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Activation of α_2 Adrenergic Receptors Suppresses Fear Conditioning: Expression of c-Fos and Phosphorylated CREB in Mouse Amygdala

M Frances Davies*[,1], Janet Tsui[2], Judy A Flannery[2], Xiangqi Li[1], Timothy M DeLorey[3] and Brian B Hoffman[2],

[1]Department of Anesthesia, Stanford University of Anesthesiology, Veterans Affairs, Palo Alto Health Care System, Palo Alto, CA, USA; [2]Department of Medicine, Stanford University of Medicine, Veterans Affairs, Palo Alto Health Care System, Palo Alto, CA, USA; [3]Molecular Research Institute, Mountain View, CA, USA

α_2 adrenergic agonists such as dexmedetomidine generally suppress noradrenergic transmission and have sedative, analgesic, and antihypertensive properties. Considering the importance of the neurotransmitter norepinephrine in forming memories for fearful events, we have investigated the acute and chronic effects of dexmedetomidine on discrete cue and contextual fear conditioning in mice. When administered before training, dexmedetomidine (10–20 μg/kg, i.p.) selectively suppressed discrete cue fear conditioning without affecting contextual memory. This behavioral change was associated with a decrease in memory retrieval-induced expression of c-Fos and P-CREB in the lateral, basolateral, and central nuclei of the amygdala. Dexmedetomidine's action on discrete cue memory did not occur in α_{2A} adrenoceptor knockout (KO) mice. When dexmedetomidine was administered after training, it suppressed contextual memory, an effect that did not occur in α_{2A} adrenoceptor KO mice. We conclude that dexmedetomidine, acting at α_{2A} adrenoceptors, must be present during the encoding process to decrease discrete cue fear memory; however, its ability to suppress contextual memory is likely the result of blocking the consolidation process. The ability of α_2 agonists to suppress fear memory may be a valuable property clinically in order to suppress the formation of memories during stressful situations.
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INTRODUCTION

Stressful situations can trigger the typical 'fight and flight' response that activates the peripheral sympathetic and central noradrenergic systems to release norepinephrine. The increased central noradrenergic release can promote memory formation evoked by the fearful stimuli (Vaandrager and de Jonge, 1996), and may explain why we remember exciting or traumatic events more vividly than everyday ones (Cahill and McGaugh, 1998). However, fear learning can be detrimental in some situations, such as for patients undergoing medical procedures, or where the persistent fear response evokes chronic stress (Svensson, 1987). Consequently, a means to selectively reduce fear learning is desirable in such circumstances.

*Correspondence: MF Davies, Department of Anesthesia, VAPAHCS, 3801 Miranda Avenue, Palo Alto, CA 94304, USA, Tel: +1 650 493 5000 ext. 64854, Fax: +1 650 852 3423, E-mail: fdavies@stanford.edu
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Fear conditioning is a form of associative learning in which defense responses can be elicited by a neutral conditioned stimulus (CS) such as an audible tone, when it has been previously paired with an aversive unconditioned stimulus (US) such as an electric footshock. The amygdala is the major brain region responsible for pairing the CS with the US and then relaying this association onto other regions (Fanselow and Kim, 1994). Discrete cue learning to an auditory tone largely occurs in the amygdala (Rogan *et al*, 1997) whereas contextual learning also involves the hippocampus (Anagnostaras *et al*, 2001). The basolateral amygdala has reported to be crucial in the formation and storage of fear memories (Maren, 1999). In the amygdala, long-term potentiation (LTP), hypothesized to be the mechanism behind some forms of learning, has been demonstrated to require a strong noradrenergic input in order to be established (Huang *et al*, 2000; Schafe *et al*, 2001), whereas norepinephrine plays mainly a modulatory role in the hippocampus LTP (Dunwiddie *et al*, 1982; Sarvey *et al*, 1989). In both areas, phosphorylation of cyclic AMP response element-binding protein (CREB) and expression of c-Fos are critical in changing gene expression that is

important to memory formation (Dragunow *et al*, 1989; Hall *et al*, 2001; Moller *et al*, 1994). The transcription factors P-CREB and c-Fos increase during memory encoding (Tischmeyer and Grimm, 1999), consolidation (Kang *et al*, 2001), and retrieval (Hall *et al*, 2001). Whenever information is being actively processed in the brain, the memory is unstable and is vulnerable to drugs that interfere with paths leading to protein synthesis (Nader *et al*, 2000). The nature by which a drug affects these processes provides insight into the mechanisms by which the drug is likely to influence memory formation.

Stimulation of α_2 adrenoceptors (ARs) decreases norepinephrine release in the amygdala (Fendt *et al*, 1994), and therefore may decrease fear learning. However, this hypothesis has not been rigorously tested. We have addressed this problem and found that the α_2 agonist dexmedetomidine decreases fear memory but not contextual memory. To further understand the mechanism of action by which α_2 ARs may influence fear memory (Hall *et al*, 2001; Moller *et al*, 1994), we have assessed dexmedetomidine's affect on c-Fos and P-CREB production in the amygdala. In doing so, we have further elucidated the α_2 adrenoceptor subtype responsible for the observed behavioral action.

METHODS

Mice

The experimental protocol was approved by the Animal Care and Use Committee at the Veterans Administration Palo Alto Health Care System. Wild-type C57BL/6J mice (Jackson Laboratories, Davis, CA), α_{2A} AR knockout (KO) adult male mice originated by Dr Brian Kobilka (Altman *et al*, 1999) and D79N mice originated by Dr Lee Limbird (MacMillan *et al*, 1996), which contains a point mutation that functionally inactivates the α_{2A} AR, were bred in house. Both were backcrossed onto a single C57BL/6J background. They were housed in groups of eight, kept on a 12 : 12 h light/dark cycle and were allowed free access to food and water.

Behavioral Apparatus

Four identical chambers ($20 \times 25 \times 22\,cm^3$) (MED Associates, Georgia, VT) were used for Pavlovian fear conditioning and testing. Each chamber contained metal floor bars through which an electrical current could be passed. Before each trial, floor bars were tested for correct amperage and correct decibel levels were assessed for both the tone generator and white noise generator.

