A 7-Tesla MRI study of the periaqueductal grey: resting state and task activation under threat

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Abstract

The periaqueductal grey (PAG) is a region of the midbrain implicated in a variety of behaviors including defensive responses to threat. Despite the wealth of knowledge pertaining to the differential functional roles of the PAG columns in nonhuman and human research, the basic functional connectivity of the PAG at rest has not been well characterized. Therefore, the current study utilized 7-Tesla MRI to characterize PAG functional connectivity at rest and task activation under uncertain threat. A sample of 53 neurologically healthy undergraduate participants (Mage=22.2, SDage=3.62) underwent structural and resting state functional MRI scans. Supporting previous work, voxel-wise analyses showed the PAG is functionally connected to emotion regulation and fear networks. Comparison of functional connectivity of PAG columns did not reveal any significant differences. Thirty-five participants from the same sample also completed an uncertain threat task with blocks of 3 conditions-- No shock, Predictable shock, and Unpredictable shock. There were no robust activity differences within the PAG columns or the whole PAG across conditions, though there was differential activity at the voxel level in the PAG and in other regions theoretically relevant to uncertain threat. Results of this study elucidate PAG connectivity at rest and activation in response to uncertain threat.

Keywords: Periaqueductal grey, PAG, resting state, functional connectivity, NPU, uncertain threat
Introduction

The periaqueductal grey (PAG) is a region of the midbrain that has been implicated in a variety of complex behaviors including defensive responses to threat, as well as integrating information from multiple systems (i.e. somatic, autonomic, and sensory systems) to coordinate and regulate emotional behavior (An et al., 1998; Bandler et al., 2000; Behbehani, 1995; Fanselow, 1991; Lindquist et al., 2012; Motta et al., 2017). Given its role in defensive behaviors, the PAG is a critical region of fear and anxiety neurocircuitry (Fanselow, 1991). Characterizing the basic functional connectivity of the PAG is important to understanding how dysfunction may underlie aberrant fear and anxiety states.

The anatomical connectivity of the PAG has been well characterized in nonhuman animal work (An et al., 1998; Bandler et al., 2000; Barbaresi & Mensa, 2016; Jansen et al., 1998; Krout & Loewy, 2000; Mantyh, 1983b, 1983a). Retrograde tracers injected in the PAG in macaque monkeys show clear connections to medial and ventrolateral prefrontal cortex (PFC), anterior cingulate cortex (ACC), caudal and lateral orbitofrontal cortex (OFC), temporal pole, ventral insula, superior temporal sulcus and gyrus, basal forebrain, hypothalamus, and amygdala (An et al., 1998). Anterograde tracers injected in the PAG in macaque monkeys show connections to medial PFC, posterior orbital cortex, and ACC (An et al., 1998; Bandler et al., 2000). More specifically, strong projections were also shown from medial PFC to the dorsolateral column of the PAG, orbital cortices more strongly projected to the ventrolateral column, and cingulate regions were more strongly connected to the lateral column (An et al., 1998; Bandler et al., 2000).

In addition to structural connectivity differences, studies with nonhuman animals have also shown columns of the PAG can be distinguished by differences in cytoarchitecture and
neurochemistry (Menant et al., 2016). The columnar organization of the PAG likely underlies the varied functional outcomes elicited in animal models. The dorsal columns have been implicated in active-coping defensive strategies such as fear, panic, and fleeing behaviors (An et al., 1998; Bandler et al., 2000; Behbehani, 1995; Bittencourt et al., 2004; Deng et al., 2016; Mendes-Gomes & Nunes-de-Souza, 2009; Molchanov & Guimaraes, 2002; Motta et al., 2017; Vianna et al., 2003; Vieira-Rasteli et al., 2018). The lateral PAG columns have also been implicated in active-coping threat reactions (Bittencourt et al., 2004; Fanselow, 1991; Faull et al., 2019). Finally, the ventral columns appear to facilitate passive-coping strategies such as freezing or quiescence behaviors (An et al., 1998; Bandler et al., 2000; Behbehani, 1995; Fanselow, 1991; Molchanov & Guimaraes, 2002; Vieira-Rasteli et al., 2018).

In humans, the distinct structural connectivity (Ezra et al., 2015) and functional roles of the PAG columns are also well-maintained, though the PAG is less frequently studied due to its inaccessibility (i.e. size and location). Most functional connectivity work of the human PAG has examined task-based effects that elicit the differential activity of the PAG columns through a wide variety of task paradigms in the MRI scanner (Faull et al., 2015, 2016; Faull & Pattinson, 2017; Hahn et al., 2013; Hashemi et al., 2019; Kragel et al., 2019; La Cesa et al., 2014; Mobbs et al., 2007; Ritter et al., 2013; Satpute et al., 2013).

Despite all of this, and to the authors knowledge, basic functional connectivity of the PAG and its columns in healthy individuals has only been characterized in two other studies (Coulombe et al., 2016; Faull & Pattinson, 2017). In addition, given its small size, conventional 3-Tesla MRI produces inadequate spatial resolution to distinguish the columns. However, use of 7-Tesla MRI has demonstrated significantly improved spatial resolution for PAG function as compared to 3T (Hahn et al., 2013). Therefore, the current study utilized high resolution 7-Tesla
MRI to characterize the functional connectivity of the PAG and its columns (ventrolateral, lateral, dorsolateral, and dorsomedial) in healthy individuals at rest (Experiment 1). To follow up the resting state analysis, PAG activity was also assessed under an uncertain threat of shock paradigm (Experiment 2).

**Experiment 1: Resting state functional connectivity of the PAG**

**Method**

**Participants**

Neurologically healthy undergraduate students from the University of Wisconsin-Milwaukee were recruited to participate in this study (n=57; 12 males, 45 females; M_age=22.2, SD_age=3.62; 56% Caucasian, 14% Asian or Pacific Islander, 12% African American, 12% Hispanic, 4% Other). The participants in the current study are part of the same dataset reported in Weis et al. (2019). Inclusion criteria included being over 18-years-old, right-handed, and English-speaking. Exclusion criteria included history of head trauma, neurologic disorder, history of psychosis or bipolar disorder, current use of antipsychotics, anticonvulsants, or mood stabilizers, and any contraindications to MRI including metal in the body, pregnancy, or claustrophobia. Note, participants were not excluded for medications such as antidepressants or anxiolytics that may have effects on anxiety neurocircuitry including the PAG (Harris & Reynell, 2017). These data were also not recorded as part of this study and thus cannot be examined as a potential confound in our results. The University of Wisconsin Milwaukee and the Medical College of Wisconsin Institutional Review Boards approved this study. Participants provided written informed consent, according to the Declaration of Helsinki, and were paid for their participation in the study.

