

The Organization of Defensive Behavior Elicited by Optogenetic Excitation of Rat Lateral or Ventrolateral Periaqueductal Gray

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Rodents display characteristic defense responses to predators that are influenced by predatory imminence. The midbrain periaqueductal gray (PAG) serves an important role controlling these responses. The most influential model states that variations in defensive topography are due to distinct PAG regions: ventrolateral PAG (VLPAG) controls postencounter defense, such as freezing and immobility, whereas lateral PAG (LPAG) controls circa-strike defense, such as escape and flight. Here we used channel rhodopsin (ChR2) stimulation to probe the structure of defensive behavior controlled by LPAG and VLPAG. Suprathreshold LPAG stimulation evoked circa-strike defense that was replaced by freezing at stimulation offset. Suprathreshold VLPAG stimulation evoked postencounter—freezing and immobility—but never circa-strike defense. More interestingly, the topography of defensive behavior evoked from LPAG scaled with variations in 465 nm light power. As light power increased, LPAG animals expressed the full defensive syntax of freezing then activity then flight characteristic of increasing predatory imminence. In contrast, the frequency, not topography, of defensive behavior evoked from VLPAG scaled with variations in light power. These findings suggest that LPAG and VLPAG can control variations in defense with increasing predatory imminence in 2 ways. First, consistent with past models, topographical variation can be assembled from different defensive responses controlled by the LPAG (circa-strike) and VLPAG (postencounter). Second, topographical variation can be assembled from variations in LPAG activity itself.

Keywords: fear, freezing, channel rhodopsin 2, anxiety, panic

Rodents display characteristic defensive responses to predators, the topography of which are determined by predatory imminence (R. J. Blanchard & Blanchard, 1971; Bolles & Fanselow, 1980; Fanselow, 1991; Fanselow & Lester, 1988). After encountering distal threats, rodents express the species-typical defense response of freezing, a crouching immobile posture to reduce probability of detection. However, as predatory imminence and the potential of attack increases, rodents switch from freezing to circa-strike defense, including escape attempts and jumps. Upon successful escape, they engage in recuperative behaviors, including quiescent immobility (Bolles & Fanselow, 1980).

The midbrain periaqueductal gray (PAG) has long been recognized as essential for these defensive behaviors (D. C. Blanchard, Williams, Lee, & Blanchard, 1981; Carrive, 1993; Depaulis & Bandler, 1991; Fanselow, 1991). The PAG is divided into four columns located dorsomedial, dorsolateral, lateral, and ventrolat-

eral to the cerebral aqueduct. Dorsally the PAG borders superior colliculus and ventrally the dorsal raphe. The PAG receives extensive descending projections from prefrontal cortex (including cingulate, prelimbic, infralimbic, and orbital), extended amygdala (especially central nucleus and bed nucleus), and ascending projections from spinal and trigeminal dorsal horn (Floyd, Price, Ferry, & Keay, 2006; Keay & Bandler, 2004; Rizvi, Ennis, Behbehani, & Shipley, 1991). The PAG, in turn, has significant ascending projections to hypothalamus, midline and intralaminar thalamus (Krout & Loewy, 2000), as well as descending projections to premotor and sensory regions in the brainstem and spinal cord (Keay & Bandler, 2004). Focal electrical stimulation of PAG elicits analgesia (Akil, Mayer, & Liebeskind, 1976; Reynolds, 1969; Richardson & Akil, 1977a, 1977b) that in humans is accompanied by feelings of dread and impending doom (Carrive & Morgan, 2003; Nashold, Wilson, & Slaughter, 1969). Human neuroimaging studies show fMRI bold signals in PAG in response to dangerous, painful, or aversive stimuli (Borras et al., 2004; Mobbs et al., 2007; Ploghaus et al., 2000). Moreover, manipulation of PAG function in rats and cats via microinjection of excitatory amino acids, GABA receptor antagonists, or focal electrical stimulation elicits profound and robust defensive behavior integrated with alterations in vocalization and autonomic function (Bandler & Depaulis, 1991; Bandler & Shipley, 1994; Bandler, Carrive, & Zhang, 1991; Carrive, 1993; Depaulis & Bandler, 1991; Fanselow, 1991). Importantly, the topography of these defensive behaviors depend on the specific PAG column manipulated. Manipulations of dorsomedial (DMPAG) or dorsolateral PAG (DLPAG), typically including superior colliculus (Brandão, de Aguiar, & Graeff,

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1982; Vianna, Graeff, Brandão, & Landeira-Fernandez, 2001), or LPAG (Bandler & Shipley, 1994; Fanselow, 1991; S. P. Zhang, Bandler, & Carrive, 1990) evoke ballistic escape and flight responses, whereas these manipulations of VLPAG evoke freezing and immobility (Bandler & Depaulis, 1991; Carrive, 1993; Fanselow, 1991).

