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Hippocampal c-Jun-N-Terminal Kinases Serve as Negative Regulators of Associative Learning

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In the adult mouse, signaling through c-Jun N-terminal kinases (JNKs) links exposure to acute stress to various physiological responses. Inflammatory cytokines, brain injury and ischemic insult, or exposure to psychological acute stressors induce activation of hippocampal JNKs. Here we report that exposure to acute stress caused activation of JNKs in the hippocampal CA1 and CA3 subfields, and impaired contextual fear conditioning. Conversely, intrahippocampal injection of JNKs inhibitors sp600125 (30 μM) or D-JNKI1 (8 μM) reduced activity of hippocampal JNKs and rescued stress-induced deficits in contextual fear. In addition, intrahippocampal administration of anisomycin (100 μg/μl), a potent JNKs activator, mimicked memory-impairing effects of stress on contextual fear. This anisomycin-induced amnesia was abolished after cotreatment with JNKs selective inhibitor sp600125 without affecting anisomycin's ability to effectively inhibit protein synthesis as measured by c-Fos immunoreactivity. We also demonstrated milder and transient activation of the JNKs pathway in the CA1 subfield of the hippocampus during contextual fear conditioning and an enhancement of contextual fear after pharmacological inhibition of JNKs under baseline conditions. Finally, using combined biochemical and transgenic approaches with mutant mice lacking different members of the JNK family (*Jnk1, Jnk2,* and *Jnk3*), we provided evidence that JNK2 and JNK3 are critically involved in stress-induced deficit of contextual fear, while JNK1 mainly regulates baseline learning in this behavioral task. Together, these results support the possibility that hippocampal JNKs serve as a critical molecular regulator in the formation of contextual fear.

Introduction

Memory formation requires a series of molecular processes, such as posttranslational protein modification, gene expression, and probably *de novo* protein synthesis, leading to functional and structural changes in cells of the CNS (Sutton and Schuman, 2006; Klann and Sweatt, 2008). Consolidation of memory is often impaired by acute stressful experiences (Diamond et al., 2005; Sandi and Pinelo-Nava, 2007) indicating that molecular cascades elicited by stress responses interfere with those involved in information processing during learning. Conversely, stress and learning may activate overlapping signaling pathways involving common protein kinases, immediate early genes and delayed response genes (Cammarota et al., 2000; Meller et al., 2003; Shen et al., 2004; Sindreu et al., 2007; Kozlovsky et al., 2008). Therefore, the understanding of stress-induced molecular changes in the brain will ultimately lead to further elucidation of mechanisms underlying learning and memory.

The mitogen-activated protein kinase family (MAPKs) represents a potential target with above-mentioned stress and learning relevant properties (Adams and Sweatt, 2002). MAPKs include the p38, c-Jun-N-terminal kinases (JNKs) and the extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) (Chang and Karin, 2001). A plethora of studies demonstrate that ERK1 and ERK2 play a central role in the development of synaptic plasticity underlying learning and memory (Kornhauser and Greenberg, 1997; Atkins et al., 1998; Impey et al., 1998; Schafe et al., 2000), whereas the contribution of p38 to associative learning is marginally described (Zhen et al., 2001; Rossato et al., 2006).

The role of JNKs in associative learning and memory, however, is unclear. The JNKs are encoded by at least three genes (JNK1, -2, and -3), and the transcripts of each of these genes are alternatively spliced to create mRNAs that encode 46 kDa and 54 kDa JNK isoforms (Gupta et al., 1995, 1996). JNKs are fully activated after phosphorylation at Thr183 and Tyr185 by MAP kinase kinases (MKK4 or MKK7, respectively). Activation and/or deactivation of JNKs pathways is regarded as a molecular switch in stress signal transduction (Manning and Davis, 2003), and the ability of the activated JNKs to phosphorylate numerous transcription factors (Gupta et al., 1995, 1996), among other targets, opens the possibility for the contribution of JNKs signaling pathways to memory formation. Considering also that various stressors activate JNKs (Manning and Davis, 2003), we hypothesized a critical involvement of JNKs in stress-induced modulation of learning and memory.

To address this question, we investigated whether the temporary impairment in contextual fear learning following exposure to acute stress (Todorovic et al., 2007) is functionally linked to changes in JNKs activity. In the current study, we first demonstrate that increased, sustained activation of hippocampal JNKs is responsible for the observed stress-induced impairment of contextual fear. Second, we demonstrate that pharmacological inhibition of hippocampal JNKs pathways under baseline conditions enhances retention of contextual fear. The latter data provide the first evidence that hippocampal JNKs might play a role as a rate-limiting factor during regulation of baseline associative learning.

Materials and Methods

Animals. Nine-week-old male and female C57BL/6J mice were obtained from Jackson ImmunoResearch Laboratories. Female mice were used as indicated. Twelve-week-old C57BL/6J wild-type, *Jnk1* (Dong et al., 1998); *Jnk2* (Yang et al., 1998), and *Jnk3* (Yang et al., 1997) single null mutant male mice were bred and genotyped at the Animal Facility of the Department of Animal Medicine, University of Massachusetts Medical Centre. All mice were individually housed in macrolon cages and maintained on a 12 h light/dark cycle (lights on at 7:00 A.M.) with access to food and water *ad libitum*. The number of mice used for each procedure is provided in the respective sections. All experimental procedures were performed in compliance with the University of Hawaii Animal Care Committee in accordance with National Institute of Health guidelines.

Drugs. sp600125 (anthra [1,9-cd] pyrazole-6 (2H)-one), a potent and selective JNK inhibitor, was obtained from EMD Biosciences. sp600125 inhibits JNKs (sp600125 acts as a reversible ATP-competitive JNK inhibitor) with an IC_{50} of $0.04-0.09$ μM (Bennett et al., 2001). sp600125 is selective for JNKs over ERKs and p38 ($IC_{50} > 10$ μM). It was dissolved in 0.1% DMSO in saline. Based on the results demonstrating its ability to downregulate phosphorylation of JNKs downstream targets c-Jun, ATF2, and Elk1 a dose of 30 μM sp600125 was selected. The D-retro-inverso form of JNK-inhibitor (D-JNKI1) (Dickens et al., 1997; Borsello et al., 2003) (H-Gly-D-Arg-D-Lys-D-Lys-D-Arg-D-Arg-D-Gln-D-Arg-D-Arg-D-Arg-D-Pro-D-Pro-D-Arg-D-Pro-D-Lys-D-Arg-D-Pro-D-Thr-D-Thr-D-Leu-D-Asn-D-Leu-D-Phe-D-Pro-D-Gln-D-Val-D-Pro-D-Arg-D-Ser-D-Gln-D-Asp-D-Thr-NH2) was obtained from GL Biochem. The D-JNKI1 was initially solubilized in DMSO to make a 6.37 mM stock solution. The same stock solution of D-JNKI1 was further diluted in saline to obtain a final concentration of 4 or 8 μM. Anisomycin (Sigma) was used as a potent activator of JNKs. It was first dissolved in 1 M HCl the pH subsequently adjusted to 7.0 with 1 M NaOH. The final concentration of 100 μg/μl was obtained by adding 0.9% saline (Wanisch and Wotjak, 2008). Human or rat CRF (h/rCRF) and the CRF_2 antagonist antisauvagine-30 (aSvg-30) were synthesized as described previously (Jahn et al., 2001). The peptides were initially dissolved in 10 mM acetic acid and diluted with twofold concentrated sterile artificial CSF (aCSF). The final pH of the peptide solutions was 7.4. The final concentrations of h/rCRF and aSvg-30 were selected on the basis of our previous experiments (Todorovic et al., 2007).

Cannula implantation. Double cannulae were implanted under 1.2% avertin anesthesia (0.4 ml/mouse) and affixed to the skull with dental cement. The injection system (C235; Plastics One) consisted of a double-guided cannula, dummy, and a cap. Cannulae were placed in the dorsal hippocampus (anteroposterior -1.5 mm, lateral 1 mm, depth 2 mm) or lateral intermediate septal area (anteroposterior $+1$ mm, lateral 0.5 mm, depth 3 mm) (Franklin and Paxinos, 2001). Animals were allowed to recover for 7–8 d before the experiments started.

Drug infusions. On the day of the experiment, mice were exposed to a light isoflurane anesthesia, the cap and the dummy were removed and solutions were delivered through an injector (CMA/Microdialysis) linked to two Hamilton microsyringes with plastic tubing. The drugs prepared in their respective vehicle (saline, aCSF) or the vehicle alone were administered bilaterally over a 15 s time period so that a 0.25 μl volume was injected in each side. No behavioral signs of epileptiform or other gross electrographic abnormalities, such as exacerbated behavioral convulsions were observed during or immediately after injections. The cannula placement was verified for each mouse following the behavior experiments by histological examination of the brains after methylene blue injection (0.25 μl/site). Only data obtained from mice with correctly placed cannulae were included in statistical analysis.

Immobilization stress. Acute stress involved taping of the mouse's limbs to a Plexiglas surface for 1 h in a supine position (Todorovic et al., 2007).

Contextual fear conditioning. The fear conditioning experiments were performed as described previously (Todorovic et al., 2007) using a computer-controlled fear conditioning system (TSE Systems). The training (conditioning) consisted of a single trial. The mouse was exposed to the conditioning context (180 s) followed by a foot shock (0.7 mA, 2 s, constant current) delivered through a stainless steel grid floor. The mouse was removed from the fear conditioning box 30 s after shock termination to avoid an aversive association with the handling procedure. Memory tests were performed 24 h after fear conditioning [context-shock (CTX-S), paired group]. Contextual memory was tested in the conditioning box for 180 s without shock presentation (with background noise). Freezing, defined as a lack of movement in addition to heartbeat and respiration, was observed in 10 s intervals and was used as an index of conditioned fear. Control groups of mice were exposed to the context alone (3 min) (CTX group) or immediate footshock (2 s, 0.7 mA, constant current) followed by context (3 min) during the training [immediate shock (ImS) group]. The mean activity [mA context (cm/s)] and activity bursts to the shock in the context (supplemental Table 1, available at www.jneurosci.org as supplemental material) were automatically recorded by a photo beam system (10 Hz detection rate) and analyzed by software developed by TSE Systems (Todorovic et al., 2007).

Hot-plate assay. Analgesia was monitored using a hot plate apparatus (TSE Systems). Mice were individually placed on the hot plate (surface temperature, 52°C) within a clear Plexiglas chamber and the latency in seconds (60 s maximum) to reach the analgesic threshold was recorded. The analgesic threshold was considered to be the latency between the time when an animal was placed on the plate and the time when it started licking its hindpaws or jumping.

