Predicting Aversive Events and Terminating Fear in the Mouse Anterior Cingulate Cortex during Trace Fear Conditioning

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A variety of studies have implicated the anterior cingulate cortex (ACC) in fear, including permanent storage of fear memory. Recent pharmacological and genetic studies indicate that early synaptic plasticity in the ACC may also contribute to certain forms of fear memory at early time points. However, no study has directly examined the possible changes in neuronal activity of ACC neurons in freely behaving mice during early learning. In the present study, we examined the neural responses of the ACC during trace fear conditioning. We found that ACC putative pyramidal and nonpyramidal neurons were involved in the termination of fear behavior (“un-freezing”), and the spike activity of these neurons was reduced during freezing. Some of the neurons were also found to acquire un-freezing locked activity and change their tuning. The results implicate the ACC neurons in fear learning and controlling the abolition of fear behavior. We also show that the ACC is important for making cue-related fear memory associations in the trace fear paradigm as measured with tone-evoked potentials and single-unit activity. Collectively, our findings indicate that the ACC is involved in predicting future aversive events and terminating fear during trace fear.

Introduction

Learning to predict or anticipate future events helps an organism adapt its behavior to impending dangers. Trace fear conditioning involves the association of a tone with a noxious shock stimulus over an interval of time (trace interval). Accordingly, the subject learns that the sound of a tone will predict the delivery of a shock. In the case of rodents, the expression of fear behavior (i.e., freezing) during the trace interval can represent the degree of expectation/prediction for the impending shock stimulus.

Convergent evidence from mouse studies in our laboratory has shown that the anterior cingulate cortex (ACC) is involved in trace fear conditioning (Zhao et al., 2005, 2006; Wu et al., 2008; Steenland et al., 2010). It has been reported that calcium/calmodulin-dependent protein kinase IV overexpressing mice have enhanced synaptic potentiation (long-term potentiation) in the ACC and increased acquisition of trace fear (Wu et al., 2008; Steenland et al., 2010). In addition, mice with deficits in ACC

synaptic plasticity due to either FMR1 knockout or chronic pain also have deficits in trace fear conditioning (Zhao et al., 2005, 2006). Studies in mice have shown that *c-fos* is elevated in the ACC following trace fear conditioning and that removal of the ACC impairs trace but not delay fear conditioning (Han et al., 2003). All of these studies suggest that the ACC may be involved in trace fear conditioning; however, no study has directly examined ACC neuron activity during the course of the trace fear conditioning.

Stimulation of the ACC induces vocalizations (Jürgens and Pratt, 1979; Tang et al., 2005b; Jürgens, 2009), fear behavior (Johansen and Fields, 2004; Tang et al., 2005a), and motor movements (Penfield and Welch, 1951; Showers, 1959; Hughes and Mazurkowski, 1962; Bancaud et al., 1976; Sinnamon and Galer, 1984). Additionally, a recent functional imaging study in humans has demonstrated that the subgenual ACC is involved in making courageous actions in the face of fearful events. In such a case, the ACC was found to be activated before making an approach to fearful stimuli and was positively correlated with the degree of subjectively reported fear. However, measures of physiological fear (i.e., skin responses) were found to decrease, suggesting that the ACC is involved in overcoming physiological/behavioral aspects of fear (Nili et al., 2010).

No field potentials or unit recordings of the ACC have been conducted during trace fear conditioning to evaluate when the ACC is active and to which behaviors it corresponds. We have previously developed an electromyogram (EMG)-based recording methodology (Steenland and Zhuo, 2009) whereby field potentials and unit recordings, for the first time, can be precisely

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registered to a physiological measure of freezing/fear behavior (Steenland and Zhuo, 2009; Steenland et al., 2010). We recorded neurons and field potentials in the mouse ACC during trace fear conditioning while measuring physiological fear. Our results are consistent with human studies, showing that the ACC is involved in predicting aversive future events (i.e., associations between shock and tone) and terminating fear behavior.

Materials and Methods

Animals. Experiments were performed on 27 male C57BL/6 mice (10–18 weeks old). Mice were maintained on a 12 h light/dark cycle (lights on at 7:00 A.M.), and had access to food and water *ad libitum*. Procedures conformed to the recommendations of the Canadian Council on Animal Care, and the University of Toronto Animal Care Committee approved the protocols. Eight animals were used for trace fear and field potential experiments: four for trace fear spike experiments, two for trace fear and home cage spike experiments, four for freely behaving, and nine for anesthesia experiments.

Preparation of electrodes for spike recording. Multiunit spike recording was done with tungsten electrodes (WE30030.5A3; Micro Probe). Electrodes were preloaded into a miniature four-channel microdrive. The microdrive was equipped with an electrode interface board (NeuroTek) to route signals and four independently manually operated drives, which could advance a minimum of 20 μm . The impedances of the electrode were lowered to 0.5 M Ω to match one another, to reduce 60 Hz interference. To lower the impedance of the electrode without impacting its profile, the tip of the electrode was first plated with a gold solution (Krohn 24K gold plating solution) at 100–500 μA (Model CCUI; Grass Instruments) with the negative terminal directed to the electrode connector. This layer of gold plating provided an interface for the platinum plating. The electrode was washed with water then plated with a platinum solution (VWR3905–0; VWR) at 100–500 μA . The electrode was continuously plated with platinum and washed until the impedance matched the reference electrode. All impedances were measured on an impedance tester (Model IMP2; BAK Electronics). A ground electrode consisted of a silver wire, which was wrapped around the body of the microdrive.

