

Periaqueductal gray activates antipredatory neural responses in the amygdala of foraging rats

Reviewed Preprint

v2 • May 21, 2024

Revised by authors

Reviewed Preprint

v1 • July 5, 2023

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Abstract

Pavlovian fear conditioning research suggests that the interaction between the dorsal periaqueductal gray (dPAG) and basolateral amygdala (BLA) acts as a prediction error mechanism in the formation of associative fear memories. However, their roles in responding to naturalistic predatory threats, characterized by less explicit cues and the absence of reiterative trial-and-error learning events, remain unexplored. In this study, we conducted single-unit recordings in rats during an ‘approach food-avoid predator’ task, focusing on the responsiveness of dPAG and BLA neurons to a looming robot predator. Optogenetic stimulation of the dPAG triggered fleeing behaviors and increased BLA activity in naive rats. Notably, BLA neurons activated by dPAG stimulation displayed immediate responses to the robot, demonstrating heightened synchronous activity compared to BLA neurons that did not respond to dPAG stimulation. Additionally, the use of anterograde and retrograde tracer injections into the dPAG and BLA, respectively, coupled with c-Fos activation in response to predatory threats, indicates that the midline thalamus may play an intermediary role in innate antipredatory defensive functioning.

eLife assessment

This study presents **valuable** findings describing how two brain regions, the midbrain periaqueductal gray matter and basolateral amygdala, communicate when a predator threat is detected. Though the periaqueductal gray is usually viewed as a downstream effector, this work contributes to a growing body of literature from this lab showing that the periaqueductal gray produces effects by acting on the basolateral amygdala. The experimental design, data collection and analysis methods provide **solid** evidence for the main claims. Although anatomical and immediately early gene results suggest the paraventricular nucleus of the thalamus may serve as a mediator of dorsolateral periaqueductal gray to basolateral amygdala neurotransmission, this finding would benefit from a functional assessment. This study will appeal to a broad audience, including basic scientists interested in neural circuits, basic and clinical researchers interested in fear, and behavioral ecologists interested in foraging.

<https://doi.org/10.7554/eLife.88733.2.sa3>

Introduction

The interaction between the dPAG and the BLA plays a pivotal role in defensive mechanisms across both animals and humans. Research on Pavlovian fear conditioning (FC), which involves pairing a conditional stimulus (CS; e.g., tones, lights, contexts) with an unconditional stimulus (US; e.g., electric shock) to produce conditional fear responses (CRs), suggests that the dPAG is part of the ascending US pain transmission pathway to the BLA, the presumed site for CS-US association formation (De Oca et al. 1998 [\[1\]](#), Gross and Canteras 2012 [\[2\]](#), Herry and Johansen 2014 [\[3\]](#), Kim, Rison, and Fanselow 1993 [\[4\]](#), Ressler and Maren 2019 [\[5\]](#), Walker and Davis 1997 [\[6\]](#)). This pathway is thought to be mediated through the lateral and dorsal-midline thalamus regions, including the posterior intralaminar nucleus and paraventricular nucleus of the thalamus (Krout and Loewy 2000 [\[7\]](#), McNally, Johansen, and Blair 2011 [\[8\]](#), Yeh, Ozawa, and Johansen 2021 [\[9\]](#); but see Brunzell and Kim 2001 [\[10\]](#)). Supporting this model, rodent studies have shown that electrical stimulation of the dPAG, which triggers robust activity bursts (jumping, running, escaping) similar to footshock-induced unconditional responses (URs) (Jenck, Moreau, and Martin 1995 [\[11\]](#), Olds and Olds 1963 [\[12\]](#)), can effectively act as a surrogate US for both auditory and contextual FC in rats (Di Scala et al. 1987 [\[13\]](#), Kim et al. 2013 [\[14\]](#)). In parallel, Johansen and colleagues (Johansen et al. 2010 [\[15\]](#)) found that pharmacological inhibition of the PAG, encompassing both dorsal (dPAG) and ventral (vPAG) regions, diminishes the behavioral and neural responses in the amygdala elicited by periorbital shock US, thereby impairing the acquisition of auditory FC. Furthermore, while dPAG neurons initially respond to the shock US, the development of fear CRs results in a reduction of US-evoked neural responses in the PAG, due to increased amygdala-PAG pathway-mediated analgesia that dampens footshock nociception (Johansen et al. 2010 [\[15\]](#)). Fundamentally, the negative feedback circuit between the amygdala and the dPAG serves as a biological implementation of the Rescorla-Wagner model (Rescorla and Wagner 1972 [\[16\]](#)), a foundational theory of associative learning that emphasizes the importance of prediction errors in reinforcement (i.e., US), as applied to FC (Fanselow 1998 [\[17\]](#)). However, the relevance of PAG-amygdala interactions observed in controlled conditioning chambers remains to be assessed in more realistic threat scenarios.

In human cases of intractable pain surgery, stimulation of the dPAG has been reported to induce intense fear and panic sensations (Carrive and Morgan 2012 [\[18\]](#), Magierek et al. 2003 [\[19\]](#)). For instance, one patient described the sensation of PAG stimulation as, “Something horrible is

coming, somebody is now chasing me, I am trying to escape from him” (Amano et al. 1982 [↗](#)). Similarly, our previous work demonstrated that electrical stimulation of either the dPAG or BLA prompted naïve rats foraging for food in a naturalistic environment to flee to a safe nest in the absence of external threats (Kim et al. 2013 [↗](#)). Notably, the fleeing behavior induced by dPAG stimulation was contingent on an intact amygdala, whereas BLA stimulation-induced fleeing did not require an intact PAG. This pattern suggests that the fear/panic sensations reported in humans may originate from an innate fear information flow from the dPAG to the BLA.

To further explore this, we employed single-unit recordings and optogenetics in an ‘approach food-avoid predator’ paradigm (Choi and Kim 2010 [↗](#), Wang, Chen, and Lin 2015 [↗](#)), focusing on the responsiveness of dPAG and BLA neurons to an approaching (looming) robotic predator. Our findings reveal that optogenetic activation of the dPAG not only precipitated escape behaviors but also significantly increased BLA neuron activity. BLA neurons activated through dPAG optogenetic stimulation exhibited immediate and heightened synchronous activity in response to the predator threat, unlike BLA neurons unresponsive to dPAG stimulation. Considering the lack of direct monosynaptic projections between dPAG and BLA neurons (Vianna and Brandao 2003 [↗](#), McNally, Johansen, and Blair 2011 [↗](#), Cameron et al. 1995 [↗](#)), we utilized anterograde and retrograde tracers in the dPAG and BLA, respectively. This was complemented by c-Fos expression analysis following exposure to predatory threats. Our anatomical findings suggest that the paraventricular nucleus of the thalamus (PVT) may be part of a network that conveys predatory threat information from the dPAG to the BLA.

Results

dPAG neurons respond to extrinsic predatory threats during a risky foraging task

Five male Long-Evans rats, maintained at 85% of their normal weight through food restriction, were implanted with microdrive arrays positioned above the dPAG. The dPAG includes dorsomedial (dmPAG), dorsolateral (dlPAG), and lateral (lPAG) regions, as designated by extensive track-tracing, neurochemical, and immunohistochemical studies (e.g., Bandler, Carrive, and Zhang 1991 [↗](#), Bandler and Keay 1996 [↗](#), Carrive 1993 [↗](#)). During baseline sessions, rats were required to procure a food pellet in an open arena (202 cm length x 58 cm width x 6 cm height; **Figure 1A** [↗](#)) and return to their nest to consume it. The electrodes were gradually lowered (<120 μ m/day) into the dPAG during these sessions (**Figure 1B** [↗](#)). Once well-isolated neural activities were detected, rats underwent predator testing with successive pre-robot, robot, and post-robot sessions (5-15 pellet attempts/session). During the robot session, the animals’ outbound foraging time (latency to reach the pellet or the predator-triggering zone) increased, while the pellet success rate decreased (**Figures 1C** [↗](#) and **1D** [↗](#)). We collected a total of 94 dPAG units (**Figure S1A** and **S1B**) during predator testing, with 23.4% ($n = 22$) showing increased firing rates ($z > 3$) specifically to the looming robot with short-latency responses (< 500 ms) during the robot session, but not during the pre- and post-robot sessions (robot cells; **Figures 1E**, **1F** [↗](#), **S1C**, and **S1D**). We focused our analysis on excited robot cells, excluding other types of units that showed food-specific and mixed robot encounter+pellet procurement responses (pellet and BOTH cells; **Figures 1F** [↗](#), **S1A**, and **S1B**), to investigate predator-related dPAG activity. One cell that exhibited decreased firing rates ($z < -3$) specifically to the robot was also excluded (**Figure S1E**). The majority of the robot cells (90.9%) did not show significant correlations between movement speed and neuronal firing rate (**Figure S1F**). These results suggest that dPAG neurons participate in detecting a looming robot and eliciting antipredatory defensive behaviors, such as fleeing to a safe nest.

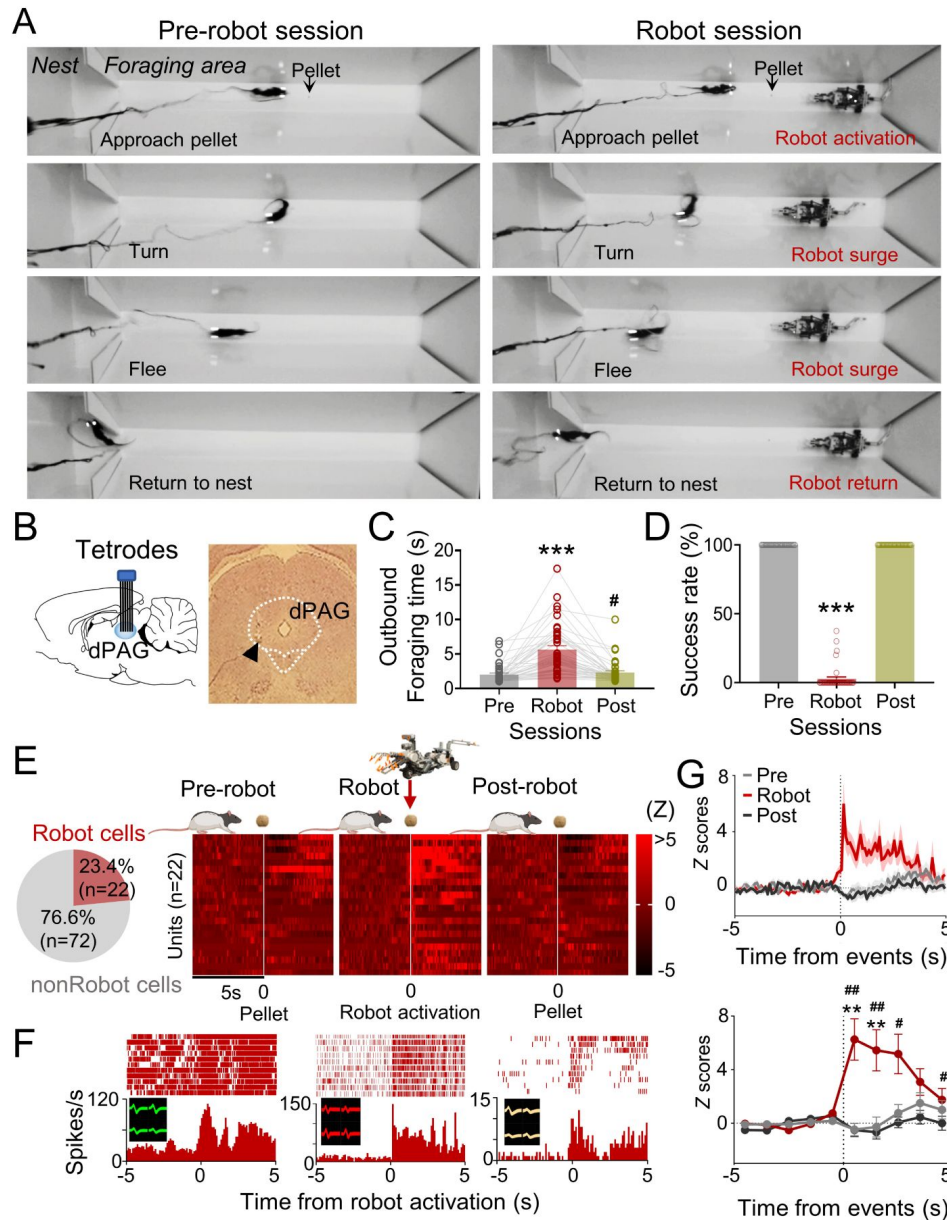


Figure 1.

dPAG single unit recordings during risky foraging.