Pavlovian Fear Conditioning

Before testing each day, the mice were moved to a holding room adjacent to the testing room and allowed to acclimate for at least 30 min. The mice were placed individually in one of the four identical experimental chambers (MED Associates, St Albans, VT) that had been scented with 1% ammonium hydroxide solution before testing. Chambers were backlit with diffuse fluorescent light and a white noise generator provided 70 dB background noise. Mice were allowed to explore the experimental chamber for 4 min, after which they were exposed to a loud tone (85 dB, 2.9 kHz) for 33 s with the last 3 s coupled with a 0.75-mA 'scrambled footshock'. This procedure was repeated for a total of three episodes with a 1-min period separating each episode. At 1 min after the final footshock, the mice were returned to their home cages. After 24 h, contextual memory was assessed by placing the mice back into the freshly rescented (1% ammonium hydroxide) conditioning chambers in which they were trained, for a 4-min test period in the absence of footshock. Conditioned fear to the context was assessed by measuring the freezing response, according to the methods of Fanselow and Bolles (Fanselow and Bolles, 1979). Freezing was defined as the absence of all visible movements of the body and vibrissae aside from those necessitated by respiration. For the discrete memory assessment, the context was altered by changing the visual, tactile, and olfactory cues. The new context consisted of chambers scented with 2.5% lemon juice concentrate and reconfigured with three white opaque plastic inserts that convert the inner dimensions of the chamber to be triangular in shape rather than the original rectangular shape. Fluorescent backlighting and white noise generation were switched off. Baseline fear to the new context was assessed by recording the freezing response for 4 min. Following this baseline exploration period, the tone originally presented in the training session was sounded for 4 min during which time freezing behavior was again recorded. An observer, blind to the genotype of the mice, scored each mouse every 8 s, for a total of 4 min, for the presence or absence of freezing. These data were transformed to a percentage of total observations. Data were analyzed by one-way analysis of variance (ANOVA) using GraphPad PRISM 3 (GraphPad Software). Separate treatment effects between groups were analyzed *post hoc* using Dunnett's comparisons.

Immunohistochemistry

At 1 h after discrete cue memory assessment, mice were euthanized with carbon dioxide and perfused with 20 ml of ice-cold phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS injected into the left ventricle of the heart. Brains were then harvested, fixed in 4% paraformaldehyde-PBS for 6 h, and cryoprotected in 30% sucrose-phosphate buffer (PB) overnight. The brains were sliced coronally into 3–4-mm thick slices in the regions of the hippocampus and amygdala using an adult mouse coronal 1 mm brain matrix (Ted Pella, Redding, CA), embedded, and stored at $-80^{\circ}C$ until sectioning. After being cut into 40-μm sections with a cryostat microtome (Leica, CM 1850 Wetzlar, 35578 Germany) and placed in 0.1 M PB, these floating sections were washed with PB twice and then blocked with a 3% solution of normal goat serum (NGS) in buffer 1 (0.1 PBS, NGS, and Triton X-100) for 5 h followed by overnight incubation on a shaker with either anti-c-Fos (1 : 10 000, Santa Cruz Biotechnology, CA) or anti-P-CREB (1 : 500, Cell Signaling, Beverly, MA) polyclonal rabbit IgG. Tissues were then washed with buffer 1 and incubated with secondary biotinylated anti-rabbit IgG (Vector Labs, Burlingame, CA) for 2 h. After washing with buffer 1, VECTASTAIN ABC reagent (Vector Labs, Burlin-

game, CA) was added and allowed to adhere to the secondary antibody for 1 h. The sections were subsequently washed with buffer 2 (0.1 PBS and Triton X-100), stained with DAB (Vector Labs, Burlingame, CA) substrate for 1–4 min, and washed in deionized water. Sections were mounted onto precoated Frost+ slides (Fisher Scientific, Pittsburgh, PA) and air-dried overnight. Slides were dehydrated in 100% alcohol for 10 min, submerged in a series of xylene washes, and covered with glass coverslips using Permount mounting medium (Fisher Scientific, Pittsburgh, PA). Slides were viewed and photographed with a light microscope connected to a SPOT RT Slider Camera (Diagnostic Instruments, Sterling Heights, MI). Amygdala and hippocampal areas were identified with the help of *Mouse Brain* atlas (2001) (Paxinos and Franklin, 2001), and positive c-Fos and P-CREB spots were counted using NIH Image. Digital photographs of every fourth section were counted and individual spots were counted and marked on the photographs. The averages were taken of all sections that corresponded to one atlas coronal section. This data point was then averaged across mice treated in the same manner.

Drugs

Dexmedetomidine (10–20 μg/kg) or saline vehicle was injected intraperitoneally in a volume of 10 ml/kg.

Statistics

Statistical significance was tested with either one-way ANOVA with Dunnett's *post hoc* test or Student's *t*-test where appropriate, using Prism version 3.0a for Macintosh (GraphPad Software, San Diego, CA, USA).

RESULTS

Changes in norepinephrine release could potentially augment fear learning in a variety of ways. Information gathered through the five senses is initially stored in a sensory buffer. If these impressions persist, they may be encoded into short-term memory storage. These memories in turn may be consolidated into long-term memories. When information from long-term storage is required to perform certain tasks, memories are retrieved. To determine the processes that are sensitive to drug administration, it is necessary to examine its effect at each phase of the memory creation process.

It is critical to memory formation that the US and CS are both perceived. Since dexmedetomidine has analgesic actions in a variety of models (Guo *et al*, 1996; Hayashi *et al*, 1995, 1996), we were concerned that it might suppress the noxious nature of the US, namely electric footshock. Consequently, we under took preliminary experiments to assess whether the footshock was sufficiently salient to elicit pain when dexmedetomidine was administered. To do this, we determined the shock threshold required to elicit flinching responses, 30 min after the test dose of dexmedetomidine (20 μg/kg i.p.) was administered. Unexpectedly, dexmedetomidine actually decreased thresholds in C57BL/6J wild-type mice (Table 1) although it had no effect in the D79N or α_{2A} AR KO mice. To ensure that the mice exhibited nociceptive behavior to the US in subsequent experiments, a supramaximal shock of 0.75 mA was used for all strains. We have demonstrated in a previous study (Davies *et al*, 2003) that applying the tone alone or shock without pairing leads to a lack of freezing to the tone when sounded during discrete cue testing.