Freely behaving spike recording preparation. Mice were anesthetized with isoflurane delivered to the mice via nose cone throughout the surgery. Oxygen (30%) was mixed with the halothane to ensure a healthy preparation. The abdomen and scalp of the mice were shaved, and then cleaned with iodine (Triadine) and alcohol. A midline scalp incision was made to expose the skull and neck muscles. The skull of the mouse was fixed into a stereotaxic adapter (Model 51625; Harvard Apparatus) mounted on a stereotaxic frame (Model 962; Kopf). Two Teflon-coated stainless steel electrodes (AS632; Cooner Wire) were sutured to the left and right nuchal neck muscles with 4.0 silk threads. Three small holes (1.19 mm diameter) were drilled into the skull for mounting screws. An additional hole was drilled in the skull overlying the ACC (AP, 0.6–1.0 mm; ML, 0.5–0.8 mm). The silver grounding wire from the microdrive was then wrapped around three mounting screws. The dura mater overlying the ACC was then reflected, and mineral oil was placed on the exposed brain. The microdrive was lowered slowly into the brain 0.5 mm below its surface to avoid the motor cortex. A small amount of a mixture of mineral oil and bone wax was packed around the electrode penetration zone. Krazy glue and dental cement were used to secure the microdrive to the mouse skull. Mice were injected, intraoperatively (subcutaneously) with buprenorphine (0.1 mg/kg) as an analgesic, and 1.0 ml of sterile saline (intraperitoneally) for hydration. Mice were placed on a warm heating pad until they showed signs of ambulation and were permitted to recover 5–8 d before recording. Neural recordings were also obtained from anesthetized animals and freely behaving animals (without trace fear conditioning). The data from these animals are only used in this manuscript for classification of putative pyramidal and nonpyramidal neurons.

Trace fear conditioning. Trace fear conditioning was performed in an isolated shock chamber (Med Associates). The conditioned stimulus (CS) is an 80 dB white noise, delivered for 15 s, and the unconditioned stimulus (US) is a 0.75 mA-scrambled footshock for 0.5 s (Wu et al.,

2008). Mice are acclimated for 60 s, and presented with 10 CS–trace–US–ITI trials [trace of 15 s, intertrial interval (ITI) of 225 s]. One day after training, mice are acclimated for 60 s and subjected to 10 CS–ITI trials (ITI of 225 s) in a novel chamber to test for trace fear memory (Huerta et al., 2000). Freezing is typically defined as the absence of movement (based on the EMG) with the exception of breathing for at least 1 s. Conventional trace fear paradigms were performed with animals during the day, when animals normally sleep. This was previously necessary because behavioral scoring and motion detection require light to determine freezing behavior. Thus, most studies have been performed when the animal would be naturally sleeping. To circumvent this potential confound, we used neck EMG recordings to examine freezing behavior in the dark (0.2 lux) when mice are naturally awake (Steenland and Zhuo, 2009). In addition, this EMG-based scoring method of freezing is the only currently available, temporally precise method to coregister the animal's muscle actions during fear with ongoing unit or field potential activity. To further confirm the validity of this measure, two animal recordings were conducted in the light with video to verify that the recorded neck EMG corresponded to observed fear behavior.

Histology. At the completion of freely behaving animal experiments, mice were killed with isoflurane, and a DC current (100–500 μA) was passed between recording electrodes and ground to lesion the brain. The brain was then removed and fixed with 10% formalin solution. After a week, the brains were transferred to 30% sucrose, and cut in 40 μm coronal sections with a cryostat (CM 1850; Leica). Sections were mounted and observed under microscope immediately after sectioning. Some brains were also Nissl stained. Lesion sites were localized and coregistered with the stereotaxic atlas of the mouse brain (Paxinos and Franklin, 2003).

Recordings. EMG signals were amplified 2000 $\times$ and filtered at 100–1000 Hz (Super-Z headstage amplifiers; CWE). Signals were digitized, rectified, and smoothed (25 ms time constant) to measure freezing behavior (Spike2 software, 1401 interface; CED), and were recorded on a computer. Spike recording was done with a custom headstage, built from a TL064 operational amplifier package in the unity gain follower configuration (Jeantet and Cho, 2003). The headstage has four channels and is powered by a +5/G/–5 V DC split battery supply. The headstage was interfaced with BNC connectors and sent to a differential amplifier (DP304; Warner Instruments). Spike recordings were filtered between 300 and 10 kHz with 1000 $\times$ amplification. The signals were then digitized at 20 kHz with an analog-to-digital converter (1401 interface; CED). Local field potentials were recorded between 1 and 100 Hz, amplified 1000 $\times$, and sampled at 200 Hz. Video recording was performed for two animal experiments with a Webcam (Logitech), which was synchronized with the Spike2 recording software in the home cage and fear recording chamber. Video data were collected at 15 frames per second.

Data analyses. Physiological signals were analyzed in 5 s epochs for the periods of wakefulness and sleep using Spike2 software. Scripts for EEG analysis (Suda-version 2.2) were obtained from CED. Custom scripts were also written in our laboratory (by H.W.S.) for neck EMGs and spike analysis. Fast Fourier transform was used to convert EEG waveforms into total power (μV^2) and binned every 5 s for the following frequency bands: δ_1 (0.5–2 Hz), δ_2 (2–4 Hz), θ (4–7.5 Hz), β_1 (7.5–13 Hz), β_2 (13–20 Hz), and α (20–30 Hz) (Steenland et al., 2010). Neck muscle recording was quantified by integrating the area under the rectified and smoothed EMG waveform.

Spike sorting was done off-line using a template-matching program provided by Spike2 software. The threshold for analyzing spikes was set at 2 $\times$ the baseline noise level. The spike template ranged from 0.5 ms before the spike peak to 1.5 ms after the peak. Following template formation, principle component analysis was performed and the templates were then subject to revised based *k*-means clustering of the spikes. Peristimulus time histograms were generated with a bin size of 200 ms. Single units from each ensemble were normalized as *z*-scores (Tsai et al., 2004). In brief, the 5 s period before stimulation was used as a baseline period. The mean firing rate and SD of the 500 bins in this baseline period were calculated. All bin values were transformed to *z*-scores according to the mean and SD of the baseline period. A 99% confidence level ($z > 2.33$ or

$z < -2.33$) was used for identifying whether a single extracellular unit responded (Kuo and Yen, 2005).

Under *in vivo* conditions, several features of extracellular recorded spikes were recorded, including firing rate, half amplitude duration, and time from trough to peak time. Attempts were made to discriminate interneurons and pyramidal neurons based on 2D plots of half-amplitude and peak-to-peak time. Following identification of putative interneurons and pyramidal neurons, cells were further classified according to their spike patterns (bursting, regular spiking, and indeterminate). Autocorrelations were performed for each neuron. Cells were classified as bursting if the maximum peak on the auto-correlogram between 3 and 6 ms was $\geq 50\%$ of the maximum bin value of the first 50 ms (Barthó et al., 2004). The criterion for regular spiking (nonbursting) neurons was that the mode of the interspike interval histogram was >35 ms. Regular spiking neurons rarely discharged in bursts, and the auto-correlogram showed an exponential rise from time zero to tenths of a millisecond. Cells that do not match either criterion were labeled as indeterminate.

