Prepronociceptin-expressing neurons in the extended amygdala encode and promote rapid arousal responses to motivationally salient stimuli

Jose Rodriguez-Romaguera
Michael R Bruchas
et al

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**Graphical Abstract**

**Highlights**

- *Prepronociceptin (Pnoc)* defines a subpopulation of GABAergic neurons within BNST
- *Pnoc* neurons encode rapid changes in arousal to motivationally salient stimuli
- Activation of *Pnoc* neurons drives arousal responses and anxiety-like behavior
- *Pnoc* neurons exhibit diversity in both anatomical connectivity and genetic identity

**Authors**

Jose Rodriguez-Romaguera, Randall L. Ung, Hiroshi Nomura, ..., Thomas L. Kash, Michael R. Bruchas, Garret D. Stuber

**Correspondence**

gstuber@uw.edu

**In Brief**

Rodriguez-Romaguera et al. find an ensemble of neurons within the extended amygdala (BNST) that expresses the *prepronociceptin* gene and functions to encode the rapid arousal responses that are triggered by motivational stimuli.
Prepronociceptin-Expressing Neurons in the Extended Amygdala Encode and Promote Rapid Arousal Responses to Motivationally Salient Stimuli

Jose Rodríguez-Romaguera,1,2 Randall L. Ung,2,3,9 Hiroshi Nomura,1,2 James M. Otis,1,2 Marcus L. Basiri,2,3 Vijay M.K. Namboodiri,1,2 Xueqi Zhu,1,2 J. Elliott Robinson,2,3 Hanna E. van den Munkhof,1,2 Jenna A. McHenry,1,2 Louisa E.H. Eckman,1,2 Oksana Kosyk,1,2 Thomas C. Jhou,4 Thomas L. Kash,5,6 Michael R. Bruchas,5,8 and Garret D. Stuber1,2,3,7,8,10,10

1Department of Psychiatry, University of North Carolina, Chapel Hill, NC 72599, USA
2Neuroscience Center, University of North Carolina, Chapel Hill, NC 72599, USA
3Neuroscience Curriculum, University of North Carolina, Chapel Hill, NC 72599, USA
4Department of Neuroscience, Medical University of South Carolina, Charleston, SC 29425, USA
5Bowles Center for Alcohol Studies, Department of Pharmacology, University of North Carolina, Chapel Hill, NC 72599, USA
6Department of Anesthesiology, Washington University Pain Center, Department of Neuroscience, Division of Biology & Biomedical Sciences; and Department of Biomedical Engineering, Washington University, St. Louis, MO 63130, USA
7Department of Cell Biology and Physiology, University of North Carolina, Chapel Hill, NC 72599, USA
8Present address: Center for the Neurobiology of Addiction, Pain, and Emotion, Department of Anesthesiology and Pain Medicine and Department of Pharmacology, University of Washington, Seattle, WA 98195, USA
9These authors contributed equally
10Lead Contact
*Correspondence: gstub@uw.edu
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SUMMARY

Motivational states consist of cognitive, emotional, and physiological components controlled by multiple brain regions. An integral component of this neural circuitry is the bed nucleus of the stria terminalis (BNST). Here, we identify that neurons within BNST that express the gene prepronociceptin (PnocBNST) modulate rapid changes in physiological arousal that occur upon exposure to motivationally salient stimuli. Using in vivo two-photon calcium imaging, we find that PnocBNST neuronal responses directly correspond with rapid increases in pupillary size when mice are exposed to aversive and rewarding odors. Furthermore, optogenetic activation of these neurons increases pupillary size and anxiety-like behaviors but does not induce approach, avoidance, or locomotion. These findings suggest that excitatory responses in PnocBNST neurons encode rapid arousal responses that modulate anxiety states. Further histological, electrophysiological, and single-cell RNA sequencing data reveal that PnocBNST neurons are composed of genetically and anatomically identifiable subpopulations that may differentially tune rapid arousal responses to motivational stimuli.

INTRODUCTION

Dysfunctional arousal responses are a core component of many neuropsychiatric disorders. For example, patients with anxiety disorders often show hyperarousal responses to negatively salient stimuli, and patients suffering from depression show hypoarousal responses to positively salient stimuli (Craske et al., 2009; Lang and McTeague, 2009; Patriquin et al., 2019; Schmidt et al., 2017; Urbano et al., 2017; Wilhelm and Roth, 2001). Elucidating the neural circuit elements that orchestrate changes in physiological arousal is, thus, essential for understanding maladaptive motivational states (Marton and Sohal, 2016; Sparta et al., 2013; Touriño et al., 2013). Rodent models have enabled intricate dissection of the neural circuits for both negative and positive motivational states by presenting stimuli that elicit aversion or reward (Calhoon and Tye, 2015; Stuber and Wise, 2016; Tovote et al., 2015). However, these studies often overlook the rapid increases in physiological arousal that characterize changing motivational states. In humans, rapid (within seconds) increases in physiological arousal, as measured by pupil size changes, follow exposure to negatively salient stimuli, such as threat-inducing images (Cascardi et al., 2015; Price et al., 2013). The same is true when humans are presented with positively salient stimuli, such as rewarding images of money or videos of caregivers (Schneider et al., 2018; Tummeltshammer et al., 2019). Thus, in addition to long-term adaptations in arousal (e.g., sleep/wake states; de Lecea et al., 2012), an important component of motivation is the rapid changes in physiological arousal upon presentation of salient stimuli.
One particular brain region that may encode rapid changes in arousal is the bed nucleus of the stria terminalis (BNST, a part of the extended amygdala). Evidence from anatomical (Dabrowska et al., 2011; Dong et al., 2001; Singewald et al., 2003), behavioral (Duvarci et al., 2009; Jennings et al., 2013a; Kim et al., 2013; Walker et al., 2009), and neuroimaging (Straube et al., 2007; Yassa et al., 2012) studies has implicated BNST as a key component of the neural circuitry that regulates motivated behavior. For instance, recent neurocircuit studies in mice have highlighted the role of BNST in reward and aversion (Giardino et al., 2018; Jennings et al., 2013a, 2013b; Kim et al., 2013), fear and anxiety-like behaviors (Crowley et al., 2016; Duvarci et al., 2009; Kim et al., 2013; Marckinkecz et al., 2016; Walker et al., 2009), and social preference and aversion (Goodson and Wang, 2006; Lei et al., 2010; Newman, 1999). Further, previous studies have identified how subsets of BNST neurons expressing certain marker genes such as corticotrophin-releasing hormone (Crh
\text{BNST} ), protein kinase C δ (Pkcδ
\text{BNST} ), and somatostatin (Som
\text{BNST} ) drive motivated behaviors (Kash et al., 2015; Koob and Heinrichs, 1999; Lebow and Chen, 2016; Toyote et al., 2015). However, whether specific neural populations within BNST drive rapid changes in physiological arousal remains unknown. This is, in part, due to the low number of studies linking the functional heterogeneity within BNST with its role in rapid changes in physiological arousal (Kim et al., 2013).

Neuropeptide gene expression patterns have identified functionally distinct subpopulations of neurons in BNST (Kash et al., 2015). Recently, neurons that express the prepronociceptin gene (Pnoc, the genetic precursor to the neuropeptide nociceptin) within the central nucleus of the amygdala, the paranginal ventral tegmental area, and the arcuate nucleus of the hypothalamus were shown to have a role in gating motivational states and reward seeking (Hardaway et al., 2019; Jais et al., 2020; Parker et al., 2019). Interestingly, Pnoc expression within BNST is among the highest within the central nervous system (Boon et al., 1998; Ikeda et al., 1998), so we set out to investigate the role of these neurons in defining motivational states, specifically in driving the physiological arousal responses that occur in response to motivationally salient stimuli.

In the present study, we used cell-type-specific optogenetic and head-fixed two-photon calcium-imaging approaches (McHenry et al., 2017; Namboodiri et al., 2019; Otis et al., 2017) to assess the role of Pnoc
\text{BNST} neurons in driving and encoding rapid physiological arousal responses to aversive and rewarding odors. We found a variety of responses among Pnoc
\text{BNST} neurons; particularly, the magnitude of correlation between neural dynamics of these neurons and pupillary responses increased with exposure to aversive and rewarding odors. We also found that excitatory responses that correlated with pupillary responses were common across both odor presentations, suggesting that motivational salience might be responsible for this response pattern. Optogenetic activation of Pnoc
\text{BNST} neurons increased physiological measurements associated with arousal (pupillary response and heart rate) and further modulated anxiety-like behavior but did not induce approach, avoidance, or locomotion. Single-cell RNA sequencing revealed that Pnoc
\text{BNST} neurons are transcriptionally diverse and can be subdivided by multiple distinct gene markers. Collectively, these results suggest that Pnoc
\text{BNST} neurons play an important role in orchestrating arousal-related responses associated with motivationally salient stimuli.

