A time-dependent role of midline thalamic nuclei in the retrieval of fear memory

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ABSTRACT

Increasing evidence indicates that the medial prefrontal cortex (mPFC) and the amygdala mediate expression and extinction of conditioned fear, but few studies have examined the inputs to these structures. The dorsal part of the midline thalamus (dMT) contains structures such as the mediodorsal nucleus, paraventricular nucleus, and paratenial nucleus that project prominently to mPFC, as well as to basal (BA) and central (Ce) nuclei of the amygdala. Using temporary inactivation with GABA agonist muscimol, we found that dMT was necessary for retrieving auditory fear memory that was 24 h old, but not 2–8 h old. However, pre-training infusions did not impair fear acquisition or extinction. To determine the possible targets of dMT that might modulate fear retrieval, we combined dMT inactivation with Fos immunohistochemistry. Rats with inactivation-induced impairment in fear retrieval showed increased Fos in the lateral division of Ce (CeL), and decreased Fos in the medial division of Ce. No differences in Fos expression were observed in the mPFC or BA. We suggest that the projections from the paraventricular nucleus to CeL are involved in retrieval of well consolidated fear memories.

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1. Introduction

In recent years, there has been renewed interest in the neural circuits mediating the regulation of conditioned fear memories. The medial prefrontal cortex (mPFC) has emerged as a key structure in emotion regulation (Etkin et al., 2011; Quirk and Beer, 2006; Sotres-Bayon et al., 2006), and can modulate fear expression bidirectionally via projections to the amygdala (McDonald, 1991; Quirk and Beer, 2006; Sotres-Bayon and Quirk, 2010; Vertes, 2004). Pharmacological inactivation and stimulation studies indicate that the prelimbic (PL) subregion of the mPFC is essential for fear expression (Corcoran and Quirk, 2007; Sierra-Mercado et al., 2011; Vidal-Gonzalez et al., 2006), whereas the infralimbic subregion (IL) is essential for fear extinction (Kim et al., 2009; Laurent and Westbrook, 2009; Milad and Quirk, 2002; Sierra-Mercado et al., 2011). PL and IL both receive strong input from the dorsal midline thalamic nuclei (dMT), including the mediodorsal nucleus (MD) and paraventricular nucleus (PV) (Hoover and Vertes, 2007; Vertes, 2006). dMT nuclei also project to the basal (BA) and central (Ce) nuclei of the amygdala (Turner and Herkenham, 1991; Vertes and Hoover, 2008). Thus, dMT nuclei are well situated to

modulate fear retrieval and/or extinction, either via the mPFC or the amygdala.

Despite anatomical as well as physiological evidence for an influence of dMT on mPFC and amygdala neurons (Ferron et al., 1984; Gigg et al., 1994; Pirot et al., 1994; Vives and Mogenson, 1985), relatively few studies have examined dMT's role in conditioned fear. Regarding fear acquisition, MD lesions reportedly had no effect on acquisition or expression of tone conditioning (Garcia et al., 2006), but impaired acquisition and expression of context conditioning (Li et al., 2004). Regarding extinction, MD lesions did not impair extinction learning or retrieval (Garcia et al., 2006), even though extinction increases mPFC potentials evoked by MD stimulation (Herry and Garcia, 2002; Herry et al., 1999).

The lesion approach often underestimates the role of a given structure, due to recovery of function by other areas (Anglada-Figueroa and Quirk, 2005; Poulos et al., 2010). In light of this, we recently used the GABA_A agonist muscimol to evaluate the contributions of prefrontal, hippocampal, and amygdalar areas to the expression and extinction of tone-induced fear (Sierra-Mercado et al., 2011). In the present study, we used the same technique to evaluate the role of the dMT in fear acquisition, retrieval and extinction. We found that while dMT was not necessary for acquisition or extinction, it was necessary for retrieval of fear memories that were 24 h old, but not 2–8 h old. We extended our approach with Fos immunohistochemistry (neural activity marker) to evaluate the effect of dMT inactivation on target areas involved in

fear regulation. Impaired fear retrieval was correlated with changes in Fos expression in Ce, but not in PL, IL or BA.

2. Materials and methods

2.1. Subjects

A total of 108 male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) weighing 270–320 g were housed and handled as described previously (Quirk et al., 2000). Rats were restricted to 18 g/day of standard laboratory rat chow, followed by training to press a bar for food on a variable interval schedule of reinforcement (VI-60). Pressing a bar for food ensures a constant level of activity against which freezing behavior can be reliably measured during long training sessions, and provides an additional measure of fear (suppression ratio) that is more sensitive than freezing (Quirk et al., 2000). All procedures were approved by the Institutional Animal Care and Use Committee of the University of Puerto Rico, School of Medicine in compliance with National Institutes of Health guidelines for the care and use of laboratory animals.

