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Research report

A role for amygdaloid PKA and PKC in the acquisition of long-term conditional fear memories in rats

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Abstract

Although there is great interest in the cellular mechanisms underlying Pavlovian conditioning, few studies have directly examined the contribution of intracellular signaling pathways in the amygdala to the acquisition and expression of conditional fear memories. In the present study, we examined this issue by infusing 1-(5′-isoquinolinesulfonyl)-2-methylpiperazine (H7), a potent inhibitor of both protein kinase C (PKC) and cAMP-dependent protein kinase (PKA), directly into the amygdala prior to fear conditioning or retention testing. We found that infusion of H7 prior to training attenuated long-term conditional fear in a dose-dependent manner (Experiment 1), but short-term fear memories were spared. The contribution of protein kinases to conditional fear was region-specific within the amygdala: infusion of H7 into the basolateral amygdala (BLA) but not the central nucleus of the amygdala (CEA) resulted in attenuated freezing (Experiment 2). Moreover, the deficits in fear conditioning produced by PKA/PKC inhibition were not modality-specific, insofar as intra-BLA H7 reduced both contextual and auditory fear. The effects of H7 on conditional freezing were not attributable to either state-dependency or performance deficits (Experiment 3). Together, these experiments suggest that amygdaloid PKA and PKC play an important role in the acquisition of fear memories.

Keywords: Amygdala; Kinase; Context; Tone; LTP; Learning; Memory; Fear conditioning

1. Introduction

Many studies indicate that the basolateral complex of the amygdala is a critical locus of plasticity during associative fear learning [5]. Neurotoxic lesions of the basolateral amygdala (BLA) result in deficits in both the acquisition and expression of conditional fear [12,18,21] and associative conditional activity develops in neurons of the BLA [26,27] during fear conditioning. A putative cellular mechanism for Pavlovian fear conditioning and the associative neuronal changes that accompany this form of learning is long-term potentiation (LTP) [19]. LTP has been demonstrated in the BLA after high-frequency stimulation of afferents that carry either contextual [22] or auditory [4,7,28] information to amygdala neurons during fear conditioning. Fear conditioning also induces LTP-like changes in the BLA [24,29] and transgenic mice deficient in amygdaloid LTP exhibit deficits in long-term fear memories [3]. Moreover, infusion of *N*-methyl-D-aspartate (NMDA) receptor antagonists into the BLA blocks both conditional freezing [11,21] and fear-potentiated startle [25], and impairs the induction of amygdaloid LTP [7,22].

Although both amygdaloid NMDA receptor activation and LTP appear to be critical steps in the acquisition of conditional fear, little is known concerning the intracellular biochemical events subsequent to NMDA receptor activation that are involved in the acquisition of conditional fear memories. It has recently been demonstrated that cAMP-dependent protein kinase (protein kinase A or PKA) activation is involved in the

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expression of an early phase of amygdaloid LTP [7]. Activation of the cAMP response element (CRE), thought to reflect PKA activity [9], has been demonstrated in the amygdala after fear conditioning [10]. Furthermore, shock-induced stress has been shown to increase protein kinase C (PKC) activity in the amygdala [32]. Given the involvement of these protein kinases in amygdaloid LTP and fear conditioning, one might expect that inhibitors of PKA and PKC activity would attenuate fear conditioning. Consistent with this prediction, it has recently been reported that PKA inhibitors impair the acquisition of fear conditioning [2,31]. However, these studies did not specifically target protein kinases in the amygdala because the inhibitors were administered intracerebroventricularly. Therefore, it is unclear whether the amygdala is a critical locus of kinase activity during fear conditioning. In the present experiments, we have addressed this question by infusing 1-(5′-isoquinolinesulfonyl)-2-methylpiperazine (H7), a potent PKA/PKC inhibitor, directly into the amygdala prior to Pavlovian fear conditioning in rats. Our results reveal that amygdaloid PKA and PKC have an important role in the establishment of long-term conditional fear memories.

