Trace and contextual fear conditioning require neural activity and NMDA receptor-dependent transmission in the medial prefrontal cortex

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The contribution of the medial prefrontal cortex (mPFC) to the formation of memory is a subject of considerable recent interest. Notably, the mechanisms supporting memory acquisition in this structure are poorly understood. The mPFC has been implicated in the acquisition of trace fear conditioning, a task that requires the association of a conditional stimulus (CS) and an aversive unconditional stimulus (UCS) across a temporal gap. In both rat and human subjects, frontal regions show increased activity during the trace interval separating the CS and UCS. We investigated the contribution of prefrontal neural activity in the rat to the acquisition of trace fear conditioning using microinfusions of the γ-aminobutyric acid type A (GABA_A) receptor agonist muscimol. We also investigated the role of prefrontal N-methyl-D-aspartate (NMDA) receptor-mediated signaling in trace fear conditioning using the NMDA receptor antagonist 2-amino-5-phosphonovaleric acid (APV). Temporary inactivation of prefrontal activity with muscimol or blockade of NMDA receptor-dependent transmission in mPFC impaired the acquisition of trace, but not delay, conditional fear responses. Simultaneously acquired contextual fear responses were also impaired in drug-treated rats exposed to trace or delay, but not unpaired, training protocols. Our results support the idea that synaptic plasticity within the mPFC is critical for the long-term storage of memory in trace fear conditioning.
impairs several forms of hippocampus-dependent memory, including trace fear conditioning (Tonegawa et al. 1996; Huerta et al. 2000; Quinn et al. 2005), but it is unknown if activation of these receptors is necessary in the mPFC for the acquisition of trace fear conditioning. Data from in vivo electrophysiology studies have shown that stimulation of ventral hippocampal inputs to prefrontal neurons in mPFC produces LTP, and the induction of prefrontal LTP depends upon functional NMDARs (Laroche et al. 1990; Jay et al. 1995). If the role of mPFC in trace fear conditioning goes beyond simply maintaining CS information in working memory, then activation of NMDAR may be critical to memory formation. We test this hypothesis by reversibly blocking NMDAR neurotransmission with 2-amino-5-phosphonovaleric acid (APV) during training to examine the role of prefrontal NMDAR to the acquisition of trace fear conditioning.

Another important question is whether mPFC contributes to the formation of contextual fear memories. Fear to the training context is acquired simultaneously with fear to the auditory CS in both trace and delay fear conditioning. Conflicting reports in the literature suggest the role of mPFC in contextual fear conditioning is unclear. Damage to ventral areas of mPFC prior to delay fear conditioning has failed to impair context fear acquisition (Morgan et al. 1993). Prefrontal lesions incorporating dorsal mPFC have in some cases been reported to augment fear responses to the context (Morgan and LeDoux 1995), while blockade of NMDAR transmission has impaired contextual fear conditioning (Zhao et al. 2005). Post-training lesions of mPFC impair context fear retention (Quinn et al. 2008) in trace and delay conditioning. Contextual fear responses were assessed in this study to determine the contribution of neuronal activity and NMDAR-mediated signaling in mPFC to the acquisition of contextual fear conditioning.

Results

The contribution of prelimbic mPFC activity and NMDA receptor-mediated signaling to the acquisition of trace fear conditioning was examined using pretraining infusions of the GABA_A receptor agonist muscimol or the NMDA receptor antagonist APV. On the day of training, rats received bilateral infusions of muscimol, APV, or saline vehicle into the prelimbic mPFC 15 min before trace (Trace saline n = 16; Trace muscimol n = 13; Trace APV n = 12) or delay (Delay saline n = 15; Delay muscimol n = 10; Delay APV n = 12) fear conditioning, or unpaired control training (Unpaired saline n = 10; Unpaired muscimol n = 9; Unpaired APV n = 11). All rats were tested for CS and context fear retention 24 h later, and freezing was used as the measure of conditional fear. During the training session, prefrontal inactivation or blockade of NMDA receptor activity did not impair the expression of freezing. This observation was confirmed by analysis of freezing during the post-acquisition period immediately following trace, delay, or unpaired training. One-way ANOVAs on each training group revealed no effect of drug treatment on post-acquisition freezing in any group (data not shown; F < 1.00, P > 0.05 for each group).

