Research Articles: Systems/Circuits

Neural correlates of fear in the periaqueductal gray.

Thomas C. Watson1,2, Nadia L. Cerminara1, Bridget M. Lumb1 and Richard Apps1

1 School of Physiology, Pharmacology and Neuroscience, Biomedical Sciences Building, University of Bristol, University Walk, Bristol BS8 1TD, UK
2 Neuroscience Paris Seine, Cerebellum, Navigation & Memory Team, F-75005, Paris France (i) Sorbonne Universities, UPMC University of Paris 06, UMR-S 8246; (ii) INSERM, UMR-S 1130; (iii) CNRS, UMR 8246.

DOI: 10.1523/JNEUROSCI.1100-16.2016

Received: 31 March 2016
Revised: 11 October 2016
Accepted: 19 October 2016
Published: 9 November 2016

Author contributions: T.C.W. and R.A. designed research; T.C.W. and N.L.C. performed research; T.C.W. and R.A. analyzed data; T.C.W., N.L.C., B.L., and R.A. wrote the paper.

Conflict of Interest: The authors declare no competing financial interests.

We gratefully acknowledge the financial support of the Biotechnology and Biological Sciences Research Council UK (BB/M019616/1), the Medical Research Council (G1100626), and The Physiological Society. We thank Rachel Bissett for her excellent technical assistance. We also thank Clement Lena and Daniela Popa for helpful discussion of the manuscript.

Corresponding author contact: Thomas.watson203@gmail.com

Cite as: J. Neurosci 2016; 10.1523/JNEUROSCI.1100-16.2016

Alerts: Sign up at www.jneurosci.org/cgi/alerts to receive customized email alerts when the fully formatted version of this article is published.
Neural correlates of fear in the periaqueductal gray.

Authors:
Thomas C. Watson¹,², Nadia L Cerminara¹, Bridget M. Lumb¹ and Richard Apps¹

Affiliations:
¹School of Physiology, Pharmacology and Neuroscience, Biomedical Sciences Building,
University of Bristol, University Walk, Bristol BS8 1TD, UK
²Neuroscience Paris Seine, Cerebellum, Navigation & Memory Team, F-75005, Paris France
(i) Sorbonne Universities, UPMC University of Paris 06, UMR-S 8246; (ii) INSERM, UMR-S 1130; (iii) CNRS, UMR 8246.

8 Figures; Words in Abstract: 245; Introduction: 483; Discussion: 1500

Corresponding author contact: Thomas.watson203@gmail.com

Conflict of Interest: The authors declare no competing financial interests.

Acknowledgements
We gratefully acknowledge the financial support of the Biotechnology and Biological Sciences Research Council UK (BB/M019616/1), the Medical Research Council (G1100626), and The Physiological Society. We thank Rachel Bissett for her excellent technical assistance. We also thank Clement Lena and Daniela Popa for helpful discussion of the manuscript.

Abstract
The dorsal and ventral periaqueductal gray (PAG) are embedded in distinct survival networks that co-ordinate, respectively, innate and conditioned fear-evoked freezing. However, the information encoded by the PAG during these survival behaviors is poorly understood. Recordings in the dorsal and ventral PAG in rats revealed differences in neuronal activity associated with the two behaviors. During innate fear, neuronal responses were significantly greater in the dorsal as compared to the ventral PAG. Following
associative fear conditioning, and during early extinction, when freezing was maximal, a field potential was evoked in the PAG by the auditory fear conditioned stimulus (CS). With repeated presentations of the unreinforced CS animals displayed progressively less freezing accompanied by a reduction in event-related field potential amplitude. During early extinction the majority of dorsal and ventral PAG units increased their firing frequency but spike-triggered averaging showed that only ventral activity during the presentation of the CS was significantly coupled to EMG-related freezing behavior. This PAG-EMG coupling was only present for the onset of freezing activity during the CS in early extinction. During late extinction, a sub-population of units in the ventral and dorsal PAG continued to show CS-evoked responses i.e. were extinction resistant. Overall, these findings support roles for the dorsal PAG in innate and conditioned fear and for the ventral PAG in initiating but not maintaining the drive to muscles to generate conditioned freezing. The existence of extinction-susceptible and extinction-resistant cells also suggests the PAG plays a role in encoding fear memories.

Significance Statement

The periaqueductal gray (PAG) orchestrates survival behaviors; with dorsal and ventral PAG concerned respectively with innate and learnt fear responses. We recorded neural activity from dorsal and ventral PAG in rats during the expression of innate fear and extinction of learnt freezing. Cells in dorsal PAG responded more robustly during innate fear but dorsal and ventral PAG both encoded the time of the conditioned stimulus during early extinction and displayed extinction sensitive and resistant characteristics. Only ventral PAG discharge was correlated to muscle activity but this was limited to the onset of conditioned freezing. The data suggest the roles of dorsal and ventral PAG in fear behavior are more complex than previously thought, including a potential role in fear memory.

Introduction

The execution of defensive responses to fearful, often life-threatening, stimuli is fundamental to survival (e.g. Fanselow, 1994). Such behavioral responses are complex and encompass active (engagement) and passive (disengagement) strategies including fight/flight and freezing respectively, which can be innate or learned (conditioned, e.g. Zhang et al., 1990; Bandler et al., 2000; Wang et al., 2015).
The neural network components for innate and conditioned fear include, but are not limited
to, the prefrontal cortex, hippocampus, amygdala, hypothalamus and different sub-divisions
of the periaqueductal grey (PAG, e.g. Furlong et al., 2016; Toyote et al., 2015). It is generally
thought that the PAG is downstream of the amygdala and prefrontal cortex, generating the
appropriate defensive response to fearful or threatening events (Toyote et al., 2016). The
networks that co-ordinate innate behaviors include dorsal regions of the PAG (dorsal PAG;
including dorsal, dorsolateral and lateral columns; Fanselow et al. 1991; Bandler and
Depaulis, 1988; Walker and Davis, 1997; De Oca et al., 1998; Vianna et al., 2001a), while
those that orchestrate learned behaviors include the ventral PAG (notably its ventrolateral
column; Bandler and Shipley, 1994; Bandler et al., 2000; Helmstetter et al., 2008; Vianna et
al., 2001b). Depending on context (e.g. proximity of threat), both innate and learned
defense behaviors include freezing (Bowen et al., 2013; Koutsikou et al., 2014).

Neuronal recordings in awake animals have provided important insights into the roles of
high-order components of survival networks in defense behaviors (e.g. the amygdala;
Muramoto et al., 1993; Quirk et al., 1995; Herry et al., 2008; Duvardi et al., 2011). However,
only limited information has been derived from equivalent neuronal recordings in the PAG
during freezing behavior evoked by innate or conditioned stimuli.

To date, studies have focused on PAG activity in relation to expectancy of an unconditioned
stimulus (Johansen et al., 2010), or retrieval of a conditioned response (Halladay and Blair,
2015; Toyote et al., 2016). Additionally, the extent of neural coupling with motor behavior,
and the differences between dorsal and ventral PAG in relation to extinction of conditioned
fear, are unknown. This is an important gap in understanding because extinction is a key
feature of associative learning, enabling an animal to adapt to a changing environment
(Bouton, 2004). Also, deficits in the normal extinction learning process are thought to
underlie anxiety disorders (Milad et al., 2008).