2. Materials and methods

2.1. Subjects

Adult male Long-Evans rats (200–224 g) were obtained from a commercial supplier (Harlan Sprague–Dawley, Indianapolis, IN) and individually housed in standard stainless-steel hanging cages on a 14:10 h light–dark cycle (lights on at 07:00 h). Food and water was provided ad libitum. Rats were handled for 30 s/day for 5 days to acclimate them to the experimenters.

2.2. Surgery

One week before behavioral testing, rats were implanted with guide cannulas (26 gauge; Plastics One, Roanoke, VA) aimed at the BLA or CEA. Rats were anaesthetized with sodium pentobarbital (65 mg/kg i.p.), and were administered atropine methyl nitrate (0.4 mg/kg i.p.) to prevent airway obstruction. After mounting in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA), the scalp was incised and retracted, and lambda and bregma were placed in the same horizontal plane. Small burr holes were drilled for bilateral placement of guide cannulas 1 mm above the BLA (2.3 mm posterior to bregma, 5.0 mm lateral to the midline, 6.3 mm ventral to dura) or CEA (2.0 mm posterior to bregma, 4.0 mm lateral to the midline, 5.7 mm dorsal to dura), and placement of three small jeweler's screws. The guide cannulas were lowered, and dental acrylic was applied to the skull to hold the cannulas in place. After surgery, obturators (33 gauge; Plastics One, Roanoke, VA) were inserted into the cannulas, and the rats were returned to their home cages. The obturators were replaced every other day during the week of recovery.

2.3. Behavioral apparatus

Eight identical observation chambers ($30 \times 24 \times 21$ cm; MED-Associates Inc., Burlington, VT) were used for all training and testing. The chambers were constructed of aluminum (two side walls) and Plexiglas (rear wall, ceiling, and hinged front door), and situated in sound-attenuating cabinets in an isolated room. The floor of each chamber consisted of 19 stainless steel rods (4 mm diameter) spaced 1.5 cm apart (center-to-center). The rods were wired to a shock source and solid-state grid scrambler (MED-Associates Inc., Burlington, VT) for the delivery of footshock unconditional stimuli (USs). A speaker for delivering acoustic stimuli was mounted to a grating on one wall of each chamber.

Each conditioning chamber rested on a load-cell platform that recorded chamber displacement in response to each rat's motor activity. The output of each chamber's load cell was amplified (vernier knob = 8) at a level that was previously determined to optimize the detection of freezing behavior. The load-cell amplifier output from each chamber was digitized at 5 Hz (yielding 300 observations per min per rat) and acquired on-line using Threshold Activity software (MED-Associates Inc., Burlington, VT). The average raw load-cell output was used to quantify activity. Freezing was quantified by calculating the number of observations below a freezing threshold [17]. Freezing was scored only if a rat was immobile for 1 s or longer; thus, freezing was scored only for five or more contiguous observations. For each session, the freezing observations were converted to a percentage of total observations.

All training occurred in Context A. The chambers were cleaned with a 5% ammonium hydroxide solution, and stainless steel pans with a thin layer of the same solution were placed under the grid floors before rats were placed in the chambers. Illumination was provided by both the room lights and a small stimulus light (15 W) in each chamber, and background noise (65 dB, A-scale) was supplied by ventilation fans in each chest. Contextual fear testing occurred in Context A and, for auditory fear conditioning experiments, tone fear testing occurred in Context B. For Context B, a 1% acetic acid solution was used to clean the chambers and placed in the pans under the grid floor. In addition, illumination was provided only by a 15-W red light in the room, and ventilation fans were turned off.

2.4. Experiment 1: dose-response analysis of the effects of intra-amygdala H7 on contextual fear conditioning

The subjects were 55 adult male rats. All rats received bilateral cannulas implants aimed at the BLA. After 1 week of recovery from surgery, pairs of rats were transported to the laboratory and given a mock intracranial infusion. On arrival, the obturators were removed and 33 gauge injection cannulas that extended 1 mm past the guide cannulas were inserted (Plastics One, Roanoke, VA). An infusion pump (Harvard Apparatus, South Natick, MA) was turned on for 2.5 min at a rate of 0.1 μl/min — no fluid was infused into the brain during the mock infusion. The pumps were then turned off, and after 1 min, the obturators were replaced and the rats were returned to their home cages.