The analyses performed for each statistical test are included in the text where appropriate. For all comparisons, differences were considered significant if the null hypothesis was rejected at $p < 0.05$ using a two-tailed test. Mixed factor (one between-subject and one within-subject variable) repeated-measures ANOVA was performed and followed by *post hoc* comparisons with the Bonferroni corrected p value to infer statistical significance. Analyses were conducted with SigmaStat (SPSS), and data were plotted with SigmaPlot (Systat Software). In some cases, where statistical tests involved multiple *post hoc* comparisons, only the ANOVA is reported in text with significance indicated in the figures. Pearson product-moment correlation was used for correlation analysis.

Results

Learning-related potentials in the ACC during trace fear conditioning

Trace fear conditioning (Fig. 1A) involves the association of a tone and shock across a time interval and is thought to engage the ACC (Han et al., 2003). Since the trace fear-conditioning paradigm involves a variety of stimuli (e.g., pain and sound) and motor responding (freezing behavior) along with learning, it is an ideal paradigm to examine the function of the ACC. Neck EMG was used, as previously described (Steenland and Zhuo, 2009; Steenland et al., 2010), to determine when the mouse was freezing (Fig. 1B). As expected, a significant alteration in freezing behavior was detected during the trace interval during conditioning (10 training trials) and memory testing (24 h later) ($F_{(20,130)} = 5.1067$; $p < 0.001$, one-way ANOVA). *Post hoc* analysis revealed that the mice both learned and remembered (24 h later) the association between shock and tone (all $t > 3.45$; all $p < 0.015$; Fig. 1C).

The prelimbic cortex and lateral amygdala exhibit enhanced tone-evoked potentials with fear conditioning (Paré and Collins, 2000; Mears et al., 2009). Trace fear conditioning was conducted in parallel with local ACC field potential recordings to examine

Figure 1. EMG-based trace fear conditioning and testing. **A**, The standard trace fear paradigm, a tone, and shock are presented, separated by a time interval (trace interval). The paradigm is repeated and the mouse learns that the tone predicts the shock (as evidenced by freezing during the trace interval). Memory can be tested 24 h later, with presentation of the tone alone. If the mouse expects to be shocked, it will freeze during and after the presentation of the tone. Highlighted green segments indicate regions of analysis in **B**. **B**, Example of EMG-based freezing in one mouse during the trace fear interval. Each panel during conditioning and memory represents one trace interval trial. Green underline indicates periods scored as freezing. **C**, Grouped data showing an increase in EMG-based freezing during both learning and memory compared with 60 s baseline recorded on day one [baseline (BL)]. *Statistical significance at $p < 0.05$. Large green dots correspond to freezing behavior in **B**. Arb., Arbitrary units; Rt/Sm, rectified and smoothed signal; S, shock; ES, expected shock.

whether changes in tone-evoked potentials or spontaneous oscillation state could be detected. We found that tone-evoked potentials could be detected during trace fear conditioning in 10 of 10 ACC recordings (Fig. 2A). An increase in EMG activity was detected in only three of eight animals in response to tone stimuli (Fig. 2A, bottom). Thus, these tone-evoked potentials appeared to be related to a sensory function of the ACC rather than a motor function per se. A significant alteration in tone-evoked potentials was detected during the course of conditioning ($F_{(9,80)} = 4.27$; $p < 0.001$, one-way ANOVA). *Post hoc* analysis revealed that a robust learning curve could be detected for the tone-evoked potentials when the 50 ms time point peak (P50) was analyzed for each potential of each trial (all $t > 3.25$; all $p < 0.015$; Fig. 2B, C). This learning curve can be seen to parallel that of freezing behavior during the trace interval. Correlation analysis of these learning curves revealed a significant covariation between fear behavior and P50 values ($r^2 = 0.79$; $p < 0.001$; Fig. 2D). Tone-evoked potentials remained elevated until the end of the conditioning trials. The results suggest that the association between the shock and tone stimuli gradually enable the ACC to help predict the future occurrence of the shock.

Figure 2. Increased ACC tone-evoked potentials with fear conditioning. **A**, Demonstrates that tone-evoked potentials could be detected in the ACC during trace fear conditioning and do not necessarily require a robust motor activation. Top, An example of an average (AVG) of 10 trials during conditioning from one mouse. Middle trace is the field potential average and SEM for all mice recorded. Notice the peak around 50 ms and the trough around 150 ms. Bottom, Rectified and smoothed EMG potential average and SEM for all animals recorded. **B**, Example temperature plots of tone-evoked potentials during 10 trials of trace fear conditioning. Arrows depict P50 after commencement of tone presentation. **C**, Tone-evoked potentials (P50 values) show a significant and robust learning curve throughout conditioning that parallels freezing behavior (gray line). **D**, P50 values and freezing quantities were significantly correlated, suggesting that tone-evoked potentials can predict freezing magnitude during the trace interval 15 s after the tone-evoked potential occurred. *Statistical significance at $p < 0.05$. Arb., Arbitrary units. Gray rectangle in **A** represents z-scores for 99% confidence.

It was previously shown that local field potentials, in the 4–7.5 Hz range, can be augmented in the ACC and the prelimbic cortex during reward anticipation (Tsujimoto et al., 2006; Paz et al., 2008) and in the amygdala and hippocampus during fear behavior (Paré and Collins, 2000; Seidenbecher et al., 2003; Narayanan et al., 2007). In addition, our recent study demonstrated that 4–7.5 Hz power is robustly augmented during the trace fear conditioning in the prelimbic cortex (Steenland et al., 2010). Power spectral analysis was performed for ACC field potentials for the tone, trace, and shock intervals of trace fear conditioning (Fig. 3A–G). Examples of the Fourier transform (before frequency binning), for spontaneous field potentials from one animal during trace fear conditioning, are depicted in Figure 3A.