In light of this lack of knowledge, we combined neuronal recordings (field potential and
single unit activity) in the dorsal and ventral PAG, before, during and after the behavioral
changes evoked by exposure to innate or conditioned fear stimuli. Simultaneous monitoring of neck muscle EMG also allowed us to relate the timing of changes in motor output associated with freezing behavior to the patterns of neural activity (Steenland and Zhuo, 2009). This allowed us to interrogate the roles of dorsal and ventral PAG in relation to motor activity associated with innate fear, and extinction of a conditioned fear stimulus.

**Materials and Methods**

**Implant procedures**

All animal procedures were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and approved by the University of Bristol Animal Welfare and Ethical Review Body. A total of 17 adult male Wistar rats (300–400 g; Charles River and Harlan, UK) were used in this study. They were housed under normal environmental conditions (20°C and 45–65% humidity), on a 12 h dark–light cycle and provided with food and water *ad libitum*.

Rats were anesthetized either with a mixture of isoflurane and O₂ (10 rats) or by intraperitoneal injection with ketamine and medetomidine (7 rats, 5 mg/100 g, Vetalar; Boehringer Ingelheim Vetmedica Inc., USA; 30 μg/100 g, Domitor; Pfizer, UK). Each animal was mounted in a stereotaxic apparatus with atraumatic ear bars and surgery was performed under aseptic conditions. Depth of anesthesia was checked regularly by testing for corneal and paw withdrawal reflexes, and the level of gaseous anesthetic adjusted or supplementary doses of ketamine given as required.

A midline scalp incision was made and a craniotomy performed to gain access to the PAG (7.5mm caudal from bregma, 1mm lateral from midline). An in-house built miniature microdrive was attached to the skull with screws and dental acrylic cement. The microdrive contained 1-4 tetrodes for LFP and single unit recordings (tungsten, 12.5 μm inner diameter, impedance 100–300 kOhm following gold plating; California Fine Wire, USA. The tetrodes were stereotaxically lowered through the craniotomy to a position just dorsal to the PAG (approximately 4mm below the brain surface). A pair of flexible, stainless steel insulated wires (Cooner, USA) were also sutured into neck muscle to record EMG as a marker of
freezing behavior (Steenland and Zhuo, 2009). These leads were fed subcutaneously to the microdrive and the skin incision closed in layers.
**Behavioral and electrophysiological recording procedures**

Prior to surgery animals were habituated to handling for at least two days. One week after surgery, rats were handled for at least another 2 days before daily recording sessions commenced in which the position of the tetrodes was adjusted in order to obtain single unit activity within either the dorsal or ventral PAG (4.0-4.5mm or 4.6-5.6mm from brain surface, respectively). Once single units were localized, the electrode was kept in the same position throughout behavioral testing (i.e. the same recording position was maintained for experimental days 0-4).

Fear conditioning and extinction testing occurred in different contexts (contexts A and B). The Skinner box (Med Associates, USA) was dimly lit and located within a soundproofed room. The walls, ceiling and floor were cleaned with 70% ethanol after each session. Context A had a clear Perspex back wall, ceiling, and front door with aluminum sidewalls and a metal grid floor. For context B, the inner structure of the chamber was altered through the addition of a white plastic floor, striped wall and a tissue impregnated with vanilla essence placed under the flooring. For habituation and fear conditioning (days 0-2), the animals were placed in context A, whereas during extinction testing they were placed in context B (day 3).

On experimental **day 0**, the rats were habituated to the conditioning chamber for 5 min. On experimental **day 1**, following a 5 min acclimatization period to context A, the rats received an auditory habituation session consisting of 7 auditory tones (each tone 10 s, 1 kHz, 80 dB). Experimental **day 2** consisted of a 5 min acclimatization period to context A followed by the fear conditioning session when the rats received 7 conditioned stimulus (CS) presentations of the same auditory tone as day 1 but with each tone co-terminating with a footshock unconditioned stimulus (0.75 mA, 1 s). On experimental **day 3**, after a 5 min acclimatization period, the rats received an extinction session consisting of CS-only trials in context B (each tone 10 s, 1 kHz, 80 dB). The CS-only trials were in blocks of 7 tones (time interval between tones in each block was 30 sec), and 7 blocks of trials were presented during the session (time interval between each block was 2 mins). In keeping with a previous study (Burgos-Robles et al., 2009) CS-only block 1 was defined as early extinction (EE; equivalent to CS retrieval), while CS-only block 7 was defined as late extinction (LE). At the end of the CS-only
session rats were returned to their home cage. After 24 hours, (experimental **day 4**), a piece of filter paper impregnated with cat odor was placed in the top of their home cage in order to test innate fear responses (exposure duration ~3 mins; Koutsikou et al. 2014).

Electrophysiological data were obtained during experimental days 1, 3 and 4 (recordings were not possible on experimental day 2 because of stimulus and movement artefacts during the acquisition phase of fear conditioning). Signals were captured using a Lynx 8 acquisition system (Neuralynx, USA) and CED Power1401 (Cambridge Electronic Design, UK). Local field potential (LFP) and EMG signals were sampled at 5 KHz and bandpass filtered between 0.1 and 600 Hz. For the majority of recordings, neural signals were sampled at 20 KHz and bandpass filtered between 600 Hz and 6 KHz. Single unit and LFP recordings were referenced to a skull screw positioned over the cerebellum, which served as the indifferent electrode. EMG signals were recorded in a bipolar configuration with wires positioned on either side of the neck (cf. Steenland and Zhuo, 2009).

Throughout all sessions behavior was monitored by video and scored offline. Time spent freezing (defined as the cessation of all movements except those associated with respiration and eye movements; Blanchard and Blanchard, 1969) was assessed using a combination of video and neck EMG recording (smoothed using a 25ms time constant and rectified; Steenland and Zhuo, 2009).

**Data analysis**

All data were tested for a Gaussian distribution using the D'Agostino & Pearson omnibus normality test and parametric or non-parametric statistical analysis performed as appropriate. Data were analyzed offline with Spike2 software (Cambridge Electronic Design, UK), Matlab (Mathworks, USA) and Neuroexplorer (Nex Technologies, USA). For extinction sessions (CS-only trials), freezing was measured as the percentage of time immobile during the **7 × 10 s** duration of the CS and the **7 × 30 s** duration of the inter-trial interval. A repeated measures ANOVA with Tukey's post hoc was used to compare freezing rates at different time points throughout the protocol (from baseline, a period of 3 minutes when animals were in the conditioning box prior to playing of the CS, through to CS block 7).
the innate fear session (cat odor presentation), freezing was measured as the percentage
time spent immobile during the 3 min exposure to cat odor.

We also recorded LFP from 7 animals during tone habituation (the presentation of the same
tone used for conditioning but presented 7 times pre-conditioning). In order to test changes
in tone evoked field potentials during habituation we constructed field potential averages
from tones 1-3 and 5-7. The amplitude of these field potentials was then compared using a
paired t test.

For field potential analysis during extinction training, average waveforms were constructed
using tone onset as the trigger (7 tones per average, n= 10 rats; 4 animals were excluded
from analysis due to poor signal to noise recording conditions). The amplitude of responses
evoked during early and late extinction were compared using a paired t test. Pearson
correlation was also used to assess the relationship between event-related field potential
amplitude and level of freezing behavior.

Spike sorting was performed using a clustering algorithm based on template matching and
principal component analysis. Single units were subdivided into two groups according to
recording depth: (i) dorsal units (depth 4.0-4.5mm from the surface of the brain); or (ii)
ventral units (4.6-5.6mm from the surface of the brain).