These findings led to an influential model of PAG function ascribing postencounter defense, such as freezing, to VLPAG, and circa-strike defense, such as escape and flight, to LPAG (Fanselow, 1991). This model is important not just because it provided a coherent account of how PAG controls variations in topography of defensive behavior following threat detection, but also because it was integrated into a broader functionalist account of Pavlovian conditioning (Fanselow & Lester, 1988). According to this model, unconditioned responses to painful stimuli are controlled by LPAG and consist of vigorous, active circa-strike behavior. In contrast, conditioned responses to stimuli that signal painful stimuli are controlled by VLPAG and typically consist of freezing and immobility. Indeed, consistent with this model, studies of neuronal activity marker expression show significant recruitment of VLPAG during expression of conditioned freezing responses (Carrive, Leung, Harris, & Paxinos, 1997; Walker & Carrive, 2003) and lesion or pharmacological manipulation of VLPAG disrupt expression of freezing as a fear conditioned response (Amorapanth, Nader, & Ledoux, 1999; De Oca, DeCola, Maren, & Fanselow, 1998; Ledoux, Iwata, Cicchetti, & Reis, 1988; Walker & Carrive, 2003).

However, some findings are inconsistent with the claims of this model regarding LPAG and VLPAG function. For example, in some studies, circa-strike behaviors are not the only defense behaviors observed following LPAG stimulation. LPAG excitatory amino acid microinjections can cause freezing (Bandler & Depaulis, 1991; Depaulis, Keay, & Bandler, 1992), although such freezing typically appears between bouts of escape, making interpretation of its place in the structure of defensive behavior difficult. Focal electrical stimulation of DLPAG, which impinged on LPAG, also elicits freezing (Vianna et al., 2001). In addition, there have been reports of circa-strike escape behavior following focal electrical stimulation of VLPAG (Vianna et al., 2001). Finally, freezing responses observed following excitation of LPAG and VLPAG can reflect different defensive profiles, with the former accompanied by hyper-reactivity and the latter by hypo-reactivity to environmental stimuli (Bandler et al., 1991; Bandler & Depaulis, 1991; Carrive, 1993).

One problem in evaluating these discrepant findings regarding LPAG and VLPAG function is that these studies have used either focal electrical stimulation or chemical excitation. The disadvantage of the former is that it cannot discriminate between effects on cell bodies versus axons passing through the various PAG columns. The disadvantage of the latter is that it lacks reversibility and hence is not always helpful in determining the temporal structure of defensive behavior controlled by LPAG and VLPAG. Recently, optogenetic approaches have been used to address these limitations and to manipulate DMPAG and DLPAG (S. Chen et al., 2015) or specific inputs to PAG (Silva et al., 2013; Wang, Chen, & Lin, 2015). Consistent with past focal electrical stimulation studies and models of PAG function, Chen et al. (2015) reported that ChR2 excitation of rat DMPAG or DLPAG evoked freezing and flight that depended on both the frequency and

intensity of stimulation (S. Chen et al., 2015). However, the frequency of these behaviors, their temporal structure before, during and after stimulation, as well as any relationships between them, were not reported. More importantly for present purposes, the effects of this stimulation in LPAG and VLPAG are unknown. Indeed, studies manipulating hypothalamic inputs to LPAG and VLPAG (Silva et al., 2013; Wang et al., 2015), have not revealed the variation in topography of defensive behavior observed in the earlier literature.

Here we used an optogenetic approach to study the organization of defensive behavior elicited by excitation of rat LPAG and VLPAG. We expressed channel rhodopsin 2 (ChR2) (F. Zhang, Wang, Boyden, & Deisseroth, 2006) in rat PAG neurons and implanted fiber optic cannulas above LPAG or VLPAG. We then determined the effects of LPAG or VLPAG optogenetic stimulation on the topography and organization of defensive behavior. There were two questions of interest. First, does ChR2 stimulation of LPAG or VLPAG elicit defensive behaviors? Second, are there differences between the LPAG and VLPAG in the topography or intensity of these behaviors?

Method

Subjects

Twenty-six male Sprague–Dawley rats (330–380 g) were used as subjects in these experiments. They were obtained from the Animal Resources Centre, Perth, Australia. Rats were housed in groups of four in Plexiglas boxes in ventilated racks located in a colony room maintained on 12:12 hr light/dark cycle. All experiments were completed during the light cycle. Rats had free access to food and water for the duration of the experiments. All procedures were approved by the University of New South Wales's Animal Care and Ethics Committee and are consistent with the NIH Guidelines on the Care and Use of Laboratory Animals.

Surgery

Rats were treated with Carprofen (5 mg/kg), procaine penicillin (0.3 ml, 300 mg/ml), and cephazolin (0.3 ml, 100 mg/ml) prior to being anesthetized with ketamine/xylazine, 0.5 μ l AAV5-hSyn-eYFP (4×10^{12} viral particles (vp)/ml titer) or AAV5-hSyn-ChR2 (H134R)-eYFP (3×10^{12} vp/ml; all vectors were obtained from UNC Vector Core). Vectors were infused unilaterally (3-min injection, 7-min diffusion) into LPAG (AP: -8.3 , ML: -1.85 , DV: -5.6) or VLPAG (AP: -8.3 , ML: -1.85 , DV: -6.6) followed by unilateral implantation of custom made fiber optic cannulas (400 μ m/0.39NA) (Sparta et al., 2012) targeting LPAG (AP: -8.3 , ML: -1.85 , DV: -5.3) or VLPAG (AP: -8.3 , ML: -1.85 , DV: -6.1) using standard stereotaxic technique. All coordinates are in mm bregma at 10° angle. Rats were allowed 21 days recovery prior to the start of the experiment.

