Neuroscience

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

Eun Joo Kim, Mi-Seon Kong, Sanggeon Park, Jeiwon Cho , Jeansok J. Kim

Department of Psychology, University of Washington, Seattle, WA 98195-1525, USA • Department of Psychiatry and Behavioral Sciences, University of Washington, Seattle, WA 98195-1525, USA • Department of Brain and Cognitive Sciences, Scranton College, Ewha Womans University, Seoul, 03760, Republic of Korea • Brain Disease Research Institute, Ewha Brain Institute, Ewha Womans University, Seoul, Republic of Korea • Program in Neuroscience, University of Washington, Seattle, WA 98195-1525, USA

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Abstract

Pavlovian fear conditioning studies propose that the interaction between the dorsal periaqueductal gray (dPAG) and basolateral amygdala (BLA) functions as a prediction error mechanism for fear memory formation. However, their roles in responding to naturalistic predatory threats, where predictive cues are ambiguous and do not afford reiterative trial-and-error learning, remain unexplored. We conducted single-unit recordings in rats engaged in an 'approach food-avoid predator' behavior, characterizing dPAG and BLA neurons responsive to a looming robot predator. Opto-stimulation of dPAG induced fleeing and increased BLA activity. Notably, BLA neurons activated by dPAG stimulation displayed an immediate response to the robot and heightened synchronous activity compared to non-responsive BLA neurons. Furthermore, anterograde and retrograde tracer injections into the dPAG and BLA, respectively, indicate that the paraventricular nucleus of the thalamus (PVT) may mediate dPAG-to-BLA neurotransmission. Our findings suggest that dPAG and BLA interactions, potentially via the PVT, underlie an innate antipredatory defensive mechanism.

eLife assessment

This study presents **valuable** findings describing how two brain regions, the midbrain periaqueductal gray matter and basolateral amygdala, communicate when a robotic predator threat is detected. While the experimental design and data collection methods are **solid**, the main claims are only partially supported by the data and would benefit from more rigorous anatomical approaches as well as functional validation of the role of the paraventricular nucleus of the thalamus as the critical connection between the periaqueductal gray and basolateral amygdala. The 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.

Introduction

The dPAG and BLA interplay is crucial for defensive mechanisms in animals and humans. For instance, Pavlovian fear conditioning (FC) research, which optimally pairs the conditional stimulus (CS) and unconditional stimulus (US) to elicit conditional fear responses (CRs), suggests that the dPAG functions as part of the ascending pain transmission pathway, providing footshock US information to the BLA, a putative site of 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). Supporting this notion, rodent studies have demonstrated that electrical stimulation of the dPAG, which results in robust bursts of activity (jumping, running, and escaping) akin to footshock-induced unconditional responses (URs) (Jenck, Moreau, and Martin 1995, Olds and Olds 1963), can effectively serve as a surrogate US for both auditory and contextual FC in rats (Di Scala et al. 1987, Kim et al. 2013). In parallel, pharmacological inhibition of PAG has been found to reduce periorbital shock US-elicited behavioral URs and neural responses in the amygdala and impair the acquisition of auditory FC (Johansen et al. 2010). Additionally, it has been shown that while dPAG neurons initially respond to the shock US, the development of fear CRs leads to a decrease in US-evoked neural responses in the PAG due to increased amygdala-PAG pathway-mediated analgesia that dampens the footshock nociception (Johansen et al. 2010). Fundamentally, this negative feedback amygdala-dPAG circuit serves as a biological implementation of the Rescorla–Wagner model (Rescorla and Wagner 1972) of FC (Fanselow 1998). Nonetheless, the significance of PAG-amygdala interactions observed in small conditioning chambers has yet to be evaluated in more realistic threat scenarios.

In humans undergoing surgery for intractable pain, dPAG stimulation has been observed to provoke intense fear/panic sensations (Carrive and Margan 2012, Magierek et al. 2003). For instance, a patient experiencing PAG stimulation described the sensation as, “Something horrible is coming, somebody is now chasing me, I am trying to escape from him” (Amano et al. 1982). Similarly, we have previously shown that electrical stimulation of either the dPAG or BLA caused naïve rats foraging for food in a naturalistic environment to escape to a safe nest in the absence of external threats (Kim et al. 2013). Moreover, the dPAG stimulation-induced fleeing was dependent on an intact amygdala, whereas the BLA stimulation-induced fleeing was independent of an intact PAG, suggesting that the fear/panic sensations reported in humans are likely due to innate fear information flow from dPAG to BLA. However, it remains unclear how dPAG neurons respond to different predatory threats and how dPAG and BLA communicate threat signals. To address this gap, we employed single-unit recording and optogenetics in our ‘approach food-avoid predator’ paradigm (Choi and Kim 2010, Wang, Chen, and Lin 2015). We characterized dPAG and BLA neurons that were responsive to a looming robot predator and found that dPAG opto-stimulation elicited fleeing and increased BLA activity. Specifically, BLA neurons that were activated by dPAG opto-stimulation demonstrated an immediate response to the robot and exhibited increased synchronous activity in comparison to BLA neurons that were unresponsive to the dPAG opto-stimulation. Additionally, injections of anterograde and retrograde tracers into the dPAG and BLA, respectively, suggest that the paraventricular nucleus of the thalamus (PVT) may serve as a mediator of dPAG to BLA neurotransmission. These results indicate that dPAG and BLA interactions, possibly via PVT, subserve antipredatory defensive mechanisms.

Results

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

We implanted a microdrive array above the dPAG of five male Long-Evans rats, which were food-restricted to maintain 85% of their normal weight. 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 μm/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 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; Figure 1E and 1F). We focused our analysis on 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. The majority of the robot cells (90.9%) did not show significant correlations between movement speed and neuronal firing rate (Figure S1C). These results suggest that dPAG neurons are involved in detecting a looming robot and eliciting antipredatory defensive behaviors, such as fleeing to a safe nest.