2.2. Surgery

After bar-press training, rats were anesthetized with intraperitoneal injections of a mixture of ketamine (80 mg/kg)-xylazine (10 mg/kg) and were stereotaxically implanted with a double 26-gauge guide cannula (Plastics One, Roanoke, VA, USA) targeting the medial dorsal nucleus of the thalamus (coordinates: anteroposterior, −2.5 mm from bregma; mediolateral, +/−0.60 mm from midline; dorsoventral, −4.5 mm from skull surface) (Paxinos and Watson, 1997). Cannulas were fixed to the skull with dental cement and two stainless steel screws. After surgery, a triple antibiotic was applied and an analgesic (Ketofen; 2 mg/kg) was injected intramuscularly. Stainless steel obturators (33-gauge) were inserted into the guide cannulas to avoid obstructions until infusions were made. After surgery, animals were allowed to recover 5–7 days before initiating experiments.

2.3. Histology

Upon completion of experiments, rats were transcardially perfused with 0.9% saline followed by 10% buffered formalin. Brains were extracted and stored in a 30% sucrose/10% formalin solution. Coronal sections were cut 40 μm thick, mounted on slides, and stained for Nissl bodies.

2.4. Drug infusions

On the day prior to commencement of the experiment (Day 0), injectors were briefly inserted without infusion and rats were acclimated for handling. Injector tips extended 1.0 mm beyond the guide cannula. Muscimol (MUS; Sigma Aldrich) was used to enhance GABA$_A$ receptor activity, thereby inactivating target structures, and was infused 15 min prior to behavioral testing. MUS or saline-vehicle (SAL) was infused at a rate of 0.2 μl/min (0.11 nmol/0.2 μl/per side) (Sierra-Mercado et al., 2011). After infusion, injectors were left in place for 1 min to allow the drug to diffuse.

2.5. Fear conditioning and extinction

Rats were conditioned and extinguished in standard operant chambers (Coulbourn Instruments, Allentown, PA) located in sound-attenuating cubicles (Med Associates, Burlington, VT). The floor of the chambers consisted of stainless steel bars that delivered a scrambled electric footshock. Between experiments, shock grids and floor trays were cleaned with soap and water, and chamber walls were cleaned with wet paper towels. The same chamber was used for conditioning, retrieval tests, and extinction training. Rats were conditioned with a pure tone (30 s, 4 kHz, 77 dB) paired with a shock delivered to the floor grids (0.5 s, 0.52 mA). All trials were separated by a variable interval averaging 3 min.

Conditioning was performed on Day 1 and consisted of 5 habituation tones followed immediately by 7 conditioning tones that co-terminated with footshocks. To evaluate the role of dMT in fear acquisition and extinction learning, dMT was inactivated prior to the conditioning session on day 1 or prior to the extinction session on day 2. In both of these experiments, 20 extinction tones were given. Extinction memory was evaluated on day 3, with a 15 tone test. To evaluate the role of dMT in fear retrieval, dMT was inactivated at one of four post-conditioning timepoints: 2, 4, 8 or 24 h post-conditioning. Each group was given two tones, 15 min after infusion.

One week after completion of fear experiments, a subset of rats were infused with either MUS or SAL to assess the effects of dMT inactivation on locomotor activity and anxiety levels. An open field square apparatus was used (l: 91.5 cm, w: 91.5 cm, h: 61 cm), which was divided into peripheral (within 15.25 cm of the walls) and central (l: 61 cm, w: 61 cm) regions of equal area. Rats were tested for 10 min each (Walsh and Cummins, 1976).