Apparatus

A transparent plastic circular bowl (35 cm depth, 40 cm diameter) with corn cob bedding served as the test chamber. It was located in a sound attenuating box with digital video cameras located above as well as behind the bowl allowing an unobstructed

view of the animal. Optical stimulation was provided by 465 nm LEDs (Doric Instruments, Quebec, Canada) controlled by a programmable LED driver (Doric Instruments, Quebec, Canada) and connected to the fiber optic cannula via 400 μm /0.39NA patch cables.

Procedure

Rats received 4 days 30 min/day pre-exposure to the test chamber. This was intended to habituate them to the test chamber. Animals were tethered via patch cables for the final 2 preexposure sessions. For stimulation sessions, rats were first exposed to the bowl for 3 min in the absence of any stimulation. They then received 4×10 s stimulations (20 Hz, 50% duty cycle with 2 min intertrial interval [ITI]). Twenty Hz was used because low frequency (5 Hz) stimulation of DMPAG or DLPAG does not yield significant increases in spike rates in vivo but higher frequency stimulation (20 Hz or 50 Hz) does (S. Chen et al., 2015). The H134R variant of ChR2 used here has excellent fidelity at 20 Hz and poor fidelity at higher frequencies (J. Y. Lin, 2011; Yizhar, Fenno, Davidson, Mogri, & Deisseroth, 2011). The intensity was 8–10 mW from the fiber tip, to yield predicted irradiances of 8.7 mw/mm^2 at 0.1 mm from the fiber tip and 3.43 mw/mm^2 at 0.3 mm from the fiber tip. A different cohort of animals was used to manipulate light power. The procedures for pre-exposure were the same, but the test session involved 10-s trials (470 nm, 20 Hz, 50% Duty cycle) with the LED driver output commencing at 25 mA, then 50 mA and incrementing in 50mA steps (30 s ITI) across trials. The protocol was terminated upon detection of flight/escape responses or when 1,000 mA was reached.

Histology

After completion of the experiment, rats were deeply anesthetized with sodium pentobarbital (100 mg/kg i.p.) and perfused transcardially with 200 ml of 0.9% saline, containing 1% sodium nitrite and heparin (5,000 i.u./ml), followed by 400 ml of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were kept in the same fixative solution for 1 hr and then transferred to 20% sucrose solution overnight fridge temperature. The brains were sectioned into 40 μm coronal sections using a cryostat (CM 1950, Leica Microsystems, Wetzlar, Germany).

Four serially adjacent sets of sections containing the periaqueductal gray were collected from each and stored in 0.1% sodium azide in 0.1 M PBS, pH 7.2 prior to staining. Free-floating sections were washed 3 times for 10 min in 0.1 M phosphate buffered saline–Triton x-100 (0.2%, pH 7.2), followed by 2 hr incubation in the blocking buffer. The blocking buffer contained 2.5% normal horse serum in phosphate buffered saline–Triton x-100 (0.2%). Sections were then incubated in the primary antibody, anti-green fluorescent protein, rabbit IgG fraction (GFP/YFP; 1:1000; A11122, Life Technologies, Sydney, Australia), diluted in the blocking buffer for 48 hr at 4° C. Excess primary antibody was removed via phosphate buffered saline–Triton washes (3×10 min), sections were then incubated for 2 hr at room temperature in secondary antibody; Alexa Fluor 488 goat anti-rabbit diluted in blocking buffer (1:750, Invitrogen, 488). Excess secondary antibody was removed via washes with phosphate buffered saline–Triton (4×10 mins). The sections were also stained with 4,

6-D-amidino-2-Phenylindole (DAPI; Sigma, D9542) for 5 min. Finally, sections were washed with phosphate buffered saline (1×10 mins), mounted onto gelatin-treated slides and cover slipped with PermaFluor (Thermo Scientific). Sections were analyzed using an Olympus BX-53 Fluorescence microscope based on the atlas of Paxinos and Watson (Paxinos & Watson, 2007).

Data Analysis

Video records of rat's behavior during stimulation sessions was scored by two observers, one of whom was unaware of the group allocations. These observers assessed the behavior of the rats every 2 s for the duration of the session and recorded the following behaviors: *freezing* (a crouching immobile posture with the absence of any movement other than that required for respiration), *flight* (explosive running and jumping/escape attempts), *rearing* and *activity* (normal locomotor activity and/or standing on hind legs), *grooming* (any self-directed licking or mouthing), and *rest* (immobile with little muscle tone). Inter-rater reliabilities exceeded 0.95. The frequencies of these behaviors were converted into a percentage of the total number of observations during the observation period. These percentages were analyzed by means of planned contrasts assessing the differences between groups. The Type I error rate (α) was controlled at the 0.05 level (Bird, 2004).

Results

Histology

Figure 1A shows representative ChR2 expression and fiber optic placements in the LPAG and VLPAG and Figure 1B shows the location of fiber tips for all animals included in the analyses. In order to be included in analyses, both ChR2 and optic fiber placement had to be located in LPAG or VLPAG. Seven animals were excluded due to either ChR2 or fiber misplacement. Three animals were excluded because fiber tips were located in the middle of the LPAG and hence optical stimulation would have affected both ventral portions of the LPAG as well as the VLPAG. Two were animals excluded because of breaks in the patch cables during the experiment. There were 5 animals in the VLPAG–ChR2 group, 7 animals in the LPAG–ChR2 group and 4 animals in the eYFP group (2 with fibers in LPAG and 2 in VLPAG).