Responding to the auditory CS

Inhibition of prelimbic activity or specific blockade of NMDAR-mediated signaling in the mPFC during training impairs the acquisition of trace, but not delay, fear conditioning. Twenty-four hours after training, rats were also tested for retention of conditional fear to the CS in a novel chamber. Figure 1 shows the mean percentage of time each group of rats spent freezing during the 6-min pre-CS baseline period and during the 5-min CS presentation during the CS retention test. Freezing during the 4-min post-CS period is not shown in this figure for the purpose of clarity, but was included in analyses. Figure 2 shows the minute-by-minute mean freezing during the CS retention test, including the post-CS period. Analysis of freezing during the retention test using three separate 3 × 3 mixed-model ANOVAs with within-subjects Period (Baseline, CS, Post-CS) and between-subjects Drug (Saline, Muscimol, APV) factors revealed a significant main effect of Period in each group: Trace F(2,70) = 31.15, P < 0.0001; Delay F(2,68) = 81.41, P < 0.0001; Unpaired F(2,53) = 11.72, P < 0.0001. Follow-up LSD post-hoc analyses on these significant main effects of Period showed that trace and delay conditioned rats froze more during the CS than during the baseline (P < 0.0001 for each group). In contrast, unpaired rats showed no difference in freezing during the baseline and CS periods (P = 0.931). All groups showed greater freezing in the post-CS period compared with baseline (all P < 0.0001). These results show that rats receiving paired, but not unpaired, presentations of the CS and UCS exhibit conditional fear to the CS during retention testing. Disruption of the mPFC during paired conditioning in trace, but not delay, impairs this CS–UCS association. The mixed-model ANOVA for the trace group revealed a significant Drug × Period interaction, F(4,70) = 2.97; P < 0.025. Follow-up LSD post-hoc analysis of this interaction showed that muscimol- and APV-infused rats froze significantly less than saline-infused rats during the CS (P < 0.001, P < 0.013, respectively). Post-CS freezing was similarly impaired in these rats (P < 0.017, P < 0.003, respectively). No significant Drug × Period interaction was revealed in the analysis of the delay or unpaired rats. Together, these data show that prefrontal activity in general and NMDA receptor activation in the mPFC are necessary for trace but not delay fear conditioning.

Responding to the training context

Inhibition of prelimbic activity or blockade of NMDAR-mediated signaling in mPFC during training impairs the acquisition of context fear during auditory-paired fear conditioning. Twenty-four hours after training, rats were also tested for retention of conditional fear to the context in the original training chamber. Figure 3 shows the mean percentage of time each group of rats spent freezing during the 15-min exposure to the training context. Trace and delay conditioned rats, but not unpaired control rats, that had received muscimol or APV infusions exhibited decreased freezing.
During the context reexposure compared with saline-infused controls, a one-way ANOVA on context freezing in the trace group revealed a significant effect of Drug treatment, \(F_{(2,33)} = 7.05, P < 0.003\). LSD post-hoc analysis showed that rats in the muscimol and APV conditions froze significantly less than saline control rats (\(P < 0.002\), \(P < 0.007\), respectively). Similarly, analysis of context freezing in the delay group revealed an effect of Drug treatment, \(F_{(2,24)} = 3.96, P < 0.029\). LSD post-hoc analysis showed that rats in the muscimol and APV conditions froze significantly less than saline control rats (\(P < 0.049\), \(P < 0.013\), respectively). In contrast, infusion of muscimol or APV had no effect on contextual fear acquisition in the unpaired group, \(F_{(2,26)} = 1.78, P = 0.189\). This was an interesting finding because the mechanisms supporting contextual fear conditioning were expected to be similar in each training group. However, these data show that general activity and NMDA receptor activation in prelimbic mPFC are necessary for the formation of fear responses to the training context in some, but not necessarily all, conditioning paradigms.