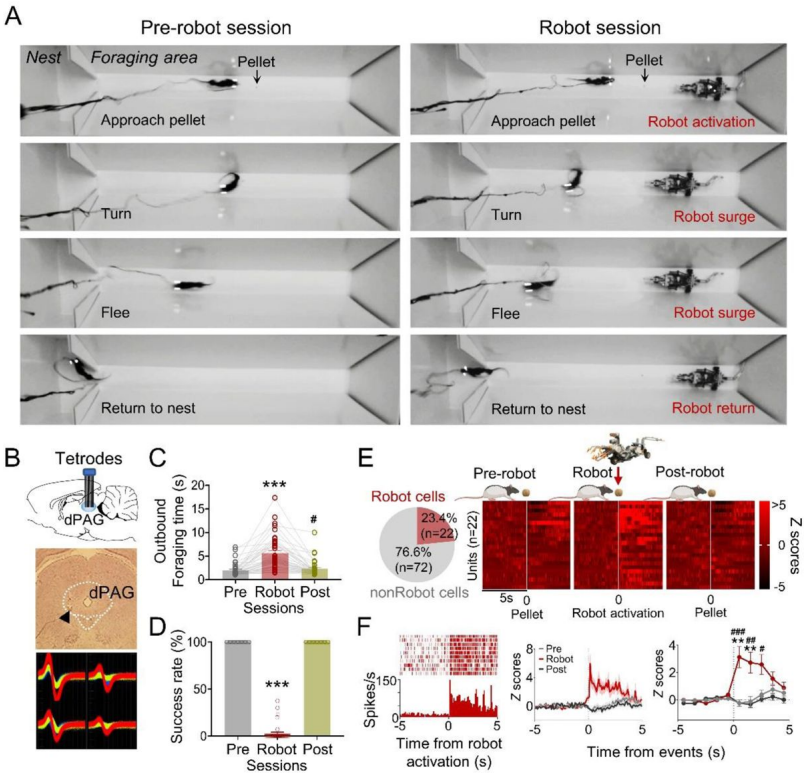

Figure 1.

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 surge. (B) Tetrode implantation in dPAG with photomicrographs of the tip (arrowhead) and unit clusters/waveforms. (C) Outbound foraging time increased during the robot session (X^2 = 64.00, P < 0.0001, Friedman test; Ps < 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) Pellet success rate decreased during robot session (X^2 = 84.00, P < 0.0001, Friedman test; Ps < 0.0001 for all comparisons, Dunn's test). ***P < 0.001 compared to pre-robot and post-robot sessions. (E) Cell type proportions showed 23.4% cells responding to robot activation (robot cells). (F) Representative dPAG robot cell raster/event histogram aligned with robot activations, and population activity of robot cells around robot activation time (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 X^2s > 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 (437 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 or EYFP 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). 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.

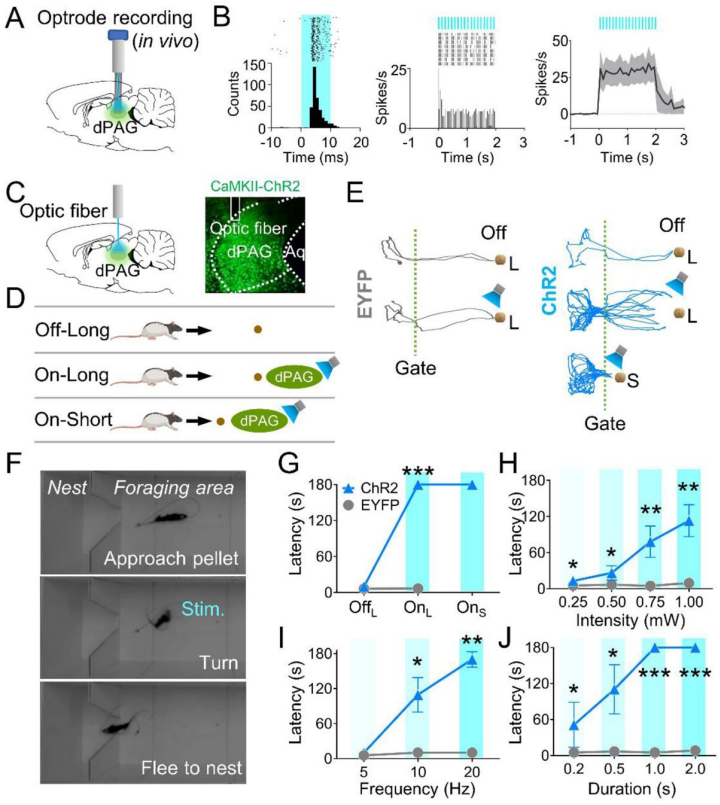

Figure 2.

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 < for all intensities < 3.5, *P*s for all intensities < 0.025; Mann-Whitney U test), frequency (I; *U*s for 10 Hz and 20 Hz < 2.5, *P*s for 10 and 20 Hz < 0.014; Mann-Whitney U test), and duration (J; *U*s for all durations < 4.5, *P*s for all durations < 0.032; Mann-Whitney U test). *, **, and *** denote $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

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. 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 322 BLA neurons. Subsets of neurons in the BLA (n=32) exhibited immediate firing increases in response to dPAG stimulation (Figures 3E and S3A-C).

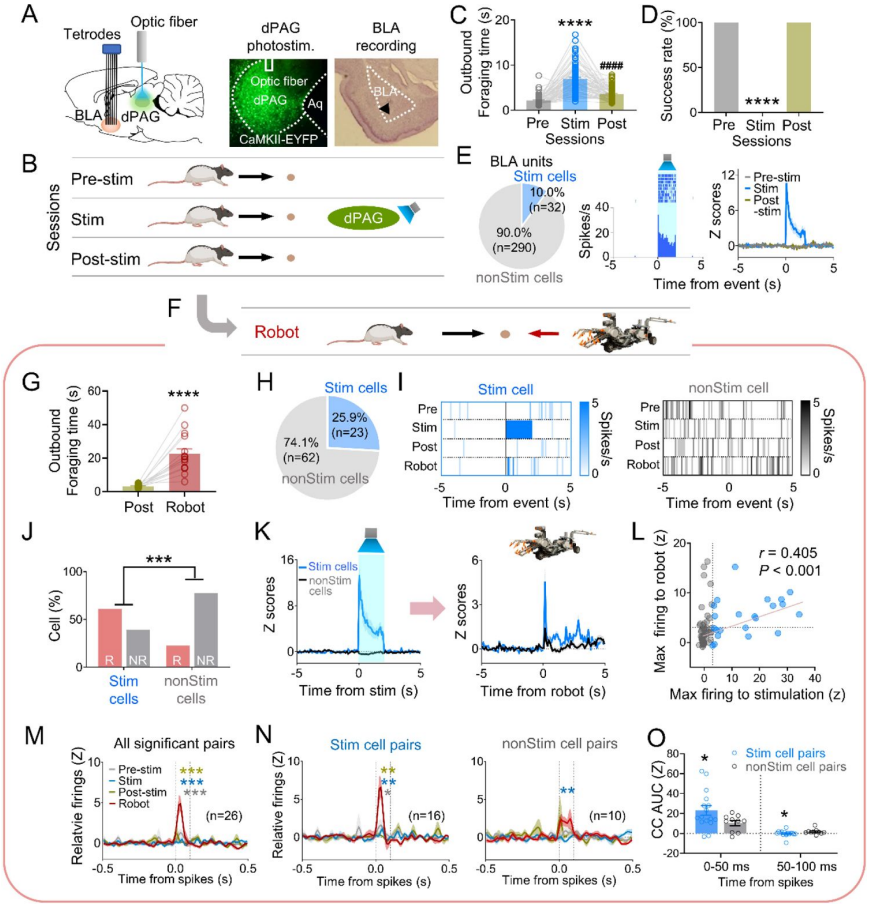

Figure 3.

dPAG optical stimulation and amygdala single-unit 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; $X^2 = 117.8$, $P < 0.0001$, Friedman test; *P*s < 0.0001, Dunn's test) and decreased success rate (D; $X^2 = 154.0$, $P < 0.0001$, 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 ($X^2 = 11.134$, $P < 0.001$; 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.