On the conditioning day, which occurred 24 h after the mock infusion, rats underwent a similar procedure, but the injection cannulas were attached to the pump with polyethylene tubing (PE-20; A-M Systems, Inc., Carlsborg, WA). Rats were administered an infusion of either vehicle (100 mM PBS, pH 7.4) or H7 in varying concentrations (0.04, 0.40, 4.00, or 8.00 mg/ml; Tocris, Ballwin, MO). This resulted in doses of 0, 0.01, 0.10, 1.00, or 2.00 μg/side. During the infusion, the rats were prevented from grooming with finger pokes, as grooming often dislodged the injection cannulas. The rats were returned to their home cages for 15 min before being transported to the conditioning chambers.

Three minutes after placement in the conditioning chambers (Context A), the rats received five unsignaled footshocks (1 s, 1.0 mA, 1-min inter-shock interval). The rats were returned to their home cages 1 min after the final shock. Fear conditioning to the context was assessed by returning the rats to the conditioning chambers 24 h later and assessing freezing during a 4-min extinction test. Eight rats were excluded from all analyses due to inaccurate cannula placement.

2.5. Experiment 2: effects of BLA versus CEA infusions of H7 on contextual and auditory fear conditioning

The subjects were 45 adult male rats. Half of the rats received bilateral cannulas implants aimed at the BLA, and the other half of the rats received bilateral cannula implants aimed at the CEA. One week after surgery, rats received a mock infusion as described in Experiment 1. Twenty-four hours later, the rats were transported to the laboratory and infused with 0.25 μl of either vehicle or an 8.0-mg/ml solution of H7 (2.0 μg infused per side), as previously described. Rats were returned to their home cages for 15 min before placement in the conditioning chambers (Context A).

Three minutes after being placed in the chambers, rats received five tone (10 s, 85 dB, 2 kHz)-footshock (2 s, 1.0 mA shock) pairings (70 s inter-trial interval). The rats were returned to their home cages 1 min after the final shock. Fear conditioning to the context was assessed by returning the rats to the conditioning chambers 24 h later and assessing freezing during an 8-min extinction test. Fear conditioning to the tone was assessed by returning the rats to a novel context (Context B) 24 h after the context test, and assessing freezing during an 8-min tone (85 dB, 2 kHz), which was delivered 2 min after placement of the rats in the chambers. Four rats were excluded from the analyses due to inaccurate cannula placement.

2.6. Experiment 3: analysis of the effects of intra-amygdala H7 on the acquisition versus expression of contextual fear conditioning

The subjects were 32 adult male rats. All rats received bilateral cannula implants aimed at the BLA. The rats were randomly assigned to a 2×2 design, receiving either vehicle or H7 prior to training, and vehicle or H7 prior to testing. Twenty-four hours before training, the rats received a mock infusion as described in Experiment 1. On the training day, the rats were infused with 0.25 μl of either vehicle or a 4-mg/ml solution of H7 (1.0 μg/side). The rats were returned to their home cages for 15 min before placement in the conditioning chambers.

Three minutes after placement in the chambers, the rats received context conditioning as described in Experiment 1. Twenty-four hours after training, rats again received an infusion of 0.25 μl of either vehicle or 4.0 mg/ml H7 15 min prior to a 4-min context extinction test. The rats were returned to their home cages for 15 min before placement in the chambers for the extinction test. Seven rats were excluded from the analyses due to either inaccurate cannula placements, or because the injectors became dislodged during the infusion process.