To detect temporal changes in single unit activity, we divided the duration of the CS tone
into 10 bins, each 1s in duration. A z-score for each of these bins was calculated relative to
10 pre tone bins of equal duration (obtained in the same session). Units were classified as
showing significant increases or decreases in response to the CS if any bins within the tone
exceeded a z-score of +1.96 or a z-score of less than -1.96, respectively (P<0.05, two tailed
test). To detect changes in firing rate, we compared peak z values (1 value per animal)
during EE and LE (independently of the time bin in which the peak in firing frequency
occurred) using Mann-Whitney U tests. Response latencies were computed using
peristimulus time histogram (PSTH) plots with 10ms time bins and calculated as the time
interval from the onset of the CS to the first bin exceeding 95% confidence level.
Group peri-event time histograms were generated by averaging z-scores of unit activity before and after tones or exposure to the cat odor. Mann–Whitney U tests were used to examine differences in unit firing rate. A chi-square test ($\chi^2$) was used to detect differences in the proportion of tone-responsive units in early extinction versus late extinction (see above), and also in relation to dorsal versus ventral recording site position. Type 1 neurons (see Results) were the most common cell type with sufficient sample size to carry out further analysis. For type 1 cells, Spearman’s correlation was used to assess the relationship between PAG firing rate and time spent freezing; and spike-triggered averages (STA) were constructed of EMG in relation to their activity.

The STA analysis was obtained for a sample of cases (n=9 rats) in relation to (i) early extinction (during the 10s of CS presentation), (ii) early extinction 10 to 20s after the CS and (iii) during quiet immobility (based on a 200s sample of recording pre-conditioning). Animals were selected for this stage of analysis based upon the quality of their EMG signals. We excluded all animals in which large amplitude artifacts contaminated the recordings. For all three conditions (during the CS, after the CS and during quiet immobility) the rectified EMG was triggered from spikes recorded from type 1 units located in either dorsal or ventral PAG. Due to variations in the EMG amplitude, the data were z-score normalized before averaging across animals. Latency to peak EMG response was calculated from individual STA plots. Mann-Whitney tests were used to compare amplitudes and latencies of the averages. All values are shown as mean ± SEM and $p < 0.05$ was selected as the criterion for statistical significance.

Histology
At the end of every experiment, animals were deeply anaesthetised (Euthatal, 200 mg ml$^{-1}$, Merial Animal Health Ltd, Harlow, UK) and electrolytic lesions performed to mark the position of tetrode tips. The animals were then perfused (4% paraformaldehyde in 0.1M phosphate buffer) and the brains extracted. Following post-fixation, the brains were cryoprotected in 30% sucrose solution and coronally sectioned at 60 $\mu$m.

Results
Freezing behavior: conditioned and innate fear
Figure 1A shows grouped data of the duration of time rats displayed freezing behavior during auditory conditioned fear acquisition and extinction (experimental days 2 and 3, n=17 rats), and also during subsequent exposure to cat odor to induce innate fear (Fig. 1C, experimental day 4, n=14 of the same rats). Consistent with previous studies (e.g. Duvarci et al. 2011; Koutsikou et al. 2014), exposure to a conditioned stimulus (CS) tone previously associated with an aversive footshock, produced a statistically significant increase in freezing during early extinction (EE, CS blocks 1-3; ANOVA with Tukey’s post hoc test between baseline and CS blocks 1,2 or 3 P<0.0001, F (8, 90) = 40.6, n =17) compared to pre-conditioning baseline. For example, during CS block 1, animals spent 82.8 ± 4 % of their time freezing compared to 27.1 ± 3 % of their time during baseline trials (Fig. 1A). The duration of maintained freezing initiated upon presentation of the first CS (an example is shown in Fig. 1B), varied considerably between animals (ranging from 18 to 470 s). However, the duration of freezing always substantially outlasted the initial 10 s tone (mean first freeze duration = 94.5 ± 41.9 s). In keeping with previous investigations, the proportion of time the animals displayed freezing gradually decreased with subsequent blocks of CS-only trials, and were similar to baseline levels by CS block 7 (late extinction, LE, 32.8 ± 4 % time spent freezing, ANOVA with Tukey’s post hoc test vs baseline, P>0.05, n=14; 3 rats did not undergo the full sequence of extinction training).

On experimental day 4 the same rats were exposed to cat odor (see Methods) and in agreement with previous studies (e.g. Koutsikou et al. 2014) they displayed a statistically significant increase in innate freezing behavior (on average they spent 54.9 ± 6 % of their time freezing, ANOVA with Tukey’s post hoc test, P<0.05, compared to baseline, n=14; Fig. 1C).

**Dorsal and ventral PAG recordings: innate fear**

The activity of single units in the dorsal and ventral PAG was examined during innate fear elicited by exposure to cat odor (Fig. 2). Grouped analysis revealed that dorsal PAG units displayed a statistically significant greater increase in firing rate than ventral PAG units during initial exposure to the innate fear-inducing stimulus (i.e. during the first 20 s following cat odor exposure; z-score 2.7 ± 0.4 versus 0.9 ± 0.12, respectively, P<0.0001, Mann Whitney U value = 68, n=14 rats, Fig 2A, B). Furthermore, individual unit analysis
revealed that 63% (14/23) of dorsal PAG units and 50% (12/24) of ventral PAG cells displayed significant increases in firing rate during the first 20 s following exposure to the cat odor (Fig. 2C, D). There were no significant reductions in firing rate in response to cat odor. To investigate the relationship between PAG firing and muscle activity, we constructed spike triggered averages (STAs) of neck EMG following exposure to cat odor. No statistically significant relationship was found for STAs constructed in relation to either dorsal or ventral PAG unit activity (Fig. 2E, F).

Dorsal and ventral PAG recordings: conditioned fear

For studying neural activity during conditioned fear, in initial experiments, we recorded field potentials in PAG evoked by the CS tone (n= 10 rats, Fig. 3A). Histological verification of example recording positions in dorsal or ventral PAG are shown in Fig 3E. Since the event related responses were similar for both dorsal and ventral recording sites the data were pooled.

On average, the onset and peak latencies of the event-related field during early extinction were 9 ± 1ms and 20 ± 2ms, respectively (Fig. 3A, B). The average duration of the event-related field was 37 ± 10ms. In cases where the event-related field was recorded simultaneously with single unit activity the time course of the field coincided with an increase in neuronal firing frequency (Fig. 3B). Field potentials mainly reflect synaptic activity so if the unit activity and field potential activity are related then it would be expected that evoked spikes would occur later than the onset of the field. In the example illustrated in Figure 3B the event-related field occurs from 8 ms to about 45ms while the onset latency of single unit firing occurs at about 35ms.

In every available case (n=10) the event-related field was reduced in amplitude in late extinction (Fig. 3A, C). The mean peak-to-trough amplitude of the response was 0.26 ± 0.04 mV during early extinction, but only 0.16 ± 0.02 mV during late extinction, representing approximately 40% reduction in size (P=0.01, paired t test, n= 10 rats). Correlation analysis revealed a statistically significant positive relationship between the amount of time spent freezing and the amplitude of the tone evoked field potential (Fig. 3D; r=0.45; P=0.04). In 7 of the same animals, we also recorded event-related field potentials during tone
habituation, prior to conditioning. In contrast to responses evoked by the same tone during extinction training, there was no significant difference in field potential amplitude during habituation (the average peak-to-trough amplitude during tones 1-3 was 0.17 ± 0.04 mV and 0.18 ± 0.04 mV during tones 5-7; P=0.56; paired t test).