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 and 3I). 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, S3H, and S3I). The higher the maximal firing rate was during the stimulation, the greater the cells fired to the actual robot predator (Figures 3L).

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 injected into the BLA in a subset of AAV-injected animals (n = 6) 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 the tracers and c-Fos activity. We found strong terminal expression in midline thalamic areas emerging from dPAG cell body 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). Subpopulations of the PVT c-Fos-positive cells also expressed CTB injected in the BLA (Figures 4G and 4H), suggesting predator-induced dPAG activity transmits information to the BLA, possibly through PVT activity.

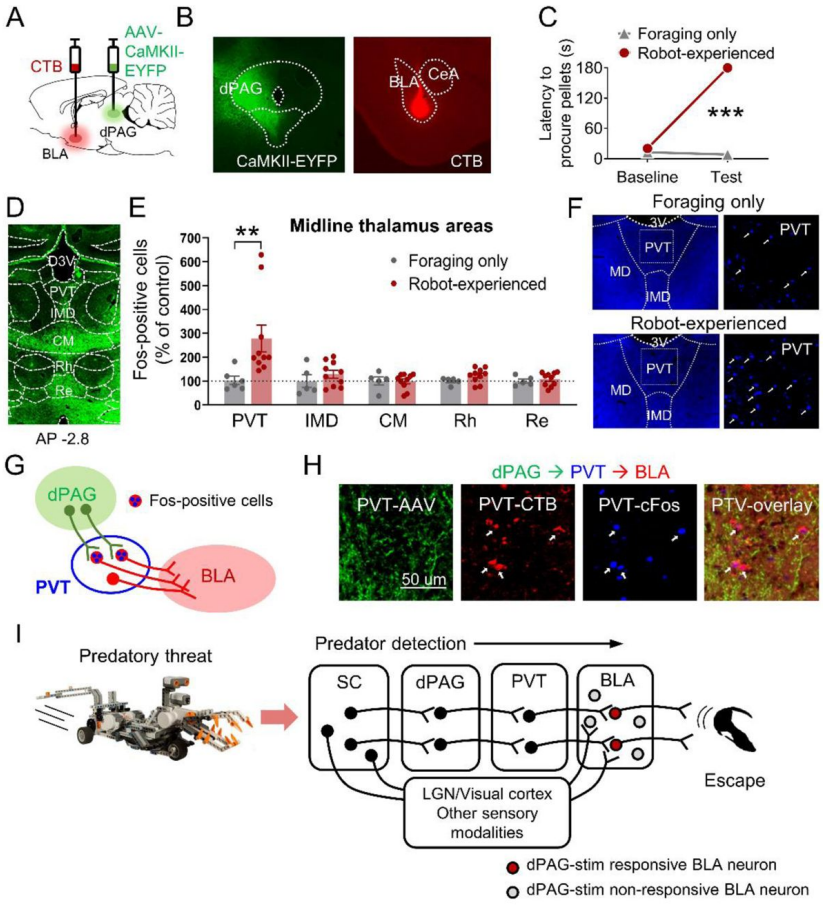

Figure 4.

PVT as a potential mediator of dPAG signals to amygdala and a hypothesized model of the dPAG-amygdala circuit in anti-predatory behaviors.

(A) Retrograde tracer CTB injected into the BLA and AAV-CaMKII-EYFP injected into the dPAG to explore candidate areas conveying dPAG signals to the BLA. (B) Representative images of AAV and CTB expressions in dPAG and BLA, respectively. (C) Rats encountering the robot predator failed to procure pellets, while foraging-only animals succeeded (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 compared to foraging-only control rats ($U = 3.0$, $P = 0.0016$; Mann-Whitney U test), while other midline thalamic areas showed no differences (Us > 12.0, Ps > 0.129; Mann-Whitney U test). **$P < 0.01$ compared to the foraging-only group. (F) Representative photomicrographs of PVT c-Fos staining from foraging-only (upper) and robot-experienced (bottom) rats. (G) Schematic of triple staining in the PVT. (H) Triple staining of AAV, CTB, and c-Fos in the PVT suggests it might mediate

predator information from the dPAG to the BLA. (I) A putative predatory fear circuit model: Predator surge detected by visual pathways, such as the superior colliculus (Furigo et al. 2010, Rhoades et al. 1989), is transmitted to the dPAG, then to the PVT, which excites the BLA. The BLA projects to regions involved in executing escape responses, such as the ventral 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).

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 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 (restrainted) with a harness (Reis et al. 2021). It has been suggested that looming stimuli may serve as simple, evolutionarily-reliable signals of danger becausegenes 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 that the original report of escape behavior in foraging rats with electrical stimulation of dPAG (Kim et al. 2013) was indeed evoked by intrinsic dPAG neurons and not the fibers of passage or spreading of currents 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).

Our study also revealed that BLA neurons respond both intrinsically to dPAG optical stimulation and extrinsically to a robot predator. Specifically, BLA cells that were responsive to dPAG stimulation showed increased firing rates and tended to be co-activated in response to the predatory threat more than dPAG stimulation-non-responsive BLA cells. Moreover, BLA firings in response to dPAG stimulation was highly correlated with their firings in response to the actual predatory agent. These findings, combined with earlier studies

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 that the dPAG-amygdala pathway is involved in processing innate fear signals and generating antipredatory defensive behavior.

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. We also observed that the PVT, but not other midline thalamic subregions, showed increased c-Fos reactivity to the robot predator, consistent with a recent study showing dPAG projections to the PVT, not the centromedial intralaminar thalamic nucleus (Yeh, Ozawa, and Johansen 2021). Subpopulations of the c-Fos-reactive PVT neurons were also found to express CTB, which was retrogradely labeled by injection into the BLA. This study's findings, combined with previous reports on the involvement 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), suggest that the dPAG→PVT→BLA pathways play a critical role in antipredatory defensive mechanisms in rats (Figure 4I) (Reis et al. 2023). Given the results from other research groups that emphasize the importance of the superior colliculus in detecting innate visual threats (Lischinsky and Lin 2019, Wei et al. 2015, Zhou et al. 2019), it is important to further investigate whether the activity of the superior colliculus changes in response to external predatory threats and to elucidate how the superior colliculus and the dPAG-BLA circuitry communicate.

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 µm 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 µl/min (total volume of 0.5 µl) 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 µm 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 three months 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 opened, and the animals explored the arena and were gradually trained to acquire a food pellet from various locations (25 cm, 50 cm, and 75 from the nest for the behavior-only experiment; additional 100 cm and 125 cm distance trials for the recording experiment).

