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MYOSIN LIGHT CHAIN KINASE REGULATES SYNAPTIC PLASTICITY AND FEAR LEARNING IN THE LATERAL AMYGDALA

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Abstract—Learning and memory depend on signaling molecules that affect synaptic efficacy. The cytoskeleton has been implicated in regulating synaptic transmission but its role in learning and memory is poorly understood. Fear learning depends on plasticity in the lateral nucleus of the amygdala. We therefore examined whether the cytoskeletal-regulatory protein, myosin light chain kinase, might contribute to fear learning in the rat lateral amygdala. Microinjection of ML-7, a specific inhibitor of myosin light chain kinase, into the lateral nucleus of the amygdala before fear conditioning, but not immediately afterward, enhanced both short-term memory and long-term memory, suggesting that myosin light chain kinase is involved specifically in memory acquisition rather than in posttraining consolidation of memory. Myosin light chain kinase inhibitor had no effect on memory retrieval. Furthermore, ML-7 had no effect on behavior when the training stimuli were presented in a non-associative manner. Anatomical studies showed that myosin light chain kinase is present in cells throughout lateral nucleus of the amygdala and is localized to dendritic shafts and spines that are postsynaptic to the projections from the auditory thalamus to lateral nucleus of the amygdala, a pathway specifically implicated in fear learning. Inhibition of myosin light chain kinase enhanced long-term potentiation, a physiological model of learning, in the auditory thalamic pathway to the lateral nucleus of the amygdala. When ML-7 was applied without associative tetanic stimulation it had no effect on synaptic responses in lateral nucleus of the amygdala. Thus, myosin light chain kinase activity in lateral nucleus of the amygdala appears to normally suppress synaptic plasticity in the circuits underlying fear learning, suggesting that myosin light chain kinase may help prevent the acquisition of irrelevant fears. Impairment of this mechanism could contribute to pathological fear learning. © 2006 Published by Elsevier Ltd on behalf of IBRO.

Key words: MLCK, fear conditioning, LTP, plasticity, memory, cytoskeleton.

The neuronal cytoskeleton controls synaptic morphology, transmission and adhesion during development (Luo, 2002). These processes are believed to be involved in memory formation (Dudai, 1989; Kandel, 2001; Lamprecht and LeDoux, 2004). However, evidence for a role of neuronal cytoskeleton in learning and memory is sparse. We therefore examined the role of a cytoskeletal-regulatory protein, myosin light chain kinase (MLCK) in fear learning. The MLCK is a calcium/calmodulin-dependent protein kinase that phosphorylates the myosin regulatory light chain (RLC), leading to contraction of the actomyosin filaments (Kamm and Stull, 2001; Somlyo and Somlyo, 2003). MLCK is involved in regulating cellular events related to synaptic transmission, such as neurotransmitter release (Mochida et al., 1994; Ryan, 1999; Polo-Parada et al., 2001), *N*-methyl-D-aspartate receptor activity (Lei et al., 2001) and potassium channel function (Akasu et al., 1993). In addition, MLCK participates in neural morphogenesis, such as in the regulation of growth cone motility (Gallo et al., 2002; Zhou et al., 2002) and dendritic branching (Ramakers et al., 2001). Similar cellular processes are believed to be involved in various stages of memory formation (Lamprecht and LeDoux, 2004). However, the role of MLCK in learning and memory has not been studied.

Classical auditory fear conditioning, a procedure in which an animal associates a neutral stimulus (such as a tone) with an aversive event (typically a mild footshock), is a useful behavioral paradigm for studying the molecular basis of learning and memory because the putative site of plasticity, the lateral nucleus of the amygdala (LA), has been identified (Fanselow and LeDoux, 1999; Schafe et al., 2001; Davis and Whalen, 2001; Maren, 2001; Rodrigues et al., 2004b). For example, damage or functional inactivation of the LA and adjacent areas during acquisition prevents the learning from taking place (e.g. LeDoux et al., 1990; Nader et al., 2001; Helmstetter and Bellgowan, 1994; Muller et al., 1997; Wilensky et al., 1999) and neural activity changes in LA during learning (Quirk et al., 1995, 1997; Collins and Pare, 2000; Repa et al., 2001). Moreover, through studies of long-term potentiation (LTP), a physiological model of the plasticity underlying learning and memory, LA synapses that receive CS information from the auditory thalamus have also been implicated in the plasticity underlying fear learning (Rogan and LeDoux, 1995; Rogan et al., 1997). Thus, the auditory fear conditioning paradigm provides a behavioral tool, anatomical site, and physiological model for assessing molecular mechanisms that might mediate changes in synaptic strength during learning and memory formation.

In the present study we assessed the contribution of MLCK in LA to synaptic plasticity and fear conditioning. We used a combination of behavioral, electrophysiological and anatomical approaches and conclude that MLCK normally suppresses fear learning at the synaptic level.

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Abbreviations: CS, conditioned stimulus; EM, electron microscopy; LA, lateral nucleus of the amygdala; LTM, long-term memory; LTP, long-term potentiation; MLCK, myosin light chain kinase; PSD, postsynaptic density; STM, short-term memory; US, unconditioned stimulus.

