controls (PH3/AcH3: $F_{(3,12)} = 10.95$, $p < 0.05$; AcH3: $F_{(3,12)} = 9.49$, $p < 0.01$; Figure 8C). We found no changes in histone H4 acetylation at the IκBα promoter after memory recall (AcH4: $F_{(3,12)} = 0.28$, $p > 0.05$; Figure 8C). As an additional control, we also determined that there were no changes in recall-mediated histone modifications at the promoter for β-Actin (Figure 8D). Together these results provide further evidence for IKKα-mediated changes in hippocampal histone H3 phosphorylation and acetylation occurring at individual gene promoters during memory reconsolidation, and identify *Zif268* as a target of chromatin modification in memory reconsolidation.

Effect of Inhibiting HDAC and IKKα/NF-κB Signaling after Memory Retrieval

Next, we sought to determine whether augmenting chromatin remodeling through triggering increased histone acetylation could affect memory reconsolidation. Thus,

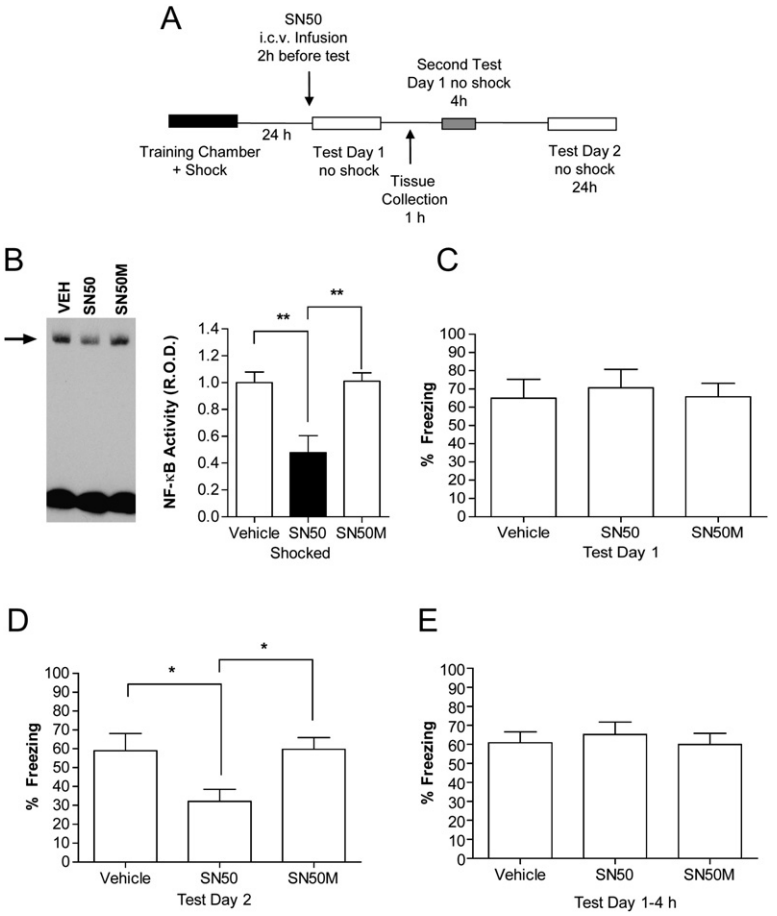

Figure 7. Effect of Direct Inhibition of the NF-κB Transcriptional Complex during Reconsolidation

(A) Outline of the experimental procedure used.

(B) Representative EMSA showing NF-κB binding activity in hippocampal nuclear extracts from animals injected with vehicle, SN50, or SN50M. Student's t test; **p < 0.01. R.O.D., relative optical density. VEH, vehicle.

(C) On Test Day 1 performance of animals infused with vehicle (n = 8), the active peptide SN50 (n = 8), or the inactive peptide SN50M (n = 7).

(D) Freezing behavior of SN50-treated animals (n = 8) compared with vehicle- treated (n = 8) or SN50M- treated (n = 7) animals on Test Day 2.

(E) Freezing behavior of vehicle-, SN50M-, or SN50-treated animals (n = 5) 4 hr after reexposure.

One-way ANOVA; **p < 0.01 compared with vehicle-shocked. Error bars are SEM.

we used the HDAC inhibitor sodium butyrate (NaB) to enhance hippocampal histone acetylation and determined if this manipulation could rescue memory reconsolidation in the presence of IKK inhibition. We speculated that inhibition of HDAC activity would enhance histone acetylation and mimic the capacity of IKKα to alter chromatin structure, and thus overcome the deficits in memory reconsolidation triggered by IKKα inhibition.

Thus, to test whether IKKα-mediated histone H3 regulation is functionally relevant to memory reconsolidation, we performed the following experiment using NaB and DDTC. Animals were trained as previously described, and on Test Day 1, animals were injected with vehicle or NaB 1 hr prior to reexposure to the training chamber (Figure 9A). At the end of the reexposure trial on Test Day 1, no differences in freezing behavior were observed in vehicle-treated animals compared with NaB-treated animals ($t_{(31)}$ = 0.24, p > 0.05; Figure 9B). These results indicate that HDAC inhibition did not disrupt retrieval of CCF memories.

Immediately after reexposure to the training chamber on Test Day 1, the vehicle- and NaB-treated animals were separated into two groups, and each group received either vehicle or DDTC injections. On Test Day 2, no differences in freezing behavior were observed with NaB treatment compared with vehicle treatment. As expected, the conditioned freezing in animals was significantly disrupted with DDTC treatment ($F_{(3,40)}$ = 6.02, p < 0.01; Figure 9C). However, no impairment of memory was observed with NaB plus DDTC treatments (i.e., freezing was observed), indicating that enhancing histone acetylation was sufficient to ameliorate the effect of inhibiting IKKα/NF-κB activity on CCF memory reconsolidation. This finding is consistent with the hypothesis that regulation of chromatin structure by IKKα is functionally relevant in memory reconsolidation.