Once the rat procured the pellet and returned to the nest, the gate closed. The behavior-only groups underwent 4 days of baseline foraging training. For the recording experiments, baseline sessions were continued until dPAG or amygdala unit activity was successfully detected.

(Testing) For dPAG recordings, single units were recorded throughout three successive pre-robot, robot, and robot sessions (5-15 trials/session). During the pre- and post-robot trials, the rat was allowed to freely procure the pellet. Before the robot trials started, the programmed robot (LEGO Mindstorms EV3 set) was placed at the end of the foraging arena. Once the gate opened, every time the rat came near (~25 cm) the pellet, the programmed robot surged 23, 60, or 140 cm toward the animal at a velocity of 20-52 cm/s and then returned backward to the original position. If the rat made another attempt within 10 s after the previous robot activation, both the previous and following trials were not included in the analyses to exclusively measure the unit properties. For optical stimulation and behavioral experiments, the testing consisted of 3 baseline trials with a 75 cm pellet location and 3 dPAG stimulation trials with sequential 75 cm, 50 cm, and 25 cm pellet locations. Each time the rat approached the pellet, 473-nm light stimulation (1-2 s, 20-Hz, 10-ms width, 0.25-3 mW) was given to the rat through a laser (Opto Engine LLC) and a pulse generator (Master-8; A.M.P.I.). If the rat failed to procure the pellet within 3 min, the gate closed, and the trial ended. For amygdala recording with optical stimulation of dPAG, pre-stim, stim, and post-stim sessions were conducted. The three consecutive procedures were the same with the pre-robot, robot, and post-robot sessions, except that the dPAG light stimulation was given instead of the robot predator. A subset of the animals (n = 3) additionally underwent the robot session following the post-stim session.

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 kΩ measured at 1 kHz. The signals were amplified (×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-stimulus baseline period (−5 s to 0 s to the pellet procurement or robot activation). 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 μA, 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 (1:500 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 S5).

Statistical analyses

Based on normality tests (Kolmogorov-Smirnov test, $P < 0.01$), 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.

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Disclosures

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

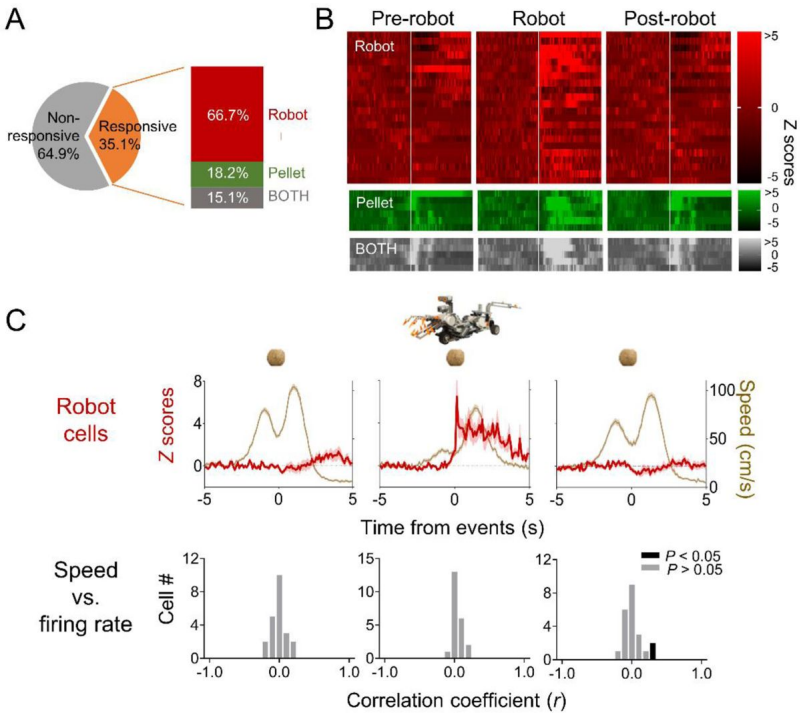

Figure S1.

Cell types of the dPAG units.

(A) A subset (35.1%) of dPAG neurons increased firing rates in response to the robot, food pellet, or both. Among them, 66.7% of dPAG neurons responded exclusively to the robot. Units were classified as "robot cells" if their z-score was greater than 3 in one or more bins during the robot phase and less than 3 during the pre-robot phase. Conversely, units were classified as "pellet cells" if their z-score was greater than 3 during the pre-robot phase and less than 3 during the robot phase. If the z-score was greater than 3 in both the pre-robot and robot phases, those units were classified as "BOTH cells." (B) Raster plots showing all robot, pellet, and BOTH cells during pre-robot, robot, and post-robot sessions. (C-E) The top row in each group of cells shows the mean firing rate and movement speed (± SEM; shaded areas) during the pre-robot, robot, and post-robot sessions. The bottom rows show the correlation coefficients between the firing rate and movement speed during each session in the robot (C), pellet (D), and BOTH (E) cell groups.

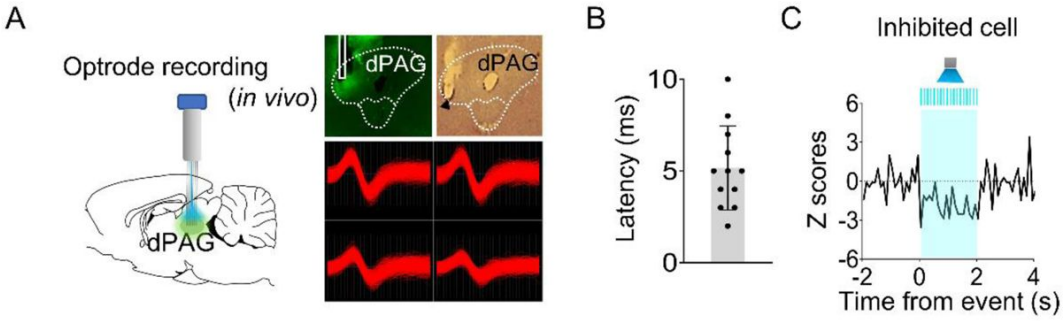

Figure S2.

Optrode recording of the dPAG.

(A) Virus was injected in the dPAG and an optrode was implanted targeting the dPAG (*left*). Photomicrographs show virus expression and optic fiber/electrode tips, as well as a representative dPAG neuron (*right*). Light stimulation was delivered while single units were recorded in anesthetized rats. (B) Response latencies of the stimulation-responsive dPAG cells. (C) There was one dPAG cell showing inhibited responses to the optical stimulation of the dPAG.

