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Memory consolidation of Pavlovian fear conditioning requires nitric oxide signaling in the lateral amygdala

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Abstract

Nitric oxide (NO) has been widely implicated in synaptic plasticity and memory formation. In studies of long-term potentiation (LTP), NO is thought to serve as a 'retrograde messenger' that contributes to presynaptic aspects of LTP expression. In this study, we examined the role of NO signaling in Pavlovian fear conditioning. We first show that neuronal nitric oxide synthase is localized in the lateral nucleus of the amygdala (LA), a critical site of plasticity in fear conditioning. We next show that NO signaling is required for LTP at thalamic inputs to the LA and for the long-term consolidation of auditory fear conditioning. Collectively, the findings suggest that NO signaling is an important component of memory formation of auditory fear conditioning, possibly as a retrograde signal that participates in presynaptic aspects of plasticity in the LA.

Introduction

A considerable amount of progress has been made in defining the neurobiological substrates of Pavlovian fear conditioning, both at the systems level, and also more recently at the biochemical and/or molecular levels. Collectively, findings suggest that fear conditioning involves transmission and integration of sensory information in the lateral nucleus of the amygdala (LA), where *N*-methyl-D-aspartate receptor (NMDAR)-mediated alterations in synaptic transmission are thought to encode key aspects of the association (Blair *et al*., 2001; Schafe *et al*., 2001; Maren, 2003).

Most recent fear conditioning studies have focused their efforts on examining the role of downstream effectors of NMDARs in LA cells, including protein kinase signaling cascades that promote long-term plastic change and memory, in part, by engaging activators of transcription and translation in the nucleus (Schafe *et al*., 2001; Lin *et al*., 2003). A number of recent findings, however, suggest that synaptic plasticity and memory formation in the LA also involves a presynaptic process. Both long-term potentiation (LTP) and fear conditioning, for example, have been shown to occlude paired-pulse facilitation (PPF), a type of short-term plasticity that is widely believed to be presynaptic (Zucker, 1989), in the LA (McKernan & Shinnick-Gallagher, 1997; Huang & Kandel, 1998; Tsvetkov *et al*., 2002). These findings suggest that both synaptic plasticity and memory formation in the LA may involve both pre- and postsynaptic alterations in cell function.

As in the LA, synaptic plasticity in the hippocampus, cortex and cerebellum is known to involve both pre- and postsynaptic changes. For example, LTP in hippocampal area CA1, which is known to be induced postsynaptically via NMDAR activation (Malenka & Nicoll, 1999), is also known to involve alterations in presynaptic function, such as the enhancement of transmitter release (Hawkins *et al*., 1993; Stevens & Wang, 1994; Malgaroli *et al*., 1995; Zakharenko *et al*., 2001). This has led to the hypothesis that a postsynaptically generated message must act retrogradely at a presynaptic locus to promote LTP expression.

Of the various candidates for a retrograde messenger, nitric oxide (NO) is perhaps the best characterized (Brenman & Bredt, 1997; Hawkins, 1996). It is a soluble gas that is synthesized in the postsynaptic cell by the enzyme nitric oxide synthase (NOS; Bredt *et al*., 1990, 1991). NOS is directly activated by calmodulin at the time of LTP induction via NMDAR-mediated elevations in Ca^{2+} (Bredt & Snyder, 1992). Once generated, NO is thought to have a variety of actions. One immediate downstream effector of NO, both pre- and postsynaptically, is soluble guanylyl cyclase (Son *et al*., 1998; Denninger & Marletta, 1999; Arancio *et al*., 2001). This enzyme leads to the activation of cGMP-dependent protein kinase (PKG), which, in turn, is thought to promote mobilization of synaptic vesicles in the presynaptic terminal, leading to enhanced transmitter release (Hawkins *et al*., 1993, 1998).

Importantly, NO signaling has been implicated in a number of hippocampal- and cerebellar-dependent learning and memory processes. This has included spatial learning in rats (Chapman *et al*., 1992; Bohme *et al*., 1993; Holscher *et al*., 1996; Suzuki *et al*., 1996; Zou *et al*., 1998), eye-blink conditioning in rabbits (Chapman *et al*., 1992), and passive avoidance learning in rats (Bernabeu *et al*., 1995, 1996, 1997) and chicks (Holscher & Rose, 1992; Holscher, 1994).

In the present series of experiments, we examined the contribution of NO signaling to synaptic plasticity and auditory fear conditioning in the LA using a combination of anatomical, electrophysiological and behavioral approaches.

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Materials and methods

Subjects

Adult male Sprague–Dawley rats (Hilltop Laboratories, Scottdale, PA, USA) were housed individually in plastic Nalgene cages and maintained on a 12 : 12-h light/dark cycle. Food and water were provided *ad libitum* throughout the experiment. All procedures were approved by the New York University Animal Care and Use Committee.

Immunohistochemistry

For immunohistochemical experiments, adult male rats ($n = 6$) were deeply anesthetized with chloral hydrate (600 mg/kg, i.p.) and perfused through the heart with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Tissue sections containing the amygdala were cut on a Vibratome, rinsed in PBS and blocked in 1% bovine serum albumin (BSA). Sections designated for electron microscopy (EM) were freeze-thawed (see Farb & LeDoux, 1997, for details). Neuronal NOS immunoreactivity (nNOS-ir) was visualized using a rabbit polyclonal antisera directed against nNOS (1 : 700–2000; Zymed, San Francisco, CA, USA, Z-RNN3; Cat. no. 61-7000), the avidin-biotin/horseradish peroxidase complex (Vector Elite Kit, Burlingame, CA, USA), and 3–3′-diaminobenzidine (DAB). α-Calcium/calmodulin-dependent protein kinase II (CaMKII)-ir was visualized using a mouse monoclonal antibody directed against total αCaMKII (1 : 1000; Upstate Biotechnology, Lake Placid, NY, USA), goat anti-mouse conjugated to gold (Ultrasmall gold; Aurion, Wageningen, The Netherlands) and silver enhancement solutions (Aurion or Amersham; Arlington Heights, IL, USA) as previously described (Rodrigues *et al*., 2004).

Antisera directed against nNOS supplied from different manufacturers were used in preliminary studies. As we found NOS staining to be highly variable, we verified the specificity of these antisera at the light and EM level using nNOS knock-out mice ($n = 3$; 8 weeks old; strain B6; 129S- $NOS1^{tm1Pl}$; Jackson Laboratories, Bar Harbor, ME, USA) and corresponding controls (strain B6; 129SF2/J; $n = 3$).

Electron microscopy

Tissue sections designated for EM were processed as previously described (Farb & LeDoux, 1997; Rodrigues *et al*., 2004). In brief, tissue sections containing the amygdala were incubated in osmium tetraoxide (OsO_4)/PB (immunogold tissue, 0.05% OsO_4 for 10 min; peroxidase tissue, 1 h in 1% OsO_4). The tissue was then dehydrated and flat- and capsule-embedded in Epon. Digital micrographs were taken from each block from which ultrathin sections (70–80 nm) were cut. Sections were collected on 8–12 nickel grids and the tissue was examined on a JEOL 1200EX electron microscope. Some grids collected from immunogold tissue were briefly counterstained with lead citrate to improve contrast.

We first examined tissue that was single-labeled for nNOS to characterize the subcellular localization of nNOS-ir. A CCD camera was used to take photographs at magnifications of 30–40 000×. Next, nNOS-ir was evaluated in relation to αCaMKII-immunogold dendritic processes. αCaMKII-ir profiles were identified by the presence of at least two silver-intensified gold particles. Analysis was confined to the surface-most portion of the vibratome section because immunogold does not penetrate effectively below the tissue surface.

Drugs

In the electrophysiological and behavioral experiments, we used the selective NOS inhibitor 7-nitroindazole (7-Ni; Calbiochem, Cat. no. 483400) or the selective NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO; Tocris, Cat. no. 0772). For behavioral experiments, each drug was dissolved in 100% DMSO to a final stock concentration of 4 μg/μL. Prior to infusion into the brain, the drug was diluted 1 : 1 in ACSF, yielding a vehicle of 50% DMSO. This vehicle has been used successfully in previous studies of fear conditioning for localized drug infusions, is well tolerated and appears to be without significant behavioral effect in control animals (Schafe *et al*., 2000).