2.7. Histology

Histological verification of cannula placement was performed after behavioral testing. Rats were perfused across the heart with 0.9% saline followed by 10% formalin. Brains were removed from the skull and post-fixed in 10% formalin for 2 days and 10% formalin/ 30% sucrose until sectioning. Coronal sections (40 μm thick, taken every 160 μm) were cut on a cryostat (-18°C) and wet mounted on glass microscope slides with 70% ethanol. After drying, sections were stained with 0.25% thionin to visualize cell bodies. Cannula placements were reconstructed on stereotaxic atlas templates [33].

2.8. Data analysis

For each session, the freezing data were transformed to a percentage of total observations, a probability estimate that is amenable to analysis with parametric statistics. These probability estimates of freezing were analyzed using analysis of variance (ANOVA). Post-hoc comparisons in the form of Fisher's Least Significant Difference tests were performed following a significant omnibus F-ratio. All data are represented as means $\pm$ the standard errors of the means (S.E.M.s).

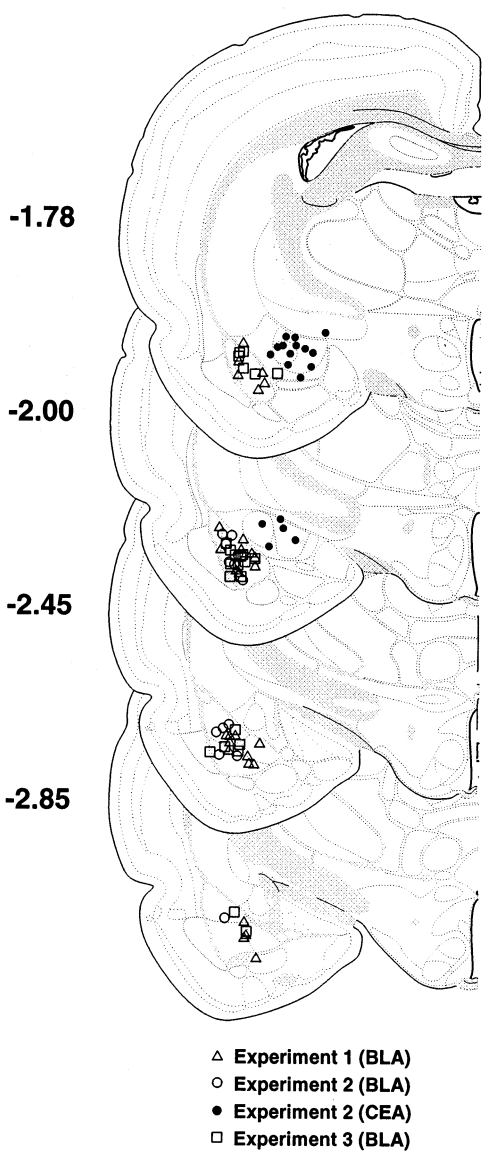

Fig. 1. Localization of injection cannula tips in the basolateral (BLA) and central (CEA) amygdala. The schematic representation of injection cannula tips is shown for all rats included in the analyses of Experiments 1–3. The numerical values to the side of the coronal half-sections (45) indicate the position of each section in millimeters relative to bregma.