Reacquisition of conditional fear responses

The observed impairments in trace and contextual fear conditioning following pretraining muscimol and APV infusions were not a result of permanent damage to prefrontal tissue. This was confirmed by retraining a subset of trace (\(n = 29\)) and delay (\(n = 22\)) rats in the absence of any drug. These rats were tested for CS and context retention 24 h later in the same manner as the original tests. A comparison of impaired retention in the first test with intact retention after retraining can be found in Figure 4. Figure 4 shows the minute-by-minute freezing during the CS retention test 24 h after initial trace conditioning (panel A) and during the CS retention test following retraining (panel B). A 3 \(\times\) 3 mixed-model ANOVA with within-subjects Period (Baseline, CS, Post-CS) and between-subjects Drug (Saline, Muscimol, APV) factors on freezing during this second test revealed a significant main effect of Period, \(F_{(2,52)} = 17.60, P < 0.0001\). Follow-up LSD post-hoc analysis showed that trace rats froze significantly more during the CS and post-CS periods compared with baseline freezing. There was no effect of drug history on freezing as neither the main effect of Drug (\(F_{(2,26)} = 0.31\)) nor the Drug \(\times\) Period interaction was significant. The same analysis was applied to the delay group during the CS retention after retraining (data not shown). There was no main effect of Drug nor a Drug \(\times\) Period interaction, but the ANOVA revealed a significant main effect of Period \(F_{(2,38)} = 50.66, P < 0.0001\). Follow-up LSD post-hoc analysis of freezing during each period showed that, regardless of drug history, delay rats froze significantly more during the CS and post-CS periods than during baseline. Just as with acquisition of fear to the CS, trace and delay rats were able to acquire contextual fear responses when retrained in the absence of drug. Figure 4 shows the mean time spent freezing during the context retention test 24 h after initial training (panel C) or 24 h after retraining (panel D). A one-way ANOVA on each group in panel D did not reach significance, \(F < 1.00\) for each group. Together, these findings demonstrate that the effects of muscimol and APV were
Twelve rats were excluded from analyses because of extensive tissue damage to the prelimbic beyond the immediate area of the guide cannulae placed correctly in the prelimbic area. Figure 5 shows the location of the guide cannulae placed correctly in the prelimbic area of the mPFC. A recent quantitative analysis of the spread of fluorescently labeled muscimol (1 μL) in the mPFC has been shown to have an average maximal spread of 1.7 mm (Martin 1991).

**Histology**

Behavioral analyses were conducted using 108 of the 120 rats in this study. All 120 rats showed reliable bilateral cannula placement in the prelimbic area of the mPFC. Figure 5 shows the location of the guide cannulae placed correctly in the prelimbic area. Twelve rats were excluded from analyses because of extensive tissue damage to the prelimbic beyond the immediate area of the cannula site (n = 10), infection (n = 1), or blocked cannulae (n = 1). This study did not directly test the spread of drug infusion; however, based on previous reports, we estimate the diffusion of drug did not extend substantially beyond the prelimbic region of the mPFC. A recent quantitative analysis of the spread of fluorescently labeled muscimol (1 μg/μL) in the mPFC showed that an infusion of 0.5 μL muscimol in saline vehicle has an effective area between 0.5 and 1.0 mm around the infusion site with white matter providing natural boundaries to the spread (Allen et al. 2008). Fluorescently labeled muscimol may, however, have a more limited spread compared with unlabeled muscimol because of its higher molecular weight. Autoradiographic analysis of [3H]muscimol may provide a better estimation of spread, and the infusion of a larger volume of 1.0 μL [3H]muscimol into cortex has been shown to have an average maximal spread of 1.7 mm (Martin 1991).

Furthermore, glucose uptake (a measure of functional inactivation) is maximally reduced within 1.0 mm of the muscimol infusion site (Martin 1991). A similar diffusion pattern of 1.0 μL APV has been reported in hippocampus (Steele and Morris 1999). Using 0.5 μL APV in CA3 hippocampus, Lee and Kesner (2002) showed that this size infusion was functionally limited to the CA3 subregion of the hippocampus as LTP was affected in CA3, but not in the neighboring CA1 and dentate gyrus subregions of the hippocampus. We have used a more conservative volume of 0.3 μL, which has been used successfully to distinguish between prelimbic and infralimbic mPFC function (LaLumiere et al. 2010). While we cannot rule out the possibility that diffusion of muscimol or APV may have extended a small amount into the more ventral IL or the more dorsal anterior cingulate areas of the mPFC, we are reasonably confident that the effective area of drug infusion in this study is within the prelimbic mPFC.

**Discussion**

This study examined the effects of temporary inactivation of prelimbic mPFC and blockade of NMDAR-mediated neurotransmission on the acquisition of trace and delay fear conditioning. Both manipulations of prefrontal function during trace fear conditioning impaired retention of fear to the CS and context. In contrast, muscimol and APV infusion had no effect on retention for the delay conditioned CS, but did impair contextual fear retention in these rats. These results suggest that the acquisition of trace and contextual fear conditioning requires cellular activity in the prelimbic mPFC and also activation of prefrontal NMDAR receptors.