DISCUSSION

In the present study, we demonstrate that the IKKα/NF-κB transcription pathway contributes to memory reconsolidation in a mammalian CCF paradigm. We observed an upregulation in hippocampal NF-κB signaling activity that was associated with increases in histone H3 phosphorylation and acetylation selectively triggered after retrieval of CCF memories. We found that inhibition of the NF-κB signaling cascade at the level of the IKKα protein kinase blocked both CCF memory reconsolidation and histone H3 posttranslational modifications in hippocampus after retrieval. This evidence links the IKKα protein kinase and its regulation of histone H3 modifications in vivo

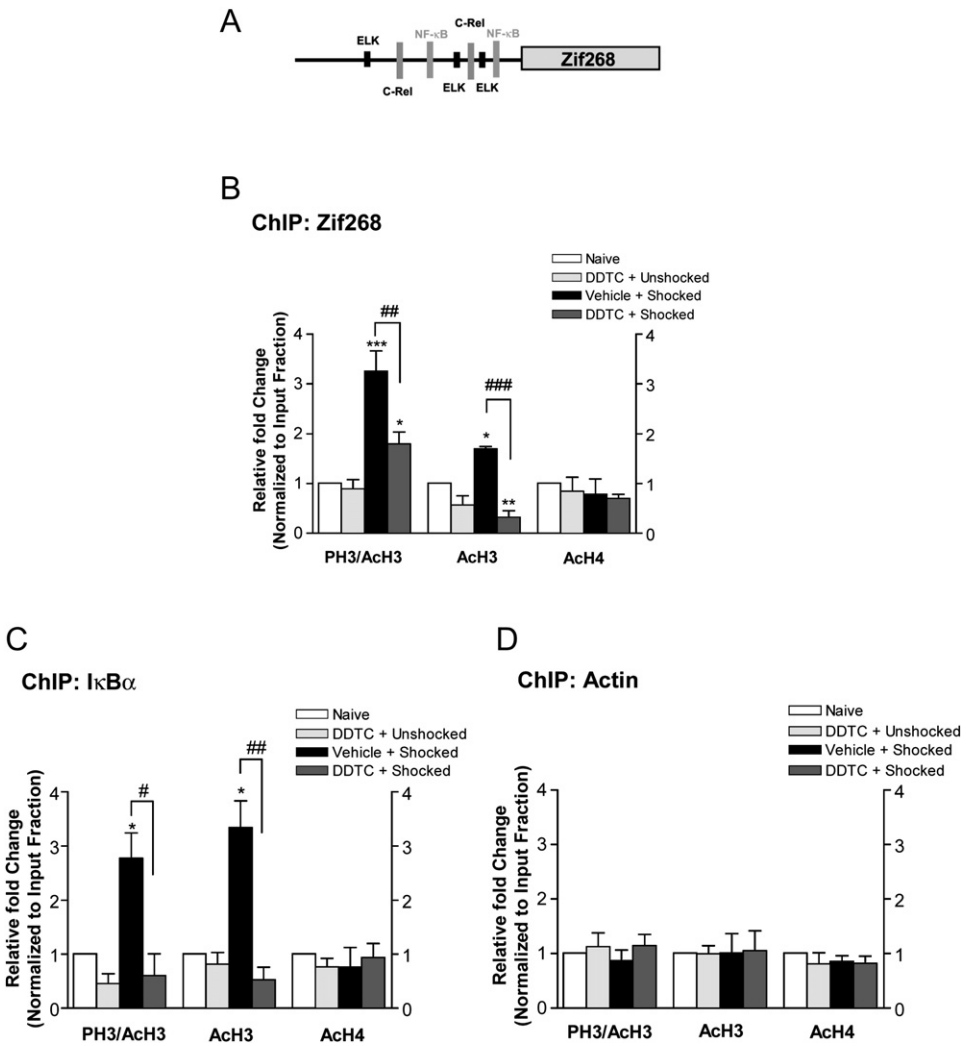

Figure 8. Inhibition of IKKα Affects Histone Modifications around Specific Gene Promoters

(A) NF-κB binding sites, including sites for the c-Rel NF-κB subunit and ELK, identified within 1 kbp promoter upstream sequences of the *Zif268* gene (GenBank accession number M18416).

(B) Histone modifications (H3 phosphoacetylation [PH3/AcH3], acetylation [AcH3], and histone H4 acetylation [AcH4]) at the *Zif268* promoter.

(C) Histone modifications at the *IκBα* promoter.

(D) Histone modifications around the β-Actin promoter.

One-way ANOVA; $^{*}p < 0.05$, $^{**}p < 0.01$ compared with naive controls, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared to shocked-DDTC, n = 4. Error bars are SEM.

to the process of memory reconsolidation. These findings provide insights into roles for both NF-κB pathway-mediated transcriptional regulation and chromatin structure regulation in the process of memory reconsolidation.

Recent investigations into the molecular mechanisms underlying formation of long-term memory have implicated the NF-κB signaling pathway in the process of initial memory consolidation (Freudenthal et al., 2005; Levenson et al., 2004a; Merlo et al., 2005; Yeh et al., 2002), and here we found that inhibition of NF-κB signaling significantly impaired memory *reconsolidation* after retrieval of CCF memories in rats. Thus far, the molecular targets of the NF-κB cascade in memory formation are not known, and we were interested in identifying these molecular marks in hippocampus during memory reconsolidation. Recent work has shown that IKKα translocates into the nucleus to mediate NF-κB-dependent and -independent gene expression (Birbach et al., 2002; Ear et al., 2005; Massa et al., 2005). Investigations into the nuclear role of IKKα have demonstrated a corecruitment of IKKα and the histone acetyltransferase CREB binding protein (CBP) onto gene promoter sites (Anest et al., 2003; Yamamoto et al., 2003). Moreover, the association between IKKα and CBP has been shown to induce IKKα-mediated phosphorylation of histone H3 at Ser-10 in vitro (Anest et al., 2003; Yamamoto et al., 2003). Thus, IKKα regulation of