**MRI Acquisition**
MR images were collected on a 7-Tesla MR950 General Electric scanner with a 32-channel Nova coil. High-resolution axial T1-weighted whole-brain anatomical images were acquired (voxel resolution = 0.43 x 0.43 x 0.80 mm, slice thickness = 0.8 mm, repetition time = 8.012 ms, echo time = 3.784 ms, inversion time = 1050 ms, flip angle = 5°, field of view = 220 mm, matrix = 276 x 276). A single-shot gradient-echo EPI sequence was used for the 8-minute resting state scan where participants were instructed to stay awake and blink normally while shown a white fixation cross on a black background (volumes = 192, repetition time = 2500 ms, echo time = 24 ms, flip angle = 73°, field of view = 220 mm, matrix = 224 x 224, number of excitations = 1, slice thickness = 1.8 mm, 30 axial slices with 0 mm gap, voxel resolution = 0.859 x 0.859 x 1.80 mm). To optimize the spatial resolution advantages of the 7T, EPI scans were acquired with partial coverage of the brain. Coverage was determined for each individual participant such that the top of the insula was covered by the most superior slices and the hippocampus covered by the most inferior slices. See Figure 1 for coverage of a representative participant. For distortion correction in the EPI preprocessing, an additional single-volume EPI scan with reverse phase encoding polarity was collected. The average signal-to-noise ratio (SNR = average[signal] / std[noise]) calculated in AFNI for the preprocessed functional data after regression across the sample was 120.93.

**Resting State fMRI analysis**

Freesurfer version 6.0 was used to segment tissue type for each individual’s anatomy (Fischl, 2012). Preprocessing and analysis of the resting state fMRI were performed using the ANATICOR (Jo et al., 2010) processing pipeline in AFNI (version AFNI_19.2.01 ‘Claudius’) (Cox, 1996). ANATICOR is a denoising protocol that removes unwanted signal from white matter and ventricles by using eroded white matter and ventricle masks extracted from
FreeSurfer to reduce any partial volume effects in the grey matter signal. Ventricular and white matter signal regressors are estimated before smoothing and projected out of the final functional dataset ahead of correlational analyses.

The first 3 volumes were removed from the EPI to remove pre-steady state artifacts. The remaining volumes were despiked and slice time corrected to the first slice. Given the greater sensitivity to distortions at ultra-high field, EPI and reverse polarity scans were warped to a middle space for distortion correction (-plusminus in 3dQwarp). Functional volumes were then co-registered to the first functional volume and aligned to the anatomy (-partial_axial in align_epi_anat.py). The anatomy and EPI were then warped to MNI space (MNI152) for group analysis. Given the close spatial proximity of the PAG columns and to preserve the high-resolution EPI, PAG column time series were extracted prior to smoothing. Functional images were then smoothed with a 3.6mm kernel (double the functional voxel size) (Satpute et al., 2013).

In the regression (3dDeconvolve), one censor file was included that indicated TRs to exclude where more than 10% of voxels in the brain were outliers and/or where excessive motion yielded a frame-to-frame Euclidean norm motion derivative greater than 0.3mm (Gorka et al., 2017; Torrisi et al., 2015). Three participants (3 females) were dropped from final analysis for exceeding the motion threshold (>15% TRs censored) leaving a final sample of 54. Six head motion parameters and their derivatives, a bandpass filter (0.01-0.1Hz), and the time series from eroded ventricle and white matter masks were also included as regressors and projected out of the final dataset. The estimated blur of the final EPI dataset was calculated with 3dFWHMx using a mask of the unblurred group PAG seed mask. To correct for multiple comparisons, resultant average auto correlation function (ACF) parameters were entered in 3dClustSim to determine the
voxel-wise $p < 0.001$, and cluster thresholds $p < 0.05$, $k > 303$ (Chen et al., 2017; Woo et al., 2014).

**Definition of PAG seeds**

Manual segmentation of the PAG was done in reference to Duvernoy’s Atlas of the Human Brain Stem and Cerebellum (Duvernoy, 2009) and in accordance with previous human PAG segmentation protocols (Kragel et al., 2019; Satpute et al., 2013). The method employed for manual segmentation involves first determining placement of the cerebral aqueduct followed by identification of the surrounding PAG. After carefully reviewing the alignment of the anatomy and EPI images for each participant, it was determined manual tracing of the aqueduct and PAG could be done on the anatomical images aligned to standard MNI space rather than the anatomy in the original subject’s space. This decision also reduces the need for transformations performed on the PAG masks and ensures resultant masks are well aligned to the high-resolution EPI.

Manual tracing began at the first axial slice where the cerebral aqueduct no longer appeared to be connected to the third ventricle. Tracing continued inferiorly until the last slice where the aqueduct is clearly surrounded by grey matter. From a sagittal view, the aqueduct should be wedged between rostral and caudal grey matter and should not continue into the cavernous 5th ventricle. With a slice thickness of 0.8 mm, in the current dataset, the aqueduct extended $\sim 15$ slices (per the average longitudinal length reported in Satpute et al., 2013). Once the aqueduct was identified, its mask was used to create the PAG by dilating out 2 voxels in all directions: + 0.86 x 0.86 x 1.60 mm (Kragel et al., 2019; Satpute et al., 2013). Then the original tracing of the aqueduct was subtracted out from the dilation, leaving only the voxels surrounding it. Resultant PAG masks were visually inspected for placement and alignment to the EPI, and
manually edited if needed (see Supplemental Figure 1 for further manual segmentation protocol details). AFNI’s dilation procedure (in 3dmask_tool) occurs across the 18 neighbors a given voxel shares either a face or an edge with, i.e. all of the neighbors in a 3x3x3 box except the 8 outer corners. Thus, depending on the initial drawing of the aqueduct, further dilation may extend too far and include voxels (especially at outer corners) likely not belonging to the PAG. Manual editing entailed removing these extraneous voxels and occurred in < 25% of the sample. One participant (female) was removed from analysis due to insufficient coverage of the PAG mask, leaving a final sample of 53 (12 males, 41 females).

Since signal from the cerebrospinal fluid in the aqueduct can contaminate our intended signal of interest, we wanted to verify voxels of the aqueduct were appropriately removed from PAG masks. To do this, we compared average signal variability between the aqueduct and PAG masks for each participant (Satpute et al., 2013). This comparison demonstrated 10 times greater variability overall in aqueduct signal intensity as compared to PAG and further validates appropriate separation of the noisy CSF versus the grey matter signal of interest (Supplemental Figure 2).

Segmentation of the 4 columns of the PAG was done one time at the group level. This decision was made to reduce the amount of bias that may result from segmenting the columns separately for each individual subject. Furthermore, there is no established protocol in the literature to segment PAG columns, and though voxel resolution is very high at 7T it is still not high enough to make judgements on PAG column delineation according to any anatomical features. First, an average group mask of all the individual manually drawn PAG masks was created. Calculation of the Sorenson-Dice coefficient (DSC) across all manually segmented PAG masks indicates imperfect overlap of masks (DSC = 0.51). Therefore, the group mask was then
thresholded to retain voxels identified as the PAG in at least 50% of the sample. This group mask was visually inspected for accuracy (see Supplemental Figure 3 for group PAG mask overlaid on an average functional scan). Next, the columns of the PAG were segmented on the group mask by dividing the PAG into dorsomedial, bilateral dorsolateral, bilateral lateral, bilateral ventrolateral, and ventromedial columns (Ezra et al., 2015). The ventromedial column was segmented to aid in the delineation of the other columns but was not included in further analyses as its thought to be part of other brainstem nuclei (Ezra et al., 2015). Left and right hemispheres were combined for dorsolateral, lateral, and ventrolateral columns (Figure 2). Then using this group mask, average time series was extracted for each participant for each of the 4 columns from the pre-smoothed distortion corrected aligned functional data. Given the size and spatial proximity of the PAG columns, for each participant Pearson correlations for all pairwise PAG column time series were calculated, Fisher Z-transformed, averaged, and back-transformed to obtain standardized group “average correlations” (Supplemental Table 1). Not surprisingly, each pair of columns was highly correlated within individual subjects (Coulombe et al., 2016).