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EXPERIMENTAL PROCEDURES

Animals

All studies involved male Sprague–Dawley rats (Hilltop Laboratories, Scottdale, PA, USA), weighing 250–300 g (unless written otherwise). The animals were housed separately in plastic Nalgene cages and placed on a 12 h light/dark cycle with *ad libitum* food and water. Every effort was made to minimize the number of animals used and any discomfort. All procedures were in accordance with the National Institutes of Health guide and were approved by the New York University Animal Care and Use Committee.

Fear conditioning procedure

Fear conditioning took place in a Plexiglas rodent conditioning chamber with a metal grid floor (model E10-10; Coulbourn Instruments, Lehigh Valley, PA, USA), dimly illuminated by a single house light and enclosed within a sound-attenuating chamber (model E10-20). The paired group was presented with five pairing of tone for 20 s (CS: conditioned stimulus; 5 kHz, 75 dB) that co-terminated with a foot shock (US: unconditioned stimulus; 0.5 s, 0.4 mA) and the unpaired group received five non-overlapping presentations of the CS and US where the US preceded the CS by 60 s and at least 180 s were required between a tone CS and the next trial.

Testing of conditioned fear memory

Rats were tested 1 h (short-term memory, STM) and 24 h (long-term memory, LTM) after conditioning for tone memory in a different chamber than the conditioning chamber. The chamber was brightly illuminated with 3 house lights and contained a flat black Formica floor that had been washed with peppermint soap. Animals were videotaped during testing for later scoring. After a 3–5 min acclimation period to the test chamber, rats were presented with three (for STM) and five (for LTM) 20 s tones (5 kHz, 75 dB; intertrial interval: 100 s). After tone testing, rats returned to their home cages and to the colony. Freezing of animals during tone presentation was scored.

Surgical procedures

Microinjection into the lateral amygdala was performed via chronically implanted cannulas. For surgery, rats were anesthetized with a mixture of ketamine (100 mg/kg, i.p.; Ketaset; Phoenix, St. Joseph, MO, USA), xylazine (6.0 mg/kg, i.p.; Xyla-Jet; Phoenix), and medetomidine (0.5 mg/kg, i.p.; Domitor; Pfizer, New York, NY, USA), restrained in a stereotaxic apparatus (Kopf, Tujunga, CA, USA), and implanted bilaterally with guide cannulas (22 gauge; Plastics One, Roanoke, VA, USA), equipped with internal cannulas that extended out 1.5 mm from the base of the guide cannulae, aimed to the LA [coordinates in reference to bregma: anteroposterior (AP), −3.0; lateral (L) ±5.3; and dorsoventral (DV), −8.0] (Paxinos and Watson, 1986). The cannulas were fixed in place with acrylic dental cement and secured by three skull screws and internal cannulas removed. A dummy cannula that was 0.5 mm longer than the chronic cannula was placed in the guide cannula to prevent clogging. After surgery, rats were administered butorphanol tartrate (2.0 mg/kg, i.p.; Torbugesic; Fort Dodge Laboratories, Fort Dodge, IA, USA) and atipamezole (1.0 mg/kg, i.p.; Antisedan; Pfizer) for analgesia and reversal of the anesthetic. Animals were allowed 1 week to recuperate before being subjected to experimental manipulations.

Microinjection

The dummy cannula was removed from the guide cannula, and a 28 gauge injection cannula was carefully inserted and lowered 1.5 mm below the tip of the guide cannula. The injection cannula was connected via polyurethane tubing, backfilled with sesame oil with a small air bubble separating the oil from the drug solution, to a 1.0 μl Hamilton syringe. Microinjection was driven by an infusion pump at a rate of 0.25 μl/min. MLCK inhibitor ML-7 (Biomol Research Laboratories, Plymouth Meeting, PA, USA) was dissolved in DMSO and diluted 1:100 in saline to give final concentration of 220 μM. After microinjection, the injection cannula was left for an additional 1 min before withdrawal to reduce efflux of injection liquid along the injection tract.

Anatomical experiments

Procedures for light microscopy (LM) were described elsewhere (Lamprecht et al., 2002; Rodrigues et al., 2004a). A mouse monoclonal antibody directed against MLCK (1:250; Sigma, St. Louis, MO, USA), biotinylated goat anti-mouse IGg avidin–biotin complex (ABC; Vector, Burlingame, CA, USA) and 3-3′-diaminobenzidine (DAB) were used to visualize MLCK. Electron microscopic (EM) and anterograde transport studies were conducted as previously described (Rodrigues et al., 2002).

