Distinct ensembles of medial prefrontal cortex neurons are activated by threatening stimuli that elicit excitation versus inhibition of movement.

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ABSTRACT

Neural circuits controlling defensive behavior were investigated by recording single units in medial prefrontal cortex (mPFC) and dorsolateral periaqueductal gray (dIPAG) while rats expressed conditioned fear responses to an auditory conditioned stimulus (CS; 20 s train of white noise pips) previously paired with an aversive unconditioned stimulus (US; 2 s train of periorbital shocks). The CS elicited conditioned movement inhibition (CMI; characterized by decreased movement speed and freezing) when rats had not recently encountered the US, whereas the CS elicited conditioned movement excitation (CME; characterized by increased movement speed and flight behavior) after recent US encounters. Many mPFC neurons were “strategy-selective” cells that changed their firing rates only when the CS elicited CME (15/71) or CMI (13/71) responses, whereas few mPFC cells (4/71) responded non-selectively to the CS during either response. By contrast, many dIPAG neurons (20/74) responded non-selectively to the CS, but most (40/74) were excited by the CS selectively during CME trials (and none during CMI trials). CME-selective neurons in dIPAG responded phasically after CS pips that elicited CME responses, whereas CME-selective neurons in mPFC showed tonically elevated activity before and after pips that evoked CME responses. These findings suggest that, at the time when the CS occurs, tonic firing rates of CME- and CMI-selective mPFC neurons may bias the rat’s choice of whether to express CME versus CMI responses, perhaps via projections to downstream structures (such as amygdala and PAG) that influence how sensory stimuli are mapped onto motor circuits that drive the expression of competing behaviors.
INTRODUCTION

Single-unit recording studies have shown that firing rates of neurons in mPFC are correlated with expression and extinction of conditioned fear in rodents (Baeg et al. 2001; Herry et al. 2008; McGinty and Grace 2008; Burgos-Robles et al., 2009; Senn et al. 2014). The prelimbic (PL) subregion of mPFC contains neurons that increase their firing rates during conditioned freezing responses (Burgos-Robles et al., 2009; Senn et al. 2014), and disruption of PL can impair conditioned freezing (Corcoran and Quirk 2007; Laurent and Westbrook 2009; but see Bravo-Rivera et al., 2014). The infralimbic (IL) subregion of mPFC contains neurons that respond to a fear conditioned CS after it has been extinguished (Burgos-Robles et al. 2007; Santini et al. 2008; Knapska and Maren 2009), and disruption of IL can impair fear extinction (Quirk et al., 2000; Laurent and Westbrook 2009; Sotres-Bayon et al. 2009; Sierra-Mercado et al. 2011; but see Garcia et al. 2006; Chang and Maren 2010). Moreover, properly timed electrical stimulation of IL can disrupt freezing responses elicited by a fear conditioned CS (Milad and Quirk, 2002; Milad et al., 2004), suggesting that IL neurons can block freezing expression.

Taken together, these findings provide compelling evidence that mPFC plays a key role in regulating the expression of conditioned fear, possibly via its reciprocal connections to the amygdala (see Sotres-Bayon and Quirk, 2010; Maren et al., 2013; Gilmartin et al., 2014; Duvarci and Pare, 2014). However, since most of these findings come from studies where freezing served as the primary behavioral index of fear, it remains unclear whether mPFC neurons regulate CS-evoked expectations of the US (which are antecedent to “fear”), or whether they instead regulate the performance of specific defensive responses to the CS, such as freezing. Based upon current evidence, PL neurons that respond to a fear conditioned CS might either signal the animal’s anticipation of danger during the CS, or they might instead promote the
expression of freezing responses to the CS, without influencing the animal’s expectation of threat. Likewise, IL neurons that respond to an extinguished CS might suppress the animal’s learned expectation of the US (thus inhibiting fear), or they might instead suppress freezing responses to the CS (and thereby disinhibit other behaviors) without inhibiting the animal’s expectation of danger.

To further investigate which aspects of fear and defensive behavior are correlated with neural activity in mPFC, we recorded responses of mPFC neurons to a fear conditioned auditory CS under conditions where it evoked two distinct different defensive responses from rats: CMI versus CME behaviors. We reasoned that if mPFC neurons excite or inhibit the rat’s fear of an aversive US, then they should respond similarly to a CS that predicts an aversive US, regardless of which defensive response is evoked by that CS. On the other hand, if mPFC neurons drive specific defensive responses, then they should respond differently to the CS depending upon whether it evokes CMI or CME behaviors, even though both are conditioned fear responses. For comparison, we also recorded neurons in dlPAG, a structure which receives afferent projections from mPFC and is thought to drive CME behaviors such as escape and avoidance (Bandler and DePaulis 1988; Fanselow 1991; Walker et al. 1997; De Oca et al. 1998; Vianna et al. 2001).

We found that more than half of neurons recorded in dlPAG responded preferentially to the CS when it elicited CME responses, and in addition, more than a quarter of dlPAG neurons responded non-selectively to the CS regardless of whether it elicited CME or CMI responses. By contrast, very few (<6%) mPFC neurons responded non-selectively to the CS, and more than 40% of mPFC neurons were “strategy-selective” cells that responded preferentially to the CS either during CMI responses (~20% of cells) or CME responses (~20% of cells). These findings suggest that rather than exciting or inhibiting the rat’s “fear” of the aversive US, mPFC neurons
may instead coordinate the selection of specific defensive responses to anticipated threats, in
accordance with existing theories that mPFC regulates the selection of behavioral response
strategies by adjusting the manner in which sensory stimuli are mapped onto behavioral
responses (Miller and Cohen 2001; Shackman et al. 2011).

MATERIALS AND METHODS

Subjects and surgery

Adult male Long-Evans rats weighing 350-400 g were housed singly and reduced to 85%
of ad-lib weight by daily limited feeding. All rats were deeply anaesthetized with isofluorane and
surgically implanted with a pair of insulated stainless steel wires (75 µm diameter) threaded into
the skin of each eyelid for delivering the periorbital shock US. Rats (n=5, of which 4 were
included in the study and one was excluded because of failure to obtain recordings during the
expression of conditioned fear) were implanted with 16 individually moveable tetrodes which
targeted mPFC and dlPAG. Four tetrodes per hemisphere were implanted in each structure of
each rat. In mPFC, the four tetrodes were arrayed from 1.7 to 2.7 mm anterior to bregma at a
lateral offset of 0.5-1.0 mm from the midline, and the tips were advanced over the course of the
experiment from a depth of 2.0 to 5.0 mm dorsal to bregma. In dlPAG, the four tetrodes were
arrayed from 6.8 to 7.8 mm posterior to bregma at a lateral offset of 0.5-1.0 mm from the
midline, and the tips were advanced over the course of the experiment from a depth of 4.0 to 6.0
mm dorsal to bregma. Rats were also implanted with a pair of 22 gauge microinjection cannula
targeted bilaterally in the basolateral amygdala (3.0 mm posterior, 5.3 mm lateral, and 8.0 mm
ventral to bregma), which were used to inactivate the amygdala with muscimol (MUS) during
neural recordings on some experiment days. We did not obtain a sufficiently large sample of recorded neurons during amygdala inactivations to report conclusive results on the effects of amygdala disruption, so inactivation sessions are excluded from the present analyses, and all data reported here were obtained during drug-free recording sessions conducted at least 48 hours after the most recent amygdala inactivation (in all graphs where data is plotted over sessions, the session number tabulates drug-free sessions only). All experimental procedures were approved by the UCLA Animal Research Committee and were conducted in accordance with USA federal guidelines.

Fear conditioning

After recovery from surgery, rats were pre-exposed for 5 days (20 min/day) to the experimental context (80 cm diameter cylindrical enclosure) before any fear conditioning sessions were conducted. To provide a baseline of motor activity against which stimulus-evoked movement and turning behavior could be measured, rats constantly foraged for 20 mg purified food pellets (Bioserv, Frenchtown, NJ) dropped from an overhead dispenser at ~30 s intervals throughout all pre-exposure and fear conditioning sessions. On every day following pre-exposure, rats received an identical regimen of fear conditioning trials: 6 CS-alone presentations (test trials) followed by 16 CS-US pairings (training trials). The present experiments were designed to investigate expression (not acquisition) of conditioned fear responses; based upon prior behavioral findings (Tarpley et al. 2010), all rats were first trained to near-asymptotic levels of CS-evoked fear behavior over five consecutive days prior to neurophysiological recordings. The CS used for fear conditioning and testing was a train of 70 dB white noise pips, each lasting 250 ms, delivered at 1 Hz for 20 s through an overhead speaker. The US was a train of 2.0 mA
shock pulses, each lasting 2.0 ms, delivered to one eyelid at a rate of 6.66 Hz for 2 s. During CS-US pairing trials, the first shock pulse was always delivered 300 ms after the offset of the final (20th) CS pip. The intertrial interval was uniformly random between 180 and 240 s.