Protein extraction and Western blot analyses. The procedure was similar to that described before (Todorovic et al., 2009). Briefly, mice were killed by cervical dislocation and their dorsal hippocampi were quickly dissected and frozen at $-80°C$. Frozen tissue was lysed in RIPA buffer containing protease and phosphatase inhibitors (Pierce). Protein samples of the cell lysates from individual mice were separated by 10% SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore). Primary phospho-state-specific antibodies were pJNKs (Thr183/Tyr185) (1:2000), pERK1/2 (Thr202/Tyr204) (1:2000), pElk1 (1:1000), pATF2 (1:1000) and p-c-Jun (Ser-63) (1:1000), and were used in conjunction with respective pan-antibodies to measure individual kinase (1:1000) (Cell Signaling Technology). The specificity of anti-pJNKs (Thr183/Tyr185) antibody was confirmed in previous studies (Kuan et al., 2003; Jaeschke et al., 2006). The immunoreactive bands were visualized by using secondary antibodies conjugated to HRP (Cell Signaling Technology, 1:5000) with an ECL Western blot detection kit (Pierce). Quantification of bands was performed by using the ImageJ 1.41 software [National Institutes of Health (NIH), Bethesda, MD]. The band density of a given phosphorylated kinase was divided by the band density of the corresponding total protein kinase and expressed as percentage of band densities obtained with samples from control mice.

Protein kinase assay. To determine JNK1 and JNK3 kinase activity, dorsohippocampal lysates (500 μg total protein) were incubated with DynaBeads Protein G (Invitrogen) for 1 h at 4°C. Using a magnet, lysates were separated from the preclearing beads and incubated with 20 μl of DynaBeads conjugated with mouse anti-JNK-1 antibody (clone G151-33, PharMingen) or rabbit anti-JNK3 antibody (clone C05T, Millipore) at 4°C for 2 h. The DynaBeads were then pelleted using a magnet and washed 3 times with PBS containing Tween 20. Immunoprecipitated (IP) complexes were eluted from the beads by adding 50 mM glycine, pH 2.8, with gentle mixing for 2 min at room temperature. JNK1 and JNK3 kinase assays were performed using c-Jun fusion protein [c-Jun residues 1-79 fused with glutathione S-transferase (GST)] (Cell Signaling Technology) as the JNK isoform substrate. To the IP complexes 50 μl of kinase

assay buffer [containing the following (in mM): 25 Tris-HCl, pH 7.5, 5-glycerol phosphate, 2 DTT, 0.1 NaVO$_3$, 10 MgCl$_2$, 200 ATP, and 2 µg of c-Jun (1-79) (c-Jun residues 1-79 fused with GST)] were added, and incubated at 30°C for 30 min. Immunoblot analysis for c-Jun phosphorylation was performed using the anti-phospho-c-Jun (Ser-63) antibody (Cell Signaling Technology). The immunoblots were reprobed with JNK1 and JNK3 antibodies. Light-chain-specific HRP-conjugated anti-rabbit IgG (Cell Signaling Technology) or anti-mouse IgG (Sigma) were used to remove masking produced by denatured IgG heavy chains. Molecular weight and densitometric calculations were performed with the computer software ImageJ 1.41 (NIH). The specificity of JNK1 and JNK3 antibodies in mouse tissue has been described previously (Kuan et al., 1999; Coffey et al., 2002; Brecht et al., 2005) and was further confirmed after testing in hippocampal whole-cell lysates obtained from the corresponding JNK-isoform deficient mice (supplemental Fig. 1, available at www.jneurosci.org as supplemental material).

c-Fos and c-Jun immunohistochemistry. Mice were anesthetized with a 1.2% avertin solution injected intraperitoneally and transcardially perfused with ice-cold PBS, pH 7.4, followed by 4% paraformaldehyde (PFA) in phosphate buffer (pH 7.4, 150 ml per mouse). Immunostaining was performed by using the standard protocol for free-floating sections (50 µm thickness) with anti-c-Fos (Oncogene Sciences, 1:15,000) anti-p(Ser-63)-c-Jun (Millipore, 1:400) primary antibodies. Biotinylated secondary antibodies (Vector Laboratories, 1:400) and ABC kit (Vector Laboratories) were used for signal amplification and diaminobenzidine (Sigma tablet set) as chromogen. A Zeiss Axioimager microscope and a Zeiss Axio Cam HRM camera were used for the analysis and photography of sections.

Single immunofluorescence staining and light microscopy. A similar method as the one used for perfusion and postfixation of mice brains was followed. A freezing microtome was used to collect serial coronal 50 µm sections through the hippocampus of fixed brains. For single immunofluorescent staining of pJNK, free-floating sections were incubated in TBST (1× PBS containing 0.03% Triton X-100) containing 5% goat serum and Avidin/Biotin blocking solution (Vector labs) following the manufacturer's protocol and then with pJNK-specific (G7) primary antibody (Santa Cruz Biotechnology, 1:500) (Gdalyahu et al., 2004) in a similar buffer solution overnight at 4°C. The sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (1 mg/ml, 1:1000) and mounted with Vectashield mounting medium (Vector Laboratories). The slides were immediately analyzed under the microscope (Axioimager, Zeiss), and microphotographs were taken with a Zeiss Axio Cam HRM with the help of the Axiovision Rel 4.5 software. We calculated relative values for immunofluorescence signals by comparison with conventional fluorescence photomicrographs taken with identical exposure times and illumination from simultaneously processed specimens. Equally sized images from different treatment groups were analyzed using AIDA imaging software. To account for differences in background staining intensity, two background intensity measurements lacking immunofluorescent profiles were taken from each section. The mean of these two measurements constituted the background intensity. The background intensity was then subtracted from the measured specific signal density to provide a final intensity immunofluorescence value. Equivalent planes of coronal sections (CA1 and CA3 subregions) were used for comparison of animal groups. Values are expressed in arbitrary units (mean integral density/area-background) (Fig. 1). For cell counting in other brain regions (e.g., supplemental Fig. 2, available at www. jneurosci.org as supplemental material) the total number of cells was determined by the number of nuclei stained with DAPI. Cells containing phosphorylated JNKs (pJNKs) were counted and calculated as the percentage of the total number of cells in the same brain region. The data are presented as the fold increase in the percentage of cells containing pJNKs in comparison with that found in the same region from control mice. The same brain areas with pJNK-positive cells were chosen based on stereotaxic coordinates and equivalent brain areas were counted from all mice.

Double immunofluorescence staining and confocal imaging. For double immunofluorescence staining free-floating sections were used. Sections were first incubated in 1× TBST containing 5% goat serum and Avidin/Biotin blocking solution (as mentioned in the single immu-

nofluorescence), and then incubated with primary antibodies overnight at 4°C. Primary antibodies used were anti-pJNK (G7) (Santa Cruz Biotechnology, 1:500) (Gdalyahu et al., 2004) and mouse anti-neuronal-specific nuclear protein (NeuN) IgG (Millipore, 1:1000). The fluorescent secondary antibodies used were biotinylated anti-rabbit (Vector Laboratories) and anti-mouse Alexa Fluor-555 (Invitrogen). The sections were rinsed and incubated with Fluorescein Avidin DCS (Vector Laboratories). Following rinsing, the sections were dried and mounted with Vectashield (Vector Laboratories). Several different titers of each antibody were tested to determine the concentration for optimal signal-to-noise results. Images were captured with a Zeiss LSM 510 confocal microscope using a 20× objective lens. All confocal images were scanned and edited using LSM 510 metasoftware (Zeiss). All presented microphotographs are composites of 5 serial optical sections scanned at a regular increment of 1 µm. Optical sections were averaged 3–4 times to reduce noise. Density filter, pinhole aperture, detector gain and offset were initially set within a linear range to obtain pixel densities, and then kept constant for experimental comparisons. Signals were acquired using sequential line scanning. Colocalization of immunosignals was confirmed in x, y, and z dimensions.

Statistical Analysis. The data for behavioral and molecular studies are expressed as mean ± SEM, and were analyzed using a one- and two-way ANOVA (StatView 5.0.1 software; SAS Institute). Scheffé's test was applied *post hoc* for individual between-group comparisons at the $p < 0.05$ level of significance.

Results

Stress-induced increase of hippocampal phospho-JNKs causes impairment of contextual fear

C57BL/6J male mice trained immediately after 1 h of immobilization stress (IS) showed significant impairment of context-dependent fear conditioning ($F_{(1,18)} = 22.3$; $p < 0.05$) measured 24 h later (Fig. 1a), consistent with previous findings (Todorovic et al., 2007). One attractive transduction system for mediation of this stress response is the JNK pathway, and activation of the JNK isoforms (JNK1, 2, and 3) is regarded as the key molecular switch (Davis, 2000). Western blot analysis revealed that 1 h IS increased phosphorylation of 46 and 54 kDa JNKs (pJNKs) in the dorsal hippocampus 0, 0.5, 1, and 3 h following 1 h IS ($F_{(6,21)} = 21.3$; $p < 0.05$; 46 kDa) ($F_{(6,21)} = 11.3$; $p < 0.05$; 54 kDa) (Scheffé test, $p < 0.05$ vs naive controls) (Fig. 1b). Moreover, when assessed 1 h after exposure to the stressor, pJNKs were strongly upregulated in the CA1 (Fig. 1c, left) and the CA3 (Fig. 1c, right) pyramidal cell somata of the hippocampus with accompanied diffusion toward the dendritic and axonal processes. Exposure to 1 h IS also elevated pJNKs in the basolateral and cortical nuclei of the amygdala, hypothalamic ventromedial and dorsomedial nuclei (Scheffé test, $p < 0.05$ vs nonstressed controls) (supplemental Fig. 2, available at www.jneurosci.org as supplemental material), but did not alter the JNKs phosphorylation pattern in lateral and medial septal areas, piriform cortex, or paraventricular nucleus of the hypothalamus, among the selected areas ($p > 0.05$) (supplemental Fig. 2, available at www. jneurosci.org as supplemental material).