(A) Rats underwent pre-robot, robot, and post-robot sessions, successfully securing pellets in pre- and post-robot trials, and failing during robot trials due to robot interference. (B) Tetrode implantation in dPAG with a photomicrograph of the tip (arrowhead). (C) Outbound foraging time increased significantly in the robot session ($\chi^2 = 64.00$, $P < 0.0001$, Friedman test; $P_s < 0.05$ for all comparisons, Dunn's test). *** $P < 0.001$ compared to pre-robot and post-robot sessions. # $P < 0.05$ compared to the pre-robot session. (D) The pellet success rate significantly decreased during robot session ($\chi^2 = 84.00$, $P < 0.0001$, Friedman test; $P_s < 0.0001$ for all comparisons, Dunn's test). *** $P < 0.001$ compared to pre-robot and post-robot sessions. (E) Cell type proportions revealed that 23.4% cells responded to robot activation (robot cells). (F) Representative dPAG robot cell raster/event histograms aligned with robot activations. (G) Population activity of robot cells around the time of robot activation ($t = 0$) with 0.1 s and 1 s bins. Firing rates of the robot cells were higher during robot session (0-3 s blocks; Friedman test, all $\chi^2_s > 6.952$, all $P_s < 0.05$; Dunn's test, all $P_s < 0.05$). Shaded areas indicate SEM. ** $P < 0.01$ compared to pre-robot session. #, ##, and ### denote $P < 0.05$, $P < 0.01$, and $P < 0.001$ respectively, compared to post-robot session.

dPAG neurons can intrinsically evoke fear in absence of external threats

We next investigated if optogenetic activation of dPAG neurons could elicit antipredatory behaviors without an external predator. To do so, we first unilaterally injected rats with ChR2 and implanted an optrode into their dPAG (**Figures 2A** and **S2A**). In two anesthetized rats, 20-Hz blue light stimulations (473 nm, 10-ms pulse width, 2 s duration) elicited excited responses in 48% of the recorded units (12 out of 25 cells; **Figures 2B** and **S2B**), while only one unit showed inhibited responses to the stimulation (Figure **S2C**), confirming the effectiveness of dPAG opto-stimulation. A separate group of rats with unilateral ChR2 ($n = 9$) or EYFP ($n = 4$) injections and optic fiber implantation (**Figure 2C**) underwent 4 days of baseline training followed by testing sessions. During testing, rats were allowed to procure a pellet without light stimulation (Off) and with light stimulation (On) (**Figure 2D**). The stimulation parameters employed were identical to those used in the anesthetized rats. During light-on trials (**Figure 2E**), the ChR2-expressing animals were not able to procure the pellet and consistently fled into the nest, whether the pellet was placed at a 76.2 cm long distance from the nest (On_L) or at a 25.4 cm short distance from the nest (On_S) (**Figures 2F** and **2G**). However, EYFP control rats did not show any defensive behaviors. Latency to procure pellets increased as a function of the stimulation intensity, frequency, and duration (**Figures 2H-2J**). These results demonstrate that optical stimulation of CaMKII-expressing dPAG neurons effectively caused naïve rats approaching a food pellet to flee to the nest without an external threat.

BLA neurons that respond intrinsically to dPAG optical stimulation also respond extrinsically to a robot predator

Our previous data indicated that PAG may transmit innate fear signals to the amygdala (Kim et al. 2013). To investigate amygdala neuron responses to dPAG activity changes, we recorded BLA neurons while optically stimulating the dPAG in foraging rats using the same parameters employed in the anesthetized rats during optrode recording (473 nm, 20 Hz, 10-ms pulse width, 2 s duration). Six rats were injected with ChR2 and implanted with an optic fiber in the dPAG and tetrode arrays in the BLA (**Figures 3A**). After the electrodes were lowered to the target structures between the baseline sessions, animals underwent testing comprising of pre-stim, stim, and post-stim sessions (**Figure 3B**). The dPAG stimulation increased outbound foraging time (**Figure 3C**) and decreased success rate (**Figure 3D**), mimicking predator-induced cautious behavior (**Figures 1C** and **1D**). While rats were escaping the foraging area without the pellet (stim session), we collected data from 320 BLA neurons. Subsets of neurons in the BLA ($n=34$) exhibited firing increases in response to dPAG stimulation (**Figures 3E** and **S3A-C**).

To further investigate how stimulation-responsive neurons respond to an actual predator, three out of the six rats were tested with a looming robot following the post-stim session (**Figure 3F**). The predatory robot increased outbound foraging time compared to the post-stim session (**Figure 3G**). Among the 85 units recorded in the BLA, robot-specific cells increased firing in response to the looming robot (robot cells; BLA, 25.9%; **Figures S3D-F**). Correlation analysis revealed that 95.4% of BLA robot cells did not show significant correlations between firing rates and movement speed (Figure **S3G**). Additionally, 23/85 BLA units were responsive to optical stimulation during the stim session but not pre/post-stim sessions (**Figures 3H**, **3I**, and **S3H**). The proportions of the robot and non-robot cells differed between stimulation-responsive and nonresponsive cells, with a lower proportion of robot cells in the stimulation-nonresponsive cells (**Figures 3J**). Stimulation-responsive cells exhibited higher firing rates to the robot predator than stimulation-nonresponsive cells (**Figures 3K**, **S3I**, and **S3J**). The higher the maximal firing rate was during the stimulation, the greater the cells fired to the actual robot predator (**Figures 3L**).

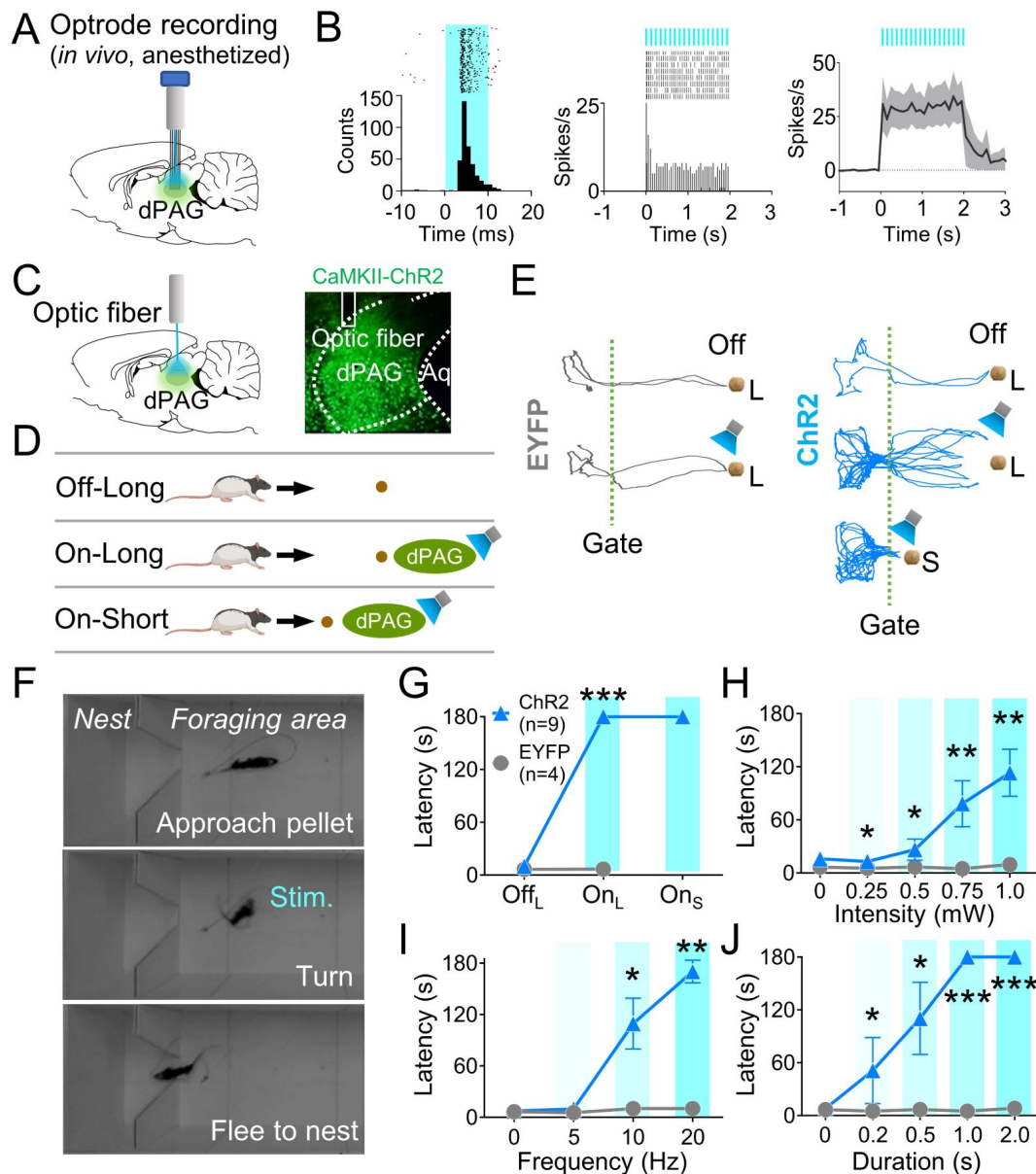


Figure 2.

dPAG optical stimulation evokes fear.

(A) Virus injection, optrode implantation in dPAG, and light stimulation during single unit recordings in anesthetized rats. (B) Raster plots and peri-event time histograms for 20-Hz light stimulations (10-ms width, *left*; 2-s duration, *center*). 48% of 25 units had increased firing during 2-s light stimulation (*right*). (C) Virus injection, expression, and optic fiber placement in dPAG. (D) Stimulation testing: baseline trials at 75-cm distance (Long) without light; stimulation trials with 2-s light as the rat approached (~25 cm) Long pellet; light applied as the rat approached 25-cm (Short) pellet if Long pellet unsuccessful. (E) Representative trajectories for EYFP- and ChR2-expressing rats during stimulation testing. (F) Rat behaviors during light stimulation trials. (G) ChR2 rats showed increased latency to procure pellet upon opto-stimulation (On_L) compared to EYFP rats (Off_L , $Z = 1.013$, $P = 0.311$; On_L , $U = 0.0$, $P < 0.001$; Mann-Whitney U test). (H-J) ChR2 group exhibited increased latency to procure pellet compared to EYFP group based on stimulation intensity (H; $U_s < \text{for all intensities} < 3.5$, $P_s \text{ for all intensities} < 0.025$; Mann-Whitney U test), frequency (I; $U_s \text{ for } 10 \text{ Hz and } 20 \text{ Hz} < 2.5$, $P_s \text{ for } 10 \text{ and } 20 \text{ Hz} < 0.014$; Mann-Whitney U test), and duration (J; $U_s \text{ for all durations} < 4.5$, $P_s \text{ for all durations} < 0.032$; Mann-Whitney U test). *, **, and *** denote $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

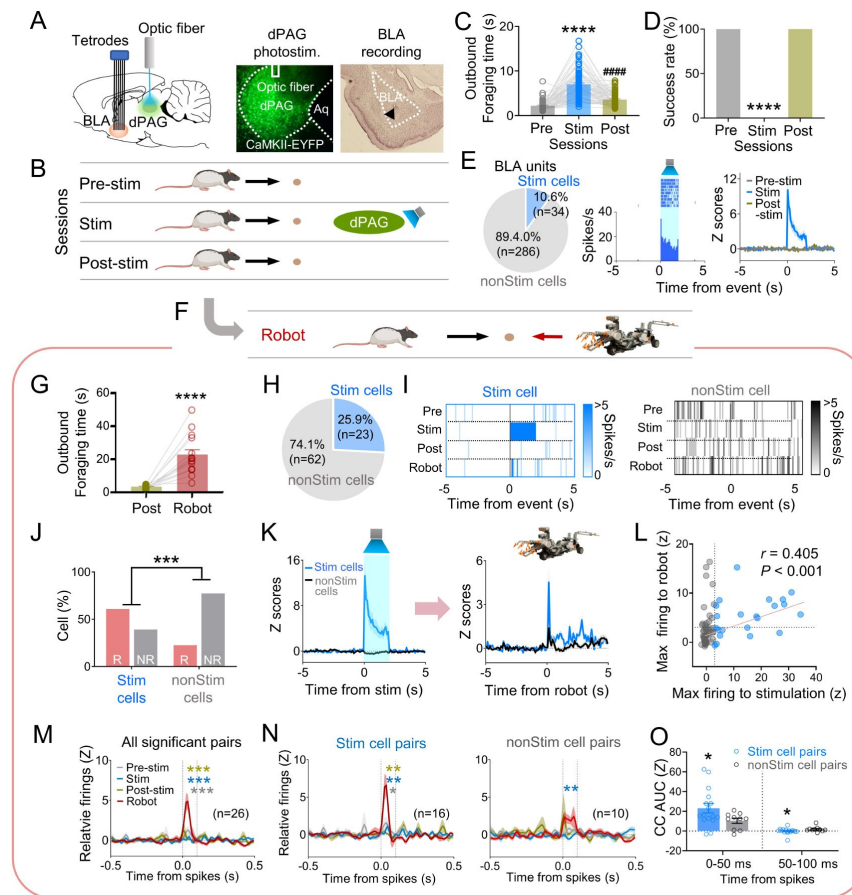


Figure 3.

dPAG optical stimulation and amygdala recordings.

