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MEDIAL PREFRONTAL CORTEX CIRCUIT FUNCTION DURING RETRIEVAL AND EXTINCTION OF ASSOCIATIVE LEARNING UNDER ANESTHESIA

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Abstract—Associative learning is encoded under anesthesia and involves the medial prefrontal cortex (mPFC). Neuronal activity in mPFC increases in response to a conditioned stimulus (CS+) previously paired with an unconditioned stimulus (US) but not during presentation of an unpaired stimulus (CS−) in anesthetized animals. Studies in conscious animals have shown dissociable roles for different mPFC subregions in mediating various memory processes, with the prelimbic (PL) and infralimbic (IL) cortex involved in the retrieval and extinction of conditioned responding, respectively. Therefore PL and IL may also play different roles in mediating the retrieval and extinction of discrimination learning under anesthesia. Here we used in vivo electrophysiology to examine unit and local field potential (LFP) activity in PL and IL before and after auditory discrimination learning and during later retrieval and extinction testing in anesthetized rats. Animals received repeated presentations of two distinct sounds, one of which was paired with footshock (US). In separate control experiments animals received footshocks without sounds. After discrimination learning the paired (CS+) and unpaired (CS−) sounds were repeatedly presented alone. We found increased unit firing and LFP power in PL and IL, and a lesser extent, IL after discrimination learning but not after footshocks alone. After discrimination learning, unit firing and LFP power increased in PL and IL in response to presentation of the first CS+, compared to the first CS−. However, PL and IL activity increased during the last CS− presentation, such that activity during presentation of the last CS+ and CS− did not differ. These results confirm previous findings and extend them by showing that increased PL and IL activity result from encoding of the CS+/US association rather than US presentation. They also suggest that extinction may occur under anesthesia and might be represented at the neural level in PL and IL.

Key words: prelimbic, infralimbic, discrimination learning, extinction, retrieval, in vivo electrophysiology.

INTRODUCTION

In certain circumstances associative learning occurs under general anesthesia. Undergoing fear learning while anesthetized can result in learned fear expression after recovery from anesthesia if epinephrine is given during learning (Weinberger et al., 1984; Gold et al., 1985). The neural mechanisms that mediate associative learning under anesthesia have begun to be elucidated. During olfactory discrimination learning in anesthetized rats, the lateral amygdala shows increased neuronal excitability in response to an odor (conditioned stimulus; CS+) previously paired with footshock (unconditioned stimulus; US), but not to another odor (CS−) presented without the US (Rosenkranz and Grace, 2002; Rosenkranz et al., 2003). We have recently shown similar results in the basolateral amygdala (BLA) during auditory discrimination learning under anesthesia, where BLA activity increases in response to CS+, but not CS−, presentation after learning (Fenton et al., 2013). These findings are comparable to changes in LA and BLA activity during discriminative fear learning (Maren et al., 1991; Collins and Paré, 2000;erry et al., 2008).

Activity in the medial prefrontal cortex (mPFC) also increases selectively during CS+ presentation after olfactory discrimination learning under anesthesia (Laviolette et al., 2005; Laviolette and Grace, 2006). This agrees with findings from similar studies showing a role for mPFC in discriminative fear learning. Neural activity in mPFC is increased during CS+, compared to CS−, presentation after successful discriminative fear learning (Likhtik et al., 2014). Temporary mPFC inactivation before testing the retention of discriminative fear learning impairs CS+/CS− discrimination (Lee and Choi, 2012). The mPFC is a heterogeneous area comprising the prelimbic (PL) and infralimbic (IL) cortex. Fear learning studies in conscious animals have shown...
dissociable roles for PL and IL in mediating different memory processes. While PL is involved in the retrieval or expression of conditioned responses, the suppression and extinction of conditioned responding involve IL (Vidal-Gonzalez et al., 2006; Sierra-Mercado et al., 2011; Fenton et al., 2014). Thus PL and IL may play different roles in memory processing related to discrimination learning. Moreover, these mPFC subregions share reciprocal connections that are functionally relevant, raising the possibility that PL–IL synchrony is also involved in discrimination learning (Jones et al., 2005; Hoover and Vertes, 2007; van Aerde et al., 2008; Ji and Neugebauer, 2012; Zelikowsky et al., 2013).