Spontaneous field potential oscillation power was averaged for every phase (baseline, tone, trace, and shock conditions) of trace fear conditioning. A significant alteration in 2–4 Hz power was detected during trace fear conditioning ($F_{(3,27)} = 4.49$; $p = 0.011$, one-way ANOVA). *Post hoc* analysis revealed that 2–4 Hz power was increased during the tone condition above that of baseline ($t = 2.70$; $p = 0.035$; Fig. 3C). This fits with the possibility that relevant sensory information may come to activate the ACC with conditioning. A significant alteration in 4–7.5 Hz power was also detected during trace fear conditioning ($F_{(3,27)} = 5.027$; $p = 0.007$, one-way ANOVA). *Post hoc* analysis revealed that 4–7.5 Hz power was increased during the trace interval above that of baseline ($t = 2.65$; $p < 0.04$; Fig. 3D) consistent with findings from other brain regions (Paré and Collins, 2000; Seidenbecher et al., 2003; Tsujimoto et al., 2006; Narayanan et al., 2007; Paz et al., 2008). Thus, these oscillations may be related to memory recall of fear behavior or attention to impending danger. A significant alteration in 13–20 Hz power was also detected during the trace fear interval ($F_{(3,27)} = 6.67$; $p = 0.002$; one-way ANOVA). *Post hoc* analysis revealed that 13–20 Hz power was increased during the trace fear interval above that of baseline ($t = 4.08$; $p = 0.001$; Fig. 3F). Interestingly, no changes in spontaneous EEG were detected following the shock stimuli, compared with baseline. However, our analysis was restricted to frequencies < 30 Hz, and stimulus artifacts occluded data from the exact point of stimulation. Thus, shock analysis was performed on the 15 s of data, which was collected immediately following the termination of the shock.

We next examined whether these oscillations covary with trace fear learning curves. A significant effect for the trace fear interval on 4–7.5 Hz power was detected ($F_{(10,88)} = 2.10$; $p < 0.032$, one-way ANOVA). *Post hoc* analysis revealed that a learning curve occurred for 4–7.5 Hz power ($t = 3.31$; $p = 0.013$; seventh time point; Fig. 3H). It was consistent with, though less robust than, our previous findings in the prelimbic cortex (Steenland et al., 2010). Correlation analysis also revealed that the 4–7.5 Hz learning curve covaried with the freezing learning curve ($r^2 = 0.25$; $p < 0.001$). In addition, a significant effect for the trace fear interval on 13–20 Hz power was detected ($F_{(10,88)} = 2.25$; $p < 0.022$, one-way ANOVA). *Post hoc* analysis revealed that a learning curve occurred for 13–20 Hz power ($t = 3.76$; $p = 0.003$; eighth time point; Fig. 3I). However, correlation analysis revealed that the 13–20 Hz curve did not covary with the freezing learning curve ($r^2 = 0.33$; $p < 0.083$). It should be noted that after the seventh to eighth conditioning trial that both 4–7.5 and 13–20 Hz spontaneous field potential power

Figure 3. Local field potential changes in the ACC accompany trace fear conditioning. **A**, Example Fourier transform computed for one mouse on spontaneous ACC field potentials during each phase of the trace fear paradigm. Comparisons are made directly to baseline (50–60 s of time before presentation of first tone). Clear power enhancements can be seen between 2 and 4 Hz for tone and 4 and 7.5 Hz for trace interval (arrows). A consistent increase was also seen between 13 and 20 Hz during the trace interval (magnified inset). Shock stimuli did not change field potential power. Below each power spectrum is a range of frequencies for which the power spectrum was binned for statistical analysis. **B–G**, Power spectral binned averages for all conditioning trials. The 2–4 Hz power was significantly increased for the tone interval, while the 4–7.5 and 13–20 Hz bands were enhanced for the trace fear interval. **H**, Four to 7.5 Hz power recorded during the trace interval shows a significant learning curve, though the effect is not robust and does not completely parallel freezing behavior (gray line). **I**, Thirteen to 20 Hz power recorded during the trace interval shows a significant learning curve, though the effect is not robust and does not completely parallel freezing behavior (gray line). **J**, The changes in 4–7.5 Hz power was strongly correlated with changes in 13–20 Hz power. *Statistical significance at $p < 0.05$ compared with baseline (BL).

Figure 4. EMG-locked ACC field potentials. Demonstrates EMG-triggered potentials during trace fear conditioning, which appear to precede motor activation. Top, Example of EMG-triggered field potentials averaged (AVG) across 10 conditioning trials from one animal. Middle, Field potential average and SEM for all animals recorded. Notice the peak activation around 70 ms. Bottom, Rectified and smoothed EMG potential average and SEM for all animals recorded. Arb., Arbitrary units. Gray rectangle represents z-scores for 99% confidence.

decreased to the preconditioning baseline level, while fear behavior was still elevated. Thus, these potentials only correlate with freezing behavior for part of the conditioning. Interestingly, the 4–7.5 and 13–20 Hz power learning curves mirrored one another, and analysis revealed a robust correlation between them ($r^2 = 0.62$; $p < 0.007$; Fig. 3J). Based on these results, we expect that the enhanced cortical 4–7.5 and 13–20 Hz rhythms may impact ongoing neural activity patterns during freezing behavior but do not appear to predict freezing behavior as well as tone-evoked potentials.

Un-freezing related potentials

When animals are trained during trace fear conditioning, they go through many periods of spontaneous freezing, which are eventually followed by spontaneous movement. These periods occur during intertrial intervals and during the conditioning trials. The execution of movement from a point of freezing (un-freezing) behavior may be an attempt to explore the safety of the environment or even to escape from that environment. We examined local field potentials at a point where freezing (lasting at least 1 s) evolves into movement. To do this, local field potentials were back-triggered and averaged from the onset of EMG activation. Figure 4, top) shows an example of an average of 10 back-triggered potentials for one animal from the time of freezing to the time of movement. Grouped data are depicted (Fig. 4, middle) in register with changes in neck EMG activity (Fig. 4, bottom). In 8 of 10 field potential recordings, we observed a significant upward deflection, which peaked between 50 and 70 ms after the movement had commenced. The waveform returned to baseline even while the EMG remained elevated. Interestingly, based on the average waveforms of all data, the spontaneous EMG-related potential started to become elevated ~25 ms before

motor movement, demonstrating that the ACC potentials may have a premotor component related to un-freezing.