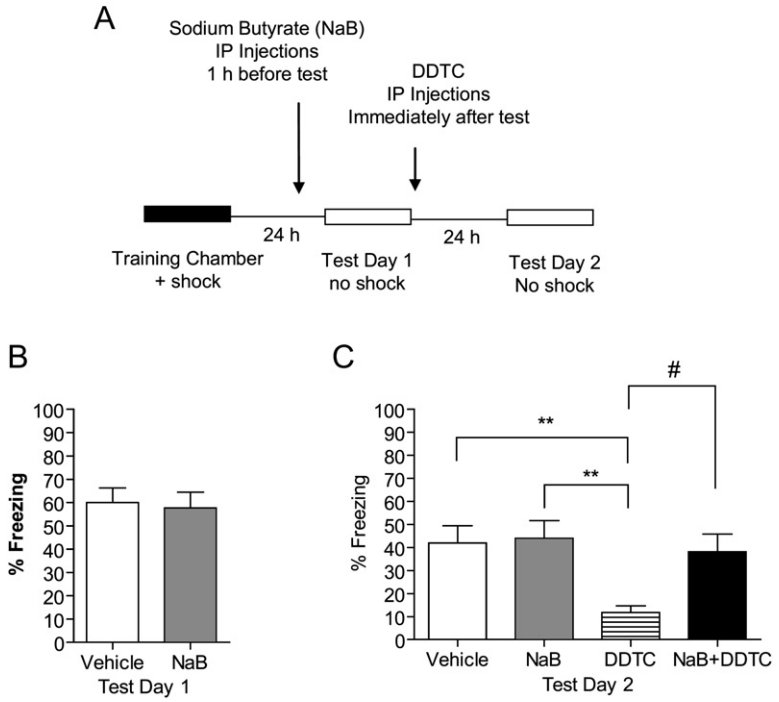

Figure 9. Effects of Inhibition of the NF-κB Signaling Pathway on Enhanced Acetylation Activity during Memory Reconsolidation

(A) Experimental design is outlined.

(B) Freezing behavior following vehicle or sodium butyrate (NaB) treatment on Test Day 1 (vehicle, n = 16; NaB, n = 17).

(C) Freezing behavior on Test Day 2 during 1 min reexposure (vehicle, n = 7; NaB, n = 7; DDTC, n = 8; NaB+DDTC, n = 6).

One-way ANOVA; *$p < 0.05$ compared with vehicle-shocked, **$p < 0.05$ compared with shocked-NaB, $^{\#}p < 0.05$ compared with shocked-DDTC. Error bars are SEM.

CBP is an appealing potential mechanism for regulating phosphorylation and acetylation of histone H3 in hippocampus in our fear conditioning reconsolidation model.

These observations prompted us to hypothesize that histone H3 regulation is a mechanism by which NF-κB signaling exerts its effects in the process of memory reconsolidation. To shed light on this question, we extended our studies to investigate chromatin regulation. After the animals' recall of CCF memories, we observed a considerable increase in histone H3 phosphorylation and acetylation in area CA1 of the hippocampus. Like CCF memory reconsolidation itself, the increases in phosphorylation and acetylation of histone H3 were blocked with inhibition of IKK complex activity. Thus, our results suggest that there are two possible mechanisms for NF-κB pathway-mediated transcriptional regulation during memory reconsolidation. One possible mechanism is that recall triggers IKKα activation to mediate release of the NF-κB DNA-binding complex for binding to κB regulatory elements within DNA and subsequently alter chromatin structure. The second possible mechanism is that recall triggers IKKα activation for direct regulation of chromatin structure, independent of NF-κB binding to DNA. Our inhibitor studies suggest that histone H3 regulation (phosphorylation and acetylation) is directly regulated at the IKKα level of the NF-κB pathway, independent of activation of the NF-κB DNA-binding complex.

Despite a large body of literature establishing a role for signaling components of the NF-κB pathway, such as IκBα and IKKα, in NF-κB-independent gene regulation, the NF-κB DNA-binding complex has often been thought of as the final target in activation of the NF-κB signaling cascade. Our data suggest a revision to this idea, as we found that IKKα activation regulates histone H3 modifications independently of NF-κB DNA binding. Thus, we expose a different mechanism for transcriptional regulation by the NF-κB pathway during memory reconsolidation: regulation of chromatin remodeling by IKKα. These studies provide insight into the role in vivo of IKKα in the regulation of histone modifications, and complement prior investigations using cell culture and construct transfection (Anest et al., 2004; Yamamoto et al., 2003).

Importantly, we found changes in histone H3 modifications across individual gene promoter regions, including *Zif268*, which were induced during memory reconsolidation through the IKKα/NF-κB signaling pathway. Although functional roles of some transcription factors are unclear in the context of memory formation, activation of *Zif268* has been found to play an important role in memory reconsolidation (Bozon et al., 2003; Hall et al., 2001; Izquierdo and Cammarota, 2004; Lee et al., 2004). Since the inhibition of IKKα/NF-κB blocked recall-induced histone H3 phosphorylation and acetylation at the *Zif268* promoter, it is possible that recall triggers IKKα/NF-κB signaling and subsequently induces *Zif268* to further modulate memory reconsolidation. Thus, our studies support previous findings that *Zif268* is involved in memory formation and expand on the mechanisms responsible for regulation of this gene in hippocampus during memory reconsolidation.

An interesting finding from these studies is that IKKα mediates both histone H3 phosphorylation and acetylation in hippocampus during memory reconsolidation. One potential outcome of histone H3 phosphorylation is