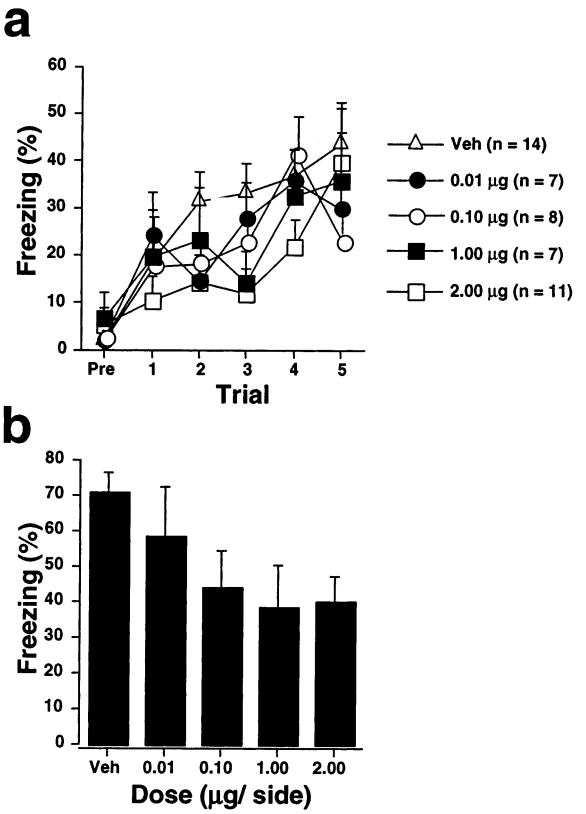

Fig. 2. Experiment 1: Dose-response analysis of the effects of intra-amygdala H7 on contextual fear conditioning. (a) Mean percentage of freezing ($\pm$ S.E.M.) exhibited on the training day, both prior to shock (Pre) and during the 1 min interval following each footshock (Trials 1–5) and during the 4-min context extinction test (b) in rats infused with varying doses of H7 15 min prior to training.

3. Results

3.1. Histology

The injection cannula tip placements for all animals included in analyses are summarized in Fig. 1. Placements were bilaterally symmetrical and localized within the target amygdaloid nuclei.

3.2. Intra-amygdala infusion of H7 blocks conditional fear in a dose-dependent manner

As shown in Fig. 2(b), intra-amygdala infusion of H7 into the BLA prior to fear conditioning attenuated conditional freezing to contextual cues in a dose-dependent manner. This observation was confirmed by a significant main effect of Dose [$F_{(4,42)} = 2.9$; $P < 0.05$] in the ANOVA. Post-hoc comparisons ($P < 0.05$) revealed that rats infused with either 0.1, 1.0, or 2.0 μg of H7 per side froze at significantly lower levels than rats infused with vehicle. Importantly, immediate post-shock freezing (Fig. 2(a)) was comparable across all doses of H7. This was confirmed in the ANOVA by the absence of a

main effect of Group [$F_{(4,42)} = 0.7$], and a non-significant Group × Trial interaction [$F_{(20,210)} = 1.5$]. Post-hoc comparisons ($P < 0.05$) revealed that all groups attained equivalent levels of asymptotic freezing. These results indicate that H7 does not affect the acquisition or expression of a short-term conditional fear memory assessed immediately following footshock, but does impair the acquisition of a long-term conditional fear memory assessed 24 h after conditioning.

3.3. Inhibition of amygdaloid PKC/PKA attenuates both context and tone freezing in a region-specific manner

In the second experiment, we examined the effects of H7 administration on the acquisition of both auditory and contextual fear. Moreover, we sought to determine whether protein kinases in the CEA also contribute to the establishment of long-term fear memories. As shown in Fig. 3, intra-BLA infusion of H7 caused a significant decrease in both context (Fig. 3(b)) and tone (Fig. 3(c)) freezing, whereas intra-CEA infusion of H7 did not affect either context or tone freezing. These observations were confirmed by a significant main effect of Group in the ANOVA for both the context [$F_{(2,38)} = 4.9$; $P < 0.01$] and tone [$F_{(2,25)} = 3.7$; $P < 0.05$] tests. Post-hoc comparisons ($P < 0.05$) revealed that rats infused with H7 in the BLA exhibited significantly lower levels of freezing than both vehicle-treated rats and rats infused with H7 in the CEA at nearly all time points during both context and tone testing; vehicle-treated rats and rats infused with H7 in the CEA did not differ from one another. Infusion of H7 into the BLA or CEA did not affect immediate postshock freezing on the training day (Fig. 3(a)), again indicating that H7 selectively affects acquisition of long-term fear memories. This was confirmed by a non-significant effect of Group in the ANOVA [$F_{(2,38)} = 2.3$], and the absence of a significant Group × Trial interaction [$F_{(10,190)} = 1.1$].