Identifying putative pyramidal and nonpyramidal neurons in the ACC

The results from the field potential studies implicate the ACC in processing cues, which predict fear behavior and possibly processing during the trace fear interval. To examine the neural correlates, which might account for alterations in field potentials, extracellular multiunit recordings were conducted. However, before the analysis of neural correlates, multiunit records needed to be sorted and neurons needed to be classified. Neurons from all *in vivo* experiments (see Materials and Methods) were sorted (Fig. 5A) and identified as putative pyramidal and nonpyramidal, based on their individual extracellular spike waveforms. A previous report in rats demonstrated that neurons, which have peak-to-peak values <0.5 ms, cluster together, and can be considered to be nonpyramidal neurons, and those above this threshold can be considered to be pyramidal neurons (Barthó et al., 2004). Consistent with this idea, peak-to-peak time provided the most obvious clustering (visually observable separation), while half-width did not show obvious clustering for both freely behaving and anesthetized conditions (Fig. 5B). The division of neuron clusters appeared around 0.5 ms peak-to-peak time and was confirmed when neuron counts were plotted against peak-to-peak time (Fig. 5C). This plot yielded a bimodal distribution confirming a division between putative pyramidal and nonpyramidal neurons. The bimodal distribution was not robust, but at least permitted some degree of separation of the two cell populations. Thus, based on our analysis and that of others (Barthó et al., 2004), putative pyramidal and nonpyramidal neurons could be identified by the peak-to-peak time of each spike waveform. For the purposes of the current experiments, neurons with peak-to-peak times <0.5 ms were considered putative nonpyramidal (interneurons) and those above this value were considered putative pyramidal neurons. To further characterize neurons, the bursting behavior versus regular spiking behavior was also identified for every cell using autocorrelation analysis (as described in Materials and Methods).

Un-freezing neurons in the ACC correlate with the termination of fear behavior

Our field potential data indicate that the ACC could be involved in sensory and motor responses during trace fear conditioning. Thus, we examined whether neural responses of the ACC were related to freezing behavior, tone, trace, and shock intervals. Every neuron was tested in response to four tones (i.e., four trials) and four shocks with each animal trained for up to 12 trials on 1 d. In between sets of training trials, animals were permitted to rest (~1–2 h), while new cells were manually isolated with small advancements of electrodes using a microdrive. In addition, two animals were trained for 12 additional trials on a second day. Throughout all experiments, EMGs were simultaneously monitored for freezing behavior.

Figure 6 shows an example of a putative pyramidal neuron (Fig. 6A) with indeterminate bursting properties (Fig. 6B). This particular neuron increased its activity after shock stimulation (Fig. 6C). However, the activation of this neuron also tended to mirror changes in neck muscle movements (Fig. 6C, bottom). Thus, we decided to examine the activity of this neuron closer. Figure 6D shows a single period of freezing followed by a period of movement in which the neuron's activity increased, corresponding with the neck muscle movement (Fig. 6D, bottom). Figure 6E shows averaged data of 18 such EMG movements in

which the spike activity was highly correlated. On average, the neuron ramped up its activity, starting 3.2 s before the motor movement, with activity peaking 200 ms just before motor movement. A significant correlation ($r^2 = 0.31$; $p < 0.001$; Fig. 6F) was detected between the EMG amplitudes and the spike rate of this particular neuron. We termed these cells “un-freezing neurons” since they appear to terminate freezing behavior. Figure 7 shows grouped data from all of the neurons recorded for trace fear experiments, which were analyzed in the same fashion as the neuron in Figure 6, A and B. Most neurons demonstrated un-freezing behavior (67.8%), followed by responding to shock (62.7%) and tone stimuli (47.5%), and finally the trace interval (42.4%). Of the proportion of motor-related neurons, a greater number had un-freezing behavior (41 of 43 neurons, 95.3%) compared with neurons that became active during the motor movement (2 of 43 neurons, 4.7%; $\chi^2 = 24.0$; $p < 0.001$). In combination with the observed EMG-triggered field potentials (Fig. 4), the data strongly suggest that the ACC has a prominent premotor function in the mouse. It is important to note that for all four animals recorded, each animal had at least 50% of neurons, which demonstrated premotor behavior. Putative pyramidal and nonpyramidal neurons were found to increase their activity by an average of 3.5 ± 0.4 and 3.1 ± 0.7 s, respectively, before un-freezing. Figure 7 shows the distributions of neurons demonstrating correlation or noncorrelation with un-freezing behavior. The mode of the correlation coefficient values for the distribution of un-freezing cells ranged from 0.5 to 0.6 (Fig. 7C). This suggests that there is still considerable variation in neuron firing, which may be accounted for by other processes (e.g., affect, sensory processing). Consistently, our tone-evoked potential data indicate that the ACC may be involved in processing tone–pain associations (Fig. 3C).

Dissecting of tone, trace, and shock responsive neurons from motor activation

There is considerable overlap between neurons that are active during un-freezing behavior and neurons activated during other phases of the trace fear paradigm (Fig. 7A,B). Since un-freezing neurons may also change their activity during the tone and trace intervals (Fig. 7, left), these presumed interval activations may actually be un-freezing behavior. To dissect out this possibility, we examined whether motor movements occurring during these intervals could account for changes in spike rates. We examined each bin of spike activity for each interval (e.g., tone) of the con-

Figure 5. *In vivo* ACC spike recording analysis. Multichannel spike recording was conducted in the mouse ACC under freely behaving and anesthesia conditions. **A**, Top, Multiunit recording from a single electrode. Bottom three panels, Principal component analysis (PCA) was used to sort the multiunit record into individual units. Once sorted, the waveforms can be averaged to yield values of the waveform half-width and the peak-to-peak time. **B**, Scatter plot of all peak-to-peak times and half-widths for each individual neuron recorded during freely behaving conditions and anesthesia. Neurons appeared to form clusters above and below 0.5 ms peak-to-peak time, consistent with others (Barthó et al., 2004). **C**, Plot of peak-to-peak time by the number of neurons. The plot appears to have some bimodality and is justification for the separation of putative pyramidal and nonpyramidal neurons.