RESULTS

Expression of Pnoc Defines a Subpopulation of GABAergic Neurons within adBNST that Can Be Monitored Using Calcium-Imaging Approaches

BNST is composed of various subnuclei that have unique molecular and functional identities (Giardino et al., 2018; Gungor and Paré, 2016). Therefore, we first assessed the distribution of Pnoc-expressing neurons across the BNST. Using fluorescence in situ hybridization (FISH), we observed high expression of Pnoc distributed throughout the BNST (Figure 1A) but particularly in the anterodorsal BNST (adBNST), as previously described (Neal et al., 1999). Further, we found that Pnoc
\text{BNST} neurons predominantly express the vesicular GABA transporter gene, Slc32a1 (Vglut1; and not the vesicular glutamate transporter 2 gene, Slc17a6 (Vglut2; Figure 1B), indicating that Pnoc expression defines a subpopulation of GABAergic neurons within BNST. Pnoc-IRES-Cre mice (Hardaway et al., 2019; Parker et al., 2019) were then used for selective targeting of Pnoc+ neurons in adBNST in conjunction with Cre-inducible viruses.

To characterize how Pnoc
\text{BNST} firing is related to calcium-mediated fluorescent dynamics, we transduced the adBNST of Pnoc-IRES-Cre mice with Cre-dependent GCaMP6s virus (Figure 1C). We then performed calcium imaging in brain slices under an epifluorescent microscope and simultaneously activated Pnoc
\text{BNST} neurons via current injections at various frequencies. We found a linear relationship between evoked action potentials and their respective fluorescent peaks (Figures 1E–1G), demonstrating that calcium dynamics track evoked firing in brain slices.

Pnoc
\text{BNST} Neurons Encode Rapid Changes in Arousal to Aversive and Rewarding Stimuli

Since the BNST is thought to coordinate motivational states essential for guiding actions of reward seeking and aversion (Calhoon and Tye, 2015; Kash et al., 2015; Lebow and Chen, 2016; Stamatakis et al., 2014; Toyote et al., 2015), we tested whether the activity of Pnoc
\text{BNST} neurons is altered by exposure to stimuli with opposing motivational salience. We exposed mice to either trimethylthiazoline (TMT, as an aversive odorant) or peanut oil (as an appetitive odorant) or peanut oil (as an appetitive odorant) or peanut oil as an appetitive odorant) as odors that induce either place aversion or place preference (Root et al., 2014), respectively. First, we demonstrated that freely moving mice reliably avoided a location with TMT and preferred a location containing peanut oil (Figures 2A–2C), consistent with the aversive and appetitive nature of these olfactory stimuli. We also observed increases in pupillary size when freely moving mice were in proximity to either TMT- or peanut-oil-containing odor swabs, as compared to a swab with water (Figures 2D–2F). Pupillary responses have been shown to reflect rapid changes in physiological arousal (Cascardi et al., 2015; Price et al., 2013).

To evaluate encoding of Pnoc
\text{BNST} neurons to these odors, we developed a head-fixed behavioral preparation compatible with two-photon microscopy to control proximity of an odor swab...
and allow us to measure pupil dilation and ambulation (Figures 2J and 2K). Odors were presented with a cotton swab that could be positioned near or far from the mouse, while pupil size was recorded through a camera aimed at one of the eyes (Reimer et al., 2014). Having animals head-fixed also allowed us to record calcium activity from individual PnocBNST neurons in awake mice via a GRIN lens under a two-photon microscope (Figures 2G and 2H). With algorithms that use constrained non-negative matrix factorization (CNMF) (Pnevmatikakis et al., 2016; Zhou et al., 2018), we extracted activity dynamics from individual PnocBNST neurons (Figure 2I; Figures S1A–S1E). We found that ~50% of PnocBNST neurons showed a significant change in response (either excitation or inhibition) to a swab with TMT or peanut oil as compared to a control (water) swab (Figures 3A–3G). Strikingly, neurons that showed significant excitation or inhibition to TMT or peanut oil swabs showed significant increases in correlation between pupillary fluctuations and their individual neural dynamics (Figures 3H–3J). We also found different proportions of neurons that were excited and inhibited between TMT and peanut oil exposure (Figure 3G), suggesting that subtypes of PnocBNST neurons may exist to encode aversive versus rewarding arousal states. Inhibitory responses appear to be specific to aversive arousal states, while excitatory responses were observed in both aversive and rewarding arousal states (Figure 3G). We next analyzed neurons from a subset of mice that were first exposed to the peanut swab and subsequently to the TMT swab (n = 162 neurons from 2 mice). We found that half of the neurons that were excited to the peanut swab were also excited to the TMT swab. Similarly, half of the neurons that showed no change to the peanut swab were significantly excited to the TMT swab, thus further suggesting that a subgroup of PnocBNST neurons encodes arousal independent of...
we found that a large proportion of responses are specific to aversive odors, whereas excitatory responses are shared across both aversive and rewarding odors, which include subgroups of *Pnoc* BNST neurons that encode arousal independent of valence.

*Pnoc* BNST activity may also relate to locomotor activity. Consistent with the valence of each odorant, the TMT swab produced an initial decrease in movement velocity when the odor was near, whereas the peanut oil swab produced a moderate increase in velocity (Figures S2A and S2B). Similar to pupil diameter, velocity and neural activity of *Pnoc* BNST neurons showed significant correlations in neurons that showed a significant change in response (either excitation or inhibition) to mice exposed to the peanut swab (Figures S2C–S2E). The increase in correlation was very modest to TMT exposure and occurred only in neurons excited by the TMT swab as compared to the control swab. In summary, we found that a large proportion of *Pnoc* BNST neurons are correlated with measurements of arousal states, and the observed heterogeneity in response dynamics that can be observed across neurons (Figure S2G) suggests that these neurons are composed of functionally distinct subtypes.

*Pnoc* BNST Neurons Drive Arousal Responses and Anxiety-like Behavior

Since we observed that excitatory responses were predominant to both motivationally salient stimuli, we next tested whether optogenetic photoactivation of *Pnoc* BNST neurons (Figures 4A–4C) can induce locomotion and motivational states. We first evaluated whether viral tools can reliably activate *Pnoc* BNST neuronal activity. Whole-cell patch-clamp slice electrophysiological recordings in adBNST within ChR2-expressing *Pnoc* BNST neurons (Figure 4B) showed that we could reliably photoactivate *Pnoc* BNST neurons at 20 Hz with 100% spike fidelity (Figures 4C and 4D). In freely moving mice, we first found that photoactivation of *Pnoc* BNST neurons did not induce changes in locomotion, as assessed by measuring velocity in freely moving mice (Figures 4E and 4F). To test whether photoactivation of *Pnoc* BNST neurons induced a place preference or aversion, freely moving mice were placed in a two-chambered arena to assess time spent in a chamber paired with photoactivation of *Pnoc* BNST neurons (real-time place preference assay). Photoactivation of *Pnoc* BNST neurons (real-time place preference assay).
neurons did not induce either place aversion or place preference (Figures 4G and 4H), indicating that Pnoc\textsuperscript{BNST} neurons may not inherently drive locomotion or approach/avoidance behaviors.

We next wanted to test whether photoactivation of Pnoc\textsuperscript{BNST} neurons was sufficient to increase physiological arousal. To accomplish this, we developed a stationary head-fixed preparation that allowed for the measurement of arousal responses in combination with optogenetics by transducing Cre-dependent channelrhodopsin into the adBNST of Pnoc-IRES-Cre mice (Figure 4I). Photoactivation of Pnoc\textsuperscript{BNST} neurons again showed that it did not alter movement, as measured by a piezoelectric sensor underneath the mice (Figure 4J), but significantly increased both pupil area (Figure 4K) and heart rate (Figure 4L). Taken together, these data suggest that the activity of Pnoc\textsuperscript{BNST} neurons increases physiological arousal responses.