Slice electrophysiology

Electrophysiological experiments in amygdala slices were conducted as previously described (see Bauer *et al*., 2002). Briefly, male Sprague–Dawley rats (3–5 weeks old) were deeply anesthetized with halothane and the brain was rapidly removed and transferred to ice-cold ACSF containing (in mM): 115 NaCl, 3.3 KCl, 1 $MgSO_4$, 2 $CaCl_2$, 25.5 $NaHCO_3$, 1.2 NaH_2PO_4 and 25 glucose, equilibrated with 95% O_2/5% CO_2. Between four and six coronal hemisections (400 μm thick) containing the amygdala were cut and recovered in a holding chamber at 32–34 °C for 30 min, and were then allowed to return to room temperature for at least another 30 min before recording. An upright microscope equipped with infrared differential interference contrast optics (IR-DIC, Olympus) was used to perform whole-cell patch recordings under visual guidance. Glass recording electrodes were filled with (in mM): 130 K-gluconate, 0.6 EGTA, 2 $MgCl_2$, 5 KCl, 10 HEPES, 2 Mg-ATP, 0.3 Na_3-GTP (pH 7.3, 290–300 mOsm). The electrodes typically had resistances of 4–8 MΩ. All cells were allowed to remain at their resting potentials.

Stimuli (150 μs duration) were delivered through bipolar stainless steel electrodes placed in the ventral striatum, just medial to LA. This stimulating protocol activates fibers that originate, at least in part, in the auditory thalamus (Weisskopf *et al*., 1999). The stimulation intensity was kept at a minimum and adjusted for each cell (between 80 and 140 μA) to produce a reliable EPSP without also recruiting polysynaptic responses or spiking. Baseline responses were monitored at 0.1 Hz for 10 min prior to tetanus. Following baseline recording, LTP was induced by a 30-Hz tetanus (100 stimuli, given twice with a 20-s inter-train interval). This LTP induction protocol is known to produce a reliable, NMDAR-dependent LTP in the LA (Bauer *et al*., 2002). For each cell, the stimulation intensity for LTP induction was the same as that used to elicit baseline EPSPs.

Picrotoxin (50 μM; Sigma) was included in the bath in all experiments to block fast GABAergic transmission, but was not observed to produce epileptiform bursting in the LA. Drugs were made up in DMSO stock solution and diluted 1000 times into the superfusing ACSF, yielding a final concentration of 7-Ni of 30 μM and of c-PTIO of 25 μM. Pilot experiments determined that these concentrations of 7-Ni and c-PTIO effectively impaired LTP in the LA without having significant effect on the slope and amplitude of evoked EPSPs alone. In each experiment, drugs were bath applied approximately 10–20 min before recordings began, making the total time between drug application and LTP induction approximately 20–30 min for each cell. Each slice was recorded from only once, and thus control and drug cells were always from different slices. Typically, both vehicle and drug conditions were run from separate slices from the same animal on the same day.

In all experiments, the initial slope of the EPSP was measured, and LTP for each time point was expressed as a percentage of the

pre-induction baseline. For analysis, EPSP slopes during the last 10 min of the recording session were compared with the last 5 min of the baseline period using Student's *t*-tests.

Behavioral procedures

Behavioral procedures were conducted as previously described (Schafe & LeDoux, 2000; Schafe *et al*., 2000). Under Nembutal anesthesia (45 mg/kg; i.p.), rats were first implanted bilaterally with 26-gauge stainless steel guide cannulas aimed at the LA (Schafe & LeDoux, 2000). The guide cannulas were fixed to screws in the skull using a mixture of acrylic and dental cement, and a 33-gauge dummy cannula was inserted into each guide to prevent clogging. All surgical procedures were conducted in accordance with the NIH *Guide for the Care and Use of Experimental Animals* and were approved by the New York University Animal Care and Use Committee. Rats were given at least 5 days to recover prior to experimental procedures.

On the day prior to conditioning, rats were habituated to the conditioning chamber for a minimum of 10–15 min and to dummy cannula removal. On the conditioning day, rats were given an intra-LA infusion of either 0.5 μL 50% DMSO (vehicle) or one of several doses of 7-Ni in 50% DMSO (1.0, 0.1 or 0.01 μg per side in 0.5 μL; 0.25 μL/min) or c-PTIO (1 μg per side in 50% DMSO). Injectors remained in the cannulas for 1 min after drug infusion to allow diffusion of the drug from the tip. Thirty minutes following drug infusions, rats were trained with either one or five conditioning trials. Single-trial fear conditioning consisted of a 30-s, 5-kHz, 80-dB tone that co-terminated with a 1.0-s, 1.5-mA foot shock. Multiple-trial fear conditioning consisted of a 20-s, 5-kHz, 80-dB tone that co-terminated with a 0.5-s, 1.0-mA foot shock [inter-trial interval (ITI) = 120 s]. Rats were allowed to explore the chamber for ~120 s before conditioning began.

Testing for conditioned fear responses (freezing) in rats conditioned with a single trial were conducted 24 h following conditioning. For this test, rats were placed in a distinctive environment (see Schafe & LeDoux, 2000, for details) and exposed to eight conditioned stimulus (CS) tones after a 120-s delay (5 kHz, 80 dB, 30 s; ITI = 120 s), respectively. For rats conditioned with five trials, testing occurred 1, 3, 6 (three tones each test) and 24 h (ten tones) later (5 kHz, 80 dB, 20 s). Total time freezing during the CS presentations were scored for each rat, and this number was expressed as a percentage of the total CS presentation time. All data were analysed with ANOVA and Duncan's *post-hoc t*-tests. Differences were considered significant at $P < 0.05$.

For reactivation and reconsolidation experiments, rats were conditioned with five pairings as before. Twenty-four hours later, they were infused with either vehicle (50% DMSO; 0.5 μL) or the highest dose of 7-Ni (1 μg per side) or c-PTIO (1 μg per side). Thirty minutes later, they were exposed to a single test (reactivation) trial (5 kHz, 80 dB, 20 s). To assess memory retention following infusion and reactivation, rats were again tested at 1 and 24 h after the reactivation session (three and eight tones, respectively).

At the end of each behavioral experiment, rats were killed by an overdose of chloral hydrate (600 mg/kg) and perfused with 10% buffered formalin. Nissl staining and light microscopy were used to verify the location of the cannula tips within the amygdala.

Results

Anatomical localization of nNOS in the LA

Previous studies have shown that nNOS is widely distributed in cerebellar, cortical and subcortical regions, including the hippocampus, hypothalamus and striatum (Bredt *et al*., 1990, 1991; Rodrigo *et al*., 1994). Early studies failed to find evidence of nNOS in CA1 pyramidal cells or dendrites, which proved troubling for the hypothesis that NO could serve as a retrograde messenger in stratum radiatum (Bredt *et al*., 1991). Later studies, however, using more gentle fixation methods did find evidence of nNOS in CA1 pyramidal cells, their apical dendrites and spines (Wendland *et al*., 1994; Burette *et al*., 2002). In this first series of experiments, we have therefore systematically examined the distribution of nNOS in the LA with respect to its localization in excitatory cells and spines. Our first goal was to examine the distribution of nNOS in the LA using light and electron microscopy. We next combined double labeling of nNOS and αCaMKII, a marker of excitatory neurons (McDonald *et al*., 2002), with EM to determine whether nNOS was localized in excitatory projection neurons in the LA, or whether it was confined to interneurons.

Localization of nNOS in the LA

The light-level distribution of nNOS within the LA is shown in Fig. 1a. Consistent with previous reports (Rodrigo *et al*., 1994), many labeled cells were observed scattered across the LA, and the number and intensity of labeled cells was similar across the different LA subregions. Staining was also observed in the basal nucleus of the amygdala, where labeling in the magnocellular division was frequently lighter than staining in the parvicellular division. Scattered cells were also observed in the intercalated cell masses, and in the amygdala–striatal transition zone. Staining was consistently absent or sparse within the central nucleus of the amygdala.