To analyze voxel-wise functional connectivity of the PAG columns, the time series for each column (extracted before smoothing) was correlated with every other voxel within the field of view and resulting correlation maps were r-to-z transformed. However, to account for the high degree of correlation among columns, a repeated measure analysis of covariance (ANCOVA) using AFNI's 3dMVM was performed with PAG column as a repeated measure (Chen et al., 2014; Coulombe et al., 2016). With a greater number of females in the sample, sex was included as a covariate in the model. For each PAG column, correlations between average seed time series (before smoothing) and the time series of every other voxel within the field of view were
calculated while controlling for the effects of the other 3 columns of interest. See Supplemental Table 2 for 3dMVM command.

Results

Resting state results

Whole PAG functional connectivity.

First, voxel-wise connectivity of the whole PAG seeds manually drawn for each participant was assessed. Results of the whole PAG seed analysis show surviving clusters were in theoretically expected regions (Figure 3). For instance, the PAG showed significant connectivity with anterior cingulate, superior orbital and hippocampal cortices, insula, and brain stem regions (see Table 1 for complete list of significant clusters).

PAG column functional connectivity

Results of the ANCOVA comparing voxel-wise resting state functional connectivity of the four PAG columns showed there were no clusters that survived correction for multiple comparisons in the overall column factor (voxel-wise \( p < 0.001 \), cluster-wise \( p < 0.05 \), \( k > 303 \)), though further examination of the main effect terms for each column revealed significant clusters. However, since the overall column factor in the ANCOVA did not yield any results that survived correction, the results from the main effects of each column are relegated to the supplement (Supplemental Table 3). Exploration of post-hoc pairwise comparisons of column functional connectivity maps further demonstrated no differences between any two pairs of columns. Individual column effects were thus only apparent after controlling for the effects of all 3 other columns. In light of these null findings, to ensure the analytic methods employed work more generally, a positive control analysis was conducted using another midbrain region, the ventral tegmental area (VTA), which has well-established connectivity patterns in the literature.
Results suggest VTA resting-state connectivity was as expected (Supplemental Table 4 and Supplemental Figure 4), providing evidence the analytic approach employed within the current study was effective and appropriate. As mentioned previously, the correlation of time series between pairwise comparisons of PAG columns showed high correlations amongst all pairs of columns. High correlations of activity amongst columns, close spatial proximity, and the absence of threat during rest may explain the lack of differential resting state functional connectivity assessed in pairwise comparisons.

**Experiment 1 Discussion**

The current study utilized high resolution 7-Tesla MRI to characterize the functional connectivity of the PAG and its columns in healthy individuals at rest. Results of the current study showed the PAG did not show differential connectivity within its columns during rest, though whole PAG functional connectivity was in theoretically expected regions within the limited field of view examined.

Previous work in non-human animal models has demonstrated functional differences in the PAG columns which has been replicated in humans under various task paradigms (Faull et al., 2015, 2016; Faull & Pattinson, 2017; Hahn et al., 2013; Hashemi et al., 2019; Kragel et al., 2019; Mobbs et al., 2007; Ritter et al., 2013; Satpute et al., 2013). However, *at rest* the current study did not find any functional connectivity differences between PAG columns across the brain within the field of view examined. While these results are inconsistent with previous work (Coulombe et al., 2016; Faull & Pattinson, 2017), despite similar sample sizes and characteristics, there are a few methodological differences to note. First, Faull & Pattinson (2017) defined PAG columns using functional activation from a breath holding task, whereas the current study utilized a rigorous manual tracing protocol based on anatomical landmarks.
Second, Coulombe et al. (2016) not only described PAG column connectivity at 3T, but also used spherical ROIs which greatly reduced spatial precision and separability of PAG columns (Coulombe et al., 2016). These key differences in study methodology and analysis approaches may explain the lack of consensus in specifically PAG column connectivity results. However, results of the voxel-wise functional connectivity analysis using the whole PAG seed are consistent with previous work demonstrating the PAG is functionally connected to the prefrontal, insular cortices, and brain stem grey matter in many human and non-human animal models (An et al., 1998; Bandler et al., 2000; Coulombe et al., 2016; Galgano et al., 2019; Harricharan et al., 2016; Jansen et al., 1998; Krout & Loewy, 2000; Mantyh, 1983b, 1983a).

Perhaps PAG column functional differences only emerge when under present threat or in anxiety-like states (Faull et al., 2016; Faull & Pattinson, 2017; Harricharan et al., 2016; Mobbs et al., 2007), but not at rest in healthy individuals. From a theoretical standpoint the PAG is involved in threat detection and initiation of defensive behaviors, both of which are processes belonging to a larger circuitry underlying fear and anxiety (An et al., 1998; Bandler et al., 2000; Behbehani, 1995; Fanselow, 1991; Harricharan et al., 2016; Lindquist et al., 2012; Motta et al., 2017; Sylvester et al., 2012; Torrisi et al., 2018). However, results of the current study would suggest that while the PAG is critical for fear and anxiety, it may not be “online” or recruited when there is no imminent threat present (Mobbs et al., 2007). In the resting state scan, participants were simply instructed to lie still and remain awake, and while the scanner may be daunting at first, most participants settle into a comfortable position within minutes. This scenario may simply not be threatening and thus necessitate recruitment of the PAG.

Moreover, we are confident PAG signal was adequately obtained and proper alignment and extraction of seeds for all participants was ensured. Therefore, we believe the observed
results are not due to technical or analytical error. Furthermore, results of the VTA positive control analysis (see Supplement) lend support to the appropriate analytic strategy used. Given the clear absence of threat and that the study sample were all neurologically healthy individuals who presumably have “normal” brain function, the functional roles of the PAG columns may not be differentiated and instead behave as a functional unit at rest. However, scan acquisition parameters likely reduced power to detect columnar connectivity (see Limitations) and thus results warrant replication.

**Experiment 2: PAG activity under uncertain threat**

As previously mentioned, most of the foundational knowledge of the functional role of the PAG has been described in terms of threat responding, and fear and anxiety behaviors in nonhuman animal models (An et al., 1998; Bandler et al., 2000; Behbehani, 1995; Bittencourt et al., 2004; Deng et al., 2016; Fanselow, 1991; Faull et al., 2019; Mendes-Gomes & Nunes-de-Souza, 2009; Molchanov & Guimaraes, 2002; Motta et al., 2017; Vianna et al., 2003; Vieira-Rasteli et al., 2018). In humans, investigation of the PAG has demonstrated differential functional roles of the PAG columns in a variety of studies including voluntary breath holding, breathlessness and conditioned respiratory threat (Faull et al., 2015, 2016), responses to painful electrical stimulation (Hahn et al., 2013), noxious heat (Ritter et al., 2013) and cold (La Cesa et al., 2014), working memory load (Kragel et al., 2019), virtual threat imminence (Hashemi et al., 2019; Mobbs et al., 2007), and passive picture viewing of aversive and neutral images (Satpute et al., 2013).

While the role of the PAG in threat detection and response has been repeatedly demonstrated in conditioning paradigms where threat is predictable, there is limited to no work on the role of the PAG in unpredictable threat. Furthermore, to follow up the null results of PAG
column connectivity at rest, we decided to evaluate the functional role of the PAG and its columns with a well validated threat of shock task comparing certain and uncertain threat.