Next, we tested whether the photoactivation of Pnoc\textsuperscript{BNST} neurons was sufficient to drive anxiety-like behaviors in an elevated plus maze (EPM), a test traditionally used to test efficacy of anxiolytic drugs. To accomplish this, we photoactivated Pnoc\textsuperscript{BNST} neurons while mice explored an EPM, which consisted of two open arms and two perpendicular closed arms. We found that photoactivation of Pnoc\textsuperscript{BNST} neurons in the EPM further decreased time in the open arms (Figures 4M and 4N). Similarly, photoactivation of Pnoc\textsuperscript{BNST} neurons in an open field arena also decreased time in the center of the arena (Figures S3A–S3C). We next tested whether Pnoc\textsuperscript{BNST} neurons were necessary to avoid the open arms of the EPM. In agreement with our photoactivation findings, photoinhibition of Pnoc\textsuperscript{BNST} neurons significantly increased time in the open arms (Figures 4O and 4P). In summary, our data indicate that Pnoc\textsuperscript{BNST} neurons are capable of driving arousal responses independent of behavior and can modulate anxiety-like states.

**Pnoc\textsuperscript{BNST} Neurons Exhibit Diversity in Both Anatomical Connectivity and Genetic Identity**

Since we found heterogeneity in response dynamics with animals exposed to both arousal-inducing aversive and rewarding

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Figure 3. Pnoc\textsuperscript{BNST} Neurons Encode Rapid Changes in Arousal to Aversive and Rewarding Stimuli

(A) Heatmap of individual data (top) and average group data (bottom) for pupil responses to control and TMT swabs (n = 4 mice).
(B) Heatmap of responses to the control and TMT swabs from all Pnoc\textsuperscript{BNST} neurons, organized by their average response to the TMT swab.
(C) Response dynamics of Pnoc\textsuperscript{BNST} neurons to the control and TMT swabs that showed significant excitatory, inhibitory, or no change in activity to the TMT swab (n = 397 neurons).
(D) Heatmap of individual data (top) and average group data (bottom) for pupil responses to control and peanut swabs (n = 3 mice).
(E) Heatmap of responses to the control and peanut swabs from all Pnoc\textsuperscript{BNST} neurons, organized by their average response to the peanut swab (n = 780 neurons).
(F) Response dynamics of Pnoc\textsuperscript{BNST} neurons to the control and peanut swabs that showed significant excitatory, inhibitory, or no change in activity to the peanut swab.
(G) Top: proportion of excitatory and inhibitory responsive cells when the TMT swab was in the Near (compared to Far) position. Bottom: proportion of excitatory and inhibitory responsive cells when the peanut swab was in the Near (compared to Far) position.
(H) Correlation between Ca\textsuperscript{2+} activity dynamics of single Pnoc\textsuperscript{BNST} neurons and pupil size when mice were exposed to the TMT swab (n = 397 neurons).
(I) Correlation between Ca\textsuperscript{2+} activity dynamics of single Pnoc\textsuperscript{BNST} neurons and pupil size when mice were exposed to the peanut swab (n = 780 neurons).
(J) Neuronal responses to TMT categorized by response to peanut oil. Data are shown as mean ± SEM. ****p < .0001.

For a description of statistical analysis and results shown in this figure, please refer to Table S1.
odors, we hypothesized that this heterogeneity may be due to the diversity of connectivity and gene expression patterns within the PnocBNST neuronal population. To investigate connectivity, we injected a Cre-dependent virus into the adBNST of Pnoc-ires-Cre mice to express both a cytotoxic marker and a presynaptic marker. We found presynaptic labeling from PnocBNST neurons within multiple compartments of BNST (evidenced by synaptophysin-mRuby expression), suggesting that these cells form local connections among various BNST subregions. Notably, we observed that presynaptic PnocBNST terminals overlapped with both mGFP-labeled and unlabeled cells within the BNST, indicating that PnocBNST neurons may form monosynaptic connections with both Pnoc+ and Pnoc- neurons (Figure 5A). Whole-cell patch-clamp electrophysiological recordings revealed that light-evoked inhibitory postsynaptic currents were detected in adBNST neurons following photostimulation of PnocBNST neurons, which was blocked by bath application of a GABA receptor antagonist (gabazine; Figure 5B), confirming local connectivity and the GABAergic phenotype of these cells. Furthermore, local inhibition arising from PnocBNST neurons (31%, Pnoc+ neurons) but still present in eYFP+ neurons (59%, putative non-PnocBNST neurons) but still present in eYFP+ neurons (31%, PnocBNST neurons) (Figure 5C). Taken together, these data demonstrate that PnocBNST neurons form local monosynaptic inhibitory connections with both putative Pnoc- and Pnoc+ BNST neurons.

To identify projection targets from PnocBNST neurons, we labeled these neurons (including their axonal projections) and assessed the expression of their fluorescent markers in distal target regions. Distal axonal labeling was observed predominantly within the medial amygdala (MeA) and medial preoptic area (mPOA), with sparse to near-absent labeling in other adBNST output regions, including the arcuate nucleus (ARC), lateral hypothalamic area (LHA), and ventromedial hypothalamus.
(D) Quantification of fiber density (% of area) across distal regions showing fiber labeling in animals expressing ChR2-eYFP in Pnoc.

(E) Confocal image depicting fibers from animals expressing eYFP in nucleus; PAG, periaqueductal gray. medial amygdala; mPOA, medial preoptic area; ARC, arcuate nucleus; LHA, lateral hypothalamic area; VMH, ventromedial hypothalamus; PB, parabrachial nucleus; PAG, periaqueductal gray.

For a description of statistical analysis and results shown in this figure, please refer to Table S1.

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Figure 5. PnocBNST Neurons Exhibit Diversity in Anatomical Connectivity

(A) Confocal image depicting the distribution of PnocBNST somata and terminals (Syn, synaptophysin-mRuby) after local injection of AAVdj-HSyn-Flex-mGFP-2A-synaptophysin-mRuby. Inset shows higher magnification of the adBNST. LV, lateral ventricle; LS, lateral septum; adBNST, anterodorsal BNST; avBNST, anteroventral BNST; ic, internal capsule; ac, anterior commissure. Scale bar, 40 μm.

(B) Left: schematic of patch-clamp electrophysiology of ChR2-expressing Pnoc+ neurons. Right: voltage-clamp traces from PnocBNST neurons showing optically evoked inhibitory postsynaptic currents (oIPSCs) being blocked by GABAzine (n = 4 neurons). aCSF, artificial cerebral spinal fluid.

(C) Proportion of Pnoc and Pnoc+ neurons exhibiting oEPSCs, no change, and optically evoked excitatory postsynaptic currents (oEPSCs).

IN THE PRESENT STUDY, WE FOUND THAT Pnoc EXPRESSION DEFINES A SUBPOPULATION OF GABAERGIC NEURONS WITHIN THE BNST THAT ARE ASSOCIATED WITH CHANGES IN PHYSIOLOGICAL AROUSAL. PnocBNST NEURONS ENCODE THE RAPID INCREASE IN AROUSAL THAT OCCURS UPON THE PRESENTATION OF SALIENT MOTIVATIONAL STIMULI. ON THEIR OWN, THEY ARE UNABLE TO ALTER LOCOMOTION OR AVOIDANCE/APPRAISAL BEHAVIORS BUT CAN BI-DIRECTIONALLY MODULATE ANXIETY-LIKE BEHAVIOR. FURTHER, THESE NEURONS FORM LOCAL MONOSYNAPTIC INHIBITORY CONNECTIONS WITH BOTH Pnoc and Pnoc+ neurons within the adBNST and project to both the MeA and mPOA. IN AGREEMENT WITH THE OBSERVED HETEROGENEITY IN PnocBNST RESPONSE DYNAMICS, WE FOUND THAT PnocBNST NEURONS CAN BE DIVIDED INTO AT LEAST 4 GENETICALLY UNIQUE CELL TYPES THAT CAN BE IDENTIFIED BY CO-EXPRESSION OF SOM, Pkcδ, Cck, and Zic1.

DISCUSSION

In the present study, we found that Pnoc expression defines a subpopulation of GABAergic neurons within the BNST that are associated with changes in physiological arousal. PnocBNST neurons encode the rapid increase in arousal that occurs upon the presentation of salient motivational stimuli. On their own, they are unable to alter locomotion or avoidance/approach behaviors but can bi-directionally modulate anxiety-like behavior. Further, these neurons form local monosynaptic inhibitory connections with both Pnoc and Pnoc+ neurons within the adBNST and project to both the MeA and mPOA. In agreement with the observed heterogeneity in PnocBNST response dynamics, we found that PnocBNST neurons can be divided into at least 4 genetically unique cell types that can be identified by co-expression of Pnoc with either Som, Pkcδ, Cck, or Zic1. Taken together, these data show that PnocBNST neurons have a critical role in driving rapid arousal responses that are characteristic of a variety of motivational states and highlight the need for future studies to further unravel the heterogeneity within this genetically identified neuron population.

