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Increasing CREB in the auditory thalamus enhances memory and generalization of auditory conditioned fear

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Although the lateral nucleus of the amygdala (LA) is essential for conditioned auditory fear memory, an emerging theme is that plasticity in multiple brain regions contributes to fear memory formation. The LA receives direct projections from the auditory thalamus, specifically the medial division of the medial geniculate nucleus (MGM) and adjacent posterior intralaminar nucleus (PIN). While traditionally viewed as a simple relay structure, mounting evidence implicates the thalamus in diverse cognitive processes. We investigated the role of plasticity in the MGM/PIN in auditory fear memory. First, we found that auditory fear conditioning (but not control manipulations) increased the levels of activated CREB in both the MGM and PIN. Next, using viral vectors, we showed that exogenously increasing CREB in this region specifically enhanced formation of an auditory conditioned fear memory without affecting expression of an auditory fear memory, formation of a contextual fear memory, or basic auditory processing. Interestingly, mice with increased CREB levels in the MGM/PIN also showed broad auditory fear generalization (in contrast to control mice, they exhibited fear responses to tones of other frequencies). Together, these results implicate CREB-mediated plasticity in the MGM/PIN in both the formation and generalization of conditioned auditory fear memory. Not only do these findings refine our knowledge of the circuitry underlying fear memory but they also provide novel insights into the neural substrates that govern the degree to which acquired fear of a tone generalizes to other tones.

Auditory fear conditioning is commonly used to probe the neural substrates of memory. In this task, an initially neutral tone (conditioned stimulus [CS]) is paired with shock (unconditioned stimulus [US]) (Kapp et al. 1979; LeDoux 2000; Davis and Whalen 2001; Fanselow and Gale 2003). Upon subsequent tone presentation, animals exhibit conditioned fear responses, including freezing (Anagnostaras et al. 2000; LeDoux 2000; Fanselow and Gale 2003). Plasticity in the amygdala, particularly the lateral nucleus (LA), is critical for long-term memory for conditioned auditory fear (Davis 1992; Fanselow and LeDoux 1999; Maren and Quirk 2004; Dityatev and Bolshakov 2005; but see Cahill et al. 1999). Accordingly, disrupting plasticity in the LA by locally perturbing transcription or translation impairs long-term memory for auditory fear conditioning (Bailey et al. 1999; Schafe and LeDoux 2000; Maren et al. 2003). On the other hand, increasing the function of the transcription factor CREB (cAMP/ Ca2+ responsive element binding protein) in the LA enhances memory for conditioned fear (Josselyn et al. 2001; Wallace et al. 2004; Han et al. 2007). The requirement of LA plasticity, however, does not exclude important contributions from additional regions.

The LA receives direct projections from auditory thalamus (medial geniculate nucleus [MGN]), specifically the medial division of MGN and adjacent posterior intralaminar nucleus (MGM/PIN) (LeDoux et al. 1990; Doron and Ledoux 2000; Radley et al. 2007). Although traditionally viewed as a relay structure, mounting evidence links the thalamus to diverse cognitive processes (Crick 1984; Komura et al. 2001; Kassubeck et al. 2005; Minamimoto et al. 2005; McAlonan et al. 2006). Indeed several findings implicate the MGM/PIN in conditioned auditory fear memory. First, this region receives both auditory and somatosensory inputs (Bordi and LeDoux 1994b; Simone et al. 2004), suggesting that it is a site of CS/US convergence. Second, auditory fear training induces associative neuronal activity in the MGM (McClellon et al. 1996; Maren et al. 2001) and high-frequency stimulation induces long-term potentiation (LTP) in the MGM (Gerfen and Weinberger 1983).

Although these studies are consistent with the notion that plasticity in the MGM/PIN plays a role in conditioned auditory fear memory, experiments designed to explicitly test this prediction are inconclusive. While several labs showed that intra-thalamic infusions of RNA synthesis inhibitors block memory for auditory fear (Apergis-Schoute et al. 2005; Parsons et al. 2006), similar infusions of protein synthesis inhibitors were reported to block (Parsons et al. 2006) or produce no effect (Maren et al. 2001; Apergis-Schoute et al. 2005; Parsons et al. 2006) on memory for conditioned auditory fear. In addition, because these intra-thalamic drug infusions may diffuse to adjacent brain regions, the precise areas that mediate potential effects are unclear. Finally, the functional significance of MGM/PIN plasticity in fear conditioning is largely unknown.

As an alternative to examining the effects of disrupting plasticity, we used a gain-of-function approach. We found that auditory fear conditioning specifically increased the activated levels...
of the transcription factor CREB (pCREB) in both the MGm and PIN. Next, we used viral vectors to exogenously increase CREB levels in this region. Increasing CREB specifically in neuronal nuclei in the MGm/PIN enhanced the formation of auditory conditioned fear memory. Similarly, increasing CREB had no effect on the expression of auditory fear memory or general auditory processing. We also found that increasing CREB in the MGm/PIN enhanced the generalization of fear responses to tones of other frequencies. This fear overgeneralization was modality (tone)-specific and did not extend to contextual fear conditioning. Together, these data implicate CREB-mediated plasticity in the MGm/PIN in the formation and generalization of conditioned auditory fear memory.