Figure 1C provides an overall summary across the categories of behaviors observed in the 3 min pre-stimulation period (pre) and the 7 min after onset of first stimulation (post). Prior to stimulation rats were resting, interrupted by occasional bouts of rearing and grooming. There were very low levels of defensive behaviors (freezing or flight). There was no difference between groups in resting, grooming, or defense, all $F(1, 13) < 1.59$, but the VLPAG group ($n = 5$), $F(1, 13) = 5.82$, $p < .05$, and not the LPAG group ($n = 7$), $F(1, 13) < 1$, $p > .05$, showed more rearing than the eYFP group ($n = 4$).

During the 7 min after the onset of first stimulation, both LPAG, $F(1, 13) = 13.44$, $p < .05$, and VLPAG groups, $F(1, 13) = 12.52$, $p < .05$, expressed significantly more defensive behavior (freezing or flight) than the eYFP group. In both regions, the dominant defensive behavior across the entire observation period was freezing. Moreover, in both regions there was significantly more freezing than flight observed during the entire observation period

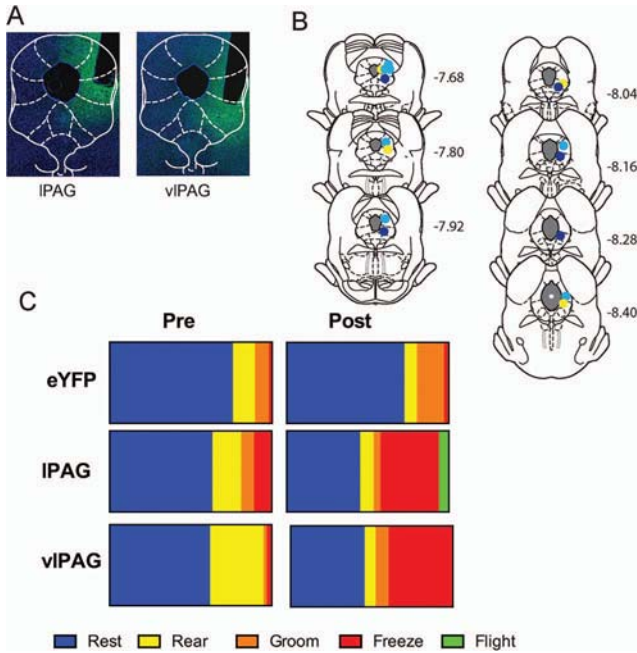


Figure 1. A: Representative ChR2-eYFP expression, fiber optic cannula placement in lateral periaqueductal gray (LPAG) or ventrolateral periaqueductal gray (VLPAG). B: Location of all fiber optic cannula tips in LPAG or VLPAG for eYFP (yellow), LPAG-ChR2 (royal blue), and VLPAG-ChR2 (navy blue). C: The overall relative frequencies of behavior observed in the 3 min prior to first stimulation and the 7 min during and after the first stimulation. See the online article for the color version of this figure.

(VLPAG paired t test, $p < .05$; LPAG paired t test, $p < .05$) (Figure 1C). The ChR2 groups also expressed significantly less resting, $F(1, 13) = 8.19$ (VLPAG vs. eYFP), $p < .05$, or 8.78 (LPAG vs. eYFP), $p < .05$, but not grooming, $F(1, 13) = 3.58$ (VLPAG vs. eYFP) $p > .05$, or 6.39 (LPAG vs. eYFP), $p > .05$, compared to eYFP animals.

To examine the immediate impact of LPAG and VLPAG ChR2 stimulation, we restricted analyses to the three 10-s epochs preceding, during, and poststimulation. First we examined the average of these three epochs across the 4 stimulation trials (Figure 2A). There was significantly more freezing for VLPAG, $F(1, 13) = 40.68$, $p < .05$, and LPAG, $F(1, 13) = 12.75$, $p < .05$, compared to eYFP, but this freezing had distinct temporal properties. For VLPAG, freezing increased significantly at stimulation onset and decreased significantly at stimulation offset, $F(1, 4) = 101.29$, $p < .05$. For LPAG, the trend toward increasing freezing at stimulation offset approached but did not reach significance averaged across the four trials, $F(1, 6) = 3.85$, $p < .05$, however it was significant on the first trial, $F(1, 6) = 9.55$, $p < .05$ (Figure 2B). LPAG ChR2 stimulation, but not VLPAG stimulation, elicited flight (LPAG vs. eYFP: $F(1, 13) = 28.36$, $p < .05$; VLPAG vs. eYFP: $F(1, 13) = 0$, $p > .05$) and this was exclusively stimulation bound, $F(1, 6) = 36.00$, $p < .05$, including on the first trial (Figure 2B). So, VLPAG ChR2 stimulation elicited freezing bound to stimulation whereas LPAG ChR2 stimulation elicited flight which was replaced by freezing at stimulation offset.

To examine behavior change we examined stimulation elicited behavior on a trial-by-trial basis (Figure 3A). For LPAG, there was significantly more flight than freezing during stimulation, $F(1, 6) = 6.34$, $p < .05$, and flight significantly decreased across trials, $F(1, 6) = 6.65$, $p < .05$ but freezing did not significantly change, $F(1, 6) = 3.37$, $p > .05$. For VLPAG, there was significantly more freezing than flight, $F(1, 4) = 109.65$, $p < .05$ and there was no significant change in either of these behaviors across trials ($F(1, 4) < 1$, $p < .05$). Nonetheless, there were individual differences in topography of defensive behavior evoked by LPAG ChR2 stimulation across trials (Figure 3B). Whereas some rats showed reductions in flight and increases in freezing across trials—suggesting conversion from circa-strike to postencounter defense across stimulations—others did not and one animal switched between the two defensive behaviors on each trial.