It is extensively documented that effects of acute stress on hippocampal-dependent tasks (i.e., trace eyeblink conditioning, learned helplessness) differ between male and female rodents (Shors, 2004). Thus, we next tested whether exposure to 1 h IS also elicits changes in contextual fear conditioning and hippocampal pJNKs in C57BL/6J female mice. Similarly to male mice, C57BL/6J female mice exposed to 1 h IS and trained immediately afterward showed significant impairment of contextual fear ($F_{(1,17)} = 33.6$; $p < 0.05$) (supplemental Fig. 3a, available at www.jneurosci.org as supplemental material). Female mice displayed a somewhat different pattern of JNKs activation compared
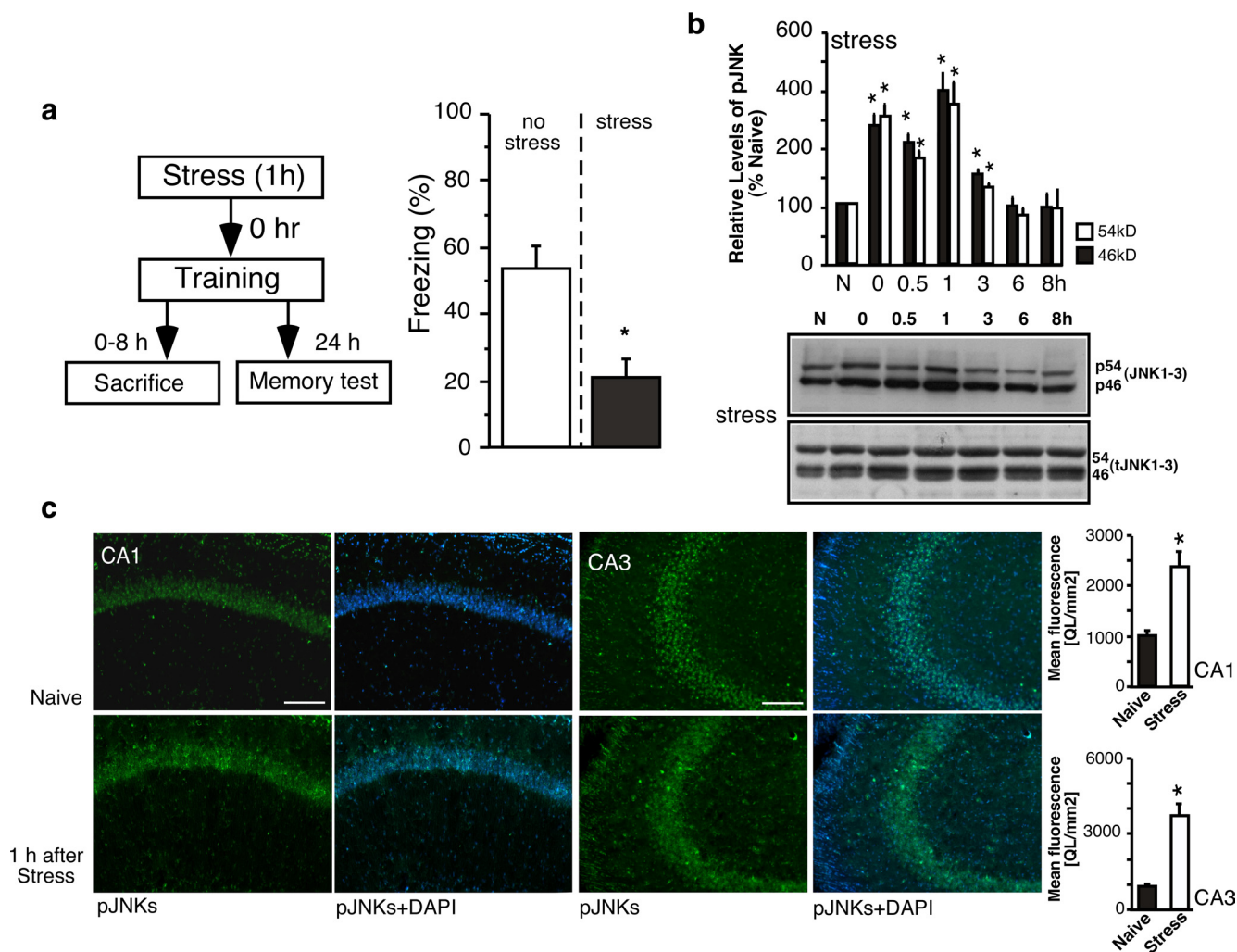
Figure 1. Stress-induced learning deficit is accompanied by increased phosphorylation of hippocampal JNKs. ***a***, C57BL/6J mice subjected to 1 h immobilization stress, trained immediately afterward and tested 24 h later for retention showed significant impairment of contextual conditioned fear (*n* = 10). ***b***, Representative Western blots of phosphorylated JNKs from naive and stress exposed mice (0 – 8 h poststress) (bottom). Densitometric analysis: The immunoblots were obtained from individual dorsohippocampal lysates isolated 0 – 8 h after exposure to stressor. p46 and p54 correspond to the molecular weights (in kilodaltons) of the phosphorylated isoforms of mammalian JNKs (pJNKs 1-3) that are recognized by the pJNKs antibody. The relative intensity of bands corresponding to phosphorylated p46 and p54 JNKs were determined and this value was divided by that obtained for corresponding bands (46 and 54 kDa) of total JNK1-3. Such calculated pJNKs levels in experimental groups were then expressed as a percentage of those in naive mice. Statistically significant differences: **p < 0.05 relative to the naive mice (top) (*n* = 4) (top). ***c***, Representative photomicrographs acquired using a conventional fluorescent microscope illustrate the strong increase in pJNKs in the CA1 (left panel) and CA3 hippocampal (right panel) subfields in mice exposed to 1 h stress; pJNKs (green), DAPI (blue). Magnification 20×. Scale bar, 400 μm.

with male mice, with a small decrease in hippocampal 54 kDa pJNKs 30 min after 1 h IS ($p < 0.05$; Scheffé test), followed by a similarly significant increase in 46 kDa JNK activation 1 and 3 h ($F_{(6,23)}$ = 19.1; $p < 0.05$; 46 kDa) and in 54 kDa JNK activation 3 h later ($F_{(6,23)}$ = 9.5; $p < 0.05$; 54 kDa) (Scheffé test, $p < 0.05$ vs naive controls) (supplemental Fig. 3b, available at www.jneurosci.org as supplemental material). Interestingly, 1 h IS significantly impaired male (Todorovic et al., 2007) but not female C57BL/6J mice in delayed tone-dependent fear conditioning (data not shown).

Because JNKs were activated in the CA1 subregion of the hippocampus, which plays a critical role in contextual fear conditioning (Abel et al., 1997; Impey et al., 1998; Rampon et al., 2000; Levenson et al., 2002), we assumed that the JNK signaling may be involved in the formation of contextual fear associated with stress. Thus, our next question was whether elevated phosphorylation of hippocampal JNKs was functionally coupled to the observed stress-induced memory deficit. To address this issue, we

injected JNK inhibitors bilaterally into the hippocampus, as follows. The small molecule JNK inhibitor sp600125 (30 μM; 0.25 μl per side), cell-permeable JNK inhibitory peptide D-JNKI1 (8 μM; 0.25 μl per side) derived from JNK-interacting protein-1 (JIP1), or saline into the CA1 subregion of the dorsal hippocampus were injected (intrahippocampally) 5 min before 1 h IS. Mice were trained immediately following 1 h IS (stress groups: sp-FC, JNKI-FC). A second group of mice received identical treatment except that the injection was performed immediately after contextual fear conditioning (stress groups: FC-sp, FC-JNKI) (Fig. 2*a*). Significant effects for treatment ($F_{(4,80)}$ = 6.3; $p < 0.05$) and stress ($F_{(1,80)}$ = 44.2; $p < 0.05$) were revealed (via two-way ANOVA, with treatment and stress as between-subject factors), demonstrating that intrahippocampal injection of sp600125 or D-JNKI1 in both mouse groups exposed to 1 h IS fully rescued stress-induced learning deficits and returned contextual conditioned fear to baseline levels (Fig. 2*a*). The dose of sp600125 was selected based on its ability to strongly reduce phosphorylation of JNKs
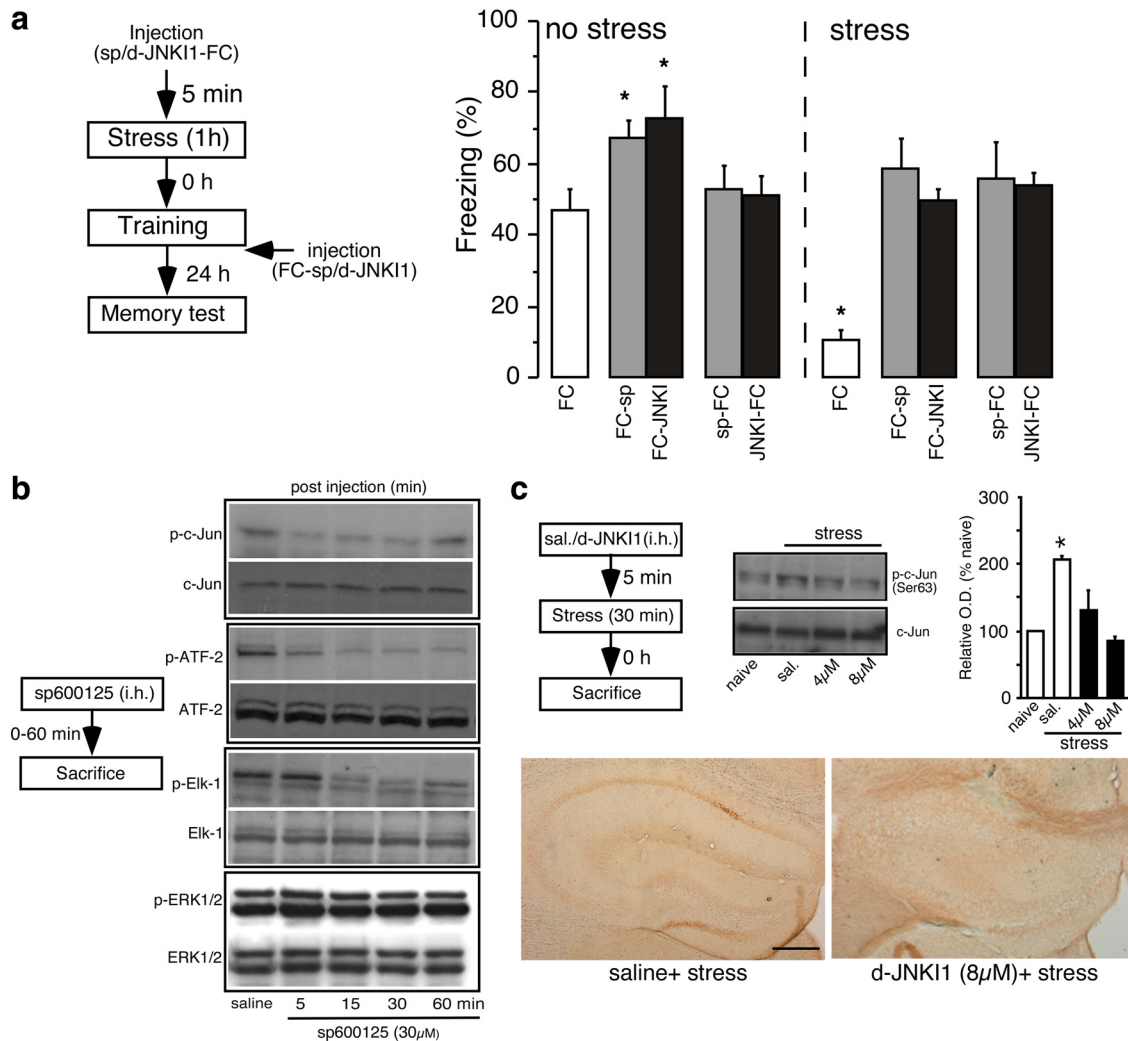
Figure 2. Injection of JNKs inhibitors into the CA1 subregion of the dorsal hippocampus before stress exposure and immediately after training prevents stress-induced memory deficit. **a**, Schematic behavioral procedure for fear conditioning and intrahippocampal injection of the JNK inhibitors, sp600125 and D-JNKI1 (left). Intrahippocampal injection of 30 μM sp600125 or 8 μM D-JNKI1 5 min before 1 h immobilization stress (stress groups: sp-FC, JNKI-FC) resulted in reversal of contextual fear deficit. Similarly, intrahippocampal injection of JNK inhibitors immediately after stress plus contextual fear conditioning (stress groups: FC-sp, FC-JNKI) blocked stress-induced memory deficit. Sp-FC and JNKI-FC no-stress control groups received injections 65 min before conditioning. FC-sp and FC-JNKI no-stress control groups received injection immediately following conditioning (right). Statistically significant differences: *$p < 0.05$ relative to the FC-no stress control group ($n = 8 – 10$). **b**, Representative immunoblots from dorsal hippocampi taken from mice at various time points after intrahippocampal injections of 30 μM sp600125. Note that injections resulted in significantly decreased phosphorylation of JNKs substrates c-Jun (Ser-63), ATF2, and Elk1 in the dorsal hippocampus up to 1 h postinfusion. Significant increases in pERK1 (44 kDa) and pERK2 (42 kDa) were observed 5 min after JNKs inhibition ($n = 5$). *$p < 0.05$ relative to the control mice (saline). **c**, Intrahippocampal injection of the selective JNKs peptide inhibitor D-JNKI1 (procedure, top, left panel) strongly decreased stress-induced phosphorylation of JNKs substrate c-Jun at Ser63 in the dorsal hippocampus. Representative Western blots of p-c-Jun (Ser-63) levels from naive and stress-exposed mice that received intrahippocampal injection of saline or D-JNKI1 5 min before exposure to 30 min IS. The immunoblots were obtained from individual dorsohippocampal lysates isolated immediately after exposure to stress (top, upper middle panel). Densitometric analysis is shown on the upper right panel: *$p < 0.05$ relative to the naive mice ($n = 5$). Photomicrographs acquired using a light microscope show pronounced blockade of stress-induced phosphorylation of c-Jun after intrahippocampal injection of 8 μM D-JNKI1 under the same conditions (bottom). Magnification 5×. Scale bar, 100 μm.