Having established that the CS could evoke substantial field potentials in PAG that were reduced by extinction training, our next step was to investigate the activity patterns of single units. Unit recordings were obtained from the PAG in 14 rats during early extinction and in 12 of the same rats during late extinction. Offline clustering (see Methods) of unit activity yielded a total sample of 73 units during early extinction (41 located within dorsal PAG and 32 located within ventral PAG, n=14 rats), and 50 units during late extinction (28 located within dorsal PAG and 22 located within ventral PAG, n=12 rats). Two animals did not undergo the full sequence of extinction training, leading to a smaller sample size in late extinction (recordings from these two animals contributed 3 dorsal and 5 ventral cells, respectively, to the total unit count during early extinction). Loss of single unit recording from individual cells during the full sequence of extinction training was 20% across all available animals.

Figure 4 shows examples of unit activity during presentation of the CS during early extinction. The sample of PAG units could be classified into 4 types. The most common type of response was an increase in firing rate (type 1, Fig. 4A, representing 67% of the total population); while no significant change in firing (type 2, Fig. 4B), or a biphasic pattern of response (type 3, Fig. 4C) each represented 14.5% of the population sample. The fourth class were units that displayed a reduction in firing rate (type 4, Fig. 4D, representing only 4% of the population sample recorded in early extinction).

The different types of activity were present for units located in both dorsal and ventral PAG (see below). To explore the activity in more detail, firing rate averages were obtained at three different time points in relation to presentation of the auditory tone: (i) in the pre-conditioning habituation phase (n=6 rats), (ii) post-conditioning during early extinction (CS block 1, n= 14 rats), and (iii) post-conditioning during late extinction (CS block 7, n=12 rats).

Figure 5 displays the data for all units located in dorsal PAG or ventral PAG grouped
separately to show population averages for the different regions of PAG as a function of
extinction training.

A total of 10 dorsal PAG units (mean baseline firing rate 2.3 ± 0.3 Hz) were recorded during
habituation to the tone and none displayed a statistically significant response (Fig. 5A). By
contrast, during early extinction grouped analysis revealed that units recorded from dorsal
PAG displayed a statistically significant increase in firing rate around the onset and offset of
the CS (onset latency 35.8 ± 6.7ms; n=41 units, left hand plot Fig. 5B). This phasic increase in
firing rate was absent in late extinction (right hand plot Fig. 5B) and the difference in peak
firing rate between early and late extinction was statistically significant (z-score 5.6 ± 0.7
versus 1.7 ± 0.6, P=0.0005, Mann-Whitney U value = 389, n = 41 cells, 14 rats during early
extinction and n = 28 cells, 12 rats during late extinction, respectively).

When the analysis was carried out on the firing patterns of individual units located in dorsal
PAG, a statistically significant increase in firing rate throughout presentation of the CS in
early extinction was found in 71% of the sample (29/41 units, left hand panel Fig. 5C); of the
remainder 27% (11/41 of units) displayed either a biphasic or no significant change in firing
rate; whereas only 2% (1/41) showed a significant reduction (left hand panel, Fig. 5C, see
also Fig. 5D, red pie chart). By comparison, during late extinction, 57% of dorsal PAG units
(16/28) significantly increased their firing rate, 29% (8/28 units) displayed a significant
decrease (Fig. 5C right hand panel), and the remainder (14%, 4/28 units) showed either a
biphasic response or no change in activity during presentation of the CS (Fig. 5D, gray pie
chart). Thus, dorsal PAG units were 29 times more likely to display an increase than a
decrease in firing rate during early extinction. This ratio reduced to a twofold difference in
late extinction. For those dorsal PAG cells that displayed a statistically significant increase in
activity during early extinction, the peak firing rate was significantly higher during the CS in
early extinction compared to the sample of dorsal PAG cells that displayed a significant
increase in late extinction (z-score 6.5 ± 0.7 versus 3.6 ± 0.7; P=0.04, Mann-Whitney U value
= 239; n=29 units and n=16 units, respectively obtained from 14 and 12 rats, Fig. 5C).

A total of 10 ventral PAG units (mean baseline firing rate 2.8 ± 0.6 Hz) were recorded during
habituation to the auditory tone and none displayed a statistically significant response (Fig.
During early extinction, grouped analysis revealed that ventral PAG units (like dorsal PAG units) displayed a statistically significant increase in firing rate during CS presentation, particularly at the onset of the tone (onset latency 34.2 ± 5.3ms; n=32 units, left hand panel Fig. 5F), and as a population, this effect was absent during late extinction (n=22 units, right hand panel Fig. 5F). The overall reduction in mean firing rate between early and late extinction was statistically significant (z-score 5.4 ± 0.9 versus 1.7 ± 0.7, P = 0.028, Mann-Whitney U value = 219; n=32 cells and n=22 cells respectively obtained from 14 and 12 rats).

The peak in activity at the onset of the CS suggests that at least some ventral PAG cells are not encoding the CS-US interstimulus interval (otherwise there would be a sustained change in firing during CS tone delivery and/or increased activity at offset when the US would be predicted to occur. However, to test this possibility would require further experiments in which the pattern of response of individual cells was recorded in relation to a range of different interstimulus intervals.

Individual unit analysis revealed that during early extinction, the majority of ventral PAG units (63%, 20/32) showed a statistically significant increase in firing rate during CS presentation, while 31% (10/32) displayed either a biphasic response or no significant change, and 6% (2/32) showed a significant decrease in firing rate (red pie chart, Fig. 5H).

During late extinction, the proportions were 41% (9/22 units) that displayed an increase; 18% (4/22) displayed a reduction and 41% (9/22) displayed either a biphasic response or no change in firing rate (gray pie chart, Fig. 5H). Thus, ventral PAG units were 10 times more likely to display an increase than a decrease in firing rate during early extinction. Like dorsal PAG units this ratio reduced to approximately a twofold difference in late extinction.

However, in contrast to dorsal PAG activity, when the ventral PAG units with a statistically significant increase in firing rate during CS presentation in early extinction were compared to the sample of ventral PAG cells that displayed a significant increase in late extinction, the increase was similar in magnitude (z-score 7.2 ± 1.0 versus 4.6 ± 1.3; ns, P = 0.17, Mann-Whitney U value = 83; n=20 units and n=9 units respectively obtained from 14 and 12 rats, Fig. 5G). In other words, those ventral PAG units that did respond with an increase in activity during late extinction did so robustly.
When the proportion of units displaying the 4 different types of response (type 1, an increase; type 2, no change in activity; type 3, biphasic response; or type 4, a decrease,) were compared there was a significant difference in early versus late extinction for both dorsal and ventral PAG units (Fig. 5D, H; for dorsal cells $\chi^2 = 30.1$, df = 3, $P < 0.001$; for ventral cells $\chi^2 = 32.1$, df = 3, $P < 0.001$). When a comparison was made between dorsal and ventral PAG units during early extinction there was no significant difference in the proportion of the different types of response (red pie charts, Fig. 5D, H, $\chi^2 = 6.1$, df = 3, $P = 0.11$). In contrast, during late extinction, dorsal and ventral PAG units displayed a statistically significant difference in prevalence of firing patterns: a greater proportion of dorsally located cells were type 1 units compared to ventrally located units (gray pie charts, Fig. 5D, H; $\chi^2 = 17.7$, df = 3, $P = 0.0005$).