In vitro field potential recordings and LTP induction

Brain slices were prepared from male Sprague–Dawley rats aged 3–5 weeks. Once deeply anesthetized with ketamine and xylazine (100 mg/kg and 10 mg/kg) animals were perfused with ice-cold ACSF (115 mM NaCl, 3.3 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 25.5 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, and 25 mM glucose, equilibrated with 95% O2/5% CO_2 to maintain pH at 7.4) then quickly decapitated. Chilled brains were blocked into coronal sections containing the entire amygdala, plunged into ice cold ACSF and then sectioned into hemi-coronal sections on a vibratome at 400 μm. Brain slices were warmed to 32 °C for 30 min and then slowly lowered to room temperature and maintained for up to two hours prior to recording. Slices were transferred to a recording chamber, flow rate 2.5 ml/min, containing ACSF. In order to position both recording and stimulating electrodes, slices and neurons were visualized with an upright fixed stage microscope equipped with infrared differential interference contrast optics. Glass recording electrodes were filled with ACSF for a resistance of 5–10 MΩ. Bipolar stimulating electrodes 75 kΩ were positioned medial to the lateral amygdala in fibers believed to contain thalamo-amygdala afferents. Orthodromic synaptic potentials were evoked via an isolated current generator (Digitimer, Hertfordshire, UK) (100 μs pulses of 0.3–0.7 mA). Evoked field potentials were recorded with an Axoclamp 2B amplifier and an Axon WCP software (Axon Instruments, Molecular Devices Corporation, Sunnyvale, CA, USA). LTP: tetanic stimulation was applied to slices (100 stimulation pulses at 100 Hz frequency were delivery three times at interval of 90 s). Evoked field potentials were filtered at 1 kHz and digitized at 4 kHz. Amygdala slices were superfused with the drug dissolved into ACSF containing a 1% DMSO vehicle. ML-7 (Biomol, Research Laboratories) was dissolved as stock solution and used at a final concentration of 220 μM. Drug and vehicle control were superfused for the 5 min immediately prior to tetanic stimulus. Data were analyzed offline using WCP PeakFit (Axon Instruments). LTP was measured as a change in the amplitude of the evoked field potential. One slice for vehicle and drug groups per animal was used except for animal number 6 where two drugs trials and one vehicle trial were tested. Thus *n* refers to slice number. Data are presented as mean±S.E.M. Vehicle and drug groups were compared with two-tailed *t*-test.

RESULTS

Inhibiton of MLCK in LA facilitates the acquisition of associative fear conditioning

The specific and cell permeable MLCK inhibitor ML-7 (e.g. Saitoh et al., 1987; Ryan, 1999; Lei et al., 2001; Jung et al.,

2004) was microinjected into the LA through chronically implanted cannulae immediately before fear conditioning (Fig. 1a). Conditioned fear memory was assessed by measuring freezing responses elicited by the CS without the US 1 h after completion of training (STM) and 24 h after conditioning (LTM). Because preliminary studies suggested that the effect of MLCK would be an enhancement of fear memory, we used a weak training protocol (see Experimental Procedures) that would produce low levels of freezing so that drug-induced increases could be observed without ceiling effects. Results were analyzed using an ANOVA that compared the effect of group (vehicle, ML-7) and time (repeated measures, STM and LTM). The analysis revealed a significant main effect for group [$F(1,10)=8.5$, $P<0.016$] indicating that memory was enhanced in the drug treated animals. There was a significant effect of time ($F(1,10)=69.3$ $P<0.001$) showing that the memory extinguished significantly as observed by the reduced fear memory at the LTM test compared with the STM test. There was no interaction between group and time variables ($P=0.21$) suggesting that the effects of ML-7 were constant over time. Consistent with the ANOVA, *t*-test analysis for the effect of drug relative to vehicle at each time point shows that microinjection of ML-7 into LA significantly enhanced both STM and LTM (Fig. 1a; $P<0.013$ and $P<0.05$, respectively). These results show that microinjection of ML-7 into the LA before conditioning significantly enhanced both STM and LTM. Fourteen days after completion of the STM and LTM tests, ML-7- and vehicle-injected animals were retrained drug free. When tested the groups did not differ ($P>0.51$), indicating that ML-7 did not produce any lasting changes in the amygdala. These results suggest that the formation of fear memory is normally suppressed by the activity of endogenous MLCK, and that inhibition of MLCK relieves this suppression, leading to the enhancement of both STM and LTM.

The effects of MLCK on fear conditioning could be due to the formation of an association between the CS and US or could be due to non-associative factors such as sensitization. To rule out the non-associative interpretation we microinjected ML-7 or vehicle into LA prior to unpaired training. In this protocol the animals receive both the CS and US but in a non-overlapping manner. Results analyzed using an ANOVA revealed no significant main effect for group ($P>0.9$) indicating that fear behavior was not enhanced in the drug-treated animals (Fig. 2a). There was a significant effect of time ($F(1,6)=12.48$ $P<0.013$) showing that the sensitized fear responses induced by non-associative factors extinguished over time. There was no interaction between group and time variables ($P=0.87$) indicating that the reduction in the generalized fear responses over time is similar for both the drug and vehicle treatment. These results indicate that the enhancing effect of MLCK on fear conditioning is not due to non-associative factors such as sensitization and instead is likely to specifically involve a facilitation of an associative link in memory between the CS and US.

Cellular events just described could be involved in the acquisition or retrieval of fear memory (LeDoux, 2000;