elective substrates, including transcriptional factors c-Jun, ATF2, and Elk1 (Scheffé test, $p < 0.05$ vs saline controls) (Fig. 2b; supplemental Fig. 4, available at www.jneurosci.org as supplemental material). Interestingly, the same dose of sp600125 also transiently increased phosphorylation of ERK1/2 MAPKs 5 min after injections. The level of the unphosphorylated ERK1/2 remained unchanged (Scheffé test, $p < 0.05$ vs saline) (Fig. 2b; supplemental Fig. 4, available at www.jneurosci.org as supplemental material). Similarly, the dose for D-JNKI1 of 8 μM was chosen based on its ability to significantly downregulate phosphorylation of c-Jun at Ser-63 after exposure to 30 min IS (Scheffé test, $p < 0.05$ vs vehicle) (Fig. 2c). It is important to note that pretraining injections of sp600125 or D-JNKI1 into the dorsal hippocampus did

not affect activity or the activity burst in response to the shock (supplemental Table 1, available at www.jneurosci.org as supplemental material).

Although phosphorylation state and hence activity of JNK in the hippocampus correlated with the stress-induced memory deficit, it may also be that exposure to 1 h IS and/or JNK inhibitors affected pain sensitivity or locomotion during fear conditioning, implying that the stress-induced learning deficit might have been due to lowered sensitivity to the foot shock or altered locomotion. We tested the first possibility by exposing the mice to a hot-plate test immediately after exposure to 1 h immobilization. One hour of IS alone did not change the response latency in the hot-plate test ($F_{(1,18)} < 1$) (supplemental Fig. 5b, available at
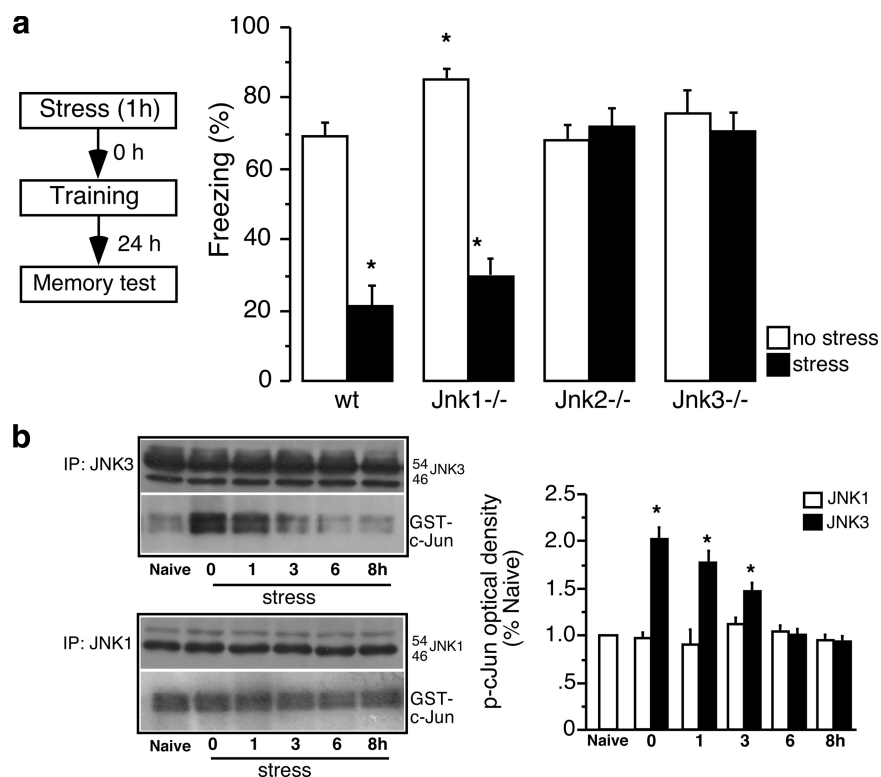
a

b

Figure 3. Selective activation of JNK 2 and JNK3 after acute stress is responsible for stress-induced memory deficit. ***a***, Targeted deletion of Jnk2 and Jnk3 genes protected mice from stress-induced memory deficit. *Jnk2*- and *Jnk3*-null mice trained immediately after 1 h immobilization stress did not display stress-induced impairment of contextual fear conditioning. As found for wild-type mice *Jnk1*-null mice did show stress-induced memory deficits. The baseline level of contextual fear, unchanged in *Jnk2*- and *Jnk3*-null mice was significantly increased in *Jnk1*-null compared with wild-type mice ($n = 8$ per group). Statistically significant differences: *$p < 0.05$ vs wild-type mice. ***b***, One hour of immobilization stress increased JNK3 kinase activity in dorsal hippocampi at the indicated time points following stress exposure. JNK1 and JNK3 were immunoprecipitated from lysates with isoform-selective JNK antibodies, and the activity was assessed with *in vitro* kinase assays using GST-c-Jun (1-79) as the substrate (left). JNK1 kinase activity in total lysates was not affected by stress (left bottom). JNK3 showed a strong increase in activity up to 3 h after stress (left top). Densitometric analysis: phospho c-Jun levels in experimental groups were expressed as percentage of those in naive mice. Phospho-c-Jun was visualized by phospho-c-Jun (Ser-63) antibody and the JNK isoforms were visualized by JNK1 or JNK3 antibodies. The double-band corresponds to mono- or biphosphorylated Jun protein. Quantified activity is displayed as percentage of control (naive mice) activity (right) ($n = 4$). *$p < 0.05$ relative to naive mice.

www.jneurosci.org as supplemental material). Thus, stress-induced analgesia did not mediate the stress-induced impairment in retention of contextual fear. Alternatively, it may be that mice injected with JNK inhibitors might display increased responsiveness to the foot shock serving as unconditioned stimulus (US), which would be responsible for rescue from stress-induced impairment of contextual fear. This possibility was rejected after finding that intrahippocampal injections of 30 μM sp600125, 8 μM D-JNKI1, or vehicle 30 min before contextual fear did not change reactivity toward the US ($F_{(1,12)} = 1.11$; $p > 0.05$) (supplemental Fig. 5a, available at www.jneurosci.org as supplemental material). Finally, exposure to 1 h IS did not affect locomotor activity measured in the fear conditioning box ($F_{(1,18)} = 1.3$; $p > 0.05$) (supplemental Table 1, available at www.jneurosci.org as supplemental material).

Specific isoforms of hippocampal JNKs are activated in response to stress linked to impairment of contextual fear

Which isoforms of JNK are involved in the observed stress-induced memory deficit was revealed via use of JNK isoform null mice. It was found that *Jnk2*- and *Jnk3*-null mice ($Jnk2^{-/-}$, $Jnk3^{-/-}$) do not display stress-induced deficits of contextual fear conditioning (main effects for genotype ($F_{(3,56)} = 13.5$; $p < 0.05$),

stress ($F_{(1,56)} = 29.4$; $p < 0.05$), geno-type × stress ($F_{(3,56)} = 14.2$; $p < 0.05$). In contrast, the stress-induced deficit was still evident in *Jnk1*-null mice (Fig. 3a). In addition, we immunoprecipitated JNK1 and JNK3 from whole-cell lysates prepared from the dorsal hippocampus of naive mice and stressed C57BL/6J mice, and then tested their activity using an *in vitro* kinase assay with a GST-c-Jun fusion protein as substrate. We detected a strong increase in JNK3 activity ($F_{(5,18)} = 14.4$; $p < 0.05$) ($p < 0.05$; Scheffé test vs naive mice) up to the 3 h poststress (top), without detectable effect of 1 h IS on JNK1 activity (bottom) (Fig. 3b). These results indicate that the increased phospho-JNK in dorsohippocampal neurons in response to stress (Fig. 1) corresponds to activation of specific isoforms. Together, these observations support a hypothesis that there is a strong difference between JNK1 and JNK3 (and likely JNK2) activity in neurons in response to stressful stimuli.