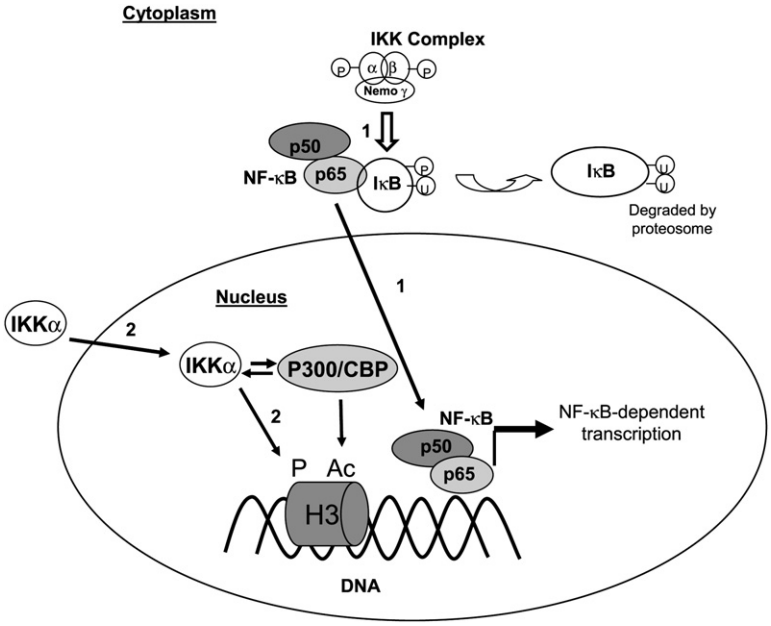

Figure 10. Model for the Role of the IKKα Kinase Protein in the Regulation of Chromatin Structure during Long-Term Memory Reconsolidation

Upon activation after memory recall, the IKKα kinase protein initiates two pathways for transcriptional regulation of genes: (1) the IKKα kinase protein at the IKK complex level functions to increase the DNA binding activity of the NF-κB DNA-binding complex for modulation of gene transcripts; and (2) the IKKα kinase protein acts independently from the IKK complex to mediate changes in chromatin structure that are apparent as an increase in phosphorylation of histone H3 and subsequent acetylation of histone H3 through its interaction with CBP. The changes in chromatin structure ultimately lead to changes in transcriptional regulation of genes relevant for restabilization of memory after retrieval.

the recruitment and activation of histone acetyltransferases (HATs) (Cheung et al., 2000). CBP, which contains HAT activity, has been shown to interact with chromatin-bound IKKα at gene promoter sites (Hoberg et al., 2006; Yamamoto et al., 2003). Therefore, we can conclude that a possible mechanism for IKKα regulation of histone H3 acetylation is through the interaction of IKKα with CBP. Interestingly, CBP has also been shown to be involved in memory formation and consolidation (Alarcon et al., 2004; Korzus et al., 2004; Wood et al., 2006).

Since inhibition of IKKα activity blocks histone H3 acetylation during memory reconsolidation, we hypothesized that enhancing histone acetylation would rescue memory reconsolidation in the face of IKKα inhibition. Indeed, results obtained using the HDAC inhibitor NaB showed that enhancing histone acetylation blocks the effect of inhibiting IKKα activity on freezing behavior. This demonstrates that IKKα inhibition produces freezing behavior deficits after memory recall that can be rescued by enhancing histone acetylation.

However, an interesting complexity to our HDAC inhibitor studies is that enhancing histone acetylation rescues the effect of inhibiting IKKα, even though our results show that IKK inhibition blocks regulation of both chromatin structure and NF-κB DNA binding during memory reconsolidation. Because NF-κB DNA binding is necessary for memory reconsolidation, one might expect that IKK inhibition would still lead to loss of memory reconsolidation even if histone acetylation was still intact. A likely explanation for this result is that increasing protein acetylation with HDAC inhibition rescues the NF-κB DNA-binding limb of the pathway as well. This would be consistent with the known capacity of direct acetylation of NF-κB subunits to promote DNA regulatory element binding (Greene and Chen, 2004; review in Perkins, 2006; Quivy and Van Lint, 2004). Thus, a plausible effect of HDAC inhibition with NaB is not only an enhancement of histone acetylation, but also an enhancement of NF-κB acetylation and enhanced NF-κB DNA binding activity, thereby leading to a rescue of the effect of IKKα inhibition on memory reconsolidation. Thus, while our study with the HDAC inhibitor rescuing memory reconsolidation is consistent with our hypothesis that increased histone acetylation is involved in memory reconsolidation, these results may also be indicative of a more general role for protein acetylation in memory formation as well (Swank and Sweatt, 2001; Yeh et al., 2004).

In conclusion, our results suggest that the NF-κB signaling pathway regulates transcription in the hippocampus during memory reconsolidation (Figure 10). Our working model is that the NF-κB signaling pathway accomplishes this through two transcriptional control mechanisms: IKK complex-mediated activation of the NF-κB DNA-binding complex for direct binding to κB regulatory sites within DNA, and IKKα regulation of histone modifications (Figure 10). One interesting possibility is that the IKKα regulation of chromatin is permissive for transcriptional control, affecting transcription triggered not only by NF-κB but also by a variety of transcription factors, including CREB. Regardless of this specific idea, the model illustrates the dynamic and complex nature of NF-κB signaling in memory processing after retrieval. In addition, there are several mechanisms involved in the regulation of chromatin structure during memory formation that also might be involved in memory reconsolidation. For example, our previous studies have also implicated the ERK/MAPK pathway in targeting histone H3 phosphorylation during memory consolidation (Chwang et al., 2006). Thus, it is possible that IKKα and ERK might target histone H3 independently during different stages of the process of memory formation

and reformation. We anticipate that a major challenge for molecular behaviorists will be to determine how the IKKα/NF-κB and ERK signaling pathways integrate signals to regulate chromatin structure in memory formation processes. While there is still much to be understood about the role of IKKα/NF-κB signaling activity in memory formation, the results presented here provide evidence that IKKα activity regulates gene transcription and chromatin structure in hippocampus to enable memory reconsolidation.

EXPERIMENTAL PROCEDURES

See Supplemental Data for additional procedures.

Animals

Adult male Sprague-Dawley rats (200–250 g) were used for all experiments. Animals were housed under light/dark 12 hr/12 hr conditions and allowed access to rodent chow and water ad libitum. Animals were allowed to acclimate to laboratory conditions and handled at least 3 days prior to use in behavioral experiments. All procedures were performed with the approval of the University of Alabama-Birmingham Institutional Animal Care and Use Committee and according to national guidelines and policies.

Fear Conditioning

Animals were handled for 5 days, and on the day of experiments they were transported to the laboratory at least 2 hr prior to fear conditioning. Animals were placed into the training chamber and allowed to explore for 2 min, after which they received an electric shock (1 s, 0.5 mA). The 2 min/1 s shock paradigm was repeated for a total of three shocks. After the last shock, animals were allowed to explore the context for an additional 1 min prior to removal from the training chamber.