2.6. Immunohistochemistry

Ninety minutes after fear retrieval, muscimol or saline infused rats were deeply anesthetized with sodium pentobarbital (450 mg/kg i.p.) and perfused transcardially with 100 ml saline (0.9%), followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were post fixed for 3 h in the same fixative solution and transferred to a solution of 30% sucrose in 0.1 M phosphate buffer at 4 °C for 2 nights. Brains were then frozen and a series of 40 μm sections were cut with a cryostat (Leica, CM 1850) in the frontal plane and collected at different levels of the medial prefrontal cortex and amygdala. One complete series of sections was processed for immunohistochemistry with anti-Fos serum raised in rabbit (Ab-5, Oncogene Science) at a dilution of 1:20,000 overnight. The primary antiserum was localized using a variation of the avidin−biotin complex system. Sections were incubated for 120 min at room temperature in a solution of biotinylated goat anti-rabbit IgG (Vector Laboratories) and then placed in the mixed avidin−biotin−horseradish peroxidase complex solution (ABC Elite kit, Vector Laboratories) for 90 min. Black immunoreactive nuclei labeled for Fos were visualized after 10 min of exposure to a chromogen solution containing 0.02% 3,3′ diaminobenzidinetetrahydrochloride with 0.3% nickel-ammonium sulfate (DAB-Ni) in 0.05 M Tris buffer (pH 7.6) followed by incubation for 5 min in a chromogen solution with glucose oxidase (10%) and D-Glucose (10%). The reaction was stopped using potassium PBS (pH 7,4). Sections were mounted in coated-gelatin slides and then dehydrated and cover slipped. Counter sections were collected, stained for Nissl bodies, cover slipped and examined in an optical microscope to determine the anatomical boundaries of each structure analyzed.

2.7. Immunoreactivity quantification

Counting of Fos positive cells was carried out at 20× magnification of an Olympus microscope (Model BX51) equipped with a digital camera. Images were generated for PL, IL, basal nucleus of the amygdala (BA), lateral/central nucleus of the central amygdala (CeL/CeC) and medial nucleus of the central amygdala (CeM). To be considered positive for Fos-like immunoreactivity, the nucleus had to be of appropriate size (area ranging from 100 to 500 μm^2) and shape (at least 50% of circularity), and be distinct from the background. Fos positive cells were automatically counted and averaged across 2 distinct rostro-caudal sections for each brain structure analyzed (Metamorph software version 6.1). The density of Fos positive cells in the CeL/CeC and CeM was calculated by dividing the number of Fos positive cells by the total area of each region.

2.8. Data collection and analysis

Behavior was recorded with digital video cameras (Micro Video Products, Bobcaygeon, Ontario, Canada) and freezing was measured using commercially available software (Freezescan, Clever Systems, Reston, VA, USA). Trials were averaged in blocks of two and compared with repeated-measures analysis of variance (ANOVA), followed by Tukey's post hoc comparisons (STATISTICA; Statsoft, Tulsa, OK). In some comparisons, Student's t-tests (two-tailed) were used. In addition to freezing, suppression of bar-pressing was used as a measure of conditioned fear (Quirk et al., 2000; Sierra-Mercado et al., 2006) and analyzed using Student's t-test. A suppression ratio comparing pre-tone press rates with tone press rates was calculated as follows: (pre-tone − tone)/(pre-tone + tone). A value of 0 represents no suppression (low fear), whereas a value of +1 represents complete suppression (high fear). For the open field, the total number of line crosses and percent time spent in the center were assessed.

3. Results

3.1. dMT is not necessary for acquisition of conditioned fear

Fig. 1A shows the location of injector tips for muscimol infusion within the mediodorsal nucleus for all experiments. Fifteen minutes prior to conditioning, rats were infused with 0.2 μl of physiological saline (SAL) or muscimol (0.11 nmol), which was the same dose we used previously to inactivate PL and IL (Sierra-Mercado et al., 2011). We estimated a spread of approximately 1 mm diameter, based on prior studies tracking labeled muscimol (Corcoran et al., 2005; Pothuizen et al., 2005). This degree of spread would affect MD, PV, part of CM, and perhaps the more rostrally located PT. As shown in Fig. 1B, inactivation of dMT prior to conditioning did not affect acquisition of fear, as repeated-measures ANOVA revealed no effect of drug ($F_{(1,50)} = 0.29$, $p = 0.59$) or interaction between drug and trial block ($F_{(5,50)} = 0.20$, $p = 0.95$). The following day (Day 2), freezing during the first trial block was
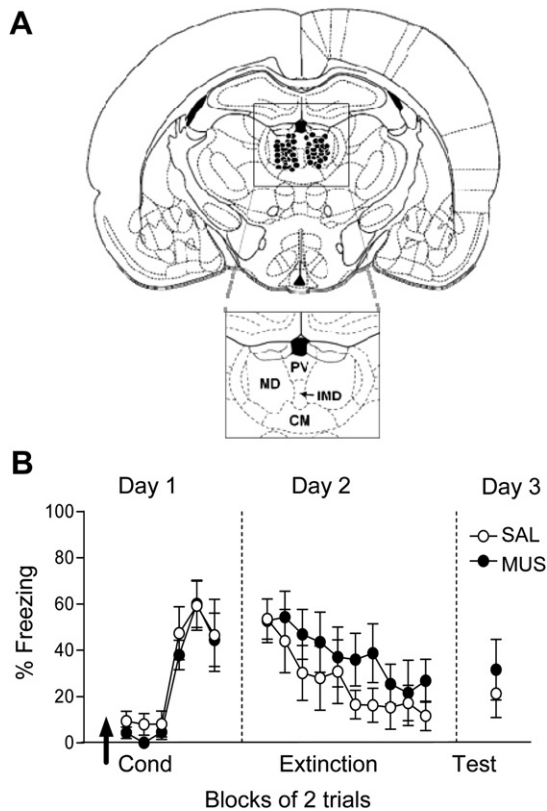
A