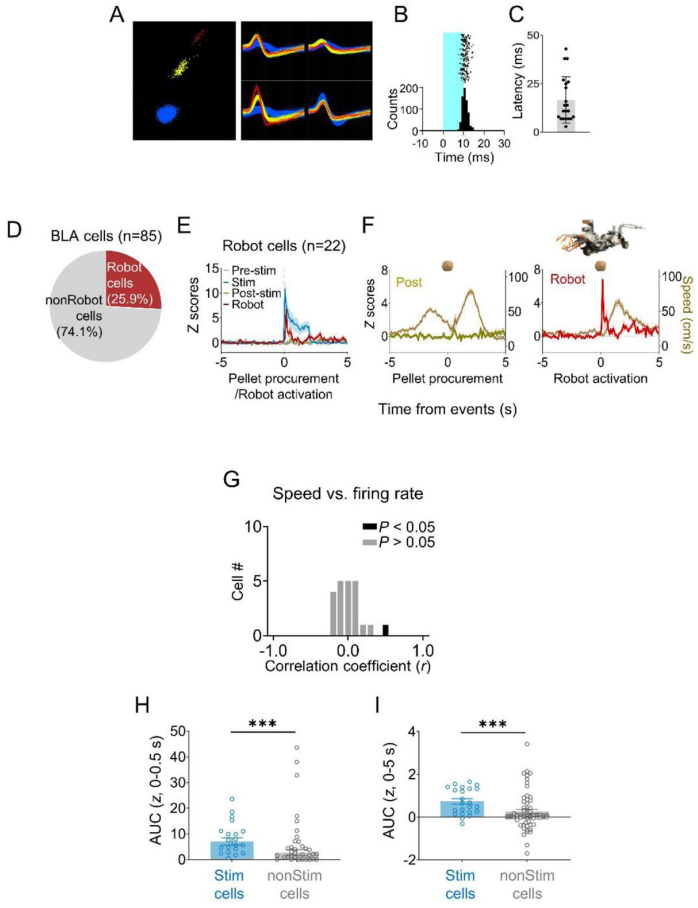

Figure S3.

Characteristics of BLA cells.

(A) Among 85 units collected from the BLA, 25.9% units immediately responded to the robot. (B) Representative waveforms of the BLA cells. (C) A representative raster plot and peri-event time histograms to the 20-Hz light stimulations (10-ms pulse width, 2-s duration). (D) Response latencies of the BLA cells that responded to the dPAG stimulation. (E) A pre-event time histogram showing the BLA activity aligned to the pellet procurement (pre-stim and post-stim sessions), dPAG stimulation (stim session), or robot activation (robot session). (F) The firing rates of the robot cells and movement speeds were plotted for the pellet procurement (post-robot session) and the robot activation (robot sessions). (G) All BLA cells, except for one, showed no significant correlation between the firing rate and the movement speed. (H, I) dPAG stimulation-responsive units showed higher levels of robot-evoked firings compared to the stimulation-non-responsive units during the short (H; 0-0.5 s after robot activation; $U = 339.0$, $P = 0.0001$; Mann-Whitney U test) and long-lasting (I; 0-5 s after robot activation; $U = 384.5$, $P = 0.0009$; Mann-Whitney U test) time windows. *** denotes P < 0.001 compared to the non-stim pairs.

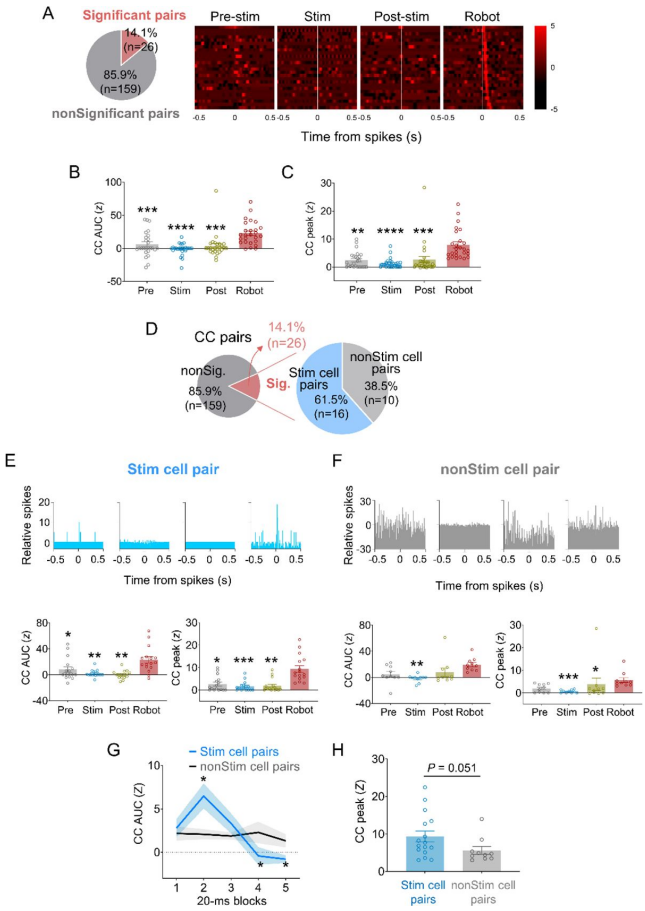

Figure S4.

Spike synchrony of the dPAG stimulation-responsive BLA neurons under predatory threats.

(A) Among the 185 CCs of the simultaneously recorded BLA neurons, 14.1% of the pairs showed significant ($z > 3$) peaks during the robot session. (B, C) The mean CC AUCs (B) and CC peak values (C) were higher during the robot session compared to the other sessions (Zs > 29.36, $P < 0.0001$, Friedman test; Ps < 0.01 for pre-stimulation vs. robot, stimulation vs. robot, and post-stimulation vs. robot sessions comparisons, Dunn's test). **, ***, and **** denote $P < 0.01$, $P < 0.001$, and $P < 0.0001$ compared to the robot session, respectively. (D) Among the BLA cell pairs that significantly synchronized during the robot session, 61.5% of the pairs (stim pairs) included dPAG stimulation-responsive BLA cells while 38.5 % of the pairs (non-stim pairs) did not. (E) Spike synchrony of the stim pairs. A representative CC of a stim pair (*top*) showed increased correlated firing during the robot session. The mean CC AUC (*bottom, left*) and CC peak (*bottom right*) of stim pairs during the robot session were higher than those during the other sessions (Zs > 17.42, $P < 0.001$, Friedman test; Ps < 0.037 for pre-stimulation vs. robot, stimulation vs. robot, and post-stimulation vs. robot sessions comparisons, Dunn's test). *, **, and *** denote $P < 0.05$, $P < 0.01$, and $P < 0.001$ compared to the robot session, respectively. (F) Spike synchrony of the non-stim pairs. The representative CCs show significant correlated firing during the robot session in the non-stim pairs (*top*). The CC AUC (*bottom left*) did not differ between the robot and pellet-only sessions (pre- and post-stim sessions) while peak area during the robot session was higher than that during the stim session ($Z = 13.84$, $P < 0.01$, Friedman test; $P = 0.0017$ for the stimulation vs. robot sessions comparison, Dunn's test). The CC peak (*bottom right*) during the robot session was higher than those during the stimulation and post-stimulation sessions ($Z = 15.92$, $P < 0.01$, Friedman test; Ps < 0.0335 for stimulation vs. robot and post-stimulation vs. robot sessions comparisons, Dunn's test), but not the pre-stimulation session. *, **, and *** denote $P < 0.05$, $P < 0.01$, and $P < 0.001$ compared to the robot session, respectively. (G) Stim pairs showed higher correlated firings than the non-stim pairs during the 20-40 ms time period after the paired cell fired ($U = 32$, $P = 0.011$; Mann-Whitney U test) while they showed lower correlated firings than the non-stim pairs during the 60-100 ms time window of the CCs (60-100 ms; Us < 37, Ps < 0.023; Mann-Whitney U test). * denotes $P < 0.05$ compared to the non-stim pairs. (H) The CC peaks of the stim pairs during the robot session tended to be higher than those of the non-stim pairs ($U = 43.0$, $P = 0.051$; Mann-Whitney U test).