(A) Virus injection in dPAG and tetrode array implantation targeting BLA. Light stimulation during single-unit recordings in freely-moving rats. (B) Stimulation testing sessions: in pre- and post-stim trials, rats freely procured pellets; in stim trials, optical stimulation prevented procurement of pellets. (C,D) During dPAG stimulation, animals showed increased outbound foraging time (C; $\chi^2 = 117.8$, $P < 0.0001$, Friedman test; $P_s < 0.0001$, Dunn's test) and decreased success rate (D; $\chi^2 = 154.0$, $P < 0.0001$, Friedman test; $P_s < 0.0001$, Dunn's test). **** $P < 0.0001$ compared to pre-robot and post-robot sessions. ##### $P < 0.0001$ compared pre-robot session. (E) Subset of BLA units (10.0%) responsive to optical stimulation (Stim cells; left), and a representative (center) and all stimulation-responsive (nonStim cells; right) raster plots with PETHs. (F) Subset of animals underwent additional robot trials following the post-stim session. (G) Increased outbound foraging time during robot session compared to post-stimulation session ($t(15) = 6.655$, $P < 0.0001$; paired t -test). **** $P < 0.0001$. (H) Twenty-two BLA units were dPAG stimulation-responsive. (I) Representative raster plots of dPAG stimulation-responsive and -nonresponsive units. (J) Proportions of robot vs. non-robot cells differed between stimulation-responsive and -nonresponsive units ($\chi^2 = 11.134$, $P < 0.001$; Chi-squared test). *** $P < 0.001$. (K) PETHs of stim and non-stim cells during stimulation and robot sessions. (L) Relationship between maximal firing rates during first 500 ms subsequent to robot activation and maximal firing rates during first 500 ms after stimulation onset ($r(85) = 0.405$, $P < 0.001$; Pearson correlation). (M) Population CCs with significant synchrony during robot session were higher than other sessions. Dotted vertical lines indicate 0-100 ms window for testing significance. Grey, blue, and dark yellow *** $P < 0.001$ compared to pre-stimulation, stimulation, and post-stimulation sessions, respectively. (N) Among synchronized BLA cell pairs, those including dPAG stimulation-responsive cell(s) (stim pairs; 61.5%) showed increased correlated firings (area under the curve, AUC, during 0-100 ms window) during the robot session compared to other sessions. In contrast, synchronized BLA cell pairs that consisted of stimulation non-responsive cells only showed no AUC differences across sessions. Grey *, blue **, and dark yellow ** $P < 0.05$ compared to pre-stimulation, $P < 0.01$ compared to stimulation, and $P < 0.001$ compared to post-stimulation sessions, respectively. (O) Comparing CCs during testing windows (0-50 ms and 50-100 ms) between stim and non-stim pairs, stim pairs exhibited higher correlated firing than non-stim pairs during the 0-50 ms block ($t(21.99) = 2.342$, $P = 0.0286$; t -test), while displaying decreased correlated firings in the second block (50-100 ms; $U = 42$, $P = 0.045$; Mann-Whitney U test). * $P < 0.05$ compared to the non-stim pairs.

We computed cross-correlations between simultaneously recorded BLA cell pairs to test how subpopulations of BLA neurons co-activate to the predatory threat differently. From 66 BLA recorded neurons, 185 BLA pairs were computed for cross-correlograms (CCs) during post-robot surge (2-s periods subsequent to robot activation; robot session), post-pellet, and post-stim (2-s periods subsequent to pellet procurement; pre- and post-stim sessions) epochs. Twenty-six CCs showed significant peaks (z scores > 3) around the paired spikes (between 0 ms to 100 ms) during the post-surge epoch (Figure S4A). Cell pairs with spike synchrony during the post-robot surge epoch did not show correlated firing during the post-pellet or post-stim epochs (Figures 3M, S4B, and S4C). This effect was prominent when pairs contained at least one of the stimulation-responsive cells (stim pairs; Figures 3N, S4D, and S4E), but not when pairs include only stimulation-nonresponsive cells (non-stim pairs; Figures 3N, S4D, and S4F). Stim pairs exhibited greater synchronous firing compared to non-stim pairs during the 0-50 ms window but had lower correlated firing during the 50-100 ms window, indicating that dPAG-stimulation responsive cells fire together more closely than dPAG-stimulation non-responsive cells (Figures 3O and S4G). Additionally, the stim pairs tended to have higher peaks of the cross-correlograms than the non-stim pairs (Figure S4H). Taken together, these results suggest that dPAG stimulation produces activity changes in subpopulations of BLA neurons, primarily in predator detection cells (robot cells), supporting the idea that PAG conveys innate fear signals to the amygdala.

The PVT interconnects the dPAG and BLA

Based on previous anatomical studies (Cameron et al. 1995, Krout and Loewy 2000) and response latency data in the present study (Figures S3B and S3C), it is likely that projections from the dPAG to the BLA are indirect. To identify potential mediators that relay predator signals from the dPAG to the BLA, we injected the anterograde tracer AAV-CaMKII-EYFP into the dPAG and investigated robot-induced c-Fos activities in AAV-expressed terminal areas of the dPAG. Cholera toxin subunit B (CTB) was also injected into the BLA in a subset of AAV-injected animals to examine potential anatomical evidence of relays between the dPAG and BLA (Figures 4A and 4B). After recovery, rats were trained to procure a pellet from the foraging arena. Animals were randomly assigned to foraging-only and robot-experienced groups. The foraging-only group quickly procured the pellet, while the robot-experienced group failed during the testing session (Figure 4C). Ninety minutes after testing, animals were perfused to analyze c-Fos activity and the tracers. Consistent with prior research (Cameron et al. 1995, Krout and Loewy 2000), we also observed strong terminal expression in midline thalamic areas emerging from dPAG cell body AAV infection (Figure 4D). Among these, the paraventricular nucleus of the thalamus (PVT) showed increased levels of c-Fos-positive cells in rats encountering the robot predator compared to controls (Figures 4E and 4F). In animals that were also injected with CTB in the BLA, the robot encounter increased the percentage of CTB-labeled PVT neurons expressing c-Fos, compared to the foraging-only control, while the density of CTB remained comparable between the groups (Figure 4G, 4H, and S5). Collectively, these results suggest predator-induced dPAG activity may convey information to the BLA, potentially through PVT activity.

Discussion

Prehistoric rock carvings and cave drawings of large carnivores from the Ice Age suggest that the fear of predation played a crucial role in the lives of early humans in their habitats (Mithen 1999). As such, the brain's fear system, which evolved to counteract unpredictable, uncontrolled threats originating from biological agents, likely influences our everyday behavior when confronted with perceived risks. In line with this view, studies using functional magnetic resonance imaging (fMRI) and an 'active escape paradigm'—in which virtually represented subjects are chased by a virtual predator and receive an electric shock when "caught"—have

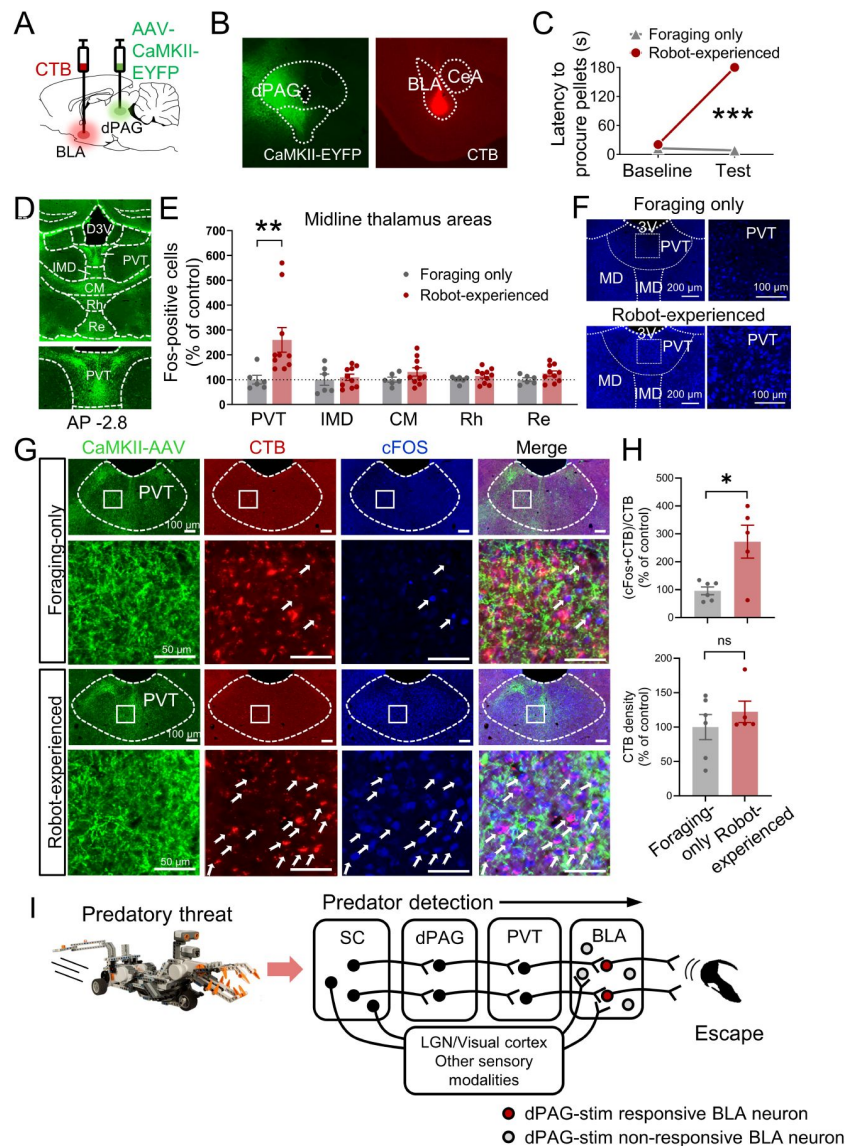


Figure 4.

PVT's hypothesized role in dPAG to amygdala signaling and anti-predatory behavior model.

(A) CTB retrograde tracer and AAV-CaMKII-EYFP used to trace dPAG signals to the BLA. (B) Representative images of AAV and CTB expressions in dPAG and BLA, respectively. (C) Robot encounters hindered pellet procurement compared to foraging-only rats (Base, $U = 26$, $P = 0.6926$; Test, $U = 0$, $P = 0.0001$; Mann-Whitney U test). *** $P < 0.001$ compared to the foraging-only group. (D) Terminal expressions of AAV injected into the dPAG cell bodies were predominantly observed in the midline nuclei of the thalamus. (E) PVT showed higher c-Fos-positive cells in robot-experienced rats ($n = 10$) compared to foraging-only control rats ($n = 6$) ($U = 4.0$, $P = 0.0027$; Mann-Whitney U test), while other midline thalamic areas showed no differences ($t(14)s < 1.611$, $P_s > 0.129$; t -test). *** $P < 0.01$ compared to the foraging-only group. Values were normalized to the mean of the corresponding control group. (F) Representative photomicrographs of PVT c-Fos staining from foraging-only (upper) and robot-experienced (bottom) rats. (G) Representative microphotographs of AAV, CTB, c-Fos, and triple staining in PVT comparing foraging-only and robot-experienced animals. (H) Robot exposure increased the percentage of CTB labelled PVT neurons expressing c-Fos ($t(9) = 3.171$, $P = 0.0113$), while CTB density levels were comparable between the two groups (robot-experienced, $n = 5$; Foraging-only, $n = 6$; $t(9) = 0.9039$, $P = 0.3896$; t -test). Values were normalized to the mean of the corresponding control group. (I) Proposed model: Predator surge detection via visual pathways, e.g., superior colliculus (Furigo et al. 2010, Rhoades et al. 1989), leads to dPAG activation, signaling through PVT to excite BLA. The BLA then projects to regions controlling escape responses, such as dorsal/posterior striatum (Li et al. 2021, Menegas et al. 2018) and ventromedial hypothalamus (Silva et al. 2013, Kunwar et al. 2015, Wang, Chen, and Lin 2015).

shown that as the threat transitions from a distant position to an impending encounter with the subject, neural activity shifts from the ventromedial PFC and amygdala towards the PAG (Mobbs et al. 2009 [↗](#), Mobbs et al. 2007 [↗](#)).

The present study investigated the neural mechanisms underlying antipredatory defensive behaviors in rats engaged in a naturalistic goal-directed behavior of foraging for food in an open arena, using a combination of electrophysiology, optogenetics, and tracing techniques. We found that neurons in the dPAG are involved in detecting predatory threats and eliciting antipredatory defensive behaviors in rats. Specifically, dPAG neurons displayed increased spiking in response to a looming robot predator as rats reflexively fled from the open foraging arena into a safe nest. However, dPAG neuronal activity did not increase when the robot was stationary, which contrasts with a previous study that reported enhanced dPAG neuronal activity in mice exposed to an anesthetized (motionless) rat separated by a wire mesh in a chamber (Deng, Xiao, and Wang 2016 [↗](#)) or a live rat tethered (restrained) with a harness (Reis et al. 2021 [↗](#)). It has been suggested that looming stimuli may serve as simple, evolutionarily-reliable signals of danger because genes are not capable of providing the brain with detailed information about all potential predatory threats, and all predators must approach their prey for consumption (Kim and Jung 2018 [↗](#)). Our study also demonstrated that optogenetic stimulation of CaMKII-expressing dPAG neurons caused naïve rats approaching a food pellet to instantly flee to the nest without an external threat (see also Tsang et al. 2023 [↗](#)). This confirmed escape behavior in foraging rats with dPAG electrical stimulation (Kim et al. 2013 [↗](#)) was evoked by intrinsic dPAG neurons, not the fibers of passage or current spread to other brain regions. In contrast to our observation of escape-to-the-nest behavior, optogenetic activation of dPAG in mice elicited various defensive behaviors, such as running, freezing and conditioned avoidance, when tested in a chamber (Deng, Xiao, and Wang 2016 [↗](#)). This is consistent with the notion that the behavioral outcome from brain stimulation is influenced by environmental settings (Kim et al. 2013 [↗](#)).