B

Fig. 1. Inactivation of dMT does not impair acquisition of fear conditioning. (A) Schematic showing the location of injector tips in dorsal midline thalamus (dMT) for muscimol-infused rats in all experiments. Abbreviations: MD: mediodorsal nucleus, PV: paraventricular nucleus, CM: centromedial nucleus, IMD: intermediodorsal nucleus. Adapted from Paxinos and Watson (1997). (B) Infusion of saline (SAL) or muscimol (MUS) into dMT 15 min prior to conditioning (arrow) did not alter freezing during conditioning, extinction (Day 2), or retrieval of extinction (Day 3). Data are shown as mean ± SEM, in blocks of two trials (SAL $n = 6$, MUS $n = 6$).

similar between SAL and MUS groups (52.71%; 53.48%, respectively; $t_{10} = 0.05$, $p = 0.95$), indicating that both groups acquired similar levels of fear during conditioning. In addition, a repeated-measures ANOVA revealed a significant effect of trial block indicating that animals extinguished ($F_{(9,90)} = 4.65$, $p < 0.001$), but no effect of drug ($F_{(1,10)} = 0.70$, $p = 0.42$). Together these data suggest that dMT is not necessary for fear acquisition.

3.2. dMT is not necessary for fear extinction

We next investigated the role of dMT in extinction of conditioned fear, by inactivating prior to extinction training on Day 2. As shown in Fig. 2, dMT inactivation prior to extinction decreased fear expression throughout the extinction session. A repeated-measures ANOVA revealed a main effect of drug ($F_{(1,17)} = 7.62$; $p = 0.013$) and an interaction between drug and trial block ($F_{(9,153)} = 3.11$, $p = 0.002$). Subsequent analysis using Tukey *post hoc* test confirmed a significant reduction in freezing by MUS during the first four blocks of extinction training ($p < 0.01$). In addition to reducing freezing, repeated-measures ANOVA revealed that MUS significantly reduced conditioned suppression of bar pressing (suppression ratio for 1st block, SAL: 0.97; MUS: 0.43; $F_{(1,17)} = 10.72$; $p < 0.01$). Subsequent analysis using Tukey *post hoc* test confirmed a significant reduction in suppression ratio during the first 5 blocks ($p < 0.001$).

Despite reduced expression of freezing, the groups significantly decreased their freezing throughout the extinction session. Repeated-measures ANOVA revealed a significant effect of trial
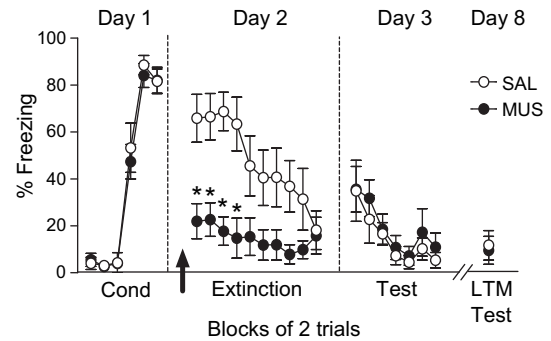

Fig. 2. dMT inactivation impaired retrieval of fear, but not extinction. Inactivation of dMT prior to extinction training (arrow) significantly decreased freezing in trial blocks 1–4. The next day, however, MUS-infused rats showed normal retrieval of extinction learned the previous day. MUS-infused rats continued to show normal extinction retrieval after an additional 5 days. Data are shown as mean ± SEM in blocks of two trials (SAL $n = 9$; MUS $n = 10$). *$p < 0.01$ Repeated-measures ANOVA followed by Tukey's post hoc test.

block ($F_{(9,153)} = 5.46$, $p = 0.001$). On day 3, both groups showed similar retrieval of extinction. Freezing during the first block of tones was similarly low in both groups (SAL: 34.94%; MUS: 35.56%, $t_{18} = -0.03$; $p = 0.97$). A repeated-measures ANOVA for the extinction test revealed a significant effect of trial block ($F_{(6,102)} = 9.95$, $p < 0.001$), but no effect of drug ($F_{(1,17)} = 0.03$, $p = 0.84$) nor interaction between drug and trial block ($F_{(6,102)} = 0.80$, $p = 0.56$). Retrieval of extinction was also unimpaired 5 days later (see Fig. 2). Together, these data suggest that dMT is necessary for retrieval of a previously acquired fear memory, but not for extinction learning.