Results

Auditory fear conditioning activates CREB in the MGm/PIN

Previous studies generally indicate that CREB plays an important role in the plasticity underlying long-term memory in both invertebrates (Dash et al. 1990; Yin et al. 1995; Wagatsuma et al. 2006) and vertebrates (Bourchuladze et al. 1994; Guzowski and McGaugh 1997; Lamprecht et al. 1997; Graves et al. 2002; Kida et al. 2002; Pittenger et al. 2002; Josselyn et al. 2004; but see Balchun et al. 2003). The ultimate products of CREB-mediated transcription are thought to contribute to the synaptic remodeling mediating long-term memory (Sheng and Greenberg 1990; Frank and Greenberg 1994; Desmedt et al. 2003). Because CREB-mediated transcription can be initiated by phosphorylation of the Ser 133 residue (Gonzalez and Montminy 1989), CREB activation (CREB that is phosphorylated at Ser 133, pCREB) is commonly used as an immunocytochemical marker of brain regions undergoing plasticity during learning (Impey et al. 1998; Desmedt et al. 2003). Accordingly, previous studies show that training that induces long-term memory for conditioned fear is associated with an increase in the levels of activated CREB in the basolateral/lateral amygdala of mice (Stanciu et al. 2001; Han et al. 2007). Here, we examined whether similar auditory fear training also increases CREB activation in the MGm and/or PIN (Fig. 1A). Figure 1B shows that pCREB levels in both the MGm and PIN are indeed increased following auditory fear conditioning compared to homecage controls.

Activation of CREB in the MGm and PIN is specific to associative tone-shock pairing

To determine whether the increase in pCREB levels in the MGm and PIN was specifically induced following auditory fear conditioning (tone-shock pairing), rather than nonassociative aspects of the procedure (such as auditory stimulation, shock, or placement in the chamber), we similarly examined pCREB levels following several control training conditions (tone alone, context fear conditioning, immediate shock training, chamber alone, and homecage).

Figure 1C, D shows that auditory fear conditioning (Tone+Shock), but not any of the control training manipulations, increased pCREB levels in both the MGm and PIN, respectively. Separate ANOVAs performed on the number of pCREB positive nuclei in the MGm and PIN using Treatment (Tone+Shock, Tone alone, Chamber+Shock, Immediate Shock, Chamber alone, and Homecage) as a between-group factor revealed a significant effect in both the MGm ($F_{(5,20)} = 20.92; P < 0.001$) and PIN ($F_{(5,20)} = 12.67; P < 0.001$). Post-hoc Bonferroni comparisons showed that auditory fear conditioning increased pCREB levels relative to all control conditions (which did not differ from each other). Interestingly, pCREB levels in the MGm and PIN were similar following auditory fear training and control manipulations ($F_{(5,20)} = 1.11; P > 0.05$, no significant interaction between Brain Region and Treatment). Together, these data indicate that CREB is normally activated in both the MGm and PIN following auditory fear conditioning and that this activation is not produced by nonassociative factors (such as auditory stimulation, shock, chamber placement, or stress).

To further investigate the role of CREB-mediated plasticity in the MGm/PIN region in auditory fear conditioning, we exogenously increased CREB levels and examined the effects on auditory fear memory. Because plasticity in the LA has been shown by many research groups to be critical for conditioned auditory fear memory, as a confirmatory step, we first examined the effects of increasing CREB levels in the LA on auditory fear memory.

Effects of shock intensity on auditory fear conditioning

To examine the effects of increasing CREB function in the LA or MGm/PIN on auditory fear conditioning memory, we first deter-
mained the training conditions that induced suboptimal memory. In this way, a potential enhancement of memory, as indicated by an increase in freezing levels, could be observed in subsequent experiments free from the potential masking by ceiling effects. We trained unoperated control mice with one tone-shock pairing but varied the intensity of the shock (0.3-, 0.4-, and 0.5-mA shock; n = 7, 10, and 8, respectively). Mice were tested 24 h later and the percentage of time spent freezing before (pre-CS freezing) and during the tone (CS freezing) was assessed. Figure 2A shows the mean (±SEM) percent time mice spent freezing during the test session, both before (pre-CS) and during (CS) the tone. As can be seen from this figure, the freezing levels did not differ between the groups before the CS was replayed. However, mice that received more intense shocks during training froze at higher levels to the CS. ANOVA with between-group factor Shock Intensity (0.3, 0.4, and 0.5 mA) and within-group factor Time (Pre-CS and CS) showed a significant effect of Shock Intensity (F_{(2,22)} = 12.12; P < 0.001), Time (F_{(1,22)} = 35.11; P < 0.001), and Shock Intensity × Time interaction (F_{(2,22)} = 6.54; P < 0.001). Post-hoc Bonferroni tests revealed that the pre-CS freezing levels did not differ between the groups (P > 0.05), but freezing levels during the CS were greater in the 0.5-mA group (P < 0.001) than in the 0.4- and 0.3-mA groups (which did not differ, P > 0.05). Therefore, in subsequent experiments, we trained mice with a 0.4-mA shock.

Increasing CREB in the LA enhances memory for auditory conditioned fear

Previously, we and others showed that increasing CREB levels in the LA, via viral-mediated gene transfer, enhanced long-term memory for fear conditioning in rats (Josselyn et al. 2001; Wallace et al. 2004) and mice (Han et al. 2007). We used a replication-defective herpes simplex virus (HSV) for these studies because, unlike many other viruses, HSV is naturally neurotropic (Fink et al. 1996). Previous studies established that infusion of this CREB vector increased both CREB levels and function (CRE-mediated transcription) (Barrot et al. 2002; Olson et al. 2005). In the present experiment, mice received intra-LA infusions of CREB or Control vector 3 d prior to auditory fear conditioning. To examine whether increasing CREB function enhanced memory, we used a minimal training protocol (0.4-mA shock) that induces only weak long-term memory.

**Histology**

Figure 2B shows an example of the distribution of GFP expression following vector infusion into the LA. Consistent with previous results from several labs, minimal tissue damage in, and around, the infusion site was observed (Carlezon and Neve 2003; Neve et al. 2005). Figure 2D shows the placement and spread of vector infusions, as determined by the highest level of GFP expression, for mice infused with the CREB and Control vectors. Only those mice with robust transgene expression bilaterally in the LA were included in subsequent statistical analysis (CREB vector n = 9, Control vector n = 10). Of the 15 mice infused with the CREB vector, one was off-target bilaterally and five were off-target unilaterally. In the Control vector group, a total of 13 mice were infused, three of which had unilateral off-target placements.