The rat’s position on the experimental platform was sampled at 30 Hz by an overhead video tracking system (Neuralynx Corporation, Bozeman, MT), which monitored the location of three light-emitting diodes (LEDs) of different colors (red, blue, and green) attached to the animal’s headstage for automated measurement of movement speed and turning behavior (see Tarpley et al., 2010; Halladay and Blair, 2012). Conditioned fear responses were assessed by comparing the animal's behavior during the context (CX) period (20 s immediately preceding CS onset) versus CS period (19 s immediately following CS onset; the 20th pip was omitted from CS period because the first shock pulse was delivered <1 s after the offset of this pip) of each trial. Each trial was classified as a CMI, CME, or no conditioned response (NCR) trial based upon the rat’s behavior during the trial. The rat’s movement speed was averaged during consecutive 1 s bins throughout the trial, and a t-test was then performed to compare mean movement speed during bins from the CX period (n=20) versus CS period (n=19). A one-tailed cutoff of p=.05 was used to classify the trial's type as either CMI (CX>CS), CME (CX<CS), or NCR (CX not different from CS).

Neurophysiological data acquisition and analysis

Single units in mPFC and dlPAG were recorded using a DigitalLynx S-series acquisition system (Neuralynx). Waveforms were isolated manually using Spikesort3D (Neuralynx) software. To be included in data analyses, spikes had to exceed a minimum amplitude threshold of 70 μV peak-to-peak (against a mean noise floor of ~25 μV peak-to-peak), and exhibit a
refractory period of at least 1 ms base upon analysis of interspike interval histograms. Spike trains recorded on different experiment days were considered to be from the same cell if the following conditions were met: 1) spikes were recorded from the same tetrode, 2) the tetrode had been advanced <80 µm between recordings, 3) cluster boundaries and waveform shapes were visually similar on all tetrode channels for each session, and 4) the effect of the CS upon the cell’s firing rate did not differ significantly across any pair of consecutive experiment days (see below, “Classification of Cell Types”). Neurophysiological data analysis only included cells that were recorded during at least 3 CMI and 3 CME trials across all sessions during which the cell was isolated. Posthoc comparisons for all ANOVAs were made using the Newman-Keuls method, unless otherwise noted.

**Classification of cell types.** Single units were classified according to how their mean firing rates changed between the CX versus CS periods during CMI and CME trials. For each recording session, a cell’s spike data from trials of a given type (CMI or CME) was used to generate a peristimulus time histogram (PSTH; bin size = 1 s) aligned to the onset of the first CS pip of each trial; the CX period spanned 20 bins before the first CS onset, and the CS period spanned 19 bins after the first CS onset. If a cell was recorded across more than one session, then spike data from each pair of consecutive sessions was analyzed by performing a 2x2x2 independent ANOVA with stimulus (CX vs CS), trial type (CMI vs CME), and session (first vs second day of the consecutive pair) on spike counts from each 1 s bin of the cell’s PSTH. If the 3-way interaction was significant for any pair of consecutive days, then data from the second day and all subsequent days was discarded from further analysis, so that only data from days across which the cell’s firing properties remained consistent were included in the analysis. The remaining spike data was collapsed across all included days, and an independent t-test compared
spike counts in PSTH bins from all included days for the CX versus CS period of each trial type (CMI or CME); a one-tailed cutoff of $p=.05$ was used to classify cells into nine categories based upon the outcome of this comparison (see “Classification of cell types” in the Results section).

**Spike waveform parameters.** To classify neurons as principal cells versus interneurons, a 3D scatterplot was generated in which each cell was plotted as a single point in a space with three dimensions: baseline firing rate (mean spike count per 1 s bin of the CX period), spike width (mean time interval between the cell’s initial departure from and subsequent return to voltage baseline), and spike area (cumulative sum of voltage across the spike width interval). A Gaussian mixture model with two components (one for principal cells, one for interneurons) was then fit to the 3D point cloud from each region (PAG or mPFC), using MATLAB’s ‘fitgmdist’ command. The resulting model was then used to classify each neuron into one of the two categories with MATLAB’s ‘cluster’ command. The validity of the classification was tested by comparing the mean distance separating pairs of points within versus between the two categories (see Results).

**Population averaged PSTH.** To generate population-averaged PSTHs for CMI or CME trials, each cell's PSTH was first normalized by converting its firing rate axis to a z-score. This was done by subtracting the mean and dividing by standard deviation of the firing rate during 20 bins from a baseline period, which was defined as the CX period of CMI trials (note that the CX period of CMI trials was used as the baseline for normalizing both CMI and CME histograms, so that population firing rates during the CX period of CMI versus CME trials could be compared). Normalized histograms were then averaged across cells to generate the population PSTH.

**Responses to pips and shocks.** Responses to auditory pips were analyzed on a short time scale by plotting PSTHs aligned to the onset of each CS pip using a bin size of 2 ms. Confidence
intervals on the spike counts in each bin were computed from a baseline period spanning 500 ms (250 bins) prior to the onset of each pip, based upon an assumption of Poisson spiking (Abeles, 1982). A neuron was considered to exhibit auditory evoked responses if there were one or more bins exceeding 99% confidence, or two or more bins exceeding 95% confidence, in the 250 ms (125 bins) time window spanning pip onset to offset. The cell’s response latency was computed as the time delay from pip onset to the first bin exceeding the 95% confidence threshold.

Responses to shock pulses were analyzed in a similar manner, except that the baseline period for computing confidence intervals spanned 50 ms (25 bins) preceding each shock onset, and a neuron was considered to be shock responsive if there were one or more bins exceeding 99% confidence, or two or more bins exceeding 95% confidence, in time window spanning 6-100 ms (47 bins) following pip onset (the 0-6 ms time period was omitted because each 2 ms shock pulse generated a brief stimulus artifact that occluded recording at the time of the pulse). The latency of shock-evoked responses was computed as the as the time delay from shock onset to the first bin in the analysis window exceeding the 95% confidence threshold.

**Freezing versus flight pips.** To classify pips based on motor behavior, movement speed was measured in 1/30 s time bins (the sampling period of the video tracker) during two 500 ms time windows: one before and one after the onset of each pip. If movement speed was significantly greater during the post-pip time window (p<.05 by a unpaired t-test, one-tailed), then the pip was classified as a 'flight' pip. If there was no change in the movement speed (p≥.05), and if the mean speed for all bins from the pre- and post-pip time windows combined (1 s total) was less than .01 standard deviation above zero, then the pip was classified as a 'freezing' pip. Pips that did not meet criterion for classification as 'flight' or 'freezing' pips were discarded from analysis. To analyze neural population activity during freezing versus flight pips, two
PSTHs (bin size = 20 ms) were computed for each cell, one triggered by freezing pips and the other by flight pips. Both PSTHs were normalized by converting their firing rate bins to z-scores using the mean and standard deviation of spike counts from a baseline period defined as the 500 ms time window preceding the onset of freezing pips (note that the pre-pip period of freezing pips was used as the baseline for normalizing both freezing and flight PSTHs, so that population firing rates during the pre-pip period of freezing versus flight pips could be compared). Statistical analyses compared z-scores at each time point, as described in Results.

Histological Procedures

At the end of the experiment, rats were intraperitoneally injected with an overdose of pentobarbital (100 mg/kg) and perfused intracardially. Brains were extracted and fixed in a formalin sucrose solution. Tissue was later sectioned into 40 μm slices and mounted on slides for electrode placement verification.