3.3. dMT has a time-dependent role in retrieval of fear memories

The pattern of results above suggests that dMT becomes involved in fear expression sometime after the initial acquisition phase. To explore the time course of dMT involvement, we conditioned four groups of rats drug-free. We then inactivated dMT at one of four timepoints post-conditioning: 2 h, 4 h, 8 h or 24 h. Fifteen minutes after each infusion, rats were given two tones to test for retrieval of fear memory (each group was only infused once).

Fig. 3 shows the conditioning levels (average of last three trials of conditioning) for all groups prior to infusions. As expected, two way ANOVA for conditioning showed no significant effect for drug ($F_{(1,51)} = 0.05$, $p = 0.82$), or timepoint ($F_{(3,51)} = 0.09$, $p = 0.07$). Subsequent dMT inactivation did not impair retrieval of fear in the 2, 4, or 8 h groups (all t's < 1.07, all p's > 0.30), but did impair retrieval in the 24 h group (SAL: 58.44%; MUS: 6.05%; $t_{17} = 5.15$; $p = 0.001$), replicating our findings from Fig. 2. In addition to reduced freezing, 24 h MUS rats showed a significant reduction in suppression ratio during fear retrieval (SAL: 0.80; MUS: 0.26; $t_{17} = 3.54$, $p = 0.002$). Therefore, the involvement of dMT in fear retrieval appears to be time-dependent, with dMT becoming involved 24 h following conditioning. The loss of memory in the 24 h group did not appear to be permanent, as a drug free test on day 3 showed equivalent levels of freezing in both groups (SAL: 27.62%; MUS: 30.54%; $t_{17} = -0.19$; $p = 0.85$) as well as similar suppression ratios (SAL: 0.63; MUS: 0.93; $t_{17} = -1.35$, $p = 0.19$). Thus, activity in dMT appears to be necessary for retrieval of fear memory, rather than consolidation or retention.

In addition to reduced tone fear, MUS rats infused 24 h after conditioning also showed reduced contextual fear, as indicated by the rates of bar pressing for food. In SAL rats, conditioning
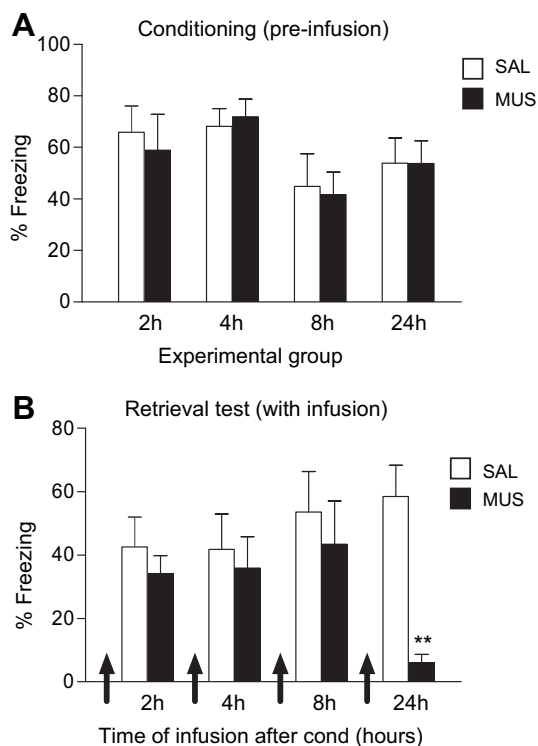
Fig. 3. Inactivation of dMT impaired retrieval of long-term, but not short-term, fear memory. (A) Freezing levels to tones in all groups during conditioning trials 5–7, on day 1, prior to infusions. (B) Freezing levels 15 min after infusion of SAL or MUS, at different post-conditioning delays (2 h, 4 h, 8 h, 24 h, each group was infused only once). Only at 24 h did MUS impair conditioned freezing (**$p < 0.01$). Data are shown as mean ± SEM in blocks of two trials (2 h: SAL $n = 7$, MUS $n = 7$; 4 h: SAL $n = 7$, MUS $n = 7$; 8 h: SAL $n = 7$, MUS $n = 7$; 24 h: SAL $n = 9$; MUS = 9).

significantly reduced the rate of spontaneous pressing prior to the first tone (pre-cond: 23.0 presses/min; pre-ext: 11.11 press/min; $t_{(1,9)} = 2.306$; $p = 0.004$). In contrast, MUS rats showed no such reduction in press rates (pre-cond: 17.66; pre-ext: 17.77; $t_{(1,9)} = 2.306$; $p = 0.973$) consistent with a deficit in retrieval of contextual fear.