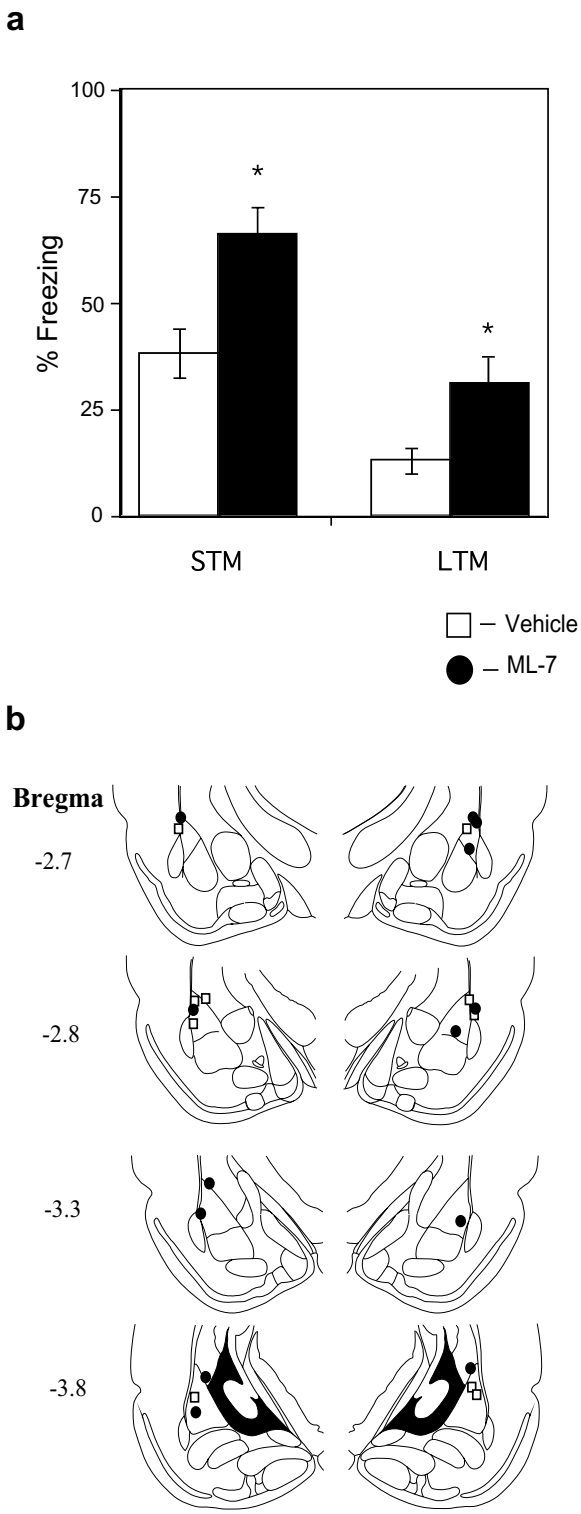

Fig. 1. Inhibition of MLCK activity in LA enhances STM and LTM formation. (a) Microinjection of MLCK inhibitor (ML-7) into the LA immediately before fear conditioning training significantly enhanced STM and LTM as compared with vehicle-injected animals ($P<0.013$ for STM and $P<0.05$ for LTM $n=5$ for ACSF, $n=7$ for ML-7). (b) Cannulas' tip placements (white squares: vehicle before training, black circles: ML-7 before training).