**Behavior**

As can be seen from Figure 2C, infusing the CREB vector into the LA of mice specifically increased the levels of freezing to the tone. This observation is supported by the results of an ANOVA (Vector [CREB vs. Control] × Time [pre-CS vs. CS]) in which significant effects of Vector (F_{(1,17)} = 5.37; P < 0.05), Time (F_{(1,17)} = 43.71; P < 0.001), and a Vector-by-Time interaction (F_{(1,17)} = 14.20; P < 0.001) were found. Mice infused with the CREB vector showed higher levels of freezing during the tone than mice infused with the Control vector (P < 0.001), but levels of freezing during the pre-CS period were not significantly different (P > 0.05), as determined by Bonferroni post-hoc comparisons of the significant interaction. Therefore, consistent with previous findings in rats (Josselyn et al. 2001; Wallace et al. 2004), mice (Han et al. 2007), and hamsters (Jasnow et al. 2005), we found that increasing CREB in the LA enhances memory.

Increasing CREB in the MGm/PIN specifically enhances memory for auditory conditioned fear

The above results show that increasing CREB in the LA enhances conditioned auditory fear memory. We used a similar strategy to examine whether increasing CREB function enhanced memory, or Control vector 3 d prior to auditory fear conditioning. To examine whether increasing CREB function enhanced memory, we used a minimal training protocol (0.4-mA shock) that induces only weak long-term memory.

**Histology**

Figure 3A shows an example of the expression pattern of GFP expression following infusion of vectors aimed at the MGm/PIN. Consistent with previous findings, minimal tissue damage in, and around, the injection site was observed (Carlezon and Neve 2003; Neve et al. 2005). Figure 3B shows the placement and spread of vector infusions, as determined by the highest level of GFP expression, for mice infused with the CREB and Control vectors. Only those mice with robust transgene expression bilaterally in the MGm/PIN were included in subsequent statistical analysis (CREB vector n = 9, Control vector n = 10). Of the 15 mice infused with the CREB vector, one was off-target bilaterally and five were off-target unilaterally. In the Control vector group, a total of 13 mice were infused, three of which had unilateral off-target placements.

**Behavior**

As can be seen from Figure 3C, infusing the CREB vector into the MGm/PIN of mice specifically increased the levels of freezing to the tone. This observation is supported by the results of an ANOVA (Vector [CREB vs. Control] × Time [pre-CS vs. CS]) in which significant effects of Vector (F_{(1,17)} = 5.37; P < 0.05), Time (F_{(1,17)} = 43.71; P < 0.001), and a Vector-by-Time interaction (F_{(1,17)} = 14.20; P < 0.001) were found. Mice infused with the CREB vector showed higher levels of freezing during the tone than mice infused with the Control vector (P < 0.001), but levels of freezing during the pre-CS period were not significantly different (P > 0.05), as determined by Bonferroni post-hoc comparisons of the significant interaction. Therefore, consistent with previous results from several labs, minimal tissue damage in, and around, the injection site was observed (Carlezon and Neve 2003; Neve et al. 2005). Nonetheless, GFP-positive neurons were observed both in the targeted region (MGm/PIN) and in neighboring regions of the auditory thalamus, including the ventral and dorsal divisions of the MGN (MgV, MgD) and suprageniculate nucleus (SG). Figure 3B shows that infusing the CREB (but not Control) vector into the MGm/PIN region indeed increases CREB levels, as assessed by immunohistochemistry for CREB. This finding is consistent with previous studies showing that...
infusion of this CREB vector increases both CREB levels and function in several brain regions (Barrot et al. 2002; Olson et al. 2005).

Figure 3D shows the placement and extent of infection for mice infused with the CREB or Control vector. The number of mice injected with the CREB or Control vector was 36 and 22, respectively. In the CREB vector group, 14 mice showed robust bilateral expression of GFP in the MGm/PIN. Three mice were excluded from subsequent analysis because of improper histology, eight mice showed unilateral infection in the MGm/PIN, and 11 mice were infused off-target bilaterally (typically in the MGd, MGv, SG, and/or dentate gyrus). In the Control vector group, 11 mice were included in the data analysis, four mice showed only unilateral expression in the target region, and seven mice showed expression that was bilaterally off-target.

**Behavior**

Figure 3C shows that infusing the CREB vector into the MGm/PIN specifically increased freezing to the CS. This observation is supported by the results of a Vector-by-Time ANOVA in which significant effects of Vector ($F_{(1,23)} = 12.32; P < 0.001$), Time ($F_{(1,23)} = 41.06; P < 0.001$), and a Vector-by-Time interaction ($F_{(2,46)} = 6.80; P < 0.001$) were found. Bonferroni post-hoc comparisons of the significant interaction revealed that freezing levels did not differ between the groups during the pre-CS period ($P > 0.05$), but that mice infused with the CREB vector froze more during the tone CS than mice infused with the Control vector ($P < 0.001$). The lack of difference between pre-CS freezing levels indicates that increasing CREB in the MGm/PIN does not increase fear or anxiety-like behavior nonspecifically. It is also important to note that infusion of the Control vector into the MGm/PIN did not appear to alter conditioned auditory fear memory. Unoperated control mice and mice infused with the Control vector showed similar levels of freezing to the tone CS (unoperated mice trained using a 0.4-mA intensity shock, CS freezing = 19.67 ± 5.18, Fig. 2A; mice with Control vector injected into the MGm/PIN, CS freezing = 22.87 ± 3.77, Fig. 3C). Together, these findings indicate that increasing CREB in the MGm/PIN specifically enhances memory for conditioned auditory fear.