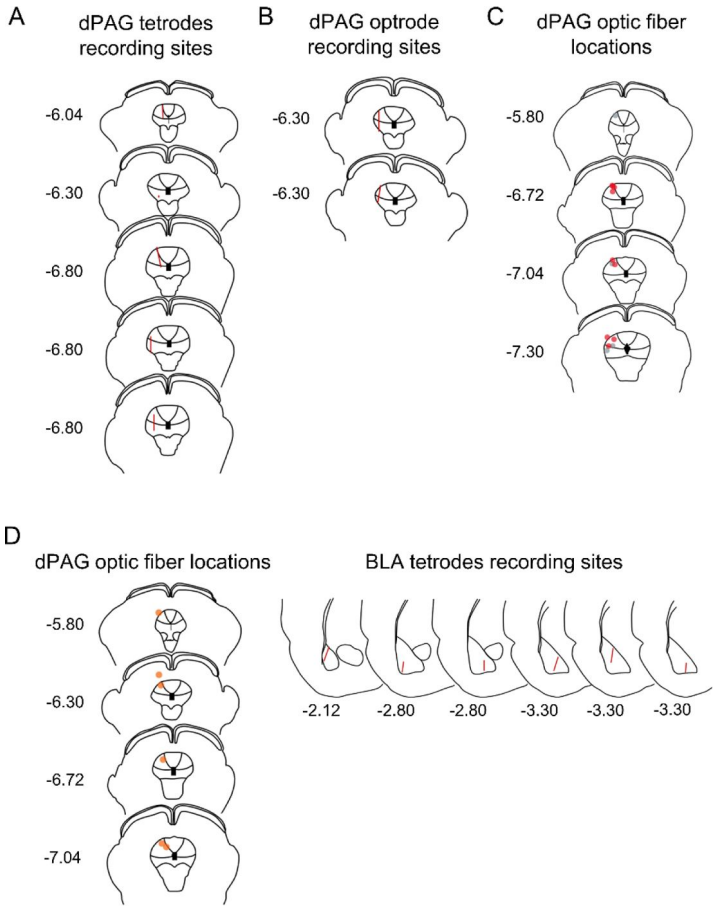

Figure S5.

Histological reconstructions of the recording sites in the dPAG and BLA, and optic fiber locations in the dPAG.

(A) Red bars indicate the trajectory of the tetrode recording sites in the dPAG. (B) Red bars indicate the trajectory of the optrode recording sites in the dPAG. (B) Red and gray circles indicate the optic fiber locations of the ChR2 and EYFP rats, respectively. (D) Orange circles and red bars indicate the optic fiber locations in the dPAG (*left*) and recording trajectories in the BLA (*right*). The numerical values represent AP coordinates relative to the Bregma.

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Author information

Eun Joo Kim
Department of Psychology, University of Washington, Seattle, WA 98195-1525, USA
ORCID iD: 0000-0002-8499-9135

Mi-Seon Kong
Department of Psychiatry and Behavioral Sciences, University of Washington, Seattle, WA 98195-1525, USA

Sanggeon Park
Department of Brain and Cognitive Sciences, Scranton College, Ewha Womans University, Seoul, 03760, Republic of Korea, Brain Disease Research Institute, Ewha Brain Institute, Ewha Womans University, Seoul, Republic of Korea

Jeiwon Cho
Department of Brain and Cognitive Sciences, Scranton College, Ewha Womans University, Seoul, 03760, Republic of Korea, Brain Disease Research Institute, Ewha Brain Institute, Ewha Womans University, Seoul, Republic of Korea
For correspondence: jelectro21@ewha.ac.kr

Jeansok J. Kim
Department of Psychology, University of Washington, Seattle, WA 98195-1525, USA, Program in Neuroscience, University of Washington, Seattle, WA 98195-1525, USA
For correspondence: jeansokk@uw.edu
ORCID iD: 0000-0001-7964-106X

Editors

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Joint 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. The authors employed single-unit recording of BLA and PAG neurons and optogenetic tools to address this question in an 'approach food-avoid predator' paradigm.

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 did not change its 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 three 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.

Strengths

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 appropriate tools to address their questions. The major impact in the field would be to add evidence to claims that the BLA can be downstream of the dPAG to evoke defensive behaviors. The study also adds to a body of evidence that the PAG mediates primal fear responses.

Weaknesses

The two most significant weaknesses relate to a) anatomical concerns related to the subregions of the BLA and PAG that were targeted by manipulations and analyses and b) the correlational nature of the PVT measures and the lack of any causal role demonstrated. Other concerns are also detailed below.

Anatomical concerns:

1. The authors claim that the recordings were performed in the dorsal PAG (dPAG), but the histological images in Fig. 1B and Supplementary S2 for example show the tip of the electrode in a different subregion of PAG (ventral/lateral). They should perform a more careful histological analysis of the recording sites and explain the histological inclusion and exclusion criteria. Diagrams showing the sites of all PAG and BLA recordings, as well as all fiber optics, would be helpful.
2. Prior studies investigating the role of BLA neurons during a foraging vs. robot test similar to the one used in this study should be also cited and discussed (e.g., Amir et al 2019, PMID: 30840520; Amir et al 2015, PMID: 26400931). These two studies demonstrated that most neurons in the basal portion of the BLA exhibit inhibitory activity during foraging behavior and only a small fraction of neurons (~4%) display excitatory activity in response to the robot (in contrast to the 25% reported in the present study). A very accurate histological analysis of BLA recording sites should be performed to clarify whether distinct subregions of the BLA encode foraging and predator-related information, as previously shown in the two described studies.

3. An important claim of this study that the PAG sends predator-related signals to BLA via the PVT (Fig. 4). The authors stated that PVT neurons labeled by intra-BLA injection of the retrograde tracer CTB were activated by the predator, but a proper immunohistochemical quantification with a control group was not provided to support this claim. To provide better support for their claim, the authors should quantify the double-labeled PVT neurons (cFos plus CTB positive neurons) during the robot test.
4. The AVV anterograde tracer deposit spread to a large part of the PAG, including dorsolateral and lateral PAG, and supraoculomotor regions (Fig. 4B). Is the projection to the PVT from the dPAG or other regions of the PAG?