To insure that the deficits in conditional freezing in rats administered H7 were not due to reduced perception of the footshock unconditional stimulus (US) during training, footshock reactivity was assessed by measuring each rat's activity during the five 2-s footshocks. No differences in shock-elicited activity were observed between any groups on this measure [$F_{(2,25)} = 0.9$]. To confirm that the long-term deficits in conditional tone freezing in the BLA H7 group were not caused by an impairment in the ability to process tone information during training, we assessed freezing to the tone CS on the conditioning day. Freezing to each 10-s tone was normalized by subtracting context freezing during the preceding 58-s interval. Freezing to the tone CS reliably increased over the conditioning session [significant effect of Tone Trial, $F_{(4,100)} = 2.5$; $P < 0.05$], and there was no effect of drug infusion on the acquisition of tone freezing [non-significant effect of Group, $F_{(2,25)} = 0.4$; non-significant Group × Tone Trial interaction, $F_{(8,100)} = 0.6$]. This suggests that administration of H7 prior to training does not impair sensory processing of the tone during conditioning. Together, these results indicate that H7 selectively affects acquisition of long-term fear memories.

3.4. The effects of H7 are not attributable to state-dependency or performance deficits

In the third experiment, we examined the possibility that deficits in freezing in rats trained after an intra-amygdala H7 infusion were due to a shift in behavioral state from conditioning to testing. We assessed the state-dependency of the H7 effect by infusing H7 into the BLA before conditioning, extinction testing, or both. As shown in Fig. 4(b), PKC/PKA inhibition in the BLA disrupted the learning, but not the performance, of conditional freezing. That is, infusion of H7 into the BLA prior to fear conditioning produced a significant attenuation of conditional freezing, but

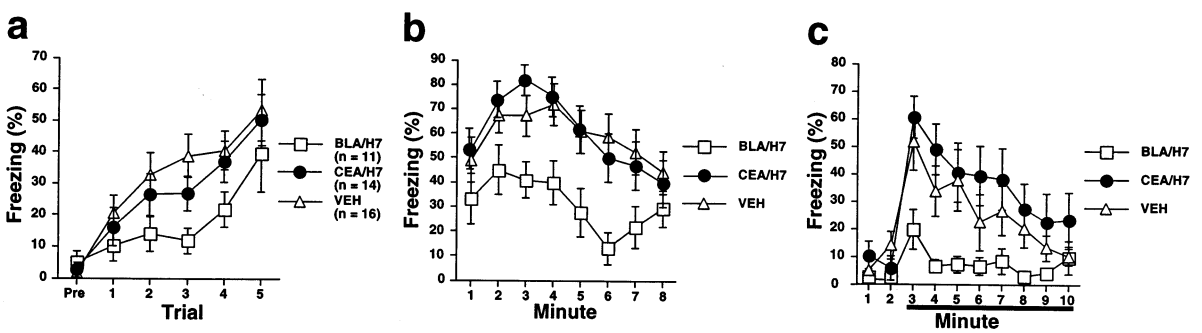

Fig. 3. Experiment 2: Effects of BLA versus CEA infusions of H7 on contextual and auditory fear conditioning. Mean percentage of freezing (±S.E.M.) exhibited on the training day (a), the 8-min context extinction test in the training context (Context A) (b), and the 8-min tone extinction test in a novel context (Context B) (c) in rats receiving a vehicle or H7 infusion into the basolateral (BLA) or central (CEA) amygdala. There were no differences observed between rats infused with vehicle in the BLA or CEA for either contextual or tone freezing, thus the two groups were collapsed into a single 'vehicle' group for both measures. The presence of the tone is indicated by the black bar below the x-axis.