**Correlating the location and number of CREB-infected neurons with memory**

*Conditioned auditory fear memory correlates with the number of MGm/PIN neurons infected with the CREB vector*

The use of HSV vectors allows precise manipulations of genes of interest in specific target regions. Because CREB is fused with GFP in our vector, it is possible to visualize the specific location of infected neuronal nuclei. Although our infusions targeted neurons with cell bodies in the MGm/PIN, neurons in neighboring regions were also infected. Indeed, a unique pattern of vector infection was observed in each mouse. We took advantage of this to determine the precise neural region supporting the observed memory enhancement. Specifically, we examined whether the number of CREB-infected neurons in the MGm/PIN or adjacent auditory thalamic regions (grouped as MGd/MGv/SG) correlated with the level of auditory fear memory. We counted the number of infected neurons (GFP-positive nuclei) in the MGm/PIN and MGd/MGv/SG regions of the auditory thalamus in mice categorized as having accurate bilateral or unilateral placements. Twenty-two mice were included in the MGm/PIN-CREB vector group (14 with bilateral placements, eight with unilateral placements).

As can be seen from Figure 4A, there was a high correlation between the number of CREB-infected neurons in the MGm/PIN and the level of freezing to the tone CS. The best fit line for this correlation was $y = 0.25x + 21.13$. Regression analysis revealed a significant correlation between the number of CREB-infected neurons and tone freezing levels ($F_{(1,20)} = 28.44; P < 0.001; R^2 = 0.59$). In contrast, the number of CREB-infected neurons in the MGm/PIN did not correlate with baseline (pre-CS) freezing levels ($F_{(1,20)} = 4.34; P > 0.05; R^2 = 0.18$; best fit line, $y = 0.909x + 16.11$). Therefore, the higher the number of MGm/PIN nuclei with the CREB vector, the greater the auditory conditioned fear memory. This increase in freezing was specific to the tone (not to baseline pre-CS freezing).

*Conditioned auditory fear memory does not correlate with the number of neurons infected with the CREB vector in surrounding regions of the auditory thalamus*

The number of neuronal nuclei in nontarget regions of the auditory thalamus (the MGd/MGv/SG) infected with the CREB vector did not correlate with either pre-CS or CS freezing levels (Fig. 4B). The lines of best fit for these correlations were $y = 0.01x + 19.84$ and $y = 0.02x + 32.98$, for pre-CS and CS freezing, respectively, and no significant correlations were found (pre-CS $F_{(1,20)} = 2.38; P > 0.05; R^2 = 0.11$ and CS $F_{(1,20)} = 0.79; P > 0.05; R^2 = 0.04$). Together, these findings indicate that the
Increasing CREB in the MGm/PIN does not enhance context fear memory

Figure 3C shows that increasing CREB in the MGm/PIN enhances memory for auditory fear conditioning. One interpretation of these data is that increasing CREB in this region facilitates the consolidation of the tone-shock association. Alternatively, increasing CREB in this region may nonspecifically enhance all fear memory. To examine this possibility, we infused an additional group of mice with CREB or Control vector into the MGm/PIN and trained them for context fear conditioning (in the absence of a tone). Mice were placed in the chamber and, 2 min later, a shock (0.4 mA) was delivered. Testing occurred 24 h later when mice were returned to the same context and the percent time mice spent assessed. Figure 5A shows that infusion of the CREB vector into the MGm/PIN does not alter memory for context fear conditioning ($F_{(1,7)} = 0.36; P > 0.05$, CREB vector [$n = 8$], Control vector [$n = 5$]). This lack of enhancement is consistent with previous reports showing that the MGm/PIN region is not critically involved in context fear conditioning (LeDoux et al. 1986; Campeau and Davis 1995a; Parsons et al. 2006). Furthermore, this finding indicates that increasing CREB levels in the MGm/PIN specifically enhances auditory fear memory, rather than conditioned fear memory in general.

Increasing CREB in the MGm/PIN does not enhance expression of conditioned auditory fear

A second possibility is that increasing CREB levels in the MGm/PIN enhances expression, rather than formation, of auditory fear memory. To examine this, we trained mice for auditory fear conditioning as before but infused the viral vectors after (rather than before) training. We tested mice 3 d following surgery, at the time of highest transgene expression. Figure 5B shows that infusion of the CREB vector into the MGm/PIN does not alter memory for context fear conditioning ($F_{(1,7)} = 0.36; P > 0.05$, CREB vector [$n = 8$], Control vector [$n = 5$]). This observation is supported by the results of a Vector by Time ANOVA in which there was a significant effect of Time only and, importantly, no significant effect of Vector or Vector-by-Time interaction ($F_{(1,11)} = 0.011; P > 0.05$) Vector [$F_{(1,11)} = 1.79; P > 0.05$], Time [$F_{(1,11)} = 16.26; P < 0.05$], CREB vector [$n = 7$], Control vector [$n = 5$]). Bonferroni post-hoc comparisons of the significant Time effect showed that freezing levels in both groups increased during the tone ($P < 0.001$). Therefore, increasing CREB in the MGm/PIN does not enhance expression of an auditory fear memory.

Increasing CREB in the MGm/PIN does not alter basic auditory processing

Although the results in Figure 3A,B, above, suggest that the effects of increasing CREB levels in the MGm/PIN are specific to the auditory thalamic regions, with freezing levels. Fifteen mice (11 with correct bilateral placements and four with correct unilateral placements) were included in this analysis. As expected, there was no significant relationship between the number of neurons in the MGm/PIN with the Control vector and freezing levels during the tone (best fit line $y = -0.04x + 34.51$; $F_{(1,13)} = 2.63; P > 0.05$; $R^2 = 0.17$) or baseline period ($y = -0.01x + 10.48$; $F_{(1,13)} = 0.65; P > 0.05$; $R^2 = 0.05$) (Fig. 4C). A similar lack of correlation was observed between the number of neurons in the MGd/MGv/SG with the Control vector and freezing during ($y = -0.01x + 10.62$; $F_{(1,13)} = 0.79; P > 0.05$; $R^2 = 0.06$) or before ($y = -0.002x + 10.62$; $F_{(1,13)} = 0.34; P > 0.05$; $R^2 = 0.03$) the tone (Fig. 4D). Together, these data indicate that infusion of the Control vector into the MGm/PIN or surrounding thalamic regions does not influence auditory fear memory or freezing behavior in general.