Here we examined PL and IL activity using a modified version of the auditory discrimination learning paradigm conducted under anesthesia that we have recently described (Fenton et al., 2013). We examined activity before and after learning given that increased mPFC activity during and after fear learning in awake animals may play a role in memory consolidation (Popa et al., 2010; Tan et al., 2011). In separate control experiments we examined activity before and after US presentations alone to further address this issue. Given the recent finding that fear extinction occurs during altered states of consciousness (Hauner et al., 2013), we also repeatedly presented the CS+ and CS− alone after learning in an attempt to examine activity during both retrieval and extinction in this discrimination learning paradigm. Assessing activity in PL and IL concurrently also allowed for the examination of functional connectivity within mPFC circuitry during these memory processes while under anesthesia.

**EXPERIMENTAL PROCEDURES**

**Animals**

All experimental protocols were performed in accordance with the Animals (Scientific Procedures) Act 1986, UK, and internal ethical approval. Male Lister hooded rats (250–350 g; Charles River, UK) were group housed on a 12-h light/dark cycle (lights on at 0700) and had free access to food and water. Every effort was made to minimize the number, and suffering, of the animals used.

**Surgery**

Anesthesia was induced under 3.5% isoflurane (IVAX Pharmaceuticals, UK) in medical air. Anesthesia was gradually reduced to and maintained at ~2.0% throughout the experimental protocol, ensuring complete lack of the hindpaw withdrawal reflex. Body temperature was maintained at ~37 °C using a homeothermic heating blanket (Harvard Apparatus Ltd., UK). Rats were placed in a stereotaxic frame with customized hollowed ear bars connected to earphones. An incision was made in the scalp, and the skull and dura over mPFC were removed. An eight-wire micro-electrode bundle (Teflon-coated stainless steel wire, 50-μm diameter/wire; NB Labs, TX) was lowered into right mPFC. The electrodes were ‘ staggered’ such that four wires were 1 mm longer than the other four, allowing for simultaneous recordings from PL and IL (2.7 mm anterior, 0.5 mm lateral to bregma; 3.3 (PL) and 4.3 (IL) mm ventral to the brain surface; (Paxinos and Watson, 1997)). The electrode was allowed to settle for 1 h before recordings began. Two 25-gauge needles connected to an electrical stimulator (Neurolog system, Digitimer Ltd., UK) were also inserted into the ventral surface of the left hindpaw, contralateral to the recording site.

**Recording procedure**

The recording protocol has been described in detail previously (Stevenson et al., 2007, 2008). The electrode was connected to a preamplifier via a headstage. Units and local field potentials (LFPs) were linked to a PC via a Plexon multichannel acquisition processor (Plexon, Inc., TX) and filtered (units: gain 1000x, bandpass filtered at 0.25–8 kHz; LFPs: bandpass filtered at 0.7–170 Hz, digitized at 1 kHz). This provided simultaneous 40-kHz A/D conversion on each channel at 12-bit resolution. Unit activity was monitored visually and aurally using a 507 analog–digital oscilloscope (Hameg Instruments, Germany) and a speaker, respectively.

**Auditory discrimination learning paradigm**

The paradigm used was adapted from our previously described auditory discriminative learning protocol (Fenton et al., 2013). Basal activity was recorded for 3 min. During learning, rats were presented with a sound (CS+) for 10 s paired with a footshock (US; 5 mA, 20 Hz, 0.5-ms pulse duration) of 5 s duration that co-terminated with the CS+. A second sound (CS−) was presented 60 s later for 10 s in the absence of footshock. The CS+/US pairings and CS− presentations were repeated four times. The two sounds (3-kHz tone or white noise, 90 dB each) were counterbalanced between the CS+ and CS− between animals. Presentations of sound and footshock were automatically controlled (Cool Edit 96, Syntrillium Software Co., AZ). After 3 min, rats were presented with 12 CS+ and 12 CS− presentations as above except that footshocks were not given (Fig. 1A). In separate control experiments, rats received four footshocks alone and activity was recorded for 3 min afterward.