Disruption of cellular activity in prelimbic mPFC during training impaired subsequent CS retention in the trace, but not the delay groups. This is consistent with previous findings, which showed that prefrontal activity is not necessary for the acquisition of standard delay fear conditioning. Permanent lesions of the ventral and dorsal areas of the mPFC have no effect on the acquisition and retention of delay fear conditioning (Morgan et al. 2003; but see Sacchetti et al. 2002). In contrast, prefrontal lesions impair acquisition and retention of trace eyelink conditioning, in which an auditory CS is paired with a puff or periorbital shock UCS separated by a 500-msec trace interval (Kronforst-Collins and Disterhoft 1998; Weible et al. 2000; McLaughlin et al. 2002). Similar impairments in trace eyelink conditioning were observed using temporary inactivation of mPFC with muscimol during training (Takehara-Nishiuchi et al. 2005). We have extended these findings to trace fear conditioning in which the trace interval is several orders of magnitude greater than in trace eyelink conditioning. Inactivation of mPFC during trace fear conditioning impaired freezing fear responses 24 h later. Disruption of prefrontal activity was temporary, as these rats were able to learn trace fear conditioning when retrained several days later. The importance of intact prefrontal function during trace, but not delay conditioning, suggests that prefrontal activity may be necessary for the association of the CS and UCS when they are separated in time.
Activity in the prefrontal cortex may be necessary to bridge the temporal gap between CS and UCS in trace fear conditioning. In order for the CS to be associated with the UCS during temporal learning such as trace conditioning, a representation of the CS may need to be present when the UCS is delivered (Wallenstein et al. 1998; Rodriguez and Levy 2001; Mongillo et al. 2003; Reutimann et al. 2004). One function of the mPFC is to maintain cue-related information in working memory for seconds or minutes in order to perform task-relevant responses (Fuster 1989). Cue-induced sustained increases in prefrontal single-neuron firing have been observed during delay periods in a number of working memory tasks in rats and primates (Fuster 1973; Funahashi et al. 1989; Sawaguchi and Yamane 1999; Chang et al. 2002). Patterns of tonic firing have also been observed during trace fear conditioning. Individual neurons recorded from prelimbic mPFC during trace fear conditioning show sustained increases during a short, 3-sec trace interval (Baeq et al. 2001) or during a long, 20-sec trace interval (Gilmartin and McEchron 2005). In contrast, prelimbic units recorded from unpaired control animals did not exhibit the same degree of post-CS sustaining activity, suggesting that trace interval firing was specific to the CS–trace–UCS association (Gilmartin and McEchron 2005). In contrast, prelimbic units recorded from unpaired control animals did not exhibit the same degree of post-CS sustaining activity, suggesting that trace interval firing was specific to the CS–trace–UCS association (Gilmartin and McEchron 2005).

Figure 5. Placement of bilateral injection cannulae in prelimbic mPFC. Symbols on each coronal diagram show the placement of injector tips in the prelimbic for rats that received infusions of saline (black circles), muscimol (gray squares), or APV (open triangles) in the trace, delay, and unpaired conditioning groups. Coronal diagrams are shown for four levels anterior to bregma. (Diagrams are adapted from Paxinos and Watson 1998, and reprinted with permission of Elsevier © 1998.)

Disruption of cellular activity in prelimbic mPFC in this study impaired contextual fear conditioning in the trace and delay groups. Contextual fear responses are acquired simultaneously with auditory CS fear responses during trace or delay fear conditioning, and retention of contextual fear is tested in the original training environment in the absence of auditory cues. Acquiring fear to the configural properties of the training environment is dependent on the hippocampus, and it is unclear what role the mPFC plays in the acquisition and consolidation of these fear responses. Electrolytic lesions of the ventral mPFC (including ventral prelimbic, infralimbic, and caudal medial orbital cortex) administered prior to delay fear conditioning failed to impair context fear acquisition (Morgan et al. 1993), and similar lesions to the dorsal mPFC (including prelimbic and anterior cingulate [ACg]) augmented fear responses to the context (Morgan and LeDoux 1995). We found that disruption of prelimbic mPFC activity during training impairs context fear conditioning in trace- and delay-conditioned rats, consistent with more recent work on this issue. Similar to our findings, Quinn and colleagues (2008) observed contextual impairments after permanent post-training lesions of mPFC following trace and delay conditioning. We also

(Kalmbach et al. 2009). Prelimbic mPFC may be the cortical structure providing these sustained CS-related responses during the trace interval in trace conditioning tasks.