As mentioned in the introduction, the dPAG is recognized as part of the ascending nociceptive pathway to the BLA (De Oca et al. 1998 [↗](#), Gross and Canteras 2012 [↗](#), Herry and Johansen 2014 [↗](#), Kim, Rison, and Fanselow 1993 [↗](#), Ressler and Maren 2019 [↗](#), Walker and Davis 1997 [↗](#)). The dPAG is also implicated in non-opioid analgesia (Bagley and Ingram 2020 [↗](#), Cannon et al. 1982 [↗](#), Fields 2000 [↗](#)). However, it is essential to emphasize that, despite its roles in pain modulation, the primary behavior observed in dPAG-stimulated, naïve rats foraging for food in an open arena was goal-directed escape to the safe nest, underscoring the dPAG's critical function in survival behaviors.

Our study also revealed that BLA neurons respond both to direct dPAG optical stimulation and to an external robot predator. Specifically, BLA cells responsive to dPAG stimulation showed increased firing rates and were more likely to be co-activated by predatory threats compared to dPAG stimulation-non-responsive BLA cells. Additionally, the firing rates of BLA cells in response to dPAG stimulation highly correlated with their responses to the actual predatory agent, indicating that BLA neurons involved in processing dPAG signals may play a crucial role in enhancing the coherence of the BLA network, thereby effectively managing predatory threat information. These findings, combined with previous research demonstrating that the amygdala is necessary for both intrinsic dPAG stimulation-induced (Kim et al. 2013 [↗](#)) and extrinsic robot-evoked (Choi and Kim 2010 [↗](#)) escape behavior, suggests the dPAG-amygdala pathway's involvement in processing innate fear signals and generating antipredatory defensive behavior. However, it is crucial to consider the recent discovery that optogenetic stimulation of CA3 neurons (3000 pulses) leads to gain-of-function changes in CA3-CA3 recurrent (monosynaptic) excitatory synapses (Oishi et al. 2019 [↗](#)). Although there is no direct connection between dPAG neurons and the BLA (Vianna and Brandao 2003 [↗](#), McNally, Johansen, and Blair 2011 [↗](#), Cameron et al. 1995 [↗](#)), and no studies have yet demonstrated gain-of-function changes in polysynaptic pathways to our knowledge, the potential for our dPAG photostimulation (320 pulses) to induce similar changes in amygdalar neurons, thereby enhancing their sensitivity to predatory threats, cannot be dismissed.

While our findings demonstrate that opto-stimulation of the dPAG is sufficient to trigger both fleeing behavior and increased BLA activity, we have not established that the dPAG is necessary for the BLA's response to predatory threats. To establish causality, it is essential to conduct experiments such as optogenetic inhibition to determine whether the dPAG is indispensable for activating BLA neurons and initiating escape behavior in the face of threats. The complexity of targeting the dPAG, which includes its dorsomedial, dorsolateral, lateral, and ventrolateral subdivisions (e.g., Bandler, Carrive, and Zhang 1991 [↗](#), Bandler and Keay 1996 [↗](#), Carrive 1993 [↗](#)), suggests the need for future studies using transgenic mouse models. Should inactivation of the dPAG negate the BLA's response to predatory threats, it would underscore the dPAG's central role in this defensive mechanism. Conversely, if BLA responses remain unaffected by dPAG inactivation, this could indicate the existence of multiple pathways for antipredatory defense mechanisms.

Lastly, we confirmed strong terminal expressions in the midline thalamic areas (Cameron et al. 1995 [↗](#), Krout and Loewy 2000 [↗](#)) following AAV injection into the dPAG using immunohistochemistry. Notably, the PVT, in contrast to other midline thalamic subregions, exhibited increased c-Fos reactivity in response to the robot predator. This observation aligns with recent findings that dPAG projections specifically target the PVT rather than the centromedial intralaminar thalamic nucleus (Yeh, Ozawa, and Johansen 2021 [↗](#)). Additionally, subpopulations of the c-Fos-reactive PVT neurons were found to express CTB, retrogradely labeled by injection into the BLA. These results, in conjunction with previous research on the roles of the dPAG, PVT, and BLA in producing flight behaviors in naïve rats (Choi and Kim 2010 [↗](#), Daviu et al. 2020 [↗](#), Deng, Xiao, and Wang 2016 [↗](#), Kim et al. 2013 [↗](#), Kim et al. 2018 [↗](#), Kong et al. 2021 [↗](#), Ma et al. 2021 [↗](#), Reis et al. 2021 [↗](#)), the anterior PVT's involvement in cat odor-induced avoidance behavior (Engelke et al. 2021 [↗](#)), and the PVT's regulation of behaviors motivated by both appetitive and aversive stimuli (Choi et al. 2019 [↗](#), Choi and McNally 2017 [↗](#)), suggest the involvement of the dPAG → PVT → BLA pathways in antipredatory defensive mechanisms, particularly as rats leave the safety of the nest to forage in an open arena (**Figure 4I** [↗](#)) (Reis et al. 2023 [↗](#)). Given the recent significance attributed to the superior colliculus in detecting innate visual threats (Lischinsky and Lin 2019 [↗](#), Wei et al. 2015 [↗](#), Zhou et al. 2019 [↗](#)) and the cuneiform nucleus in the directed flight behavior of mice (Bindi et al. 2023 [↗](#), Tsang et al. 2023 [↗](#)), further exploration into the communication between these structures and the dPAG-BLA circuitry is warranted.

Future studies will also need to delineate the downstream pathways emanating from the BLA that orchestrate goal-directed flight responses to external predatory threats as well as internal stimulations from the dPAG/BLA circuit. Potential key structures include the dorsal/posterior striatum, which has been associated with avoidance behaviors in response to airpuff in head-fixed mice (Menegas et al. 2018 [↗](#)) and flight reactions triggered by auditory looming cues (Li et al. 2021 [↗](#)). Additionally, the ventromedial hypothalamus (VMH) has been implicated in flight behaviors in mice, evidenced by responses to the presence of a rat predator (Silva et al. 2013 [↗](#)) and upon optogenetic activation of VMH Steroidogenic factor 1 (Kunwar et al. 2015 [↗](#)) or the VMH-anterior hypothalamic nucleus pathway (Wang, Chen, and Lin 2015 [↗](#)). Investigating the indispensable role of these structures in flight behavior could involve lesion or inactivation studies. Such interventions are anticipated to inhibit flight behaviors elicited by amygdala stimulation and predatory threats, confirming their critical involvement. Conversely, activating these structures in subjects with an inactivated or lesioned amygdala, which would typically inhibit fear responses to external threats (Choi and Kim 2010 [↗](#)), is expected to induce fleeing behavior, further elucidating their functional significance.

The innate predator-fear functions of the dPAG and BLA are distinct from their learned fear functions, as observed in FC studies. In FC, the dPAG is proposed to play a role in “US-processing,” transmitting footshock (pain) information to the amygdala, thus enhancing the CS pathway's ability to activate the amygdalar fear system (Fanselow 1998 [↗](#), Maren 2001 [↗](#), Almeida, Roizenblatt, and Tufik 2004 [↗](#)). This hypothesis is supported by research demonstrating that

muscimol inactivation of the dPAG hinders FC to the footshock US (Johansen et al. 2010 [↗](#)), while electrical stimulation of dPAG neurons effectively serves as a US surrogate for FC (Ballesteros et al. 2014 [↗](#), Kim et al. 2013 [↗](#)). As conditioning progresses, amygdalar fear may suppress footshock-evoked dPAG's teaching signals, thereby moderating FC in the amygdala, potentially through conditioned analgesia that dampens the footshock US's painfulness (Fanselow 1981 [↗](#), Fanselow and Bolles 1979 [↗](#)). Consistent with this amygdalar-dPAG negative feedback notion, research has revealed that as CS-evoked responses of amygdalar neurons increase with CS-US pairings, footshock-evoked neural activity in the dPAG decreases (Johansen et al. 2010 [↗](#), Ozawa et al. 2017 [↗](#), Quirk, Repa, and LeDoux 1995 [↗](#)). It remains to be determined whether there are distinct populations of innate predator-responsive and pain-responsive dPAG neurons or if the same dPAG neurons react to a wide range of aversive stimuli. Furthermore, it is worth noting that the same dPAG stimulation resulted in an activity burst UR in a small operant chamber, as opposed to a goal-directed escape to a nest UR in a large foraging arena (Kim et al. 2013 [↗](#)). Additionally, FC to shock or predator US does not readily occur in naturalistic settings (Zambetti et al. 2022 [↗](#)).

Overall, this study enhances our understanding of the neural basis of antipredatory defensive behaviors in rats, emphasizing the important roles of the dPAG and BLA in processing and responding to threat-related stimuli. The study also suggests the possibility of an innate fear mechanism, which may complement the commonly studied learned fear mechanism in the research of anxiety and fear-related disorders. Further research into these mechanisms could lead to the development of novel therapeutic interventions that target both innate and learned fear mechanisms, improving the treatment of anxiety and fear-related disorders.

Materials and Methods

Subjects

Male and female Long-Evans rats (initial weight 325-350 g) were individually housed in a climate-controlled vivarium (accredited by the Association for Assessment and Accreditation of Laboratory Animal Care), with a reversed 12-h light/dark cycle (lights on at 7 PM) and placed on a standard food-restriction schedule with free access to water to gradually reach ~85% normal body weights. All experiments were performed during the dark cycle in compliance with the University of Washington Institutional Animal Care and Use Committee guidelines.

Surgery

Under anesthesia (94 mg/kg ketamine and 6 mg/kg xylazine, intraperitoneally), rats were mounted in a stereotaxic instrument (Kopf) and implanted with a Microdrive of tetrode bundles (formvar-insulated nichrome wires, 14 μ m diameter; Kanthal) into the dPAG (AP, -6.8; ML, +0.6; from Bregma), or BLA (AP, -2.8; ML, +5.2; from Bregma). For optogenetic stimulation, anesthetized male rats were injected with Adeno-associated viruses (AAVs; serotype 5) to express Channelrhodopsin-EYFP (AAV5-CaMKIIa-hChR2(H134R)-EYFP, UNC Vector Core) or EYFP only (AAV5-CaMKIIa-EYFP) in the dPAG at a rate of 0.05 μ l/min (total volume of 0.5 μ l) via a microinjection pump (UMP3-1, World Precision Instruments) with a 33-gauge syringe (Hamilton). To avoid backflow of the virus, the injection needle was left in place for 10 min. After virus injection, an optic fiber attached to ferrules (0.22 NA, 200 μ m core; ferrule diameter: 2.5 mm; Doric Lenses) or an optrode was implanted 0.4 mm dorsal to the injection sites. The microdrive and/or ferrule were secured by Metabond and dental cement with anchoring screws. Behavioral and recording experiments started after at least one week of recovery while optogenetic stimulation sessions started after at least 4 weeks after the surgery to allow for sufficient viral expression. For tracing experiments, male and female rats were injected with AAV-CaMKII-EYFP into the dPAG and CTB into the BLA > 5 weeks and one week prior to the predator testing, respectively.

Foraging apparatus

A custom-built foraging arena comprised a nest (29 cm x 57 cm x 60 cm height) and a foraging area (202 cm length x 58 cm width x 6 cm height) with a V-shaped gate connecting the two areas. The animals' movement was automatically tracked via two video tracking systems (Neuralynx and Any-maze).

Behavioral procedures

Habituation

Animals were placed in the nest area, where they were acclimated to the experimental room and chamber and allowed to eat food pellets (0.5 g; F0171, Bio-Serv) for 30 min/day for 2 consecutive days.

Baseline foraging

After the animals were placed in the nest, the gate to the foraging area was opened, allowing them to explore the arena. The animals were gradually trained to acquire a food pellet from various distances (25 cm, 50 cm, and 75 cm from the nest for the behavior-only experiment; an additional 100 cm and 125 cm for the recording experiment) and then return to their nest to consume it. Once the rat returned to the nest with the pellet, the gate was closed. The behavior-only groups underwent 4 days of baseline foraging training. For the recording experiments, baseline sessions continued until unit activity in the dPAG or amygdala unit was successfully detected.