**Method**

**Uncertain Threat Task Paradigm Acquisition**

The same participants from the resting state analysis also completed the following uncertain threat task paradigm. Prior to scanning, two electrodes for electrical stimulation were placed on participants’ left ankle. A shock work-up procedure was then completed to establish each participants’ individual level of shock intensity. Participants were instructed to determine a shock level that was “painful but tolerable” to ensure adequate aversion to the stimulus. Shocks were administered for 500ms where applicable during the task. Skin conductance was continuously collected using a BIOPAC System (MP-160) with MR-safe disposable electrodes attached to the index and middle finger of the left hand.

The uncertainty task was modeled directly from the NPU task by Gorka et al., (2017) (see Figure 4 for trial and block structure). The task is a block design consisting of 3 conditions: no shock (N), predictable shock (P), and unpredictable shock (U). There were 3 runs of the task during which each condition (N/P/U) was presented once per run with order of condition presentation counterbalanced across runs. Order of runs was counterbalanced across participants. Each condition block consisted of 6 trials lasting a total of 78 seconds, as such a full run lasted 234 seconds (93 TRs). Interstimulus intervals (ISI) within a condition block ranged from 5-7 sec.

Each trial began with a fixation cross and text at the bottom of the screen serving to remind participants of the current experimental block condition. Following fixation, a variable 8-second countdown was shown. In the N condition, no shocks were delivered at any point in the trial, in the P condition a shock was delivered when the countdown reached 1, and in the U
condition a shock was delivered randomly at any point during the countdown. In total, participants received 36 electric shocks across all 3 runs, 18 in the P condition, 18 in the U condition. Participants were not instructed to make any responses during the task.

Uncertain Threat (NPU) Task Paradigm Analysis

Preprocessing of the NPU task was done in a similar manner as the resting state. Briefly, the first 3 volumes were removed from the EPI and remaining volumes were slice time corrected to the first EPI slice. Reverse polarity scans were again used for distortion correction. Functional volumes were then co-registered to the first functional volume and aligned to the anatomy. The anatomy and EPI were then warped to MNI space (MNI152) for group analysis. Again, to preserve the high-resolution EPI and enhance signal within our PAG seed during the task, functional images were minimally smoothed with a 3.6mm kernel. Six head motion parameters were included as nuisance regressors, and one censor file was included into the regression that excluded TRs where more than 10% of voxels in the brain were outliers and where motion exceeded 0.3mm. Shock delivery was also modeled in the regression to account for shock-related variability in brain activation. Of the original 57 participants analyzed in the resting state analysis, 35 completed all runs of the task and were retained according to motion and censor thresholds (12 males, 23 females, Mage=21.71, SDage=2.78). BOLD signal was modeled over the course of the full condition block using duration modulated basis functions in AFNI.

First, activation of the PAG between experimental conditions, at the block level, was evaluated in a voxel wise manner (i.e. across all voxels within the whole PAG irrespective of column delineations) using AFNI’s 3dMVM with sex as a covariate. Given that the average size of the PAG across participants was only ~1,100 voxels, a small volume correction (Faull & Pattinson, 2017), using the average group PAG mask in 3dClustSim, was applied to correct for
multiple comparisons (Chen et al., 2017; Woo et al., 2014). Furthermore, for all NPU analyses, unless otherwise stated, we opted to use a more liberal voxel-wise threshold (voxel-wise: \( p < 0.01 \), cluster: \( p < 0.05, k > 47 \)).

Next, to compare voxel-wise PAG activation within columns across experimental conditions, voxel-wise contrasts were evaluated for each of the 4 PAG columns in separate ANCOVA’s, using AFNI’s 3dMVM with sex as a covariate. In addition, average beta weights for each participant for each column were compared across conditions using repeated measures ANOVAs. To examine fluctuations in activation, time series data from each column were also extracted, averaged across participants, and plotted by condition.

For completeness, average whole PAG and whole brain activity were compared across conditions though details and results of these analyses are reported in the Supplement.

Results

Uncertain Threat Task Results

Analysis of skin conductance level is described in the supplemental material. At the block level, results of a one-way repeated measures ANOVA showed an effect of experimental condition on skin conductance level such that participants had significantly higher skin conductance levels during U blocks compared to P, with marginal effects of U > N and P > N (Supplemental Figure 5). These results affirm the validity of the NPU paradigm (Gorka et al., 2017).

Voxel-wise whole PAG

No clusters survived correction for any condition comparisons in the voxel-wise activation analysis within the whole PAG mask. However, further examination showed greater activation for U > P for 2 small clusters within the right ventrolateral and lateral columns that
just missed the cluster-size threshold ($p < 0.05$, $k > 47$; Figure 5). The rostral cluster (MNI coordinates: 1.5, 27.8, -3.8) lies primarily in the right ventrolateral column with some overlap in the right lateral column ($t = 3.19$, $k = 25$). The caudal cluster (MNI coordinates: 3, 31.5, -10.5), bridges both right ventrolateral and lateral columns evenly ($t = 3.09$, $k = 15$). No other clusters showed marginal effects for U vs. N, or P vs. N.

**Voxel-wise PAG columns.**

Results of the separate PAG column ANCOVAs showed no significant voxels in the overall task condition factor that survived correction for any of the 4 columns. Therefore, there were no voxel-wise activation differences within individual columns for any task condition comparisons. However, average beta weights for each participant for each column were compared using repeated measures ANOVAs (Figure 6). Results of this analysis indicate a significant effect of column during P blocks ($F(1.41, 47.86) = 4.46$, $p = 0.02$) such that the dorsolateral column showed greater activation than ventrolateral ($t(35) = 27$, $p_{\text{Holm}} = 0.01$). In addition, there was a significant effect of condition within the ventrolateral column ($F(2, 68) = 3.01$, $p = 0.05$) such that there was greater activation during U compared to P blocks, though this effect did not survive correction for multiple comparisons ($t(35) = 27$, $p_{\text{uncorrected}} = 0.09$).

Examination of time series data by column across all runs for each condition shows no clear differentiation of PAG column activity for any condition (Figure 6). As reported in the supplement, the whole PAG also showed no clear differences in average activation (Supplemental Table 5) or pattern of activation in time series plots across conditions (Supplemental Figure 6). Despite these largely null findings, there were other expected brain regions, beyond the PAG, that showed robust activation to each condition as well as differential
activation between conditions (Supplemental Figure 7, Supplemental Table 6), evidence of the validity of the NPU task in regions other than the PAG.

**Experiment 2 Discussion**

Given the lack of robust results from the resting state analysis, we were keen on investigating PAG activity in our sample to certain and uncertain threat of shock. Despite a strong rationale for expecting PAG engagement in response to threat, results did not turn out as expected. At the block level, though there were marginal effects in the voxel wise analysis of the whole PAG and in the comparison of average beta weights within PAG columns, there were no robust activity differences within the PAG columns or in the PAG as a whole. In addition, an examination of the time course of PAG activity did not show any meaningful patterns of transient activity in the PAG columns or in the whole PAG over the course of the task.

