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Stephanie A. Flavin, Vanderbilt University
Robert T. Matthews, Vanderbilt University
Qin Wang, University of Alabama at Birmingham
E. Muly, Emory University
Danny G. Winder, Vanderbilt University

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α2A-Adrenergic Receptors Filter Parabrachial Inputs to the Bed Nucleus of the Stria Terminalis

Stephanie A. Flavin, Robert T. Matthews, Qin Wang, E. Chris Muly, and Danny G. Winder

1Department of Molecular Physiology & Biophysics, 2John F. Kennedy Center for Research on Human Development, and 3Neuroscience Program in Substance Abuse, Vanderbilt Brain Institute, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0615, 4Department of Psychiatry, Emory University School of Medicine, Atlanta, Georgia 30322, 5Department of Cell, Developmental and Integrative Biology, University of Alabama at Birmingham, Birmingham, Alabama 35294

α2-adrenergic receptors (AR) within the bed nucleus of the stria terminalis (BNST) reduce stress–reward interactions in rodent models. In addition to their roles as autoreceptors, BNST α2A-ARs suppress glutamatergic transmission. One prominent glutamatergic input to the BNST originates from the parabrachial nucleus (PBN) and consists of asymmetric axosomatic synapses containing calcitonin gene-related peptide (CGRP) and vGluT2. Here we provide immunoelectron microscopic data showing that many asymmetric axosomatic synapses in the BNST contain α2A-ARs. Further, we examined optically evoked glutamate release ex vivo in BNST from mice with virally delivered channelrhodopsin2 (ChR2) expression in PBN. In BNST from these animals, ChR2 partially colocalized with CGRP, and activation generated EPSCs in dorsal anterolateral BNST neurons that elicited two cell-type-specific outcomes: (1) feedforward inhibition or (2) an EPSP that elicited firing. We found that the α2A-AR agonist guanfacine selectively inhibited this PBN input to the BNST, preferentially reducing the excitatory response in ex vivo mouse brain slices. To begin to assess the overall impact of α2A-AR control of this PBN input on BNST excitatory transmission, we used a Thy1-COP4 mouse line with little postsynaptic ChR2 expression nor colocalization of ChR2 with CGRP in the BNST. In slices from these mice, we found that guanfacine enhanced, rather than suppressed, optogenetically initiated excitatory drive in BNST. Thus, our study reveals distinct actions of PBN afferents within the BNST and suggests that α2A-AR agonists may filter excitatory transmission in the BNST by inhibiting a component of the PBN input while enhancing the actions of other inputs.

Key words: adrenergic receptors; bed nucleus of the stria terminalis; excitatory transmission; extended amygdala; norepinephrine; optogenetics

Introduction

Risk of relapse to drug-seeking behavior in addicts remains high even after treatment (Weiss and Koob, 2001), and stress increases relapse risk (Brown et al., 1995; Sinha et al., 1999; Sinha et al., 2011). In recent studies, α2-AR agonists have shown promise in curbing cravings in drug-addicted individuals (Sinha et al., 2007; Jobes et al., 2011; Fox and Sinha, 2014; Fox et al., 2014). These clinical data are supported by rodent data demonstrating that α2-AR agonists reduce stress-induced reinstatement of drug-seeking behavior in rats (Erb et al., 2000; Shaham et al., 2000; Highfield et al., 2001; Mantsch et al., 2010).

Noradrenergic signaling in the bed nucleus of the stria terminalis (BNST) plays an important role in stress-induced relapse to drug-seeking behavior (Aston-Jones et al., 1999; Erb et al., 2001; Flavin and Winder, 2013). Direct administration of α2-AR agonists into the BNST reduces stress-induced reinstatement of drug-seeking behavior (Wang et al., 2001), as well as conditioned place aversion from morphine withdrawal (Delfs et al., 2000). α2A-ARs are widely expressed in the BNST (Shields et al., 2009). In addition to their autoreceptor function, α2A-ARs in the BNST can heterosynaptically modulate excitatory transmission (Shields et al., 2009; Krawczyk et al., 2011). The BNST receives many glutamatergic inputs from which α2A-ARs may modulate excitatory transmission. A better understanding of which excitatory inputs to the BNST are selectively modulated by α2A-ARs may help elucidate neural circuitry underlying the ability of α2-AR agonists to block stress-induced reinstatement of drug-seeking in rodent models.

One of the more prominent glutamatergic inputs to the BNST is an ascending input from the parabrachial nucleus (PBN) (Shimada et al., 1985; Shimada et al., 1989; Dobolyi et al., 2005) that forms axosomatic synapses onto dorsal BNST neurons (Shimada et al., 1989; Dobolyi et al., 2005). These axosomatic inputs contain both the neuropeptide calcitonin gene-related peptide (CGRP) as well as vGluT2 (Shimada et al., 1985; Shimada et al.,...
1989; Dobolyi et al., 2005; Niu et al., 2010; Kaur et al., 2013). The PBN input to the extended amygdala, which includes both the BNST and the central nucleus of the amygdala (CeA), has been implicated in a wide range of behaviors, including pain sensitization (Han et al., 2005, 2010), taste aversion (Mungrar et al., 2006), fear conditioning (Sink et al., 2013a), hypercapnic arousal (Kaur et al., 2013), and feeding (Carter et al., 2013). Previous studies using electrical stimulation to target glutamatergic inputs suggest that α2-ARs may differentially modulate the PBN and basolateral amygdala (BLA) inputs to the CeA (Delaney et al., 2007).