ditioning paradigm. If neurons fired significantly (as judged by z -score) within 1 s of a significant EMG activation, they were classified as related to motor activation. The remaining neurons will be a conservative estimate of cells that are independent of motor activation. Figure 8A shows the number of neurons that were capable of demonstrating independent activation from motor movement. The results confirm that subsets of neurons in the ACC can change their firing rate in response to different phases of trace fear conditioning without also being related to un-freezing. The timing properties of these neurons were further examined. However, two-way between-subjects ANOVA did not reveal a significant main effect for the latency to onset of cell firing among tone, trace, and shock conditions ($F_{(2,38)} = 1.86$; $p < 0.169$) or between putative pyramidal and nonpyramidal neurons ($F_{(1,38)} =$

Figure 6. ACC neurons demonstrate un-freezing behavior. **A**, Example of spike-sorted putative pyramidal neuron. **B**, Autocorrelation demonstrating this neuron is of the indeterminate type. **C**, Top, This neuron became active prominently after the application of the shock stimuli. Bottom, Reduction in EMG activity during tone and trace intervals. EMG activity dramatically raises after the shock stimuli coincident with the neural activation. Thus, it appears that this cell not only responds to pain but may be involved in motor behavior. **D**, Example of single-unit activity during freezing behavior and during spontaneous neck muscle activation. **E**, Top two panels show trial-by-trial activations (temperature plot) and the average of this neuron during freezing and during spontaneous neck muscle activation. Bottom two panels show trial-by-trial activations (temperature plot) and average of neck EMG. Notice the neuron develops premotor activation and its peak activity precedes the motor movement (arrow). **F**, Correlation analysis revealed a significant relationship between spontaneous motor movement and neuron firing frequency. Green-colored bins represent significant responses above baseline (excitatory) based on z -score for 99% confidence (gray bar is confidence). Yellow-colored bins represent significant responses below baseline (inhibitory) based on a z -score for 99% confidence.

1.86; $p < 0.312$). Based on our conservative estimate, it is likely that neurons in the ACC can process information during the various conditions of the trace fear paradigm, independent of un-freezing behavior.

Putative pyramidal neuron inhibition during the trace interval

We found that there was an increase in 4–7.5 Hz potentials in the ACC concomitant with a rise in 13–20 Hz power during the trace

Figure 7. ACC neurons respond to trace fear conditioning and demonstrate un-freezing activation. **A**, Neuron responses to particular phases of the trace fear paradigm and motor-related activation. Excitatory responses are represented by green boxes, while inhibitory responses are represented by yellow. If both excitatory and inhibitory responses happened during a particular phase the box is a yellow-green intermediate. It is easy to see that the vast majority of recorded neurons demonstrate premotor activity. In addition, regular spiking neurons are often inhibited during trace conditioning. **B**, Example of binned neural responses to the different phases of the trace fear paradigm and neural activations back-triggered from the neck EMG. Each phase occurs at the time points between the vertical lines. **C**, Proportions of neurons that were significantly or not significantly correlated with motor movements. BL, Baseline.

Figure 8. Independence from un-freezing, reduced cell firing during freezing and motor learning. **A**, The proportions of neurons that demonstrated selective changes in activity during different phases of trace fear conditioning and were not related to un-freezing behavior. The average onset of inhibition or excitation is represented as a number within each bar in seconds. **B**, When all putative pyramidal neurons (excluding bursting cells) are averaged, there is a decrease in firing during the trace fear interval without any change in nonpyramidal neuron firing. Nonpyramidal neurons show higher rates of firing than pyramidal neurons. **C**, Temperature plot (2.0 spikes/bin = red) for an example neuron, which developed premotor-related activity across four conditioning trials (for the second block of conditioning, trials 4–8). Twenty-four sweeps of data were taken during periods of spontaneous freezing behavior, regardless of training trial. **D**, Percentage of all neurons that became locked to EMG activity. **E**, Temperature plot (7.0 spikes/bin = red) for an example neuron, which changed its tuning to EMG activity across four conditioning trials (for the first block of conditioning). Twenty-four sweeps of data were taken during periods of spontaneous freezing behavior, regardless of training trial. **F**, Percentage of all neurons that changed their tuning to EMG activity. BL, Baseline. *Statistical significance at $p < 0.05$.

fear interval. The change in these oscillations may be reflected at the neuron level as well. Thus, we investigated whether neuron types were differentially activated across the different phases of fear conditioning. The rate of recording responses during conditioning did not depend on whether pyramidal neurons or nonpyramidal neurons were recorded, with 40 of 47 (85.1%) responsive pyramidal neurons and 10 of 12 (83.3%) responsive nonpyramidal neurons ($\chi^2 = 0.09$; $p = 0.766$). Examination of

inhibitory and excitatory responses shows that regular spiking pyramidal neurons were significantly more (6 of 10 = 60%) inhibited during trace fear conditioning as compared with indeterminate cells (3 of 34 = 8.8%; $\chi^2 = 9.49$; $p = 0.002$). However, close inspection of these results shows that this inhibition could occur during the tone, trace, or shock intervals. We next examined whether pyramidal neurons were generally excited or generally inhibited compared with nonpyramidal neurons across the different phases of trace fear conditioning (Fig. 8B). The average of each neuron for each conditioning phase was computed (baseline, tone, trace interval, and shock). Bursting neurons were excluded from the analysis to keep the comparison symmetric (for nonpyramidal vs pyramidal neurons). A significant main effect of neuron type ($F_{(1,54)} = 4.67$; $p < 0.035$, two-way repeated-measures ANOVA) and conditioning phase was found ($F_{(3,162)} = 4.67$; $p < 0.035$, two-way repeated-measures ANOVA). *Post hoc* analysis revealed that, during the trace interval, pyramidal neurons significantly reduced their activity compared with baseline ($t = 2.89$; $p = 0.026$). Since putative pyramidal neuron activity did not decrease concomitant with an increase in putative nonpyramidal neuron activity, it is possible that some of the putative nonpyramidal neurons may have been misclassified. However, in contrast to this possibility, our nonpyramidal neurons had significantly higher firing rates ($t = 2.87$; $p = 0.035$; Fig. 8B) compared with putative pyramidal neurons. Based on these findings, it is more likely that the change in 4–7.5 and 13–20 Hz power and the reduced pyramidal activity, during the trace fear interval, reflect the removal of excitatory afferent input rather than local inhibition. These findings likely rule out the possibility that the ACC produces a short-term memory representation or attention during the trace interval. Rather, the decrease in ACC activity seems to reflect freezing behavior, and any increase in activity appears to be related to the termination of freezing.