Though marginal, the current results suggest there is some degree of activation in the PAG in response to uncertain threat. For U compared to P, greater activation at the voxel level along the ventrolateral column as well as greater average ventrolateral column activity suggest there may be something unique to function of the ventrolateral PAG in humans in response to uncertain threat. These results broadly correspond with the results in Satpute et al., (2013) that showed similar activation to aversive image viewing in small clusters of the lateral and ventrolateral columns. Though there are clear differences between aversive image viewing and threat of shock paradigms, the concordance of activation in similar regions to threatening stimuli suggests the lack of robust results in the current study may simply be due to reduced power (see Limitations) or, from a conceptual standpoint, that PAG involvement in specifically uncertain threat is more nuanced. Nonetheless, the lack of robust task activation differences were rather surprising, as such we offer up a few potential explanations.
While the PAG has been previously shown to play a role in the initiation of fear-related behaviors, it’s possible it may not play a role in perception or appraisal of uncertainty (Aupperle & Paulus, 2010; Drabant et al., 2011; Grupe & Nitschke, 2013; Harricharan et al., 2016). Neuroimaging studies of uncertainty have shown notable activity of the amygdala (Alvarez et al., 2011; Herrmann et al., 2016; Herry et al., 2007; Janak & Tye, 2015; Sarinopoulos et al., 2010; Torrisi et al., 2018), anterior cingulate cortex (ACC; Alvarez et al., 2015; Herrmann et al., 2016; Shankman et al., 2014), ventromedial prefrontal cortex (PFC; Aupperle & Paulus, 2010; Herrmann et al., 2016), BNST (Alvarez et al., 2011, 2015; Herrmann et al., 2016; Torrisi et al., 2018) and insula (Aupperle & Paulus, 2010; Sarinopoulos et al., 2010; Shankman et al., 2014) in response to various uncertain stimuli and periods of anticipation. This body of literature has suggested that uncertainty may “prime” the fear system to over-respond to the threat stimulus. The results of the voxel-wise contrasts within the limited field of view across NPU conditions lend support to this theory by demonstrating recruitment of regions beyond the PAG in each of the experimental conditions. Most notable of these results was the robust activation of the insula and caudate in response to unpredictable compared to predictable threat of shock.

While there is a reasonable explanation for the lack of PAG activity during the uncertain condition, it is also surprising that PAG activity did not emerge in response to the predictable threat condition. A fair amount of work has detailed the PAG’s response to threat (Faull et al., 2015, 2016; Faull & Pattinson, 2017; Hahn et al., 2013; Hashemi et al., 2019; Kragel et al., 2019; La Cesa et al., 2014; Mobbs et al., 2007; Ritter et al., 2013; Satpute et al., 2013); however, a notable difference between this body of work and the current study is the use of the temporal countdown ahead of the threat presentation. This period of time was explicit and directed. Predictable threat or not, the countdown introduces an element of anticipation that has not been
clearly examined with regard to the PAG. The anticipation period may impede a clear response from the PAG due to the recruitment of regions, as demonstrated in the whole brain voxel-wise contrasts (Supplemental Table 4), including the insula, ACC, PFC, and hippocampus which in the literature have more theoretical relevance to anticipation (Alvarez et al., 2011, 2015; Aupperle & Paulus, 2010; Herrmann et al., 2016; Herry et al., 2007; Sarinopoulos et al., 2010; Shankman et al., 2014; Torrisi et al., 2018).

At its core, the NPU task is one aimed to understand the effects of uncertainty rather than the effects of threat or fear (Gorka et al., 2017). While these concepts can be hard to disentangle definitively, the point is the PAG may not be recruited during the current NPU task because it is not involved in the network of regions responsive to uncertainty or anticipation such as the amygdala, ACC, vmPFC, BNST, (Aupperle & Paulus, 2010; Drabant et al., 2011; Grupe & Nitschke, 2013; Janak & Tye, 2015; Torrisi et al., 2018). This explanation is likely too nuanced in the true effects at play here but may serve as one potential theory to follow up in future studies. Given the lack of robust results in the resting state analysis, the lack of results in the NPU task should be more thoroughly explored before discounting the PAG’s involvement in anticipation of threatening stimulus as there may be some inherent biases unique to the current study or sample. Nevertheless, our confidence in the validity of the null findings is increased due to the rigorous analytic approach employed to ensure results were not artifacts.

**Limitations**

The current study is not without limitation. Most notably, the current study only had partial coverage of the brain for the functional MRI acquisition. As 7-Tesla MRI is well known to be susceptible to increased artifact and distortion, the decision was made for partial acquisition to optimize the signal and resolution from the regions of greatest interest to the current study.
However, given the limited range of functional acquisition our reported results of voxel-wise resting state functional connectivity with the PAG may not represent the full connectivity results. Furthermore, the power to detect connectivity and activity was significantly reduced due to a combination of factors including the partial spatial acquisition, the signal reduction afforded to voxels <1mm, and the positioning and thickness of slices not specifically tailored to optimize PAG column signal. Moreover, despite careful preprocessing and analysis, the whole brain functional connectivity analyses should be interpreted with caution as results indicated connectivity in orbitofrontal regions and across distributed brain stem grey matter, regions in the field-of-view that typically exhibit dropout at ultra-high resolution (see Supplemental Figure 3 for average FOV in the current sample). In addition, there are a few limitations to hand-drawing the PAG as done in the current study, rather than using previously derived masks, that include bias in implementing the drawing protocol and margins for error when drawing from subject-to-subject. However, manual tracing at the individual level and back projection of the group-level PAG columns to individuals was rigorously checked for each participant.

There were also a few notable limitations to the design of the overall study. The NPU task as described accompanied 2 other tasks in the scanner that were part of a larger study. These tasks also used shock as a learning contingent in their respective paradigms, and the order of the task administration was counterbalanced across participants. This means, the NPU task did not standalone as the sole task with shock as a potential aversive stimulus and was not always the first task participants experienced. Having the additional tasks during the scan session, that were beyond the scope of the current study, may have led to a habituation of the uncertain threat in the NPU task making the overall experimental paradigm less salient. In addition, while initially the presentation of the electric shock for some individuals is aversive, most participants anecdotally
report it as only mildly aversive by the end of the scan session suggesting clear habituation to its presentation (Klorman, 1974). The combination of these factors and the shock presentation only being a quick 500ms together may not prove to be very threatening (Klorman, 1974; Shankman et al., 2011). Thus, the PAG may not be recruited under these conditions or at least may need a higher threat threshold to initiate any defensive behaviors despite the uncertainty of threat within the experimental paradigm.

**General Conclusion**

Despite the limitations to the current study, the results of the current study are still rather interesting. At rest and in healthy individuals, there are no differences in the voxel-wise functional connectivity patterns of the PAG’s columns. In addition, there were no robust differences of PAG activity in response to predictable and unpredictable threat suggesting the PAG may not play a role in processing uncertain threat. The results of the current study suggest nuances within the role of the PAG, including rest and threat paradigms in which the PAG is not responsive, that have not been described previously. The continuation of PAG research in healthy individuals is important for the understanding of the PAG’s role in anxiety neurocircuitry.
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Disclosures

The authors have no conflicts of interest to disclose.
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Figure Legends

Figure 1. Depiction of partial EPI acquisition coverage for a representative participant shown in native space.

Figure 2. Illustration of group level unsmoothed PAG seed with columns—ventromedial (red), ventrolateral (purple), lateral (teal), dorsolateral (lime green), dorsomedial (yellow). Note that the ventromedial region was segmented but not used in analysis.
Figure 3. Results of voxel-wise functional connectivity using the whole PAG as a seed. Warm-colored clusters indicate regions that show increased connectivity with the PAG. Images are overlaid on a standard MNI template at voxel-wise threshold ($p<0.001$) and cluster thresholds ($p<0.05, k>303$). Bright grey regions indicate average coverage of the axial partial acquisition functional scan.