**Histology**

At the end of each experiment rats were culled by isoflurane overdose. A current (0.1 mA) was briefly passed through a pair of electrodes in PL and IL, depositing ferric ions at the electrode tips. Brains were removed and stored in a solution of 4% paraformaldehyde/4% potassium hexacyanoferrate (Sigma, UK), marking the recording sites by the Prussian blue reaction. Electrode placements were later confirmed by obtaining mPFC sections of 200-μm thickness (Fig. 1B, C).
Fig. 1. (A) Schematic representation of the discrimination learning paradigm used. Animals were anesthetized and subjected to CS+ shock pairings and CS− alone presentations (four of each) followed 3 min later by repeated presentations of the CS+ and CS− alone (12 of each). Neuronal activity was analyzed before and after discrimination learning, and during the first and last CS+ and CS− alone presentations after learning. (B) Schematic representation of multi-electrode array placements in PL and IL. Distance (mm) anterior to bregma is indicated beside each section. (C) Representative electrode placements in PL (dorsal) and IL (ventral) indicated by the arrows. (D) Cumulative (black and gray) and resulting average (white and black) waveform of discriminated unit activity recorded from two neurons on one microwire of an electrode array. (E) Cluster analysis of unit activity from two neurons (black and gray dots) using principal component analysis. (F) Unit and LFP activity recorded from PL and IL under basal conditions. Unit activity was characterized by irregular burst firing and LFP activity was characterized by deflections in potential corresponding to unit firing.
Unit sorting
The parameters used have been described in detail previously (Stevenson et al., 2007, 2008). Briefly, unit discrimination was performed using Offline Sorter (Plexon Inc., TX). Noise artefacts were removed manually before using the automated k-means clustering algorithm to sort the units. Waveforms not consistent with the shape of action potentials and occurring within the refractory period (1 ms) were removed manually (Fig. 1D). Only waveforms consistent with the shape (biphasic) and basal firing rate (0.1–10 Hz) of units originating from putative glutamatergic pyramidal neurons were included in the data analysis. Clusters were further scrutinized manually after using principal component analysis to display the waveforms in 3D space and were only classified as separate units if their borders did not overlap (Fig. 1E).

Data analysis
Basal and post-learning activity was defined as activity during the 3-min periods before and after learning, respectively, and analyzed. Activity during the first (i.e. retrieval) and last (i.e. extinction) CS+ and CS− presentations alone after learning was also analyzed. In the control experiments, activity during the 3-min periods before and after footshocks alone was analyzed.

Changes in unit firing rate were analyzed using NeuroExplorer software (NEX Technologies, TX). Differences in mean firing rate before and after learning were analyzed using a two-tailed paired t-test. Differences in mean firing rate during the first and last CS+ and CS− presentations were analyzed using a two-way analysis of variance (ANOVA) with CS type (i.e. CS+ or CS−) and time (i.e. first or last) as within subject measures; post hoc analysis was conducted using the Tukey’s Honestly Significant Difference (HSD) test. In the control experiments, differences in mean firing rate before and after footshocks were analyzed using a two-tailed paired t-test.

The burst analysis parameters used have been described in detail previously (Stevenson et al., 2007). The Poisson surprise method was used to calculate unit bursting given the irregular activity pattern observed, which was characterized by periods of low tonic activity together with phasic bursting (Fig. 1F). The percentage of units firing as bursts (% bursting) was calculated using a surprise value of $s = 5$. Differences in mean % bursting before and after learning were analyzed using a t-test as above. Differences in mean % bursting during the first and last CS+ and CS− presentations were analyzed using a two-way ANOVA and post hoc testing as above. In the control experiments, differences in mean % bursting before and after footshocks were analyzed using a t-test as above.