The requirement of prefrontal neuronal activity for trace fear conditioning is consistent with the well-established role for this structure in working memory. However, recent work has demonstrated a role for prefrontal plasticity in long-term memory (i.e., 24 h or longer) (e.g., Jung et al. 2008) and there is some evidence that mPFC may contribute to the long-term stability of trace fear memories. Pre- or post-training disruption of MAPK intracellular signaling or post-training inhibition of protein synthesis in the mPFC impairs the formation of trace fear memory (Runyan et al. 2004; Blum et al. 2006b). The mechanisms during training that may contribute to long-term changes in prefrontal plasticity are poorly understood. This study tested whether NMDAR activation during the acquisition period is important for the formation of long-term trace fear memory. NMDAR-dependent mechanisms have been shown to be critical for several forms of LTP in vitro, a cellular model of long-term memory (Bliss and Collingridge 1993). NMDAR neurotransmission is also critical for memory formation in vivo for a number of learning paradigms, including in the amygda for delay fear conditioning and in the hippocampus for trace fear conditioning (Kim et al. 1992; Maren et al. 1996; Huerta et al. 2000; Wanisch et al. 2005). Our results show that NMDAR neurotransmission is also necessary in the mPFC for trace fear conditioning. This finding corroborates similar results from trace eyeblink conditioning: Daily blockade of prefrontal NMDARs during trace eyeblink conditioning impaired the acquisition of eyeblink conditional responses (Takehara-Nishiuchi et al. 2005). Given that induction of LTP at hippocampal–prefrontal synapses requires NMDARs (Jay et al. 1995), it is possible that hippocampally driven activation of these receptors in prelimbic mPFC triggers intracellular signaling and the subsequent synthesis of new protein important for cellular plasticity. It is important to note that a requirement for NMDAR neurotransmission does not confirm prefrontal plasticity in the formation of trace fear conditioning. Ca\(^{2+}\) signaling via activation of NMDARs and L-type calcium channels has been proposed to provide a mechanism supporting persistent neuronal activity similar to that observed in the mPFC during working memory tasks (Lisman et al. 1998; Egorov et al. 2002; Fransen et al. 2006). Because NMDAR activation is sensitive to postsynaptic voltage changes, NMDAR activation may serve to select a specific subset of neurons to maintain persistent activity in working memory (Lisman et al. 1998).

Prefrontal NMDA and trace fear...
show that blockade of NMDAR-mediated signaling in the prelimbic mPFC impairs contextual fear responses acquired during paired conditioning. This result is consistent with a recent study in which selective blockade of the NR2B subunit of the NMDA receptor in the ACg mPFC impaired both cingulate LTP and the formation of contextual fear memory during a one-trial auditory fear conditioning session (Zhao et al. 2005). Together, these findings suggest that dorsal areas of mPFC contribute to the formation of contextual fear memory.

These findings demonstrate a role for mPFC in the formation of contextual fear memory when it is acquired simultaneously with fear to a discrete auditory cue during paired auditory fear conditioning. We did not find impairments in contextual fear retention in the unpaired group, where the context, but not the auditory CS, predicts the UCS. This suggests that the mPFC may be necessary to associate the training context with the UCS when the context has a weaker predictive relationship with the UCS relative to the foreground auditory CS–UCS association. When the context–UCS association is in the foreground, as in unpaired training, prefrontal activity may not be required. However, other regions of the mPFC may be important for foreground contextual fear learning. Blockade of NMDA receptors in the ventral mPFC (predominantly infralimbic) immediately before a context retention test impaired the expression of a foreground contextual memory (Rest et al. 2008). Clearly, more work is needed to determine the circumstances underlying a prefrontal role in the acquisition and expression of contextual fear conditioning.