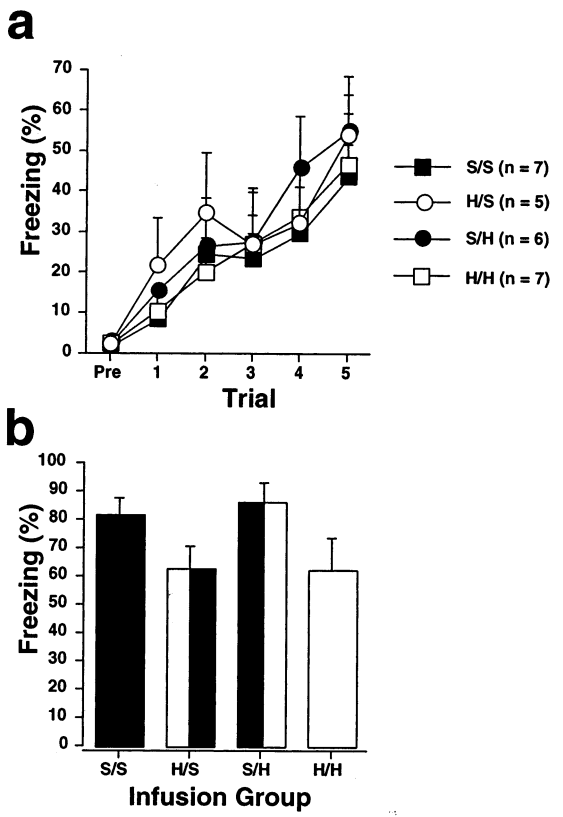

Fig. 4. Experiment 3: Analysis of the effects of intra-amygdala H7 on the acquisition vs. expression of contextual fear conditioning. Mean percentage of freezing (± S.E.M.) exhibited on the training day (a) and during the 4-min context extinction test (b) in rats infused with saline (S) or H7 (H) prior to training, testing, or both. S/S = Saline–Saline, S/H = Saline–H7, H/V = H7–Saline, H/H = H7–H7.

freezing was unaffected by an infusion of H7 prior to extinction testing. Thus, H7 does not impair the processing of contextual information per se, or the performance of freezing behavior. This observation was confirmed in the ANOVA by a significant main effect of Training [$F_{(1,21)} = 6.1$; $P < 0.05$], and a non-significant main effect of Testing [$F_{(1,21)} = 0.2$]. The non-significant Training × Testing interaction [$F_{(1,21)} = 0.01$] indicated that the conditional freezing deficits produced by intra-amygdaloid H7 were not state-dependent. As in Experiments 1 and 2, commensurate levels of immediate post-shock freezing were observed for all groups on the training day (Fig. 4(a)). This was confirmed by the absence of both a main effect of Group [$F_{(3,21)} = 0.4$] and a Group × Trial interaction [$F_{(15,105)} = 0.3$] in the ANOVA. Again, this indicates that the differences in conditional freezing observed on the testing day were likely due to effects of the drug on long-term memory consolidation. In none of the experiments did intra-amygdaloid H7 affect motor activity prior to footshock or footshock sensitivity (data not shown). Thus, our experiments reveal that inhibition of PKA and PKC activity in the BLA, but not CEA, produces a selective deficit in the acquisition of long-term memories for both auditory and contextual fear.

4. Discussion

In the present study, we examined the role of amygdaloid protein kinases in the acquisition and expression of fear memories by using direct intra-amygdaloid infusion of H7, a PKC/PKA inhibitor. The results revealed that infusion of H7 into the BLA produces a dose-dependent attenuation of contextual fear conditioning (Experiment 1) and that H7 attenuates the acquisition of both auditory and contextual fear (Experiment 2). Intra-amygdala infusion of H7 impaired the learning as opposed to the performance of conditional fear to the extent that pre-testing infusions of H7 did not affect the expression of conditional freezing (Experiment 3). Moreover, the effects of H7 were selective for long-term fear memories insofar as immediate postshock freezing, a measure of short-term fear memory, was not affected by intra-amygdala H7 infusion. Our results also demonstrate that the BLA is the critical locus of PKA and PKC activity within the amygdala during fear conditioning — infusion of H7 into the CEA did not affect the acquisition of fear conditioning (Experiment 2). Collectively, these findings extend earlier work using intracerebroventricular administration of protein kinase inhibitors [2,31] and identify a critical role for protein kinase activity in the BLA in the acquisition of long-term conditional fear memories.