**Relationship between unit activity and freezing behavior/EMG during extinction**

The heatmaps of Figure 6A and B illustrate the change in firing pattern of two example units recorded from dorsal and ventral PAG respectively, during the full sequence of extinction training, together with the time spent freezing within each trial (right hand bar charts). Consistent with the data as a whole (Fig. 5), during early extinction (CS block 1) both units displayed a clear change (in this case an increase) in firing rate during presentation of the CS (Fig. 6, red arrowheads) but during late extinction (CS block 7) the same units showed little or no change in activity during presentation of the CS (Fig. 6, black arrowheads).

Visual inspection of the individual examples illustrated in Figure 6 suggests that changes in cell firing during extinction training are not tightly coupled to the concomitant changes in freezing behavior. For example, when the dorsal unit shown in Figure 6A showed an increase in firing in extinction block 1 there was also a high level of freezing behavior, but by extinction training block 3 the same unit displayed a smaller increase in firing while the animal still displayed a high level of freezing behavior. Such findings have implications for the role of PAG in driving motor output. Therefore, to further investigate the apparently weak coupling between PAG activity and behavior we constructed CS-triggered peri stimulus time histograms (PSTHs) and CS triggered EMG plots for a sample of dorsal and ventral PAG units, all of which showed a significant increase in activity (type 1 units) during presentation of the CS in early extinction (triggered by the first CS; Fig. 7). Significant increases in unit
firing in both dorsal and ventral PAG were time locked to the duration of the CS (falling below significance levels by CS offset; Fig. 7A & B). In contrast, neck EMG amplitude (which is a reliable marker of freezing, Steenland and Zhuo, 2009) remained significantly reduced after the CS had ended, consistent with a sustained period of freezing that outlasts the CS (Fig. 7C; *, P<0.05, Mean Rank diff = 13.3 for -10 to 0s bin vs 0 to +10s bin; ***, P<0.001, Mean Rank diff = 25.0 for -10 to 0s bin vs 10 to +20s bin; ***, P<0.001, Mean Rank diff = 21.3 for -10 to 0s bin vs 20 to +30s bin, Kruksal- Wallace test with Dunn’s post hoc; n = 9 rats).

The data in Figure 7 therefore indicate at a population level, that changes in EMG activity induced by the CS during early extinction outlast the associated increases in firing rate of both dorsal and ventral PAG units. However, group analysis may obscure effects at the level of single units. Figure 8A and B therefore plot for individual type 1 units, the change in normalized firing rate between pre- and post-conditioning, as a function of freezing for dorsal and ventral PAG units, respectively. For type 1 units located in dorsal PAG, we found no significant correlation between change in firing rate and the extent of freezing in either early or late extinction (Fig. 8A upper panel; Spearman correlation P=0.39, r=0.32 during early extinction, lower panel, P=0.07, r=0.57 during late extinction). However, for type 1 units recorded from ventral PAG we found a significant positive correlation between change in firing rate and freezing level during early extinction (Fig. 8B upper panel; Spearman correlation P=0.03, r=0.62) but not during late extinction (Fig. 8B lower panel; Spearman correlation P=0.23, r=0.52).

To further investigate the relationship between PAG activity and behavior we constructed spike-triggered averages of neck EMG. Figure 8C and D show the averaged data, respectively for dorsal units (n=20) and ventral units (n=20). The plots show the results when rats were: (i) displaying immobility prior to conditioning (baseline, black plots), (ii) during early extinction and the 10 second period in which the CS was presented, when conditioned freezing starts to occur (red plots); and (iii) during early extinction and the 10-20s period after the CS when conditioned freezing continues to occur (blue plots, cf. Fig. 8C). For dorsal units the spike-triggered EMG was similar for the baseline sample and during both CS time periods in early extinction training. The small peak during early extinction, approximately 45
ms after the onset of the CS, was not significantly different compared to peaks during baseline or post-CS (baseline z-score 0.97 ± 0.3 versus early extinction peak z-score 1.8 ± 0.3; ANOVA with Tukey’s posthoc tests between peak in early extinction spike-triggered average (red) vs baseline (black) or post CS (blue), P=0.07, F (2, 24) = 2.9, n= 9 rats; Fig. 8C).

In contrast, for ventral units there was a statistically significant peak (asterisk. Fig. 8D) in the spike-triggered EMG compared to baseline, during the 10 sec time period of the CS in early extinction but not during the 10 sec time period immediately after the CS (baseline z-score 0.68 ± 0.4, early extinction peak z-score 2.05 ± 0.4; ANOVA with Tukey’s posthoc tests between peak in early extinction spike-triggered average (red) vs baseline (black), P=0.02, F (2, 24) = 4.4, n= 9 rats; Fig. 8D). The significant peak in cross correlation occurred approximately 10 ms after the onset of the CS.
Discussion

The key findings from the present study are: (i) in response to an innate fear stimulus, dorsal PAG (dPAG) units show more robust increases in their activity than ventral PAG (vPAG) units, (ii) the pattern of response of dPAG and vPAG units during extinction of a fear conditioned response can be divided into 4 types: type 1 (increase), type 2 (no change), type 3 (biphasic) and type 4 (decrease ), (iii) for dPAG and vPAG units, the proportion of the different types of response is similar during early extinction (EE; mainly type 1) but differs during late extinction (LE), when, (a) despite there being a general increase of type 2 and 4 cells, the majority of dPAG cells remain as type 1 and (b) type 1 vPAG units in EE continue to respond robustly in LE (i.e. a sub population of both dPAG and vPAG units are extinction resistant) and (iv) the activity of vPAG units, but not dPAG units, is cross correlated with EMG during the initial stage of conditioned freezing.

Comparison to previous studies

Most PAG studies to date have involved behavioral observations following localized lesions or stimulation, or charting regions of cellular activation with FOS (Vianna et al. 2001b; Bandler and Depaulis, 1988; Carrive et al., 1997; Bittencourt et al., 2005). A general consensus has emerged that dPAG directs motor outputs to innate fear, including flight (Carrive, 1993; Bandler and Shipley, 1994; Vianna et al., 2001b; Kim et al., 2013; Halladay and Blair, 2015; Wang et al., 2015), while vPAG is required for the expression of fear conditioned freezing i.e. its activation leads to a general cessation in movements and a fixed motionless posture (LeDoux et al., 1988; Kim et al., 1993; De Oca et al., 1998). Conditioned fear is related to a marked increase in FOS expression in vPAG (Carrive et al., 1997). Thus, dorsal and ventral columns of PAG are thought to drive mainly opposing types of behavior.

Our finding that dPAG cells fire more robustly than ventral ones to an innate fear stimulus of predator odor is consistent with this functional distinction. It should however be noted that the cat odor was always presented after the footshock conditioning and extinction training which raises the possibility that the innate responses may have been modified by this previous experience. Further studies would be required to explore any interaction between innate and conditioned fear on neural activity in the PAG. In relation to fear conditioning we found that during EE (when retrieval of the conditioned freezing response is maximal), both
dorsal and vPAG neurons display similar patterns of response. In relation to vPAG our findings show that neural activity in this region of PAG is time-locked to a CS that elicits freezing behavior, particularly the onset of the conditioned tone. Importantly, vPAG cells did not respond to the same auditory tone when it was unconditioned. Taken together this provides evidence that vPAG activity is related to the associative conditioning rather than the sensory stimulus. Moreover, since vPAG cells displayed significant coupling to conditioned EMG activity during EE (with spikes preceding the peak in EMG activity by approximately 10 ms) this is consistent with vPAG cells driving the initial motor response, although it should be emphasized that this does not exclude the possibility that such cells might also encode fear memory.