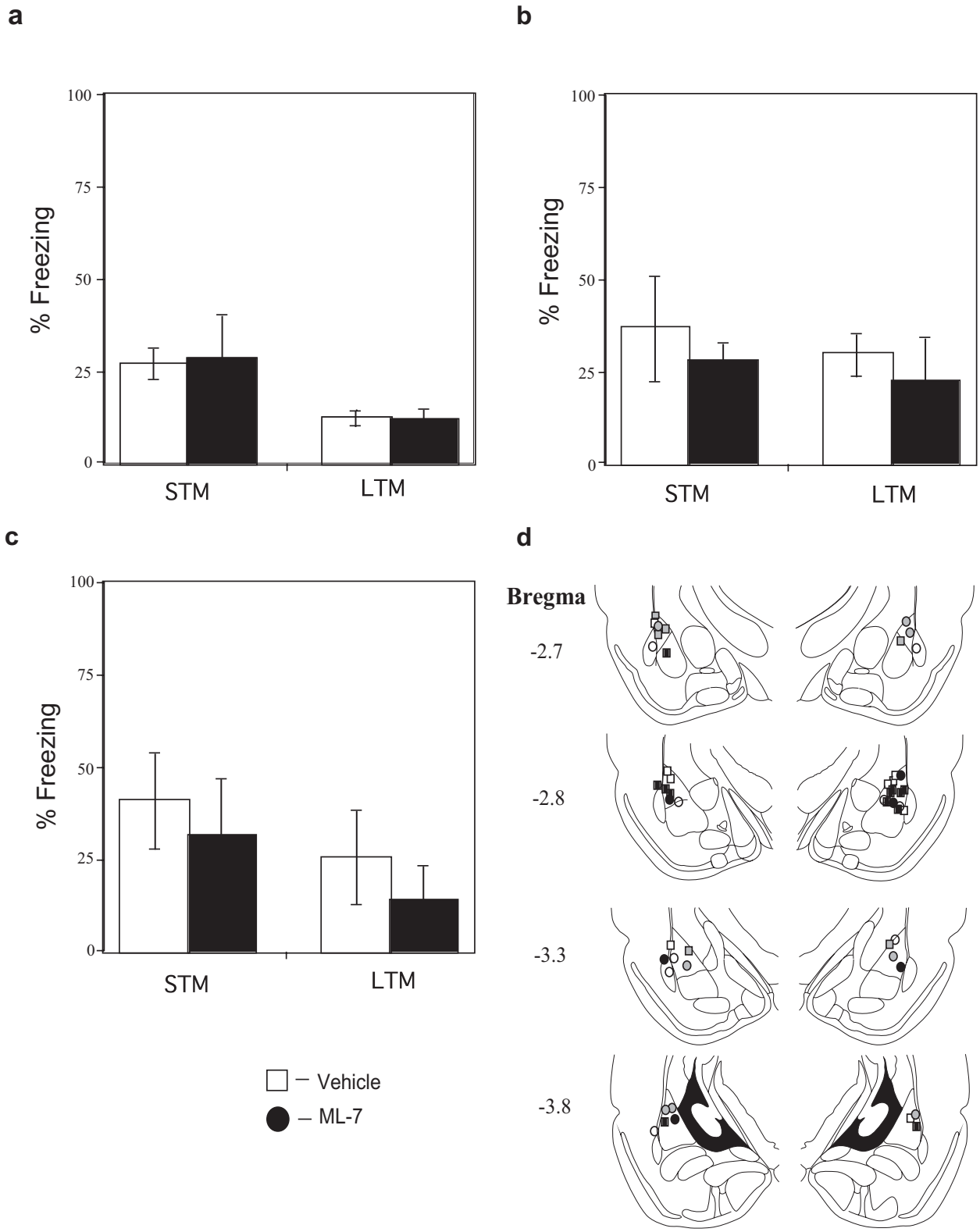

Fig. 2. Microinjection of ML-7 into LA does not affect sensitization, retrieval or postacquisition events. (a) Inhibition of MLCK in LA immediately before unpaired training protocol had no effects on freezing responses as compared with vehicle-injected animals ($P>0.9$; $n=5$ for vehicle $n=3$ for ML-7). (b) Microinjection of ML-7 into the LA immediately before STM test had no effect on memory retrieval ($P=0.5$; $n=5$ each group). (c) Microinjection of ML-7 into the LA immediately after fear conditioning had no effect on fear memory ($P=0.51$; $n=4$ each group). (d) Cannula placements (white squares: vehicle before unpaired training; black circles: ML-7 before unpaired training; white circles: vehicle before test, black squares: ML-7 before test; gray squares: vehicle postconditioning; gray circles: ML-7 postconditioning).

Rodrigues et al., 2004b). To assess this, we microinjected ML-7 or vehicle into LA immediately before the STM test. Results were analyzed using an ANOVA and revealed no significant main effect for group ($P=0.5$) indicating that memory was not enhanced in the drug-treated animals (Fig. 2b). There was no significant effect of time ($P=0.46$) and there was no interaction between group and time variables ($P=0.93$). These results show that MLCK does not participate in regulating cellular functions in LA underlying memory retrieval and also indicate that MLCK is not necessary for routine synaptic transmission through the amygdala circuitry, a conclusion supported by the physiological studies described below.

Because fear conditioning occurs quickly, drugs given prior to training could affect memory by either altering the acquisition of the fear association during training or the consolidation of the fear memory after training (McGaugh, 2000). To distinguish between these possibilities, we injected ML-7 or vehicle into LA immediately following fear conditioning. Results were analyzed using an ANOVA and revealed no significant main effect for group ($P=0.51$) indicating that memory was not enhanced in the drug-treated animals (Fig. 2c). There was no significant effect of time ($P=0.15$) and no interaction between group and time variables ($P=0.92$). Thus, MLCK is involved in regulating cellular events underlying acquisition rather than the consolidation of associative fear memory. These results are consistent with the view that MLCK participates in regulating cellular activities (such as synaptic transmission, membrane conductance and/or vesicle release) involved in memory acquisition (Akasu et al., 1993; Mochida et al., 1994; Ryan, 1999; Lei et al., 2001; Polo-Parada et al., 2001).

MLCK is present in LA synapses that receive auditory conditioned stimuli

We examined the localization of MLCK in the LA using immunocytochemical staining. Light microscopic examination showed MLCK-labeled cells throughout the LA (Fig. 3a). Higher-power (40×) optics revealed that labeled cells were round or ovoid in shape, with dendrites emanating from some of the cell bodies (Fig. 3a, inset).

Labeled tissue was also examined by EM to characterize the subcellular localization of MLCK. Ultrastructural analysis showed that MLCK immunoreactivity was seen in somata, large and small dendrites, dendritic spines, axon terminals, and glia (Fig. 3b). MLCK-labeling in axon terminals occurred as small, discreet patches of immunoreactivity that rimmed the synaptic vesicles and were localized to sites away from the synapse. Labeled terminals frequently formed asymmetric synapses onto dendritic spines (Fig. 3b). MLCK labeling in spines was frequently prominent at the postsynaptic density (PSD) but labeling was also seen away from the PSD and within the spinoplasm.

Because we are specifically interested in the role of MLCK in auditory fear conditioning we next examined whether MLCK is present at sites postsynaptic to auditory inputs to LA. CS information reaches the LA from auditory processing regions in the thalamus and cortex (LeDoux, 2000). To determine whether MLCK is localized to sites postsynaptic to the afferents that transmit CS information to LA, we microinjected the anterograde tracer BDA into the auditory thalamus and examined the LA by EM. Many BDA terminals formed asymmetric synapses onto dendritic spines or small dendrites that expressed MLCK (Fig. 3c, d), indicating that MLCK in LA is postsynaptic to the thalamic auditory-LA afferents.