Elevated anxiety is a maladaptive state that is associated with many neuropsychiatric conditions (Calhoon and Tye, 2015; Le-Doux and Pine, 2016; Perusini and Fanselow, 2015). The manifestation of anxiety-like states includes both behavioral and physiological responses that need to occur rapidly in order to guide actions necessary for survival. Past research has developed an expansive literature on the neural circuits governing anxiety-like behavioral actions (for reviews, see Calhoon and Tye, 2015; Harris and Gordon, 2015; Shin and Liberzon, 2010;...
yet physiological arousal has received less attention. Excessive arousal responses as measured by increases in pupil size during threat exposure is commonly observed in patients suffering from anxiety disorders (Cascardi et al., 2015; Price et al., 2013). The presentation of negative emotionally arousing images increases both pupil size and the spatial resolution to identify subregions and, more important, individual neurons. In this study, we showed that changes in the activity of individual Pnoc neurons has remained elusive. fMRI lacks both the temporal resolution needed to match the rapid changes in pupil size and the spatial resolution to identify subregions and, more important, individual neurons. In this study, we showed that changes in the activity dynamics of individual Pnoc neurons are correlated with changes in pupil size and that these neurons are able to modulate anxiety-like behaviors. Therefore, Pnoc neurons may be a critical component for orchestrating excessive physiological arousal responses in pathological anxiety.

Our findings highlight a diversity of response dynamics to salient olfactory stimuli and genetically identifiable subtypes within the Pnoc neuronal population. Therefore, future studies may want to address whether diverse responses are still present during spontaneous states in the absence of motivational stimuli. Furthermore, experiments aimed at understanding the role of Pnoc subtypes during specific time windows following presentation of arousal-inducing stimuli such as rewarding stimuli, omission of reward, and cues that predict them may also aid in understanding the role of this ensemble in orchestrating rapid arousal responses during more complex motivational states. It is important to note that studies that directly inhibit Pnoc neurons (and functional subtypes) are needed to make causal conclusions about the role of this ensemble in driving rapid arousal responses. We found that Pnoc neurons consist of an interconnected microcircuit of GABAergic neurons within the BNST that may be classified by the expression of distinct genetic markers (Som, Prkcd, Cck, and Zic1). This further suggests that either functionally distinct subtypes of Pnoc neurons exist or molecularly distinct subtypes of BNST neurons share a similar function. Therefore, future studies are needed to systematically assess the causal role of Pnoc neuronal subtypes and their role in rapid arousal responses. For example, co-expression of Npy and Som has been previously reported throughout the entire amygdala (McDonald, 1989), suggesting that both of these markers identify a single neuronal cell type. Our data identify a similar neuronal cluster characterized by the co-expression of Npy and Som. It was previously shown that Npy-expressing neurons have a specific projection output to the preoptic region of the hypothalamus (Pompolo et al., 2005). Our data show that at least a subset of Pnoc neurons share this projection. Taken together, perhaps these three genetic markers (Pnoc, Npy, and Som) may be used to target the sub-population of Pnoc neurons that project from the BNST to the mPOA. Considering the role of mPOA in social avoidance/approach behavior (McHenry et al., 2017), this projection may be important for social arousal.

A recent study showed that local photoactivation of all Som neurons drives anxiety-mediated avoidance in the EPM (Ahrens et al., 2018). Therefore, how Pnoc/Som and Pnoc/Som neurons might differ in the regulation of anxiety-like behavior and arousal responses deserves further investigation. Additionally, future studies using intersectional genetic
approaches to target Pnoc and either PKCd, Cck, or Zic1 neurons could help characterize the heterogeneity of activity responses we observed in distinct Pnoc\textsuperscript{BNST} subsets. It is also equally important to delineate how Pnoc\textsuperscript{BNST} neurons may interact with other local Pnoc\textsuperscript{+} neurons and local Pnoc\textsuperscript{–} neuron clusters, such as those expressing Foxp2, Penk, Pdyn, Calb2, Crh, Nts, and Vglut3 (Gafford and Ressler, 2015; Hammack et al., 2009; Kash et al., 2015; Lebow and Chen, 2016; McClelligott and Winder, 2009; Nguyen et al., 2016).

BNST neurons have also been distinguished by their projection targets in previous studies. For instance, PB-projecting neurons regulate autonomic arousal states as measured by respiratory, whereas neurons projecting to the LHA regulate anxiety-like behavior in the EPM (Kim et al., 2013; Kodani et al., 2017). A recent study showed that BNST neurons that project to the LHA can be further subdivided by the expression of the neuropeptidergic genes Crh and Cck. These neurons show an increase in average calcium activity specific to a rewarding (female mouse urine) or aversive odorant (TMT), respectively (Giardino et al., 2018). Furthermore, chemogenetic activation of Vgat-expressing neurons within the BNST increases anxiety-like behavior and leads to activation of the locus coeruleus (LC) (Mazzone et al., 2018). Although these phenotypes are similar to our findings with Pnoc\textsuperscript{BNST} neurons, we did not observe appreciable projections from Pnoc\textsuperscript{BNST} neurons to either the PB, LHA, or LC, indicating that Pnoc\textsuperscript{BNST} neurons may be distinct from both PB-, LHA- and LC-projecting neurons. Nonetheless, whether local interactions between Pnoc\textsuperscript{BNST} neurons and either PB-, LHA-, or LC-projecting neurons within the BNST exist remains an open question that warrants further investigation.

Using advanced tools to probe neurons with single-cell resolution, we discovered that Pnoc\textsuperscript{BNST} neurons encode rapid changes in physiological arousal responses that can drive anxiety-like behaviors. However, these neurons are likely only a piece of the complex mosaic of cell types within the BNST that contribute toward arousal responding and motivational states. Further investigations into how Pnoc\textsuperscript{BNST} neurons and other BNST cell types differentially and synergistically control rapid arousal responses will shed light onto how the BNST and the larger network of brain regions that regulate motivational states contribute to the development and perpetuation of neuropsychiatric disorders characterized by maladaptive motivational states.

**STAR Methods**

Detailed methods are provided in the online version of this paper and include the following:

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  - Tissue Isolation and Single-cell cDNA Library Preparation

**Supplemental Information**

Supplemental information can be found online at [https://doi.org/10.1016/j.celrep.2020.108362](https://doi.org/10.1016/j.celrep.2020.108362).

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**Author Contributions**


**Declaration of Interests**

The authors declare no competing interests.

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**STAR METHODS**

**KEY RESOURCES TABLE**

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**RESOURCE AVAILABILITY**

**Lead Contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Garret D. Stuber (gstuber@uw.edu).
**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Animals**
Adult (25-30 g) male prepronociceptin-IRES-Cre (Pnoc-IRES-Cre) or wild-type mice (C57 BL6/J) were independently housed and maintained on a reverse 12-hr light-dark cycle (lights off at 08:00 AM) with ad libitum access to food and water. Behavior was tested during the dark cycle. All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, as adopted by the National Institute of Health, and with the approval of the Institutional Animal Care and Use Committee from the University of North Carolina at Chapel Hill.

**METHOD DETAILS**

**Fluorescent In Situ Hybridization**
For processing tissue samples for in situ hybridization, mice were anesthetized with isoflurane (3.5%–4.0%) vaporized in pure oxygen (1 L/min), rapidly decapitated and brains immediately extracted, and flash frozen on dry ice. 18 µm thick coronal sections were collected with a cryostat under RNase-free conditions, fixed in 4% PFA for 15 min at 4°C, dehydrated in serial concentrations of ethanol (50%–100%), and processed according to instructions provided in the RNAscope kit (Advanced Cell Diagnostics, Newark, CA). Sections were hybridized with the following mixed probes: Pnoc (Mm-Pnoc, cat. no. 437881), Vgat (Mm-Slc32a1, cat. no. 319191), Vglut2 (Mm-Slc17a6-C2, cat. no. 319171), CaMKIIα (Mm-Camk2a-cust-C2, cat. no. 411851). Hybridization probes used can also be found in supplementary information (Table S1). Following amplification, sections were counterstained with DAPI and coverslipped for subsequent confocal microscopy and counted using ImageJ software.