We next assessed whether topography of defensive behavior during PAG ChR2 stimulation was influenced by stimulation power. In a separate cohort of animals ($n = 5$ LPAG and $n = 5$ VLPAG, Figure 4A), we manipulated LED power to determine the threshold at which defensive behaviors were observed and changes in these behaviors as power was increased (see Figure 4). We commenced at 25 mA, then 50 mA and incremented thereafter in

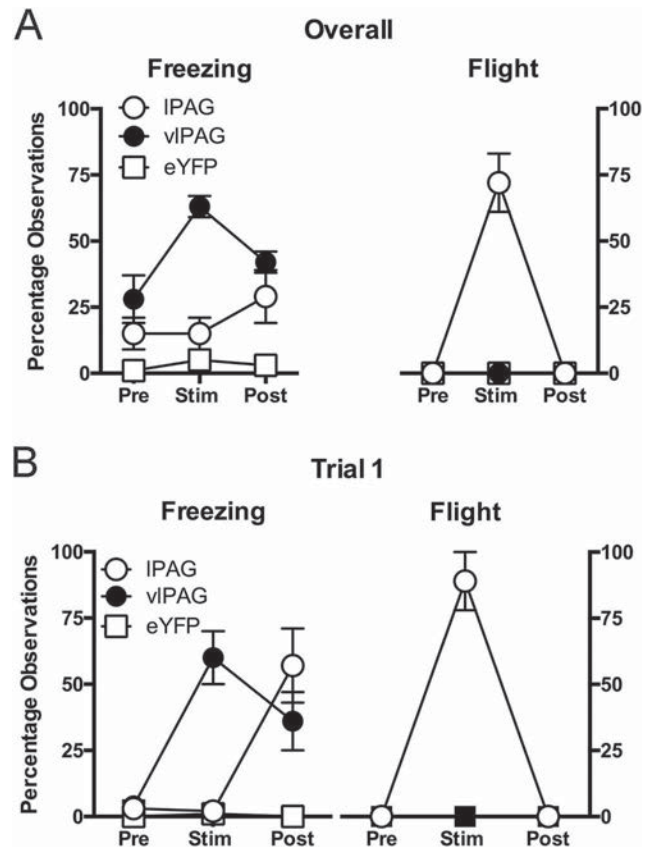


Figure 2. A: Mean ($\pm$) SEM percentages of observations scored as freezing or flight during the 10 s preceding ChR2 stimulation, the 10s of ChR2 stimulation and the 10 s post stimulation, averaged across the four stimulation trials. B: Mean ($\pm$) SEM percentages of observations scored as freezing and flight during the first stimulation trial. LPAG = lateral periaqueductal gray; VLPAG = ventrolateral periaqueductal gray.

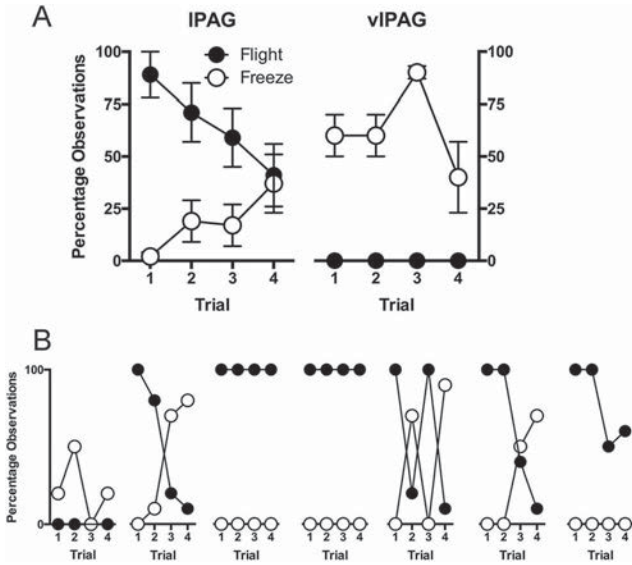


Figure 3. A: Mean ($\pm$) SEM percentages of observations scored as freezing or flight during the 10 s of ChR2 stimulation across the four stimulation trials. B: Individual profiles of defensive behavior across the four stimulation trials for LPAG. LPAG = lateral periaqueductal gray; VLPAG = ventrolateral periaqueductal gray.

50 mA steps. The protocol was terminated upon detection of flight/escape responses or when 1000mA was reached. We recorded the power at which the first instance of defensive behavior was observed, regardless of the frequency of this behavior during stimulation (i.e., the threshold), and we also scored the frequency of behavior at this threshold stimulation.