The important role for amygdaloid protein kinase activation in establishing long-term fear memories is not surprising given the sensitivity of fear conditioning to NMDA receptor blockade [11,21,25]. Unlike NMDA receptor blockade, however, protein kinase inhibition in the amygdala produced a selective deficit in the acquisition, but not expression, of conditional fear memories. This suggests that the effects of NMDA receptor antagonists on both the acquisition and expression of fear conditioning are more likely related to reductions in cell excitability produced by the antagonists [13,22] rather than a specific impairment in LTP induction. Although we did not assess the effects of H7 on amygdaloid cell excitability, it is unlikely that H7 produced its effects on fear conditioning through a non-specific attenuation of amygdaloid activity. That is, in contrast to neurotoxic amygdala lesions [17,18,20], H7 did not affect either the expression of immediate-postshock freezing on the conditioning day or the expression of conditional freezing during retention testing.

Based on the available evidence, it seems reasonable to speculate that H7 attenuated the acquisition of long-term conditional fear memories by impairing the induction of amygdaloid LTP. Several recent reports are

consistent with a role for LTP in the acquisition of Pavlovian fear conditioning (see Ref. [19] for a review). Indeed, the selective effect of H7 on the acquisition of long-term conditional fear is consistent with the role PKA and PKC have been demonstrated to play in the induction of the late phase of LTP (L-LTP) in the CA1 region of the hippocampus [6,8,15] (see Ref. [34] for a review). Although it is not known whether protein kinase activity in the amygdala is involved in the induction of L-LTP, there is some evidence for a role for PKA in the expression of an early phase of amygdaloid LTP [7]. Further work is required to understand the specific contribution of protein kinase activity to amygdaloid LTP. Nonetheless, current data are consistent with the view that intra-amygdala kinase inhibitors attenuate long-term memory formation by disrupting the induction of L-LTP in amygdala neurons.

In contrast to the important role for PKA and PKC in the induction of LTP, these enzymes do not appear to be involved in the expression or maintenance of L-LTP, at least in the hippocampus [14,15]. Based on this evidence, one would not expect PKA/PKC inhibitors to affect the expression of long-term conditional fear memories, which are presumably dependent on late phase LTP in the amygdala [19]. Consistent with this prediction, we found that intra-amygdala H7 infusions did not affect the expression of conditional freezing when administered 15 min prior to a retention test, which was conducted 24 h after fear conditioning. It has also been reported that PKA/PKC activity is not required for the induction or expression of either post-tetanic (<10 min) or short-term (<1 h) potentiation. This finding is consistent with our observation of normal immediate post-shock freezing in rats trained after infusion of H7 into the BLA. Thus, our results support the view that PKA/PKC-independent forms of plasticity underlie the short-term fear memories required for immediate post-shock freezing [16]. Considering all of our results, it is apparent that the temporal profile of the conditional fear deficits we observed in rats infused with H7 closely parallels the temporal profile of synaptic plasticity deficits observed after inhibition of PKA and PKC.

It should be noted that other protein kinases, including mitogen-activated protein kinase (MAPK) [1,30] and Ca^{2+}-calmodulin-dependent kinase (CaMKII) [23] have been implicated in Pavlovian fear conditioning. The involvement of these other kinase pathways in fear conditioning might explain the partial attenuation of conditional freezing that we have observed after PKA/PKC inhibition in the BLA. Thus, the MAPK and CaMKII pathways may be capable of supporting some degree of fear conditioning in the absence of PKA and PKC activity. Work is currently underway in our laboratory to determine the relative contributions of amygdaloid PKA, PKC, MAPK, and CaMKII to fear conditioning using inhibitors selective for each of these kinases.

In sum, the present results reveal an important role for amygdaloid PKA and PKC in the acquisition of long-term, but not short-term, conditional fear memories. The involvement of amygdaloid PKA and PKC in the acquisition of conditional fear provides additional support for a role for amygdaloid LTP in this form of learning. Collectively, these results provide important new insight into the intracellular biochemical cascades underlying amygdala-dependent memory formation during Pavlovian fear conditioning in rats.

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