RESULTS

Single-unit activity was recorded in dlPAG and mPFC while rats (n=4) expressed conditioned fear responses to an auditory CS. A total of 74 neurons were recorded from dlPAG, with a mean baseline firing rate of 2.96±.36 Hz and a mean peak-to-peak spike height of 93.6±1.0 μV. Most dlPAG cells (n=57) were recorded from the dorsal PAG (dPAG), which is comprised of the dorsomedial and dorsolateral columns, and the remaining cells (n=17) were recorded from the lateral column (lPAG). Reconstructions of dPAG and lPAG recording sites are shown in Figure 1A. A total of 71 neurons were recorded from mPFC, with a mean baseline firing rate of 2.93±.32 Hz and a mean peak-to-peak spike height of 108.8±3.1 μV. These cells
were distributed among three mPFC subregions: anterior cingulate (ACC; n=31), prelimbic (PL; n=29), and infralimbic (IL; n=11) subdivisions of mPFC (Figure 1B).

Classification of trial types

Each experimental session consisted of 6 presentations of the CS alone followed by 16 CS-US pairings (Figure 2A). Rats were trained on this regimen for five consecutive days prior to their first recording session, so that CS-evoked CMI and CME responses would be at or near asymptote when recordings began (confirmed by data analysis presented below). The same daily regimen of 6 CS alone trials and 16 CS-US pairing trials then continued throughout the duration of the neural recording experiments, for up to 15 recording sessions. After the initial five-day training period, fear conditioning sessions were only conducted on days when well-isolated neurons were observed on the electrodes. Consequently, the number of recording sessions differed for each rat because of variability in cell yields: rat 1 was run for 15 sessions, rat 2 was run for 14 sessions, rat 3 was run for 10 sessions, and rat 4 was run for 6 sessions.

Within each trial, we define the CX period as the 20 s time window preceding onset of the first CS pip, the CS period as the 19.6 s time window between CS and US onset, and the US period as the 2 s period following US onset. On each experiment day, ‘pre-shock trials’ were defined as the 7 trials during which both the CX and CS preceded the day’s first US presentation (all 6 CS alone trials, plus the first CS-US pairing trial), whereas ‘post-shock trials’ were defined as the remaining 15 CS-US pairing trials. Figure 2A shows line graphs plotting the rats’ mean movement speed (averaged first over trials, then over rats) during pre- and post-shock trials across recording sessions, up to the maximum of 15 sessions. Because each of the four rats was...
run for a different number of sessions (as explained above), the number of included rats
decreased as the session number increased: n=4 for sessions 1-6, n=3 for sessions 7-10, n=2 for
sessions 11-14, and n=1 for session 15.

The rats’ movement speed was suppressed by the CS during pre-shock trials, but
enhanced by the CS during post-shock trials, in agreement with prior studies showing that a fear
conditioned CS evokes freezing responses in rats that have not recently been shocked, and flight
responses in rats that have recently been shocked (Tarpley et al. 2010; Halladay and Blair 2012).
Confirming this, a 6x2x2 repeated measures ANOVA with session (first six sessions only, since
these were the only sessions that included all four rats), phase (pre- versus post-shock), and
stimulus (CX versus CS) as factors yielded a significant 2-way interaction between phase and
stimulus ($F_{1,3}=14.6, p=.032$). There was no main effect of session ($F_{5,15}=0.74, p=.60$), nor was
there any significant 2-way or 3-way interaction between session and any other variable ($p>.1$ for
all interactions), supporting the conclusion that rats had reached behavioral asymptote during the
pre-training phase prior to the first recording session, so that their conditioned behavior did not
change significantly across recording sessions.

Movement speed data in Figure 2A indicates that during recording sessions, rats exhibit
two distinct modes of defensive responding to the same CS: CMI responses during pre-shock
trials, and CME responses during post-shock trials. To analyze relations between neural activity
and defensive behavior, each experimental trial was classified into one of three types, depending
upon the rat’s behavior during that trial (see Methods): 1) CMI trials were those during which the
rat’s movement speed was suppressed during the CS when compared against CX, 2) CME trials
were those during which the rat’s movement speed was enhanced during the CS when compared
against CX, and 3) NCR trials were defined as those during which the rat’s movement speed did
not differ during the CS versus CX. Pie graphs in Figure 2B show that across all sessions and rats, 59.9% of the pre-shock trials were CMI trials and only 6.3% were CME trials (the rest were NCR trials). Conversely, 66.4% of the post-shock trials were CME trials and only 6.8% were CMI trials (again, the rest were NCR trials). Line graphs in Figure 2B show that this differential distribution of pre- versus post-shock trial types persisted across all recording sessions.

Figure 2C (top graph) shows mean linear movement speeds during CMI, CME, and NCR trials, averaged over all trials of each type during which at least one neuron was recorded (total of 990 trials from 45 recording sessions in 4 rats). Figure 2C (bottom graph) shows the rats’ mean turning velocity towards versus away from the shocked eyelid during each trial type. A 3×2 ANOVA of turning velocity with trial type (CMI, CME, NCR) and stimulus (CX, CS) as independent factors yielded a significant interaction effect ($F_{2,896}=21.48; p<.00001$), and Scheffe corrected posthoc comparisons revealed that turning during the CS for CME trials was greater than all other conditions ($p<.00001$ for all comparisons), but other conditions did not differ significantly from one another ($p>.66$ for all comparisons). These results show that during the CS period of CME trials, rats turned away from the eyelid where shock delivery was anticipated, in accordance with prior work showing that these CS-evoked movements are flight CRs directed away from the eyelid where shock is expected (Tarpley et al. 2010; Halladay and Blair 2012).

**Classification of cell types**

Neurons were classified into nine types, with each type corresponding to an entry in a 3x3 table (Figure 3, panels A1 and B1) where rows denote the sign of the cell's firing rate difference between the CX versus CS periods of CME trials ($ΔHz$ CME), and columns denote
the sign of the cell's firing rate difference between the CX versus CS periods of CMI trials (ΔHz CMI). When a single cell was recorded during more than one session (see example, Figure 2D), its type was classified based upon spike data collapsed across consecutive sessions during which the cell met criteria for inclusion in the study (see Methods). The nine cell types fell into four functional categories: *strategy-selective* cells that responded to the CS differently depending upon the rat’s behavioral response to the CS, *stimulus-selective* cells that responded to the CS in the same way regardless of the rat’s behavior, *speed-selective* cells that fired in correlation with the rat’s movement speed regardless of whether the CS was present, and *non-responsive* cells that did not exhibit any clear task-related activity.

### Strategy-selective cell types

Four cell types were classified as *strategy-selective neurons*, because their firing rates during the CS depended upon the rat’s defensive response strategy during the CS. If a neuron's firing rate changed during the CS period of CMI but not CME trials, then the neuron was classified as a CMI+ cell if its firing rate increased (CS>CX) during the CS period, or as a CMI-cell if its firing rate decreased (CS<CX) during the CS period of CMI trials. Conversely, if a neuron's firing rate changed during the CS period of CME but not CMI trials, then the neuron was classified as either a CME+ or CME- cell, depending upon whether its firing rate increased or decreased, respectively, during the CS period of CME trials.

### Stimulus-selective cell types
Two cell types were classified as stimulus-selective neurons, because these neurons always responded to the auditory CS in the same way, regardless of the rat’s defensive response strategy during the CS. A neuron was classified as a CS-responsive (CSR) cell if CS presentations evoked firing rate changes of the same sign during both CMI and CME trials; CSR+ cells always increased their firing rate during the CS, and CSR- cells always decreased their firing rate during the CS. Since the CS predicted the same threat (the eyelid shock US) during both CMI and CME trials, it was not possible to dissociate whether stimulus-selective neurons were selective for the CS’s auditory sensory properties, motivational valance properties, or some other uncontrolled aspect of the CS.

Speed-selective cell types

Two cell types were classified as speed-selective neurons, because they were always modulated by the rat’s movement speed in the same way, regardless of other factors. As shown in Figure 2A, the CS evoked decreases in movement speed during CMI trials, and evoked increases in movement speed during CME trials. A neuron was classified as a movement cell (MOV) if CS presentations likewise evoked firing rate changes of opposite sign during CMI versus CME trials. MOV+ cells changed their firing rate in the same direction as the change in the rat’s movement speed (decreased firing during CMI trials and increased firing during CME trials), whereas MOV- cells changing their firing rate in the opposite direction from the rat’s movement speed (increased firing during CMI trials and decreased firing during CME trials).
Non-responsive neurons

Non-responsive (NR) cells were neurons that did not change their firing rate between the CX versus CS period of either CMI or CME trials. The firing rates of these neurons were not related in any clear way to the rat’s behavior during the task.