Higher magnification optics revealed that many nNOS-ir cells in the LA are multipolar, with several dendrites emanating from their cell bodies, morphological characteristics that are common to interneurons (Fig. 1b). Many punctate processes were also seen. Using EM, nNOS-ir was seen in somata, large and small dendrites, dendritic spines and axon terminals. Endothelial cells were occasionally stained, but glial labeling was not observed. Many nNOS-ir somata had the morphological features of interneurons, e.g. invaginated nuclei and abundant cytoplasm (Carlsen, 1988). In other cases, however, nNOS-ir cells were observed to have a thin rim of cytoplasm and their nuclei lacked folds, features associated with excitatory projection cells. Sometimes, nNOS-ir was observed in the spine but not the dendrite to which it belonged. nNOS-ir terminals were also observed to form symmetric and asymmetric synaptic junctions onto dendrites, spines and somata. Furthermore, some nNOS-ir terminals formed synaptic associations with NOS-ir-labeled dendrites.

nNOS and αCaMKII dual labeling

To examine further the distribution of nNOS in the LA at the EM level, we combined nNOS labeling with that of αCaMKII, which has been shown in previous studies to be a reliable marker of excitatory neurons (McDonald *et al*., 2002). To perform dual immunolabeling, we used peroxidase methods for nNOS and immunogold for αCaMKII (Rodrigues *et al*., 2004). By lower-power EM, nNOS and αCaMKII staining appeared largely segregated into two different populations of cells. At higher magnification, however, dual label was also observed in large and small dendrites. Dual labeled dendritic spines were also observed (Fig. 1c). This latter finding provides the first evidence that nNOS is anatomically localized in spines of LA pyramidal neurons, and supports the possibility that retrograde release of NO from LA spines may promote presynaptic aspects of plasticity and memory formation.

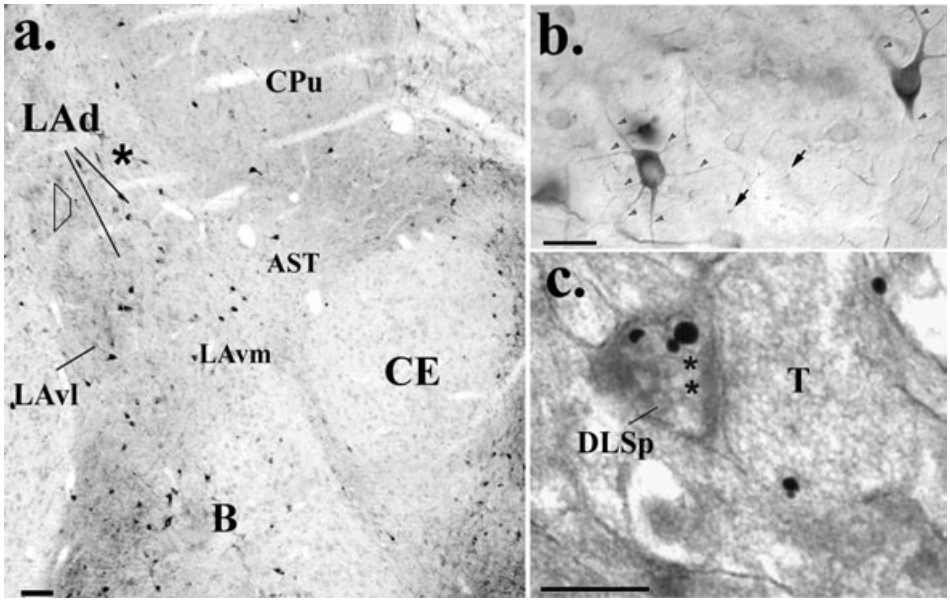

FIG. 1. Localization of nNOS in the LA. (a) Low-power micrograph showing the distribution of nNOS-ir cells in the LA and adjacent regions. The trapezoid depicts the region that was sampled for EM analysis. The asterisk corresponds to area shown at higher magnification in (b). (b) Higher-power Nomarski optics reveal labeled nNOS-ir cells in the LA, their dendrites (arrowheads) and punctuate processes (arrows). (c) A terminal (T) forms an asymmetric synapse (asterisks) onto an nNOS- and αCaMKII-ir dually labeled spine (DLSp). Scale bars, 100 μm (a), 50 μm (b) and 250 nm (c).

Bath application of an inhibitor of NOS or of a scavenger of NO impairs LTP at thalamic inputs to the LA

Previous studies using both pharmacological and molecular–genetic methods have shown that nNOS is required for synaptic plasticity, including LTP and LTD, in the hippocampus, cortex and cerebellum. Pharmacological inhibition, for example, of NOS activation impairs LTP in hippocampal area CA1 (Zhuo *et al*., 1994; Doyle *et al*., 1996; Son *et al*., 1998; Lu *et al*., 1999; Monfort *et al*., 2002). Conversely, bath application of exogenous NO combined with weak tetanic stimulation, which would not produce LTP alone, induces long-lasting LTP (Zhuo *et al*., 1994; Son *et al*., 1998; Lu *et al*., 1999; Lu & Hawkins, 2002). Inhibition of NOS activity is equally effective at impairing LTP whether the NOS inhibitor is injected directly into the postsynaptic cell or perfused over the entire slice, suggesting that the critical activation of NOS occurs postsynaptically (Schuman & Madison, 1991; Arancio *et al*., 1996; Ko & Kelly, 1999). However, NO is thought to act presynaptically, at least in part, because bath application of membrane-impermeable scavengers of NO also impairs LTP in area CA1 (Schuman & Madison, 1991; Haley *et al*., 1992; Ko & Kelly, 1999).

In this second series of experiments, we used *in vitro* recording methods to examine the role of NO signaling in LTP at thalamic input synapses to the LA. To that end, we used two distinct pharmacological compounds: the NOS inhibitor 7-Ni (30 μM) and the selective NO scavenger c-PTIO (25 μM), which is reportedly membrane-impermeable (Ko & Kelly, 1999). 7-Ni or c-PTIO were applied to amygdala slices prior to delivering a 30-Hz tetanus, a type of LTP induction protocol that, in the LA, is known to be NMDAR-dependent (Bauer *et al*., 2002). In these experiments, we stimulated fibers coursing through the ventral striatum just medial to the LA that contain, in part, projections from the auditory thalamus (Fig. 2a; Weisskopf *et al*., 1999). Furthermore, to evaluate the effect of LTP on PPF in this pathway, we examined PPF in control cells before, 30 and 60 min after LTP induction. At each time point, cells received 8–10 paired pulses separated by 10 s. The interpulse interval was 50 ms.

Results showed that both 7-Ni and c-PTIO impaired LTP at thalamic inputs to the LA. In the 7-Ni experiment, the control group showed 146.8 ± 6.8% potentiation, which was significantly different from baseline ($t_7 = 8.35$, $P < 0.01$). Cells treated with 30 μM 7-Ni showed 95 ± 6.4% potentiation, which was not significantly different from baseline ($t_7 = 0.96$, $P > 0.05$), but was significantly different from controls ($t_{14} = 5.47$, $P < 0.01$; Fig. 2d). In the c-PTIO experiment, the control group showed 166.9 ± 18% potentiation, which was significantly different from baseline ($t_6 = 3.35$, $P < 0.05$). Cells treated with 25 μM c-PTIO showed 110 ± 13% potentiation, which was not significantly different from baseline ($t_6 = 0.76$, $P > 0.05$), but was significantly different from controls ($t_{12} = 2.52$, $P < 0.05$; Fig. 2e). In addition, LTP induction in control cells was shown to lead to a significant occlusion in PPF at thalamic inputs to the LA (Fig. 2b). Before LTP induction, PPF was 1.34 ± 0.09 (a 34% increase in the second pulse relative to the first). At 30 min after LTP induction, PPF was 1.01 ± 0.06, which was significantly different from PPF before LTP ($t_{11} = 3.52$, $P < 0.01$). Similarly, at 60 min after LTP induction, PPF was 1.00 ± 0.05, which was also significantly different from PPF before LTP ($t_{11} = 3.85$, $P < 0.01$). To examine the relationship between PPF and LTP further, we performed a regression analysis on the amount of LTP and the percentage of occlusion of PPF in each cell 60 min after LTP induction. The findings revealed a significant correlation between the two measures ($r = 0.60$, $F_{1,10} = 5.61$, $P < 0.05$; Fig. 2c). Collectively, this pattern of findings suggests that LTP at thalamic inputs to the LA has a presynaptic component (Zucker, 1989).