Figure 4B shows the mean $\pm$ SEM threshold (in mW from an unimplanted fiber tip) for emergence of defensive behavior during PAG ChR2 stimulation. Table 1 shows the LED driver current (mA), light power from fiber tip (mW), and predicted irradiance values (mW/mm²) in PAG for the appearance of each behavior. For LPAG there was a clear relationship between light power and topography of defensive behavior. At low powers, ChR2 stimulation elicited freezing in all animals and, as power increased, freezing was replaced by stimulation bound bursts of locomotor activity in all animals. This locomotor activity was distinguished from flight due to the absence of jumping/escape attempts. Finally, for four or five animals, locomotor activity was replaced by flight and escape jumping at higher power. The overall power eliciting these behaviors were very low. For all LPAG animals, freezing was evoked at significantly lower power than activity, $F(1, 4) = 26.00$, $p < .05$, and for the animals expressing both activity and flight, activity was evoked at significantly lower power than flight, $F(1, 3) = 8.65$, $p < .05$. So, for LPAG animals expressing the full sequence of freezing then activity then flight ($n = 4$), there was a significant linear relationship between topography of behavior and intensity of stimulation, $F(1, 3) = 26.42$, $p < .05$. Thus, when systematically manipulating intensity of LPAG stimulation there was very little, if any, variation between animals. Rather, there was an orderly transition from postencounter to circa-strike defense. In marked contrast, VLPAG ChR2 stimulation elicited freezing and very occasional bouts of locomotor activity but never flight (Fig-

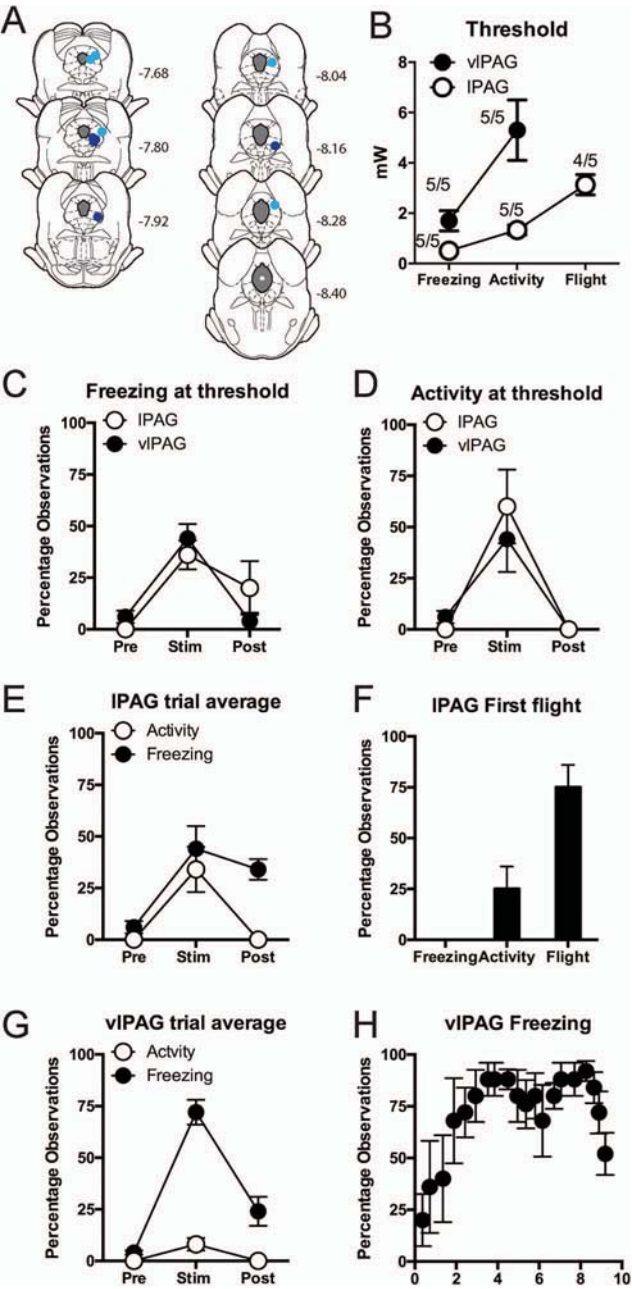


Figure 4. A: Fiber optic cannula placement in LPAG (royal blue) or VLPAG (navy blue). B: Mean ($\pm$) SEM 465 nm light power required to elicit the first bout of freezing, activity, and flight for VLPAG and LPAG. Numbers above and below symbols show number of animals in each group expressing that behavior. C: Mean ($\pm$) SEM percentages of observations scored as freezing at threshold stimulation for LPAG and VLPAG. D: Mean ($\pm$) SEM percentages of observations scored as activity at threshold stimulation for LPAG and VLPAG. E: Mean ($\pm$) SEM percentages of observations scored as freezing or activity across all trials prior to the trial that elicited flight, for LPAG. F: Mean ($\pm$) SEM percentages of observations scored as freezing, activity, or flight at threshold stimulation for flight. G: Mean ($\pm$) SEM percentages of observations scored as freezing or activity across all trials for VLPAG. H: Mean ($\pm$) SEM percentages of observations scored as freezing across variations in 465 nm light power for VLPAG stimulation. LPAG = lateral periaqueductal gray; VLPAG = ventrolateral periaqueductal gray. See the online article for the color version of this figure.