PL–IL cross-correlation analysis was conducted using custom Matlab scripts (Mathworks, MA). Unit cross-correlograms were calculated for all unit pairs (10-ms bins, ±500-ms lead/lag), normalized to the firing rate of the reference unit, averaged, and expressed as firing rate/s (Fenton et al., 2013). Peak and mean (i.e. mean of the correlogram bins) cross-correlation values were taken as measures of temporal synchrony. Differences in peak and mean cross-correlation before and after learning were analyzed separately using t-tests as above. Differences in peak and mean cross-correlation during the first and last CS+ and CS− presentations were analyzed separately using a two-way ANOVA and post hoc testing as above. In the control experiments, differences in peak and mean cross-correlation before and after footshocks were analyzed separately using t-tests as above.

Unit firing rate, % bursting, and peak and mean cross-correlation data are plotted as the mean ± SEM. For the sake of clarity only the bin means are plotted in the cross-correlograms. The level of statistical significance for all unit analyses was set at $P < 0.05$.

LFP activity was analyzed in the frequency domain using multi-taper spectral analysis as previously described (Fenton et al., 2013). Briefly, spectral estimates were devised using custom Matlab scripts by splitting the appropriate sections from each record into disjoint segments of equal length and applying the same number of multitaper windows to each segment. Further averaging across segments and animals was used to produce spectral estimates of LFP power in PL and IL during the basal and post-learning periods and during the first and last CS+ and CS− presentations. Differences in LFP power before and after learning were determined using the log ratio difference of spectra test and quantified statistically using 95% confidence intervals. Differences in LFP power during the first and last CS+ and CS− presentations were quantified statistically using 99% confidence intervals to correct for multiple pairwise comparisons. In the control experiments, differences in LFP power before and after footshocks alone were quantified statistically using 95% confidence intervals.

Synchronization of LFP activity between PL and IL was determined in the frequency domain using coherence analysis (Stevenson et al., 2007, 2008). Coherence spectra were estimated using multi-taper analysis as above for LFP power. Differences in LFP coherence between PL and IL before and after learning were determined using the comparison of coherence test and quantified statistically using 95% confidence intervals. Differences in LFP coherence during the first and last CS+ and CS− presentations were quantified statistically using 99% confidence intervals to correct for multiple pairwise comparisons. In the control experiments, differences in LFP coherence between PL and IL before and after footshocks alone were quantified statistically using 95% confidence intervals.

RESULTS
Only data from rats with histologically verified electrode placements in both PL and IL were used in the analysis (Fig. 1B, C). In the discrimination learning paradigm $n = 11$ rats met criteria, with activity recorded from $n = 33$ PL and $n = 37$ IL neurons. In the control experiments $n = 8$ rats met criteria, with activity
recorded from \( n = 21 \) PL and \( n = 20 \) IL neurons. Both PL and IL showed similar unit activity under basal conditions, with intermittent highly synchronized phasic bursting activity. LFP oscillations showed similar activity with periods of low activity coupled with brief periods of high activity, corresponding with the bursts of unit activity (Fig. 1F). We have previously observed this pattern of mPFC activity under isoflurane anesthesia (Stevenson et al., 2007, 2008).

mPFC activity after discrimination learning

Unit activity in PL and IL before and after learning is shown in Fig. 2. In PL, unit firing rate was significantly increased after, compared to before, learning (\( t(32) = 2.77, P < 0.01 \); Fig. 2A). There was no difference in unit bursting before and after learning in PL (\( t(32) = 0.63, P > 0.05 \); Fig. 2B). Unit firing rate was also increased after, compared to before, learning in IL but this difference did not reach significance (\( t(30) = 1.55, P > 0.05 \); Fig. 2C). There was no difference in unit bursting before and after learning in IL (\( t(30) = 0.36, P > 0.05 \); Fig. 2D). Unit cross-correlations between PL and IL (\( n = 116 \) unit pairs) before and after learning are shown in Fig. 2E. There were no differences in peak (\( t(115) = 0.77, P > 0.05 \); Fig. 2F) or mean (\( t(115) = 0.52, P > 0.05 \); Fig. 2G) cross-correlation before or after learning.