Table 1 Evaluation of Pain Perception for C57BL/6, D79N, and α_{2A} AR KO Mice Injected with Dexmedetomidine

	Flinch threshold (mA)
C57BL/6	
Saline	0.17 ± 0.02
Dexmedetomidine	0.16 ± 0.01
D79N	
Saline	0.18 ± 0.01
Dexmedetomidine	0.20 ± 0.02
α_{2A} AR KO	
Control	0.21 ± 0.01
Dexmedetomidine	0.21 ± 0.01

Mice were injected with either dexmedetomidine (20 μg/kg i.p.) or saline and then placed in the experimental chambers 30 min after injection. The intensity of the footshock was increased in increments of 0.05 mA and the intensity required to evoke flinching was recorded. $N = 4$.

Acute Effect of Dexmedetomidine on Fear Conditioning

In assessing the effect of a drug on fear memory, it is first necessary to ascertain that the drug does not alter the perception of either the context or tone used in the training period. Figure 1a shows the exploratory behavior of the mice when they are first exposed to the training cage and for the three intervals after the tone–shock pairing. The footshock itself significantly reduced exploratory behavior in saline-treated mice during all three intershock intervals (Figure 1a), indicating that it was salient enough to be remembered in the short term. Mice administered dexmedetomidine (20 μg/kg) exhibited a significant decrease in the initial chamber exploration prior to the footshock when compared to saline-treated mice (Figure 1a). However, after the first shock, the dexmedetomidine-treated group moved significantly less than the saline-treated nonshocked and shocked mice. It was clear that the exploratory behavior of dexmedetomidine-treated mice was likewise decreased by the first footshock, indicating that the mice experienced both pain and demonstrated short-term memory.

When saline- and dexmedetomidine-treated mice are placed in a novel contextual environment, different than the context in which they were trained, they both exhibited less than 10% freezing (Figure 1b) suggesting that neither group has generalized their fear of the original training context to the new test context. Mice placed in the novel environment but not shocked during the training period exhibited minimal freezing in the novel context whether with (2.6 ± 1.5%) or without (1.6–1.6%) the tone. Wild-type mice that were fear conditioned to a tone froze approximately 50% of the total possible time when re-exposed to

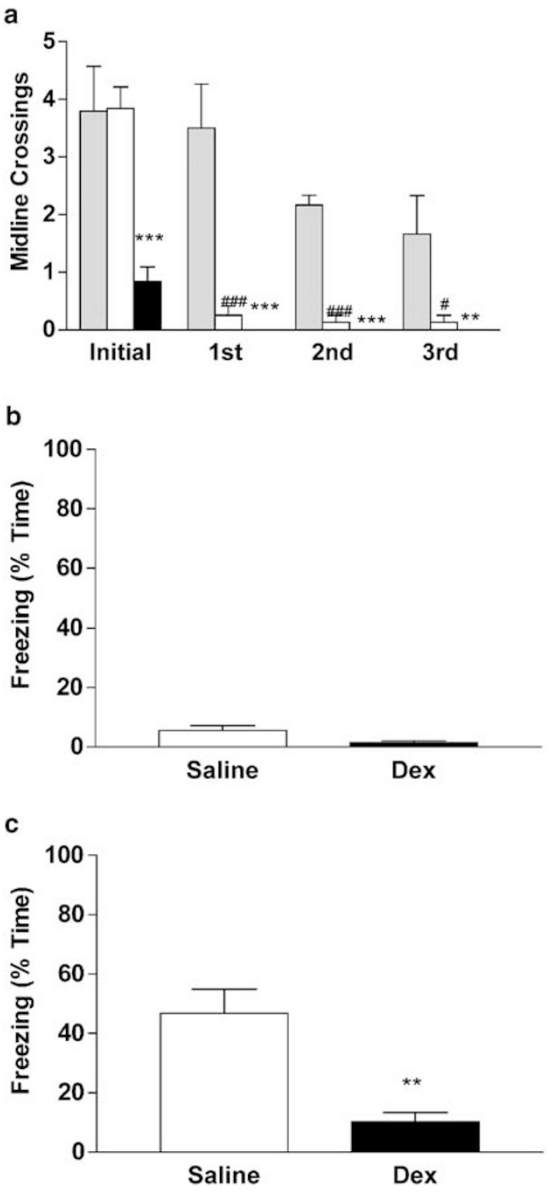

Figure 1 Effect of preadministering dexmedetomidine (20 μg/kg i.p.) on fear-conditioned C57BL/6 wild-type mice. (a) Chamber exploration during the initial 4-min acclimaton period in the training chamber was significantly decreased by administration of dexmedetomidine (20 μg/kg i.p.) 30 min before presentation of the first shock. The initial exploration data are expressed as the average centerline crosses per minute. During subsequent 1-min intervals following each tone and shock presentation, saline-treated mice that were shocked (white bars) exhibited a significantly reduction in exploration compared to saline-treated mice that were not shocked (light gray bar) (### $p<0.001$, # $p<0.05$, $N=6$–8). Dexmedetomidine-treated mice that were shocked (black bars) explored significantly less than saline-treated mice that were shocked (white bars) (***$p<0.001$, **$p<0.01$, $N=6$–8). (b) Dexmedetomidine had no effect on freezing when 24 h after training the mice were put into a novel environment different than the one trained in and allowed to freely explore for 4 min. (c) Dexmedetomidine reduced freezing in the novel environment when the tone was presented for 4 min. Bars represent mean + SEM. Statistically significant difference to saline-treated controls: **$p<0.01$, $N=6$.

the tone when placed in a novel context (discrete cue) 24 h after training (Figure 1c). Mice injected with dexmedetomidine (20 μg/kg i.p.) 30 min before footshock tone pairing, froze significantly less to the tone presentation compared to