Table 1
Threshold Stimulation Parameters for Appearance of Defensive Behaviors From LPAG and VLPAG

Parameter	Freezing		Activity		Flight	
	LPAG	VLPAG	LPAG	VLPAG	LPAG	VLPAG
LED current (mA)	35	140	100	500	275	N/A
mW from fibre tip	.5	1.74	1.36	5.3	3.7	N/A
Predicted irradiance (mw/mm ²)	.48	1.61	1.31	5.12	3.57	N/A

Note. LPAG = lateral periaqueductal gray; VLPAG = ventrolateral periaqueductal gray; LED = light-emitting diode. Predicted irradiance values .1 mm below implanted fiber tip were determined using the calculator at <http://web.stanford.edu/group/dlab/cgi-bin/graph/chart.php> for a 400- μ m .39NA Fibre.

ure 4B). Freezing was elicited by significantly lower power than activity $F(1, 4) = 8.44, p < .05$. Despite both VLPAG and LPAG ChR2 stimulation eliciting freezing and activity, significantly more power was required to elicit the first bout of freezing, $F(1, 8) = 6.73, p < .05$, and first bout of activity, $F(1, 8) = 8.77, p < .05$, after VLPAG ChR2 stimulation compared to LPAG ChR2 stimulation (Figure 4B).

The first time freezing or activity were evoked from stimulation, there were no obvious differences between LPAG and VLPAG (Figures 4C and 4D). The frequency of these defensive behaviors at their respective thresholds did not differ between LPAG and VLPAG, $F_s(1, 8) < 1, p > .05$, and for both regions freezing, $F(1, 8) = 38.53, p < .05$, and activity, $F(1, 8) = 18.78, p < .05$, were stimulation bound.

We continued increasing intensity of stimulation until animals expressed flight or stimulation reached 1000mA. For LPAG we examined the frequencies of freezing and activity across all trials prior to the trial that elicited flight (Figure 4E). Average freezing and activity did not differ, $F(1, 4) = 4.48, p > .05$, across these trials, both increased during stimulation and decreased poststimulation, $F(1, 4) = 115.6, p < .05$, at the same rate, $F(1, 4) < 1, p > .05$ (i.e., although freezing tended to persist more into the post-stimulation period more than activity this did not reach significance). On the first LPAG trial that animals expressed flight (and the last trial for the experiment) there was no stimulation-elicited freezing and low levels of stimulation-elicited activity (Figure 4F), so that there were significantly more flight attempts than activity or freezing, $F(1, 3) = 10.97, p < .05$. Each animal that expressed flight showed a transition from quiescence to activity then flight during the threshold stimulation.

For VLPAG, averaged across all intensities, there was significantly more freezing than activity, $F(1, 4) = 64.43, p < .05$ (Figure 4G), both increased during stimulation and decreased poststimulation, $F(1, 4) = 68.97, p < .05$, and this increase and decrease were greater for freezing than activity, $F(1, 4) = 61.99, p < .05$. Interestingly, the frequency of freezing changed across stimulation intensity (Figure 4H). There was a quadratic relationship between freezing and power of VLPAG ChR2 stimulation, $F(1, 4) = 12.23, p < .05 (R^2 = 0.75)$. So, although the topography of defensive behavior evoked by VLPAG ChR2 stimulation was not altered by variations in light power, the frequency of that behavior was.

Discussion

Here we examined the organization of defensive behavior elicited by optogenetic excitation of rat PAG. There are three main findings. First, consistent with previous focal electrical and chemical stimulation studies, PAG ChR2 stimulation evoked pronounced defensive behaviors and the topography of these behaviors was determined, in part, by the PAG column stimulated. ChR2 stimulation of LPAG and VLPAG both evoked freezing whereas stimulation of LPAG additionally evoked escape/flight. Moreover, some animals alternated between these two defensive behaviors across stimulation trials.

Second, the topography of defensive behavior evoked from LPAG, but not VLPAG, ChR2 stimulation scaled with stimulation intensity. At low intensity, LPAG ChR2 stimulation evoked freezing. As stimulation power increased, freezing was replaced by locomotor activity and then by flight/escape. This defensive syntax of freezing then activity then flight is a defining characteristic of defense in response to increasing predatory imminence (Fanselow, 1991; Fanselow & Lester, 1988). These findings from LPAG are similar to those reported for ChR2 stimulation of DMPAG and DLPAG (S. Chen et al., 2015), suggesting a possible functional relationship between lateral and dorsal PAG regions in defense. In contrast, no such variations in topography were observed for VLPAG.

Third, there were differences between LPAG and VLPAG. At suprathreshold intensity, behavior evoked from VLPAG was relatively stable across trials whereas that evoked from LPAG was not. At these suprathreshold levels, some LPAG animals switched defensive behavior between flight and freezing across trials whereas others did not. The reason for this variation is unclear. It could reflect individual differences in response strategies when threat or predatory imminence is high. The overall decrease in flight across trials could reflect differences in learning, for example that the experimenter would always return the animal to the testing chamber if flight/escape was successful and/or the conditioning of fear to the apparatus. According to this possibility, ChR2 LPAG stimulation serves as an aversive unconditioned stimulus to condition fear to the apparatus cues. This could also explain the persistence of freezing into the poststimulation period because this tended to be observed on later stimulation trials (Figure 4E) rather than the first stimulation trial. The merit of this explanation is unclear. Animals were extensively pre-exposed to the apparatus to reduce any conditioning that might occur.