3.4. dMT inactivation modulates Fos expression in Ce, but not in BA or mPFC

To investigate the neural circuit through which dMT might influence fear, we assessed Fos expression in rats infused with SAL or MUS 24 h after conditioning. Again, replicating our findings above (Figs. 2 and 3), inactivation of dMT 24 h after conditioning impaired the retrieval of fear memory, as evidenced by significantly reduced freezing during a two-tone test (MUS: 78.5%; SAL: 17.4%; $t_7 = 4.17$, $p = 0.005$) (see Fig. 4A). Ninety minutes after the test, rats were sacrificed and the brains were processed for Fos immunoreactivity. dMT inactivation had no effect on Fos expression in PL, IL or BA (all t's < 0.31, all p's > 0.76) (see Fig. 4B–C). In contrast, inactivation of dMT significantly increased Fos expression in CeL (SAL: 7.67; MUS: 25.25, $t_7 = -2.73$; $p = 0.04$), and significantly decreased Fos expression in CeM (SAL: 7.75; MUS: 3.75, $t_7 = 2.82$; $p = 0.03$) (see Fig. 4D). The density of Fos positive cells in the saline group showed a trend toward higher counts in CeM compared to CeL (CeM: 5.3; CeL: 2.87, $t_6 = -2.23$; $p = 0.08$), consistent with fear retrieval (inset Fig. 4D). In contrast, under muscimol, CeL showed a significantly higher density of label than CeM (CeL: 9.4; CeM: 2.4, $t_8 = 3.34$; $p = 0.01$). Given that CeL contains GABAergic neurons that inhibit CeM output neurons (Ciocchi et al., 2010; Martina et al.,

1999), our findings suggest that dMT inactivation reduced fear by increasing feed-forward inhibition of CeM.

3.5. Inactivation of dMT does not affect locomotion, anxiety, or motivation

Given the marked effects of dMT inactivation on freezing, we sought to determine if dMT inactivation produced other behavioral effects that might potentially confound freezing measurements. Inactivation of dMT did not affect motivation to press a lever for food, as indicated by similar rates of spontaneous pressing in SAL and MUS-infused rats prior to conditioning (MUS: 14.3 presses per min, SAL: 12.05 presses per min; $t_{13} = -0.54$, $p = 0.59$). To assess open field behavior, a subset of rats was re-infused one week following the fear experiments. Inactivation of dMT did not increase locomotion in the open field, as indicated by the number of line crosses (MUS: 253.4, SAL: 235.0; $t_{13} = -1.12$; $p = 0.28$). Neither did dMT inactivation alter anxiety levels, since the amount of time spent in the center of the open field was similarly low for both MUS and SAL groups (14.0%, 13.4%, respectively; $t_{13} = -0.22$, $p = 0.82$).

4. Discussion

Previous studies using permanent lesions to assess the role of dorsal midline thalamic areas in conditioned fear have produced conflicting results. Here, we used the GABA$_A$ agonist muscimol to induce temporary inactivation of dMT during different phases of conditioning and extinction. We found that: (i) inactivation of dMT prior to conditioning or extinction training did not impair these processes; (ii) dMT was necessary for retrieval of long-term, but not short-term fear memory, and (iii) dMT inactivation did not alter Fos expression in mPFC or BA, but increased and decreased, respectively, Fos expression in CeL and CeM nuclei. These findings suggest that retrieval of a well consolidated fear memory involves dMT modulation of Ce activity.

4.1. The role of dMT in acquisition and extinction

Our negative results with respect to acquisition of fear agree with a previous finding that electrolytic lesions of MD did not affect acquisition of auditory fear conditioning (Garcia et al., 2006). Thus, dMT does not appear to be an essential site of plasticity for fear learning, at least for auditory conditioning. We also observed that inactivation of dMT did not alter extinction learning. These results were unexpected, given the extensive reciprocal connections between MD and infralimbic-mPFC (Hoover and Vertes, 2007), a key region in recall of fear extinction (Laurent and Westbrook, 2009; Quirk et al., 2006), and the fact that extinction potentiates MD-mPFC evoked potentials (Herry and Garcia, 2002; Herry et al., 1999). The critical factor here may be time, as a previous study showed that MD-mPFC evoked potentials increased 7 days after extinction training, (Hugues and Garcia, 2007). We observed stable extinction memory 7 days after extinction with pre-extinction infusions, however, the effect of MD inactivation 7 days after extinction training on recall of extinction is not known.