Distribution of cell types in PAG

Out of 74 neurons recorded in dlPAG, 40 cells (54%) were classified as CME+ cells, 20 cells (27%) were classified as CSR+ cells, 8 cells (11%) were classified as MOV+ cells, 5 cells (8%) were classified as NR cells, and one cell was classified as a CMI- cell (Figure 3, panels A1 and A2). Population averages and example neurons for three prevalent dlPAG cell types (CME+, CSR+, and MOV+) are shown in Figure 4A. Notice that CME+ cells exhibited sustained firing throughout the CS period of CME trials but not CMI trials (which is why they were classified as CME+ cells), but CME+ cells responded to the initial onset of the CS during both CME and CMI trials; possible explanations for this shall be addressed in the Discussion.

Baseline firing rates of dlPAG neurons during the CX period were analyzed using a $4 \times 2$ ANOVA with cell type (CME+, CSR+, MOV+, NR) as an independent factor and trial type (CMI, CME) as a repeated factor (Table 1); there was a significant main effect of trial type ($F_{1,70}=5.28$, $p=.02$) but not cell type ($F_{1,70}=1.09$, $p=.36$) with no interaction ($F_{3,69}=1.53$, $p=.22$). Posthoc comparisons revealed that only cells belonging to the MOV+ category showed significantly lower baseline firing rates during the CX period of CME trials versus CMI trials ($p=.047$). This supports the conclusion that speed was the primary determinant of firing rates for
MOV+ cells, since movement speed was also lower during the CX period of CME than CMI trials (see Figure 2C). All other cell types exhibited similar baseline firing rates during the CX periods of CME versus CMI trials.

We performed a series of 2x2 chi square tests to examine whether specific types of cells were more common in the IPAG vs dPAG subregions. Only MOV+ cells were unevenly distributed, constituting 30% of IPAG cells but only 5% of dPAG cells ($\chi^2_{1,74} = 7.92, p = .005$, uncorrected). To examine whether certain cell types became more or less prevalent over the course of the experiment, we analyzed whether a cell’s type classification was dependent upon the session number of the first day on which it was recorded (columns in Figure 3, panel A3). About half of the PAG neurons were first recorded during or before session 7 (early cells), while the remaining half were first recorded during or after session 8 (late cells). CSR+ cells constituted 18% of cells recorded early in the study and 38% of cells recorded late in the study ($\chi^2_{1,74} = 4.01, p = .045$, uncorrected), but this difference was not significant after correction for multiple comparisons, and no other cell type was differentially distributed across early versus late recording sessions ($p>.05$ for all uncorrected chi-square tests). To examine the influence of data sampling upon cell types, we tested whether a cell’s type classification was contingent upon the number of days across which is was held (rows in Figure 3, panel A3). PAG neurons were held across a median of 2 sessions per neuron; no cell type was more or less prevalent among neurons that had been recorded for more versus fewer than the median number of sessions ($p>.05$ for all chi-square tests). We also examined whether PAG cell type classifications were contingent upon whether the cell was recorded in the hemisphere ipsilateral versus contralateral from the shocked eyelid (see Table 1), and found no evidence for such a contingency in any cell type. Finally, we tested whether any cell type was more common in specific rats. PAG neurons...
were recorded from 3 of the 4 rats in the study, and a series of 3x2 chi-square tests yielded no
evidence that any cell type was significantly more or less common in specific rats (p>.05 for all
chi-square tests).

To test whether PAG cells could be subdivided into principal cells versus interneurons, a
3D scatter plot was generated from their spike area, spike width, and baseline firing rate
parameters (Figure 3, panel A4), then a two-component Gaussian mixture model was used to
cluster spikes into two categories (see Methods). The mean between-category distance was only
8% larger than the mean within-category distance separating points in the scatterplot, so the
spike parameters did not separate into two well-distinguished clusters. Hence, PAG neurons
were not easily classifiable into categories corresponding to principal cells versus interneurons.

Distribution of cell types in mPFC

Neurons recorded in mPFC exhibited a broader diversity of type classifications than those
in dlPAG. Figure 3 (panels B1 and B2) show that out of 71 neurons recorded in mPFC, 23 cells
(32%) were classified as NR cells, 15 (23%) cells were classified as MOV+ cells (with one
additional neuron classified as a MOV- cell), 14 (20%) cells were classified as CME+ cells (with
one additional neuron classified as a CME- cell), 6 cells (8%) were classified as CMI+ cells, 7
cells (10%) were classified as CMI- cells, and 3 cells (4%) were classified as CSR+ cells (with
one additional neuron classified as a CSR- cell). Figure 4B shows population averages and
example neurons for prevalent mPFC cell types (CME+, CMI+, and MOV+). Notice that like
CME+ cells in PAG, CME+ cells in mPFC tended to respond to the initial onset of the CS during both CMI and CME trials (see Discussion).

A 5x2 ANOVA of baseline firing rates for the most prevalent cell types recorded in mPFC (NR, CME+, CMI+, CMI-, MOV+) revealed a significant main effect of trial type ($F_{1,68}=5.44$, $p=.02$) but not of cell type ($F_{1,68}=0.54$, $p=.66$) with no interaction ($F_{4,68}=1.8$, $p=.16$). Posthoc comparisons revealed that, as in PAG (see above), only cells belonging to the MOV+ category trended toward lower baseline firing rates during the CX period of CME versus CMI trials ($p=.054$), again reinforcing the conclusion that movement speed was the primary determinant of firing rates for MOV+ cells. All other cell types exhibited stable baseline firing rates during the CX periods of CME versus CMI trials.

We performed a series of 3x2 chi square tests to investigate whether cells in ACC, PL, or IL were more likely to be of a specific type. As in PAG, only MOV+ cells were unevenly distributed across subregions, constituting 32% of ACC cells and 27% of IL cells, but only 7% of PL cells ($\chi^2_{2,71} = 6.08$, $p = .048$, uncorrected). All CSR+ and CSR- cells were recorded in PL, and none in ACC or IL, but the small sample of such cells (n=4) did not supply sufficient statistical power to test whether CSR neurons were more concentrated in PL than other subregions. About half of the mPFC cells were first recorded during or before session 4 (early sessions), and the remainder were recorded during or after session 5 (late sessions). No cell type in mPFC was differentially distributed across early versus late recording sessions ($p>.05$ for all uncorrected chi-square tests). Individual mPFC neurons were held for a median of 2 recording sessions (Figure 3, Panel B3), and a 2x2 chi-square test indicated that MOV+ cells constituted 40% of neurons that had been held for more than the median number of sessions, but only 11%
of neurons that had been held for less than or equal to the median number of sessions ($\chi^2_{2,71} = 8.25, p = .004$, uncorrected). No other cell type’s prevalence was contingent upon the number of sessions over which the cell was held ($p > .05$ for all chi-square tests). We examined whether mPFC cell type classifications were contingent upon which hemisphere the cell was recorded in (ipsilateral versus contralateral from the shocked eyelid, see Table 1), and found no evidence for lateralization of any cell type. We also tested whether any cell type was more common in specific rats; mPFC neurons were recorded from all 4 rats in the study, and a series of 4x2 chi-square tests indicated that MOV+ cells were more prevalent in rat number 2 than in the other three rats ($\chi^2_{3,71} = 15.5, p = .001$, uncorrected). No other cell type was significantly more common in any given rat, but it should be noted that small sample sizes made this difficult to assess for some cell types.

To test whether mPFC cells were separable into principal cells versus interneurons, a 3D scatter plot was generated from their spike area, spike width, and baseline firing rate parameters (Figure 3, panel B4), then a two-component Gaussian mixture model was used to cluster spikes into two categories (see Methods). The mean between-category distance was 90% larger than the mean within-category distance separating points in the scatterplot, so mPFC spikes were readily classifiable into categories corresponding to principal cells (with low firing rates and large spike widths) versus interneurons (with high firing rates and narrow spike widths). Of the 71 mPFC cells we recorded, 58 were classified as principal cells and 13 were classified as interneurons (Figure 3, panel B4). We examined whether mPFC cell type classifications were contingent upon whether the cell was a principal cell or interneuron, and found that CMI+ cells constituted 23% of interneurons but only 5% of principal cells ($\chi^2_{1,71} = 5.54, p = .019$, uncorrected). Moreover, the few CMI+ principal cells (n=3) we did observe were all found in the PL
subregion, which is consistent with prior evidence (Burgos-Robles et al. 2009; Sotres-Bayon et al. 2012) suggesting that PL principal cells may promote freezing behavior (see Discussion). No other cell type differed in prevalence among principal cells versus interneurons.