Conditioned auditory fear memory does not correlate with the number of neurons infected by the Control vector in the auditory thalamus

As a control, we also correlated the number of neurons with the Control vector in the target region, or surrounding auditory thalamus. No significant correlation was observed between the number of neurons infected with the Control vector in the auditory thalamus and the mean percent time spent freezing during the test. (best fit line $y = -0.002x + 10.48$; $F_{(1,13)} = 0.65; P > 0.05$; $R^2 = 0.05$) (Fig. 4C). A similar lack of correlation was observed between the number of neurons in the MGd/MGv/SG with the Control vector and freezing during ($y = -0.01x + 10.62$; $F_{(1,13)} = 0.79; P > 0.05$; $R^2 = 0.06$) or before ($y = -0.002x + 10.62$; $F_{(1,13)} = 0.34; P > 0.05$; $R^2 = 0.03$) the tone (Fig. 4D). Together, these data indicate that infusion of the Control vector into the MGm/PIN or surrounding thalamic regions does not influence auditory fear memory or freezing behavior in general.
formation of an auditory fear memory, it could be that increasing CREB levels in this important region of the auditory pathway enhances the impact of the CS during training. That is, the observed enhancement of auditory fear memory may be due to an increase in salience of the auditory CS during training. To closely examine this possibility, we used the auditory startle response to test the effects of similarly increasing CREB in the MGm/PIN on hearing and sensorimotor processing. First, we found that infusion of the CREB vector did not alter the habituation of the auditory startle response to repeated presentations of a 120-dB auditory stimulus (Fig. 5C). This observation is supported by the results of a Vector-by-Time ANOVA in which there was a significant effect of Time only and, importantly, no significant effect of Vector or Vector by Time interaction (Vector by Time interaction $F_{(3,36)} = 0.036; P > 0.05$, Vector $F_{(1,12)} = 0.049; P > 0.05$, Time $F_{(3,36)} = 16.26; P < 0.05$, CREB vector $\eta = 7$), Control vector ($\eta = 7$). Bonferroni post-hoc comparisons of the significant Time effect showed that the startle response decreased over trials ($P < 0.001$). Therefore, increasing CREB in the MGm/PIN did not affect habituation of the acoustic startle response, a nonassociative form of learning (Duer and Quinn 1982; Hawkins et al. 1998). There was also no effect of increasing CREB on overall startle responding (Fig. 5D). This finding further indicates that this manipulation does not increase auditory startle or anxiety-like behavior.

We used prepulse inhibition to examine the effects of increasing CREB in the MGm/PIN on sensorimotor gating of the auditory startle response. We tested mice with three different prepulse intensities (70, 75, and 80 dB) prior to a 120-dB startle pulse. As can be seen in Figure 5E, the CREB vector did not alter sensorimotor gating. Mice infused with Control or CREB vector showed greater inhibition of the startle response with higher prepulse intensities. The results of an ANOVA supported this interpretation, showing a significant effect of Prepulse Intensity only and, importantly, no significant effect of Vector or Vector by Prepulse Intensity interaction (Vector by Prepulse Intensity interaction $F_{(2,24)} = 1.09; P > 0.05$, Vector $F_{(1,12)} = 0.002; P > 0.05$, Prepulse Intensity $F_{(2,24)} = 145.79; P < 0.05$).

Finally, to examine whether increasing CREB in the MGm/PIN lowered the auditory threshold for startle responding, we tested the startle amplitude of mice over a range of different intensities of auditory stimuli (ranging from 0 to 120 dB, Fig. 5F). Increasing CREB in the MGm/PIN did not alter the threshold of startle responding, a significant effect of Startle Intensity only and, importantly, no significant effect of Vector or Vector by Startle Intensity interaction (Vector by Startle Intensity interaction $F_{(3,10,120)} = 0.30; P > 0.05$, Vector $F_{(1,12)} = 0.008; P > 0.05$, Startle Intensity $F_{(3,10,120)} = 171.66; P < 0.05$). Bonferroni post-hoc comparisons of the significant Startle Intensity effect showed that the startle response in both groups of mice increased at 100 dB ($P < 0.001$). Together, these data demonstrate that increasing CREB in the MGm/PIN did not alter numerous aspects of basic central auditory processing. Therefore, the enhancement of auditory fear memory in mice with higher levels of CREB in the MGm/PIN cannot be attributed to changes in simple auditory processing.

Increasing CREB in the MGm/PIN increases the generalization of conditioned auditory fear

Our findings that (1) auditory fear conditioning increases CREB activation in the MGm and PIN and (2) exogenously increasing CREB levels in this region enhances auditory fear conditioning converge to suggest that CREB-mediated plasticity in the MGm/PIN is important for the formation of conditioned auditory fear memory. However, the functional contribution of this plasticity is unknown.

The LA receives both direct and indirect projections from the auditory thalamus. Previous results show that either the thalamo-amygdala pathway (direct projection from the MGm/PIN to the amygdala) or the thalamo-cortico-amygdala pathway (indirect pathway from regions of the auditory thalamus to the cortex to the amygdala) is sufficient to support auditory fear conditioning to a single tone CS (Romanski and LeDoux 1992; Campeau and Davis 1995b). However, each of these pathways may make a unique contribution. Several lines of evidence suggest that the direct thalamo-amygdala pathway processes auditory information in a rapid but crude manner, while the thalamo-cortico-amygdala pathway processes more detailed representations of sound stimuli (LeDoux 1995). First, many cells in the MGm/PIN are broadly tuned (Bordi and LeDoux 1994a,b), in
contrast to the sharply tuned cells in the auditory cortex and MGv, a region of the auditory thalamus that directly projects to the auditory cortex, which then projects to the LA (Miller et al. 1972; Rauschecker et al. 1995). Second, post-training lesions of the auditory cortex disrupt performance on a complex sound processing (tone discrimination) task (Thompson 1960; Jarrell et al. 1987). It is somewhat surprising, therefore, that pretraining lesions of the auditory cortex failed to influence tone fear generalization in both a computational model and behaving rats (Armony et al. 1997). These apparently discrepant results could be due to differences between mechanisms that mediate tone discrimination and generalization, the timing of lesion (preversus post-training), and potential functional compensation by other intact brain areas following the lesion. As an alternative strategy, we examined the effects by using a gain-of-function approach.