For experiments investigating the effect of inhibition of the NF-κB signaling pathway on long-term memory after recall, animals received an injection of either saline (vehicle) or DDTC (200 mg/kg; Sigma Chemical Co., St. Louis, MO) intraperitoneally (i.p.). DDTC was freshly prepared in 0.9% saline solution (final pH 7.6). For experiments investigating the effect of IKK inhibition on long-term memory after recall, SSZ (5 or 10 mM; Sigma Chemical Co.) was infused at a rate of 1.0 μl/min. SSZ was freshly dissolved in saline solution with 10 mM HEPES (pH 7.6) plus 20% DMSO (final pH 7.6). All injections were performed immediately following the retrieval session in the training chamber.

For experiments investigating the effect of NF-κB DNA-binding complex inhibition on long-term memory after recall, the SN50 active peptide or SN50M inactive peptide (50 μg; Calbiochem) was infused at a rate of 2.5 μl/min. SN50 infusions were performed 2 hr prior to the retrieval session in the training chamber. SN50 and SN50M were dissolved in saline at 10 μg/μl.

For experiments investigating the effect of NaB on long-term memory, animals were injected with 1.2 g/kg of NaB 1 hr prior to the retrieval session.

For behavioral experiments, freezing behavior was measured at either 24 hr (DDTC, SSZ, or SN50) or 7 days (DDTC) after fear conditioning and 24 hr after reexposure. Freezing behavior was measured by observing the animals for 5 min.

For biochemistry studies, age-matched animals that were handled but did not receive any experimental manipulations (unshocked) were used as controls in all fear conditioning experiments.

Intracerebroventricular Cannulae

During surgery, each rat was implanted with a 23G single-guide cannula (Plastics One) from which the injector extended 1 mm to end in the left or right lateral ventricle. Stereotaxic coordinates used were as follows: −1.0 mm from bregma, ±1.2 mm lateral from the midline, and −3.5 mm from dura (Paxinos and Watson, 1998), measured from the tip of the cannulae guide. Cannulae placements for all experimental animals were verified by giving each rat an intracerebroventricular infusion of angiotensin II (50 ng/2 ml; Sigma Chemical Co.) and by observing subsequent drinking behavior. Placements were considered to be accurate if a rat drank within 1 min of the infusion and sustained drinking over 2–3 min (Sakai et al., 1995). Animals were habituated to dummy cannula removal and given 5 days of recovery and handling before the start of behavioral fear conditioning.

Isolation of Area CA1

Whole brains were immersed in oxygenated (95%/5% O_2/CO_2), ice-cold cutting saline (CS) (containing 110 mM sucrose, 60 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 28 mM $NaHCO_3$, 0.5 mM $CaCl_2$, 7 mM $MgCl_2$, 5 mM glucose, and 0.6 mM Ascorbate) prior to isolation of CA1 subfields. Area CA1 of hippocampus was microdissected and immediately frozen with dry ice and stored at –80°C until protein extracts were prepared.

Histone Extraction

As previously described (Chwang et al., 2006; Levenson et al., 2004b), tissue homogenates were centrifuged at 7700 × g for 1 min. Pelleted nuclei were resuspended in 250 μl of 0.4 N H_2SO_4, incubated on ice for 30 min, and then centrifuged at 4°C again for 10 min at 14,000 × g. The protein in supernatant was precipitated with trichloroacetic acid containing 4 mg/ml deoxycholic acid (Na^+ salt, Sigma), collected by centrifugation, and dried with acetone. The resulting purified proteins were resuspended in 10 mM Tris (pH 8.0) and stored at −80°C. Protein concentrations were determined using the Bio-Rad protein assay reagent.

Western Blotting

For assessment of histone phosphorylation and acetylation, histone protein extracts (1 μg) were separated by SDS-PAGE on a 12% resolving gel with a 4% stacking gel and transferred onto a PVDF membrane. For assessment of NF-κB signaling components, protein extracts (20 μg) were separated by SDS-PAGE on a 10% resolving gel with a 4% stacking gel and transferred onto a PVDF membrane as previously described (Chwang et al., 2006; Levenson et al., 2004b).

Nuclear Protein Extraction

Nuclear extracts were prepared as previously described by Lubin et al. (2005).

EMSA

Biotinylated double-stranded oligonucleotides containing the NF-κB binding site 5-TGGGGAATCCCGC-3 were used. Relative NF-κB DNA binding activity was assessed using the LightShift Chemiluminescent EMSA kit (Pierce) as previously described (Lubin et al., 2005).

ChIP Assay

Rats were decapitated and whole hippocampi were removed by gross dissection. The CA1 regions of hippocampus were then microdissected on ice and held in ice-cold PBS solution containing protease inhibitors (1 mM phenylmethysulfonyl fluoride, 1 μg/ml of protease inhibitor cocktail, and phosphatase inhibitors [1 mM Na_3VO_4 and 20 mM NaF]). Isolated CA1 was minced to ~1.0 mm sized pieces and immediately incubated in 1% formaldehyde in PBS at 37°C for 10 min. The tissue was washed six times with an ice-cold PBS solution containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 1 μg/ml of protease inhibitor cocktail). Tissue was homogenized in SDS lysis buffer (50 mM Tris [pH 8.1], 10 mM EDTA, and 1% SDS) supplemented with protease inhibitors. Chromatin was sheared by sonication using a Branson Sonifier 250 at 1.5 power and constant duty cycle. Lysates were centrifuged to pellet debris and then diluted 1:10 in ChIP dilution buffer (16.7 mM Tris [pH 8.1], 0.01% SDS, 1.1% Triton X-100, 167 mM NaCl, 1.2 mM EDTA). Extracts were precleared for 30 min with 40 μl of a 50% suspension of salmon-sperm-saturated protein A.