Comparison of cell types in PAG versus mPFC

A 4x2 chi-square test indicated that cell types in different functional categories (strategy-selective, stimulus-selective, speed-selective, or non-responsive) were differentially distributed between dlPAG versus mPFC ($\chi^2_{3,145} = 27.3, p < .00001$). This remained true even when non-responsive cells were omitted ($\chi^2_{2,117} = 12.4, p = .002$), so the effect was not wholly attributable to the larger number of NR cells in mPFC. Stimulus-selective neurons were more prevalent in dlPAG (27% of cells, all of which were CSR+ cells) than mPFC (6% of cells), whereas speed-selective neurons (all but one of which were MOV+ cells) were more prevalent in mPFC (22% of cells) than dlPAG (10% of cells).

Strategy-selective neurons constituted a large proportion of cells in both dlPAG (55% of cells) and mPFC (39% of cells), but different types of strategy selective neurons were observed in each area ($\chi^2_{3,69} = 22.4, p = .00006$). Almost all (97.5%) of the strategy-selective neurons in dlPAG were CME+ cells, whereas only half (50%) of the strategy-selective neurons in mPFC were CME+ cells; another one quarter (25%) were CMI- cells and one fifth (21%) were CMI+ cells. This suggests that dlPAG may be specifically involved in the execution of CME responses, in accordance with prior evidence that this area orchestrates escape and avoidance behavior (Bandler and DePaulis 1988; Fanselow 1991; De Oca et al. 1998). By contrast, the
diversity of strategy selective neurons in mPFC suggests involvement in selecting between competing defense strategies, such as CME versus CMI responses, in accordance with prior theories positing that mPFC mediates behavioral strategy selection by altering how stimuli are mapped onto responses in other brain regions (Miller and Cohen, 2001). To further investigate the roles of dlPAG and mPFC in defensive responding, we analyzed their spike activity on a shorter time scale (see below).

**Pip-evoked responses**

Short-latency responses to CS pips were analyzed on a fast time scale to assess whether neurons exhibited sensory evoked responses to the auditory pips. About half of the dlPAG cells (34 of 74, or 46%) exhibited excitatory responses that were time locked to pip onset, with a mean onset latency of 32.7 ± 1.7 ms (no dlPAG cells showed evidence of being inhibited by pip onset). None of the pip responsive cells in dlPAG belonged to the MOV+ category, supporting the interpretation that motor rather than sensory influences were the primary determinant of firing rate changes for MOV+ cells. Pip responses were observed for 58% (23/40) of CME+ cells (Figure 5A) and 50% (10/20) of CSR+ cells (Figure 5B) in dlPAG; in addition, 1 of 5 NR cells was pip responsive. Pip responsiveness was observed 36% (12/33) of PAG cells recorded contralateral and 51% (21/41) of cells recorded ipsilateral to the shocked eyelid, which was not a significant difference ($\chi^2_{1,74} = 2.2, p = .14$). Unlike dlPAG neurons, none of the individual mPFC neurons exhibited significant short-latency CS-evoked responses (either excitatory or inhibitory) that were time locked to the CS onset. However, pip-evoked inhibition was observed for population averaged responses of CMI+ cells in mPFC, as discussed further below.
To further dissociate sensory, behavioral, and motivational influences on CS-evoked firing of recorded neurons, individual CS pips from post-shock trials were parsed for membership in two categories, *freezing* or *flight*, based upon the animal's behavior during each pip (pre-shock trials were not included, to eliminate the confounding influence of recent shock upon behavior and neural activity). Freezing pips were defined as those during which the animal remained motionless before and after the pip, whereas flight pips were defined as those for which the animal's movement speed was significantly greater during the post- than pre-pip period (note that pips not belonging to either category and were discarded from the analysis).

During flight pips, averaged movement speed became significantly elevated above the pre-pip baseline at a motor response latency of ~140 ms after pip onset (dashed vertical lines in Figures 5C-E). Both freezing and flight pips elicited phasic spiking responses (concentrated in the 20-40 ms bin) from CME+ and CSR+ cells in dlPAG (Figure 5D). For CME+ cells, the initial phasic response was similar in magnitude for both freezing and flight pips. The phasic response was followed by a sustained elevation of firing, which was significantly larger during flight than freezing pips at time points beyond (but not prior to) the 140 ms motor response latency (black dots in Figure 5D, top graph). A pair of binomial tests confirmed that this result, whereby the flight pip response exceeded the freezing pip response only at time points after (but not before) onset of movement, was extremely unlikely to arise by chance ($p<.00001$).

Firing rates of CME+ cells in mPFC were also larger for flight pips than freezing pips, but at different time points (black dots in Figure 5E, top graph). When flight pips were compared against freezing pips, CME+ cells in mPFC showed higher firing rates during flight pips for the 13 time bins (260 ms) prior to pip onset ($p<.0001$, based on a binomial test for 7/13...
successes at 5% success rate), as well as for the 12 time bins (240 ms) after pip offset (p<.001, based on 6/12 successes), but not during the pip period itself (0/13 successes). This lack of elevated firing rate during the pip period of flight pips appeared to be caused by a phasic inhibition of mPFC CME+ cells at ~80 ms after the onset of flight pips, which reduced their firing rate to a level similar to that seen during freezing pips (Figure 5E).

One possible explanation for the elevated firing rates of mPFC CME+ cells prior to the onset of flight pips could be that CME+ cells in mPFC were sensitive to movement speed, since the rats' average movement speed was slightly elevated before the onset of flight pips compared with freezing pips (Figure 5C). However, movement speed was even more elevated after the offset than prior to the onset of flight pips (Figure 5C), and yet, CME+ cells in mPFC exhibited similar firing rates both before onset and after offset flight pips (Figure 5E, top graph). Moreover, movement speed was lower during the CX period of CME than CMI trials (see Figure 2B), but baseline firing rates of CME+ cells did not decrease along with movement speed during the CX period of flight trials (Figure 3D, left graph). These results imply that movement speed was not a primary influence upon the firing rates of CME+ cells in mPFC.

In contrast with CME+ cells, population-averaged responses of CMI+ cells in mPFC were smaller for flight pips than freezing pips, mainly at time points clustered after pip onset and offset (black stars in Figure 5E, bottom graph). Binomial tests did not reveal a significant overall reduction in firing rate during the flight pip period (p=.14, 2/13 successes), but did indicate a significantly reduced firing rate during the time period spanning 250 ms before onset to 250 ms after offset of flight pips when compared against freezing pips (p=.04, 5/38 successes). By definition, CMI+ cells increased their firing rates during the CS of CMI trials, so it would be
desirable to test whether these cells increased their firing rates during a subset of pips that
evoked a decrease in movement speed (in the same way that flight pips evoked an increase in
movement speed). Unfortunately, it was not possible to perform such an analysis, because CMI
behavior tended to be expressed tonically over a time scale of seconds, and thus, movement
cessation was not a phasically evoked by pips in the same manner as CME behavior. Since very
few pips were followed at short latency by a significant decrease in movement speed, it was not
possible to analyze neural activity during pips that evoked movement cessation.

Shock-evoked responses

Shock-evoked responses of dIPAG and mPFC neurons were analyzed on a short time
scale by plotting PSTHs triggered by individual shock pulses. In dIPAG, more than half of the
recorded cells (41 of 74) were excited by shock pulses (see example in Figure 6A, left) with a
mean onset latency of 15.4±1.1 ms, whereas about one quarter of the cells (17 of 74) were
inhibited by shock pulses (see example in Figure 6A, right). Shock responsiveness in dIPAG was
not contingent upon a cell’s type classification (Fisher’s exact test, p=.66), but was contingent
upon whether a cell was recorded in dPAG versus lPAG (Fisher's exact test, p=.02). Binomial
tests revealed that this was because cells inhibited by shock were found exclusively in dPAG and
not in lPAG (p=.02), whereas cells excited by shock were evenly distributed between dPAG and
lPAG (p=.43), as were cells that were non-responsive to shocks (p=.60). A 3x2 chi-square test
revealed that shock responsiveness was also asymmetric across hemispheres ($\chi^2_{1,74} = 7.16, p =
.028$); while excitatory responses to shock were similar among cells recorded in the dIPAG
ipsilateral (58%) versus contralateral (54%) to the shocked eyelid, inhibitory responses were
more prevalent in the ipsilateral (33%) than contralateral (15%) hemisphere, and non-shock responsive cells were rarer in the ipsilateral (9%) than contralateral (32%) hemisphere.