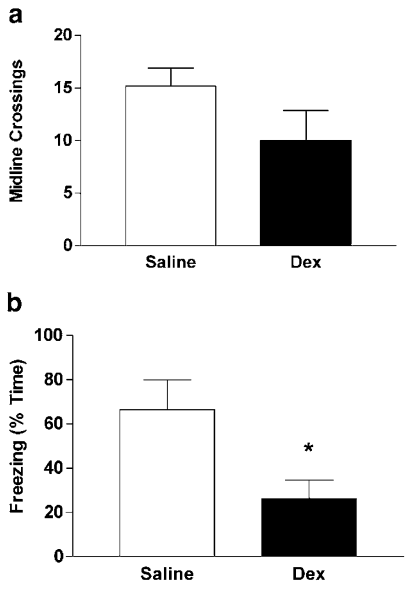

Figure 2 Effect of preadministration of a nonsedating dose of dexmedetomidine before fear-conditioned training in C57BL/6 wild-type mice. Dexmedetomidine (10 μg/kg i.p.) was administered 30 min before fear-conditioned training. (a) Chamber exploration during training was not affected by administration of this dose of dexmedetomidine. (b) When tested 24 h after training, the mice treated with dexmedetomidine froze significantly less in response to the tone. Bars represent mean + SEM. Statistically significant difference to saline-treated controls: *$p<0.05$, $N=6$.

controls when tested 24 h after training. To determine whether the sedative action of dexmedetomidine (20 μg/kg) was responsible for the suppression of the discrete cue fear conditioning, a lower, nonsedating dose of 10 μg/kg dexmedetomidine was also tested. This dose did not significantly affect the initial exploration of the chamber (Figure 2a), but significantly reduced freezing behavior compared to control in the presence of the tone when presented 24 h after training (Figure 2b). This result suggests that the sedative action of dexmedetomidine administration is not responsible for its fear reducing action.

To determine whether dexmedetomidine suppression of discrete cue freezing is attributable to an effect on the encoding or on the consolidation phase of fear memory, dexmedetomidine (20 μg/kg) was also given immediately after training to a separate set of wild-type mice. As expected, the exploratory behavior during training in the two groups of mice was the same (Figure 3a). Under these conditions, the drug had no effect on discrete cue freezing behavior compared to control (Figure 3b). Taken together with results presented in Figure 2, these findings support the hypothesis that dexmedetomidine influences the encoding rather than the consolidation processes.

Induction of P-CREB Immunoreactivity and c-Fos Immunoreactivity Within the Amygdala by Fear Conditioning

Transcription factors regulating gene expression in the lateral, basolateral nucleus, and the central nucleus of the amygdala, are likely to be important to the development of fear memory (Ferry *et al*, 1999b). Auditory and other

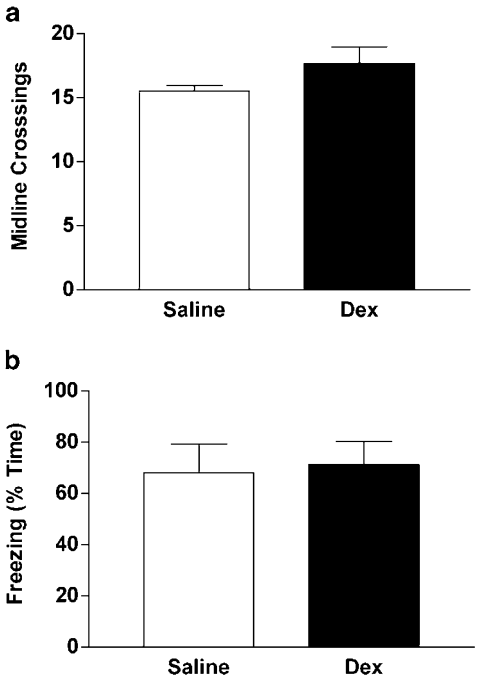

Figure 3 Effect of dexmedetomidine (20 μg/kg i.p.) administered immediately after training on fear-conditioned C57BL/6 wild-type mice. Dexmedetomidine (20 μg/kg i.p.) was administered immediately after training. (a) No significant difference between dexmedetomidine treated and untreated mice was observed during the initial chamber exploration period prior to sounding of the tone and presentation of the shock. (b) There was no significant difference between the two groups in freezing response to the tone during discrete cue assessment tested one day after drug administration and training. Bars represent mean + SEM. Statistically significant difference to saline-treated controls: $*p < 0.05$, $**p < 0.01$, $N = 6$.

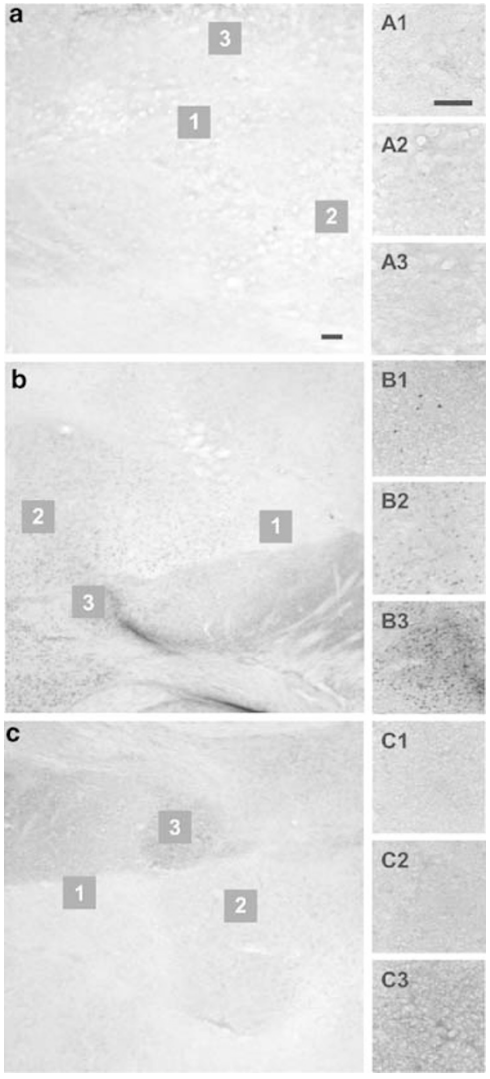

Figure 4 Effect of preadministration of dexmedetomidine on the number of cells expressing P-CREB in the amygdala evoked by discrete cue retrieval tested 24 h later. Immunohistochemical staining of P-CREB in a coronal section of the amygdala of C57BL6 mice (a) exposed to the training box and tone but not given a footshock, (b) given saline then trained, or (c) given dexmedetomidine (20 μg/kg, i.p.) 30 min before fear conditioning and then tested on the following day. Mice were killed 1 h after discrete cue testing. The numbers in the gray boxes refer to the areas that are enlarged in the side figures labeled. The (1) lateral, (2) basolateral, and (3) central nucleus of the amygdala of dexmedetomidine-treated mice had fewer P-CREB positive cells than saline-treated mice. Bregma −1.58 mm; interaural 2.22 mm; Bar = 100 μm.