Any significant increases in vPAG activity were short lasting (typically 1-2 sec in duration) and did not match the longer lasting EMG activity associated with the conditioned freezing response (which in EE lasted at least 18 sec). Based on the sample of cells we recorded, the pattern of neural activity observed in vPAG is therefore inconsistent with maintaining freezing behavior. This is contrary to the model proposed by Burgos-Robles et al. (2009), which predicts that PAG neurons would show sustained conditioned responses. Instead, our data suggest vPAG cells could drive initial aspects of the conditioned motor response, but other CNS structures may be involved in sustaining the behavior. Further studies would be required to explore this possibility, for example, by studying the effect of delivering brief stimulus pulses to vPAG.

One candidate structure for sustaining the conditioned response is the prelimbic cortex (Blum et al., 2006; Sierra-Mercado et al., 2011). Prelimbic cells can show tonic increases in firing activity that mirror the time course of freezing to a conditioned tone (Burgos-Robles et al., 2009). However, prelimbic activity starts 100ms after the onset of a conditioned tone, while changes in PAG activity occur sooner (~30-35ms in the current study; ~30ms in Halliday and Blair, 2015). By comparison, responses in lateral amygdala occur ~15ms after a conditioned tone (Quirk, et al. 1997), suggesting that both vPAG and prelimbic activity are initiated by the amygdala. However, in rats, the prefrontal cortex is also the major recipient of cortico-petal projections arising from the PAG (Herrero et al., 1991), raising the additional
possibility that vPAG contributes directly to driving the conditioned responses in prelimbic
cortex.

Our finding that dPAG neurons display a similar pattern of response as vPAG cells to the CS
during EE is surprising. Carrive et al. (1997) found less FOS expression in this region of PAG
as a result of conditioned fear, and lesions of dPAG do not block the expression of
conditioned fear behavior (Leman et al., 2003). Also, Halladay and Blair (2015) found that
dPAG neurons were responsive to a CS that led to movement excitation (i.e. increased
movement, including flight) but not inhibition (i.e. suppression of movement, including
freezing). However, one important difference from our study is that, rather than extinction
learning, Halladay and Blair (2015) examined retrieval of conditioned responses in well
trained animals that received daily reinforcement of the fear conditioning paradigm. As a
consequence, their animals may have been in a more aroused/fearful state with a greater
expectancy of the unconditioned stimulus, which may influence the pattern of PAG activity.

However, consistent with our findings, about half of their dPAG cells increased activity
during the initial part of the auditory CS, irrespective of whether the conditioned behavioral
response was excitatory or inhibitory. Chemical or optogenetic activation of dPAG can
induce both flight and freezing (Krieger and Graeff, 1985; Deng et al., 2016). Our finding that
dPAG cell responses are time-locked to a CS associated with freezing is therefore consistent
with the possibility that they may be related to fear memory and/or freezing behavior. Like
vPAG cells, dPAG cells did not respond to the same auditory tone when it was
unconditioned. But unlike vPAG cells they did not display a significant coupling to
conditioned EMG activity during EE.

Taken together these results therefore suggest that dPAG activity during EE may be more
related to the recall of fear memory than sensory or motor aspects of the behavioral
response. Consistent with this is the finding that lesions of dPAG can enhance both the
acquisition and expression of conditioned fear responses, suggesting an inhibitory role in
learning and memory (De Oca et al., 1998).

Types of response

20
Studies of neural activity in central circuits associated with fear have focused on the amygdala (e.g. Muramoto et al., 1993; Quirk et al., 1995). Since the direct amygdalo-PAG pathway is thought to be inhibitory, it has been predicted that target neurons in PAG will reduce their activity during fear conditioned behavior (Duvarc et al., 2011). However, our results indicate that most PAG cells increase their activity during fear retrieval which implies that the primary influence of amygdala on the PAG may be on inhibitory interneurons, leading presumably to disinhibition of other PAG cells. A recent optogenetic study in mice provides direct evidence to support this interpretation (Toyote et al., 2016).

**Extinction susceptible and resistant cells**

During LE there was an increased incidence of type 2 and type 4 cells in both dorsal and vPAG i.e. an increased proportion of cells that displayed either no change or a reduction in response to the unreinforced CS. Such activity is consistent with neural plasticity associated with extinction and is strikingly similar to the pattern described previously for the amygdala (Hobin et al., 2003; Herry et al., 2008; An et al., 2012). A substantial population of dorsal and vPAG cells (almost half) also responded during LE. In particular, type 1 cells in vPAG displayed an increase in firing rate that was not significantly different compared to those responses observed during EE (i.e. were extinction resistant). According to general learning theory, extinction is not the erasure of the associative memory but is a form of context dependent inhibitory learning that temporarily suppresses expression of the conditioned response (Todd et al., 2014). The extinction resistant cells reported here may therefore contribute to the persistence of fear memory after extinction, analogous to the pattern of activity in the amygdala and prefrontal cortex (Burgos-Robles et al., 2009; An et al., 2012).

Our results therefore suggest that PAG is not, as widely considered, merely the final executor for top down drive of fear-related motor output, but may also be concerned with maintaining the memory trace of conditioned fear.
Figure Legends

Figure 1. Freezing levels during conditioned and innate fear.
A, Percentage of time freezing during tone habituation (day 1); auditory fear conditioning (day 2); pre-CS baseline and auditory conditioned fear extinction (day 3). Color coded data points indicate times when single unit and LFP data were analyzed. Early extinction (EE; 17 rats, red circle), late extinction (LE; 14 rats, filled circle). ns, P>0.05; ***, P<0.001, ANOVA with Tukey’s post test against baseline. B, example of immobility detection before (baseline) and freezing detection during exposure to an auditory CS (CS block 1, onset of each tone is indicated by red lines). F1, the initial period of maintained freezing following presentation of the first CS. In this example this is interrupted by a brief period of movement prior to a subsequent extended period of further freezing. C, Freezing time in the home cage following extinction training and during exposure to cat odor (day 4; n= 14 rats; ***, P<0.001, paired t test).

Figure 2. PAG cell responses to cat odor
A, Grouped z-score plots of dorsal PAG unit activity during exposure to cat odor (onset at time zero, average of n=23 units). B, Same as A but grouped responses of ventral PAG units (average of n=24 units). Black line represents mean; shaded area indicates S.E.M. 1 s bins. Dotted horizontal lines indicate P=0.05. Pie charts (C, dorsal; D, ventral units) to indicate proportions of response patterns to the stimulus. E, F, spike-triggered EMG averages using spike activity of dorsal and ventral PAG units, respectively. EMG data were rectified, smoothed (0.025s) and expressed as a z-score. Shading indicates S.E.M. Dotted horizontal lines indicate confidence level at P=0.05.