Another important difference between LPAG and VLPAG is that the topography of defensive behaviors evoked from LPAG and the frequency of defensive behavior evoked from VLPAG scaled with stimulation intensity. Moreover, even when LPAG and VLPAG stimulation elicited the same behavior (freezing) there were differences. For example, significantly higher intensities were required to elicit freezing from VLPAG compared to LPAG. It is unlikely that these differences between LPAG and VLPAG were due to differences in ChR2 expression because there were high levels of ChR2 expression in all animals included in the analyses. Nor are they readily attributable to penetration of the 465 nm light from LPAG into VLPAG for animals with LPAG canulas (this would predict the opposite pattern of results). Rather, the differences between these columns likely reflect differences in the structure of defensive behavior controlled by them. They are

reminiscent of the distinction between reactive and hyporeactive immobility following chemical excitation of PAG (Bandler et al., 1991; Bandler & Depaulis, 1991; Carrive, 1993). Reactive immobility (LPAG) is characterized by increased reactivity to other stimuli, emission of ultrasonic vocalizations, and autonomic arousal; hyporeactive immobility (VLPAG) is characterized by somatomotor and autonomic inhibition (Carrive, 1993). This distinction speaks to the function of VLPAG in defense (Bolles & Fanselow, 1980; Fanselow, 1991; Fanselow & Lester, 1988) versus recuperation (Carrive, 1993). It will be of interest to combine ChR2 stimulation of LPAG and VLPAG with measurements of autonomic function to further assess this.

Together, these findings offer support for the standard model of PAG function (Fanselow, 1991) but suggest an important caveat. In this model, LPAG controls circa-strike defense such as escape and VLPAG controls postencounter defense such as freezing and immobility (Fanselow, 1991). Our findings at suprathreshold intensity are generally consistent with this model and with past findings. LPAG stimulation evoked circa-strike defense that was replaced by freezing at stimulation offset. Across trials, circa-strike defense decreased at the group level, with freezing observed for some animals on some trials (Vianna et al., 2001). In contrast, stimulation of VLPAG evoked postencounter defense—freezing and immobility—but never circa-strike defense. This differential profile of responding is consistent with the standard model of LPAG and VLPAG function. However, our findings also suggest a more nuanced understanding. At low intensity, rats expressed postencounter defense and as stimulation intensity increased they became active, eventually engaging in circa-strike defense. As noted, this defensive syntax is a defining characteristic of behavior in response to increasing predatory imminence (Fanselow, 1991; Fanselow & Lester, 1988). This shows that activity in LPAG itself is sufficient to control topographical variations in defensive behavior.

The critical inputs to PAG for these responses remain unclear. Amygdala projections, especially from central nucleus, are likely important, but other inputs are also relevant. Prefrontal cortex has extensive projections to each PAG column (Floyd et al., 2006; Keay & Bandler, 2004) and, in humans, distal threat is associated with recruitment of ventromedial prefrontal cortex (Mobbs et al., 2007). The role of prefrontal inputs to PAG in controlling defensive behaviors is worth investigation. Ventromedial hypothalamus (VMH) also has a well documented role in fear and defense (Gross & Canteras, 2012; Silva et al., 2013) and is a major source of inputs to most PAG columns (Canteras, Simerly, & Swanson, 1994; Lindberg, Chen, & Li, 2013). Notably, a similar scalability of defensive behavior was observed following ChR2 stimulation of cell bodies in mouse VMHdm/c (Kunwar et al., 2015; Wang et al., 2015). However, stimulation of the VMH → PAG pathway only elicited freezing, even at high stimulation intensities. Escape did not depend on the VMH → PAG pathway. It was mediated by the VMH → anterior hypothalamus pathway (Wang et al., 2015). Thus, although a VMH → LPAG pathway may be sufficient to cause postencounter defense, additional inputs may be needed to drive LPAG-based switching to circa-strike defense as predatory imminence increases.

The relevant PAG microcircuitry controlling expression of defensive behaviors also remains unclear. Our findings from LPAG are similar to those previously reported for DMPAG and DLPAG

(S. Chen et al., 2015). Chen et al. reported that ChR2 stimulation excitation of rat DMPAG or DLPAG evoked freezing and flight and that these depended on both the frequency and intensity of stimulation. So, the topography of defensive behavior evoked by ChR2 stimulation of DMPAG, DLPAG, or LPAG are each scalable with variations in stimulation intensity. In contrast, no such variation was observed for VLPAG. This suggests important interactions between DMPAG, DLPAG, and LPAG in the organization of defensive behavior and marks VLPAG as different to these other columns. It could be interesting to determine the impact of variations in frequency of stimulation on expression and topography of defensive behavior evoked from LPAG or VLPAG. Here we used 20 Hz stimulation. Low frequency (5 Hz) stimulation of DMPAG or DLPAG does not yield significant increases in spike rates in vivo, but higher frequency stimulation (20 Hz or 50 Hz) does (S. Chen et al., 2015). However, the H134R variant of ChR2 used here has low fidelity at higher stimulation frequencies (J. Y. Lin, 2011; Yizhar et al., 2011) and a faster spiking variant of ChR2 (e.g., ChETA, ChIEF) would be more appropriate to address this. Here we used the hSYN promoter to express ChR2 in PAG neurons. The majority of LPAG and VLPAG output neurons are glutamatergic (Oka, Tsumori, Yokota, & Yasui, 2008) but these are likely subject to regulation by networks of GABAergic neurons (Bagley et al., 2011). The roles of different PAG cell types in defensive behavior, and whether these roles are similar across different PAG columns, remains to be determined.

In conclusion, these findings show that LPAG and VLPAG can control variations in defense with increasing predatory imminence in two ways. First, consistent with past models, topographical variation can be assembled from the different defensive responses controlled by the LPAG (circa-strike) and VLPAG (postencounter). Second, topographical variation can be assembled from variations in LPAG activity itself.

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