Because many cells in the MGm/PIN are broadly tuned, we examined whether increasing CREB in this region increased tone fear generalization. To this end, a random subset of mice injected with the CREB vector into the MGm/PIN (n = 7, from Fig. 3C) was given two additional tone generalization tests during which freezing levels to different frequency tones (700 and 10,000 Hz, rather than CS training tone [2800 Hz]) were measured. The order of tone presentation (700, 10,000 Hz) in these generalization tests was counterbalanced across mice. Because mice that were infused with Control vector did not show high levels of freezing to the training CS tone (see Fig. 3C), we did not test them further for tone fear generalization. Instead, we compared tone generalization in the CREB vector group to an additional unoperated control group (n = 19) trained using a protocol that produced similar levels of CS freezing (2800-Hz tone paired with a 0.5-mA shock; see Fig. 2A).

Figure 6 shows the percent time mice spent freezing to the training tone (2800 Hz), as well the low-(700 Hz) and high-(10,000 Hz) frequency tones. As can be seen from this graph, the control mice froze much less to the high- and low-frequency tones than to the training tone, showing little tone fear generalization. In contrast, mice infused with the CREB vector froze at equally high levels to the training and the other tones, showing enhanced tone fear generalization. The results of an ANOVA (Group × Tone Frequency) supported this finding, revealing a significant interaction of Group-by-Tone Frequency (F(2,48) = 4.84; P < 0.05) as well as significant main effects of Group (F(1,2,48) = 25.10; P < 0.001) and Tone Frequency (F(2,48) = 7.86; P < 0.05). Bonferroni post-hoc analysis of the significant interaction indicated that freezing to the training tone was similar in both groups. Furthermore, whereas the control mice froze significantly less to the low- and high-frequency tones than to the training tone, mice infused with the CREB vector froze at similarly high levels to all tone frequencies. Therefore, increasing CREB in the MGm/PIN broadens the generalization gradient for conditioned auditory fear.

**Discussion**

The present experiments examined the role of the CREB-mediated plasticity in MGm/PIN in conditioned auditory fear memory. We targeted this region based on three key findings. First, antidromic stimulation of the LA activates cells in the MGm/PIN, indicating that this region sends direct projections to the LA (Bordi and LeDoux 1994b). Second, cells in the MGm/PIN receive inputs from both auditory and somatosensory pathways (Bordi and LeDoux 1994b), providing an anatomical substrate for possible CS/US convergence. Surrounding regions of the auditory thalamus do not share these important characteristics. For instance, cells in the MGv fail to respond to both auditory and somatosensory stimulation and few or no antidromically activated cells are observed in the MGv, SG, or MGd following LA stimulation (Bordi and LeDoux 1994b). Finally, labeling studies reveal that the PIN is the primary source of the direct thalamo–LA pathway (Doron and Ledoux 2000).

It is well established that enduring forms of memory depend on mRNA and protein synthesis. Accordingly, previous studies examined the role of plasticity in the MGm/PIN on conditioned auditory fear memory by locally infusing drugs that interfere with these processes. Intra-thalamic infusions of RNA synthesis inhibitors either before or after training specifically block long-term memory for auditory fear conditioning (Apergis-Schoute et al. 2005; Parsons et al. 2006). However, the effect of anisomycin (a drug used to interfere with translation) seems to vary with the time of infusion. Long-term memory is impaired when anisomycin is infused before training (Parsons et al. 2006), but not after training (Maren et al. 2003; Apergis-Schoute et al. 2005). These contrasting results may be due to several factors, such as the nonspecific effects of anisomycin (Zechner et al. 1997) or a disparity between the time course of protein synthesis disruption produced by this manipulation and the time window of protein synthesis necessary to support long-term memory in this region. Furthermore, because anisomycin may disrupt protein synthesis in neurons both within and surrounding the MGm/PIN, it is difficult to localize the precise anatomical regions mediating any potential effect.

We used a gain-of-function approach to examine the role and behavioral significance of plasticity in the MGm/PIN in conditioned auditory fear memory. We found that auditory fear conditioning increased the levels of activated CREB in both the MGm and PIN. Control training manipulations did not produce similar increases in pCREB, thus highlighting the specificity of the observed increase in CREB activation. These findings indicate that training that induces long-term memory for auditory fear conditioning normally activates CREB in the MGm and PIN. Next, we therefore examined the effects of exogenously increasing CREB in this region.

We showed that increasing CREB levels in the MGm/PIN enhanced memory for auditory fear conditioning. We trained mice using a low intensity of shock that normally produced weak memory for auditory conditioned fear. However, mice infused with the CREB vector into the MGm/PIN showed robust auditory fear memory. Tagging the CREB transgene with GFP allowed us to localize the specific brain regions mediating this memory enhancement. We found that the number of neuronal nuclei in the
MGr/PIN (but not surrounding regions of the auditory thalamus) with the CREB vector was positively correlated with the level of auditory fear memory. Therefore, the memory enhancement produced by increasing CREB levels can be specifically attributed to neurons with nuclei located in the MGr/PIN.

In a series of control studies, we examined the specificity of this memory enhancement. We showed that increasing CREB in the MGr/PIN did not alter (1) expression of an auditory fear memory, (2) hearing or auditory processing (as measured by habituation, prepulse inhibition, or threshold of the acoustic startle response), or (3) formation of a context fear memory. Therefore, the auditory fear memory enhancement produced by increasing CREB in the MGr/PIN cannot be attributed to an increase in overall fear memory, a nonspecific increase in anxiety, or a general change in auditory processing.