To determine whether either drug affected baseline synaptic transmission in the LA, we examined the effects of bath application of 30 μM 7-Ni ($n = 6$ cells) or 25 μM c-PTIO ($n = 5$ cells) on the initial slope and amplitude of EPSPs induced by thalamic stimulation (data not shown). Each drug was added to the bath after a baseline period of at least 10 min. An analysis of the initial slope of the EPSPs 25–30 min after 7-Ni application showed no significant effect of the drug (101.6 ± 7% of baseline), which was not significantly different from baseline ($t_5 = 0.17$, $P > 0.05$). Bath application of c-PTIO also

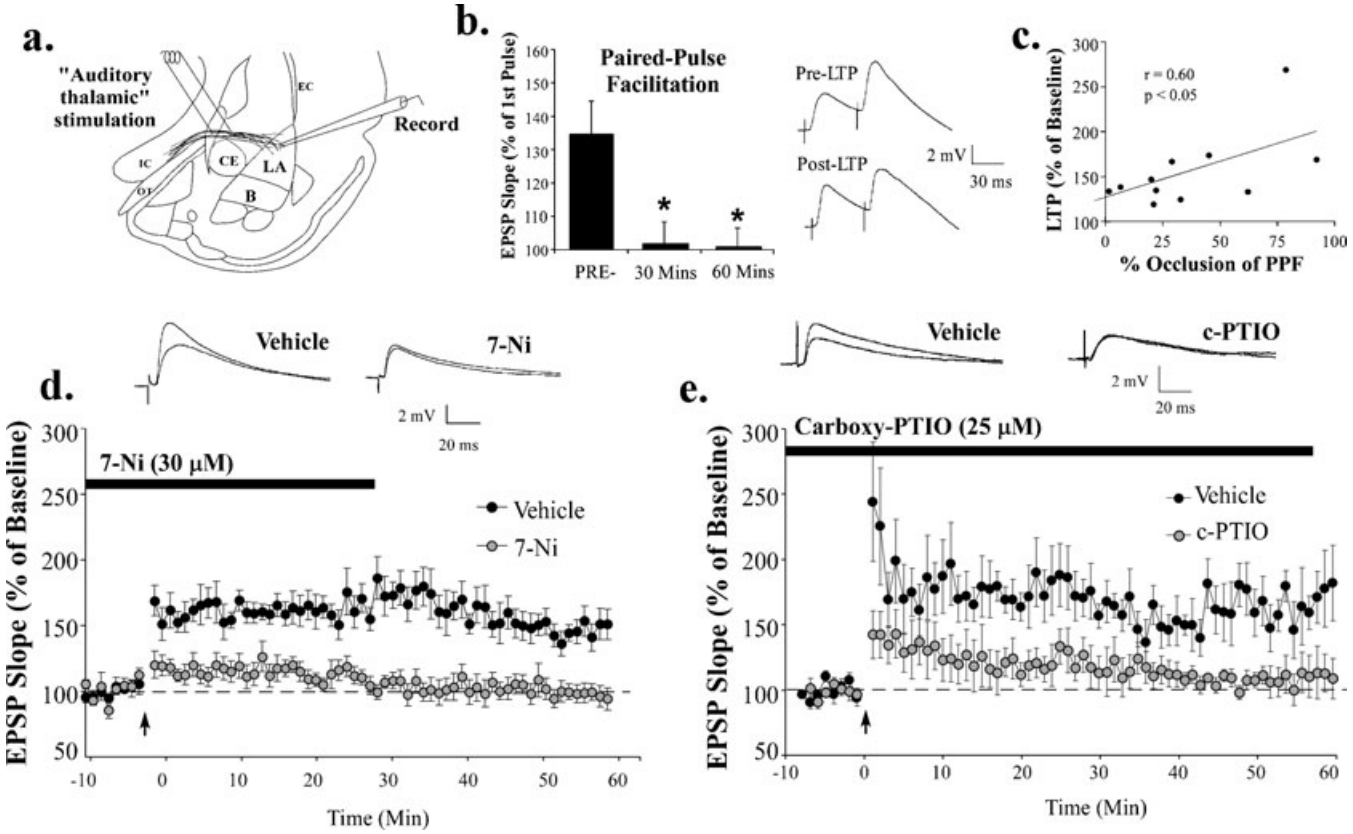

FIG. 2. LTP at thalamic inputs to the LA requires NO signaling. (a) Schematic of the amygdala slice preparation, showing placement of stimulating and recording electrodes. Recordings were made just below the site of termination of auditory thalamic fibers terminating in the LA. IC, internal capsule; OT, optic tract; EC, external capsule. (b) Paired-pulse facilitation at thalamic inputs to the LA before (PRE-), 30 min and 60 min after LTP induction (ten at each time point; $n = 12$ cells). Each cell was given two stimulations that were spaced 50 ms apart, and the second pulse was expressed as a percentage of the first pulse. Representative traces are shown on the left. (c) Correlation between LTP (% of baseline) and percentage occlusion of PPF. (d) Mean (± SEM) percentage EPSP slope (relative to baseline) in cells treated with vehicle ($n = 8$; black circles) or 30 μM 7-Ni ($n = 8$; grey circles). Traces from an individual experiment before and 50 min after tetanic stimulation are shown in the inset. (e) Mean (± SEM) percentage EPSP slope (relative to baseline) in cells treated with vehicle ($n = 7$; black circles) or 25 μM c-PTIO ($n = 7$; grey circles). Traces from an individual experiment before and 50 min after tetanic stimulation are shown in the inset.

failed to affect significantly the slope of EPSPs (96.7 ± 6.8% of baseline, $t_4 = 0.04$, $P > 0.05$). Thus, 7-Ni and c-PTIO impair LTP at thalamic inputs to the LA, without affecting baseline transmission alone.

Inhibition of NO signaling in the LA impairs memory formation of auditory Pavlovian fear conditioning

To examine the role of NO signaling in Pavlovian fear conditioning, we gave rats intra-amygdala infusions of either 7-Ni or c-PTIO prior to auditory fear conditioning. In preliminary experiments, we determined the optimal dose of 7-Ni that was necessary to impair fear conditioning (Fig. 3a). Rats in that experiment were given intra-amygdala infusions of 50% DMSO vehicle or multiple doses of 7-Ni (0.01, 0.1 or 1.0 μg per side) 30 min prior to a single tone–shock pairing. Retention of auditory fear conditioning was assessed the following day (~24 h later). Findings revealed that 7-Ni dose-dependently impaired retention of auditory fear conditioning ($F_{3,32} = 5.08$, $P < 0.05$). *Post-hoc* *t*-tests showed that both the 0.1- and 1.0-μg doses of 7-Ni significantly impaired retention relative to vehicle controls and the low dose ($P < 0.05$), but did not differ from each other ($P > 0.05$).