In mPFC, about one third of the cells (24 of 71) were excited after the onset of shock pulses (see example in Figure 6B, left), whereas only 4% were inhibited by shock (3 of 71 cells, all CMI+ cells recorded ipsilateral to the shocked eyelid; see example in Figure 6B, right), and the remaining cells (44 of 71) were non-responsive to shocks. A chi-square test indicated that the proportion of cells excited by shock differed significantly among mPFC subregions (χ² = 8.8, p = .01). Approximately half of the cells in ACC (15 of 31) were excited by shock with an onset latency of 16.1 ± 1.5 ms, and half of the cells in IL (5 of 11) were excited by shock with an onset of 13.2 ± 2.3 ms. By contrast, only fourteen percent (4 of 29) of cells in PL were excited by shock, with a longer mean onset latency of 28.8 ± 9.3 ms. A 3x2 chi-square test revealed that shock responsiveness of mPFC neurons was not contingent upon whether they were recorded ipsilateral versus contralateral from the shocked eyelid (χ² = 3.83, p = .15).

Longer lasting effects of the shock US were analyzed by averaging behavior and neural activity during CS-US pairing trials throughout a 160 s time window beginning with the onset of the CX period (that is, 20 s prior to CS onset), and ending 2 min after offset of the shock US (Figure 7). Rats exhibited turning away from the shocked eyelid during the CS and US, but turning ceased within a few seconds following US offset (Figure 7A, top graph). By contrast, movement speed remained elevated above the CX baseline for nearly 2 min following shock offset (Figure 7A, bottom graph). Visual observation indicated that during this time period, animals exhibited bouts of freezing (which became longer in duration with increasing latency from shock offset) punctuated by rapid scurrying among different locations (which became less
frequent with increasing latency from shock offset), as if the shock made them uncertain of which location in the environment was the safest place to freeze. In dlPAG, firing rates of CME+, CSR+, and MOV+ cells remained elevated above baseline throughout much of this post-shock period of motor activity (Figure 7B). CSR+ cell firing rates fell below the CX baseline near the end of the motor activity period.

In mPFC, firing rates of CME+, CMI-, and MOV+ cells were elevated above baseline throughout much of the post-shock period of motor activity (Figure 7C). Conversely, the population average firing rate for CMI+ cells decreased during the post-shock period. Post-shock firing rate changes for CMI+ and CMI- cells did not reach the p<.05 significance level at most time points, probably because the sample size was smaller than for other cell types.

**DISCUSSION**

Here we recorded neural activity from mPFC and dlPAG while rats expressed two distinct defensive behaviors—CMI versus CME responses—elicited by the same fear conditioned CS. During each experimental session, shock delivery served as a catalyst for inducing a transition in the rat’s defensive response strategy: the CS elicited only CMI responses during pre-shock trials, but then began to elicit CME responses during post-shock trials. This behavioral transition may be interpreted as a rightward shift along the “predatory imminence continuum,” whereby an animal’s defensive response strategy changes as a function of its proximity to danger (Bolles, 1970; Fanselow and Lester, 1988). CMI responses may be classified as a “post-encounter” defensive behavior that is expressed when danger is present but not proximal, whereas CME responses may be classified as a “circa-strike” (or in this case,
“circa-shock”) defensive behavior that is expressed when danger is in close proximity. Hence, a CS that has not recently been paired with shock it may signal a distal threat that elicits CMI responses, whereas after recently being paired with shock, the same CS may evoke a greater sense of proximity to danger, and thus begin to trigger CME responses (Tarpley et al., 2010).

Our single-unit recording experiments indicated that about 40% of mPFC neurons were strategy-selective cells that responded differentially to the CS, depending upon whether it elicited CMI or CME responses from the rat. This result is consistent with prior evidence that mPFC participates in regulating the selection of defensive response strategies, as a function of proximity to danger (Mobbs et al. 2007, 2009). However, a limitation of our experimental design was that the rat’s behavioral strategy was confounded with other variables such as recency of shock delivery, and perhaps also the degree of fear during the CS (since the rat’s expectation of the shock may have been greater during post-shock trials than pre-shock trials).

As discussed below, even when these confounding factors are considered, our findings suggest that some mPFC neurons may indeed participate in regulating the animal’s selection of defensive actions as a function of predatory imminence.

Stimulus-selective cells

Stimulus-selective neurons (those that responded to the CS regardless of whether it elicited CMI or CME behavior) constituted 27% (20/74) of the neurons recorded in dIPAG, all of which were CSR+ cells excited by the CS, but less than 6% (4/71) of the neurons recorded in mPFC. Interestingly, all of the stimulus-selective mPFC neurons (3 CSR+ cells and 1 CSR- cell) were located in the PL subregion, so that 15% (4/29) of PL neurons were stimulus-selective. Since the CS predicted the same threat (the eyelid shock US) during both CMI and CME trials, it
was not possible to dissociate whether stimulus-selective neurons were tuned for the CS’s auditory properties or its motivational valence properties. But clearly, if any recorded neuron functioned as a low-threshold “fear cell” that fired whenever the rat’s anticipation of shock exceeded the minimum required for eliciting CMI behavior, then that neuron should be expected to behave as a stimulus-selective cell in our experiment (since the cell’s low threshold for shock anticipation would presumably be exceeded during both CMI and CME trials). By this reasoning, the paucity of stimulus-selective neurons in mPFC (especially in the ACC and IL subregions) implies a paucity of cells that simply responded whenever the rat’s anticipation of shock exceeded the minimum threshold for eliciting CMI behaviors. One possible explanation for this lack of low-threshold fear cells in mPFC could be that prefrontal activity did not simply signal the rat’s anticipation of shock during the CS, but more specifically regulated the selection of appropriate defensive response strategies as function of predatory imminence. As discussed below, this interpretation is further supported by our observations that strategy-selective neurons were more abundant than stimulus-selective neurons in mPFC.

**Strategy-selective cells**

Strategy-selective cells were prevalent in both dlPAG, where they constituted more than half (40/74) of the recorded neurons, and mPFC, where they constituted 39% (28/71) of the recorded neurons. However, the distributions and firing properties of strategy selective cells differed in dlPAG versus mPFC.

**CME+ neurons**

All of the strategy-selective neurons in dlPAG (except for one) were CME+ cells that
increased their firing rates during the CS when it elicited CME but not CMI responses. By contrast, only half (14/28) of the strategy-selective neurons in mPFC were CME+ cells (the rest were mostly CMI- or CMI+ cells, discussed further below). Prefrontal CME+ cells could have been involved in regulating the rat’s behavioral strategy to express CME rather than CMI responses during the CS. Alternatively, CME+ cells might instead have been “high-threshold fear cells” that were insensitive to the rat’s behavior during the CS, but only responded to the CS when it evoked a strong anticipation of shock (during post-shock trials) but not when it evoked a weaker anticipation of shock (during pre-shock trials). It is difficult to dissociate between these possibilities based solely upon the neurons’ selectivity for CME trials. However, since CME+ neurons were the only cell type (other than MOV+ cells) that was abundant in both dlPAG and mPFC, it was possible to compare the time course of CME+ cell activity across the two structures. Following CS pips that elicited short-latency movement responses, the firing rates of CME+ cells in dlPAG increased after the onset of the pip, at about the same latency as the movement response evoked by the pip. It is thus possible that CME+ neurons in dlPAG were involved in motor activation to drive the CME response, in accordance with prior evidence that the dorsal PAG orchestrates active escape and avoidance behaviors (Bandler and DePaulis 1988; Fanselow 1991; De Oca et al. 1998). By contrast, firing rates of CME+ cells in mPFC were already significantly elevated prior to the onset of CS pips that evoked short-latency movement responses, and did not increase further after the onset of the pip. Hence, it was the tonic firing rates of mPFC CME+ cells (both before and after the pip), rather than their phasic responses to the pip, that appeared to differ depending whether the pip evoked a movement response. This suggests that CME+ cells in mPFC did not drive CME responses on their own (since their firing rates did not change dynamically along with motor behavior). Instead, the tonic activity of
CME+ cells in mPFC may have influenced how downstream structures, such as amygdala and PAG, mapped sensory inputs encoding the CS onto motor circuits that controlled competing defensive behaviors, thereby biasing the rat’s defensive response strategy toward the selection of CME responses and away from CMI responses during states of high predatory imminence. This interpretation accords well with theories proposing that mPFC participates in behavioral strategy selection by altering how sensory stimuli are mapped onto motor responses in downstream structures (Miller and Cohen, 2001).