Figure 4. (A) Instruction screen presented to participants at the beginning of each experimental run. (B) Schematic of example trial structure for an unpredictable (U) trial. (C) Structure of task runs. Each run consisted of 1 block of each condition. Order of condition presentation across runs was counterbalanced. Order of runs was counterbalanced across participants.
Figure 5. Two marginally significant clusters showing greater activation during U block compared to P. Clusters are marginal at the following thresholds (p<0.01, k>47 corrected). Note, the image depicts PAG columns, but this is only a means to orient spatial location of the significant clusters (grey). Indeed, the voxel-wise analysis was run with all voxels in the whole PAG seed.
Figure 6. A) Average activation (beta) by condition and PAG column. Black dots represent individuals, boxplots designate mean values and interquartile ranges. Results of repeated measures ANOVAs show the dorsolateral column had greater activation than ventrolateral during P blocks, and a marginal effect within the ventrolateral column such that there was greater activation during U compared to P blocks. B) Average time series of activation by column for each condition. All runs are concatenated separated by vertical black lines. Time series plots show no clear differentiation in PAG activation by condition.
| Brain stem   | X   | Y   | Z   | T  | k    |
|-------------|-----|-----|-----|----|------|
| Right insula| -43.5 | -21 | 1.5 | 5.71 | 3098 |
| Right anterior cingulate | -6   | -48.8 | -6.8 | 6.35 | 2732 |
| Left insula  | 41.2 | -12.8 | -9  | 6.82 | 1845 |
| Right superior orbital gyrus | -15  | -18  | -19.5 | 5.72 | 1003 |
| Left calcarine gyrus | 13.5 | 59.2 | 17.2 | 6.35 | 2732 |
| Left thalamus | 10.5 | 18  | -3.8 | 5.63 | 503  |
| Left cuneus  | -3   | 87  | 39  | 5.38 | 378  |
| Left cerebellar cortex | 26.2 | 45  | -21 | 6.00 | 377  |
| Right insula | -42  | 12  | -3  | 5.24 | 348  |
| Left fusiform | 24.8 | 29.2 | -27 | 5.45 | 316  |
| Left superior temporal gyrus | 50.2 | 30  | 16.5 | 4.44 | 310  |

Voxel-wise threshold p<0.001, cluster threshold k>303. T, t-statistic. k, number of voxels.
Supplementary Material

Supplemental Figure 1. Protocol for the manual segmentation of the PAG.

1. Find the first axial slice where the cerebral aqueduct is no longer connected with the third ventricle.
2. Trace the aqueduct down inferiorly. Continue until you get to the last slice where the aqueduct is clearly surrounded by grey matter tissue.
3. From a sagittal view the ROI should be wedged between rostral and caudal grey matter and should not continue into the cavernous 5th ventricle. With a slice thickness of 0.8mm, in our dataset, the aqueduct should only extend ~10-15 slices (per the average longitudinal length reported in Satpute et al., 2013).
4. Once the aqueduct has been identified, this mask can be used to create the PAG by dilating five-voxels (for a total of 2 voxels in all directions: +0.86x0.86x1.60mm) surrounding the aqueduct as has been done in similar protocols (Kragel et al., 2019; Satpute et al., 2013). After dilation, the resultant mask can then be restricted to only grey matter voxels by using FreeSurfer tissue segmentation masks output for each subject (Fischl, 2012). Then remove the original aqueduct ROI so that PAG voxels are not contaminated by the high intensity CSF signal.
*Supplemental Figure 2.* Side by side comparison of participants’ average signal intensity values (prior to blurring) from the manual tracing of cerebral aqueduct voxels and the subsequent construction of PAG mask. Each bar represents signal from a given participant for each of the aqueduct and PAG.
Supplemental Figure 3. Group PAG mask (red) overlaid on average distortion corrected MNI-aligned EPI images before smoothing. Top panel: sagittal slices, middle panel: coronal slices, bottom panel: axial slices.
Supplemental Table 1

Fisher transformed pairwise correlations of PAG column time series (averaged across subjects)

|        | R-Z-R Transformation |
|--------|----------------------|
| dm     | dl                   | 0.42 |
| dm     | l                    | 0.21 |
| dm     | vl                   | 0.20 |
| l      | dl                   | 0.42 |
| l      | vl                   | 0.40 |
| vl     | dl                   | 0.31 |

dm=dorsomedial, dl=dorsolateral, l=lateral, vl=ventrolateral PAG
### Supplemental Table 2

**AFNI 3dMVM command for resting state ANCOVA analysis**

```bash
3dMVM -prefix ####
  -jobs 24 \\
  -bsVars "sex" \\
  -wsVars ROI \\
  -mask mean_epi_mask+tlrc \\
  -num_glt 10 \\
  -gltLabel 1 dmPAG -gltCode 1 'ROI : 1*dmPAG' \\
  -gltLabel 2 lPAG -gltCode 2 'ROI : 1*lPAG' \\
  -gltLabel 3 vlPAG -gltCode 3 'ROI : 1*vlPAG' \\
  -gltLabel 4 dlPAG -gltCode 4 'ROI : 1*dlPAG' \\
  -gltLabel 5 vlPAGvdmPAG -gltCode 5 'ROI : 1*vlPAG -1*dmPAG' \\
  -gltLabel 6 lPAGvdmPAG -gltCode 6 'ROI : 1*lPAG -1*dmPAG' \\
  -gltLabel 7 dlPAGvdmPAG -gltCode 7 'ROI : 1*dlPAG -1*dmPAG' \\
  -gltLabel 8 vlPAGvlPAG -gltCode 8 'ROI : 1*vlPAG -1*lPAG' \\
  -gltLabel 9 dlPAGvlPAG -gltCode 9 'ROI : 1*dlPAG -1*lPAG' \\
  -gltLabel 10 dlPAGvvlPAG -gltCode 10 'ROI : 1*dlPAG -1*vlPAG' \\
```