sensory inputs generally terminate in the lateral amygdala (LeDoux *et al*, 1990; Romanski *et al*, 1993), and destruction of this area interferes with discrete cue conditioning (LeDoux *et al*, 1990). The lateral amygdala is the region proposed to be responsible for associating the CS, the auditory tone, with the nociceptive information generated by the US, the footshock (LeDoux *et al*, 1990). The central nucleus is essential for autonomic and emotional responses to stressful stimuli (LeDoux *et al*, 1988). The basolateral amygdala appears to play a role in conditioned aversion (Amorapanth *et al*, 2000) or inhibitory avoidance (Wilensky *et al*, 2000). Long-term memory has been reported to be facilitated by CREB protein overexpression in the basolateral amygdala (Josselyn *et al*, 2001). Moreover, fear memory retrieval has been shown to induce phosphorylation of CREB and expression of c-Fos in the basolateral amygdala (Hall *et al*, 2001) as well as the induction of a protein-synthesis-dependent reconsolidation of memories within the amygdala (Nader *et al*, 2000).

We wondered if the fear suppressive action of acute administration of dexmedetomidine given before training is associated with changes in phosphorylation of CREB and c-Fos expression in the three amygdala nuclei implicated in fear conditioning following retrieval. In untrained wild-type mice, there was no indication of P-CREB (Figure 4a) or c-Fos (Figure 5a) positive neurons in the lateral, basolateral, or central nucleus of the amygdala. In fear-conditioned mice killed 1 h after discrete cue testing, to stimulate fear retrieval, the number of P-CREB (Figure 4b) and c-Fos (Figure 5b) positive neurons significantly increased in all three amygdaloid nuclei. When dexmedetomidine was administered before training, the number of P-CREB (Figure 6a–c) and c-Fos (Figure 6d–f) positive neurons were significantly reduced in all three amygdaloidal regions.

As the hippocampus is thought to play a role in contextual memory, we endeavored to examine P-CREB and c-Fos expression in the hippocampal upon retrieval. We observed no expression of either transcription factor in the hippocampus following context re-exposure in the presence or absence of dexmedetomidine (data not shown).

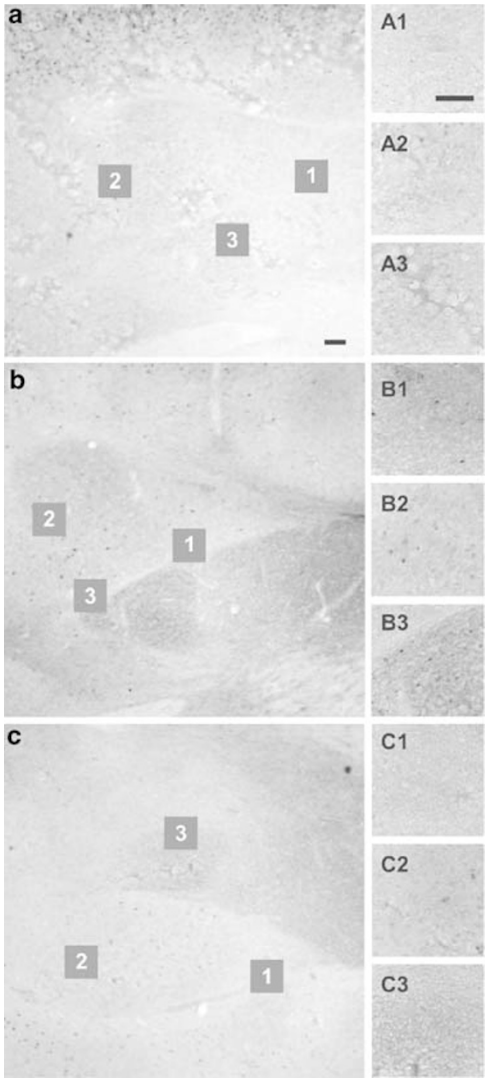

Figure 5 Effect of preadministration of dexmedetomidine on the number of cells expressing c-Fos in the amygdala evoked by discrete cue retrieval tested 24 h later. Immunohistochemical staining of c-Fos in a coronal section of the amygdala of C57BL6 mice (a) exposed to the training box and tone but not given a footshock, (b) given saline then trained, or (c) given dexmedetomidine (20 μg/kg) 30 min before fear conditioning and then tested on the following day. Mice were killed 1 h after discrete cue testing. The numbers in the gray boxes refer to the areas that are enlarged in the side figures labeled. The (1) lateral, (2) basolateral, and (3) central nucleus of the amygdala of dexmedetomidine-treated mice had fewer c-Fos positive cells than saline-treated mice. Bregma −1.58 mm; interaural 2.22 mm; Bar = 100 μm.

Effect of Dexmedetomidine on Discrete Cue Memory in Mice Deficient in α_{2A} ARs

There are three α_2 AR subtypes in the brain *A*, *B* and C (Blaxall *et al*, 1994; Flordellis *et al*, 1991; Regan *et al*, 1988), which could potentially mediate the amnestic effects of the nonselective α_2 agonist dexmedetomidine. As many of the central effects of α_2 agonists are mediated by the α_{2A} AR (Lakhlani *et al*, 1997), we sought to determine whether this AR subtype was also the major mediator of the fear amnestic effect by which dexmedetomidine acted. To test this, dexmedetomidine (20 μg/kg) was administered to α_{2A} AR KO mice prior to training. This dose was found not to affect the initial chamber exploration compared to saline (Figure 7a). At 24 h after training, mice were placed in a novel context, different from the one they were trained in. Both dexmedetomidine-treated α_{2A} AR KO mice and wild-type mice exhibited similar low levels of freezing in the novel context prior to tone (Figure 7b). In the presence of the tone dexmedetomidine-treated α_{2A} AR KO mice exhibited robust freezing in the discrete cue test compared to the control (Figure 7c). To further test the role of the α_{2A} AR, the effect of dexmedetomidine was also assessed in transgenic D79N mice that are functionally deficient in the α_{2A} AR (MacMillan *et al*, 1998). A similar behavioral pattern was observed in the transgenic D79N mice with dexmedetomidine having no effect on initial chamber exploration prior to training (Figure 8a), there was no change in freezing behavior in response to a context different from training (Figure 8b), and failed to reduce freezing behavior to the tone presentation in comparison to wild-type mice receiving dexmedetomidine (Figure 8c). Combined, these results demonstrate that the inhibitory effects of dexmedetomidine on discrete cue freezing are mediated through α_{2A} ARs.