Inhibition of MLCK facilitates synaptic plasticity at CS input synapses in LA

LTP in LA is a means of studying plasticity at synapses involved in fear conditioning (McKernan and Shinnick-Gallagher, 1997; Weisskopf et al., 1999; Huang et al., 2000; Schafe et al., 2001; Bauer et al., 2002; Tsvetkov et al., 2002; Rodrigues et al., 2004a). For example, induction of LTP in the auditory thalamic pathway increases auditory-evoked field potentials in the LA (Rogan and LeDoux, 1995) and fear conditioning alters CS-evoked responses in LA the same way as LTP induction (Rogan et al., 1997). Given that we showed that MLCK is present in the CS pathway from the auditory thalamus to the LA, we therefore examined whether MLCK contributes to LTP in this pathway.

We were interested to examine whether MLCK constrains the induction of LTP in LA. A high frequency tetanus (three trains of 100 pulses at 100 Hz frequency with interval of 90 s; Fig. 4a) was delivered to the thalamic afferents that project to LA. This stimulation protocol failed to produce LTP (Fig. 4b). However, when the MLCK inhibitor ML-7 was added to the bath 5 min immediately prior to LTP induction, a significant synaptic potentiation was induced in LA when compared with the control vehicle ($P<0.001$ and $P<0.005$ at 0–5 min and 55–60 min after tetanic stimulation respectively; Fig. 4b). Application of ML-7 alone without tetanic stimulation did not induce LTP in LA: responses were not different from vehicle controls (Fig. 4c; $P=0.6$). ML-7 thus does not increase the potentiation of synaptic responses in LA independent of associative tetanic stimulation.

DISCUSSION

In the present study, we show that inhibition of MLCK in LA enhances the acquisition of auditory fear conditioning, that MLCK is present in synapses of the auditory CS pathway in LA and that inhibition of MLCK enhances LTP induction in this same pathway. These findings show that MLCK plays an important role in regulating synaptic plasticity underlying fear learning in LA.

Infusion of MLCK inhibitor into LA prior to CS–US pairing led to an enhancement of fear behavior elicited by CS the next day (Fig. 1a) but did not affect the amount of fear elicited by a CS that was presented in an unpaired relation to the US during training (Fig. 2a). Moreover, the same treatment that enhanced fear learning when given before training had no effect when given immediately after training (Fig. 2c). These findings indicate that MLCK affects associative rather than non-associative conditioning