Activation of septal CRF$_2$ mimics effects of acute exposure to a stressor on contextual fear conditioning and hippocampal JNKs activation

Our previous findings showed that the stress-induced increases of anxiety and decreases of contextual fear are fully prevented by injections of CRF$_2$ antagonist in the lateral septum. In addition, pharmacological activation of CRF$_2$ in the lateral septum mimics the effects of acute stress on anxiety and memory processes (Todorovic et al., 2007). Thus, to address the possibility that CRF$_2$ serves upstream of JNK activation, our next experiment tested whether pharmacological activation of CRF$_2$ in the lateral septum impaired contextual conditioned fear, and if so, whether such impairment, as in the case of exposure to 1 h IS, was accompanied by elevated hippocampal pJNKs levels. For that purpose, mice were injected with 100 ng (20 pmol; 0.25 μl per side) of h/rCRF into the lateral septum and subjected to contextual fear conditioning 30 min later (Fig. 4a, left). Indeed, this treatment produced a significant impairment in the retention of contextual fear 24 h later ($F_{(2,21)} = 22.3$; $p < 0.05$) (Fig. 4a, right). Moreover, intraseptal injection of 400 ng (110 pmol; 0.25 μl per side) of the CRF$_2$-selective antagonist aSvg-30 alone facilitated contextual fear (Scheffé test, $p < 0.05$ vs aCSF controls) (Fig. 4a, right). Paralleling the memory test results, the phosphorylation levels of 46 kDa and 54 kDa JNKs in the hippocampus increased significantly in septal h/rCRF-injected mice 30 min after contextual fear conditioning (Scheffé test, $p < 0.05$ vs aCSF) (Fig. 4b). Furthermore, memory-facilitating blockade of CRF$_2$ in the lateral septum by aSvg-30 resulted in a decrease of hippocampal 54 kDa pJNKs (Scheffé test, $p < 0.05$ vs aCSF) (Fig. 4b). This finding suggested that during contextual fear conditioning the hippocampal response to 1 h IS was mediated by septal CRF$_2$ using the circuitry of the septo-hippocampal system (Gray and McNaughton, 1983). Activation of septal CRF$_2$ would then
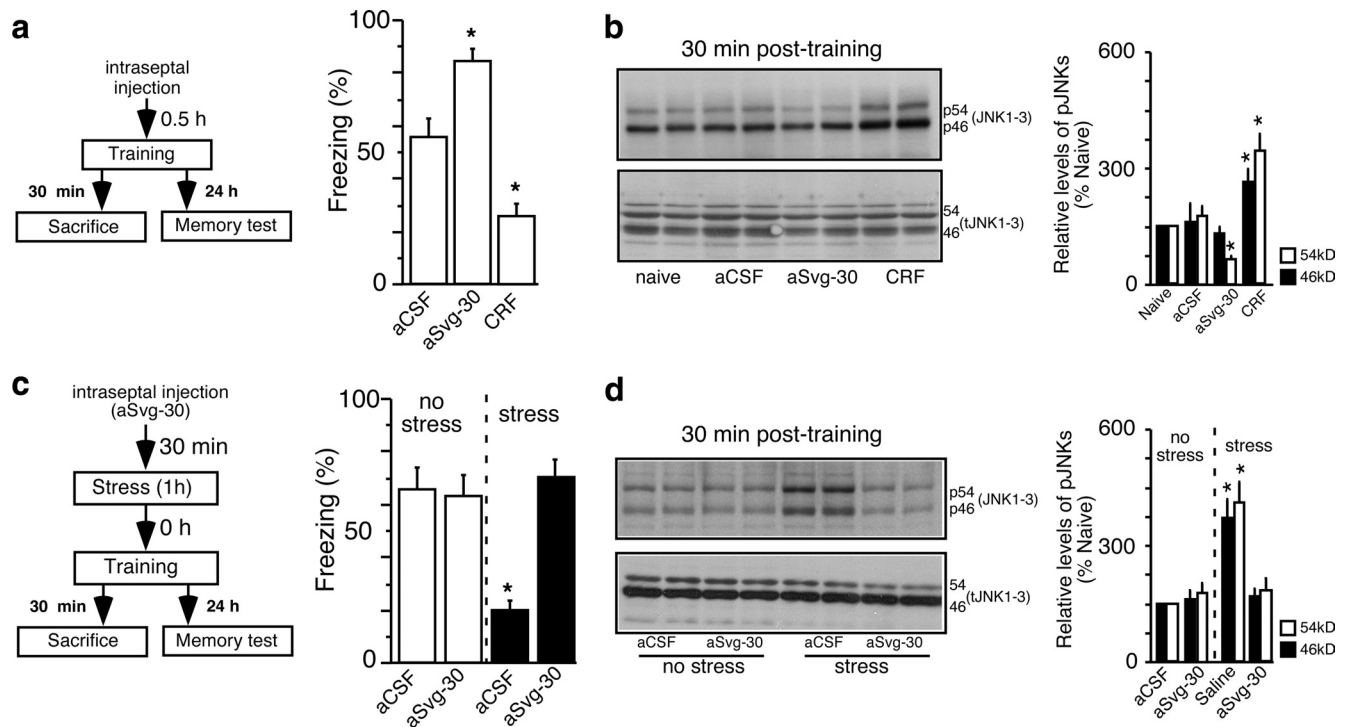
Figure 4. Septal CRF$_2$-mediated modulation of contextual conditioned fear correlates with changes of hippocampal JNKs phosphorylation levels. ***a***, Activation of CRF$_2$ in the lateral septum by 100 ng of h/rCRF or antagonism of septal CRF$_2$ by 400 ng of CRF$_2$-selective antagonist aSvg-30 resulted in impairment and enhancement of contextual fear, respectively ($n = 8$). ***b***, The same pharmacological procedures triggered signal transduction changes in the hippocampus responsible for the observed impairment or enhancement of contextual fear. The phosphorylation level of JNKs (46 and 54 kDa) increased significantly in septal h/rCRF-injected mice 0.5 h after training. Memory-facilitating blockade of CRF$_2$ in the lateral septum after injection of aSvg-30 resulted in decreased phosphorylation of 54 kDa JNKs. Representative immunoblots of pJNKs levels and amounts from aCSF, aSvg-30, and CRF-treated mice (0.5 h post-training) (left). Densitometric analysis: activated kinase levels in experimental groups were expressed as percentage of those in naive mice. Before the calculation, activated kinase levels were normalized to total kinase levels. Statistically significant differences: *$p < 0.05$ relative to the naive mice ($n = 4$) (right). ***c***, Stress-induced impairment of context-dependent fear conditioning was fully antagonized by 400 ng (110 pmol) of aSvg-30 per mouse injected intraseptally 30 min before immobilization ($n = 7–9$). ***d***, Intraseptal injection of aSvg-30 30 min before 1 h immobilization blocked stress-induced increase of hippocampal phospho-JNKs (46 and 54 kDa). Immunoblots were obtained from individual dorsohippocampal lysates 30 min after the end of 1 h immobilization or from respective nonstressed control groups (left). Densitometric analysis: phosphokinase levels in experimental groups were expressed as percentage of those in aCSF-treated nonstressed mice (right) ($n = 4$). Statistically significant differences: *$p \leq 0.05$ vs control (nonstressed mice + aCSF).

lead to activation of hippocampal JNKs, which would be responsible for stress-induced impairment of contextual fear memory consolidation.

Further evidence supporting this hypothesis was provided by the finding that intraseptal administration of 400 ng (110 pmol) of aSvg-30 30 min before 1 h IS (Fig. 4c, left) completely prevented stress-induced impairment of contextual fear (two-way ANOVA: treatment, $F_{(1,34)} = 11.6$; $p < 0.05$; stress, $F_{(1,34)} = 8.1$; $p < 0.05$; interaction, $F_{(1,34)} = 16.6$; $p < 0.05$) (Fig. 4c, right). The same treatment completely prevented stress-induced increase in hippocampal pJNKs (Scheffé test; $p > 0.05$ compared with aCSF) (Fig. 4d).

Anisomycin-induced amnesia is prevented by selective blockade of hippocampal JNKs pathways
Our initial data (Fig. 1) suggested that exposure to 1 h IS leads to a strong and sustained (0–3 h) increase in hippocampal pJNKs levels. Anisomycin is a potent activator of JNKs (Cano et al., 1994; Hazzalin et al., 1998). The next experiment was designed to investigate whether bilateral intrahippocampal administration of anisomycin (100 µg/µl; 0.25 µl per side) would mimic the effects of acute stress on retention of long-term contextual fear and hippocampal JNKs activation. As shown in Figure 5a, freezing during a memory test 24 h after contextual fear conditioning was significantly lower in mice injected with anisomycin into the CA1 subregion of the hippocampus 15 min before training than in

vehicle-injected controls. Thus, the anisomycin treatment successfully produced a memory deficit 24 h after training ($F_{(2,21)} = 19.0$; $p < 0.05$). Interestingly, a similar deficit was observed when mice were tested 90 min ($F_{(2,21)} = 40.1$; $p < 0.05$) and 360 min ($F_{(2,21)} = 7.9$; $p < 0.05$), but not when tested immediately ($F_{(2,21)} = 1.8$; $p > 0.05$) or 180 min ($F_{(2,21)} = 2.1$; $p > 0.05$) after contextual fear conditioning (Fig. 5a). Similar to pretraining, post-training intrahippocampal administration of anisomycin produced a memory deficit 360 min ($F_{(2,24)} = 14.64$; $p < 0.05$) and 24 h following training ($F_{(2,24)} = 35.34$; $p < 0.05$) as well as a short-term memory deficit 90 min after conditioning ($F_{(2,24)} = 5.6$; $p < 0.05$) (Fig. 5b). Importantly, intrahippocampal application of sp600125 (30 µM; 0.25 µl per side) 30 min before (Fig. 5a), or immediately after (Fig. 5b) contextual conditioning completely prevented the anisomycin effect on retention of contextual fear memory at all time points tested (Scheffé test; $p > 0.05$ vs saline). As expected, anisomycin also caused increased phosphorylation of hippocampal JNKs. Representative immunoblots in Figure 5c demonstrated a significant increase in both 46 kDa and 54 kDa pJNKs levels after intrahippocampal injection of anisomycin ($F_{(2,9)} = 29.4$; $p < 0.05$; 46 kDa) ($F_{(2,9)} = 21.2$; $p < 0.05$; 54 kDa). This increase was prevented by prior treatment with sp600125 (30 µM; 0.25 µl per side) (Scheffé test; $p > 0.05$ vs saline) (Fig. 5c).