Effect of Dexmedetomidine on Contextual Memory: The Role of α_{2A} ARs

Contextual memory differs from discrete cue memory in that the hippocampus plays a crucial role in contextual memory (Anagnostaras *et al*, 2001). Dexmedetomidine (10 μg/kg i.p.) administered 30 min before training had no effect on contextual memory when assessed 24 h later (Figure 9a). Unexpectedly, we found that administration of dexmedetomidine (20 μg/kg i.p.) to wild-type mice immediately following training decreased their freezing behavior in the contextual memory test (Figure 9b). To determine if the α_{2A} AR was responsible for this retrograde amnestic effect of dexmedetomidine, this experiment was repeated using α_{2A} AR KO mice (Figure 9c) and D79N mice (Figure 9d). Both α_{2A} AR KO and D79N mice given dexmedetomidine and placed in the same context froze to the same extent as controls not given dexmedetomidine, indicating that the α_{2A} AR mediates this retrograde amnestic action.

DISCUSSION

This study demonstrates that activation by dexmedetomidine of α_{2A} AR suppresses discrete cue fear conditioning only when administered prior to training, but not when given immediately after training. This suggests that α_2 AR agonists modulate the encoding rather than the consolidation process of memory formation. The lack of effect of dexmedetomidine on contextual memory when administered prior to training supports the concept that the noradrenergic system is not essential for the encoding of contextual memory. However, its inhibitory effect when administered after training points to a role for the noradrenergic system in the consolidation phase of spatial memory as has been observed by others when exogenous noradrenergic agents were administered after training (Cahill, 2000; Hatfield and McGaugh, 1999). Our results

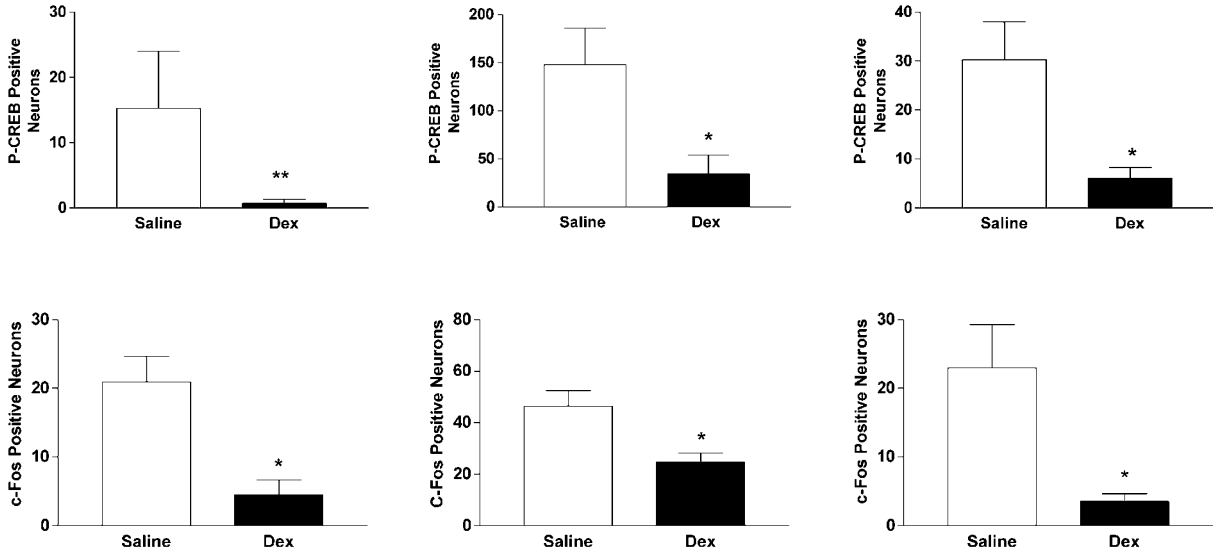

Figure 6 Effect of dexmedetomidine (20 μg/kg i.p.) administered 30 min before fear-conditioning training session on retrieval-induced expression of P-CREB (a–c) and c-Fos (d–f) in the lateral (a, d), basolateral (b, e), and central (c, f) nuclei. On day 1 wild-type mice were either injected with saline or dexmedetomidine (20 μg/kg i.p.) 30 min before training, on day 2 the mice were placed in a novel environment and presented with the conditioning stimulus tone in the absence of footshock for 4 min. Mice were then removed from the chamber and killed 1 h after discrete cue testing. Bars represent mean + SEM. Statistically significant difference to saline-treated controls $^{*}p<0.05$, $^{**}p<0.01$, $N=3$–4.

strongly suggest that the α_{2A} AR is largely responsible for the fear suppressing action of α_2 adrenergic agonists as shown by the studies performed in the α_{2A} AR KO and D79N mice. Furthermore, when dexmedetomidine was administered prior to training, we observed a reduction in phosphorylation of CREB and expression of c-Fos in the lateral, basolateral, and central nuclei of the amygdala evoked by memory retrieval in the discrete cue test, suggesting that it was able to reduce the establishment of long-term memory formation in these structures.