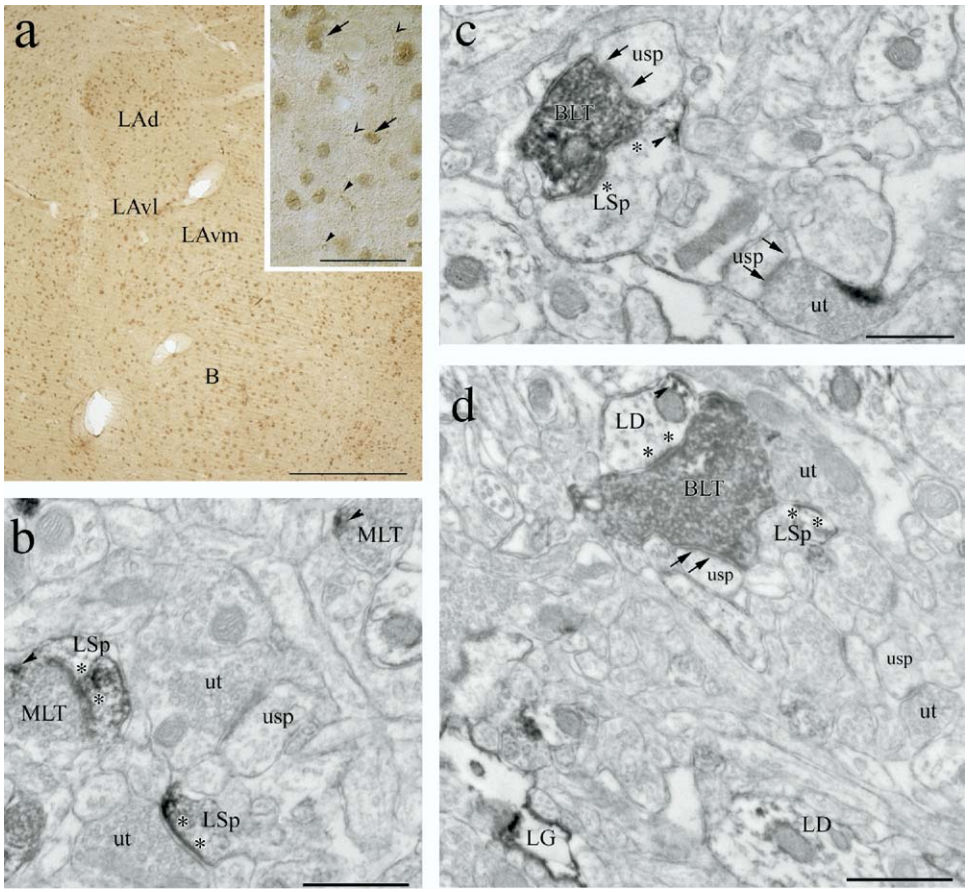

Fig. 3. Micrographs that depict the distribution of MLCK immunolabel within the amygdala. (a) Low-power micrograph shows that MLCK-labeled cells are distributed throughout the amygdala. Inset: higher-power Nomarski optics show labeled cells (arrows), their proximal dendritic processes (open arrowheads), and labeled punctate processes (arrowheads). Scale bars=250 μm and 50 μm in inset. (b) Electron micrograph demonstrates that MLCK immunolabel is localized to both pre- and postsynaptic sites. A labeled terminal (MLT) forms an asymmetric synapse (**) on labeled dendritic spine (Lsp). Arrow points to the patch of immunoreactivity within the terminal. Another labeled spine (LSp) receives an asymmetric synapse (**) from an unlabeled axon terminal (ut). Also shown are a labeled terminal (arrowhead; arrow points to the patch of immunoreactivity within the terminal), an ut and an unlabeled spine (unp). Scale bar=25 nm. (c) A BDA-labeled terminal (BLT) synapses simultaneously on two dendritic spines; one is labeled (LSp) and the other is unlabeled (usp). Arrowheads point to a patch of immunolabel within the labeled spine and the asterisks indicate the labeled asymmetric synapse formed by the BDA terminal. Arrows point to the asymmetric synapse the BDA terminal forms with the unlabeled spine. An ut forming an asymmetric synapse onto an unlabeled spine is also shown. (d) A BDA-terminal simultaneously forms two asymmetric synapses (asterisks), one on a small labeled dendrite (LD) and another (arrows) on an unlabeled spine (usp). Arrowhead in labeled dendrite points to a patch of immunolabel. A labeled spine (LSp) apposed to the BDA terminal receives an asymmetric synapse (asterisks) from an ut. Also shown are a labeled glial process (LG), a labeled dendrite (LD) and an ut synapsing on an unlabeled spine (usp). Scale bars in b, c and d=500 nm.

processes and that its main contribution is to the acquisition or learning of fear rather than in memory storage.

The involvement of MLCK in LA in fear conditioning is interesting in light of the fact that this protein has been shown to play a central role in the molecular regulation of synaptic transmission, and in the morphogenesis of dendrites and axons (Akasu et al., 1993; Mochida et al., 1994; Ryan, 1999; Lei et al., 2001; Polo-Parada et al., 2001; Ramakers et al., 2001; Gallo et al., 2002; Zhou et al., 2002), events believed to be involved in synaptic efficiency during memory formation (Lamprecht and LeDoux, 2004; Rodrigues et al., 2004b). Our results, showing that inhibition of MLCK before acquisition (Fig. 1a), but not immediately after training or before retrieval (Fig. 2), affects fear memory suggest that MLCK is involved in the integration of the CS and US information during acquisition. This brief time of involvement of MLCK in fear conditioning is consistent with its ability to rapidly regulate synaptic transmission (Ryan, 1999; Lei et al., 2001). In addition, the anatomical findings (Fig. 3) showing that MLCK is located in LA presynaptic terminals and in postsynaptic densities suggest that MLCK might be involved in regulating events in these sites such as vesicle release (Ryan, 1999) or receptor activity (Lei et al., 2001). The finding that MLCK inhibition did not affect fear memory retrieval (Fig. 2b) implies that MLCK does not regulate transmission during memory activation, but specifically during acquisition. Consistent with these findings is the observation that application of ML-7 onto amygdala slices has no effect on basal transmission but rather specifically on the induction of associative LTP (Fig. 4). Given that inhibition of MLCK enhances conditioning and the synaptic plasticity underlying conditioning, we conclude that endogenous MLCK normally inhibits fear learning.