LFP activity in PL and IL before and after learning is shown in Fig. 3. In general, a similar increase in LFP activity occurred after learning as was observed for unit activity. In PL, LFP power was significantly increased after, compared to before, learning across the entire frequency range examined (\( P < 0.05 \); Fig. 3A). LFP power was also significantly increased after, compared to before, learning in IL, albeit to a lesser extent than in PL (\( P < 0.05 \); Fig. 3B). There was little difference in LFP coherence between PL and IL before and after learning, with slight increases and decreases observed across the frequency range examined (Fig. 3C).

mPFC activity after footshocks alone

Increased mPFC activity after learning might be indicative of a short-term memory consolidation process (Popa et al., 2010; Tan et al., 2011), although this may also have been observed in response to the footshocks independently of any learning that occurred. To address this issue we also examined the effects of footshocks alone on later mPFC activity in separate control experiments.

Unit activity in PL and IL before and after footshocks alone is shown in Fig. 4. In PL, there were no differences in unit firing rate (\( t(20) = 0.38, P > 0.05 \); Fig. 4A) or bursting (\( t(20) = 0.95, P > 0.05 \); Fig. 4B) before or after footshocks alone. The same was also observed for unit firing rate (\( t(19) = 0.33, P > 0.05 \); Fig. 4C) and bursting (\( t(19) = 0.15, P > 0.05 \); Fig. 4D) in IL. However, both peak (\( t(68) = 2.19, P < 0.05 \); Fig. 4E) and mean (\( t(68) = 7.59, P < 0.0001 \); Fig. 4F) cross-correlation between PL and IL (\( n = 69 \) unit pairs) were significantly decreased after, compared to before, footshocks alone.

LFP activity in PL and IL before and after footshocks alone is shown in Fig. 5. Again, the pattern of LFP activity observed was generally similar to that reported....
for unit activity. There was little difference in LFP power before and after footshocks alone in PL (Fig. 5A) or IL (Fig. 5B). However, as was observed for unit synchrony, LFP coherence showed a significant decrease after, compared to before, footshocks at certain frequencies \((P < 0.05; \text{Fig. 5C})\).

**mPFC activity during repeated CS+ and CS− presentations after learning**

Mean firing rate histograms of unit activity in PL and IL during the first and last CS+ and CS− presentations after learning are shown in **Fig. 6**. Despite activity increasing the most at CS+ and CS− onset (and offset), unit firing was observed to some extent throughout the duration of the CS+ and CS−. Differences in unit firing rate during the first and last CS+ and CS− presentations were thus calculated as the mean of each 10 s period. In general, there were differences in unit firing rate during the first, but not the last, CS+ and CS− presentations observed in both mPFC subregions.

In PL, the statistical analysis of unit firing rate showed a significant CS \(\times\) time interaction \((F_{(1,32)} = 5.04, P < 0.05)\). Post-hoc analysis revealed that unit firing rate was significantly decreased during the first CS−, compared to the first CS+ and last CS−, presentation \((P < 0.05; \text{Fig. 7A})\). For unit bursting, there were significant main effects of CS \((F_{(1,32)} = 16.74, P < 0.001)\) and time \((F_{(1,32)} = 5.18, P < 0.05)\). Post-hoc analysis revealed that unit bursting in PL was significantly increased during CS+, compared to CS−, presentations and during the last, compared to the first, CS presentations \((P < 0.05; \text{Fig. 7B})\). In IL, the statistical analysis of unit firing rate also showed a significant CS \(\times\) time interaction \((F_{(1,36)} = 6.67, P < 0.05)\). Post-hoc analysis revealed that unit firing rate was significantly decreased during the first CS−, compared to the first CS+, presentation \((P < 0.05; \text{Fig. 7C})\). For unit bursting, there was a significant main effect of CS \((F_{(1,36)} = 16.74, P < 0.001)\). Post-hoc analysis revealed that unit bursting in IL was significantly increased during CS+, compared to CS−, presentations \((P < 0.05; \text{Fig. 7D})\). There were no differences in peak correlation between PL and IL \((n = 116 \text{ unit pairs})\) during the first and last CS+ and CS− presentations (Fig. 7E). However, the statistical analysis of mean correlation showed a significant CS \(\times\) time interaction \((F_{(1,115)} = 16.42, P < 0.0001)\). Post-hoc analysis revealed that mean correlation was significantly increased during the last CS+, compared to the first CS+ and the last CS−, presentation \((P < 0.05; \text{Fig. 7F})\).