**Immunohistochemistry**
For processing tissue samples for immunohistochemistry, mice were euthanized with pentobarbital (50 mg/kg, 1.p.) and transcardially perfused with 0.01 M phosphate-buffered saline (PBS) and 4% paraformaldehyde (PFA). Tissue was fixed overnight in PFA at 4°C, cryoprotected with 30% sucrose in PBS, and 40 µm thick coronal sections were collected with a cryostat. Immunohistochemistry was performed in Pnoc-IRES-Cre mice using the following primary (kept overnight at 4°C) and secondary (kept at room temperature for 2 h) antibodies: chicken-anti-GFP (1:1,000; Aves labs, Tigard, OR), donkey anti-chicken 488 (1:500; Jackson Immuno Research Labs, West Grove, PA), mouse anti-PKCδ (1:500; BD Biosciences, Fanklin Lakes, NJ), donkey anti-mouse 647 (1:500; Jackson Immuno Research Labs, West Grove, PA), rabbit anti-Somatostatin (1:2,000; BMA Biomedicals, Switzerland), and donkey anti-rabbit 647 (1:500; Jackson Immuno Research Labs, West Grove, PA). Antibodies used with dilutions can also be found in supplementary information (Table S1). Immunoprocessing procedures were done as previously described (Jennings et al., 2013a), and sections were counterstained with DAPI and coverslipped for subsequent confocal microscopy and counted using ImageJ software.

**Confocal Microscopy**
A confocal microscope (Zeiss LSM 780, Carl Zeiss, San Diego, CA) with either a 20x (air), 40x (air), or a 63x (oil) objective was used to capture images. Software (Zen Software, Carl Zeiss, Jena, Germany) settings were optimized for each experiment. In general, z stacks were acquired in less than 1 µm increments and the maximum intensity projection of tiled images were used for representative images or for quantification purposes.

**Viral Constructs**
All viral constructs [Cre-inducible AAVdj-EF1α-DIO-GCaMP6s (3.1 × 10^{12} infectious units/mL), AAV5-EF1α-DIO-hChR2(H134R)-eYFP (4.3 × 10^{12} infectious units/mL), AAV5-EF1α-DIO-eNhHR3.0-eYFP (8.0 × 10^{12} infectious units/mL), AAV5-EF1α-DIO-eYFP (6.0 × 10^{12} infectious units/mL), and AAVdj-hSyn-Flex-mGFP-2A-synaptophysin-mRuby (5.0 × 10^{8} infectious units/mL)] were packaged by the UNC Vector Core and can also be found in supplementary information (Table S1).

**Surgery and Histology**
Mice were anesthetized with isoflurane (0.8%–1.5%) vaporized in pure oxygen (1 L/min) and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). Ophthalmic ointment (Akorn, Lake Forest, IL) and topical anesthetic (2% lidocaine; Akorn, Lake Forest, IL) were applied during surgeries, and subcutaneous administration of saline (0.9% NaCL in water) were administered to prevent dehydration. Microinjections using injection needles (33 gauge) connected to a 2 uL syringe (Hamilton Company, Reno, NV) were used to deliver viruses into the anterior dorsal portion of the bed nucleus of the stria terminalis (adBNST; 500 nL per side; relative
to bregma: +0.14 AP, +/−0.95 ML, DV −4.20 DV). For calcium imaging studies, unilateral virus injections were made into adBNST. To allow subsequent imaging of BNST neurons, a microendoscope [a gradient refractive index (GRIN) lens, 0.6 mm in diameter, 7.3 mm in length; Incscopix, Palo Alto, CA] was implanted 0.2 mm dorsal to the adBNST target site.

For optogenetic studies, bilateral virus injections were made into BNST, and an optical fiber was implanted with a 10° angle approximately 0.5 mm above the BNST. For experiments involving head-fixed behavior, a custom-made head-ring (stainless steel; 5 mm ID, 11 mm OD) was attached to the skull during surgery to allow head-fixation. Following surgeries, mice were given acetaminophen in their drinking water for 2 days and were allowed to recover with access to food and water ad libitum for at least 21 days. Following behavioral experiments, all cohorts were euthanized and perfused, tissue was extracted and 40 μm thick coronal sections collected with a cryostat, counterstained with DAPI and overlaid for verification of viral expression and fiber/lens placement.

**Patch-Clamp Electrophysiology**

Mice were anesthetized with pentobarbital (50mg/kg) before transcardial perfusion with ice-cold sucrose cutting solution containing the following (in mM): 225 sucrose, 119 NaCl, 1.0 NaH2PO4, 4.9 MgCl2, 0.1 CaCl2, 26.2 NaHCO3, 1.25 glucose, 305 mOsm. Brains were then rapidly removed, and 300 μm thick coronal sections containing BNST were taken using a vibratome (Leica, VT 1200, Germany). Sections were then incubated in aCSF (32°C) containing the following (in mM): 119 NaCl, 2.5 KCl, 1.0 NaH2PO4, 1.3 MgCl2, 2.5 CaCl2, 26.2 NaHCO3, 15 glucose, ~306 mOsm. After an hour of recovery, slices were constantly perfused with aCSF (32°C) and visualized using differential interference contrast through a 40x water-immersion objective mounted on an upright microscope (Olympus BX51WI, Center Valley, PA). Recordings were obtained using borosilicate pipettes (3–5 MΩ) back-filled with internal solution containing the following (in mM): 130 K gluconate, 10 KCl, 10 HEPES, 10 EGTA, 2 MgCl2, 2 ATP, 0.2 GTP (pH 7.35, 270–285 mOsm).

Current-clamp recordings were obtained from GCaMP6s-expressing PnocBNST neurons to identify how action potential frequency correlated with GCaMP6s fluorescence as previously described (Otis et al., 2017). Specifically, to determine how elevations in action potential frequency influence GCaMP6s fluorescence, a 1 s train of depolarizing pulses (2 nA, 2 ms) was applied at a frequency of 1, 2, 5, 10, or 20 Hz. During electrophysiological recordings, GCaMP6s fluorescence dynamics were visualized using a mercury lamp (Olympus U-RFL-T, Center Valley, PA) and a microscope-mounted camera (QImaging, optiMOS, Canada). Imaging data were acquired using Micro-Manager and extracted through hand-drawn ROIs for each recorded neuron using ImageJ. In addition to these experiments, we also performed current-clamp recordings to determine the spike fidelity of PnocBNST ChR2-expressing neurons during optogenetic stimulation. To do so, neurons were held at resting membrane potential (n = 7), and a blue LED (490nm; 1 mW) was presented in a series of 10 pulses (5 ms per pulse) at 1, 5, 10, and 20 Hz. We found that every pulse evoked an action potential for all neurons, suggesting 100% spike fidelity across cells.

Voltage-clamp recordings were obtained from BNST ChR2-expressing neurons (Pnoc+), and BNST non-ChR2 expressing neurons (Pnoc−) to identify local synaptic innervation of PnocBNST neurons. To determine if a neuron was Pnoc+, we held all cells at −70 mV and tested for the presence of ChR2 by displaying a blue LED (490nm; < 1mW) for 1 s. In the case that a long, stable inward current was evoked for the duration of that sweep, the neuron was confirmed to be Pnoc+ and ChR2+ (n = 26). Otherwise, the neuron was assumed to be Pnoc− and ChR2− (n = 37). We did not detect the presence of any transient, optically-evoked excitatory postsynaptic current (oeEPSC) during these sweeps, suggesting that PnocBNST neurons do not release excitatory transmitters within this circuit. Next, we held all neurons at the reversal potential for ChR2 (+5 to +15 mV for Pnoc+ neurons; +10 mV for Pnoc− neurons) and tested for the presence of an optically-evoked inhibitory postsynaptic current (oIPSC) by displaying the blue LED for 5 ms. In a subset of cells, we tested whether the oIPSC was mediated by GABA receptors by bath-applying GABAzine (10 μM) for 10 minutes. For all voltage-clamp experiments, data acquisition occurred at a 10 kHz sampling rate. All patch-clamp recordings were made through a MultiClamp 700B amplifier connected to a Digidata 1440A digitizer ( Molecular Devices, San Jose, CA) and analyzed using Clampfit 10.3 (Molecular Devices, San Jose, CA).

**Odor Preference in Freely-moving Mice**

Mice were habituated to have a square block holder in their home cage for 2 days prior to testing. The day of testing, a cotton swab, followed by placing either 2.5 L of TMT or 2.5 L of Peanut oil (same as head-fixed experiment) in the cotton swab. Distance to odor (cm, max: 25 cm), time spent freezing (s), and velocity (cm/s) were calculated using automated tracking software (Ethovision XT 11, Noldus, Leesburg, VA). Similar to head-fixed odor exposure experiments, a low dose of TMT was used to minimize freezing responses and maintain ambulation.

Pupil recordings were made in freely moving animals using the same camera system used for head-fixed experiments but using a triangle shape arena of similar size to the home cage with one of the corners having a 45-degree angle where the cotton swab was placed. A transparent plexiglass wall would allow viewing of the pupil when mice explored the cotton swab at close proximity. Images of the pupil where captured during the first 10 s bouts of exploration.