Central release of norepinephrine plays an important role in the reinforcement of fear memory likely through activation of β (Ferry and McGaugh, 1999) and α_1 ARs (Ferry *et al*, 1999b). It has been established that locus coeruleus noradrenergic neurons are activated by the US of the footshock (Ishida *et al*, 2002; Sara, 1985) as well as other stressful stimuli (Abercrombie and Jacobs, 1987). Activation of either the β_2 (Ferry and McGaugh, 1999) or α_1 (Ferry *et al*, 1999b) ARs increases attention and arousal, and enhances memory. CREB-dependent transcription is required for cellular events underlying long-term memory in Aplysia, drosophila, mice, and rats (Impey *et al*, 1998; Lamprecht *et al*, 1997). Both the behavioral signs of fear freezing and the increase in P-CREB in the amygdala are sensitive to administration of β adrenergic antagonists (Przybyslawski *et al*, 1999). A cascade of intracellular events that culminate in increased intracellular $[Ca^{2+}]$ or [cAMP] can lead to phosphorylation and activation of CREB (Lin *et al*, 1998; Takuma *et al*, 1997). Norepinephrine is ideally suited to initiate phosphorylation of CREB as it can activate G_s-coupled β ARs to increase cAMP levels (Reisine *et al*, 1983) and activate α_1 ARs to increase intracellular $[Ca^{2+}]$ (Nelemans and den Hertog, 1987). P-CREB bound to promoter CRE sites may then enhance gene transcription including the *fos* gene (Simpson and McGinty, 1994). Furthermore, it has been demonstrated by immunocytochemical studies in lateral amygdala that both repeated tetanizing stimulation and forskolin administration, which enhances adenylyl cyclase activity, stimulate the phosphorylation of CREB (Huang *et al*, 2000). The ability of dexmedetomidine to suppress discrete fear memory and inhibit retrieval-induced P-CREB and c-Fos expression in the amygdala is consistent with its ability to suppress norepinephrine release or by functionally antagonizing the β AR mediated increase in cAMP (Mori-Okamoto *et al*, 1991).

The site of action of dexmedetomidine in the neuronal circuits involved in fear conditioning is unclear. Our results point to the α_{2A} AR being the prime receptor subtype mediating the suppressant action of dexmedetomidine on discrete cue and contextual fear learning. α_{2A} ARs are expressed in the amygdala where they are located on both the postsynaptic amygdala neurons or presynaptically on noradrenergic terminals (Talley *et al*, 1996). Immunocytochemical studies of the rat brainstem have shown that α_{2A} receptors are found on the axon terminals and dendrites of noradrenergic locus coeruleus neurons that project to the lateral and basolateral amygdala nuclei (Lee *et al*, 1998). Noradrenergic neurons within the nucleus tractus solitarius (NTS) project to the central nucleus (Asan, 1998), and the activation of the NTS (Zardetto-Smith and Gray, 1990) contributes to an increased norepinephrine release in the amygdala and enhances memory (Williams *et al*, 2000). Norepinephrine release from the ascending noradrenergic tracts is also under presynaptic control of both α_{2C} and α_{2A} ARs (Bucheler *et al*, 2002), where they act as an autoreceptors on noradrenergic terminals. The absence of α_{2A} AR-mediated presynaptic inhibition in α_{2A} AR−defi-deficient mice would likely promote a stronger release of norepinephrine evoked by the US to promote discrete cue

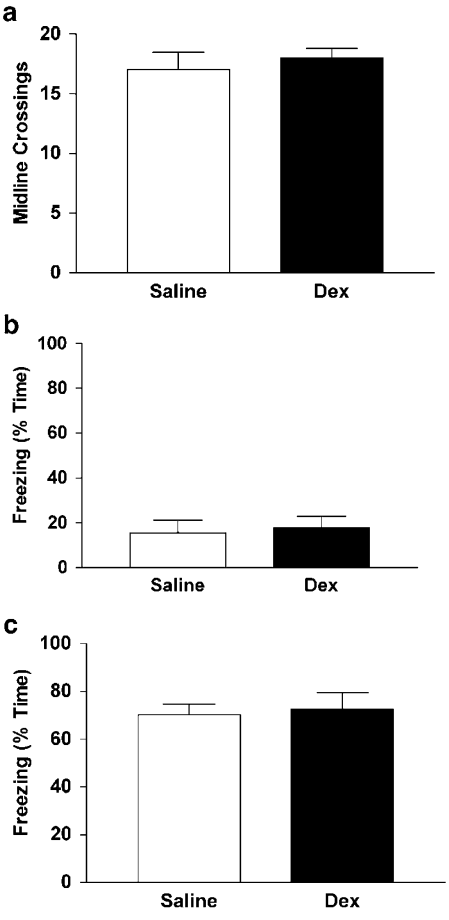

Figure 7 Effect of preadministration of dexmedetomidine administered 30 min before training on fear-conditioned α_{2A} AR KO mice. Dexmedetomidine (20 μg/kg) administered 30 min before training on α_{2A} AR KO mice and discrete cue memory was tested the next day (a) Cage exploration during training was not significantly affected by dexmedetomidine. (b) Dexmedetomidine did not significantly affect freezing behavior of α_{2A} AR KO mice compared to wild-type controls placed in the novel environment. (c) There was no significant difference in freezing response between the two mouse groups to the tone during the discrete cue assessment. Bars represent mean + SEM; $N = 4$.

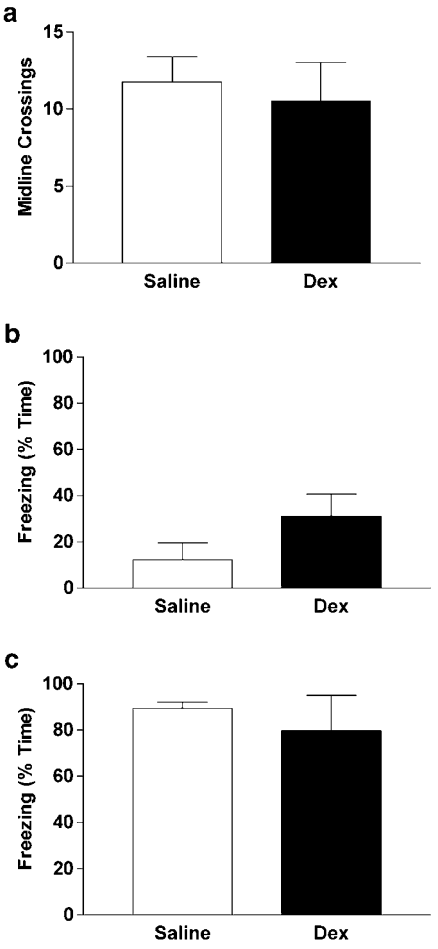

Figure 8 Effect of preadministration of dexmedetomidine administered 30 min before training on fear-conditioned D79N mice. Dexmedetomidine (20 μg/kg i.p.) was administered 30 min before fear conditioned-training began. (a) Dexmedetomidine did not significantly decrease initial chamber exploration during the training session. (b) There was no difference in the freezing behavior between saline- and dexmedetomidine-treated mice placed 24 h later in a new context. (c) There was no significant difference in freezing response to the tone presentation during discrete cue assessment. Bars represent mean + SEM; $N = 4$.

fear encoding (Bucheler *et al*, 2002). Indeed, we have found that α_{2A} AR-deficient mice exhibit a higher level of freezing than controls in the discrete test (Davies *et al*, 2003).