- dataTable ........
## Supplemental Table 3

*Main Effects of PAG columns from resting state PAG column ANCOVA*

| Peak Coordinates | X  | Y  | Z  | T   | Voxels |
|------------------|----|----|----|-----|--------|
| **Dorsomedial**  |    |    |    |     |        |
| Brain stem       | -0.8 | 36 | -8.2 | 26.06 | 13958  |
| **Dorsolateral** |    |    |    |     |        |
| Brain stem       | 1.5 | 36 | -9.8 | 22.16 | 19114  |
| Bilateral anterior cingulate cortex | 1.5 | -53.2 | -0.8 | 5.26 | 1637  |
| Left thalamus    | 3  | 19.5 | 12.8 | 4.56 | 839  |
| Left cingulate gyrus | 22.5 | 42.8 | 20.2 | 5.68 | 361  |
| **Lateral**      |    |    |    |     |        |
| Brain stem       | 2.2 | 33 | -11.2 | 4.76 | 16127  |
| Left calcarine gyrus | 14.2 | 56.2 | 18.5 | 4.98 | 4444  |
| Left thalamus    | 9  | 16.5 | 12 | 6.12 | 1750  |
| Right precuneus  | -6 | 52.5 | 34.5 | 4.80 | 1400  |
| Right thalamus   | -6 | 18 | 6.8 | 6.23 | 1383  |
| Right insula     | -42.8 | 15.8 | -10.5 | 5.84 | 627  |
| Left anterior cingulate cortex | -0.8 | -40.5 | 4.5 | 4.69 | 545  |
| Right middle orbital gyrus | -12 | 53.2 | -3 | 5.08 | 462  |
| **Ventrolateral**|    |    |    |     |        |
| Brain stem       | -1.5 | 30 | -8.2 | 23.20 | 21454  |
| Right middle orbital gyrus | -10.5 | -46.5 | -6 | 4.93 | 2210  |
| Right middle temporal gyrus | -62.2 | 46.5 | 4.5 | 6.16 | 1401  |
| Right calcarine gyrus | -12.8 | 82.5 | 3.8 | 5.76 | 934  |
| Right superior temporal gyrus | -48 | 28.5 | 2.2 | 6.28 | 634  |
| Left caudate     | 9.8 | -15.8 | 5.2 | 5.72 | 607  |
| Left insula      | 30 | -27.8 | 5.2 | 5.60 | 507  |
| Right caudate    | -12.8 | -18 | 7.5 | 5.35 | 501  |
| Right inferior frontal gyrus | -30.8 | -36.8 | -11.2 | 4.92 | 480  |
| Right caudate    | -11.2 | -19.5 | -5.2 | 4.58 | 472  |
| Left insula      | 31.5 | -14.2 | -9.8 | 5.72 | 409  |
| Right insula     | -49.5 | -15.8 | -4.5 | 4.50 | 375  |
| Right calcarine gyrus | -19.5 | 59.2 | 6 | 4.76 | 339  |
| Left middle temporal gyrus | 63.8 | 57.8 | 10.5 | 3.81 | 336  |
| Left thalamus    | 13.5 | 28.5 | 13.5 | 5.69 | 317  |
| Right calcarine gyrus | -12.8 | 69.8 | 12.8 | 5.13 | 303  |

*all results survived correction for multiple comparisons: voxel-wise \( p<0.001\), cluster-wise \( p<0.05\), \( k>303\)

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**Resting State Positive Control Analysis**
In response to the null findings of differential PAG column resting state functional connectivity and to bolster the strength of the analytical methods used in the current study, we conducted a positive control analysis with another midbrain region, the ventral tegmental area. The VTA seed was derived from the Harvard Ascending Arousal Network (AAN) atlas (https://www.nmr.mgh.harvard.edu/resources/aan-atlas) (Edlow et al., 2012).

Identical to the methods described in the main text, the time series of the VTA seed for each participant was extracted from the pre-smoothed distortion corrected MNI-aligned functional data. VTA time series was then correlated with every other voxel within the field of view and resultant correlation maps were r-to-z transformed. Group level VTA functional connectivity was assessed using AFNI’s 3dttest++ with Sex as a covariate. To correct for multiple comparisons the same thresholds as the PAG analysis were applied (voxel-wise, $p<0.001$, cluster-wise $p<0.05$, $k>303$).

Voxel-wise connectivity of the VTA is shown in Supplemental Figure 4 (see Supplemental Table 4 for complete list of significant clusters). Results indicate robust VTA connectivity with expected regions including the caudate, thalamus, insula, prefrontal and occipital cortices, and other brain stem regions including the PAG. Importantly, though there is some expected degree of overlap, functional connectivity of the VTA and PAG is qualitatively different lending support for the analytic method employed.
Supplemental Figure 4. Results of voxel-wise functional connectivity using the ventral tegmental area (VTA) seed derived from the Harvard Ascending Arousal Network (AAN) Atlas (Edlow et al., 2012). The red bordered box inlays depict sagittal (x=0, top) and coronal (y=26, bottom) slices of the VTA seed region. Warm-colored clusters indicate regions that show increased connectivity with the VTA. Images are overlaid on a standard MNI template at voxel-wise threshold \((p<0.001)\) and cluster thresholds \((p<0.05, k>303)\). Bright grey regions indicate average coverage of the axial partial acquisition functional scan.
Supplemental Table 4
Coordinates of peak voxels for whole ventral tegmental area (VTA) functional connectivity

| Peak Coordinates                  | X  | Y  | Z  | T  | k  |
|-----------------------------------|----|----|----|----|----|
| Brain stem                        | 0  | 23.2 | -17.2 | 39.5 | 76105 |
| Bilateral anterior cingulate/medial prefrontal cortex | -2.2 | -45.8 | 3 | 8.02 | 14221 |
| Left middle occipital gyrus       | 40.5 | 74.2 | 30.8 | 6.79 | 12888 |
| Right middle temporal gyrus       | -54 | 50.2 | 21 | 6.86 | 9145 |
| Right thalamus                    | -5.2 | 17.2 | 12 | 6.75 | 3957 |
| Left inferior occipital gyrus     | 28.5 | 88.5 | -6.8 | 5.4 | 1635 |
| Left inferior temporal gyrus      | 53.2 | 57 | -13.5 | 5.9 | 1634 |
| Left caudate                      | 9 | -7.5 | 8.2 | 5.41 | 1282 |
| Left insula                       | 27.8 | -24.8 | -0.8 | 6.59 | 1137 |
| Right caudate                     | -14.2 | -19.5 | 6.8 | 5.96 | 1130 |
| Right insula                      | -39 | 27 | 18 | 5.8 | 1009 |
| Left temporal pole/insula         | 35.2 | -3 | -17.2 | 5.85 | 969 |
| Left middle temporal gyrus        | 56.2 | 54 | 18 | 5.36 | 895 |
| Right insula                      | -41.2 | -2.2 | 14.2 | 6.4 | 767 |
| Left middle temporal gyrus        | 52.5 | 5.2 | 15.8 | 4.83 | 675 |
| Right superior temporal gyrus     | -47.2 | 24 | 15 | 5.8 | 636 |
| Right superior temporal gyrus     | -58.5 | 9 | -9.8 | 5.58 | 613 |
| Right hippocampus                 | -33 | 91.5 | 21.8 | 4.73 | 537 |
| Left superior occipital gyrus     | 18.8 | 91.5 | 21.8 | 5.34 | 528 |
| Right fusiform gyrus              | -28.5 | 33.8 | -21.8 | 5.65 | 484 |
| Right calcarine gyrus             | 11.2 | 75 | 9.8 | 5.65 | 483 |
| Left fusiform gyrus               | 30.8 | 65.2 | -13.5 | 3.83 | 483 |
| Left inferior temporal gyrus      | 55.5 | 45.8 | -14.2 | 4.73 | 447 |
| Left calcarine gyrus              | 3 | 79.5 | 9.8 | 5.72 | 443 |
| Right middle occipital gyrus      | -33.8 | 92.2 | 16.5 | 5.05 | 424 |
| Left postcentral gyrus            | 58.5 | 3.8 | 13.5 | 4.17 | 401 |
| Right cerebellum                  | -16.5 | 53.2 | -13.5 | 3.85 | 386 |
| Right hippocampus                 | -23.2 | 25.5 | -10.5 | 4.45 | 375 |
| Right middle temporal gyrus       | -69.8 | 44.2 | 1.5 | 5.33 | 348 |
| Left hippocampus                  | 26.2 | 21.8 | -15.8 | 5.51 | 344 |
| Right middle temporal gyrus       | -53.2 | 69.8 | 9.8 | 5.19 | 341 |
| Left inferior occipital gyrus     | 39.8 | 77.2 | -8.2 | 4.96 | 331 |
| Left inferior frontal gyrus       | 23.2 | -34.5 | -9 | 4.58 | 317 |
| Left middle temporal gyrus        | 61.5 | 19.5 | -7.5 | 4.13 | 313 |

Voxel-wise threshold $p<0.001$, cluster threshold $k>303$ ($p<0.05$), T, t-statistic, $k$, number of voxels
NPU Skin Conductance Analysis and Results

To match the analysis strategy of the fMRI data, skin conductance was also analyzed at the block level. First, the full time series of skin conductance level for each NPU block for all 3 runs was extracted. Next, all blocks for each participant were concatenated and normalized (z-scored) for each participant. Lastly, normalized skin conductance level was averaged within conditions (across all 3 runs) across all subjects (N=35).