Immunoprecipitations were carried out at 4°C overnight with primary antibodies specific to (1) AcH3, which recognizes acetylated Lys14 (Upstate Biotechnology); (2) PH3/AcH3, which recognizes phosphorylated Ser-10 and acetylated Lys14; or (3) AcH4, which recognizes acetylated Lys5, Lys8, Lys12, and Lys16 (Upstate Biotechnology); or (4) an equivalent amount of control IgG (rabbit; Santa Cruz). Immune complexes were collected with protein A and sequentially washed with low-salt buffer (20 mM Tris [pH 8.0], 0.1% SDS, 1% Triton X-100, 2 mM EDTA, and 150 mM NaCl), high-salt buffer (20 mM Tris [pH 8.1], 0.1% SDS, 1% Triton X-100, 500 mM NaCl, and 1 mM EDTA), LiCl immune complex buffer (0.25 M LiCl, 10 mM Tris [pH 8.1], 1% deoxycholic acid, 1% IGEPAL-CA630, 500 mM NaCl, and 2 mM EDTA), and TE buffer. Immune complexes were extracted in 1 × TE containing 1% SDS, and protein-DNA cross-links were reverted by heating at 65°C overnight. After proteinase K digestion (100 μg; 2 hr at 37°C), DNA was extracted by phenol/chloroform/isoamyl alcohol and then ethanol-precipitated.

Immunoprecipitated DNA was subjected to quantitative real-time PCR using primers specific for 150–200 bp segments corresponding to promoters upstream of the rat *Zif268*, *IκBα*, or β-*actin* (used as a negative control): *Zif268* sense, 5′-ATGGGCTGTTAGGGACAGTG-3′; antisense, 5′-TTGGGGATTTAGCTCAGTGG-3′; *IκBα* sense, 5′-CGCT AAGAGGAACAGCCTAG-3′; antisense, 5′- CAGCTGGTCGAAACA TGGC-3′; β-*actin* sense, 5′-CTCTCTCCCAGGAGTTGTGC −3′; antisense, 5′-GCTACAGCAGGGGATCAGAG-3′. The cumulative fluorescence for each amplicon was normalized to input amplification.

Transcription Factor Analysis

Transcription factor analysis was performed as previously described (Levenson et al., 2004a). Briefly, upstream sequence information of *Zif268* was obtained form the University of California, Santa Cruz Genome Browser (http://www.genome.ucsc.edu/). To identify putative NF-κB transcription factor binding sites, 1000 bp upstream of the immediate upstream sequence was examined using MatInspector (Geomatix, Munich, Germany) (Cartharius et al., 2005).

Statistics

The experimenter was blind to treatment groups when taking all measurements. For experiments using SSZ or SN50 (Figure 5, Figure 6, Figure 7, and Figure S2), area CA1 was isolated from one hippocampus, alternating left (n = 4–9) or right (n = 4–9), from each animal per experiment. For all other experiments both hippocampi were pooled from each animal per experiment (n = 4–9). Effects of DDTC on recall of CCF memories with accompanying immunoreactivity (Figure 2B and Figure 4) were analyzed by two-way ANOVA with Bonferroni corrections. Effects of SSZ or SN50 on freezing behavior after recall with accompanying immunoreactivity were analyzed by one-way ANOVA with Tukey post hoc test (Figure 5, Figure 6, Figure 7, and Figure S2). For evaluation of DNA binding of nuclear NF-κB (Figure 3D, Figure 5F, and Figure 7B), specific comparisons between vehicle- and DDTC-, SSZ-, or SN50-treated shock groups were made using Student's t test for each of the experiments. Separate comparisons between freezing behaviors for drug- and vehicle-treated groups were made using Student's t tests for each of the Test Days (Figure 1, Figure 5, and Figure 9B). Immunoreactivity was quantified by densitometry (Scion) and GraphPad Prism software was used for statistical analysis of the data. Data are shown as the mean ± standard error of mean (SEM).

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/55/6/942/DC1/.

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Supplemental Data for D. Lubin et al.

Document S1. Supplemental Experimental Procedures, Figures, and References (PDF 279 kb)

Summary of this Article Full Text of this Article

Neuron, volume 55

Supplemental Data

The IκB Kinase Regulates Chromatin Structure during Reconsolidation of Conditioned Fear Memories

Farah D. Lubin and J. David Sweatt

Supplemental Experimental Procedures

Western blotting. After transfer, PVDF membranes were briefly rinsed with 100% methanol, air-dried for 15 min, and washed briefly with 25 ml TTBS (0.1% Tween 20, 50 mM, Tris-HCl, pH 7.5, 150 mM, NaCl) for 5 min at room temperature. This was followed by incubation in 25 ml of blocking buffer (0.1% Tween 20, 50 mM, Tris-HCl, pH 7.5, 150 mM, NaCl, and 5% w/v non-fat dry milk or bovine serum albumin) for 1 hour at room temperature. The membranes were then washed with 25 ml TTBS and incubated overnight with a primary antibody in primary buffer (0.1% Tween 20, 50 mM, Tris-HCl, pH 7.5, 150 mM, NaCl, and 5% w/v non-fat dry milk or bovine serum albumin). This was followed by three washes in TTBS and incubation for 1 hour with a HRP-conjugated secondary anti-rabbit antibody in primary buffer (1:10,000). The membranes were washed in TTBS and blots were developed using enhanced chemiluminescence (ECL) and exposed to X-ray film. *Antibodies*—The anti-rabbit primary antibodies used, and their dilutions were as follows: anti-IKKα/β (1:1000, Cell Signaling Technology, Beverly, CA), anti-phospho- IKKα and IKKβ (1:500, Cell Signaling Technology, Beverly, CA), (IKKα at Ser 180, IKKβ at Ser-181;1:1000, Cell Signaling Technology, Beverly, CA), anti-Histone H3 (1:1000, Upstate Biotechnology Inc.), anti-Phosphorylated H3 (Ser-10; 1:1000, Upstate Biotechnology Inc.), anti-acetyl

Histone H3 (Lys-14, 1:1000, Upstate), anti-acetyl Histone H4 (Lys-5/Lys-8/Lys-12/Lys16; 1:1000, Upstate).