Pooled LFP activity in PL and IL during the first and last CS+ and CS− presentations after learning is shown in **Fig. 8**. LFP power increased the most at CS+ and CS− onset (and offset), although some activity was observed throughout for each. Differences in LFP power between the first and last CS+ and CS− presentations were thus analyzed over their entire 10 s durations.
Again, differences in LFP power were generally observed during the first, but not the last, CS+ and CS− presentations.

In PL, LFP power during the first CS− presentation was significantly decreased compared to during the first CS+ and the last CS− presentation ($P < 0.01$; Fig. 9A, B). LFP power during the first CS− presentation was also significantly decreased compared to during the first CS+ and the last CS− presentations in IL; there was also a significant decrease in LFP power during the last compared to the first CS+ presentation ($P < 0.01$; Fig. 9C, D). In contrast to unit cross-correlation, there was a significant decrease in LFP coherence during the first CS+, compared to the first CS−, presentation; LFP coherence also showed a significant decrease during the last CS+, compared to the first CS+ and last CS−, presentation ($P < 0.01$; Fig. 9E, F).

**DISCUSSION**

We examined neuronal activity in PL and IL during the retrieval and extinction of auditory discrimination learning in anesthetized rats. After learning we found that activity increased in PL and, to a lesser extent, IL. In contrast, there was little change in PL or IL activity after footshocks alone. During retrieval we found increased PL and IL activity during CS+, compared to CS−, presentation. However, activity in PL and IL in response to CS+ and CS− presentations did not differ after extinction, due to increased activity during CS− presentation. These results confirm previous findings showing that discrimination learning under anesthesia occurs at the neural level in PL and IL. They also suggest that increased PL and IL activity after learning results from encoding of the CS+/US association rather than US presentations. Finally, our results suggest that extinction of discrimination learning may occur under anesthesia, which might also be encoded by activity in PL and IL neurons.

In this study we used a modified version of our recently described auditory discrimination learning paradigm (Fenton et al., 2013). In that study we waited 1 h after learning before examining BLA activity in response to a single presentation of the CS+ and CS−. However, previous studies examining mPFC activity using a similar olfactory discrimination learning procedure waited only a few min between the end of learning and retrieval testing (Laviolette et al., 2005;
Fig. 5. LFP activity before and after footshocks alone. (A) Power spectra in PL during the 3-min periods before (gray) and after (black) footshocks. (B) Log ratio plot showing little difference in PL power before and after footshocks. (C) Power spectra in IL before (gray) and after (black) footshocks. (D) Log ratio plot showing little difference in IL power before and after footshocks. (E) PL–IL coherence spectra before (gray) and after (black) footshocks. (F) Comparison of coherence plot showing decreased LFP coherence after, compared to before, footshocks ($P < 0.05$).

Fig. 6. Mean firing rate histograms (100 ms bins; bin SEMs not shown) showing unit activity 5 s before, during, and 5 s after the first and last CS+ and CS-/C0 presentations after learning in (A) PL and (B) IL. Unit firing increased the most at CS+ and CS– onset and offset but some activity was also observed throughout the CS+ and CS– presentations.
Laviolette and Grace, 2006). Therefore to make our results more comparable with these previous studies we used a similar duration after learning before examining PL and IL activity during CS+ and CS− presentations. We also used repeated CS+ and CS− presentations after learning in this study in an attempt to examine PL and IL activity during both the retrieval and extinction of auditory discrimination learning.

We found that unit firing increased in PL after learning. There was also a non-significant increase in unit firing after learning in IL. Similarly, LFP power increased after learning in both mPFC subregions, with a greater increase observed in PL. Interestingly, studies in conscious animals suggest that elevated mPFC activity is involved in fear memory consolidation. LFP power increases in mPFC after fear conditioning (Popa et al., 2010). PL inactivation prevents potentiated fear memory encoding caused by cannabinoid receptor activation in BLA (Tan et al., 2011). This short-term increase in mPFC activity may, in turn, facilitate the induction of local synaptic plasticity mechanisms involved in long-term memory consolidation, such as brain-derived neurotrophic factor signaling (Choi et al., 2010, 2012). However, in the present study, increased mPFC activity may also have occurred in response to footshocks independently of associative learning. To address this issue we examined the effects of footshocks alone on later PL and IL activity. We found little increase in unit firing or LFP power after footshocks alone. These findings suggest that increased mPFC activity after learning was due to the CS+/US association being encoded and not simply to US presentations.