**Two-Photon Calcium Imaging in Head-fixed Mice**

A two-photon microscope (FVMPE-RS, Olympus, Center Valley, PA) was used to visualize activity dynamics of Pnoc+ neurons in BNST in vivo in head-fixed mice while they underwent odor exposure with pupillometry. A virus encoding the Cre-dependent calcium
indicator GCaMP6s (AAVdj-EF1α-DIO-GCaMP6s; 3.1 × 10^{12} infectious units/mL) was injected into BNST of Pnoc-ires-Cre mice (see Surgery and histology section). After a minimum of 8 weeks to allow sufficient time for virus transport and infection, mice underwent the head-fixed freely moving odor exposure assay described above, during which GCaMP6s-expressing neurons were visualized using two-photon microscopy.

The two-photon microscope used was equipped with the following to allow imaging of BNST in vivo: a hybrid scanning core set with galvanometers and fast scan resonant scanners (which allows up to 30 Hz frame-rate acquisition; set at 5 Hz), GaAsP-PMT photo detectors with adjustable voltage, gain, and offset features, a single green/red NDD filter cube, a long working distance 20x objective (air) designed for optical transmission at infrared wavelengths (LCPLN20XIR, 0.45 NA, 8.3 mm WD, Olympus, Center Valley, PA), a software-controlled modular xy stage loaded on a manual z-deck, and a tunable Mai-Tai Deep See laser system (laser set to 955 nm, ~100 fs pulse width, Spectra Physics, Santa Clara, CA) with automated four-axis alignment. Prior to testing, the optimal field of view (FOV) was selected by adjusting the imaging plane (z-axis). Two-photon scanning was triggered by an Arduino microcontroller and video was collected for each testing epoch (baseline, water or odor). Data were both acquired and processed using FluoView FV1200 and CellSens software packages (Olympus, Center Valley, PA). Following data acquisition, videos were motion corrected using a planar hidden Markov model (SIMA v1.3) (Kaufosh et al., 2014), calcium transients and deconvolved events were extracted from individual ROI’s using constraint non-negative matrix factorization algorithms (CNMF) (Zhou et al., 2018) and data was analyzed using custom data analysis pipelines written in Python (see Quantification and Statistical Analysis section).

**Head-fixed Odor Swab Exposure with Pupillometry**

For exposing odors in head-fixed freely moving mice, experimental events and behavioral recordings were orchestrated using custom-designed hardware interfaced with microcontrollers (Arduino) and Python using custom code. Odor delivery relied on a custom-made conveyor system that carried a cotton swab with odor source along a 25-cm track over 6 s to and from the animal. The cotton swab remained in close proximity to the animal for a 10 s bout. We assessed locomotor activity of head-fixed animals using a custom-made running disc. The disc was fixed under the head-fixed animal, which allowed movement similar to a rodent flying saucer wheel. Rotational changes were measured by a rotary encoder (Sparkfun, Boulder, CO) every 50 ms without regard to direction of rotation. Pupil recordings were made using a monochromatic CMOS camera with macro zoom lens (MVL7000 & DCC1545M, ThorLabs, Newton, NJ) at 10 frames per second. An infrared light (Thorlabs, Newton, NJ) was used to illuminate the eye in optogenetic experiments. For two-photon experiments, the illumination light from the objective was sufficient to visualize the eye (here the light transmitted through nervous tissue and out the pupil, thus the pupil was brighter than the cornea). An ultraviolet light (Thorlabs, Newton, NJ) was used to adjust the pupil size to avoid a ceiling or floor effect of pupil changes as necessary.

Experimentation began after minimal pupillary responses were observed to the approaching of a dry cotton swab (6 days). The day of testing, mice were exposed to 3 epochs (5 minutes each) that consisted of 5 baseline bouts (dry cotton swab), 5 control bouts (cotton swab with 2.5 μL of distilled H₂O), and 5 odor bouts (cotton swab with wither 2.5 μL of TMT or 2.5 μL of Peanut oil). The first 2 bouts of each epoch were used for analysis to assess responses. A low dose of TMT was used to minimize freezing responses and maintain ambulation.

Pupil changes were assessed offline after experimentation. A median filter was applied to each pupil recording frame before pupil diameter was measured. We used OpenCV to identify the pupil within each frame and morphological processing (erosion and dilation) to further filter noise from the image. The diameter of the pupil was measured by fitting a bounding box, and the length of its horizontal sides were used as the pupil diameter since this also measured pupil diameter fairly well during mid-blink. Calculated diameter measures were then filtered using a rolling 1 s median filter.

**Optogenetics**

Optogenetic experiments were performed as previously described (Sparta et al., 2011). Briefly, a virus encoding the Cre-inducible channel-rodopsin-2 (AAV5-ef1α-DIO-hChR2(H134R)-eYFP; 5.0 × 10^{12} infectious units per ml) was injected into BNST of either Pnoc-ires-Cre mice or their wild-type littermates as controls. For photoactivation manipulations in ChR2 or control mice, the laser (473 nm; 8–10 mW) was turned on for 5-ms pulses (20 Hz) during a 3 min period, followed or preceded by 3 min periods the laser was off. All mice were habituated to the tether for 3 days prior to behavioral testing. Following behavioral experiments, histological verification of fluorescence and optical fiber placement were performed.

**Real-Time Place Preference**

Mice were placed into a rectangular two-compartment arena (52.5 × 25.5 × 25.5 cm) as previously described (Jennings et al., 2013a). Mice were allowed to freely explore the arena for 20 min. Entry into one of the compartments triggered constant 20 Hz photostimulation (473 nm; 8–10 mW). Entry into the other chamber ended the photostimulation. The side paired with photostimulation was counterbalanced across mice. Time spent in the stimulation side was calculated using automated tracking software (Ethovision XT 11, Noldus, Leesburg, VA).

**Head-fixed Stationary Assay with Pupillometry**

Mice were head-fixed as previously described (Otis et al., 2017). Physiological and licking measures were obtained using a custom designed apparatus. A piezo sensor under the mouse monitored general movement in the tube. A pulse oximeter placed near the
neck was used to measure heart rate. Mice received unpredictable drops of sucrose (10% in water, 2.0-2.5 μL, ~1 drop/min) for 30 min using a gravity-driven solenoid through a ~18-G steel tube. Mice were habituated to the setup for 6 days. Measurements were recorded using a LabJack data acquisition box (U12 Series, LabJack Corp., Lakewood, CO). Once mice habituated to the apparatus, as evident by a reduced heart rate as compared to Day 1 (6 days), optogenetic experiments were performed while pupil videos, movement (piezo sensor) and heart rate (pulse oximeter) was tracked with an Arduino microcontroller and recorded with custom software (written in Python) during a single laser off (3 min) and laser on (3 min) period.

**Elevated Plus Maze**

Mice were placed in a standard elevated plus maze (EPM; 13.5 in height; 25 in x 2 in for each arm, 7 in tall wall for closed arms, 0.5 in tall/wide ledge for open arms). Mice were placed in the center of the EPM and allowed to freely explore it for 10 min. Distance to center (cm) and time spent in each arm (s) were calculated using automated tracking software (Ethovision XT 11, Noldus, Leesburg, VA).

**Tissue Isolation and Single-cell cDNA Library Preparation**

Mice were anesthetized with 390 g/kg sodium pentobarbital, 500 mg/kg phenytoin sodium and transcardially perfused with 20 mL in ice-cold sodium-substituted aCSF (NMDG-aCSF: 96 mM NMDG, 2.5 mM KCl, 1.35 mM NaH2PO4, 30 mM NaHCO3, 20 mM HEPES, 25 mM glucose, 2 mM thioiurea, 5 mM Na+ascorbate, 3 mM Na+pyruvate, 0.6 mM glutathione-ethyl-ester, 2 mM N-acetyl-cysteine, 0.5 mM CaCl2, 10 mM MgSO4; pH 7.35–7.40, 300-305 mOsm) modified from Ting et al. (2014). Brains were isolated and three 300 μm sections beginning at ~0.45 mm Bregma were collected in ice-cold NMDG-aCSF on a vibratome (Leica, VT 1200, Germany). Sections from 6 mice at a time (total of 4 batches with 24 mice) were recovered in NMDG-aCSF supplemented with 500 nM TTX, 10 μM APV, 10 μM DNOX (NMDG-aCSF-R) for 15 minutes after the addition of the last slice. The BNST was then isolated with 0.75 and 0.50 mm Palkovitz punches and digested in NMDG-aCSF-R containing 1.0 mg/mL pronase for 30 minutes at room temperature. Tissue was then triturated with a patch pipet fire-polished to an internal diameter of 300 μm in 1.0 mL of NMDG-aCSF-R supplemented with 0.05% BSA (NMDG-aCSF-BSA) to dissociate. The suspension transferred to 12 mL NMDG-aCSF-BSA and sedimented at 220 g for 6 minutes at 18°C to wash. The supernatant was removed, and cells were resuspended in 1 mL NMDG-aCSF-BSA. To fix the cells (Alles et al., 2017), 4.0 mL of ice-cold 100% methanol was added dropwise to the suspension while gently swirling the tube. Cells were then incubated for 30 minutes on ice and transferred to –80°C. To rehydrate suspensions prior to Drop-seq, cells were removed from –80°C and incubated on ice for 15 minutes. Cells were then sedimented at 500 x g for 5 minutes at 4°C, resuspended in 5 mL of PBS supplemented with 0.01% BSA (PBS-BSA), and incubated for 5 minutes on ice. The suspension was then sedimented at 220 x g for 6 minutes at 18°C and resuspended in 1.0 mL of PBS-BSA for a final concentration of ~2.6-3.2 × 10^5 cells/mL. Rehydration and droplet generation was performed on fixed samples within 3 weeks of fixation.