ACC neurons learn un-freezing behavior

Since the largest proportion of neurons recorded corresponded with un-freezing activity, we examined whether neurons gradually develop this activity (Fig. 8C,D) and whether they alter their tuning with the EMG during conditioning (Fig. 8E,F). Figure 8D shows the percentage of neurons recorded that demonstrated signs of learning un-freezing behavior. The proportions of learning to nonlearning neurons were similar ($\chi^2 = 2.67$; $p = 0.263$). Neurons may also change their tuning to motor behavior. Figure 8F shows the percentage of neurons recorded that did and

did not change their tuning to motor movements. The proportion of tuning to nontuning neurons were similar ($\chi^2 = 0.016$; $p = 0.992$). Collectively, the results suggest that a variety of neuron types in the ACC are capable of developing un-freezing activity and changing their tuning to motor behavior throughout the course of conditioning.

Un-freezing EMG consists of head movements

Although measurements of neck EMG activity provide excellent temporal resolution for when a motor movement of the head occurs, they do not provide an intuitive visual tool to evaluate the behavior of the mouse. In two additional animals, chronically implanted with EMG electrodes and microdrives, we compared the neck EMG record with that of a video recording in both the trace fear recording and the home cage environment. In both environments, neck EMG activity was perfectly correlated with nearly every video-monitored behavior and was silent only when the animal ceased movement. Figure 9A shows the typical behavior of a mouse during trace fear conditioning. In Figure 9, the mouse is seen in the video to lift his head toward the wall of the shock chamber after a period of freezing. The movement from the video file was precisely in frame with the animal's EMG activity. Moreover, neuron activity can be seen correlating with this movement, confirming our earlier findings (Fig. 6).

Two of our previous studies have used EMG-based freezing methods to demonstrate that this measure can be used to precisely time motor outflow with electrophysiological brain measures (Steenland and Zhuo, 2009; Steenland et al., 2010). Indeed, we previously showed that EMG-based freezing measures are 88% similar to automated-camera-based measures and analyses (Steenland and Zhuo, 2009). In addition, using the EMG-based method, we were able to reproduce the enhanced freezing phenotype of CaMKIV mice (Steenland et al., 2010), which was originally discovered with an automated-camera method (Wu et al., 2008). To further verify the EMG-based freezing method, we compared a video score of freezing time to an EMG-based score of freezing time for every 15 s of trace fear conditioning in two animals. Freezing behavior as measured by neck EMG and the video was constructed and scored independently. Figure 9B shows, with a sample of 124 events, that EMG-based freezing scores account for 94% ($p < 0.001$) of the variability seen in the video-based score. The remaining 6% appears to be related to experimenter variability, since co-replay of the video behavior with the EMG activity appeared to be almost perfectly synchronized.

Figure 9. Visual confirmation of EMG-based freezing and selectivity of ACC neurons to un-freezing. **A**, Example of parallel video, neck EMG, and unit recording during the freezing to un-freezing transition. Video frames have been traced over to improve clarity. Freezing behavior (blue) transitions into a head raise (purple) and is followed by an inspection of the shock chamber wall (red; shock chamber wall not shown). Unit activity from an individual neuron is synchronized to the behavior. The signal is rectified and smoothed (Rt/Sm). **B**, Correlation of video- and EMG-based freezing scores. Data are combined from two animals for one training trial of freezing. **C**, Three categories of un-freezing behaviors. Simple head movements (mvt) are the most frequently observed. Behaviors appear consistent with and effort to escape. **D**, Left, Temperature plot (4.0 spikes/bin = red) of neurons, which are selectively active during un-freezing motor movements but not active during home cage motor movements. Right, Home cage motor activity was compared with a preceding period of quiet wakefulness.

In the trace fear conditioning environment, three major behaviors could be observed, which we classify as un-freezing. These behaviors consist of simple head movement (up, left, right), head movements toward the edges of the cage, and head movements followed by locomotion in the shock chamber. Figure 9C shows that the predominant behavior was a simple head movement. Video-recorded movements in the home cage were also well correlated with neck muscle activity (data not shown). The respective movements consisted of grooming, eating, lifting the head, and locomotion.

ACC neurons are selective for un-freezing compared with other motor behaviors

Since ACC neurons appear to learn the association with un-freezing behavior, with continued fear training these neurons are not likely to be just generic motor cells. However, a significant population of neurons did not appear to develop this association (Fig. 8*D*), indicating that they may be involved in more than one motor behavior. To investigate whether neurons involved in un-freezing behavior could be involved in other motor activities, we examined the relationship of the ACC neuron activity to the EMG activity in both the home cage environment and the trace fear-conditioning environment in two animals. Figure 9*D* shows an example of an indeterminate neuron that was related to un-freezing behavior in the trace fear conditioning environment, but was not related to motor behavior in the home cage environment. Of the 23 neurons recorded in these two animals, 26.0% were exclusively related to un-freezing behavior, 8.7% were exclusively related to movements in the home cage, 43.5% were related to movements in both environments, and 21.8% did not appear to be related to motor movements in either environment. These results argue that there are neurons in the ACC that are selective to un-freezing motor movements. These findings also support the notion that the ACC is multifunctional in its motor behavior. The latter finding is consistent with the reported role of the ACC in motor processing related to reward (Koyama et al., 2001; Shidara et al., 2005).

Recording sites

All recording sites were verified following completion of trace fear experiments. Lesion sites could be easily found throughout the ACC (Fig. 10*A*). Coordinates of lesions were matched to a standard template (Paxinos and Franklin, 2003) of the mouse brain for both spike recording (Fig. 10*A, B*) and local field potential studies (Fig. 10*C*). Since the ACC of the mouse is relatively small (1–1.5 mm DV), we do not make specific claims about which layers the neurons were located in or speculate about the layers generating specific field potentials. Nonetheless, it will be helpful for future investigations to delineate the downstream effectors of specific ACC locations to examine how these regions might be tied to other effectors controlling un-freezing behavior.