4.2. The time-dependent role of dMT in fear retrieval

Despite these negative findings, dMT was necessary for retrieval of fear memory learned the previous day. To our knowledge, this is the first evidence that dMT activity is essential for retrieval of a tone-shock association. In addition to impaired retrieval during the tone, we also observed impaired contextual fear, reflected in bar press rates, which agrees with prior studies examining lesions of MD made prior to (Antoniadis and McDonald, 2006; Li et al., 2004),
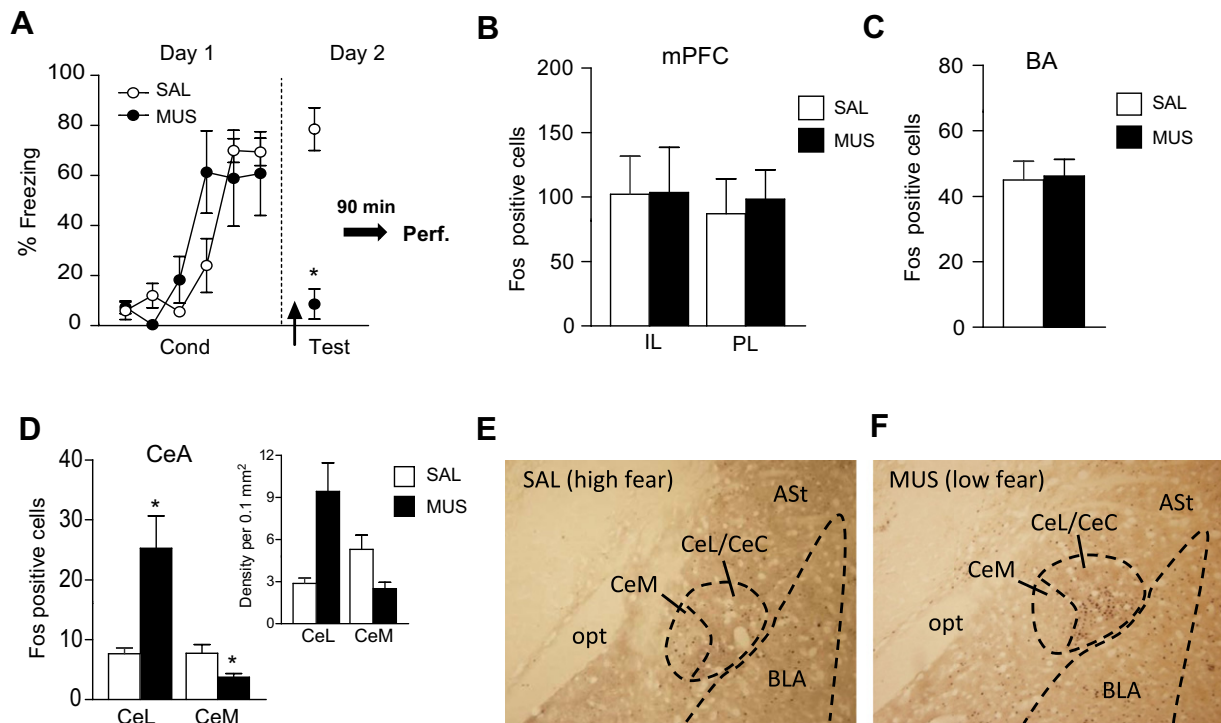
Fig. 4. Inactivation of dMT altered Fos expression in the amygdala central nucleus. (A) Replicating our findings above (see Fig. 2 and Fig. 3B), dMT infusion of MUS 24 h after conditioning (arrow) impaired fear retrieval. 90 min later, brains were processed for Fos immunohistochemistry. (B–C) dMT inactivation did not alter Fos counts in IL, PL, or BA. (D) In contrast, dMT inactivation increased Fos counts in CeL and decreased Fos counts in CeM. The density of Fos positive cells in CeM and CeL is shown in the inset. *$p < 0.05$ *t*-test between MUS and SAL groups (SAL $n = 3$; MUS $n = 4$). (E–F) Photomicrographs showing Fos labeled neurons in the amygdala of SAL and MUS-infused rats (10× magnification). Note the shift of label from CeM to CeL with MUS infusion.

or after (Li et al., 2004) contextual fear conditioning. A surprising finding was the time dependence of our effects: inactivation of dMT 2, 4 or 8 h after conditioning did not impair fear retrieval. This suggests that thalamic circuits may be recruited sometime between 8 and 24 h after training for retrieval of fear conditioning. Late phase involvement of dMT in fear retrieval has not yet been reported, but similar observations have been made for other structures. For example, 12 h after inhibitory avoidance training, there is a sudden increase in Fos expression in the hippocampus (Katche et al., 2010).