Testing

For dPAG recordings, single units were recorded across three successive sessions: pre-robot, robot, and post-robot (7-14 trials/session; 4-14 recording days; $n = 5$ rats, 96 units). The number of trials varied among subjects because trials were excluded if rats attempted to procure the pellet within 10 s post-dPAG stimulation or robot activation. This exclusion criterion was implemented to ensure an accurate characterization of unit responsiveness. In the pre- and post-robot trials, rats were permitted to freely procure the pellet. Before initiating the robot trials, a programmed robot (LEGO Mindstorms EV3 set) was placed at the end of the foraging arena. Upon the gate's opening, whenever the rat came near (~ 25 cm) the pellet, the programmed robot would surge forward 23, 60, or 140 cm towards the animal at a velocity of ~ 75 cm/s, before returning to its original position. If the rat made another attempt to procure the pellet within 10 s after a robot activation, both the preceding and subsequent trials were excluded from the analyses to isolate and measure the unit properties accurately. For optical stimulation and behavioral experiments, the procedure included 3 baseline trials with the pellet placed 75 cm away, followed by 3 dPAG stimulation trials with the pellet locations sequentially set at 75 cm, 50 cm, and 25 cm. During each approach to the pellet, rats received 473-nm light stimulation (1-2 s, 20-Hz, 10-ms width, 1-3 mW) through a laser (Opto Engine LLC) and a pulse generator (Master-8; A.M.P.I.). Additional testing to examine the functional response curves was conducted over multiple days, with incremental adjustments to the stimulation parameters (intensity, frequency, duration) after confirming that normal baseline foraging behavior was maintained. For these tests, one parameter was adjusted incrementally while the others were held constant (intensity curve at 20 Hz, 2 s; frequency curve at 3 mW, 2 s; duration curve at 20 Hz, 3 mW). If the rat failed to procure the pellet within 3 min, the gate was closed, and the trial was concluded. For amygdala recordings with optical stimulation of the dPAG, pre-stim, stim, and post-stim sessions (5-15 trials/session) were carried out successively on the same day. These three consecutive phases mirrored the pre-robot, robot, and post-robot sessions, except that the dPAG light stimulation was given instead of the robot predator (6-18 recording days; $n = 6$ rats, 320 units). A subset of these animals ($n = 3$ out of 6 rats, 85 out of 320 units) also participated in a robot session following the post-stim session on selected days (1-16 recording

days). Sessions involving the robot predator were repeated until habituation was observed or recordings were deemed invalid due to limitations with the microdrive or failure to detect units. Note that the mean success rate for the dPAG recordings was $2.8\% \pm 1.31$, and animals subjected to the BLA recordings did not succeed in retrieving pellets during any of the robot trials.

Optrode recording under anesthesia

Upon optrode implantation, the animal was kept under anesthesia and moved to a unit recording setup (Neuralynx). The electrode was slowly lowered, and dPAG activity was monitored. Once dPAG activity was detected, eight light stimulations (2 s, 20-Hz, 10-ms width) were given with 30-s inter-stimulus intervals. Ten and fourteen stimulation sessions were repeated in the two anesthetized rats, respectively. After the recording was completed, the animal was immediately perfused under an overdose of Beuthanasia. Only the unit data collected in the dPAG were included in the analyses after histological verification.

Single-unit recording and analyses

Extracellular single unit activity was recorded through a 24-channel microdrive array loaded with a bundle of six tetrodes. The electrode tip was gold-plated to 100-300 k Ω measured at 1 kHz. The signals were amplified ($\times 10,000$), filtered (600-6000 kHz), and digitized (32 kHz) using a Cheetah data acquisition system (Neuralynx). A spike-sorting program (SpikeSort 3D; Neuralynx) and additional manual cutting were used for cluster isolation. Peri-event time histograms (PETH) were generated using NeuroExplorer (version 5.030; Nex Technologies) and further analyzed with custom MATLAB programs. The unit and speed data were binned at 0.1 s or 1 s and aligned to the time when the robot was activated, when the pellet was procured, or when the rat turned its body immediately before fleeing to the nest. All PETH data were normalized (z-scored) to the pre-event baseline period (from -5 s to 0 s prior to pellet procurement or robot activation, across 50 bins). Pellet retrieval was indicated by the animal entering a designated zone 19 cm from the pellet, a behavior driven by hunger. To classify the responsiveness of the unit responses, the first five bins (0.1-s bins) were analyzed. If one or more bins within the 500-ms period showed z-scores higher than 3 ($z > 3$) exclusively during the robot session, then the unit was classified as a 'robot cell'. The rest of the cells were further classified as food-specific ($z > 3$ exclusively during pre-robot session), BOTH ($z > 3$ during both pre-robot and robot sessions), or none (non-responsive) cells. In the light stimulation and recording experiments, the same criteria were applied to the unit classification except that the stimulation-responsive units (stim cells) were defined as cells showing significant activity ($z > 3$) during the stim session when aligned to the stimulation onset instead of the robot activation.

Cross-correlation

BLA cells that were simultaneously recorded from rats undergoing the four successive sessions (pre-stim, stim, post-stim, and robot sessions) were analyzed to generate cross-correlograms (CCs). Units firing less than 0.1 Hz were excluded due to the possible false peaks in their CCs. The shift predictors (100 random trial shuffles) were subtracted from the raw CCs. The correlated firing (in 10-ms bins) during the 0-2 s period following robot activation was calculated, and the CCs that showed significant peaks ($z > 3$) within the 0-100 ms window during the robot session were further analyzed. The peak values of the CCs and the peak areas under CC curves during the 100-ms window were compared across sessions (pre-stim, stim, post-stim, and robot sessions).

Histology

To verify the electrode placement, rats were overdosed with Beuthanasia, given electrolytic currents (10 μ A, 10 s) in the target regions through the tetrode tips, and perfused intracardially with 0.9% saline and then 10% formalin. For rats injected with the virus, phosphate-buffered saline (PBS) and 4% paraformaldehyde were used as perfusates. Extracted brains were stored in the fixative at 4 °C overnight followed by 30% sucrose solution until they sank. Transverse sections

(50 μm) were mounted on gelatin-coated slides and stained with cresyl violet and Prussian blue dyes to examine the recording sites. To confirm viral expression, 30- μm sections were cut, mounted, and cover-slipped with Flouromount-GTM with DAPI (eBioscience). Immunohistochemistry was performed on some of the sections to visualize the viral and c-Fos expressions. In short, the sections were washed with 0.1M phosphate buffered saline (PBS) for 10 min three times, followed by three rinses with PBS with Triton X-100 (PBST). After two hours of blocking (using normal goat serum), the sections were incubated in primary antibodies (1:500 mouse anti-GFP and rabbit anti-c-Fos; Abcam) overnight. The following day, additional PBST washes and secondary antibodies (1:250 anti-mouse Alexa 488 and anti-rabbit Alexa 405; Abcam) were applied. The sections were examined under a fluorescence microscope (Keyence BZ-X800E) and analyzed using ImageJ (NIH). Only rats with correct viral expression or electrode/optic fiber tip locations in the target structure(s) were included for the statistical analyses (Figure S6).

Statistical analyses

Based on normality tests (Kolmogorov-Smirnov test, $P < 0.01$; Table S1), non-normally distributed variables were analyzed using non-parametric tests, while parametric tests were applied to normally distributed variables. Statistical significance across sessions was determined using the Friedman test, followed by Dunn's multiple comparison test with correction when needed. Pearson's correlation coefficients evaluated variable relationships, and Chi-square tests compared percentages of distinct unit types. Group comparisons involved independent t -tests or Mann-Whitney U tests, and within-group comparisons employed paired t -tests or Wilcoxon signed rank tests. Statistical analyses and graph generation were performed using SPSS (ver. 19), custom MATLAB codes, GraphPad Prism (ver. 9.00), and NeuroExplorer (ver. 5.030).

Data availability

The data that support the findings of this study and the relevant analysis code will be available from the Dryad data repository upon the acceptance of the article.

Acknowledgements

This study was supported by the National Institutes of Health grants MH099073 (J.J.K.), AG067008 (E.J.K.), and F32MH127801 (M.K.), and the Ministry of Science and ICT through the National Research Foundation of Korea grant: Brain Science Research Program NRF-2022M3E5E8018421 (J.C.) and NRF-2022R1A2C2009265 (J.C.). Cartoons in **Figures 1E**, 2D, 3B, and 3F were created with BioRender.com.

Disclosures

All authors report no biomedical financial interests or potential conflicts of interests.

Author Contributions

E.J.K., J.J.K., and J.C. conceived the study. E.J.K. and M.K. performed behavioral, neurophysiological, optogenetic, and tracing experiments. E.J.K., S.G.P., J.C., and J.J.K. analyzed the data. E.J.K., J.C., and J.J.K. wrote the manuscript. J.C. and J.J.K. supervised all aspects of the study.

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Reviewer #1 (Public Review):

In the presence of predators, animals display attenuated foraging responses and increased defensive behaviors that serve to protect them from potential predatory attacks. Previous studies have shown that the basolateral nucleus of the amygdala (BLA) and the

periaqueductal gray matter (PAG) are necessary for the acquisition and expression of conditioned fear responses. However, it remains unclear how BLA and PAG neurons respond to predatory threats when animals are foraging for food. To address this question, Kim and colleagues conducted *in vivo* electrophysiological recordings from BLA and PAG neurons and assessed approach-avoidance responses while rats search for food in the presence of a robotic predator.

The authors observed that rats exhibited a significant increase in the latency to obtain the food pellets and a reduction in the pellet success rate when the predator robot was activated. A subpopulation of PAG neurons showing increased firing rate in response to the robot activation didn't change their activity in response to food pellet retrieval during the pre- or post-robot sessions. Optogenetic stimulation of PAG neurons increased the latency to procure the food pellet in a frequency- and intensity-dependent manner, similar to what was observed during the robot test. Combining optogenetics with single-unit recordings, the authors demonstrated that photoactivation of PAG neurons increased the firing rate of 10% of BLA cells. A subsequent behavioral test in 3 of these same rats demonstrated that BLA neurons responsive to PAG stimulation displayed higher firing rates to the robot than BLA neurons nonresponsive to PAG stimulation. Next, because the PAG does not project monosynaptically to the BLA, the authors used a combination of retrograde and anterograde neural tracing to identify possible regions that could convey robot-related information from PAG to the BLA. They observed that neurons in specific areas of the paraventricular nucleus of the thalamus (PVT) that are innervated by PAG fibers contained neurons that were retrogradely labeled by the injection of CTB in the BLA. In addition, PVT neurons showed increased expression of the neural activity marker cFos after the robot test, suggesting that PVT may be a mediator of PAG signals to the BLA.

Overall, the idea that the PAG interacts with the BLA via the midline thalamus during a predator vs. foraging test is new and quite interesting. The authors have used appropriated tools to address their questions.

In this revised version of the manuscript, the authors have made important modifications in the text, inserted new data analyses, and incorporated additional references, as recommended by the reviewers. These modifications have significantly improved the quality of the manuscript.

<https://doi.org/10.7554/eLife.88733.2.sa2>

Reviewer #2 (Public Review):

The authors characterized activity of the dorsal periaqueductal gray (dPAG) - basolateral amygdala (BLA) circuit. They show that BLA cells that are activated by dPAG stimulation are also more likely to be activated by a robot predator. These same cells are also more likely to display synchronous firing.

The authors also replicate prior results showing that dPAG stimulation evokes fear and the dPAG is activated by a predator.

Lastly, the report performs anatomical tracing to show that the dPAG may act on the BLA via the paraventricular thalamus (PVT). Indeed, the PVT receives dPAG projections and also projects to the BLA. However, the authors do not show if the PVT mediates dPAG to BLA communication with any functional behavioral assay. Furthermore, the authors also do not thoroughly characterize the activity of BLA cells during the predatory assay.

The major impact in the field would be to add evidence to their prior work, strengthening the view that the BLA can be downstream of the dPAG.

Reviewer #3 (Public Review):

In the present study, the authors examined how dPAG neurons respond to predatory threats and how dPAG and BLA communicate threat signals. The authors employed single-unit recording and optogenetics tools to address these issues in an 'approach food-avoid predator' paradigm. They characterized dPAG and BLA neurons responsive to a looming robot predator and found that dPAG opto-stimulation elicited fleeing and increased BLA activity. Importantly, they found that dPAG stimulation produces activity changes in subpopulations of BLA neurons related to predator detection, thus supporting the idea that dPAG conveys innate fear signals to the amygdala. In addition, injections of anterograde and retrograde tracers into the dPAG and BLA, respectively, along with the examination of c-FOS activity in midline thalamic relay stations, suggest that the paraventricular nucleus of the thalamus (PVT) may serve as a mediator of dPAG to BLA neurotransmission. Of relevance, the study helps to validate an important concept that dPAG mediates primal fear emotion and may engage upstream amygdalar targets to evoke defensive responses. The series of experiments provide a compelling case for supporting their conclusions. The study brings important concepts revealing dynamics of fear-related circuits particularly attractive to a broad audience, from basic scientists interested in neural circuits to psychiatrists.

<https://doi.org/10.7554/eLife.88733.2.sa0>

Author response:

The following is the authors' response to the previous reviews.

Recommendations for the authors:

We sincerely value the insightful and constructive feedback (italicized) provided by the reviewers, which has been instrumental in identifying areas of our manuscript that required further clarification or amendment. In response to these valuable comments, we have significantly revised the manuscript to enhance clarity and accuracy. Specifically, we have corrected an oversight related to the robot's velocity and secondary antibody ratios, and addressed previously missing values in Figs. 3E and 4E. Importantly, these corrections did not alter the outcomes of our results. Additionally, we have enriched our manuscript with new data analyses, as reflected in Figures 1B, 1F, 2H-J, 4D, 4F-H, S1A, S1C-E, S3H, S5, and Table 1, ensuring a more comprehensive presentation of our findings. Below are our responses detailing each comment and explaining the modifications integrated into the revised manuscript.