Results of a one-way repeated measures ANOVA indicate a significant effect of experimental condition $F(2, 68) = 12.43, p < 0.001$ (Supplemental Figure 5). Post-hoc comparisons with Holm correction show higher skin conductance for U > N ($p < 0.001$ corrected), and marginal effects of P > N and U > P (both $p$’s = 0.08 corrected).

Supplemental Figure 5. N=35. Violin plots of normalized skin conductance level for each experimental condition in NPU task. Black dots are individual participants, with box plots depicting mean and interquartile ranges. Post comparisons with Holm correction indicate U > N ($p < 0.001$ corrected), and marginal effects of P > N and U > P (both $p$’s = 0.08 corrected).
Whole PAG NPU activation

To evaluate whole PAG activity across experimental conditions, average activity was extracted and compared at the block level for each condition. Paired two-sample t-tests were used to evaluate average signal intensity of the PAG across all runs by condition using AFNI’s 3dttest++. No condition comparisons showed significant differences in average PAG activity (Supplemental Table 5).

| Condition | Average Signal Intensity |
|-----------|--------------------------|
| N         | -0.028                   |
| P         | -0.035                   |
| U         | -0.019                   |

Supplemental Table 5

Average PAG activity by Threat Condition

|                         | T   | P   |
|-------------------------|-----|-----|
| N vs. P                 | 0.42| 0.66|
| P vs. U                 | -0.98| 0.32|
| N vs. U                 | -0.56| 0.57|

N, no shock; P, predictable shock; U, unpredictable shock; T, t-statistic; p, p-value.

To examine more transient activity, PAG activation in each condition was examined across all runs (Supplemental Figure 6). Examination of the PAG time course suggests there were no robust differences in transient PAG activity through the course of the experiment. However, qualitative comparison of PAG activation suggests activity in the conditions with threat of shock (P & U) follow a similar time course throughout the experiment and appear distinct from the safe (N) condition. This lack of separable activity in the PAG between task conditions may further support the lack of findings between conditions within the individual columns.
**Supplemental Figure 6.** Time series of PAG activity by condition: red=No Shock (N), green=Predictable shock (P), blue=unpredictable shock (U). All runs are concatenated in this plot separated by vertical black lines. Error bars represent standard error over a local polynomial regression line fit over each condition separately.

**NPU voxel-wise activation beyond PAG**

Finally, voxel-wise activation across experimental conditions beyond the PAG was evaluated using AFNI’s 3dMVM with sex as a covariate corrected for multiple comparisons using 3dClustSim (voxel-wise $p < 0.001$, cluster-wise $p < 0.05$, $k > 303$). Results indicate that while there were no strong differences in PAG activity across conditions, there was robust differential activation in other brain regions. Most notably, the insula (30, 28, 1; $t = 4.94$, 545 voxels) and caudate (21, 8, 6; $t = 5.46$, 326 voxels) showed robust increased activity for U
compared to P that survived correction (Supplemental Figure 7). Though no other results survived correction for any other pairwise condition comparisons (i.e. U vs. N, N vs. P), there were surviving clusters when each condition was examined separately (Supplemental Table 6). Furthermore, there were surviving clusters when comparing both shock conditions to N (i.e. 0.5*U +0.5*P -1*N). Bilateral insula and inferior frontal gyrus showed greater activation during U and P compared to N (Supplemental Table 6).

Supplemental Figure 7. Voxel-wise comparison of unpredictable (U) versus predictable (P) shock. Image is overlaid on a standard MNI template at voxel-wise threshold ($p<0.001$) and cluster thresholds ($p<0.05, k>303$).
Supplemental Table 6

*Coordinates of peak voxel activations by NPU condition*

| No Shock                  | X    | Y    | Z    | T    | Voxels |
|---------------------------|------|------|------|------|--------|
| Right inferior frontal gyrus | -42  | -19.5|  9   | -6.98| 4690   |
| Right thalamus            | -30.8| -16.5| -2.2 | -7.3 | 1416   |
| Right lingual gyrus       | -64.5|  46.5| -2.2 | -6.15| 1108   |
| Right middle temporal gyrus | -39.8|  77.2| 28.5 | -6.26| 1034   |
| Left middle temporal gyrus |  0.8 |  35.2|  2.2 | -5.31|  704   |
| Left insula               | -24.8|   72 | -2.2 |  -4.99|  614   |
| Right inferior frontal gyrus | -0.8 |   15 | -5.2 |  -5.2 |  608   |
| Right precuneus           | -35.2| -51.8| -2.2 |  -6  |  473   |
| Right putamen             |  12.8|  76.5|  9   |  -5.84|  441   |

| Predictable Shock         | X    | Y    | Z    | T    | Voxels |
|---------------------------|------|------|------|------|--------|
| Right inferior frontal gyrus | -42  | -19.5|  9   | -6.19| 1408   |
| Right putamen             | -30.8| -16.5| -2.2 | -5.67| 1043   |
| Right middle temporal gyrus | -64.5|  46.5| -2.2 | -5.02| 1033   |
| Right middle occipital gyrus | -39.8|  77.2| 28.5 | -5.11|  578   |
| Right lingual gyrus       |  0.8 |  35.2|  2.2 | -6.11|  453   |
| Right lingual gyrus       | -24.8|   72 | -2.2 | -5.31|  419   |
| Right thalamus            | -0.8 |   15 | -5.2 | -5.66|  400   |
| Right middle occipital gyrus | -35.2| -51.8| -2.2 | -5.55|  385   |
| Left calcarine gyrus      |  12.8|  76.5|  9   | -4.61|  377   |

| Unpredictable Shock       | X    | Y    | Z    | T    | Voxels |
|---------------------------|------|------|------|------|--------|
| Right insula              | -38.2|  20.2| 16.5 |  5.68| 1014   |
| Left insula               |  38.2|   0  | -6.8 |  7.13|  568   |
| Left posterior cingulate cortex |  2.2 |  48.8| 28.5 |  5.24|  464   |
| Right superior temporal gyrus | -49.5|  18.8| 12   |  5.32|  437   |
| Left lingual gyrus        |   21 |  73.5| -6   | -4.96|  367   |

| Unpredictable and Predictable vs. No Shock | X    | Y    | Z    | T    | Voxels |
|--------------------------------------------|------|------|------|------|--------|
| Left insula                                |  39.8|  8.2 | -0.8 |  4.8 |  743   |
| Right insula                               | -35.2|  21.8| 13.5 |  4.04|  675   |
| Right inferior frontal gyrus               | -57  | -9.8 |  4.5 |   4.9 |  463   |
| Left inferior frontal gyrus                |  54.8|   -9 | 12.8 |  3.76|  326   |
| Left insula                                |  34.5| -0.8 | 16.5 |  2.66|  303   |

*all results survived correction: voxel-wise *p*<0.001, cluster-wise *p*<0.05, *k*>303
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