To further examine the effect of NO signaling on memory formation of auditory fear conditioning, rats were given intra-amygdala infusions of vehicle, the highest dose of 7-Ni or c-PTIO (1 μg) 30 min prior to five tone–shock pairings. Rats were then tested for retention of auditory fear conditioning 1, 3, 6 and 24 h after training (see Fig. 3b). The findings revealed that the level of post-shock freezing obtained during training was not affected by either 7-Ni or c-PTIO (Fig. 3c). The ANOVA (drug by trials) showed a significant effect of trials ($F_{4,104} = 47.28$, $P < 0.01$), and a significant drug by trials interaction ($F_{8,104} = 2.52$, $P < 0.05$). Duncan's *t*-test showed that relative to vehicle controls there was a modest yet significant reduction in post-shock freezing in 7-Ni- and c-PTIO-treated rats after the first trial ($P < 0.05$) and in c-PTIO-treated rats on the second trial ($P < 0.05$), but no differences were observed on subsequent trials ($P > 0.05$). Consistent with that finding, auditory fear memory tested 1 or 3 h later was found to be intact (Fig. 3d and e). The ANOVA (drug by trials) for fear memory at 1 h showed a non-significant effect of drug ($F_{2,26} = 1.41$), trials ($F_{2,52} = 0.60$) and for the interaction ($F_{4,52} = 0.41$). The ANOVA for fear memory at 3 h showed a significant effect of trials ($F_{2,52} = 3.2$, $P < 0.05$), but the effects for drug ($F_{2,26} = 1.28$) and the interaction ($F_{4,52} = 0.40$) were not significant. At 6 h following conditioning, however, differences began to emerge in the group treated with the highest doses of 7-Ni or c-PTIO (Fig. 3f). The ANOVA for fear memory at 6 h revealed a significant effect of dose ($F_{2,26} = 4.25$, $P < 0.05$), and a significant effect of trials ($F_{2,52} = 3.46$, $P < 0.05$). The interaction was not

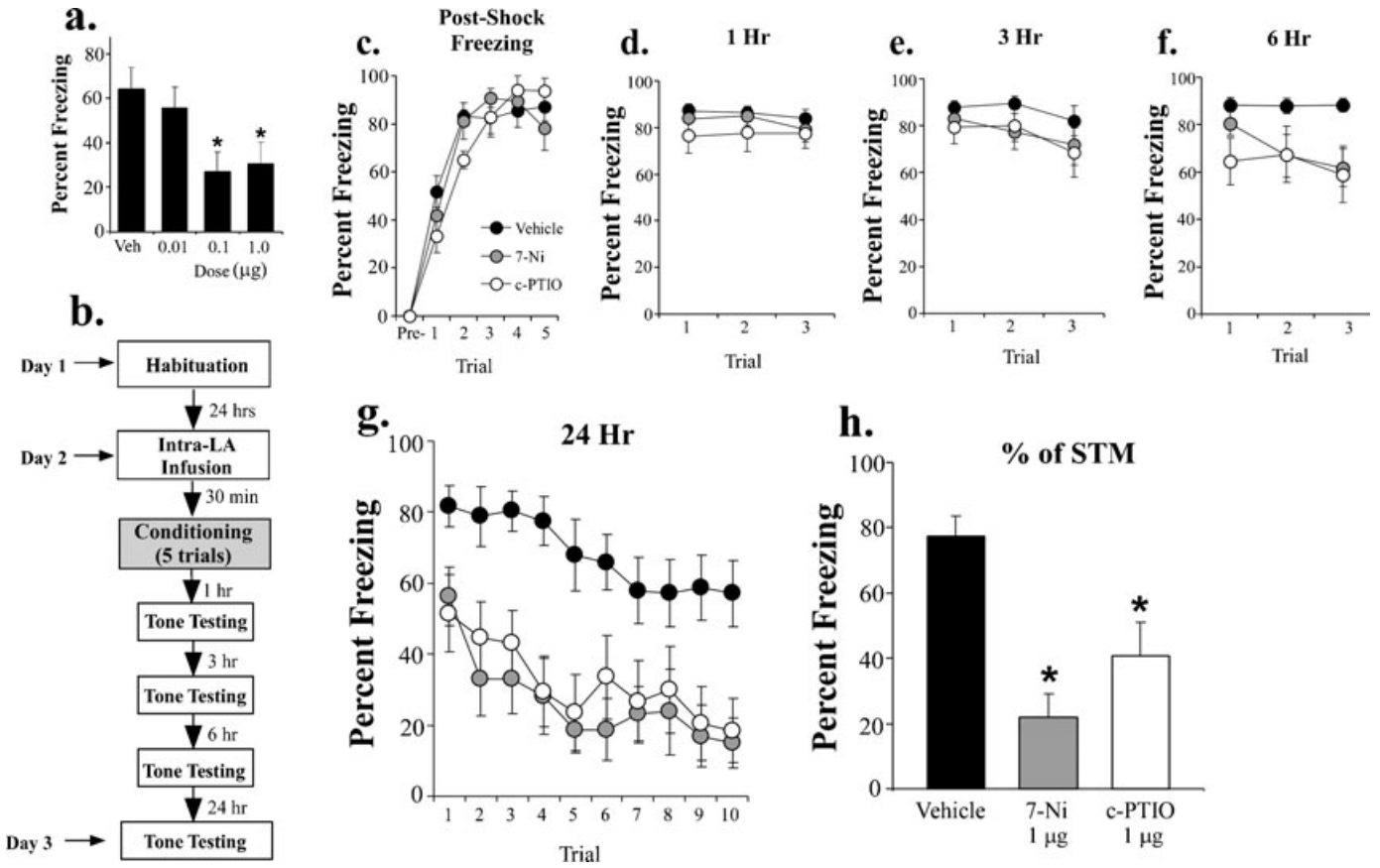

FIG. 3. Auditory fear conditioning requires NO signaling in the LA. (a) Dose–response for 7-Ni. Rats were given intra-LA infusion of vehicle ($n = 9$) or different doses of 7-Ni (0.01, 0.1 or 1.0 μg per side; $n = 9$ each) 30 min prior to single-trial fear conditioning and tested for retention of auditory fear conditioning 24 h later. *$P < 0.05$, relative to the vehicle group. (b) Schematic of the behavioral protocol. Rats were given intra-LA infusion of vehicle, the highest dose of 7-Ni (1 μg) or 1 μg c-PTIO 30 min before five tone–shock pairings. They were then tested for retention of auditory fear conditioning at 1, 3, 6 and 24 h. (c) Mean (± SEM) post-shock freezing between conditioning trials in rats given intra-LA infusions of 50% DMSO (vehicle; $n = 12$), 1.0 μg 7-Ni ($n = 7$) or 1.0 μg c-PTIO ($n = 10$). (d) Mean (± SEM) auditory fear memory assessed at 1 h following conditioning in the rats from (c). (e) Mean (± SEM) auditory fear memory assessed at 3 h following conditioning in the rats from (c). (f) Mean (± SEM) auditory fear memory assessed at 6 h following conditioning in the rats from (c). (g) Mean (± SEM) auditory fear memory assessed at 24 h following conditioning in the rats from (c). (h) Percentage of STM in each group. Each rat's 24-h memory score is expressed as a percentage of its 1-h memory score. *$P < 0.05$ relative to vehicle.

significant ($F_{4,52} = 2.22$). This difference became more pronounced the following day (Fig. 3g). The ANOVA for long-term memory (LTM) scores revealed a significant effect for group ($F_{2,26} = 10.70$, $P < 0.01$) and trials ($F_{9,234} = 9.67$, $P < 0.01$), but not for the interaction ($F_{18,234} = 0.80$). Duncan's *post-hoc* *t*-tests revealed that significant differences existed between vehicle controls and rats infused with either 7-Ni or c-PTIO on every trial but the second ($P < 0.05$). Thus, intra-LA administration of the NOS inhibitor 7-Ni or the selective NO scavenger c-PTIO impairs memory formation of auditory fear conditioning. The effect, however, is specific to LTM; short-term memory (STM), measured within 1 h after training, is not affected. This pattern of findings is consistent with the interpretation that NO signaling in the LA is required for the long-term retention, or consolidation, of fear memories rather than the initial acquisition of conditioned fear.

Histological verification of cannula placements for the first two behavioral experiments can be viewed below in Fig. 5a and b (see figure legend for details). Cannula tips were observed to lie throughout the LA at various rostro-caudal levels. Only rats with cannula tips at or within the boundaries of the LA were included in the data analysis. In these experiments, we used a total of 59 rats, of which three were removed from the analysis for improper cannula placement.