Our findings lend further support for a prefrontal role in the initial formation of memory, expanding upon known roles for this structure in working memory, extinction, fear expression, and systems consolidation (Takehara et al. 2003; Frankland et al. 2004; Santini et al. 2004; Corcoran and Quirk 2007; Jung et al. 2008). Much of the previous work on the contribution of frontal regions to long-term memory demonstrated that anterior cingulate and prelimbic mPFC participates in the systems consolidation of some memories—the gradual transition of memory from dependence on subcortical structures to cortical sites (Frankland and Bontempi 2005). Permanent lesions or temporary inactivation of mPFC, including ACg or PL, impair contextual fear and spatial memories when administered weeks after training, but not when administered 1 or 2 days after training (Frankland et al. 2004; Teixeira et al. 2006; Ding et al. 2008). Similar results have been observed for trace eyelink and trace fear paradigms (Takehara et al. 2003; Quinn et al. 2008; but see Blum et al. 2006a). These manipulations occur post-training, and the mPFC is intact during the initial acquisition. Our findings demonstrate a need for an intact prelimbic mPFC for the initial formation of memory. Taken together, these two lines of evidence suggest that the PL mPFC may participate in multiple phases of memory formation. The mPFC is necessary for the encoding of trace fear conditioning and may store information about the CS–UCS association; however, the information initially stored in this structure may not be critical to the expression of the memory when the memory is still recent. As the memory ages, a separate mechanism may engage to consolidate the memory in cortical circuits. Trace fear conditioning may provide a useful means to examine multiple phases of prefrontal mechanisms of memory.

In conclusion, acquisition of hippocampus-dependent trace fear conditioning requires cellular activity in mPFC. Prefrontal NMDAR-mediated signaling is necessary for the association of the CS and UCS when they are separated by a 20-sec trace interval. Given that LTP at hippocampus–prelimbic synapses requires NMDAR activation (Jay et al. 1995), it is likely that the hippocampus and mPFC interact to encode the CS–trace–UCS association. Future investigations of the cooperative nature of hippocampal and prefrontal mechanisms of acquisition and consolidation in trace fear conditioning will provide new insights into the systems-level control of memory formation.

Materials and Methods

Subjects and surgery
Surgery was performed on a total of 120 adult male Long–Evans rats in this study (325–400 g; Harlan, IN). All rats were housed individually and received food and water ad libitum. All surgical procedures were in accordance with the National Institutes of Health and the University of Wisconsin–Milwaukee Institutional Animal Care and Use Committee. Rats were anesthetized with either 2.5 mg/kg sodium pentobarbital (n = 44) or 1%–2% isoflurane in 100% O2 (n = 76) during surgery. Each rat was positioned in a stereotaxic frame and the skull was exposed. Two skull screws (#0-80-02-M, Small Parts, Inc.) were inserted ~1 mm into the skull to anchor the final acrylic cement head assembly. Holes of 4-mm diameter were drilled in the skull above the left and right medial prefrontal cortex. Stainless steel guide cannulae (26 ga; Plastics One, Inc.) were stereotaxically lowered to the dorsal border of the prelimbic area of the mPFC, bilaterally at a 15° angle to vertical (AP + 3.2 mm; ML ± 1.6 mm; DV −3.2 mm from the skull). Acrylic cement was used to secure the cannulae to the skull and 33-ga dummy cannulae were screwed into the guide cannulae to prevent clogging.

Infusion procedure
Following recovery from surgery, rats received 3 d of acclimation to transport from their home cages to the procedure rooms. During this time, the rats were also acclimated to gentle restraint in a towel and to the sound of the infusion pump that would be used for intracranial injections. No infusions were delivered during these acclimation sessions. On the day of conditioning, rats received bilateral infusions (0.3 µL/side) of the GABA A agonist muscimol (1 µg/µL; 5-aminomethyl-3-hydroxyisoxazole, MP Biomedicals), the NMDA receptor antagonist D-APV (10 µg/µL; Tocris), or sterile saline vehicle 15 min prior to training. Drug or vehicle was infused at a rate of 0.5 µL/min through 33-ga injection cannulae, which extended 0.5 mm below the end of the guide cannulae. Injectors were left in place for 90 sec following the completion of the infusion to allow for diffusion of the drug or vehicle away from the cannulae. Rats were returned to their home cages immediately after the infusion procedure. The time points for infusion were based on reports that muscimol and APV take effect within minutes of infusion and may be cleared from the system within 2−3 h post-infusion (Martin 1991; Steele and Morris 1999). Training is complete within 1 h of drug infusion.