It was surprising that dMT inactivation did not reduce Fos immunoreactivity in PL, given dMT's strong reciprocal connections with PL (Groenewegen, 1988; Moga et al., 1995; Van der Werf et al., 2002; Vertes and Hoover, 2008), and the role of PL in expression of conditioned fear (Burgos-Robles et al., 2009; Choi et al., 2010; Laurent and Westbrook, 2009; Sierra-Mercado et al., 2011). At face value, this suggests that dMT modulates fear via targets other than the mPFC. PL involvement, however, cannot be completely ruled out based on these findings, because Fos may not be sufficiently sensitive to detect changes in tone responses in PL neurons, given their high baseline firing rate (Burgos-Robles et al., 2009). The critical test of PL involvement will be to record PL unit responses in rats with dMT inactivation, to determine the extent to which PL tone responses are dependent on dMT inputs. Regarding BA, our negative Fos findings agree with prior observations that small lesions (Onishi and Xavier, 2010) or inactivation (Herry et al., 2008) of BA did not impair retrieval of auditory fear conditioning.

4.3. dMT modulation of amygdala central nucleus

In contrast to PL and BA, Ce showed pronounced Fos changes after dMT inactivation. Fos expression increased in CeL and

decreased in CeM. It is well established that CeM neurons mediate amygdala control of fear via subcortical projections to midbrain and hypothalamic targets (Antoniadis and McDonald, 2006; Davis, 2000; De Oca et al., 1998; LeDoux et al., 1988), and that CeL sends inhibitory projection to CeM output neurons (Lopez de Armentia and Sah, 2004; Petrovich and Swanson, 1997). This CeL-CeM network has received increased attention recently for its role in fear acquisition and expression (Ehrlich et al., 2009; Pape and Paré, 2010; Wilensky et al., 2006). Retrieval of auditory fear memory was correlated with excitatory tone responses in CeM neurons, together with inhibitory tone responses in CeL neurons, consistent with disinhibition of CeM (Ciocchi et al., 2010; Duvarci et al., 2011). Furthermore, two subpopulations of reciprocally connected CeL neurons were identified (Haubensak et al., 2010), suggesting gating of CeL projections to CeM. Thus, dMT could drive fear responses by exerting feed-forward inhibition of the CeL neurons that project to CeM.

Given known projections of the midline thalamus (Li and Kirouac, 2008; Moga et al., 1995; Vertes and Hoover, 2008), there are several possible circuits that are consistent with the pattern of Fos immunoreactivity we observed. MD does not project to CeL, but the paraventricular nucleus (PV) does. PV could activate intra-CeL GABAergic neurons, which in turn would inhibit CeL-CeM inhibitory projections. It was previously proposed that thalamic inputs to CeL involved in retrieval of fear conditioning originated in the sensory thalamus (Ciocchi et al., 2010), but our findings suggest that PV might play this role. Consistent with this, stimulation of PV induces feed-forward inhibition of CeL neurons (Veinante and Freund-Mercier, 1998), and retrieval of conditioned fear activates Fos in PV (Beck and Fibiger, 1995). Alternatively, PV and paratenial (PT) nuclei both project to CeM (Vertes and Hoover, 2008), and could drive CeM directly. Lastly, PT projects to the dorsal

intercalated cells of the amygdala (Royer and Paré, 2002; Vertes and Hoover, 2008), which could inhibit CeL neurons that project to CeM. Additional experiments assessing conditioned responses in different parts of dMT, and connections with Ce are needed to distinguish between these possibilities.

In further support of CeL as the likely target of dMT inactivation, Duvarci et al. (2011) observed that the number of CeL neurons with inhibitory tone responses was increased 24 h after conditioning, compared to immediately after conditioning. Together with our findings, this suggests that 8–24 h after conditioning, there may be potentiation of auditory inputs to PV (from PFC, BA, or midbrain), which would increase the inhibitory responses in CeL, thereby disinhibiting CeM. A testable prediction from this scenario is that PV would show a time-dependent development of tone responses after conditioning. One possible advantage of recruiting midline thalamic areas such as PV and PT is that they target diverse areas (and functions) such as dorsal striatum (avoidance), bed nucleus of stria terminalis (anxiety), entorhinal cortex (spatial function), and accumbens (reward). In this way, retrieval of fear could be coordinated with other functions to generate the most adaptive behavioral response to a threatening stimulus.

Acknowledgments

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