Inhibition of NO signaling in the LA has no effect on retrieval or reconsolidation of fear memory

Our initial behavioral experiments showed that NO signaling in the LA was critical for memory formation of auditory fear conditioning. Recent studies, however, have shown that protein synthesis in the amygdala is required to maintain a fear memory once it is retrieved (Nader *et al.*, 2000), a process that has become known as 'reconsolidation'. In this final series of experiments, we examined the role of NO signaling in retrieval and reconsolidation of auditory fear conditioning.

The protocol for the reconsolidation experiment is shown in Fig. 4a. Rats were first habituated and conditioned with five tone–shock pairings as before. Twenty-four hours after conditioning, rats received intra-amygdala infusions of vehicle (50% DMSO; 0.5 μL), the highest dose of 7-Ni (1 μg) or c-PTIO (1 μg) 30 min prior to a single reactivation (retrieval) test. To examine the effects of blockade of NO signaling on subsequent retention of conditioned fear, rats were then tested for retention of auditory fear conditioning at 1 and 24 h after the reactivation trial. To maintain consistency with previous terminology (Nader *et al.*, 2000), we refer here to the 1-h test as post-reactivation STM (PR-STM) and the 24-h test as post-reactivation LTM (PR-LTM).

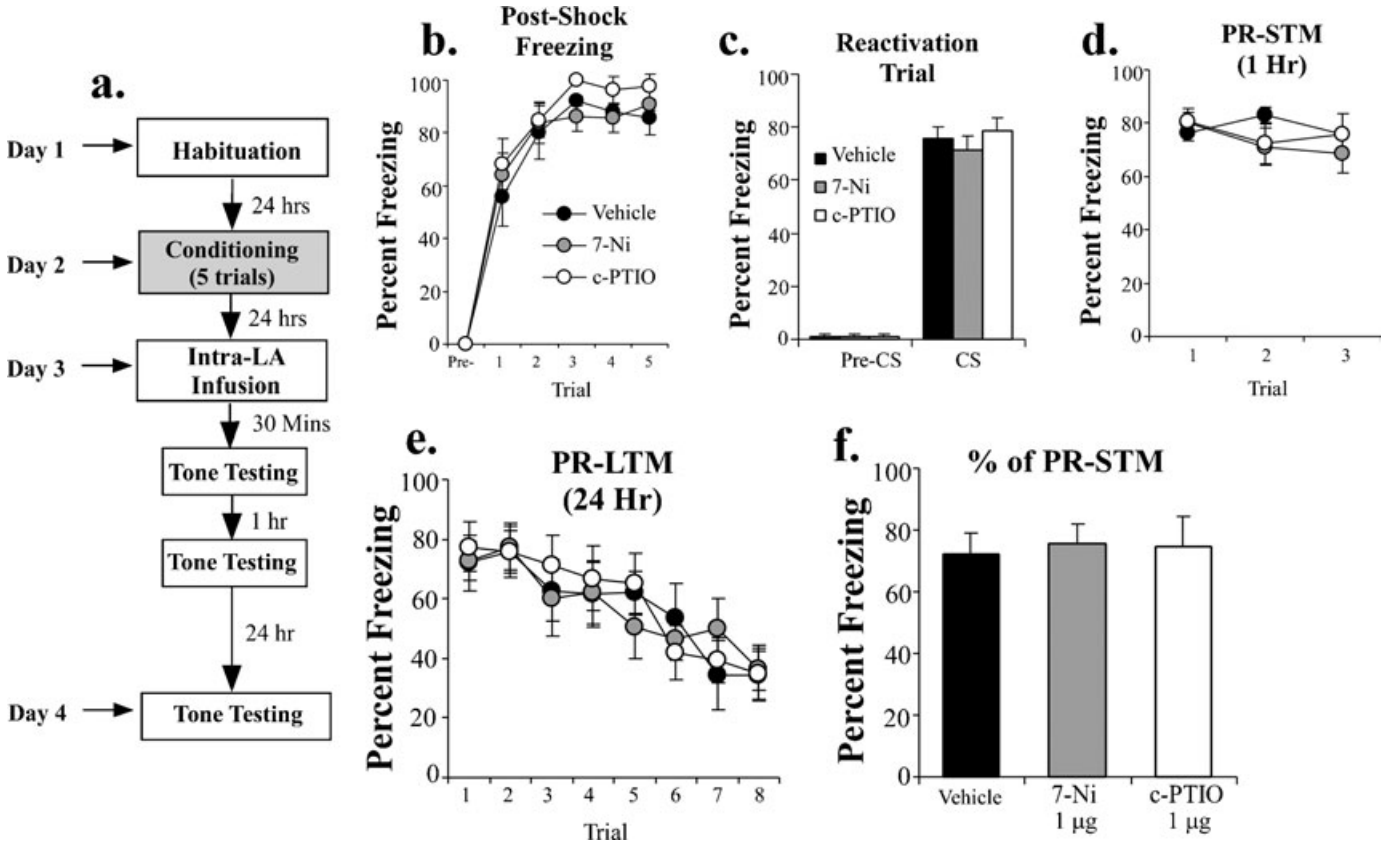

FIG. 4. Retrieval and reconsolidation of auditory fear conditioning do not require NO signaling in the LA. (a) Schematic of reconsolidation behavioral protocol. Rats were trained with five tone–shock pairings. Twenty-four hours later they received an intra-LA infusion of vehicle ($n = 7$), the highest dose of 7-Ni (1 μg; $n = 8$) or 1 μg c-PTIO ($n = 8$) 30 min before a single tone retention (reactivation) test. They were then tested for retention of auditory fear conditioning at 1 and 24 h after the reactivation test. (b) Mean (± SEM) post-shock freezing between conditioning trials. (c) Mean (± SEM) auditory fear memory assessed during the reactivation trial ~24 h after training. (d) Mean (± SEM) auditory fear memory assessed at 1 h following the reactivation trial ('post-reactivation short-term memory', PR-STM). (e) Mean (± SEM) auditory fear memory assessed at 24 h following the reactivation trial ('post-reactivation long-term memory', PR-LTM). (F) Percentage of PR-STM in each group. Each rat's 24-h PR-LTM score is expressed as a percentage of its 1-h PR-STM score.

The findings revealed that neither 7-Ni nor c-PTIO impaired retrieval or reconsolidation of auditory fear conditioning. Rats in each group conditioned normally, as evidenced by post-shock freezing (Fig. 4B), and each group showed equivalent retrieval of the fear memory during the reactivation trial on the following day after infusion of either drug (Fig. 4c). The ANOVA for post-shock freezing scores showed only a significant effect for trials ($F_{4,80} = 17.09$, $P < 0.01$). The effects for drug ($F_{2,20} = 1.25$) and the interaction ($F_{8,80} = 0.42$) were not significant. The ANOVA for the reactivation trial also showed a significant effect for trials (freezing during the pre-CS period vs. freezing during the CS; $F_{1,20} = 711.28$, $P < 0.01$), but the effect of drug ($F_{2,20} = 0.62$) or the interaction ($F_{2,20} = 0.62$) were not significant. Thus, NO signaling in the LA is not required for normal expression or retrieval of auditory fear conditioning. Furthermore, fear memory was intact on subsequent retrieval tests. The ANOVA for the PR-STM test revealed no effects for the drug ($F_{2,20} = 0.21$), for trials ($F_{2,40} = 1.39$) or for the interaction ($F_{4,40} = 1.18$). The ANOVA for the PR-LTM test showed only an effect of trials ($F_{7,140} = 16.81$, $P < 0.01$). The effect for drug ($F_{2,20} = 0.02$) and the interaction ($F_{14,140} = 0.71$) were not significant.

Histological verification of cannula placements for the reconsolidation experiment is given in Fig. 5c (see figure legend for details). As before, only rats with cannula tips at or within the boundaries of the LA were included in the data analysis. In these experiments, we used a total of 23 rats, all of which had correct placements.