To control for changes in protein synthesis, we measured training-induced changes in c-Fos protein production in the
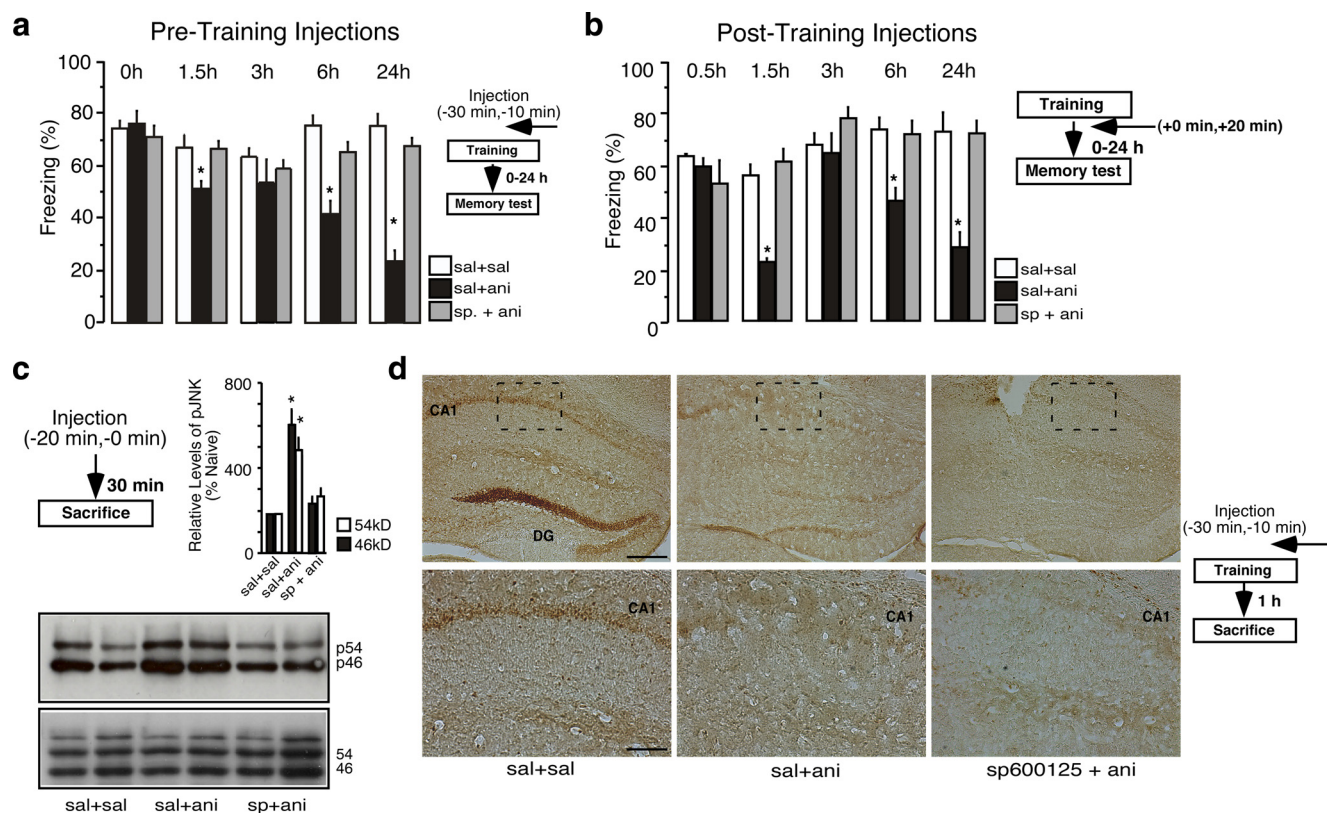
Figure 5. Inhibition of hippocampal JNKs signaling pathways rescues anisomycin-induced deficit of contextual fear. **a**, Effects of pretraining intrahippocampal infusions of anisomycin on contextual fear memory tested 0, 1.5, 3, 6, and 24 h after training. The treatment impaired memory at 1.5, 6, and 24 h but not at 0 and 3 h after training. Intrahippocampal administration of JNKs-selective inhibitor sp600125 (30 μM) prevented observed memory deficits. **b**, Post-training intrahippocampal infusions of anisomycin impaired contextual fear retention 1.5, 6, and 24 h after training. Intrahippocampal injections of sp600125 (30 μM) prevented anisomycin-induced memory deficits. **c**, Western blot analysis indicated that intrahippocampal injection of anisomycin produced a strong increase in JNKs phosphorylation. Pretreatment with sp600125 reduced this increase in pJNKs levels. The immunoblots were obtained from individual dorsohippocampal lysates isolated 30 min after injections. **d**, Contextual fear conditioning-induced c-Fos expression following intrahippocampal administrations of either saline (left), anisomycin (middle) or coadministration of sp600125 with anisomycin (right). The photomicrographs were taken just below the cannulae tracks. c-Fos expression following anisomycin infusion and fear conditioning was markedly reduced, in the principal hippocampus subfields. The additional pretreatment with sp600125 did not affect c-Fos inhibition caused by intrahippocampal anisomycin treatment. Magnification 10×. Scale bar, 200 μm (top). For comparison, a CA1 subregion of the hippocampus (indicated with dashed rectangle) is shown at higher magnification (bottom). Magnification 20×. Scale bar, 400 μm (bottom). Statistically significant differences: *$p < 0.05$ relative to the saline-treated mice.

presence or absence of anisomycin. [Note that the turnover rate of c-Fos transcripts is remarkably fast: their accumulation peaks at ≈30 min after the onset of neuronal activity and decreases to background level ≈30 min after the offset of neuronal activity (Takahata et al., 2009).] Such a measure, although qualitative, is accepted as sensitive enough to determine the degree of protein synthesis (Lamprecht and Dudai, 1996; Inda et al., 2005; Frankland et al., 2006; Canal et al., 2007). Immunohistochemistry for c-Fos was performed on sections of brains removed 60 min after the end of contextual fear conditioning (Canal and Gold, 2007). Figure 5d shows representative photomicrographs of c-Fos immunoreactivity in the dorsal hippocampus after vehicle, anisomycin or combined sp600125 and anisomycin injections. Positive c-Fos-immunoreactivity detectable after contextual fear conditioning was essentially eliminated by intrahippocampal injections of anisomycin before training, providing evidence for effective inhibition of protein synthesis. Moreover, c-Fos immunostaining remained unchanged in mice that received sp600125 before anisomycin (Fig. 5d).

The role of hippocampal pJNKs in contextual fear conditioning under baseline conditions

To determine whether activation of JNKs plays a role in the acquisition of baseline conditioned fear, we measured the phos-

phorylation of JNKs in the CA1 subregion of the dorsal hippocampus at different time points after context-dependent fear conditioning. Control groups consisted of naive mice, mice exposed to the context without foot shock (CTX group, nonshocked controls), and mice exposed to an immediate foot shock followed by context (ImS group, nonpaired control mice). As expected, the latter training conditions did not result in associative learning ($F_{(3,28)} = 14.9$; $p < 0.05$) (Fig. 6a) and were therefore used to delineate the impact of associative learning on JNKs activation from the effects of nonassociative learning and unconditioned stress responses to foot shock. Unlike immobilization (Fig. 1b,c), the novel context or immediate foot shock did not upregulate pJNKs (Fig. 6d) (Scheffé test; $p > 0.05$), indicating that JNKs activation depended on the type of stressor (Shen et al., 2004). Importantly, exposure of mice to a paired presentation of context and shock (CTX-S group, paired mice) resulted in stimulus-, time- and region-specific increase in phosphorylation of JNKs (Fig. 6b–d). In the CA1 hippocampal subregion 46 kDa and to lesser extent 54 kDa pJNKs were significantly elevated 60 min after training in the CTX-S group when compared with naive mice (Scheffé test; $p < 0.05$ vs naive controls) (Fig. 6b–d). Absence of pJNKs changes in the control groups indicated that under these conditions, the observed effect was specific for associative learning.
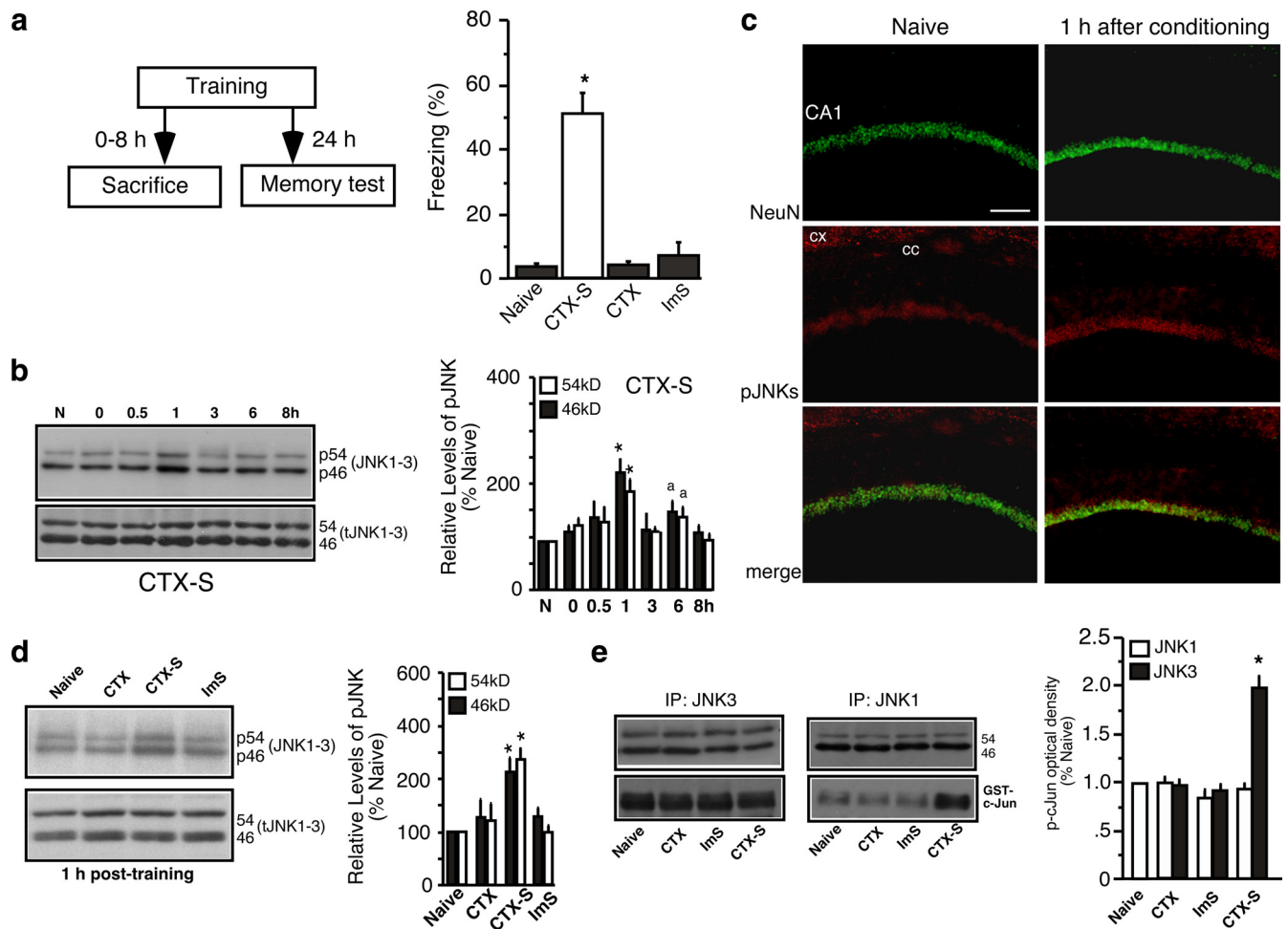
Figure 6. Contextual fear conditioning is accompanied by temporally increased JNK activity in the CA1 subregion of hippocampus. ***a**,* Mice were trained using a contextual fear conditioning protocol and pretraining baseline freezing behavior (naive group) was compared with freezing when mice were placed in the context on testing day. ***b**,* Representative Western blot of pJNKs levels from naive mice and context-shock (CTX-S) (0–8 h post-training) experimental groups (left). Densitometric analysis: phosphorylated kinase levels in experimental groups are expressed in percentage relative to pJNK levels in naive mice. Before the calculation, pJNK levels were normalized to total kinase levels (tJNK1-3) (right). Densitometric analysis revealed significantly increased 46 kDa and 54 kDa pJNKs levels 1 h after contextual fear conditioning. No changes in total JNKs amounts were observed across experimental groups. ***c**,* Representative confocal images of pJNKs immunoreactivity showed an increased density of pJNK-labeled cells 1 h following contextual fear conditioning only in the CA1 subregion of the hippocampus. No such changes were observed in the corpus callosum or cortex. pJNKs (red), NeuN (green); Scale bar, 400 μm. ***d**,* Western blot analysis also provided evidence that the exposure to context only (CTX group) or immediate-shock (ImS group) did not change hippocampal pJNK levels. The immunoblots were obtained from individual dorsohippocampal lysates isolated 1 h after training. ***e**,* Contextual fear conditioning increases JNK1 activity in the dorsal hippocampus. Total JNK1 and JNK3 were precipitated from whole-cell lysates by respective antibodies and were analyzed for kinase activity. Representative blots of c-Jun phosphorylation by JNK1 (middle) and JNK3 (left) 1 h after fear conditioning or control conditions are shown. Dorsohippocampal lysates were obtained from naive mice or 1 h after exposure to context only (CTX group), or immediate-shock (ImS group) and context-shock (CTX-S) experimental conditions ($n = 4$). Phospho-c-Jun was visualized by anti-phospho-c-Jun (Ser-63) antibody and the JNK isoforms were visualized by JNK1 or JNK3 antibody. Quantified activity is displayed as percentage of control (naive mice) activity (right). Statistically significant differences: *$p < 0.05$ relative to naive mice. CA1, CA1 subregion of hippocampus; cc, corpus callosum; cx, cortex.