Drop-seq was performed as previously described in with modifications (Macosko et al., 2015). Single-cell capture was performed on a glass microfluidics device (Dolomite Microfluidics, United Kingdom) with aqueous flow at 40 μL/min and oil at 200 μL/min. Beads were loaded at ~200 beads/μL. Reverse transcription, Exol digestion, and PCR were performed as previously described, but with 11 cycles for second stage of amplification. PCR products were pooled by batch, purified on SPRI beads (Axygen, Union City, CA), and indexed using Nextera XT with 800 pg input per batch. Purified tagmentation products were pooled by mass according to the estimated number of cells per pool member as quantified by a Qubit dsDNA HS Assay. Sequencing was performed at the UNC High Throughput Sequencing Facility on an Illumina HiSeq2500 using Paired-End 2x50 Rapid Run v2 chemistry.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Behavioral Optogenetics and Electrophysiology Data Analysis**

For data obtained from the optogenetic and patch-clamp electrophysiology experiments, data were analyzed using Prism 7 (GraphPad Sotware Inc., La Jolla, CA). Mean values are accompanied by SEM values. Comparisons were tested using paired or unpaired t tests. Two-way ANOVA tests followed by either Tukey’s post hoc tests or Bonferroni post hoc comparisons were applied for comparisons with more than two groups, n.s. p > 0.05, *p < 0.05, **p < 0.01, ***p < 0.001.

**Calcium Imaging Analysis**

Calcium imaging recordings were first motion corrected using a planar hidden Markov model (Kaifosh et al., 2014). Neurons were identified, and their calcium signals were extracted using a modified version of constrained nonnegative matrix factorization (CNMF) (Zhou et al., 2018), allowing us to segregate spatially overlapping signals. This extracted signal was adjusted (scaled) to account for variations in fluorescence intensities among cells by the standard deviation of a neuron’s fluorescence throughout the Control odor exposure. For head-fixed, odor-presentation experiments, neuronal activity was aligned to the presentation of the odor. Neurons were classified as excitatory or inhibitory to proximity of TMT or Peanut oil if the fluorescence values for frames differed between near and far location—defined using a Mann-Whitney U test with Bonferroni correction. Correlations in activity and behavior were calculated using the Spearman correlation coefficient.
Single-Cell Sequencing Clustering and Analysis

Demultiplexing was performed allowing 1 mismatch with Illumina bcl2fastq v2.18.0.12. Initial processing and generation of digital expression matrices was performed with Drop-seq_tools v1.12 and Picard Tools v2.2.4 (Macosko et al., 2015). Alignment was performed using STAR v2.4.2a with 72 GB of RAM and 16 threads. Clustering was performed in R using Seurat v1.4.0.16 unless otherwise noted. Prior to clustering, cells were filtered by ≥ 500 unique genes, ≤ 20,000 unique molecules, and ≤ 5 percent mitochondrial reads. Filtered data was scaled to the median number of unique molecules and log(x+1) transformed. Zero-variance genes were removed from the data, and batch correction was performed with ComBat (Johnson et al., 2007) from SVA v3.220 (Leek et al., 2012) using parametric adjustment on a model matrix containing number of unique genes and molecules, and percent mitochondrial reads. Four batches were included, each containing six animals that were pooled during tissue isolation. Relative log expression by cell and mean expression correlation across batches were used to assess the correction. Only genes detected in all batches were included in the analysis.

Variable genes were selected with a cutoff of 0.5 standard deviations from the mean dispersion within a bin (Macosko et al., 2015). Variable genes were used as the basis for principal components analysis, and cluster calling was performed on principal components using the Louvain algorithm with multilevel refinement under default settings. Principal components were reduced and visualized via t-distributed stochastic neighbor embedding (tSNE) using the first 20 components and a resolution of 1.3. Clusters were reordered on a hierarchically-clustered distance matrix based on all genes. Features were identified using a single-cell likelihood-ratio test6 implemented in Seurat. To identify cluster-specific features, genes in each cluster were tested against those in either the nearest cluster or node in the hierarchically-clustered dendrogram. Analysis from pre-processing to digital expression matrices were run on a Dell blade-based cluster running RedHat Enterprise Linux 5.6. Cluster calling and tSNE were run on a similar cluster running RedHat Enterprise Linux 7.3. All other steps were run on macOS 10.13.3.
Supplemental Information

Prepronociceptin-Expressing Neurons in the Extended Amygdala Encode and Promote Rapid Arousal Responses to Motivationally Salient Stimuli

## STATISTICAL ANALYSIS

<table>
<thead>
<tr>
<th>Figure</th>
<th># of Samples</th>
<th>Test</th>
<th>Test statistic</th>
<th>p value</th>
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<tbody>
<tr>
<td>Figure 1</td>
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<tr>
<td>G; Evoked fluorescent/spike relationship</td>
<td>n = 10 Pnoc* neurons</td>
<td>Pearson correlation coefficient</td>
<td>r = 0.996</td>
<td>p&lt;0.001</td>
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<tr>
<td>Figure 2</td>
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<tr>
<td>B; Time in odor zone when exposed to the control and TMT swabs</td>
<td>n = 4 wild type mice</td>
<td>Paired t-test</td>
<td>t₂=6.518</td>
<td>p=0.0073</td>
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<tr>
<td>C; Time in odor zone when exposed to the control and peanut swabs</td>
<td>n = 4 wild type mice</td>
<td>Paired t-test</td>
<td>t₂=6.277</td>
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<td>E; Pupil size when exposed to the control and TMT swabs</td>
<td>n = 4 wild type mice</td>
<td>Paired t-test</td>
<td>t₂=3.604</td>
<td>p=0.0367</td>
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<td>F; Pupil size when exposed to the control and peanut swabs</td>
<td>n = 4 wild type mice</td>
<td>Paired t-test</td>
<td>t₂=4.298</td>
<td>p=0.0232</td>
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<td>Figure 3</td>
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<tr>
<td>H; Correlation between Ca²⁺ activity and pupil size when mice were exposed to the TMT swab (responses defined by exposure to the TMT swab)</td>
<td>n = 397 Pnoc* neurons</td>
<td>Mann-Whitney U test, significance level adjusted using Bonferroni correction</td>
<td>Excited vs No Change: U=2527.0</td>
<td>p=6.5808e-31</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Inhibited vs No Change: U=1671.0</td>
<td>p=1.0754e-25</td>
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<td>I; Correlation between Ca²⁺ activity and pupil size when mice were exposed to the control Swab (responses defined by exposure to the TMT swab)</td>
<td>n = 397 Pnoc* neurons</td>
<td>Mann-Whitney U test, significance level adjusted using Bonferroni correction</td>
<td>Excited vs No Change: U=11474.0</td>
<td>p=2.9406</td>
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<td>Inhibited vs No Change: U=7683.0</td>
<td>p=1.6152</td>
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<td>J; Correlation between Ca²⁺ activity and pupil size when mice were exposed to the peanut swab (responses defined by</td>
<td>n = 780 Pnoc* neurons</td>
<td>Mann-Whitney U test, significance level</td>
<td>Excited vs No Change: U=24140.0</td>
<td>p=8.8104e-56</td>
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<tr>
<td>Exposure to the peanut swab</td>
<td>Adjusted using Bonferroni correction</td>
<td>U=971.0</td>
<td>p=8.9200e-8</td>
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**Figure 4**