After learning we found that unit firing in PL and IL were increased in response to the first CS+, compared to the first CS−. We also found that unit bursting in PL and IL increased during presentation of the first CS+, compared to the last CS−, although this did not reach significance. These results generally agree with previous findings showing increased unit firing and bursting in mPFC selectively during CS+ presentation.

![Unit activity during the first and last CS+ and CS− presentations after learning.](image)

Fig. 7. Unit activity during the first and last CS+ and CS− presentations after learning. (A) Unit firing rate in PL was decreased during the first CS− presentation, compared to the first CS+ and the last CS− presentation (*P < 0.05). (B) Unit burst firing in PL was increased during CS+, compared to CS−, presentations (*P < 0.05). (C) In IL, unit firing rate was increased during the first CS+, compared to the first CS−, presentation (*P < 0.05). (D) Unit burst firing in IL was increased during CS+, compared to CS−, presentations (*P < 0.05). (E) There was no difference in peak cross-correlation during the first and last CS+ and CS− presentations. (F) Mean cross-correlation was increased during the last CS+ presentation compared to the first CS+ and the last CS− presentation (*P < 0.05).
after olfactory discrimination learning under anesthesia (Laviolette et al., 2005; Laviolette and Grace, 2006). We also found increased LFP power in PL and IL in response to the first CS+, compared to the first CS−. These results confirm and extend previous findings showing that memory retrieval is represented by mPFC activity in anesthetized animals.

Recent evidence indicates that fear extinction is potentiated during slow-wave sleep, suggesting that extinction can occur during altered states of consciousness (Hauner et al., 2013). To determine if the extinction of associative learning can occur under anesthesia we examined PL and IL activity in response to repeated presentations of the CS+ and CS− after learning. In contrast to the first CS+ and CS− presentation, we found no differences in unit firing in PL or IL in response to the last CS+ and CS−. This lack of difference was due to increased unit firing during presentation of the last CS−, compared to the first CS−, although this did not reach significance in IL.

Similarly, there was no difference in LFP power in PL or IL during the last CS+ and CS− presentation due to increased LFP power in PL and IL during the last, compared to the first, CS− presentation. It should be noted that a previous study found that fear extinction does not occur under anesthesia. Animals fear conditioned while conscious and extinguished under anesthesia showed no extinction retention when later tested while awake (Park and Choi, 2010). Methodological differences between that report and our study may account for this discrepancy (e.g. anesthetic type, state-dependency of learning, etc.). It is also possible that anesthesia permits extinction learning but not its later consolidation. Extinction memory consolidation requires neuronal activation and synaptic plasticity in mPFC (Santini et al., 2001, 2004, 2008; Herry and Garcia, 2002; Herry and Mons, 2004). Interestingly, the increase in mPFC Fos expression that is normally induced by extinction is blocked when extinguishing under anesthesia (Park and Choi, 2010).

**Fig. 8.** Pooled LFP power 5 s before, during, and 5 s after the first and last CS+ and CS− presentations after learning in (A) PL and (B) IL. Power (in dB) is represented by different colors as indicated in the adjacent color bars (dark blue: low; dark red: high). Power increased the most at CS+ and CS− onset and offset but activity also occurred at other times during CS+ and CS− presentations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
This may partly explain why we found no difference in mPFC unit firing, and no change (PL) or decreased (IL) LFP power, during the last, compared to the first, CS+ presentation. Another possibility may relate to when extinction occurred after learning. Evidence indicates that extinction conducted shortly after conditioning, as was the case in our study, decreases conditioned responding during extinction learning but without maintaining this suppression at later retention intervals (Maren, in press). Moreover, this immediate extinction deficit involves mPFC function. The increase in Fos expression that normally occurs in mPFC with delayed extinction (i.e. 24 h after conditioning) is not observed after immediate extinction (Kim et al., 2010).