The present results are consistent with previous findings showing that intra-thalamic infusion of U0126, a MEK inhibitor, prior to training impaired long-term memory for auditory fear conditioning (Apergis-Schoute et al. 2005). Because MEK phospho-rates CREB via MAPK and RSK (Roberson et al. 1999), it is tempting to speculate that the memory-impairing effects of U0126 were mediated by disrupting CREB function.

The present findings are also in agreement with the notion that the MGr/PIN region stores critical aspects of a tone-reward memory. Following tone-reward pairing in mice, presentation of the tone alone produced a region-specific increase in high-frequency firing in MGr/PIN neurons (Komura et al. 2001). Extinction training gradually decreased this neuronal firing. However, a single retraining trial was sufficient to rapidly restore the previously observed high-frequency firing in the MGr/PIN. The rapidity of this response suggests that the MGr/PIN stores some aspect of the tone-reward memory. Together, these results show a conserved role for the MGr/PIN in learning during which a tone acquires emotional significance (either aversive and appetitive).

The present findings that the MGr/PIN is important for auditory fear conditioning memory do not diminish the role of plasticity in the LA. Indeed, we found that increasing CREB function in the LA produced a similar robust memory enhancement. Although the role of LA plasticity in the consolidation of auditory conditioned fear memory has been clearly established (Davis 1992; Fanselow and LeDoux 1999; LeDoux 2000; Maren and Quirk 2004; Dityatev and Bolshakov 2005, but see Cahill et al. 1992; Fanselow and LeDoux 1999; LeDoux 2000; Maren and Quirk 2004; Dityatev and Bolshakov 2005), our recent findings indicate that plasticity in multiple regions may be necessary for long-term auditory fear memory (Pare et al. 2004; Wilensky et al. 2006).

Finally, we investigated the functional role of MGr/PIN plasticity in conditioned auditory fear. We found that increasing CREB in the MGr/PIN increased tone fear generalization. Control mice showed a sharp tone fear generalization gradient (they froze robustly to the CS tone frequency but much less to other tones). In contrast, mice infused with the CREB vector froze at the CS tone frequency but much less to other tones. In contrast, the more broadly tuned cells in the MGr develop a generalized multi-peaked curve that responds to many tone frequencies (Edeline and Weinberger 1992, 1993). Our current findings suggest that increasing CREB in the MGr/PIN broadens tone fear generalization in agreement with the broad retuning curves normally observed in this region.

The neural substrates underlying the acquisition and consolidation of memory for auditory fear conditioning have been well studied. In contrast, relatively little is known about the neural mechanisms that determine the degree to which conditioned auditory fear generalizes to similar tones. The present findings suggest that CREB-mediated plasticity in the MGr/PIN is important for long-term memory for auditory fear conditioning and regulating the extent of auditory fear generalization.

Materials and Methods

Mice

Adult F1 hybrid (C57Bl/6NTac × 129S6/SvEvTac) mice were group-housed (three to five mice per cage) on a 12-h light/dark cycle. Food and water were available ad libitum throughout the experiment. All procedures were approved by the Hospital for Sick Children Animal Care and Use Committee.

Immunocytochemistry

Male mice aged 3–5 mo were handled for six consecutive days and randomly assigned to the following treatment groups: (1) Tone+Shock (Tone fear conditioning [Tone FC, n = 6]), (2) Tone alone (n = 4), (3) Chamber+Shock (Context FC, n = 4), (4) Immediate Shock (n = 4), (5) Chamber alone (n = 4), and (6) Home cage (n = 4). The shock intensity for all mice receiving shock in these immunocytochemical experiments was 0.5 mA. Mice in the Tone+Shock group were placed in a conditioning chamber (Context A) and, 2 min later, presented a tone (2800 Hz, 85 dB, 30 sec) that coterminal with a shock (2 sec, 0.5 mA). Mice in the Tone alone group were treated identically, except that no shock was delivered. Two minutes following placement in the chamber, mice in the Chamber+Shock (context fear conditioning) group received the shock (without tone). The Immediate shock group received the shock 5 sec after being placed in the chamber and was not exposed to the tone. The Chamber alone group did not...
receive the tone or the shock following placement in the chamber. The Homecage mice were taken directly from their homecages and not exposed to the conditioning chamber, tone, or shock.

Thirty minutes following training, mice were perfused transcardially with 4% paraformaldehyde. Brains were sliced coronally (50 µm) and prepared for immunocytochemistry using anti-pCREB primary rabbit polyclonal antibody (1:1000 dilution, Upstate Cell Signaling Solutions). We chose this 30-min time-point based on previous studies (Trifilieff et al. 2006). A biotinylated goat anti-rabbit antibody (1:1000 dilution, Vector Laboratories) was used as a secondary antibody. Staining was visualized using the avidin-biotin peroxidase method (Vectorstain Elite ABC kit, Vector Laboratories) coupled to diaminobenzidine (DAB, Sigma) as a chromogen. No staining was detected in the absence of the primary or secondary antibodies. Quantitative analysis of pCREB-positive nuclei was performed using the NIH image processing system by two experimenters unaware of the treatment condition. The total number of immunoreactive cells in the MGm and PIN (as defined by Paxinos and Franklin 2003) were counted bilaterally from at least six sections from comparable anterior-posterior levels from each mouse. The number of pCREB positive nuclei (per 0.1 mm² of MGm and PIN tissue, respectively) was calculated per mouse and averaged for each group.

HSV vectors
Two vectors were used, HSV-GFP-CREB (CREB vector) and HSV-GFP-LacZ (Control vector). Genes of interest (CREB, LacZ) were cloned into the HSV amplicon (HSV-PrpUC) and packaged using the IHG image processing system by two experimenters unaware of the treatment condition. The total number of immunoreactive cells in the MGm and PIN (as defined by Paxinos and Franklin 2003) were counted bilaterally from at least six sections from comparable anterior-posterior levels from each mouse. The number of pCREB positive nuclei (per 0.1 mm² of MGm and PIN tissue, respectively) was calculated per mouse and averaged for each group.