Figure 3. Tone-evoked PAG local field potentials during conditioned fear behavior
A, Example of event related field potential recorded in the PAG obtained from one animal (average of 7 trials triggered relative to tone onset, arrow heads). Event related field response recorded from same recording site during early and late extinction. Solid lines, mean, light red and gray lines indicate S.E.M. B, Simultaneously recorded event related field potential (average of 7 trials; upper trace) and single-unit (raster and PSTH) in dorsal PAG during early extinction. Tone onset indicated by arrowhead. PSTH bins 2ms). C, Relationship between amplitude of tone-evoked field and percentage of recording session time displaying
freezing (n=10 animals, 2 data points per animal (EE, red and LE, black). D, Correlation plot of evoked field amplitude as a function of time spent freezing. Solid line indicates linear regression and dotted lines represent 95% confidence intervals. E, Coronal sections of the PAG showing examples of electrolytic lesions performed at the end of the experiments to mark recording sites. The photomicrograph to the left shows a case with a lesion in ventral PAG (white arrowhead) while the photomicrograph to the right shows a different case with lesions in dorsal PAG (black arrowhead). Approximate position of section relative to bregma is indicated. Abbreviations: dm, dorsomedial periaqueductal gray; dl, dorsolateral periaqueductal gray; lat, lateral periaqueductal gray; vl, ventrolateral periaqueductal gray.

Figure 4. Different types of response of units in PAG during early extinction.

Examples of the different patterns of response to the CS during early extinction (tone block 1). A, type 1, increased firing. B, type 2, no significant change in firing. C, type 3, biphasic response. D, type 4, decreased firing. CS, conditioned tone stimulus. Examples in A and C were units located in the dorsal PAG while examples in B and D were units located in the ventral PAG.

Figure 5. Changes in firing pattern during extinction training.

A, E, Firing patterns of cells recorded in dorsal and ventral PAG during presentation of an initially neutral auditory tone (n = 10 dorsal and n = 10 ventral cells, 6 rats). B, F, Pooled firing rate plots of all dorsal (left panel, n = 41 cells, 14 rats; right panel, n = 28 cells, 12 rats) and ventral (left panel, n = 32 cells, 14 rats; right panel, n = 22 cells, 12 rats) cells during early (red) and late extinction (black), respectively. C, G, Average of a subset of dorsal (left panel, n = 30 cells, 14 rats; right panel, 24 cells, 12 rats) and ventral (left panel, n = 22 cells, 14 rats; right panel, n = 13 cells, 12 rats) PAG cells with statistically significant increases or decreases (P = ≤0.05) in activity during the conditioned stimulus (CS). D, H, Pie charts showing proportion of dorsal and ventral cells with different response types during early (red) and late (black) extinction, respectively. For z-score plots, average is represented by solid lines and shading indicates the SEM. Dotted horizontal lines indicate confidence level at P=0.05. Bins are 1s. ***P <0.001 **, P<0.01 , *, P<0.05, n.s., non-significant, Mann Whitney test between peak responses. For pie charts, ***, P<0.001. n.s., non-significant, χ² test. CS, conditioned stimulus.
**Figure 6. Changes in PAG firing patterns during extinction training.**

Example heat maps displaying changes in dorsal (A) and ventral (B) PAG unit firing frequency as a peri-stimulus plot throughout the complete sequence of extinction training (from CS block 1 to 7). Hotter colors indicate higher firing rates. Red and black arrowheads indicate early (CS block 1) and late (CS block 7) extinction blocks, respectively. Bin size =100ms. Time zero indicates onset of conditioned stimulus (CS) and gray horizontal bar at top of each heat map shows CS duration. Percentage time spent freezing during each trial (7 trials per block) is shown to the right of the heat map.

**Figure 7. PAG unit and EMG activity during early extinction**

A, PSTH constructed from a sample of 20 dorsal PAG units (n= 9 animals) in which neck EMG was simultaneously recorded with unit activity. B, Same as A but PSTH constructed from a sample of 20 ventral PAG neurons (n= 9 animals). For A and B bin size = 1s; horizontal dotted line indicates P=0.05. C, Histogram of neck EMG amplitude in 10s bins. Analysis in A- C based on response to first presentation of CS in early extinction. In all plots, time zero indicates CS onset and gray horizontal bar in A and B shows CS duration. **P<0.001, *P<0.05, Kruskal-Wallis test with Dunn’s post hoc, n=9 rats. CS, conditioned stimulus.

**Figure 8. Relationship between PAG and EMG activity**

A and B, Spearman correlation between the average CS-evoked changes in firing rate for type 1 dorsal and ventral PAG units as a function of freezing levels. Sample of dorsal PAG units obtained from n= 9 rats during early extinction and n=10 rats during late extinction. Sample of ventral PAG units obtained from n= 11 rats during early extinction and n=7 rats during late extinction). Z-score change computed for each cell and averaged per rat before correlation against freezing level (dashed line). Dotted gray lines indicate 95% confidence interval. C, spike-triggered EMG average using spiking activity of 20 dorsally located PAG units recorded from 9 rats during 10 sec sample of immobile behavior pre-conditioning (black line); during the 0-10 sec period of CS delivery in early extinction (red line), and 10-20s post CS (blue line). EMG data were rectified, smoothed (0.025s) and expressed as a z-score. Shading indicates S.E.M. D, same as C but constructed using spikes from ventrally located units (n=20 units, 9 rats during early extinction and 7 during late extinction). *,
P<0.05, ANOVA with Tukey’s posthoc test between peak in early extinction spike-triggered average (red) vs baseline (black).
References

An B, Hong I, Choi S (2012) Long-term neural correlates of reversible fear learning in the lateral amygdala. J Neurosci 32:16845–16856.

Bandler R, Depaulis A (1988) Elicitation of intraspecific defence reactions in the rat from midbrain periaqueductal grey by microinjection of kainic acid, without neurotoxic effects. Neurosci Lett 88:291–296.

Bandler R, Keay KA, Floyd N, Price J (2000) Central circuits mediating patterned autonomic activity during active vs. passive emotional coping. Brain Res Bull 53:95–104.

Bandler R, Shipley MT (1994) Columnar organization in the midbrain periaqueductal gray: modules for emotional expression? Trends Neurosci 17:379–389.

Bittencourt AS, Nakamura-Palacios EM, Mauad H, Tufik S, Schenberg LC (2005) Organization of electrically and chemically evoked defensive behaviors within the deeper collicular layers as compared to the periaqueductal gray matter of the rat. Neuroscience 133:873–892.

Blanchard RJ, Blanchard DC (1969) Crouching as an index of fear. J Comp Physiol Psychol 67:370–375.

Blum S, Hebert AE, Dash PK (2006) A role for the prefrontal cortex in recall of recent and remote memories. Neuroreport 17:341–344.

Bouton ME (2004) Context and Behavioral Processes in Extinction. Learn Mem 11:485–494.

Bowen MT, Kevin RC, May M, Staples LG, Hunt GE, McGregor IS (2013) Defensive aggregation (huddling) in Rattus norvegicus toward predator odor: individual differences, social buffering effects and neural correlates. PLoS One 8:e68483.

Burgos-Robles A, Vidal-Gonzalez I, Quirk GJ (2009) Sustained conditioned responses in prelimbic prefrontal neurons are correlated with fear expression and extinction failure. J Neurosci 29:8474–8482.

Carrive P (1993) The periaqueductal gray and defensive behavior: functional representation and neuronal organization. Behav Brain Res 58:27–47.