Concerns about the strength of the evidence supporting a role for the PVT:

5. The authors conclude in the discussion section that the dPAG-amygdala pathway is involved in generating antipredatory defensive behavior. However, the current results are entirely based on correlational analyses of neural firing rate and there is no direct demonstration that the PAG provides information about the robot to the BLA. Therefore, the authors should tone down their interpretation or provide more evidence to support it by performing experiments applying inhibitory tools in the dPAG > PVT > BLA pathway and examining the impact on behavior and downstream neural firing.

Other concerns:

6. One of the main findings of this study is the observation that BLA neurons that are responsive to PAG photostimulation are preferentially recruited during the foraging vs. robot test (Fig. 3). However, the experimental design used to address this question is problematic because the laser photostimulation of PAG neurons preceded the foraging vs. robot test. Prior photoactivation of PAG may have caused indirect short-term synaptic plasticity in BLA cells, which would favor the response of these cells to the robot. Please see Oishi et al, 2019 PMID: 30621738, which demonstrated that 10 trains of 20Hz photoactivation (300 pulses each) was sufficient to induce LTP in brain slices.
7. The authors should perform a longitudinal analysis of the behavioral responses of the rats across the trials to clarify whether the animals habituate to the robot or not. In Figure 1E, it appears that PAG neurons fire less across the trials, which could be associated with behavioral habituation to the predator robot. If that is the case, the activity of many other PAG and BLA neurons will also most likely vary according to the trial number, which would impact the current interpretation of the results.
8. In Figure 1, it is unclear why the authors compared the activity of neurons that respond to the robot activation against the activity of the neurons during the retrieval of the food pellets in the pre-robot and post-robot sessions. The best comparison would be aligning the cells that were responsive to the activation of the robot with the moment in which the animals run back to the nest after consuming the pellets during the pre-robot or post-robot sessions. This would enable the authors to demonstrate that the PAG responses are directly associated with the expression of escaping behavior in the presence of the robot rather than associated with the onset of goal-directed movement in direction to the next during the pre- and post-robot sessions. A graphic showing the correlation between PAG firing rate and escape response would be also informative.

Author Response

Joint Public Review

Strengths

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 appropriate tools to address their questions. The major impact in the field would be to add evidence to claims that the BLA can be downstream of the dPAG to evoke defensive behaviors. The study also adds to a body of evidence that the PAG mediates primal fear responses.

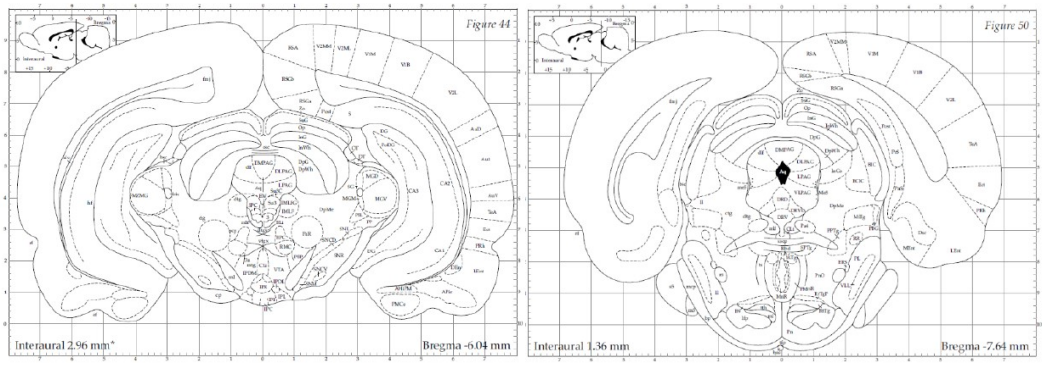
Weaknesses

(Anatomical concerns)

1. *The authors claim that the recordings were performed in the dorsal PAG (dPAG), but the histological images in Fig. 1B and Supplementary S2 for example show the tip of the electrode in a different subregion of PAG (ventral/lateral). They should perform a more careful histological analysis of the recording sites and explain the histological inclusion and exclusion criteria. Diagrams showing the sites of all PAG and BLA recordings, as well as all fiber optics, would be helpful.*

The PAG is composed of dorsomedial (dm), dorsolateral (dl), lateral (l), and ventrolateral (vl) columns that extend along the rostro-caudal axis of the aqueduct. The term “dorsal PAG” (dPAG) generally encompasses dmPAG, dlPAG, and lPAG, as substantiated by track-tracing, neurochemical, and immunohistochemical techniques (e.g., Bandler et al., 1991; Bandler & Keay, 1996; Carrive, 1993). As 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. (2005) clarified in their review that, “According to this parcellation…the defensive behaviors (freezing, flight or fight) and aversion-related responses (switchoff behavior) were ascribed to the DMPAG, DLPAG, and LPAG (usually named the ‘dorsal’ PAG).” In our study, all recordings were conducted within the dPAG. Also, Figures 1B and S2 in our manuscript correspond to the -6.04 mm template from Paxinos & Watson’s atlas (1998), which is shown in the left panel in Author response image 1 and is considerably anterior to the location where the vlPAG emerges, as shown in the right panel. In our revised manuscript, we will provide a detailed definition of the dPAG, inclusive of dmPAG, dlPAG, and lPAG, and support this with the referenced literature.

Author response image 1.



> 1. *Prior studies investigating the role of BLA neurons during a foraging vs. robot test similar to the one used in this study should be also cited and discussed (e.g., Amir et al 2019; Amir et al 2015). These two studies demonstrated that most neurons in the basal portion of the BLA exhibit inhibitory activity during foraging behavior and only a small fraction of neurons (~4%) display excitatory activity in response to the robot (in contrast to the 25% reported in the present study). A very accurate histological analysis of BLA recording sites should be performed to clarify whether distinct subregions of the BLA encode foraging and predator-related information, as previously shown in the two described studies.*

In the revised manuscript, we will discuss papers by Amir et al. (2015) and Amir et al. (2019) that utilized a similar 'approach food-avoid predator' paradigm. These studies found a correlation between the neuronal activities in the basolateral amygdala (BL) and the velocity of animal movement during foraging, regardless of the presence or absence of predators. Specifically, the majority of BL neurons were inhibited in both conditions, with only 4.5% being responsive to predators. Consequently, Amir et al. posited that amygdala activity predominantly aligns with behavioral output such as foraging, rather than with responses to threats.

In contrast, our body of work (Kim et al., 2018; Kong et al., 2021; the present study) reveals that the majority of neurons in the BA/BLA displayed distinct responses in pre-robot and robot sessions. Kong et al. (2021) discussed in depth several factors that may account for this discrepancy, given that both Amir et al. and our research used similar behavioral paradigms. Differences in apparatus features, experimental procedures, and data analysis methodologies (refer to Amir et al., 2019) could be contributing to the conflicting results and interpretations concerning the significance of amygdalar neuronal activities.