Validation of vectors
To ensure that infusion of the CREB vector indeed enhanced CREB levels in the MGm/PIN, we performed immunocytochemistry using an anti-CREB primary mouse monoclonal antibody (1:1000, Upstate Cell Signaling Solutions). A biotinylated goat anti-mouse antibody (1:1000, Jackson ImmunoResearch) was used as a secondary antibody. Immunoreactivity was visualized using a DAB reaction as above.

Surgery
Mice were pretreated with atropine sulfate (0.1 mg/kg, i.p.), anesthetized with chloral hydrate (400 mg/kg, i.p.), and placed in a stereotaxic frame. The skin was retracted and holes drilled in the skull bilaterally above the LA (AP = −1.4, ML = ± 3.5, V = −5.0 mm from bregma) or MGm/PIN (AP = −3.0, ML = ± 2.1, V = −3.1 mm from bregma) according to Paxinos and Franklin (2003). Bilateral microinjections (1.5 µL) of the CREB or Control vector were delivered over 20 min through glass micropipettes. Microinjections were left in place an additional 10 min to ensure diffusion of the vector. Because transgene expression using this viral system peaks 3 d following surgery (Barrot et al. 2002), we trained mice 3 d following surgery, except in the expression experiment. To examine the effects of increasing CREB in the MGm/PIN on expression of an auditory fear memory, mice were trained and surgery was performed 2 d later. Mice were tested 3 d following surgery.

Auditory (tone) fear conditioning
Training consisted of placing mice in a conditioning chamber (Context A) and, 2 min later, presenting a tone (2800 Hz, 85 dB, 30 sec) that coterminated with a shock (2 sec, 0.3, 0.4, or 0.5 mA, depending on experiment). Mice remained in the chamber for an additional 30 sec. Testing for auditory fear conditioning occurred 24 h later. Mice were placed in a novel chamber (Context B) and 2 min later the tone CS was presented (for 1 min). Our index of memory, freezing (the cessation of all movement except for respiration), was assessed via automated procedures (Actimetrics).

Tone fear generalization test
A subset of CREB-infused and unoperated control mice was further tested for generalization of tone fear conditioning using tones of frequencies different than the training frequency. The control mice used in this generalization experiment were trained with a single tone-shock pairing as above, using a 0.5-mA shock. Twenty-four hours following the initial CS tone test (2800 Hz), mice were placed back in Context B and presented with a similar 85-dB tone that was either higher (10,000 Hz) or lower (700 Hz) in frequency than the training tone. Mice were then transported back to their homecages. Six hours later, mice were once again placed in Chamber B and the tone of the other frequency was played. The order of tone presentation (high versus low frequency) in the tone generalization tests was counterbalanced across mice.

Context fear conditioning
CREB- or Control vector-infused mice were placed in Context A and 2 min later presented with a shock (2 sec, 0.4 mA). Mice remained in the context for an additional 30 sec. Twenty-four hours later, mice were replaced in the context and the amount of time spent freezing during the 3-min test was assessed.

Auditory startle response
Startle testing was conducted using a SR-LAB startle testing system (San Diego Instruments). Mice were placed in a Plexiglas testing cylinder (3.2 cm internal diameter). Acoustic startle stimuli and prepulse stimuli were delivered via a high-frequency speaker, placed 15 cm from the testing cylinder. Background white noise was generated by a standard speaker. The testing cylinder was mounted on a sensor platform. A piezoelectric acelerometer, attached to the base of the sensor platform, detected and transduced cage movements that were then digitized by and stored in a computer. The startle amplitude was taken to be the maximal response that occurred in the 100 msec after presentation of the startle stimulus. The sound levels for background noise and startle/prepulse stimuli were calibrated with a digital sound level meter. The speakers, testing cylinder, and sensor platform were housed within a sound-attenuated chamber.

Habitation
Mice were placed in the testing cylinder and, 5 min later, presented with 80 startle pulses of 120 dB each (15 sec interstimulus interval [ISI]).

Prepulse inhibition
The next day, mice were tested for prepulse inhibition of the startle response. Following a 5-min acclimation period where no stimuli were delivered, mice were presented with 20 habitation trials (120 dB, ISI 15 sec). In the prepulse inhibition phase, mice were presented with a total of 90 trials. Three prepulse intensities were tested: 70, 75, and 80 dB. Prepulses were 20 msec in duration with a rise/fall time of <1 msec. For each prepulse intensity, there were three types of trial: prepulse alone, prepulse/startle stimulus, and startle stimulus alone. In the prepulse/startle stimulus trial, the onset of the prepulse preceded the onset of the startle stimulus by 100 msec. All startle stimuli were presented in a pseudorandom sequence with the constraint that each stimulus intensity occur only once in each consecutive four-trial block. The % PPI was calculated per mouse for each of the three prepulse conditions.
Startle threshold

The following day, mice were given a startle threshold test session. Following an acclimation period of 5 min, mice were presented with a total of 99 trials (15-sec ISI). There were 11 trial types: no stimulus (NS), and 10 types of startle trials in which the intensity of the startle stimulus varied from 75 to 120 dB (with 5 dB increments). The startle stimuli were 40 msec noise bursts with a rise/fall time of <1 msec. The 11 trial types (NS, startle stimuli) were presented in a pseudorandom order such that each trial type was presented once within a block of 11 trials. Startle threshold was defined as the minimal intensity at which responding was significantly greater than in the NS trials.

Histology

Placement and extent of the viral infection were determined by GFP-immunofluorescence. Only those mice that showed robust bilateral expression of GFP in the target region (LA or MGN/PIN, depending on the experiment) were included in subsequent statistical analysis.

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