Reviewer 1:

(1) To address the question of whether PAG photostimulation biases the cells that respond to the robot, a counterbalanced experiment, in which the BLA activity is initially recorded during the foraging vs. robot test and the PAG stimulation happens at the end of the session, should have been performed.

In our study, we investigated fear behavior and BLA cell responses to intrinsic dPAG photostimulation (320 pulses) in naïve animals, followed by their reactions to an extrinsic predatory robot. We recognize the reviewer's concern regarding the potential influence of initial dPAG photostimulation on BLA neuron responses to the robot. We address this issue in our discussion (pg. 13) as follows: "However, it is crucial to consider the recent discovery that

optogenetic stimulation of CA3 neurons (3000 pulses) leads to gain-of-function changes in CA3-CA3 recurrent (monosynaptic) excitatory synapses (Oishi et al., 2019). Although there is no direct connection between dPAG neurons and the BLA (Vianna and Brandao 2003, McNally, Johansen, and Blair 2011, Cameron et al. 1995), and no studies have yet demonstrated gain-of-function changes in polysynaptic pathways to our knowledge, the potential for our dPAG photostimulation (320 pulses) to induce similar changes in amygdalar neurons, thereby enhancing their sensitivity to predatory threats, cannot be dismissed.”

(2) In Figure 3, it is unclear which criteria (e.g. response latency, minimum Z score, spike fidelity) was used to identify the BLA neurons that were indirectly activated by PAG stimulation. A graphic containing at least the distribution of the response latencies for each BLA neuron after PAG laser activation is needed.

We have specified the criteria for determining the responsiveness of BLA neurons to dPAG stimulation on page 22. This involves analyzing the first 500-ms post-stimulation across five 0.1-s bins. Units were classified as ‘stim cells’ if they showed z-scores greater than 3 ($z > 3$) in any of the bins during the initial 500-ms period post-stimulation. Neurons activated by both pellet procurement and dPAG stimulation were not included in the ‘stim cell’ category. Additionally, we have included a graphic in the revised manuscript (Fig. S3C) that presents the distribution of response latencies of BLA neurons to dPAG stimulation.

(3) To strengthen the claim that it is a BLA-PVT-PAG circuit that carries information about predatory threat, a new experiment using CTB and cFos could be used to demonstrate that PAG neurons that project to PVT are recruited during the robot exposure.

Our study primarily aimed to explore the transmission of threat signals between the dPAG and BLA. We acknowledge that our evidence for the PVT’s intermediary role, derived from CTB injections in the BLA and subsequent CTB+cFos co-labeling analysis in the PVT (Fig. 4G and 4H), is limited. Accordingly, we have moderated the emphasis on the PVT’s involvement in both the abstract and introduction. We now present the PVT’s role as a promising direction for future research in the discussion section of our revised manuscript.

(4) In Fig 2, the authors’ interpretation is that photostimulation of PAG neurons elicits fleeing responses in the rats. However, there is a vast literature demonstrating that the PAG is also involved in nociception. Although this is recognized by the authors in the first part of the introduction and briefly described in the discussion, the authors should more explicitly explain that PAG stimulation produces analgesia and thus is unlikely to underlie the escaping responses observed. This may not be intuitive for a broader audience.

We appreciate the reviewer’s insightful suggestion to elaborate on the PAG involvement in nociception and analgesia, as supported by the literature. While our initial manuscript acknowledged these functions, we have now expanded our discussion to address the PAG’s multifaceted roles (pg. 12): “As mentioned in the introduction, the dPAG is recognized as part of the ascending nociceptive pathway to the BLA (De Oca et al. 1998, Gross and Canteras 2012, Herry and Johansen 2014, Kim, Rison, and Fanselow 1993, Ressler and Maren 2019, Walker and Davis 1997). The dPAG is also implicated in non-opioid analgesia (e.g., Bagley and Ingram 2020, Cannon et al. 1982, Fields 2000). However, it is essential to emphasize that, despite its roles in pain modulation, the primary behavior observed in dPAG-stimulated, naive rats foraging for food in an open arena was goal-directed escape to the safe nest, underscoring the dPAG’s critical function in survival behaviors.” Note that this aligns with human studies on PAG stimulation (e.g., Carrive and Morgan 2012, Magierek et al. 2003), particularly those by Amano et al. (Amano et al. 1982), which reported patients feeling an urge to escape, similar to being chased, upon PAG stimulation.

(5) To truly demonstrate the functional links between the PAG and BLA, more experiments are needed. For example, one could record from BLA neurons during the robot surge while performing optogenetic inhibition of the PAG neurons. There is also no evidence that activity in the indirect pathway that connects the PAG to the BLA is indispensable for the expression of defensive responses towards the robot (e.g., causality tests using chemogenetic or optogenetic inactivation).

We agree that incorporating optogenetic inhibition of PAG neurons while simultaneously recording from BLA neurons during a robot surge would strengthen the evidence for the functional connectivity between the PAG and BLA. Such an experiment would necessitate the transfection and photoinhibition of a wide array of dPAG neurons responsive to predatory threats. This procedure is technically more viable in transgenic mouse models, given their suitability for genetic manipulation. In light of this, and in response to the suggestions in the Joint Public Review, we have revised the abstract, introduction, and discussion to offer a more cautious interpretation of our findings. This revision reflects a careful consideration of both the evidence and the limitations inherent in our study (pg. 13): “While our findings demonstrate that opto-stimulation of the dPAG is sufficient to trigger both fleeing behavior and increased BLA activity, we have not established that the dPAG is necessary for the BLA’s response to predatory threats. To establish causality, it is essential to conduct experiments such as optogenetic inhibition to determine whether the dPAG is indispensable for activating BLA neurons and initiating escape behavior in the face of threats. The complexity of targeting the dPAG, which includes its dorsomedial, dorsolateral, lateral, and ventrolateral subdivisions (e.g., Bandler, Carrive, and Zhang 1991, Bandler and Keay 1996, Carrive 1993), suggests the need for future studies using transgenic mouse models. Should inactivation of the dPAG negate the BLA’s response to predatory threats, it would underscore the dPAG’s central role in this defensive mechanism. Conversely, if BLA responses remain unaffected by dPAG inactivation, this could indicate the existence of multiple pathways for antipredatory defense mechanisms.”

(6) The manuscript lacks information about the number of rats and trials that were used across the experiments (e.g. Fig 2G-J). In some occasions, the authors start the experiments with a specific number of animals and then reduce the N by half without providing a rationale (e.g. Fig. 3). Equally confusing is the experimental timeline. For example: a) Were the pre-robot, robot, and post-robot sessions always performed within the same day? b) It was described that microdrivable arrays were used, but did the same rats experienced the robot test more than one time? c) How many bins were used for normalization during the Z-score calculation and when were the data binned at 100 ms versus 1 s? d) How many trials were used for each analysis? For example, to identify robot cells, did the authors establish a minimum number of trials per animal to calculate the peristimulus time histograms? Having a significant number of trials is critical to make sure that the observed neuronal responses are replicable across the trials. e) How was the neuronal activity related to “pellet retrieval” aligned during robot sessions? Was the activity aligned with the moment in which the rat touches the pellet or when the animal returns to the nest with the pellet? f) How did the authors control for trials in which the rat consumed the pellets in the same local vs. those in which they returned to the nest to eat it? All these points are extremely important for future replicability.

We apologize for any confusion caused by the initial lack of detail in our experimental procedures. The revised manuscript has been updated with comprehensive methodological details:

(i) The study involved thirteen rats (ChR2, n = 9; EYFP, n = 4), subjected to dPAG stimulation using fixed light parameters (473 nm, 20 Hz, 10-ms pulse width, 2 s duration) during Long

and Short pellet distance trials (refer to Fig. 2E-G). The stimulation intensity was adjusted to each animal's response (fleeing behavior), ranging from 1-3 mW. Additional testing occurred over multiple days, with incremental adjustments to stimulation parameters (intensity, frequency, duration) after confirming normal baseline foraging behavior (Fig. 2H-J, at $x = 0$). These details are now clearly depicted in the manuscript.

(ii) The primary objective was to investigate BLA neuron responses to dPAG opto-stimulation. Six rats were initially tested, with three later assessed for their reactions to dPAG stimulation in the presence of an actual predator, to gauge behavioral effects.

(iii) Regarding the experimental timeline:

a) Pre-robot, robot, and post-robot sessions were conducted successively on the same day.

b) Sessions with the robot predator were repeated until habituation occurred or when unit recordings were deemed invalid due to microdrive limitations or the absence of unit detection. Throughout these sessions, the success rate for pellet retrieval remained consistently low. Specifically, the mean success rate for the dPAG recordings was $2.803\% \pm 1.311$. For the BLA recordings, animals did not succeed in retrieving pellets during any of the robot trials. To provide a more detailed account of the methodology, the manuscript has been updated to include the number of recording days and the units recorded in the "Behavioral Procedures" section.

c) As described in Materials and Methods, unit recording data were binned at 0.1-s intervals and normalized against a 5-s pre-event baseline (50 bins). For statistical analyses in Figure 1F's rightmost column, 1-s bins were used to simplify post-hoc analysis corrections.

d) Each recording session consisted of 5-15 trials. Trials were excluded if rats attempted to procure the pellet within 10 s post-dPAG stimulation or robot activation, ensuring accurate characterization of unit responsiveness. Consequently, the number of trials varied among subjects.

e) Pellet retrieval was indicated by the animal entering a designated zone 19 cm from the pellet, driven by hunger.

f) Animals were trained to retrieve pellets and return to their nest for consumption prior to robot testing sessions, as elaborated in the "Baseline foraging" section.

(7) In the abstract, the authors mention that predictive cues are ambiguous during naturalistic predatory threats, but it is not clear what do they mean by ambiguous. In addition, in the introduction section, the authors describe that the present study will investigate how the dPAG and BLA communicate threat signals. However, the author should clarify right in the beginning that these two regions are not monosynaptically connected with each other and cite the proper references.

The abstract's original sentence, "...where predictive cues are ambiguous and do not afford reiterative trial-and-error learning..." has been refined to "...characterized by less explicit cues and the absence of reiterative trial-and-error learning events ..." This adjustment more accurately reflects that cues in natural settings often lack the clear and consistent quality of those in controlled experimental settings, which is necessary for the straightforward process of trial-and-error learning.

Regarding the dPAG and BLA connectivity, the revised introduction (pg. 5) now states: "Considering the lack of direct monosynaptic projections between dPAG and BLA neurons (Vianna and Brandao 2003, McNally, Johansen, and Blair 2011, Cameron et al. 1995), we utilized anterograde and retrograde tracers in the dPAG and BLA, respectively. This was complemented by c-Fos expression analysis following exposure to predatory threats. Our

anatomical findings suggest that the paraventricular nucleus of the thalamus (PVT) may be part of a network that conveys predatory threat information from the dPAG to the BLA.”

(8) In the introduction section, the authors should clarify that the US information is conveyed from the PAG to BLA via the lateral thalamus (posterior intralaminar nucleus, medial geniculate nucleus) or dorsal midline thalamus (paraventricular nucleus of the thalamus). The statement regarding how "the PAG functions as part of the ascending pain transmission pathway, providing footshock US information to the BLA" is misleading because the PAG does not send monosynaptic projections directly to the BLA.

The revised text (pg. 3) now reads: “...suggest that the dPAG is part of the ascending US pain transmission pathway to the BLA, the presumed site for CS-US association formation (De Oca et al. 1998, Gross and Canteras 2012, Herry and Johansen 2014, Kim, Rison, and Fanselow 1993, Ressler and Maren 2019, Walker and Davis 1997). This pathway is thought to be mediated through the lateral and dorsal-midline thalamus regions, including the posterior intralaminar nucleus and paraventricular nucleus of the thalamus (Krout and Loewy, 2000; McNally, Johansen, and Blair, 2011; Yeh, Ozawa, and Johansen, 2021; but see Brunzell and Kim, 2001).”

(9) The author's assumption that threat information flows from the PAG to the BLA, rather than BLA to PAG, based on electrical stimulation and lesion experiments performed in previous studies is problematic for at least three reasons: a) Electrical stimulation can activate fibers of passage as well as presynaptic neurons antidromically. b) The lesion approach may not have targeted 100% of the neurons in PAG, which extends anatomically along the antero-posterior axis of the midbrain for several millimeters in rats. This observation also disagrees with more recent studies using optogenetics and imaging tools demonstrating that the PAG is the downstream target of the BLA-CeA pathway. c) The authors cited prior reports describing the role of the amygdala-PAG pathway in dampening the US response and providing a negative signal to the PAG. However, a series of previous studies demonstrating that the PAG serves as the downstream target of the central nucleus of the amygdala for the expression of defensive response are completely ignored by the authors. Here are just some examples: Massi et al, 2023, PMID: 36652513; Tovote et al 2016, PMID: 27279213; Penzo et al, 2014 PMID: 24523533).