<table>
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<tr>
<th>F; Velocity</th>
<th>n = 8 ChR2-Cre mice, n = 9 ChR2-Cre+ mice</th>
<th>Two-way ANOVA</th>
<th>Group: F&lt;sub&gt;(1,15)&lt;/sub&gt;=0.3553 Laser: F&lt;sub&gt;(1,15)&lt;/sub&gt;=16.23 Interaction: F&lt;sub&gt;(1,15)&lt;/sub&gt;=0.103</th>
<th>p=0.5600 p=0.0011 p=0.7527</th>
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<tr>
<td>H; Time in stimulation side during real time place preference</td>
<td>n = 8 ChR2-Cre mice, n = 10 ChR2-Cre+ mice</td>
<td>Two-sample unpaired t-test</td>
<td>t&lt;sub&gt;16&lt;/sub&gt;=0.9711</td>
<td>p=0.3459</td>
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<tr>
<td>J; Movement</td>
<td>n = 4 ChR2-Cre mice, n = 4 ChR2-Cre+ mice</td>
<td>Two-sample unpaired t-test</td>
<td>t&lt;sub&gt;6&lt;/sub&gt;=0.240</td>
<td>p=0.8185</td>
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<td>K; Pupil size</td>
<td>n = 4 ChR2-Cre mice, n = 4 ChR2-Cre+ mice</td>
<td>Two-sample unpaired t-test</td>
<td>t&lt;sub&gt;6&lt;/sub&gt;=2.616</td>
<td>p=0.0398</td>
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<td>L; Heart rate</td>
<td>n = 4 ChR2-Cre mice, n = 4 ChR2-Cre+ mice</td>
<td>Two-sample unpaired t-test</td>
<td>t&lt;sub&gt;6&lt;/sub&gt;=2.928</td>
<td>p=0.0264</td>
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<td>N; Time in open arms</td>
<td>n = 7 ChR2-Cre mice, n = 6 ChR2-Cre+ mice</td>
<td>Two-way ANOVA</td>
<td>Group: F&lt;sub&gt;(1,11)&lt;/sub&gt;=4.417 Laser: F&lt;sub&gt;(1,11)&lt;/sub&gt;=4.927 Interaction: F&lt;sub&gt;(1,11)&lt;/sub&gt;=23.12</td>
<td>p=0.0594 p=0.0484 p=0.0005</td>
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<tr>
<td>P; Time in open arms</td>
<td>n = 9 NpHR-Cre mice, n = 6 NpHR-Cre+ mice</td>
<td>Two-way ANOVA</td>
<td>Group: F&lt;sub&gt;(1,13)&lt;/sub&gt;=3.455 Laser: F&lt;sub&gt;(1,13)&lt;/sub&gt;=5.845 Interaction: F&lt;sub&gt;(1,13)&lt;/sub&gt;=5.667</td>
<td>p=0.0859 p=0.0310 p=0.0333</td>
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**Figure 5**

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<th>B; GABAzine block</th>
<th>n = 4 Pnoc+ neurons</th>
<th>Paired t-test</th>
<th>t&lt;sub&gt;3&lt;/sub&gt;=4.83</th>
<th>p=0.0064</th>
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**Figure S2**

<p>| D; Correlation between Ca&lt;sup&gt;2+&lt;/sup&gt; activity and velocity when mice were exposed to the TMT swab (responses defined by n = 397 Pnoc+ neurons) | Mann-Whitney U test, significance level adjusted | Excited vs No Change: U=8790.0 Inhibited vs No Change: U=7438.0 | p=0.0013 p=0.9216 |</p>
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<th>Exposure to the TMT swab)</th>
<th>Mann-Whitney U test, significance level adjusted using Bonferroni correction</th>
<th>Excited vs No Change: ( U=101550.0 ) Inhibited vs No Change: ( U=7818.0 )</th>
<th>( p=0.6720 ) ( p=2.0868 )</th>
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<tr>
<td>( n = 397 \text{ Pnoc}^{+} ) neurons</td>
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<tr>
<td>E; Correlation between ( \text{Ca}^{2+} ) activity and velocity when mice were exposed to the control Swab (responses defined by exposure to the TMT swab)</td>
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<tr>
<td>( n = 780 \text{ Pnoc}^{+} ) neurons</td>
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<tr>
<td>F; Correlation between ( \text{Ca}^{2+} ) activity and velocity when mice were exposed to the peanut swab (responses defined by exposure to the peanut swab)</td>
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<tr>
<td>( n = 8 \text{ ChR2-Cre}^{-} ) mice, ( n = 10 \text{ ChR2-Cre}^{+} ) mice</td>
<td>Two-way ANOVA ( F(1,16)=2.44 ) Laser: ( F(1,16)=0.06083 ) Interaction: ( F(1,16)=9.761 )</td>
<td>Laser Off Laser On</td>
<td>( p=0.9999 ) ( p=0.0281 )</td>
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<td>Figure S3</td>
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<td>( n = 9 \text{ ChR2-Cre}^{-} ) mice, ( n = 8 \text{ ChR2-Cre}^{+} ) mice</td>
<td>Two-way ANOVA ( F(1,15)=2.123 ) Laser: ( F(1,15)=1.817 ) Interaction: ( F(1,15)=0.103 )</td>
<td>Laser Off Laser On</td>
<td>( p=0.4172 ) ( p=0.2962 )</td>
</tr>
<tr>
<td>C; Time freezing</td>
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</table>
Figure S1, Extracting activity dynamics from $Pnoc^{BNST}$ neurons. Related to Figure 2. (A-E)
Analysis pipeline of calcium imaging data using constrained nonnegative matrix factorization (CNMF) for extracting single-cell fluorescence signals from imaging data.
Figure S2, Correlations between velocity and neural activity of Pnoc\textsuperscript{BNST} neurons. Related to Figure 2. (A) Heat map of individual data (top) and average group data (bottom) for velocity responses to the control and TMT swabs. (B) Heat map of individual data (top) and average group data (bottom) for velocity responses to the control and peanut swabs. (C) Top: Proportion of excitatory and inhibitory responsive cells when the TMT swab was in the Near position (compared to Far position). Bottom: Proportion of excitatory and inhibitory responsive cells when the peanut swab was in the Near position (compared to Far position). Same as Figure 3G. (D) Correlation between Ca\textsuperscript{2+} activity dynamics of single Pnoc\textsuperscript{BNST} neurons and velocity when mice were exposed to the TMT swab (n=397 neurons). (E) Correlation between Ca\textsuperscript{2+} activity dynamics of single Pnoc\textsuperscript{BNST} neurons and velocity when mice were exposed to the control swab (excited and inhibited as defined by their response to the TMT swab, n=397 neurons). (F) Correlation between Ca\textsuperscript{2+} activity dynamics of single Pnoc\textsuperscript{BNST} neurons and velocity when mice were exposed to the peanut swab (n=780 neurons). (G) Individual traces for pupil size, velocity and Ca\textsuperscript{2+} activity from single neuron responses from two representative mice that were exposed to either TMT (left) or peanut (right) swabs. Data shown as mean ± SEM. **p<0.01, ****p<0.0001. For a description of statistical analysis and results shown in this figure please refer to Table S1.
Figure S3, Photoactivation of $Pnoc^{BNST}$ neurons increases anxiety-like behavior in an open field test. Related to Figure 2. (A) Sample heat map illustrating the location of a mouse during photoactivation of $Pnoc^{BNST}$ neurons during an open field test (OFT). (B) Group average for time spent in center of the OFT with photoactivation of $Pnoc^{BNST}$ neurons (n=8-10 per group). (C) Group average for time spent freezing with photoactivation of $Pnoc^{BNST}$ neurons (n=8-9 per group). Data shown as mean ± SEM. *$p<0.05$. For a description of all statistical analysis and results shown in this figure please refer to Table S1.
Figure S4, Genetic clustering of cell types across BNST neurons. Related to Figure 6. (A) Schematic of tissue isolated from BNST (n=2492 cells, n=24 mice). (B) Relative log expression normalization across cells and (C) mean gene expression correlations across batches following parametric batch correction. (D) Percent mitochondrial reads distribution across clusters. (E) Fraction of significant genes marking each cluster as determined using a likelihood-ratio test for single-cell data. (F) Individual cells plotted by the number of unique genes and unique transcripts detected. (Median = 1435 unique genes, 2257 unique transcripts). (G) Gene mean expression across all cell clusters. The 11 neuronal clusters are highlighted. (H) Distribution of all cells across clusters. (I) Heat map depicting expression of candidate marker genes for each neuronal cluster. (J) Expression of Vgat and Vglut2 across all neuronal clusters. Color represents normalized gene expression level. Size corresponds to proportion of neurons that expressed the gene.