Carrive P, Leung P, Harris J, Paxinos G (1997) Conditioned fear to context is associated with increased Fos expression in the caudal ventrolateral region of the midbrain periaqueductal gray. Neuroscience 78:165–177.

De Oca BM, DeCola JP, Maren S, Fanselow MS (1998) Distinct regions of the periaqueductal gray are involved in the acquisition and expression of defensive responses. J Neurosci 18:3426–3432.

Deng H, Xiao X, Wang, Z (2016) Periaqueductal Gray Neuronal Activities Underlie Different Aspects of Defensive Behaviors. J Neurosci 36:7580-8.
Duvarci S, Popa D, Paré D (2011) Central amygdala activity during fear conditioning. J Neurosci 31:289–294.

Fanselow MS (1994) Neural organization of the defensive behavior system responsible for fear. Psychon Bull Rev 1:429–438.

Fanselow MS, Kim JJ, Young SL, Calcagnetti DJ, DeCola JP, Helmstetter FJ, Landeira-Fernandez J (1991) Differential effects of selective opioid peptide antagonists on the acquisition of pavlovian fear conditioning. Peptides 12:1033–1037.

Furlong, TM, Richardson R, McNally GP (2016) Habituation and extinction of fear recruit overlapping forebrain structures. Neurobiology of Learning and Memory 128:7-16.

Halladay LR, Blair HT (2015) Distinct ensembles of medial prefrontal cortex neurons are activated by threatening stimuli that elicit excitation vs. inhibition of movement. J Neurophysiol 114:793–807.

Herrero MT, Insausti R, Gonzalo LM (1991) Cortically projecting cells in the periaqueductal gray matter of the rat. A retrograde fluorescent tracer study. Brain Res 543:201–212.

Herry C, Ciocchi S, Senn V, Demmou L, Müller C, Lüthi A (2008) Switching on and off fear by distinct neuronal circuits. Nature 454:600–606.

Hobin JA, Goosens KA, Maren S (2003) Context-dependent neuronal activity in the lateral amygdala represents fear memories after extinction. J Neurosci 23:8410–8416.

Johansen JP, Tarpley JW, LeDoux JE, Blair HT (2010) Neural substrates for expectation-modulated fear learning in the amygdala and periaqueductal gray. Nat Neurosci 13:979–986.

Kim EJ, Horovitz O, Pellman BA, Tan LM, Li Q, Richter-Levin G, Kim JJ (2013) Dorsal periaqueductal gray-amygdala pathway conveys both innate and learned fear responses in rats. Proc Natl Acad Sci U S A 110:14795–14800.

Kim JJ, Rison RA, Fanselow MS (1993) Effects of amygdala, hippocampus, and periaqueductal gray lesions on short- and long-term contextual fear. Behav Neurosci 107:1093–1098.

Koutsikou S, Crook JJ, Earl E V, Leith JL, Watson TC, Lumb BM, Apps R (2014) Neural substrates underlying fear-evoked freezing: the periaqueductal grey-cerebellar link. J Physiol 592:2197–2213.

Krieger JE, Graeff FG (1985) Defensive behavior and hypertension induced by glutamate in the midbrain central gray of the rat. Brazilian J Med Biol Res.
LeDoux JE, Iwata J, Cicchetti P, Reis DJ (1988) Different projections of the central amygdaloid nucleus mediate autonomic and behavioral correlates of conditioned fear. J Neurosci 8:2517–2529.

Leman S, Dielenberg RA, Cariolle P (2003) Effect of dorsal periaqueductal gray lesion on cardiovascular and behavioural responses to contextual conditioned fear in rats. Behav Brain Res 143:169–176.

Milad MR, Orr SP, Lasko NB, Chang Y, Rauch SL, Pitman RK (2008) Presence and acquired origin of reduced recall for fear extinction in PTSD: results of a twin study. J Psychiatr Res 42:515–520.

Muramoto K, Ono T, Nishijo H, Fukuda M (1993) Rat amygdaloid neuron responses during auditory discrimination. Neuroscience 52:621–636.

Quirk GJ, Repa C, LeDoux JE (1995) Fear conditioning enhances short-latency auditory responses of lateral amygdala neurons: parallel recordings in the freely behaving rat. Neuron 15:1029–1039.

Quirk GJ, Armony JL, LeDoux JE (1997) Fear conditioning enhances different temporal components of tone-evoked spike trains in auditory cortex and lateral amygdala. Neuron 19:613-624.

Sierra-Mercado D, Padilla-Coreano N, Quirk GJ (2011) Dissociable roles of prelimbic and infralimbic cortices, ventral hippocampus, and basolateral amygdala in the expression and extinction of conditioned fear. Neuropsychopharmacology 36:529–538.

Steenland HW, Zhuo M (2009) Neck electromyography is an effective measure of fear behavior. J Neurosci Methods 177:355–360.

Todd TP, Vurbic D, Bouton ME (2014) Behavioral and neurobiological mechanisms of extinction in Pavlovian and instrumental learning. Neurobiol Learn Mem 108:52–64.

Tovote P, Fadok JP, Lüthi A (2015) Neuronal circuits for fear and anxiety. Nat Rev Neurosci 16:317–331.

Tovote P, Esposito MS, Botta P, Chaudun F, Fadok JP, Markovic M, Wolff SBE,

Ramakrishnan C, Fermo L, Deisseroth K, Henry C, Arber S, Lüthi A (2016) Midbrain circuits for defensive behaviour. Nature 534: 206-12.

Vianna DM, Graeff FG, Brandão ML, Landeira-Fernandez J (2001a) Defensive freezing evoked by electrical stimulation of the periaqueductal gray: comparison between dorsolateral and ventrolateral regions. Neureport 12:4109–4112.

Vianna DM, Landeira-Fernandez J, Brandão ML (2001b) Dorsolateral and ventral regions of the periaqueductal gray matter are involved in distinct types of fear. Neurosci Biobehav Rev 25:711–719.

Walker DL, Davis M (1997) Involvement of the dorsal periaqueductal gray in the loss of fear-potentiated startle accompanying high footshock training. Behav Neurosci 111:692–702.
Wang L, Chen IZ, Lin D (2015) Collateral Pathways from the Ventromedial Hypothalamus Mediate Defensive Behaviors. Neuron 85:1344–1358.

Zhang SP, Bandler R, Carrive P (1990) Flight and immobility evoked by excitatory amino acid microinjection within distinct parts of the subventrional midbrain periaqueductal gray of the cat. Brain Res 520:73–82.
A

Freezing (% Time)

Day 1  Day 2  Day 3
Hab. Conditioning Extinction Training

Pre-CS  EE

B

Baseline  CS block 1

EMG

Immobility  Freezing

F1

60s

C

Freezing (% Time)

Day 4

***

Homecage  Cat Odor
A. Dorsal Cells

Firing Rate (Z-score)

-200 -100 0 100 200

Time (s)

B. Ventral cells

Firing Rate (Z-score)

-200 -100 0 100 200

Time (s)

C. 37% Increase 63% No change

D. 50% 50%

E. Normalised EMG Amplitude (Z-score)

-2 -1 0 1 2 3

Time (s)

F. Normalised EMG Amplitude (Z-score)

-2 -1 0 1 2 3

Time (s)
A. Dorsal PAG

B. Ventral PAG

Freezing (% Time of each 30s trial)

- Block 1
- Block 2
- Block 3
- Block 4
- Block 5
- Block 6
- Block 7

Rate (Hz)

Time (s)