Because the increased activation of pJNK mediated a stress-induced deficit of contextual fear, we were interested in what the functional significance of transiently increased pJNKs following contextual conditioning might be. As strong and prolonged (0–3 h; Fig. 1b) activation of pJNKs under stressful conditions leads to a memory deficit, it seemed plausible that a briefer and weaker activation of hippocampal JNKs might limit the amount of acquired memory, guarding the animal from overlearning the contextual fear after a single conditioning trial. To test this idea, mice were injected intrahippocampally with JNKs inhibitors sp600125 (30 μM, 100 μM), D-JNKI1 (4 μM, 8 μM), or saline (0.25 μl per side) immediately after contextual fear conditioning. Inhibition of the JNK pathway resulted in enhanced contextual fear when mice were tested 24 h later ($F_{(4,33)} = 4.13$; $p < 0.05$) (Fig. 7a). Moreover, in mice injected intrahippocampally with 30 μM sp600125 (0.25 μl per side) 30 min before, immediately and 30,

60 and 180 min after contextual fear conditioning, all but the 60 min post-training group, froze significantly more when tested 24 h later than mice injected intrahippocampally with saline ($F_{(5,40)} = 12.2$; $p < 0.05$) (Fig. 7b). The finding that Jnk1-null mice ($Jnk1^{-/-}$), but not $Jnk2^{-/-}$ or $Jnk3^{-/-}$, displayed increased baseline contextual fear (Scheffé test; $p < 0.05$ vs wild-type littermates) (Fig. 3a) indicated that the JNK1 activity negatively regulates contextual fear conditioning. It should be noted that none of the three JNK-null mouse lines displayed changes in locomotor activity or in response to the shock when compared with their wild-type littermates (supplemental Table 1, available at www. jneurosci.org as supplemental material).

To further examine whether JNK1 is the predominantly activated isoform after contextual fear conditioning, we analyzed dorsohippocampal lysates obtained 1 h after exposure to training or control conditions [naive, CTX-S (paired), CTX alone, and
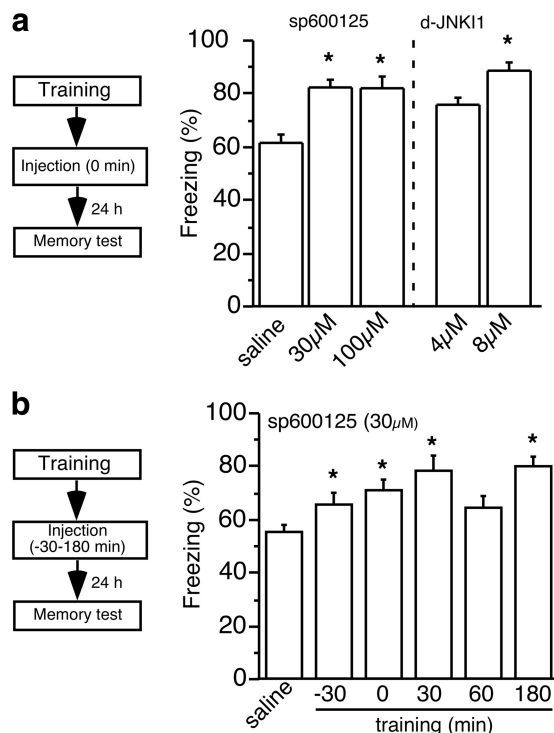
Figure 7. Intrahippocampal administration of JNKs inhibitors sp600125 and D-JNKI1 results in enhancement of context-dependent fear conditioning. *a*, Mice injected immediately after training with JNK inhibitors sp600125 (30 or 100 μM), D-JNKI1 (4 or 8 μM) showed increased contextual fear when assessed for conditioned freezing 24 h later ($n = 7$–8). *b*, Separate groups of mice were injected with 30 μM sp600125, or saline 30 min before, immediately, or 30, 60, and 180 min after contextual fear conditioning. All the groups, except sp600125 1 h post-training group, showed enhanced contextual freezing during the memory test 24 h later ($n = 8$–9). Statistically significant differences: *$p < 0.05$ relative to saline controls.

ImS mouse groups]. This time point was selected because pJNKs levels were increased in C57BL/6J mice after contextual fear conditioning (Fig. 6*b,c*). Consistent with the *Jnk1*$^{-/-}$ data, JNK1 activity, as measured by c-Jun activation was elevated in the dorsal hippocampi of CTX-S mice ($F_{(3,12)} = 4.2; p < 0.05$) ($p < 0.05$; Scheffé test vs naive mice). CTX alone and ImS mouse groups did not display a similar increase in JNK1 activity. This observation suggested learning-specific activity for JNK1. The JNK3 isoform showed no significant change in activity under all three experimental conditions (Fig. 6*e*). In sum, along with findings that *Jnk2*$^{-/-}$ and *Jnk3*$^{-/-}$ mice do not display stress-induced memory deficits (Fig. 3), and that stress induces a sustained increase in JNK3 activity, our findings that JNK1 activity is primarily responsible for baseline regulation of contextual fear conditioning is in agreement with previous studies that demonstrated preferential involvement of JNK isoforms in basal- and stress-induced signaling activity. Specifically, it has been shown that JNK1 is the primary source of the high level of basal hippocampal JNK activity, whereas JNK2 and JNK3 are critical components of stress-induced JNK neuronal signaling (Coffey et al., 2002; Kuan et al., 2003; Brecht et al., 2005). Since increased levels of pJNKs were observed only up to 1 h postconditioning, the finding that enhanced retention is observed even after JNKs inhibition 180 min post-training also raised the intriguing possibility that such enhancement might be explained by increased activation of the ERK pathway following JNKs inhibition. Overall, these results open the possibility that the hippocampal JNKs pathways alone, or acting via a dynamic crosstalk with the ERK pathway, might
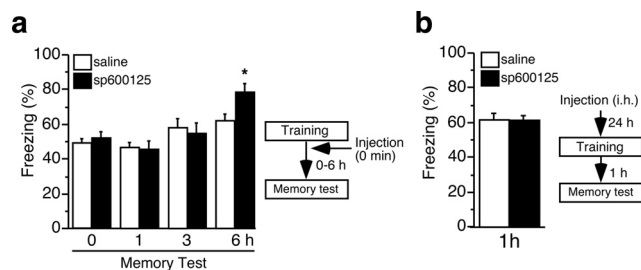

Figure 8. Enhancement of contextual conditioned fear produced by blockade of the hippocampal JNKs signal is not attributable to nonspecific effects of sp600125 on general activity level. *a*, Mice were injected into the dorsal hippocampus with 30 μM sp600125 or vehicle (saline) immediately after contextual conditioning, and assessed for immediate postshock freezing (0 min), short-term memory at 1 and 3 h (left panel), and presumably long-term memory 6 h after conditioning. *b*, Context-dependent short-term memory in mice given intra-hippocampal (i.h.) injection of saline 30 μM sp600125 24 h before conditioning and tested for short-term memory 24 h later (see adjacent schematic of behavioral procedures, right) ($n = 5$). Statistically significant differences: *$p < 0.05$ relative to saline controls.

serve as a limiting factor in fear conditioning under baseline conditions.

Since sp600125 might have some off-target actions (Bain et al., 2007), to further control for its possible nonspecific effects on cognitive or performance factors related to baseline fear memory, two additional experiments were conducted. First, separate groups of mice were injected intrahippocampally with 30 μM sp600125 or vehicle before training and tested 0, 1, 3, or 6 h afterward. Two-way ANOVA with the drug and time as between-subject factors revealed that mice treated with sp600125 had intact short-term memory (STM) for the context 0, 1 or 3 h after training (time: $F_{(3,58)} = 1.9; p > 0.05$; drug: $F_{(1,58)} \leq 1$; interaction: $F_{(3,58)} = 3.2; p < 0.05$) (Fig. 8*a*). Memory-enhancing effects of JNKs inhibition were observed for the first time 6 h after fear conditioning (Scheffé test; $p < 0.05$ vs saline). These results suggest that the enhanced retention of contextual fear memory observed following JNKs inhibition was not due to improved short-term memory. Second, infusion of 30 μM sp600125 24 h before conditioning had no effect on the expression of STM 1 h after training, which was assessed at approximately the same time as LTM in the previous experiment ($F_{(1,8)} < 1$) (Fig. 8*b*). Thus, it is unlikely that the freezing enhancement observed in the LTM test was attributable to nonspecific effects of sp600125 on general activity levels (i.e., hypoactivity) that might compete with normal behavioral expression 24 h after the infusion. Taking also into account that neither sp600125 nor D-JNKI1 altered baseline retention of contextual fear by affecting foot shock sensitivity (supplemental Fig. 3*b*, available at www.jneurosci.org as supplemental material) or locomotor activity (supplemental Table 1, available at www.jneurosci.org as supplemental material), collectively, these control studies strongly favor the conclusion that JNKs inhibition enhances fear memory retention by improving memory consolidation processes.