Population averaged responses (Figure 4) show that CME+ cells exhibited sustained firing throughout the CS during CME but not CMI trials (which is why they were statistically classified as CME+ cells), but they also exhibited brief (<1 s) transient responses to the first pip at the onset of the CS period during both CME and CMI trials. This was true in both dlPAG (Figure 4A) and mPFC (Figure 4B). One possible explanation for this could be that CME+ cells were in competition with other neurons (such as CMI+ cells) for control of the rat’s behavior; if so, then both CME+ and CMI+ cells might have responded to the initial onset of the CS during all trials, but only continued to show sustained firing during trials where they “won” the competition for behavioral control (which, in the case of CME+ cells, would of course occur only during CME trials). Another possible interpretation could be that CME+ cells were involved in conveying a temporal difference (TD) prediction error for aversive events (see McNally et al. 2011). By definition, a TD prediction error signal is generated at moments when the rat’s expectation of the US increases. Because of this, a TD error signal responds transiently to a sudden increase in expectation of the US, but responds in a sustained manner when expectation of the US increases gradually over time. It is thus possible that during pre-shock CMI trials, the rat’s expectation of the US increased suddenly at the CS onset (thus generating a
brief TD prediction error signal), but then expectation remained constant and did not continue to increase, so that the TD error signal ended and did not persist throughout the CS. By contrast, during post-shock CME trials, the rat’s expectation of the US might have continued to ramp up throughout the CS period, resulting in a sustained TD error signal as the rat’s expectation of the US grew steadily from one moment to the next. Midbrain dopamine (DA) neurons (which are thought to signal TD errors for rewarding rather than aversive events) have similarly been observed to exhibit either transient or sustained responses to an appetitive CS, depending upon experimental conditions (Fiorillo et al., 2003). Like DA neurons, dlPAG neurons are located in the midbrain, and have been implicated in signaling prediction errors during aversive conditioning (Johansen et al., 2010). Hence, CME+ neurons in dlPAG (and possibly mPFC as well) could play some role in signaling aversive prediction errors.

**CMI+ neurons**

Prior studies have reported that 25-30% of neurons in the PL subregion of mPFC increase their firing rates during conditioned freezing responses (Burgos-Robles et al. 2009; Sotres-Bayon et al. 2012; but for conflicting results see Chang et al. 2010). Accordingly, we observed that 35% (10/29) of PL neurons belonged to one of the three cell types (CMI+, CSR+, MOV-) that increased their firing rates during the CS when it elicited movement suppression behavior (that is, during CMI trials). Neurons belonging to these three cell types would likely have been classified as ‘freezing cells’ in prior studies, where freezing was the only measure of conditioned fear. But in the present study, a comparison between CMI versus CME trials made it possible to further discern that 10% (3/29) of PL neurons were CMI+ cells that were excited by the CS only during CMI (but not CME) trials; another 10% (3/29) of PL neurons were stimulus-
selective CSR+ cells excited by the CS during both CMI and CME trials (see above), and one PL neuron was a MOV- cell that always fired during low movement speed, regardless of whether the CS was present or absent. Interestingly, only half (3/6) of the CMI+ cells we recorded in mPFC were located in PL subregion, and these were all classified as principal cells, whereas CMI+ cells recorded outside of PL (two in ACC, one in IL) were all classified as interneurons. It is difficult to draw firm conclusions from such a small sample of cells, but these findings are consistent with prior evidence that output relayed by PL projection neurons could promote freezing (Corcoran and Quirk 2007; Burgos-Robles et al., 2009; Laurent and Westbrook 2009; Senn et al. 2014), whereas inhibitory interneurons might conversely suppress ACC and IL activity during freezing. CMI+ cells did not increase their firing rates during the CS period of CME trials, so it seems unlikely that their firing rates were directly correlated with the rat’s anticipation of shock (if anything, the rat’s anticipation of shock should have been greater during CME than CMI trials). Thus, if CMI+ cells signaled anticipation of shock, they must have done so non-monotonically, firing only when the shock was weakly but not strongly anticipated. A simpler interpretation is that CMI+ firing rates were monotonically correlated with expression of CMI responses, rather than non-monotonically correlated with fear or anticipation of shock.

CMI- neurons

Another type of strategy-selective cell we observed was CMI- neurons, which were inhibited by the CS during CMI (but not CME) trials, and constituted 8% (6/71) of the neurons recorded in mPFC. Courtin at al. (2014) have reported that prefrontal cells with similar firing properties (which suppress their activity during freezing behavior) are predominantly interneurons, but only one of the CMI- cells we recorded here (located in ACC) was classified as
an interneuron, whereas the rest were classified as principal cells (all located in IL or PL). As discussed above for CMI+ cells, the fact that CMI- cell firing rates were suppressed during CMI but not CME trials implies that the suppression was correlated with the expression of CMI behaviors, rather than with fear or anticipation of shock (because shock anticipation during CME trials should have been equal or greater than during CMI trials). Hence, like CMI+ cells, CMI- cells might project to downstream targets in amygdala or PAG, where they could participate in disinhibiting behavioral circuits that promote the expression of CMI responses (or alternatively, in inhibiting circuits that promote CME responses).

**Shock-evoked responses**

Many neurons in both mPFC and dPAG exhibited short latency responses to shock pulses (Figure 6). In mPFC, a disproportionate number of shock-responsive neurons were recorded from the ACC subregion, which participates in processing nociceptive sensory information in rodents (Johansen et al., 2001). In dPAG, more than half of the neurons were shock responsive, in accordance with the fact that PAG is a major relay center that conveys ascending nociceptive information from the spinal cord to higher structures (see Willis and Westlund, 1997), and also exerts descending modulatory influences upon nociceptive processing in the spinal cord (see Basbaum and Fields, 1984). We did not find evidence that shock responsiveness was more prevalent among some cell types than others; instead, shock responsiveness was similarly prevalent among all cell types in both mPFC and dPAG. Hence, it appears that there was ample opportunity for the shock stimulus to widely and directly influence neural processing in both structures. Several cell types shifted their baseline firing rates for up to two minutes after shock delivery, in conjunction with changes in the rats’ tonic motor activity.
(Figure 7). Hence, shock delivery may have affected the activity of multiple cell types in mPFC and dlPAG, in ways that might have promoted the transition in behavioral strategy from CMI to CME responses following recent shock delivery.

Summary and conclusions

Our findings suggest that strategy-selective mPFC neurons may participate in regulating which defensive response strategy a rat chooses to perform in the presence of a fear conditioned CS, in accordance with prior theories positing that mPFC controls behavioral strategies by modulating how downstream structures map sensory stimuli onto specific motor responses (Miller and Cohen, 2001). One such downstream structures could be the amygdala, which stores memories of the association between the CS and US during fear conditioning (Davis, 1992; Maren, 2003; Johansen et al. 2011; Herry and Johansen, 2014), and may also participate in selecting appropriate defensive responses to the CS since different output pathways from the amygdala appear to mediate distinct modes of defensive responding (Killeross et al. 1997; Amorapanth et al. 2000; Gozzi et al. 2010). PAG is another downstream target of mPFC neurons that could mediate the selection of appropriate defensive responses to a threat. The ACC and PL subregions of mPFC project to the dorsal columns of PAG, whereas the PL and IL subregions project to the ventral PAG (Floyd et al. 2000). It has been proposed that the dorsal and ventral columns of PAG may compete with one another to drive CME versus CMI defenses, respectively (Bandler and DePaulis 1988; Fanselow 1991; Walker et al. 1997; De Oca et al. 1998; Vianna et al. 2001). Hence, mPFC is well positioned to mediate the outcome of competition between PAG columns, and thereby regulate which defensive behavior is expressed to a threatening stimulus. Recent evidence also suggests that inhibitory outputs from mPFC to
the ventral striatum might mediate avoidance behavior (Lee et al., 2014), so the ventral striatum is another target where mPFC might exert an influence over the mapping from threatening sensory stimuli onto specific behavioral defense responses. Further study is warranted to more deeply investigate how defensive action selection might be mediated by interactions between strategy-selective mPFC neurons, such as those recorded here, and downstream regions.