Additionally, our studies uniquely monitored the same set of amygdalar neurons during pre-robot and robot sessions, affording us the opportunity for a direct comparison of neuronal activities under different threat conditions.

Another salient difference lines in the foraging success rates, which were markedly higher in Amir et al (~80%) compared to our studies (<3-4%). We hypothesize that there may be an inverse relationship between the pellet procurement rate and the intensity of fear. The high foraging success rate in Amir et al., which correlates with subdued amygdalar activity, stands in contrast to our findings of heightened amygdalar activity associated with a lower foraging success rate. Supporting this notion, optogeneticallyinduced amygdalar activity led naïve rats to abandon foraging and escape to the nest (Kong et al., 2021, the present study).

> 1. *An important claim of this study that the PAG sends predator-related signals to BLA via the PVT (Fig. 4). The authors stated that PVT neurons labeled by intra-BLA injection of the retrograde tracer CTB were activated by the predator, but a proper immunohistochemical quantification with a control group was not provided to support this claim. To provide better support for their claim, the authors should quantify the doublelabeled PVT neurons (cFos plus CTB positive neurons) during the robot test.*

As recommended, we will include a revised Fig. 4 in the manuscript to present the quantification of neurons that are double-labeled with c-Fos and CTB in the PVT. This updated figure will provide a more rigorous analysis and visual representation of the data.

> 1. *The AVV anterograde tracer deposit spread to a large part of the PAG, including dorsolateral and lateral PAG, and supraoculomotor regions (Fig. 4B). Is the projection to the PVT from the dPAG or other regions of the PAG?*

As previously addressed in response to Comment #1, the dPAG comprises the dmPAG, dlPAG, and lPAG. In the revised manuscript, we will acknowledge the diffusion of the AAV to the adjacent deep gray layer of the superior colliculus. Additionally, we are considering conducting more restricted AAV injections into the dPAG to verify terminal expressions in the PVT.

> *(Concerns about the strength of the evidence supporting a role for the PVT)*

> 1. *The authors conclude in the discussion section that the dPAG-amygdala pathway is involved in generating antipredatory defensive behavior. However, the current results are entirely based on correlational analyses of neural firing rate and there is no direct demonstration that the PAG provides information about the robot to the BLA. Therefore, the authors should tone down their interpretation or provide more evidence to support it by performing experiments applying inhibitory tools in the dPAG > PVT > BLA pathway and examining the impact on behavior and downstream neural firing.*

As suggested, we will moderate the assertions about the functional implications of the PVT, based on the data from anterograde and retrograde tracers, to present a more measured interpretation in the manuscript.

> *(Other concerns)*

> 1. *One of the main findings of this study is the observation that BLA neurons that are responsive to PAG photostimulation are preferentially recruited during the foraging vs. robot test (Fig. 3). However, the experimental design used to address this question is problematic because the laser photostimulation of PAG neurons preceded the foraging vs. robot test. Prior photoactivation of PAG may have caused indirect shortterm synaptic plasticity in BLA cells, which would favor the response of these cells to the robot. Please see Oishi et al, 2019 PMID: 30621738, which demonstrated that 10 trains of 20Hz photoactivation (300 pulses each) was sufficient to induce LTP in brain slices.*

After approximately eight photostimulation trials of the dPAG, with 40 pulses each, the animals entered a post-photostimulation testing phase (referred to as "Post"; Fig. 3C), lasting 10-15 minutes over an average of eight trials before robot testing. Although the PAG does not directly project to the BLA, the remote possibility of trans-synaptic plasticity in the BLA cannot be completely excluded and will be acknowledged. Additionally, it is noteworthy that Oishi et al's (2019) study applied a total of 3,000 pulses (i.e., 10 15-s trains of 20-Hz pulses) and investigated CA3-CA3 synaptic plasticity, as opposed to a total of 320 pulses (i.e., 8 2-s trains of 20-Hz pulses) in our study.

> 1. *The authors should perform a longitudinal analysis of the behavioral responses of the rats across the trials to clarify whether the animals habituate to the robot or not. In Figure 1E, it appears that PAG neurons fire less across the trials, which could be associated with behavioral habituation to the predator robot. If that is the case, the activity of many other PAG and BLA neurons will also most likely vary according to the trial number, which would impact the current interpretation of the results.*

In Figure 1E, the y-axis represents the Z scores of individual dPAG neurons, instead of representing repeated tests of the same neuron across multiple trials. The raster plot in Figure 1F clearly depicts that the same dPAG neurons consistently display heightened neural activity in response to the approaching robot across successive trials.

> 1. *In Figure 1, it is unclear why the authors compared the activity of neurons that respond to the robot activation against the activity of the neurons during the retrieval of the food pellets in the pre-robot and postrobot sessions. The best comparison would be aligning the cells that were responsive to the activation of the robot with the moment in which the animals run back to the nest after consuming the pellets during the prerobot or post-robot sessions. This would enable the authors to demonstrate that the PAG responses are directly associated with the expression of escaping behavior in the presence of the robot rather than associated with the onset of goal-directed movement in direction to the next during the pre- and post-robot sessions. A graphic showing the correlation between PAG firing rate and escape response would be also informative.*

Figure 1E compares the dPAG neural activity when animals enter a designated pellet zone (time-stamped by camera tracking) during both pre-robot and post-robot trials to the dPAG neural activity when entering the robot trigger zone (time-stamped by robot activation). We wish to clarify that rats carry the large (0.5 g) pellet back to the nest for consumption rather than consume it in the open arena before returning to the nest.

In our study, we aimed to investigate the direct response of dPAG neurons to the looming predator and explore the communication between dPAG and BLA in relation to antipredatory defensive responses. To build upon our previous research that suggests a potential role of dPAG in conveying such responses to the BLA (Kim et al., 2013) and the immediate firing of BLA neurons in response to predatory threats (Kim et al., 2018; Kong et al., 2021), we chose to narrow our testing window to a short latency period (< 500 ms) following robot activations. This specific time window allowed us to focus on the initial stages of the threat stimulus processing and minimize potential confounding factors such as the presence of residual firing activity triggered by the robot during the animals' escape or any activity changes induced by the animals' behavior.

Furthermore, Figure S1C clearly demonstrates that (i) increased activity of dPAG robot cells preceded the animals' actual turning and fleeing behavior toward the nest, as indicated by the peak values of movement speed (dark yellow), and (ii) the presence of pellets did not affect activity changes of the robot cells during pre- and post-robot sessions. These observations suggest that the heightened activity of dPAG robot cells was not due to movement changes or pellet motivation.

Lastly, as stated in the original manuscript, the vast majority of robot cells (90.9%) did not show significant correlations between movement speed and firing rates, lending further support to the interpretation that the dPAG activity observed was not merely a reflection of movement changes.

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