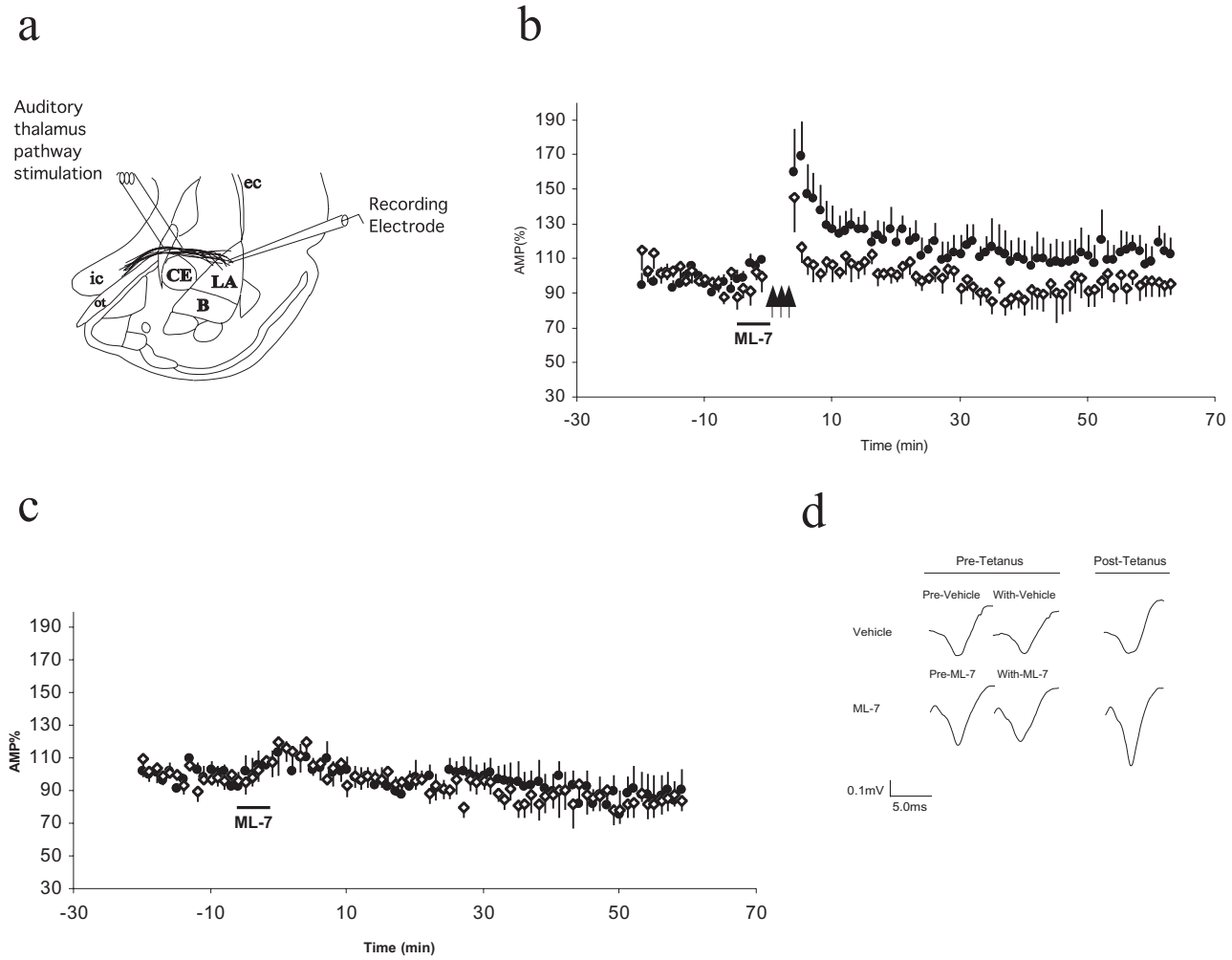

Fig. 4. Inhibition of MLCK facilitates LTP in LA after stimulation of the auditory thalamus pathway to LA. (a) Schematic of the amygdala slice preparation showing the placement of the stimulating electrode in the auditory thalamus pathway and the recording electrode in the dorsal lateral amygdala (LA). (b, c) Mean±S.E.M. percentage of EPSP amplitude (relative to baseline) in slices treated in the following ways: (b) tetanized in the presence of vehicle (n=5, diamonds); tetanized in the presence of ML-7 in vehicle (n=6; circles) (P<0.001 and P<0.005 at 0–5 min and 55–60 min after tetanic stimulation respectively); (c) vehicle (diamonds; n=5) or ML-7 in vehicle (circles; n=4) without tetanic stimulation (P=0.6); (d) representative average traces (n=30 over 10 min) are taken from individual experiments before tetanic stimulation, with or without drug/vehicle, or 10 min following tetanic stimulation.

Fear conditioning is mediated by way of projections of the tone CS from medial geniculate body and the adjacent posterior intrathalamic nucleus (MGm/PIN) to LA (LeDoux, 2000). High frequency stimulation of this pathway leads to LTP in LA and also results in enduring increase of field potentials elicited by natural acoustic stimulation in LA (Rogan and LeDoux, 1995). Furthermore, fear conditioning alters auditory CS-evoked responses in LA in the same ways as LTP induction (Rogan et al., 1997). Collectively, these studies suggest that fear conditioning is encoded in LA by LTP-like mechanisms. The finding that inhibition of MLCK facilitates LTP in LA after auditory thalamus afferent stimulation (Fig. 4b) suggests that suppression of MLCK activity could regulate fear-conditioning acquisition by facilitating the induction of LTP in LA, thus leading to an increase in CS-evoked responses in LA.

Interestingly, it has been shown that dopamine enables the induction of LTP in LA (Bissiere et al., 2003) similarly to the results shown in our study. MLCK, which is involved in regulation of presynaptic vesicle recycling, affects dopamine activities by regulating its uptake (Uchikawa et al., 1995). In addition, it has been suggested that dopamine exerts its effects on actin rearrangements by inhibition of MLCK activity (Vitale and Carbajal, 2004). Thus, neuromodulators could inhibit MLCK and facilitate synaptic plasticity in LA. Consistent with this hypothesis is the finding that MLCK activity is inhibited by phosphorylation via protein kinases such as PKA (Conti and Adelstein, 1981) that are downstream to neuromodulators (including dopamine) and involved in fear conditioning formation in LA (Schafe and LeDoux, 2000). Thus, further studies should explore the possibility that the MLCK activity is involved in neuromodulators effects on synaptic plasticity in LA.

Although ML-7 is a selective inhibitor of MLCK, it might also inhibit PKA or PKC at much higher concentrations (Saitoh et al., 1987; Zhou and Cohan, 2001). However, inhibition of PKA and PKC in the lateral amygdala has previously been shown to impair fear conditioning (Schafe and LeDoux, 2000; Goosens et al., 2000), whereas we found that injection of ML-7 into the LA facilitates fear conditioning. The effects we observed are therefore unlikely due to inhibition of PKA and PKC, but rather of MLCK.

The present study shows that MLCK in LA determines the efficacy of fear learning. Normally, MLCK may suppress fear learning. This may be particularly important for preventing the development of fear to stimuli that are not threatening or that are marginally threatening, or for preventing the generalization of fear to stimuli similar to the learned fear stimuli. Disruption of this regulatory mechanism could contribute to the development of pathological fear and anxiety. Drugs that enhance MLCK could therefore be useful in treating fear disorders.

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