We recognize the complexities in interpreting findings from electrical stimulation and lesion studies. Our prior work (Kim et al. 2013) supports the conclusion that predatory threat information directionally flows from the dPAG to the BLA, as evidenced by distinct behavioral outcomes from experimental manipulations of dPAG and BLA. Specifically, dPAG stimulation-induced fleeing behavior was blocked by BLA lesions (as well as muscimol inactivation), whereas BLA stimulation-induced fleeing was unaffected by dPAG or combined dPAG+vPAG lesions (refer to Fig. 5A), suggesting a flow from dPAG to BLA. Our manuscript further clarifies that dPAG optostimulation results confirmed that escape behavior in foraging rats, induce by dPAG electrical stimulation (Kim et al. 2013), was activated by intrinsic dPAG neurons rather than by fibers of passage or current spread to other brain regions.

Furthermore, the PAG’s anatomical and functional diversity, with distinct segments along its longitudinal axis associated with different defensive behaviors, reinforces our conclusions. The dPAG is implicated in flight responses, while the vPAG is associated with freezing behavior (e.g., Bandler and Shipley 1994, Kim, Rison, and Fanselow 1993, Lefler, Campagner, and Branco 2020, Morgan, Whitney, and Gold 1998). The critiques' referenced studies primarily focus on the BLA-CeA-vPAG circuit's role in freezing during Pavlovian fear

conditioning, contrasting with our emphasis on the dPAG-PVT-BLA circuit and its mediation in escape behavior in response to naturalistic predatory threats.

We also note that different invasive procedures can yield varying behavioral outcomes. For example, both acute (e.g., optogenetic and muscimol inactivation) and chronic (e.g., surgical ablation) manipulations within the same brain circuit have shown diverse effects across species (Otchy et al. 2015). Moreover, optogenetics comes with its own set of conceptual and technical challenges (Adamantidis et al. 2015), including the difficulty of targeting, quantifying and photo-inhibiting 100% of PAG neurons. Despite the limitations of each technique, our collective evidence from lesions, inactivation, electrical stimulation (Kim et al. 2013), optostimulation, and single-unit recordings (the present study) supports the premise that the dPAG acts upstream of the BLA in processing predatory threat information.

(10) In the discussion, the authors suggest that the PVT may be the interface between the PAG and the BLA for the expression of antipredatory defensive behavior during their foraging vs. robot test, but previous studies looking at the role of PVT in antipredator defensive behavior and/or approach-avoidance conflict tasks are not cited and discussed in the manuscript (Engelke et al, 2021, PMID: 33947849; Choi et al 2019, PMID: 30979815; Choi and McNally 2017, PMID: 28193686).

We thank the reviewer for pointing out these pivotal studies, which we have carefully reviewed and integrated into the revised manuscript (pg. 14): “These results, in conjunction with previous research on the roles of the dPAG, PVT, and BLA in producing flight behaviors in naïve rats (Choi and Kim 2010, Daviu et al. 2020, Deng, Xiao, and Wang 2016, Kim et al. 2013, Kim et al. 2018, Kong et al. 2021, Ma et al. 2021, Reis et al. 2021), the anterior PVT’s involvement in cat odor-induced avoidance behavior (Engelke et al. 2021), and the PVT’s regulation of behaviors motivated by both appetitive and aversive stimuli (Choi and McNally 2017, Choi et al. 2019), suggest the involvement of the dPAG→PVT→BLA pathways in antipredatory defensive mechanisms, particularly as rats leave the safety of the nest to forage in an open arena (Figure 4I) (Reis et al. 2023).”

(11) The authors use the expression "looming robot predator" in many cases throughout the manuscript. However, it is unclear whether the defensive responses observed in the rats are elicited by the looming stimulus produced by the movement of the robot towards the rats. The authors describe that rats do not respond to a stationary robot, but would the sound produced by the movement of the robot elicit defensive responses? Would non-approaching lateral or dorsoventral movements (not associated with looming) be sufficient to induce defensive behavior in the rats? There is a vast literature in the field about defensive behaviors induced by looming stimuli. The authors should empirically demonstrate that the escaping responses induced by the robot are mediated by looming or refrain to use the looming terminology to avoid confusion.

Our use of "looming robot predator" is based on empirical evidence from a prior parametric study, which identified the forward, or 'looming,' motion of the Robogator as the key stimulus eliciting a flight response in rats (Kim, Choi, and Lee 2016). This reaction significantly decreased when the robot moved backward from the same starting position, producing a similar sound, and was absent when the robot remained stationary. This suggests that neither sound alone nor the mere presence of a novel object provokes goal-directed escape behavior (Kong et al. 2021). This aligns with studies indicating that simulated looming stimuli, like an expanding disk, induce flight or freezing responses in mice (De Franceschi et al. 2016, Yilmaz and Meister 2013).

It should be noted that the 2013 study by Yilmaz & Meister (Yilmaz and Meister 2013) on the looming disk paradigm showed that not all mice responded to the stimuli (e.g., Figs. 2A and 3A), with those that did exhibiting rapid habituation by the second exposure. This contrasts

with our predatory robot paradigm (Choi and Kim 2010), where all rats consistently fled from the looming robotic predator across multiple trials, underscoring the critical role of looming motion in simulating predator attacks that trigger flight behavior in rats.

Thus, the term "looming" accurately captures the nature of the robot's movement and its effect on eliciting defensive responses in rats. Nonetheless, should the editors agree with the reviewer's suggestion to minimize potential confusion, we are willing to substitute "looming" with "approaching," although we consider the terms to be synonymous in the context of our study.

(12) If the authors are citing the Rescorla-Wagner model, they should include at least one additional sentence to explain it, as many people in the field are not familiar with this model.

In response to the request for clarification on the Rescorla-Wagner model, we have added an explanatory sentence (pg. 4): "Fundamentally, the negative feedback circuit between the amygdala and the dPAG serves as a biological implementation of the Rescorla-Wagner (1972) model, a foundational theory of associative learning that emphasizes the importance of prediction errors in reinforcement (i.e., US), as applied to FC (Fanselow 1998)."

(13) The authors need to include the normality test used to determine whether a parametric or non-parametric statistical analysis was the most appropriate test for each experiment.

We have included the outcomes of the normality tests, detailed in Table S1.

(14) In Fig. 1F, the authors show a representative PAG neuron with peristimulus-time histogram and rasters reaching frequencies higher than 100 Hz and sustained firing rates of >50 Hz following robot activation. The authors should include a firing rate analysis (e.g., average firing rate and maximum firing rate before and after robot activation) of the 22 robot-responsive PAG neurons recorded during the session to clarify whether this high firing rate, which is atypical in other brain regions, is commonly observed in the PAG. Showing the isolated waveforms of some representative neurons would help to clarify whether the activity is being recorded from a single-isolated unit instead of multiple units within the same channel.

In response to the critique, we have expanded our analysis to include both average and maximum firing rates before and after robot activation for the 22 robot-responsive PAG neurons. This detailed firing rate analysis, illustrating their distribution, has been incorporated into the revised manuscript (refer to Figure S1C and S1D). Furthermore, to alleviate concerns regarding the identification of single-unit activity versus potential multi-unit recordings, we have included peri-event raster plots and waveforms for two additional representative neurons in Figure 1F.

(15) In Figure 2, the authors should indicate when the recordings are performed on anesthetized vs. freely-moving awake animals.

In the original manuscript, we specified that the optrode recordings depicted in Figure 2B were conducted on anesthetized rats. To enhance clarity and directly address the critique, we have now clearly indicated this condition in Figure 2A as well.

(16) The optogenetic stimulation parameters used in Fig 2H indicate that 0.5 mW was sufficient to induce behavioral changes. This is surprising because most optogenetic experiments in the field use much higher intensities (> 5mW). If much lower intensities

are sufficient to drive PAG-mediated behaviors, this may be a very important observation that should be conveyed to the field. I recommend the reviewers clarify if they in fact used 0.5 mW and then discuss that the laser intensity used in the experiments was 10X lower than that required for other brain regions

In our study, we indeed observed that 0.5 mW of dPAG stimulation increased the latency to procure the pellet without completely preventing the action. Notably, at 1 mW, more than half of the animals ($n = 5/9$ rats; Fig. 2H) and at 3 mW, all rats (9/9) failed to procure the pellet and fled from the foraging area to the nest (Fig. 2G). These results indicate that even lower intensities were sufficient to elicit behavioral changes through dPAG stimulation in a large foraging arena, highlighting the dPAG's sensitivity to optogenetic manipulation. This finding is consistent with our earlier research on dPAG electrical stimulation, which required significantly lower intensities to provoke defensive behaviors compared to the BLA. Specifically, the stimulation intensity needed for aversive behavior in the dPAG was substantially lower (dPAG: $65.0 \pm 6.85 \mu\text{A}$) than for the BLA (BLA: $275.0 \pm 24.44 \mu\text{A}$) (Kim et al. 2013). Furthermore, Deng et al. (Deng, Xiao, and Wang 2016) showed that 1 mW of blue light could elicit a 60% freezing response, with 2 mW triggering flight behavior within a latency of 0.6 seconds.

(17) In Fig 2 G-J, how many animals are being used per group and how was the sequence of the experiments performed? This is very important for replicability.

A total of three rats were utilized for the robot testing experiments depicted in Fig. 2 G-J. The experimental sequence for these animals consisted of successive pre-stimulation, stimulation, post-stimulation, and robot sessions. We have updated the manuscript to include this information.

(18) For the photostimulation of PAG neurons in Figs. 2 and 3, the authors need to clarify if the same parameters of laser stimulation used during the anesthetized recordings were also used during the behavioral tests. Also, the wavelength corresponding to the blue laser should be 473 nm instead of 437 nm.

We thank the reviewer for identifying the error. We confirm that the opto-stimulation parameters (473 nm, 10-ms pulse width, 2 s duration) were consistently applied across both anesthetized recordings and behavioral tests. This consistency has been explicitly stated in the revised manuscript to ensure clarity regarding our experimental approach.

(19) In Fig. 3I, how was the representative trials selected? Instead of picking up the most representative trials, the authors should demonstrate the response of the cell during the entire session.

In response to the critique, we clarify that the color-coded PETH shown in Fig. 3I represents averaged BLA activity across a comprehensive set of trials. This includes 8 pre-stimulation, 10 stimulation, and 8 post-stimulation trials for the robot-activated sessions, with a similar distribution for non-stimulated sessions. This approach was chosen to provide a representative overview of the cell's response throughout the entire session. To address the request for more detailed data, we have added traditional PETHs to the revised manuscript (see Fig. S3H), which depict the cell's response across all trials.

(20) Fig 4 D should demonstrate a colabeling between the anterograde PAG fibers in the PVT and the retrogradely labeled neurons from BLA instead of PAG fibers only.

We wish to clarify that Fig. 4D is intended to show the distribution of dPAG terminals within the midline thalamic nuclei, as noted in prior research (Krout and Loewy 2000). Although

dPAG terminals are distributed throughout the midline thalamus, our observations have specifically highlighted a notable increase in c-Fos expression within the paraventricular nucleus of the thalamus (PVT) in rats subjected to the robotic predator stimulus, in contrast to those in the foraging-only control condition (Fig. 4E). Addressing the reviewer's point, we direct attention to Fig. 4G, which includes images labeled "Robot-experienced" and "Merge." This figure demonstrates a subset of PVT neurons that were retrogradely labeled with CTB injected into the BLA, anterogradely labeled with AAV injected into the dPAG, and activated (as indicated by c-Fos expression) in response to the robotic predator. This provides specific colabeling evidence between anterograde PAG fibers in the PVT and retrogradely labeled neurons from the BLA, directly addressing the critique.

(21) *The resolution of the cFos images is very low and makes it hard to appreciate.*

We have updated Figs. 4F and 4G with high-resolution versions to ensure the details are more clearly visible. Furthermore, should there be a need for even greater clarity, we are prepared to supply the images as TIFF files, which are known for preserving high image quality.

Reviewer 2:

(1) *The text is clearly written, and I appreciated the inclusion of interesting citations, such as the one about paintings by cavemen. The authors also do a good job of discussing the underlying theoretical framework and the figures are easy to understand. Although the topic is very interesting, the amount of novel work is somewhat low. Figure 1 shows that dPAG cells are activated by the predator, and this has been shown by many prior reports. Similarly, Figure 2 shows that dPAG activation creates defensive responses, and this too has been shown by many prior reports.*

We appreciate the reviewer's positive remarks. We acknowledge the rich body of research documenting dPAG neuronal activation by various predator cues such as odors (e.g., fox urine) (Lu et al. 2023), and scenarios involving anesthetized or spontaneously moving rat/cat predators, either physically partitioned or harness-restrained (Bindi et al. 2022, Deng, Xiao, and Wang 2016, Esteban Masferrer et al. 2020). Nevertheless, our study distinguishes itself by examining dPAG neuronal responses to a robotic predator, uniquely designed to replicate consistent looming motions across multiple trials and subjects within an environment that simulates natural foraging conditions, inclusive of a safe nest (cf. Choi and Kim, 2010). This approach allowed us to not only reveal the immediate activation of dPAG neurons in response to a rapidly approaching predator but also to explore the consequent fleeing behavior towards safety, thereby providing new insights into the dPAG's role in mediating goal-directed defensive responses in a more ecologically-relevant setting. Furthermore, our investigation extends beyond these findings to assess the impact of dPAG activation on BLA neuronal responses and their functional connectivity during predator-prey interactions, offering a fresh perspective on the neural circuits that support survival behaviors in animals when confronted with naturalistic threats.