Conditioning
Fifteen minutes after the end of the infusion, rats were placed in the conditioning chambers and each received six trials of paired trace (n = 48) or delay (n = 40) fear conditioning, or 12 trials of unpaired control training (n = 32) following a 6-min baseline period. Each trial consisted of a 10-sec white noise conditional stimulus (CS; 72 dB) and a 1-s footshock unconditioned stimulus (UCS; 1 mA) separated by an empty 20-s trace interval. The intertrial interval (ITI) for this session was 240 ± 20 sec. Delay fear conditioning (ITI = 260 ± 20 sec) was the same as trace fear conditioning, except that the UCS was delivered at CS offset. Unpaired control training (120 ± 20 sec) consisted of the same number of CSs and UCSs as the paired training, but the UCSs and CSs were presented explicitly unpaired. This training served as a control for nonassociative responding to the white noise CS. All training sessions lasted 33 min. Conditioning occurred in a set of four Flexiglas and stainless steel conditioning chambers (internal dimensions: 21 × 28 × 21 cm), each housed in a sound attenuating outer chamber and illuminated with a white incandescent house lamp. Ventilation fans
in each outer chamber provided 63–65-dB background noise and the white noise CS was delivered through a speaker centered in the end of each conditioning chamber. Stainless steel bars (4-mm diameter, spaced 12 mm apart) on the floor of each chamber served to deliver the footshock UCS. The chambers were cleaned and wiped down with 5% ammonium hydroxide solution between each set of rats.

Twenty-four hours after training, rats received a single CS retention test in a novel chamber and a single Context retention test in the original training chamber. The CS retention test consisted of a 6-min baseline period, followed by a 5-min continuous presentation of muscimol and were able to learn the CS–UCS association, without these three outliers is 16.52%. While we cannot rule out the possibility that these three rats received a complete infusion of muscimol-infused trace group. These rats showed near ceiling levels of freezing during the CS test (internal dimensions: 20.5 × 26.5 × 21 cm) were each housed in a sound-attenuating outer chamber with S8–60-dB background noise. These chambers were in a separate room and differed from the training chambers in illumination (infrared house lamp), texture (solid floor), and odor (5% acetic acid solution).

Twenty-four hours after testing, trace and delay rats were retrained in the absence of drug. Retraining and testing procedures were identical to the original training and testing procedures for each rat. Whenever possible, all rats were retrained; however, because of large group sizes, a random selection of rats was not retrained.

Analyses
Freezing, the cessation of all movement except that needed for respiration, was used as the measure of conditional fear during all training and testing sessions (Fanselow and Bolles 1979). Freezing was scored automatically in real-time using the FreezeScan 1.0 detection software (Clever Sys, Inc.). All statistical analyses were performed with Statistica version 9 (Statsoft, Inc.). Each training group was analyzed separately and drug differences were analyzed using one-way ANOVAs (immediate post-acquisition; context retention) or mixed-model ANOVAs with repeated measures (CS retention), which included the following factors: a repeated measure of Period (Baseline, CS, and Post-CS periods) and a between factor of Drug (saline, muscimol, and APV). Fisher LSD post-hoc tests were used to test the significance of mean differences. An α level of 0.05 was required for significance in all analyses. Three rats in the trace muscimol group were flagged as statistical outliers using a standard outlier test in Statistica based on percentiles and were excluded from analyses. These rats showed near ceiling levels of freezing during the CS retention test, levels that were greater than those of most control rats. The remaining muscimol-infused rats all showed consistently low CS freezing levels below 35%. The median freezing without these three outliers is 16.52%. While we cannot rule out the possibility that these three rats received a complete infusion of muscimol and were able to learn the CS–UCS association, these rats are clearly different from the majority of the muscimol-infused trace group.

Histology
At the end of the experiment, rats were deeply anesthetized with 5% isoFL and placed in 100% O2, trancalectally perfused with 0.9% saline followed by 10% buffered formalin, and the brains were placed in a 10% formalin solution (in 0.9% saline). Brains were transferred to a 20% sucrose/formalin solution prior to processing for histology. Brains were then frozen, sectioned coronally, mounted on glass slides, and stained with cresyl violet.

Acknowledgments
This research was supported by the National Institute of Mental Health (NIMH) grant ROI MH069558 to F.J.H. and NIMH grant F32MH083422 to M.R.G. We thank Janine Kwapis and Mary Lonergan for assistance with the drug microinfusions.

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Received August 17, 2009; accepted in revised form April 22, 2010.