Nuclear protein extraction. Hippocampal CA1 regions from experimental animals were homogenized with a Dounce homogenizer using 10 strokes in 400 μl of ice-cold PBS supplemented with 0.5 mM PMSF, 20 mM NaF, and 1 μg/ml of protease inhibitor cocktail. Homogenates were centrifuged at 4 ^{o}C for 30 s at 12,000 g. Pellets were resuspended in 200 μl of lysis buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 20 mM NaF, 20 mM p-nitrophenyl phosphate, 1 mM Na_3VO_4 , 0.5 mM PMSF, 1 μg/ml of protease inhibitor cocktail) and incubated on ice for 10 min. Then 10 μl of 10% IGEPAL solution was added, and resuspended pellets were vigorously mixed for 30 s and recentrifuged for 30 s at 12,000 g at 4 ^{o}C. The supernatant from this centrifugation was taken as the cytoplasmic fractions. Pelleted nuclei were resuspended in 50 μl of extraction buffer (20 mM HEPES, 25 % glycerol, 1.5 mM $MgCl_2$, 300 mM NaCl, 0.25 mM EDTA, 0.5 mM DTT, 20 mM NaF, 20 mM p-nitrophenyl phosphate, 1 mM Na_3VO_4, 0.5 mM PMSF, 1 μg/ml of protease inhibitor cocktail), incubated on ice for 20 min, and then centrifuged again for 20 min at 12,000 g at 4 ^{o}C. The supernatant contained nuclear proteins. Protein concentration was determined by using Bio-Rad protein assay reagent to control for protein loading.

Electrophoretic mobility shift assays (EMSA). Briefly, 10 μg of nuclear extracts were incubated in 20 μl of binding reaction mixture (10 mM Tris, pH 7.5; 50 mM KCl, 1 mM DTT, 50 ng/μl Poly (dI-dC), 5 mM $MgCl_2$, 2.5% glycerol, 10 mM EDTA, and 0.05%

NP-40) for 2 min before the addition of the double-stranded DNA biotin labeled probe (6 ng) for 20 min at room temperature. Samples were subjected to electrophoretic separation at a voltage of 150 V for 1 h on a 5% non-denaturing polyacrylamide gel in 0.5X TBE diluted from a 5X TBE stock buffer solution (450 mM Tris, 450 mM boric acid, and 10 mM EDTA). The protein–DNA complexes were then transferred to a Biodyne B membrane in 0.5X TBE buffer pre-cooled to 10 °C and cross-linked to membrane using a Stratalinker® 1800 UV Crosslinker (Strategene, La Jolla, CA). The biotinylated probes were visualized using the EMSA kit according to the specifications of the manufacturer followed by exposure to X-ray film.

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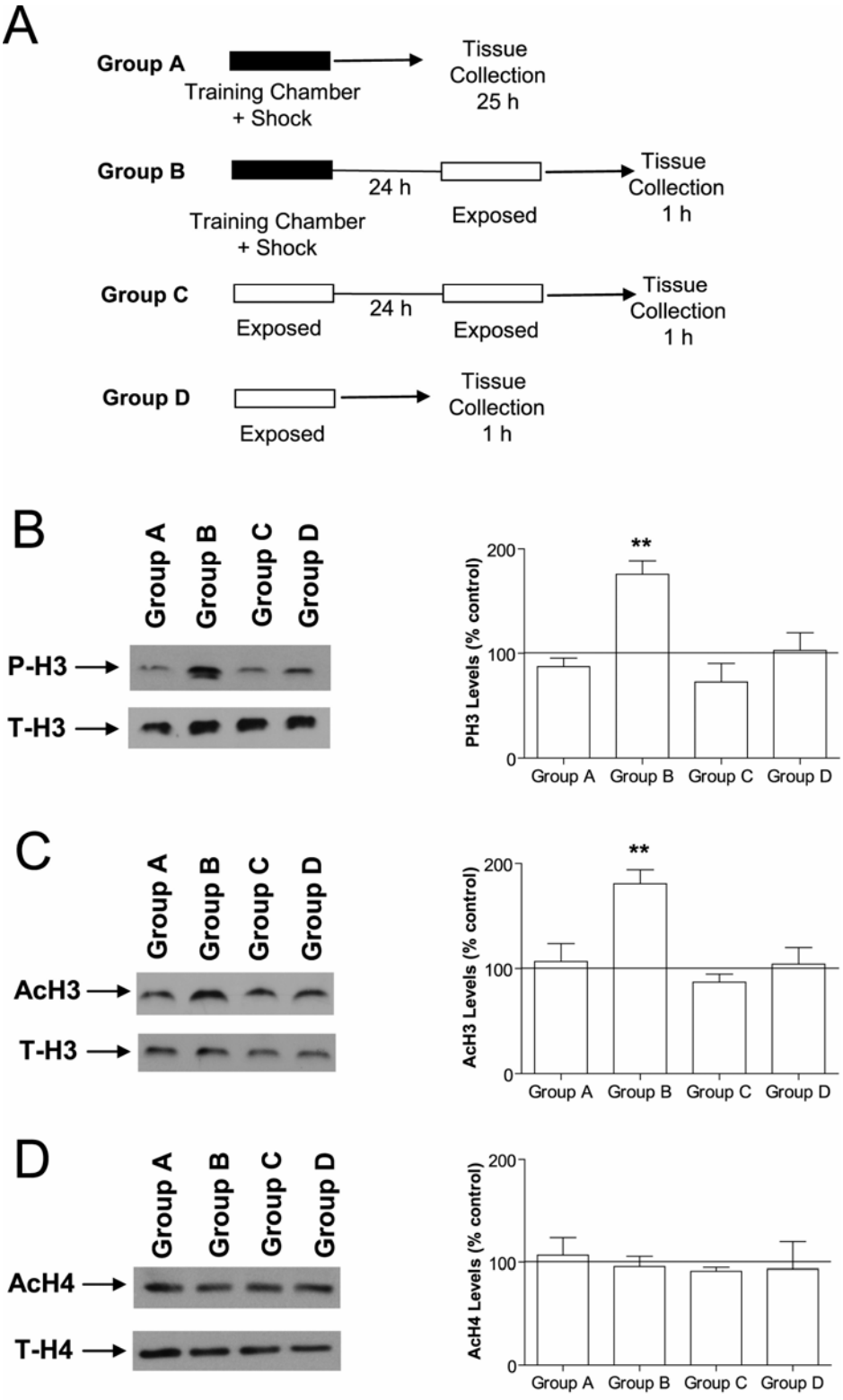
Supplemental Figures