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**FIGURE LEGENDS**

**Figure 1.** Histological reconstruction of recording sites in dlPAG and mPFC. Reconstructed recording sites of dlPAG neurons (A; n=74) and mPFC neurons (B; n=71) are overlaid upon coronal templates (with coordinates in mm relative to bregma) from the atlas of Paxinos and Watson (1997). Symbol colors indicate each cell’s type classification (see Section 3) and symbol shapes indicate how the cell responded to CS pips or shock pulses (* excited by pip and
shocks, ▲ excited by shocks but not pips, ■ excited by pips but not shocks, ♦ excited by pips and inhibited by shocks, ▼ no pip response and inhibited by shocks, ● no response to pips or shocks). Left and right sides of the midline correspond to hemispheres ipsilateral (IPSI) and contralateral (CONTRA) to the eyelid where periorbital shocks were delivered.

Figure 2. Behavioral and neural activity during fear conditioning trials. A, Each session consisted of 6 CS alone trials followed by 16 CS-US pairings, so there were 7 pre-shock and 15 post-shock trials per recording session; line graphs show mean movement speeds during the CX and CS periods of Pre-versus Post-shock trials. B, Line graphs show percentage of trials (for all rats combined) that were classified as CMI, NCR, or CME per session (session 1 is the first session after 5 days of initial training); pie charts show proportions of trial types from all sessions combined. C, Movement speed (top) and turning bias (bottom) during the CX (unshaded area), CS (light gray shaded area), and US (dark gray shaded area) periods of each trial; data is averaged separately for CMI, NCR and CME trials over all sessions. D, Example of spike data from a dlPAG neuron recorded over two days; color of shading beneath spike rasters indicates the rat’s movement speed during each trial, and rate histograms (1 s bins) show the cell’s mean firing rate averaged over all CMI and CME trials.

Figure 3. Cell type classifications in dlPAG (A) and mPFC (B). A1 & B1, Summary table provides a color-coded key for the number of cells of each type; labels denote which cell types belong to the strategy, stimulus, and speed selective categories. A2 & B2, Large pie chart shows proportion of cells from each region that were classified in each type category, small pie charts show proportions of cell classifications by subregion. A3 & B3, Table shows distribution of
recording days across which cells were held (vertical axis) and of which session cells were first encountered in (horizontal axis, session #1 is the first session after 5 days of initial training); type category is indicated by symbol color, and rat is indicated by symbol shape (table at upper right summarizes total number of cells in each category by rat). A4 & B4, Distributions of waveform parameters for all cells, with symbol shapes denoting which subregion the cell was recorded from (NR cells are plotted as small dots).

Figure 4. Dominant cell types in dlPAG and mPFC neurons. A, Each panel shows population averaged firing rates (top graph) and a PSTH for one example cell (bottom graphs) for the three most prevalent cell types recorded in dlPAG: CME+ cells (n=40), CSR+ cells (n=20), and MOV+ cells (n=8). B, Population averaged firing rates (top graph) and a PSTH for one example cell (bottom graphs) for three prevalent cell types recorded in mPFC: CME+ cells (n=14), CMI+ cells (n=6), and MOV+ cells (n=15). For all graphs, bin size is 1 s and vertical gray bars indicate individual CS pips.

Figure 5. Pip-evoked responses of dlPAG and mPFC neurons. A, Short latency pip-evoked responses of CME+ neurons in dlPAG; top graph show population-averaged firing rates (n=40), bottom graphs show rasters and PSTHs during CMI and CME trials for one example of a pip-responsive CME+ cell (bin size = 2 ms; gray shaded region indicates time window of the 250 ms pip presentation). B, Same as 'A' except data is shown for the population of CSR+ cells in dlPAG (n=20, top graph), and one example of a pip-responsive CSR+ cell (bottom graphs). C, Mean movement speed calculated in 20 ms bins within a time window surrounding all freezing and flight pips (see Section 2 for definitions) during which neurons were recorded; gray shading
indicates the 250 ms pip, and dashed line marks the time at which the mean speed first exceeds
the pre-pip baseline during flight trials (z-test p<.05 of individual time points against all samples
from the 500 ms period prior to pip onset). \textbf{D}, Population-averaged responses of CME+ and
CSR+ cells in dlPAG during freezing versus flight pips (20 ms bins); colored dots (green for
freezing and orange for flight) mark time points at which the population z-score exceeded zero
(p<.05 against the mean pre-pip firing rate for freezing pips), whereas black dots mark time
points at which the z-score for flight pips exceeded that for freezing pips (p<.05). \textbf{E}, Same as 'D'
except data is shown for CME+ and CMI+ cells in mPFC; in the bottom graph, colored stars
mark time points at which the population z-score fell significantly below zero (p<.05 against the
mean pre-pip firing rate for freezing pips).

\textbf{Figure 6.} \emph{Shock-evoked responses of dlPAG and mPFC neurons. A,} Spike rasters and PSTHs
for two example cells recorded in dlPAG that were excited (left) and inhibited (right) by shock
pulses; t=0 marks onset of the 2 ms shock pulse, and gray shading indicates the ~6 ms time
window during which spike recording was occluded by stimulus artifact (see Section 2). \textbf{B,}
Same as 'A' except data is shown for two example cells recorded in mPFC.

\textbf{Figure 7.} \emph{Post-shock responses of dlPAG and mPFC neurons. A,} Mean turning velocity (top)
and movement speed (bottom) averaged over all CS-US pairings (CMI & CME trials combined)
during which neurons were recorded; blue and red shaded regions indicate the CS and US
periods, respectively, while green dots indicate time points at which the plotted value is
significantly (p<.05) above or below the mean from the CX baseline period. \textbf{B,} Population-
averaged firing rates during CS-US pairings for CME+, CSR+, MOV+ cells recorded in dlPAG.
C, Population-averaged firing rates during CS-US pairings for CME+, CMI+, CMI-, and MOV+ cells recorded in mPFC.
Table 1. “Trial type” columns show mean CX firing rates during CMI versus CME trials for main cell types recorded in dIPAG and mPFC (*p<.05 and †<.10 for two tail paired t-test comparing CMI versus CME trials). “Hemi%” columns show the percentage of cells that were classified as a given type in the brain hemisphere ipsilateral (IPSI) versus contralateral (CONTRA) from the shocked eyelid (41 IPSI and 33 CONTRA cells were recorded in dIPAG; 41 IPSI and 30 CONTRA cells were recorded in mPFC).

<table>
<thead>
<tr>
<th>Site</th>
<th>CELL TYPE</th>
<th>N</th>
<th>CMI</th>
<th>CME</th>
<th>IPSI</th>
<th>CONTRA</th>
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<tr>
<td>dIPAG</td>
<td>CME+</td>
<td>40</td>
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<td>MOV+</td>
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<td>3.6 ± 0.7</td>
<td>3.1 ± 0.7*</td>
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<td>9.1</td>
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<tr>
<td>dIPAG</td>
<td>NR</td>
<td>5</td>
<td>3.1 ± 1.3</td>
<td>2.9 ± 1.2</td>
<td>2.4</td>
<td>12.1</td>
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<tr>
<td>mPFC</td>
<td>NR</td>
<td>23</td>
<td>2.0 ± 0.7</td>
<td>1.7 ± 0.5</td>
<td>29.2</td>
<td>36.7</td>
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<tr>
<td>mPFC</td>
<td>MOV+</td>
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<td>3.5 ± 1.0</td>
<td>2.5 ± 0.8†</td>
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<tr>
<td>mPFC</td>
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<td>1.9 ± 1.0</td>
<td>2.0 ± 1.0</td>
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<td>5.7 ± 1.5</td>
<td>5.7 ± 2.6</td>
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<tr>
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<td>6.5 ± 5.7</td>
<td>0.0</td>
<td>10.0</td>
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