Nevertheless, our finding of a difference in mPFC activity between presentations of the first CS+ and CS+/C0, but not the last CS+ and CS+/C0, suggests that extinction learning was observed at the neural level in our study.

We found few differences between PL and IL activity throughout this study. This has been reported in similar studies examining mPFC activity during the retrieval of olfactory discrimination learning under anesthesia (Laviolette et al., 2005; Laviolette and Grace, 2006). This may seem unexpected as studies in conscious animals have shown that PL and IL mediate fear memory retrieval and extinction, respectively. Whereas PL inactivation reduces conditioned freezing during fear retrieval, IL inactivation impairs the reduction in freezing that normally occurs during fear extinction (Sierra-Mercado et al., 2011).

Similarly, PL activity decreases and IL activity increases with reduced conditioned freezing during fear extinction (Fenton et al., 2014). However, these studies used a single CS paired with the US. Studies which also included an unpaired CS+/C0 have shown that mPFC is involved in discriminating between the CS+ and CS−. Inactivation of mPFC before retention testing impairs CS+/C0 discrimination by increasing conditioned freezing during CS− presentation rather than decreasing freezing in response to the CS+ (Lee and Choi, 2012). Animals demonstrating successful fear discrimination learning show greater mPFC activity in response to the CS+, compared to the CS−, whereas animals showing stimulus generalization show no difference in mPFC activity during CS+ and CS− presentations (Likhtik et al., 2014). Although the findings from studies examining mPFC activity during discrimination learning under anesthesia suggest the involvement of both PL and IL in this process, the extent to which distinct mPFC subregions play different roles in mediating fear discrimination learning while conscious remains unclear (Powell et al., 1994).

In addition to investigating PL and IL activity, we examined the possibility that functional interactions between these reciprocally connected mPFC subregions are involved in discrimination learning under anesthesia (Jones et al., 2005; Hoover and Vertes, 2007; van Aerde et al., 2008; Ji and Neugebauer, 2012). We found decreases in both unit correlation and LFP coherence between PL and IL after footshocks alone, but not after learning, suggesting that encoding of the CS+/US association might also involve synchrony within the PL–IL circuit. However, during retrieval and extinction we...
observed different or opposing patterns of changes in unit correlation and LFP coherence. During retrieval there was little difference in unit correlation in response to CS+ or CS− presentation, whereas LFP coherence was decreased during CS+, compared to CS− presentation. During extinction unit correlation was increased, while LFP coherence was decreased, in response to the CS+, compared to the CS−. The reasons for this divergence in measures of unit and LFP synchrony are unclear but might reflect differences in functional coupling at the single neuron vs neural population levels. It is worth noting that the LFP coherence reported here is much greater than in our recent study in conscious animals (coherence < 0.1 throughout (Fenton et al., 2014)). Nonetheless, our results add to evidence implicating PL−IL interactions in certain memory processes (Żelikowsky et al., 2013).

This study confirms and extends previous findings showing that mPFC activity encodes associative learning and its short-term retrieval under anesthesia. It also provides preliminary evidence suggesting that extinction learning can occur under anesthesia and that this is encoded by mPFC activity. Future studies examining mPFC activity using longer intervals between learning and extinction using this paradigm may provide novel insights on the neurophysiological mechanisms involved in the immediate extinction deficit. Future studies examining the functional connectivity between mPFC and other relevant brain regions, such as BLA, may also prove useful in clarifying if the neural circuitry underlying memory retrieval and extinction learning in conscious animals is also involved in memory processing under anesthesia (Rosenkranz et al., 2003; Herry et al., 2008; Park and Choi, 2010; Popa et al., 2010; Tan et al., 2011; Likhtik et al., 2014).

CONTRIBUTIONS

RM and CWS designed the experiments. GEF conducted the experiments. GEF and CWS analyzed the data. DMH provided essential data analysis tools. GEF and CWS wrote the paper.

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