Discussion

We have demonstrated an increase in hippocampal JNK phosphorylation and function in response to environmental stimuli that result in a strict regulation of context-dependent fear conditioning. More specifically, exposure to acute stress, acting via CRF$_2$ in the lateral septum, caused overactivation of the hippocampal JNKs pathway and a profound impairment in contextual fear conditioning. To our knowledge, these data provide the first evidence that JNK pathways play an important role during

stress-induced impairment of associative learning. In addition, contextual fear conditioning without prior stress exposure, produced a shorter, learning-dependent activation of JNKs in the CA1 subregion of the dorsal hippocampus. The selective blockade of the JNK signal under these conditions resulted in the enhancement of contextual fear. Together, these findings indicate that an optimal time window, level and type of hippocampal JNK activation is required to fine-tune contextual fear conditioning.

Our results were also first to demonstrate that different JNK isoforms differentially affect retention of contextual fear, both under baseline conditions and after exposure to stress. In particular, we found that *Jnk2*- and *Jnk3*-null mice did not show stress-induced impairment of contextual fear, while *Jnk1*-null mice displayed enhanced contextual fear under stress-free baseline conditions. Moreover, exposure to stress selectively increased hippocampal JNK3 (and presumably JNK2) activity, whereas contextual fear conditioning alone led to briefly elevated JNK1 activity. Overall, our data obtained from *Jnk* mutant mice and *in vitro* kinase assays suggest that stress-induced activation of JNK2 and JNK3 may serve as a functional switch, transitioning from a JNK1-dominated baseline state to a stress-driven phenotype (Coffey et al., 2002; Kuan et al., 2003).

In view of previous observations reporting JNKs activation in the adult CNS mainly after more invasive or longer lasting manipulations, such as chronic kindling epileptogenesis (Cole-Edwards et al., 2006), inflammation (Jara et al., 2007), kainic acid-induced excitotoxicity (Yang et al., 1997; Brecht et al., 2005), ischemic apoptosis (Kuan et al., 2003) or in models for Alzheimer's (Morishima et al., 2001; Marques et al., 2003) and Parkinson's disease (Saporito et al., 1999; Hunot et al., 2004), these acute effects of JNKs activity might seem unexpected. However, in the adult mouse brain the biological function of JNK signaling seems to be integrative, interlinking various physiological responses to stress. In support of our findings that JNKs regulate learning and memory, JNK activation has been shown to block hippocampal long-term potentiation (LTP) (Wang et al., 2004), and to be involved in low frequency stimulation-dependent long-term depression (LTD) in the dentate gyrus (Curran et al., 2003). Furthermore, JNKs are also critically involved in synaptic AMPA receptor trafficking (Zhu et al., 2005; Thomas et al., 2008) and contribute to metabotropic glutamate receptor-dependent LTD in the CA1 subregion (Li et al., 2007). These *in vitro* studies may provide a functional basis for the observed effects of JNKs on associative learning.

On the other hand, the molecular mechanisms by which JNKs contribute to neuronal plasticity underlying learning and memory are not yet clear. Potential downstream candidates regulated by JNKs during learning are numerous and diverse. Hippocampal JNKs are localized both presynaptically and postsynaptically in the hippocampus and can thus regulate synaptic vesicle proteins such as synaptotagmin-4 (Mori et al., 2008), second messenger systems such as cytosolic phospholipase A (Van Putten et al., 2001), cytoskeletal elements (i.e., MAP2, tau) (Bogoyevitch and Kobe, 2006), receptors such as AMPA (Zhu et al., 2005; Thomas et al., 2008), nuclear hormone receptors (i.e., glucocorticoid receptor) (Bruna et al., 2003), MAPK-activated protein kinase (i.e., RSK2) or transcription factors, including c-Jun, an activator protein-1 (AP-1) member, activator transcription factor (ATF-2), CREB (calcium/cAMP response element binding protein), and Elk-1 (Gupta et al., 1995, 1996). All of these substrates could potentially be targeted by JNKs during learning. It is evident that future experiments studying these biochemical

markers during learning are necessary to understand how JNKs contribute to memory formation.

Previous studies using acute restraint (Meller et al., 2003; Liu et al., 2004) or forced swim stress (Liu et al., 2004; Shen et al., 2004) have already demonstrated that exposure to acute stress leads to significant early increase of pJNKs in various brain regions (i.e., prefrontal cortex, discrete nuclei of the hypothalamus and the amygdala) (Liu et al., 2004; Shen et al., 2004), indicating that activation of the JNKs signaling pathway might be involved in initiation of stress responses. Although authors reported that two forms of acute stress yielded somewhat different regional patterns of JNKs activation, it might have been that these differences resulted from the different rodent species and stress procedures used. More importantly, such dependence of the pJNK expression pattern on the nature, and particularly, on the relative strength of acute stress (Liu et al., 2004) may explain why exposure to immediate foot shock alone did not elevate hippocampal pJNK levels in a manner similar to prolonged immobilization stress. This assumption is further corroborated by data showing selective activation of JNKs in the hippocampal formation, and in discrete nuclei of the hypothalamus and the amygdala, which represent brain regions that play a critical role in the induction and regulation of stress responses (McEwen, 2007). Moreover, that all the aforementioned studies demonstrate activation of JNKs in the hippocampus reinforces the likelihood that JNKs signaling is crucially involved in the formation of stress-associated emotional memories (Liu et al., 2004; Kim et al., 2006).

We have used anisomycin as a potent agonist of JNK (Cano et al., 1994; Hazzalin et al., 1998; Pagès et al., 2000), and with such an approach, we corroborated our findings that both exposure to acute stress and activation of septal CRF_2 receptors results in hippocampal JNK activation, which, in turns, leads to an impairment in learning. The fact that administration of a JNK inhibitor blocked the observed anisomycin-induced memory deficit indicated a dissociation between hippocampal JNK activation and anisomycin-induced translational arrest in the regulation of memory formation. In other words, our experimental setting raises the intriguing possibility that the behavioral effects elicited by anisomycin are mainly mediated via the JNK signaling pathway and not via inhibition of protein synthesis. Similarly, previous studies have demonstrated that noradrenergic agonists or antagonists, when administered intracerebroventricularly before treatment with protein synthesis inhibitor cycloheximide (Gold and Sternberg, 1978), or when applied into the basolateral amygdala (BLA) with appropriate timing relative to the abnormal BLA noradrenergic response to anisomycin, attenuate amnesia despite reduced protein synthesis (Canal et al., 2007). It is important to stress that neither these studies nor our study directly challenge the extensive evidence of changes in gene and protein expression patterns that support memory consolidation (Alberini, 2008; Klann and Sweatt, 2008). What they do show is the importance of distinguishing between problems that might be raised when interpreting the results obtained with general protein synthesis inhibitors such as anisomycin (Gold, 2008; Hernandez and Abel, 2008; Rudy, 2008) and the specific issue of the role of protein synthesis in memory formation (Alberini, 2008; Klann and Sweatt, 2008). Additional experiments with other protein synthesis inhibitors and protein kinase inhibitors are needed to determine the extent to which neurochemical actions mediate the memory deficit produced by inhibitors of global protein synthesis and by inhibitors of specific protein phosphorylation.

As previously noted, it is well established that acute stress activates hippocampal protein kinase pathways that are critically involved in baseline synaptic plasticity, learning and memory

formation (Blank et al., 2002; Revest et al., 2005). Therefore, it was not surprising that we found a rapid and temporally specific increase in pJNKs to occur in the CA1 subregion of the dorsal hippocampus after paired stimuli that supported baseline contextual fear conditioning but not after exposure to the context or foot shock alone. This finding is in accordance with observations that kinases critically involved in the formation of contextual fear do not exhibit changed activation under control conditions (Atkins et al., 1998; Fischer et al., 2002; Sindreu et al., 2007; Isosaka et al., 2008). However, the question may be raised as to why a specific increase in pJNKs was not observed in mice exposed to foot shock alone (ImS control group), since they were exposed to some context prior the conditioning box. Thus, operationally, the mice should have been subjected to contextual conditioning as well. Although still speculative, several possibilities could be considered. First, discrete salient stimuli (i.e., tactile, auditory) that occur in the close temporal contiguity with foot shock may compete for associative strength with contextual stimuli preceding foot shock delivery, thereby promoting formation of a simple (unimodal) CS-US association that does not involve the CA1 subregion of the hippocampus (Pearce and Bouton, 2001). Also, if one assumes that the mouse cage may serve as a contextual CS in the ImS control group, it is plausible that its representation was formed long before exposure to immediate foot shock. Under such a scenario, a previously established memory trace may be retrieved during fear conditioning but not subjected to further consolidation processing (Biedenkapp and Rudy, 2007), or alternatively may be stored in some other brain region that now serves as a primary source for the retrieved information (Frankland and Bontempi, 2005).

We also found that selective inhibition of JNK signaling resulted in the enhancement of baseline contextual fear. This indicated that activation of hippocampal JNKs pathways during contextual fear conditioning in the absence of stress may serve as a memory "break" protecting the subject from overconsolidating conditioned fear after a single conditioning trial. Mechanistically, studies using cultured hippocampal neurons suggest that different JNKs, p38 and ERK1/2 may either complement or oppose each other during synaptic depotentiation, LTD and LTP (J. J. Zhu et al., 2002; Y. Zhu et al., 2005), thereby contributing to the bidirectional control of synaptic strength. Moreover, it is demonstrated that the memory-promoting ERK pathway (Maher et al., 2006) and the memory-limiting JNKs pathway act in dynamic balance *in vitro*, with the ERK pathway acting to inhibit the JNK pathway and vice versa (Masuda et al., 2003; Shen et al., 2003; Tamagno et al., 2009). For example, in cortical neurons treated with D-JNKI1, ERK1, as well as MEK1, the ERK upstream kinase are strongly activated (Repici et al., 2009), suggesting that inhibition of JNKs induces compensatory activation of the ERK pathway. Whether such dynamic antagonistic crosstalk between ERK and JNKs pathways exists in the dorsal hippocampus so that the memory enhancing effect of JNK inhibition may also be due to the activation of ERK pathway and not only to the reduction of JNKs activity remains to be determined.

In conclusion, tightly regulated JNK activity is involved in memory consolidation during associative learning under stressful and baseline conditions. Characterization of the upstream induction mechanisms and downstream targets of JNK isoforms within the hippocampus will facilitate the delineation of the mechanisms by and which short-term JNKs activation transiently impairs memory, whereas prolonged JNKs activation may be a contributing factor to memory deficits and even neurodegeneration (Manning and Davis, 2003).

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