The ability of dexmedetomidine to suppress c-Fos and P-CREB expressions in the amygdala of mice fear conditioned to a discrete cue tone suggests that the norepinephrine release is also reduced in all three amygdaloid nuclei. α_2 Adrenergic agonists have been found to block fear-startle when locally injected into the lateral nucleus of the amygdala (Schulz *et al*, 2002), whereas local injection of the nonselective α_2 antagonist atipamezole into the junction of the central and lateral amydaloid nucleus induces c-Fos expression in the amygdala (Stone *et al*, 1997). Afferents from the central nucleus project to and affect brainstem structures involved in the control of the autonomic nervous system (LeDoux *et al*, 1988). These reports taken together with our results support the idea that dexmedetomidine affects both the emotional and autonomic expression of fear (Asan, 1998).

We have found that dexmedetomidine selectively decreases discrete cue freezing without affecting encoding of contextual memory. This implies that administration of an α_2 adrenergic agonist selectively affects auditory cue memory but not contextual memory. This is consistent with studies in the mice deficient in tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of norepinephrine, in which these mice displayed impairment in a water-finding task, active avoidance, cued fear conditioning, and conditioned taste aversion but exhibited normal spatial learning and hippocampal LTP (Kobayashi *et al*, 2000). Others have found that blockade of β ARs by propranolol affects spatial memory when given after training (Cahill, 2000). Therefore, suppression of the noradrenergic system may cause retrograde spatial amnesia. There has been a ongoing controversy on whether the noradrenergic system affects acquisition or retention of memory, where auditory cued fear conditioning points to a noradrenergic influence on acquisition (Wilensky *et al*, 1999), the more spatially

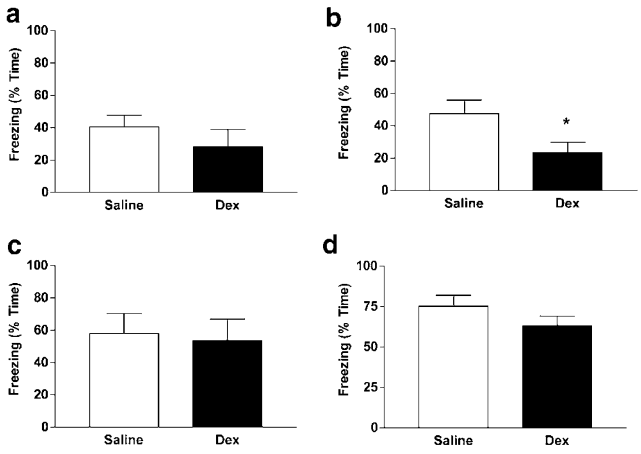

Figure 9 Effect of dexmedetomidine on contextual memory: (a) dexmedetomidine (10 μg/kg i.p.) administered 30 min before training on fear-conditioned C57BL/6 wild-type mice had no effect on contextual memory when tested 24 h later; (b) dexmedetomidine (20 μg/kg i.p.), administered to mice immediately after training which were then tested 24 h later in the same context, significantly reduced freezing compared to saline-treated C57BL/6 wild-type mice; (c) in α_{2A} AR KO mice, dexmedetomidine (20 μg/kg i.p.) administered immediately after fear-conditioned training had no effect on freezing induced by exposure to the training environment when tested 24 h; (d) in D79N mice, dexmedetomidine (20 μg/kg i.p.) administered immediately after training had no effect on freezing when tested 24 h. Bars represent mean + SEM. Statistically significant difference to saline-treated controls: $*p < 0.05$, $N = 5$–6.

oriented task of inhibitory avoidance indicates that consolidation is affected (LaLumiere *et al*, 2003; Weisskopf *et al*, 1999). Our results support the concept that the noradrenergic system influences the acquisition of auditory cued memory while alternatively influencing the consolidation of contextual memory.

Attempts have been made to enhance memory by modulating the adrenergic system to affect memory with most of the attention focused on the use α_2 antagonists, such as yohimbine (O'Carroll *et al*, 1999), idazoxan (Coull *et al*, 1996), and dexefaroxan (Chopin *et al*, 2002). Unfortunately, yohimbine has been reported to increase anxiety in PTSD patients (Southwick *et al*, 1999b) and to enhance fear memory when infused into rat amygdala (Ferry *et al*, 1999a). On the other hand, amnesia to stressful situations such as surgical procedures is a valuable effect of anesthetic agents and many anesthetic agents such as halothane (Rosman *et al*, 1992), propofol (Pang *et al*, 1993), midazolam (Barros *et al*, 1998), isoflurane (Dutton *et al*, 2002), and thiopental do cause anterograde but not retrograde amnesia (Pandit, 1992). However, α_2 agonists appear to behave differently in that they preferentially suppress discrete fear memory over contextual memory. Therefore, α_2 agonists such as dexmedetomidine may reduce anxiety and the responses to the memories of a stressful situation. This may be one of the additional benefits of dexmedetomidine administration in the ICU environment.

There is mounting evidence that the noradrenergic system is dysregulated in post-traumatic stress disorder (PTSD), where traumatic events are vividly recalled with concomitant emotional and autonomic responses (Southwick *et al*, 1999a). The ability of α_2 adrenergic agonists to limit the production of transcription factors generated in response to memory retrieval indicates that they may be effective in breaking the vicious cycle of PTSD. Clearly, the ability of α_2 agonists to curb noradrenergic transmission may ultimately be useful in many clinical settings where fear memory is detrimental to the health of the patient.

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