Discussion

The current study examined the role of the ACC in the trace fear conditioning paradigm. Our data demonstrate that the ACC is involved in sensing or learning about pain-related information and involved in un-freezing motor movements. We suggest that the ACC is one of the brain's major systems used to integrate predictions of aversive stimuli with the appropriate motor behavior.

Tone-evoked potentials become enhanced with fear conditioning in the prelimbic cortex (Mears et al., 2009). Similarly, visually evoked signals are enhanced in aversive conditioning in the human ACC (Büchel et al., 1998). Consistently, we find that the tone-evoked potentials are robustly enhanced with increased conditioning and parallel the behavioral learning curve remarkably well. These potentials are not locked to motor behaviors, suggesting that they are related to sensory or affective processing. Moreover, it was commonly observed that neurons were activated with the presentation of the tone stimulus. Thus, the ACC seems to be important for the ability of an animal to remain receptive to biologically relevant information (e.g., tone), which signals the future occurrence of an aversive stimulus (e.g., shock). Whether this is a mediator to focus attention to the impending

Figure 10. Histological verification of recording sites in the ACC. *A*, Nissl stain and the effect of passing current across four electrodes in the ACC (four round lesions clustered together). *B*, Each line represents the locations of two to four spike electrodes in the ACC from six mice during trace fear conditioning. Electrodes were found in two different coronal sections of the ACC. *C*, Each line represents the deepest location of a bipolar electrode in the ACC from eight mice (tips had a 0.5 mm off-set) for field potential recording. CG, Cingulate; PL, prelimbic; CC, corpus callosum; M, motor cortex.

danger or retrieve memory of previous aversive events remains to be discovered.

Lesions to the ACC block trace fear conditioning, but do not block delay fear conditioning in mice (Han et al., 2003). However, the ACC was shown to be equally active in delay fear conditioning as it was in trace fear conditioning in humans (Knight et al., 2004). Our results show that the activity of ACC neurons is reduced during the trace interval. These deactivations tended to occur in pyramidal cells, with the highest percentage occurring in

regular spiking neurons. However, when motor movements were observed during the tone or trace interval (un-freezing), they tended to be robust and correlated with neuron firing frequency. Thus, it is possible that reducing the activity of ACC neurons during periods of freezing may permit these neurons to rebound into a firing mode associated with motor movements. This would account for why many neurons demonstrate trace interval activations (usually with motor movements) while the population averages show a net decrease in pyramidal activity during these periods. Our findings support the notion that the ACC is not involved in attention processes during the trace interval. Rather, this niche may be filled by the prelimbic cortex, which demonstrates elevated activity during the trace interval of trace fear conditioning (Baeg et al., 2001; Gilmartin and McEchron, 2005). This concept is corroborated by the finding that reversible inactivation of the prelimbic cortex blocks trace but not delay fear conditioning (Gilmartin and Helmstetter, 2010).

While we found a decrease in ACC neuron activity during the trace interval, other studies show that *c-fos* is elevated selectively in the ACC by trace fear (Han et al., 2003). In addition, a recent report showed that blocking or lesioning the ACC prevents cued delay fear acquisition (Bissière et al., 2008). The major difference between those studies and the current one is that our unit and field potential recording have excellent temporal resolution, while lesions, pharmacology, and immediate early gene studies do not. It should be emphasized that the ACC tone-evoked potentials were augmented in a learning-dependent fashion. This suggested that the ACC is permitting information flow through its network. Thus, previous studies may have blocked tone-evoked potentials or measured increases in *c-fos* related to these potentials. Experiments need to be conducted to selectively turn off neuron activity during tone-evoked potentials to examine whether these potentials have any influence over behavior or are epiphenomena of an already sensitized network.

We also characterized EMG un-freezing in parallel with video monitoring to examine what behaviors this EMG activity would correspond to. The data suggest that this behavior may be related to trying to find a method to escape the operant shock chamber. This is consistent with a recent human imaging study (Nili et al., 2010), which shows that the ACC may be involved in overcoming fear (i.e., courage). Since we do not give the mouse an opportunity to escape the operant chamber, the animal may only execute a small part of an escape motor plan (e.g., a lifting of the head or movements to inspect the confines of the operant chamber). Moreover, since un-freezing behavior occurs throughout the whole training paradigm. We think it is likely that the neural circuits controlling un-freezing behavior may be used across different fear paradigms. This result is consistent with the recent observation that the ACC is involved in modulation of the acquisition of basic cued fear (Bissière et al., 2008).

After doing an extra set of experiments in two animals, we found that only 26% of the recorded un-freezing units were exclusively related to the trace fear paradigm compared with the home cage motor movements. Indeed, many neurons showed motor-related activity in both the home cage and trace fear paradigm (43.5%). The results argue for the involvement of ACC neurons in basic motor movements and more specialized movements related to the termination of freezing.

The seemingly multimodal behavior of neurons, overlapping with motor movements and pain, makes sense if an animal is to avoid pain in the future through motor behavior, and fits nicely with recent predictions for the overlap of pain and movement systems (Dum et al., 2009) and the involvement of the ACC in

courageous behavior (Nili et al., 2010). However, it is surprising that ACC neuron activity would correlate with motor behavior even when the animal is not experiencing pain (i.e., during the trace fear interval). One explanation for this is that the ACC learns information important for escape from painful situations. Consistently, we found that a significant number of neurons developed premotor activity and changed their tuning throughout conditioning, implicating their sensitivity to the conditioning paradigm. Moreover, if the ACC is involved in an affective (Devinsky et al., 1995; Rainville et al., 1997) or a motivational component to escape aversive stimuli (Gabriel et al., 1991a,b; Freeman et al., 1996; Kubota et al., 1996; Koyama et al., 1998, 2000, 2001; Takenouchi et al., 1999; Johansen and Fields, 2004; Ding et al., 2008), premotor activation may reflect these processes, rather than direct motor activations per se. Our working model is that ACC selectively learns the relationship between the shock and tone, and thus helps assess the degree of danger and whether it is escapable. If freezing behavior is appropriate, then the ACC reduces its output permitting the amygdala, hippocampus, and prelimbic cortex to maintain freezing behavior during the trace interval or until escape is possible. Should escape be perceived as possible or worthwhile, the ACC may become active to terminate fear behavior (un-freezing) and engage appropriate motor systems involved in behavioral escape.

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