Discussion

The present study examined the contribution of NO signaling to fear conditioning. In other memory systems, NO has been hypothesized to serve as a retrograde messenger that engages presynaptic aspects of plasticity. Using a multifaceted approach, we have shown that nNOS, the enzyme which generates NO, is localized in LA neurons, and that NO signaling is required for synaptic plasticity and memory formation of auditory fear conditioning in the LA.

Nearly all of the studies that have examined the cellular mechanisms underlying fear conditioning have focused on postsynaptically mediated events in the LA, including the roles of membrane receptors and channels (NMDARs, L-type VGCCs), protein kinases (αCaMKII, Akt, PKA, MAPK), transcription factors (CREB), and RNA and protein synthesis (Miserendino *et al.*, 1990; Kim *et al.*, 1991; Campeau *et al.*, 1992; Bailey *et al.*, 1999; Goosens *et al.*, 2000; Schafe & LeDoux, 2000; Schafe *et al.*, 2000; Josselyn *et al.*, 2001; Lin *et al.*, 2001; Rodrigues *et al.*, 2001; Bauer *et al.*, 2002; Rodrigues *et al.*, 2004). There is growing evidence, however, that suggests that synaptic plasticity and memory formation in the LA involves a presynaptic

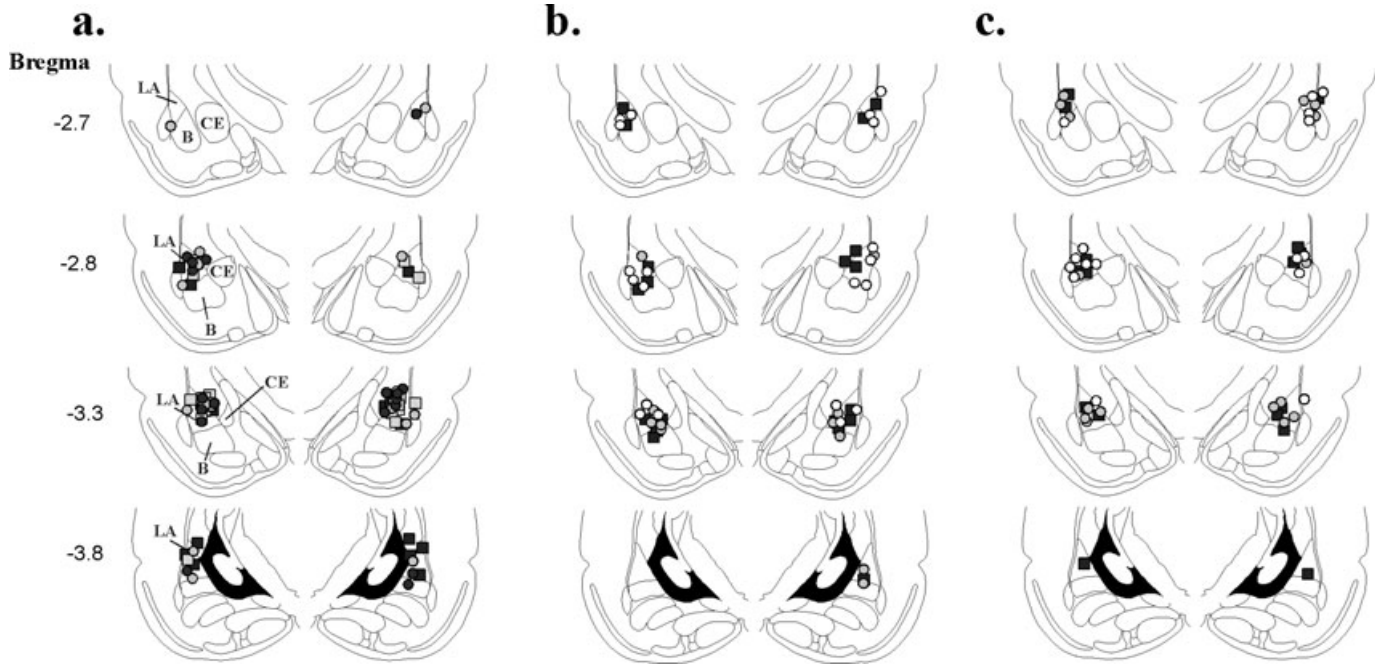

FIG. 5. Histological verification of cannula placements. (a) Cannula tip placements from rats trained with a single pairing and tested for LTM 24 h later (see Fig. 3a). Rats were infused with ACSF (black squares), 0.01 μg 7-Ni (grey squares), 0.1 μg 7-Ni (grey circles) or 1.0 μg 7-Ni (black circles). (b) Cannula tip placements from rats in Fig. 3c–h. Rats were infused with ACSF (black squares), 1.0 μg 7-Ni (grey circles) or 1.0 μg c-PTIO (white circles). (c) Cannula tip placements from rats in the reconsolidation experiment (see Fig. 4). Rats were infused with ACSF (black squares), 1.0 μg 7-Ni (grey circles) or 1.0 μg c-PTIO (white circles). Panels adapted from Paxinos & Watson (1997).

process. Huang & Kandel (1998), for example, observed that LTP occludes PPF at cortical inputs to the LA, a type of short-term plasticity that is largely believed to be presynaptic (Zucker, 1989). They also showed that bath application, but not postsynaptic injection, of a protein kinase A (PKA) inhibitor impaired LTP in LA neurons (Huang & Kandel, 1998). Conversely, bath application of forskolin, a PKA activator, in the presence of antagonists of postsynaptic NMDAR and AMPAR receptors, induced LTP and occluded PPF at cortical inputs (Huang & Kandel, 1998), suggesting that the presynaptic component of LTP in this pathway is PKA-dependent. More recently, Tsvetkov *et al*. (2002) showed that auditory fear conditioning itself, in addition to LTP, occludes PPF at cortical inputs to LA, a finding consistent with that of McKernan & Shinnick-Gallagher (1997). Collectively, these findings suggest that both synaptic plasticity and memory formation in the LA involve a presynaptic component in addition to a postsynaptic component, and that a complete understanding of the cellular and biochemical basis of fear conditioning will require attention to both of these processes.

Anatomical localization of nNOS in the LA

The anatomical basis for a role of NO in hippocampal synaptic plasticity has been controversial. The conclusions of early studies pointing to a role for NO signaling in LTP in area CA1 were tempered by anatomical studies that failed to find evidence of nNOS in CA1 pyramidal cells (Bredt *et al*., 1991). Later studies, however, using more gentle fixation protocols did find evidence of nNOS in CA1 pyramidal cell dendrites and spines (Wendland *et al*., 1994; Burette *et al*., 2002). Consistent with both sets of findings, our anatomical experiments revealed that the most abundant nNOS label in the LA appeared to be confined to medium to large multipolar cells that have the morphological characteristics of interneurons. However, at the EM level, we also observed nNOS label in cells that had the characteristics of pyramidal cells. Furthermore, our double-label EM analysis showed that at least in some cases nNOS was co-localized in LA dendrites and spines with αCaMKII, a marker of excitatory neurons. Although we were able to find fewer examples of dual labeled NOS and αCaMKII processes, two methodological issues should be kept in mind when considering these findings. First, because immunogold does not adequately penetrate below the tissue surface, our analysis of αCaMKII-ir synapses was restricted to the surface-most portion of the tissue section, which constrains our sample considerably. Second, as in the hippocampus, it remains possible that the use of the stronger fixative that is required for αCaMKII immunoreactivity and EM analysis reduced our ability to detect NOS-ir in excitatory cells and spines. These considerations aside, our anatomical observations provide at least partial support for the possibility that NO is generated in spines of LA pyramidal cells during synaptic plasticity and fear conditioning and serves as a retrograde messenger to engage presynaptic aspects of plasticity.