(2) *The results in Figure 3 are novel and interesting, but the characterization of BLA activity is incomplete. For example, what are the percentages of BLA cells that are inhibited or activated by all major behaviors observed? These behaviors include approach to pellet, escape from robot, freezing, stretch-attend postures, etc. These same analyses should also be added to dPAG activity in Figure 1. How does BLA single cell encoding of these behaviors relate to their responsivity to dPAG stimulation? And, finally, it is unclear what is the significance of BLA correlated synchronous firing. Is the animal more or less likely to be performing certain behaviors when correlated BLA firing occurs?*

Our analysis, as presented in Figs. 3I, 3K, and S3D-F, selectively focused on BLA cell responses during distinct behaviors such as approaching a pellet and escaping from the robot. These behaviors were selected because their precise temporal markers allow for accurate correlation with BLA cell activity, building on the findings of our previous research (Kim et al. 2018, Kong et al. 2021).

The robot's motion, programmed to advance a fixed distance before retreating to its starting position, is designed to repeatedly elicit foraging, thus facilitating analysis of neural changes during conflict situations involving food approach and predator avoidance. However, this also leads to the rapid diminution of freezing and stretch-attend postures inside the nest as animals quickly adapt to the robot's movement pattern, rendering a time-stamped analysis of these behaviors unfeasible under our experimental conditions. While the inclusion of these behaviors in our analysis would be insightful, especially in extended interaction scenarios where the robot advances to the nest opening and remains before returning in a less predictable manner, such conditions would likely reduce foraging behavior due to increased fear, deviating from our study's primary objective of elucidating the interactions between the dorsal periaqueductal gray (dPAG) and the basolateral amygdala (BLA) functions.

Regarding the significance of BLA correlated synchronous firing, our findings, particularly in Figures 3M-O and S4, demonstrate significant synchronous activity among BLA neuronal pairs during encounters with the robot, as opposed to pre-stim, stim, and post-stim sessions. This synchrony is notably prominent among neurons responsive to dPAG stimulation, indicating that BLA neurons involved in processing dPAG signals may play a crucial role in enhancing BLA network coherence to effectively manage predatory threat information (pg. 13).

(3) In Figure 4, the authors identify the PVT as a potential region that can mediate dPAG to BLA communication via anatomical tracing. However, functional assays are missing. For example, if the PVT is inhibited chemogenetically, does this result in a smaller number of BLA cells that are activated by dPAG stimulation? Does activation of the dPAG-PVT or the PVT-BLA projections cause defensive behaviors? Functionally showing that the dPAG-PVT-BLA circuit controls defensive actions would be a major advance in the field and would greatly enhance the significance of this paper. It would also provide an anatomical substrate to support the view that the BLA is downstream of the dPAG, which was first demonstrated by the authors in their elegant 2013 PNAS paper.

We appreciate the reviewer's constructive critique and valuable suggestions on the necessity for functional validation of the dPAG-PVT-BLA circuit's involvement in mediating defensive behaviors. In light of these comments, we have carefully considered and included a discussion on the importance of these proposed experiments as a direction for future research in our manuscript revision (also see response to Reviewer 1's critique #5).

Our initial work in 2013 (Kim et al. 2013) laid the groundwork for identifying BLA neurons responsive to dPAG stimulation and suggested the PVT as a potential relay in this neural circuit. Recognizing the limitations of our current study, which does not include direct functional assays, we have adjusted our manuscript to convey the speculative aspect of the dPAG-PVT-BLA circuit's role more accurately. Moreover, we have enriched our discussion by citing relevant studies that lend support to our proposed circuit mechanism. These references serve to place our findings within the broader context of existing research and highlight the imperative for subsequent studies to empirically confirm the functional significance of the dPAG-PVT-BLA pathway in driving defensive behaviors.

Reviewer 3:

(1) The Introduction refers to a negative feedback amygdala-dPAG from a study of the Johansen group, but in this case, the authors were referring to the ventrolateral and not the dorsal PAG.

We thank the reviewer for pointing out the need to distinguish between the dPAG and vPAG regions in our introduction. While Johansen et al. (2010) investigated the roles of PAG (including both dPAG and vPAG regions; see their Supplementary Figs. 4, 5, and 10), the differentiation between their specific contributions to the amygdala's negative feedback mechanism was not explicitly detailed in their initial publication. This distinction was further elaborated upon in later work by the same group (Yeh, Ozawa, and Johansen 2021), which specifically illuminated the dPAG's role in conditioned fear memory formation and its neural pathways to the PVT that influence fear learning. To reflect this nuanced understanding, we have revised our introduction (pg. 3): "In parallel, Johansen et al. (2010) found that pharmacological inhibition of the PAG, encompassing both dPAG and vPAG regions, diminishes the behavioral and neural responses in the amygdala elicited by periorbital shock US, thereby impairing the acquisition of auditory FC."

(2) In the experiments recording dPAG in response to the predator threat, the authors mentioned cells activated by the predator threat, referred to as "robot cells." Were these cells inhibited in response to threat?

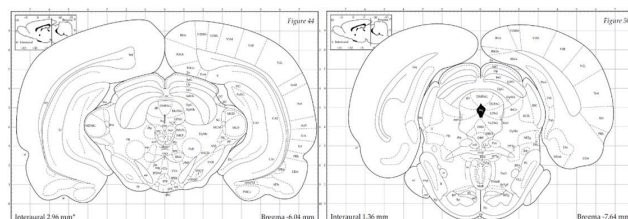
In the Result and Materials and Methods sections, we report that 23.4% (22 out of 94) of dPAG neurons, termed "robot cells," showed a significant increase in firing rates ($z > 3$) within a latency of less than 500 ms during exposure to the looming robot threat, but not during the pre- and post-robot sessions. These cells are highlighted in Figures 1E-G. In contrast, we identified only a single unit exhibiting a decrease in activity (z -score < -3) in response to the robot threat. Given the overwhelming prevalence of cells with excitatory responses to the threat, our discussions and analyses have primarily centered on these excited cells. Nevertheless, to ensure a full depiction of our observations, we have included data on the inhibited unit in the revised manuscript, specifically in Figure S1E.

(3) The authors claim that tetrodes were implanted in the dorsal PAG; however, the electrodes' tips shown in the figures are positioned more ventrally in the lateral PAG (see Figures 1B, S5A).

The PAG is anatomically organized into dorsomedial (dmPAG), dorsolateral (dlPAG), lateral (lPAG), and ventrolateral (vlPAG) columns along the rostro-caudal axis of the aqueduct. The designation "dorsal PAG" (dPAG) traditionally encompasses the dmPAG, dlPAG, and lPAG regions, a classification supported by extensive track-tracing, neurochemical, and immunohistochemical evidence (e.g., (Bandler, Carrive, and Zhang 1991, Bandler and Keay 1996, Carrive 1993)). As Bandler and Shipley (Bandler and Shipley 1994) summarized, "These findings suggest that what has been traditionally called the 'dorsal PAG' (a collective term for regions dorsal and lateral to the aqueduct), consists of three anatomically distinct longitudinal columns: dorsomedial and lateral columns...and a dorsolateral column..." Similarly, Schenberg et al. (Schenberg et al. 2005) clarified in their review that, "According to this parcellation...the defensive behaviors (freezing, flight or fight) and aversion-related responses (switch-off behavior) were ascribed to the DMPAG, DLPAG, and LPAG (usually named the 'dorsal' PAG)." In our study, electrode placements were strictly within these specified dPAG regions. The electrode tip locations depicted in Figures 1B and S5A correspond with the -6.04 mm template (left panel below) from Paxinos & Watson's atlas (Paxinos and Watson 1998), situated anteriorly to the emergence of the vlPAG (right panel below). To enhance clarification in our manuscript, we provide a detailed definition of the dPAG that

includes the dmPAG, dIPAG, and lPAG, and support our electrode placement rationale with references to established literature (pg. 5).

Author response image 1.



(4) It would be nice to include a series of observations applying inhibitory tools (i.e., optogenetic photo inhibition) in the dPAG and BLA and see how they affect the behavioral responses in the 'approach food-avoid predator' paradigm. Moreover, it would be interesting to explore how inhibiting the dPAG to PVT pathway influences the flee response during the robot surge.

We appreciate the suggestion to explore the effects of optogenetic inhibition in the dPAG and BLA on behavioral responses within the 'approach food-avoid predator' paradigm, as well as the potential impact of inhibiting the dPAG to PVT pathway on flee responses during robot surge incidents. As mentioned in our response to Reviewer 1's critique #5, the application of optogenetic inhibition necessitates transfecting, quantifying, and photoinhibiting a comprehensive set of dPAG neurons activated by predatory threats. This approach is more viable in future studies that can leverage transgenic mouse models for their genetic tractability. Following the Joint Public Review's recommendations, we have revised our manuscript to ensure a more measured interpretation of our data, carefully balancing the evidence from tracer studies against the limitations of our current methodology.

Furthermore, referencing Reviewer 1's critique #9, it is important to consider that various invasive techniques can yield different behavioral outcomes. For instance, research by Olveczky and colleagues (Otchy et al. 2015) demonstrated that acute manipulations (i.e., optogenetic and muscimol inactivation) and chronic surgical ablation of the same brain circuit can produce distinct effects in rats and finches. Despite these methodological constraints, our collective results from lesion, inactivation, electrical stimulation (Kim et al. 2013), optostimulation, and single-unit recording (present) studies cohesively suggest that the dPAG functions upstream of the BLA in processing predatory threat signals.

(5) The authors should also examine whether 'synaptic' appositions exist between the anterogradely labeled terminals from the dPAG and the double labeled CTB and cFOS neurons in the PVT.

We appreciate the suggestion to investigate the presence of synaptic appositions, which could potentially offer valuable insights into the synaptic connections and functional interactions within this neural circuit. However, due to the specialized nature of electron microscopy required for these examinations and the extensive resources it entails, this line of inquiry falls beyond the scope of our current study. We hope to address this aspect in future studies, where we can dedicate the necessary resources and expertise to conducting these intricate analyses.

(6) It is odd to see the projection fields shown in Fig. 4D, where the projection to the PVT looks much sparser compared to other targets in the thalamus and hypothalamus. If the projection to the PVT has such an important function, why does it seem so weak? This

should be discussed. Also, because the projection to the PVT seems sparse, the authors should consider alternative paths like the one involving the cuneiform nucleus. The cuneiform nucleus is an important region responding to looming shadows with strong bidirectional links to the dorsolateral periaqueductal gray, providing strong projections to the rostral PVT.

The perceived scarcity of the dPAG-PVT pathway might not reflect its functional significance accurately. The PVT's small size could make its projections appear less dense in broad anatomical studies. To address this, we have updated Figure 4D with a high-resolution image that offers a detailed view of the PVT region. This enhancement (refer to the updated Fig. 4, bottom) more accurately depicts the projection density within the PVT. It is also critical to consider that the functional impact of neural pathways is not solely dependent on the quantity of projecting neurons. For instance, work by Deisseroth and colleagues (Rajasethupathy et al. 2015) has shown that even relatively sparse monosynaptic projections from the anterior cingulate cortex to the hippocampus can exert significant effects on neural circuit dynamics. Additionally, we have expanded our discussion to consider the potential roles of other circuits, such as the cuneiform nucleus, in driving the behavioral responses observed in our study (pg. 15): "Given the recent significance attributed to the superior colliculus in detecting innate visual threats (Lischinsky and Lin 2019, Wei et al. 2015, Zhou et al. 2019) and the cuneiform nucleus in the directed flight behavior of mice (Bindi et al. 2023, Tsang et al. 2023), further exploration into the communication between these structures and the dPAG-BLA circuitry is warranted."

(7) Finally, in the Discussion, it would be nice to comment on how the BLA mediates flee responses. Which pathways are likely involved?

This excellent suggestion has been incorporated in the discussion (pg. 15): "Future studies will also need to delineate the downstream pathways emanating from the BLA that orchestrate goal-directed flight responses to external predatory threats as well as internal stimulations from the dPAG/BLA circuit. Potential key structures include the dorsal/posterior striatum, which has been associated with avoidance behaviors in response to airpuff in head-fixed mice (Menegas et al. 2018) and flight reactions triggered by auditory looming cues (Li et al. 2021). Additionally, the ventromedial hypothalamus (VMH) has been implicated in flight behaviors in mice, evidenced by responses to the presence of a rat predator (Silva et al. 2013) and upon optogenetic activation of VMH Steroidogenic factor 1 (Kunwar et al. 2015) or the VMH-anterior hypothalamic nucleus pathway (Wang, Chen, and Lin 2015). Investigating the indispensable role of these structures in flight behavior could involve lesion or inactivation studies. Such interventions are anticipated to inhibit flight behaviors elicited by amygdala stimulation and predatory threats, confirming their critical involvement. Conversely, activating these structures in subjects with an inactivated or lesioned amygdala, which would typically inhibit fear responses to external threats (Choi and Kim 2010), is expected to induce fleeing behavior, further elucidating their functional significance."

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<https://doi.org/10.7554/eLife.88733.2.sa4>