Supplemental Figure 1. Histone H3 phosphorylation and acetylation is regulated after re-exposure to the context. **A**, Outline of the experimental procedure performed on the following four groups of animals: Group A; trained animals whose hippocampal tissue was collected 25 h later, Group B; trained animals that were re-exposed to training chamber and their hippocampal tissue collected 1 h later, Group C; animals exposed to the training chamber with no shock, re-exposed 24 h later with no training or shocks, and tissue collected 1 h later, Group D; animals exposed once to the training chamber with no shock and hippocampal tissue collected 1 hr later. Western blots and graph summary of data for phosphorylated Histone H3 (**B;** P-H3) and acetylated Histone H3 (**C;** AcH3) normalized to total Histone H3 (T-H3) levels are shown. In Group B, phosphorylated and acetylated histone H3 levels significantly increased compared to Group A or C (*Student's t-test*; **$p<0.01$ compared with naive controls). **D**, Western blots for acetylated histone H4 (AcH4) normalized to total histone H4 (T-H4) are shown. Summary quantification of histone H4 showed no significant effect on acetylated histone H4 in all three groups. (*Student's t-test*; $p>0.05$ compared with naive controls). *Error bars* are SEM; *Solid lines* represent normalized vehicle naïve control levels.


A
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Training Chamber
+ Shock
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25 h
Group B
24 h
Training Chamber
+ Shock
Exposed
Tissue
Collection
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Exposed
Exposed
Tissue
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1 h
Group D
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PH3 Levels (% control)
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AcH3 Levels (% control)
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Group A
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Group C
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Group A
Group B
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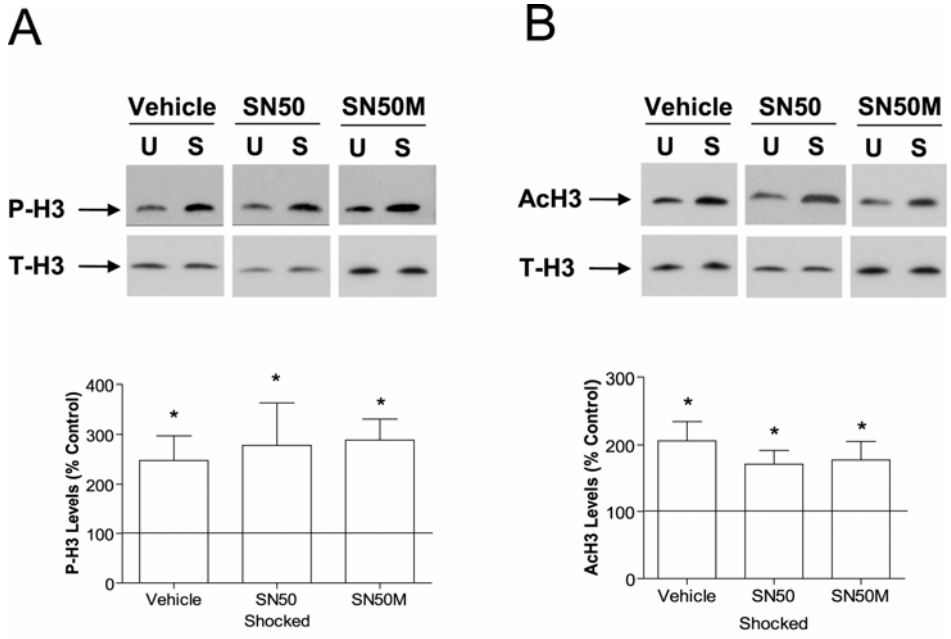
Group D
AcH4
T-H4
AcH4 Levels (% control)
200
100
0
Group A
Group B
Group C
Group D

Supplemental Figure 2. Histone H3 phosphorylation and acetylation is not effected by direct inhibition of the NF-κB transcriptional complex. After 2 h of pre-treatment with either vehicle, SN50 or SN50M animals were re-exposure to the training chamber. Histone extracts were prepared from area CA1 of hippocampus 1hr after re-exposure to the training chamber. (unshocked, *n*=4; shocked-vehicle, *n*=4; shocked-SN50, *n*=4; shocked-SN50M, *n*=4). **A**, Western blot analysis show no significant difference in increased Histone H3 phosphorylation (P-H3) levels following SN50 pre-treament prior to re-exposure to the training chamber. **B**, Quantitative analysis of western blots for Histone H3 actelylation (AcH3) showed no significant change in increased levels following vehicle, SN50, or SN50M pre-treatment. **C**, Acetylated Histone H4 (AcH4) was determined by western blotting following vehicle, SN50, or SN50M pre-treatment and showed no significant change. (*One-way ANOVA*; *$p<0.05$, compared with shocked-vehicle). *Error bars* are SEM; *Solid lines* represent normalized unshocked-vehicle control levels.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P-H3 Levels (% Control)
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SN50
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Shocked
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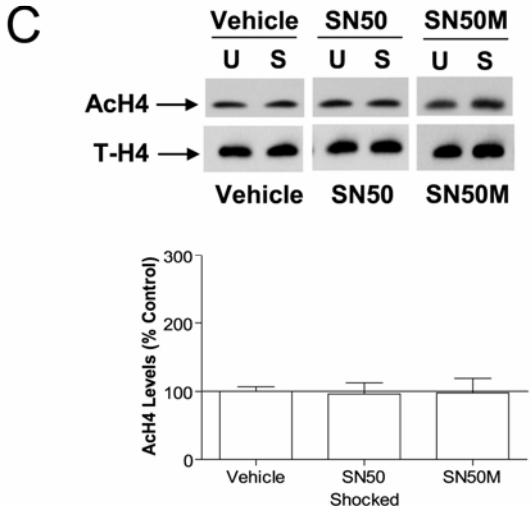
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