NO signaling and amygdala LTP

The NO–cGMP–PKG signaling pathway has been widely implicated in synaptic plasticity in the hippocampus and cerebellum (Zhuo *et al*., 1994; Doyle *et al*., 1996; Son *et al*., 1998; Lu *et al*., 1999; Monfort *et al*., 2002), although its exact role in the hippocampus has remained controversial. Although NO signaling has clearly been implicated in CA1 in many studies, others have observed that the effects of NOS inhibitors are sensitive to temperature and age (Williams *et al*., 1993) or the intensity of the LTP induction protocol (Haley *et al*., 1993). In our electrophysiological experiments, we examined the role of NO signaling in amygdala LTP using an

NMDAR-dependent 30-Hz tetanus protocol (Bauer *et al*., 2002). Our findings revealed that this induction protocol occluded PPF at thalamic input synapses to the LA, suggesting that LTP at these synapses has a presynaptic component. In addition, bath application of either the NOS inhibitor 7-Ni or the NO scavenger c-PTIO effectively impaired LTP at thalamic inputs (Fig. 2), without having effect on baseline EPSPs alone. Given that c-PTIO is reportedly membrane-impermeable (Ko & Kelly, 1999), these findings strongly imply that both NOS activation and extracellular release of NO is required for synaptic plasticity in the LA. Although we contend that our PPF and LTP deficits were observed at 'thalamic' inputs to the LA, it should be kept in mind that this conclusion is based on observations that our site of stimulation, the ventral striatum, contains fibers that originate, at least in part, in the auditory thalamus (Weisskopf *et al*., 1999). Additional *in vivo* experiments utilizing actual stimulation of the auditory thalamus will be required to make definitive statements about the role of NO signaling at thalamic input synapses to the LA.

Our electrophysiological findings stand in contrast to at least one early study that failed to find a role for NO signaling at cortical inputs to the LA (Watanabe *et al*., 1995). Thus, although that study used only a single dose and a different LTP induction protocol (100 Hz), our ability to impair LTP at thalamic inputs might suggest a fairly specific role for NO signaling at thalamo-LA synapses. This is also the case in the hippocampus, where LTP at apical, but not basilar, synapses on CA1 pyramidal cells requires NO signaling (O'Dell *et al*., 1994; Son *et al*., 1996). If such pathway specificity were true, it would appear to stand in contrast to an earlier study indicating that both the thalamo-LA and cortical-LA input pathways are equipotential for fear conditioning (Romanski & LeDoux, 1992). However, it also remains possible that our ability to impair amygdala LTP using NOS inhibitors is unique to our use of the relatively weak, NMDAR-dependent 30-Hz LTP induction protocol. Stronger protocols, including 100-Hz and pairing protocols that are less sensitive to NMDAR blockade (Huang & Kandel, 1998; Bauer *et al*., 2002), may not be NO-dependent. Additional experiments carried out in each input pathway will be needed to examine these questions further.

As in previous studies that have directly examined the relationship between LTP and memory in the amygdala, LTP in our slices treated with either 7-Ni or c-PTIO decayed with a much faster time course than fear memory after the same manipulations. This is not a novel finding but is in fact quite common in the LTP–behavior literature (Schafe *et al*., 2001). Many compounds that impair LTM but not STM have been shown to block LTP within minutes after induction. In the *in vitro* amygdala preparation, for example, bath application of inhibitors of protein synthesis, PKA or ERK/MAPK activity begins to impair LTP immediately after induction (Huang *et al*., 2000), whereas intra-amygdala infusion of the same compounds in behavioral experiments produces memory deficits that emerge between 6 and 24 h after conditioning (Schafe & LeDoux, 2000; Schafe *et al*., 2000). In thinking about this discrepancy, it is important to note that induction of LTP in the LA using artificial high-frequency stimulation of afferent input pathways is not a substitute for fear conditioning. Rather, fear conditioning and LTP are two types of stimulation that induce plasticity in the LA that happen to share a common biochemical mechanism. In this sense, LTP is a useful tool to uncover biochemical mechanisms that might underlie fear conditioning. However, it is clear that a complete understanding of how synaptic plasticity in the LA and fear conditioning are related will require attention to the relationship between naturally occurring plasticity in the LA and fear memory formation.

NO signaling and fear memory

Nitric oxide and/or its downstream effectors have been implicated in a number of hippocampal- and cerebellar-dependent learning and memory tasks (Chapman *et al*., 1992; Holscher & Rose, 1992; Bohme *et al*., 1993; Holscher, 1994; Bernabeu *et al*., 1995, 1996, 1997; Holscher *et al*., 1996; Suzuki *et al*., 1996; Zou *et al*., 1998). Few studies, however, have examined the role of NO signaling in amygdala-dependent learning, although Maren (1998) failed to find effects on contextual fear conditioning using a systemically administered NOS inhibitor. In our behavioral experiments, we examined the role of NO signaling specifically in auditory fear conditioning and in the amygdala. We found that intra-LA infusion of either the NOS inhibitor 7-Ni or the NO scavenger c-PTIO impaired fear memory formation. Specifically, LTM was impaired, whereas STM, measured within hours of training, was intact. The fact that fear acquisition and STM formation was left intact after NO manipulations rules out possible non-specific explanations of the drug effect, including the possibility that 7-Ni or c-PTIO disrupted fear acquisition by impairing sensory and/or performance factors, including the ability of the amygdala to process tone and shock information. Intact acquisition and STM formation also rules out potential effects of intra-LA infusion of NOS inhibitors on motivational factors that might interfere with fear conditioning. A previous study has shown that intra-LA infusion of an NOS inhibitor produces an anxiogenic state in rats as measured by the elevated plus maze (Monzón *et al*., 2001). Given the fact that anxiogenic drugs, including dopamine receptor agonists (Sullivan *et al*., 2003), adrenergic agonists (Davis *et al*., 1979) and acutely administered SSRIs (Burghardt *et al*., 2004), are all known to facilitate, rather than impair, fear conditioning, our results are not consistent with this finding. Rather, our findings suggest that memory formation of auditory fear conditioning, like LTP at auditory thalamic inputs to the LA, requires NOS activation and the extracellular release of NO in the amygdala. Furthermore, when considered together with the results of Maren (1998), our findings might suggest that there is some specificity with respect to the biochemical mechanisms that underlie auditory and contextual fear conditioning. Additional experiments will be required to explore this possibility further.

In our experiments, inhibition of NO signaling in the LA impaired the initial formation of a fear memory, but not reactivation or reconsolidation of auditory fear conditioning. These latter findings provide an important additional series of controls for the initial behavioral experiments. Under the influence of both compounds, rats exhibited normal retrieval of the memory, indicating that neither compound interferes with auditory processing in the amygdala. In addition, the fact that memory remained intact 24 h later suggests that infusion of either of these compounds did not produce memory deficits in the initial behavioral experiments by somehow non-specifically competing with freezing 24 h after infusion. Importantly, these are also the first data, of which we are aware, that suggest that initial memory formation and reconsolidation of fear conditioning can be distinguished at the biochemical level. Previous studies have suggested that the mechanisms that underlie consolidation and reconsolidation might be similar (for a review see Nader, 2003). These studies, however, have all focused on signaling pathways in the postsynaptic cell, including MAP kinase (Duvarci *et al*., 2005), protein synthesis (Nader *et al*., 2000; Duvarci *et al*., 2005) and the transcription factor CREB (Kida *et al*., 2002). This pattern of findings might suggest that the reconsolidation process is exclusively postsynaptic, whereas initial memory formation involves plasticity at both sides of the synapse.

The results of the present study clearly suggest that NO signaling underlies synaptic plasticity and fear memory formation in the LA.

These findings provide a potential biochemical mechanism whereby postsynaptically mediated events in the LA can engage presynaptic aspects of plasticity during fear conditioning and make an additional contribution towards understanding the cellular and molecular processes underlying emotional memory formation in the amygdala.

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Abbreviations

7-Ni, 7-nitroindazole; CaMKII, calcium/calmodulin-dependent protein kinase II; c-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; EM, electron microscopy; LA, lateral nucleus of the amygdala; LTM, long-term memory; LTP, long-term potentiation; NMDAR, *N*-methyl-D-aspartate receptor; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; PKA, protein kinase A; PKG, protein kinase G; PPF, paired pulse facilitation; STM, short-term memory.

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