Here we demonstrate that the α2A-AR agonist guancaine selectively regulates a PBN input to the BNST. We also show that optical PBN afferent stimulation in the BNST generates two different responses and that these responses are differentially inhibited by guancaine. Conversely, we show that, in a mouse line where ChR2 is widely expressed but not colocalized with BNST 2A-AR KI) mice that label the N terminus of the α2A-AR receptor with HA antibodies to more easily visualize the localization of CGRFP or ChR2-YFP fluorescence in the BNST of both Thy1-COP4 mice and mice that had PBN injections of AAV5-CaMKII-ChR2:YFP. We did this as a means of assessing the relative expression of ChR2 on PBN terminals in the BNST of these two types of mice. We used ImageJ (Fiji) software to visualize the colocalization of CGRF containing axosomatic synapses are found in projections from the PBN to the dorsal BNST (Dobolyi et al., 2005). We then drew a line through the selected cell body as well as the neuropil immediately surrounding the soma. We then individually plotted the gray value (g/axis) versus distance (μm) for each of the different fluorescent markers (CGRF, ChR2, or Neun) along the line that we drew through the soma and the immediately surrounding neuropil. Next, we overlaid each of the fluorescent profiles using PRISM software to visualize the level of overlap in the localization of CGRF, ChR2:YFP, or Neun.

Electron microscopy. For immunoelectron microscopy, mice were killed and perfused with 4% PFA, 0.2% glutaraldehyde, and 0.2% picric acid in PBS. We perfused hemagglutinin (HA) α2A-AR knock-in (HA α2A-AR KI) mice that label the N terminus of the α2A-AR with an HA tag. This mouse was developed by Q.W. The HA-tag on the α2A-AR allows us to use HA antibodies to visually visualize the α2A-AR expression, so α2A-AR antibodies show poor specificity.

In some cases, the mice were pretreated with the α2A-AR agonist clonidine to induce internalization of the HA-tagged α2A-AR. Animals were given intraperitoneal clonidine (1 mg/kg) 30 min before death. The brains were blocked and postfixed in 4% PFA for 4 h. Coronal, 50-μm-thick vibratome sections of the brain were cut and stored frozen at −80°C in 15% sucrose until immunohistochemical experiments were performed. The care of the animals and all anesthesia and sacrifice procedures in this study were performed according to the National Institutes for Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Emory University.

Single-label immunoperoxidase labeling was performed using a mouse monoclonal antibody against the HA tag at a 1:500 dilution (Covance, clone 16B12). The single-label immunoperoxidase labeling was performed as described previously (Muly et al., 2003). Briefly, sections were thawed, incubated in blocking serum (3% normal goat serum, 1% BSA, 0.1% glycine, 0.1% BSA in 0.01 M PBS, pH 7.4) for 1 h and then placed in primary antisera diluted in blocking serum. After 36 h at 4°C, the sections were rinsed and placed in a 1:200 dilution of biotinylated horse anti-mouse IgG (Vector) for 1 h at RT. The sections were then rinsed, placed in avidin-biotinylated peroxidase complex (ABC Elite,
Vector) for 1 h at RT, and then processed to reveal peroxidase using DAB as the chromagen. Sections were then postfixed in osmium tetroxide, stained en bloc with uranyl acetate, dehydrated, and embedded in Durcupan resin (Electron Microscopy Sciences). Selected regions of the BNST were mounted on blocks, and ultrathin sections were collected onto pioloform-coated slot grids and counterstained with lead citrate. Control sections processed as above, except for the omission of the primary immunoreagent, did not contain DAB label upon electron microscopic examination.

Ultrathin sections were examined with a Zeiss EM10C electron microscope and immunoreactive elements were imaged using a DualVision cooled CCD camera (1300 × 1030 pixels) and Digital Micrograph software (version 3.7.4, Gatan). Images selected for publication were saved in TIFF format and imported into an image processing program (Canvas 8; Deneba Software). The contrast was adjusted, and the images were cropped to meet size requirements.

In considering the results of our immunoelectron microscopic examination of α2A-AR, there are several caveats. First, it is possible that the presence of the HA tag on the knock-in transgenic mouse resulted in an alteration in the subcellular distribution of the receptor. However, other studies of α2A-AR receptor localization have found it is localized in dendritic, axonal, and glial compartments in the locus ceruleus (Lee et al., 1998), the ventrolateral medulla (Milner et al., 1999), and hippocampus (Milner et al., 1998), so if the presence of the HA tag had an effect on localization, it appears to have been of a qualitative nature, and not such as to qualitatively change the localization pattern. A second issue was our use of clonidine pretreatment. We found this necessary because the HA tag is located on an extracellular portion of the receptor and in untreated animals the majority of the label appeared to be extracellular, outlining adjacent structures. It is possible that the clonidine-induced internalization also induced transport from axon terminals and dendritic spines to more proximal positions in the dendritic and axonal arbors. In clonidine-treated animals, we observed that dendritic shafts and preterminal axons were the most commonly labeled structures; however, it is possible that spines and axon terminals would have been more common without clonidine treatment.

**Electrophysiology recordings in the BNST: brain slice preparation.** Brain slice preparation methods were used as previously described (Grueter and Winder, 2005; Grueter et al., 2006; Shields et al., 2009). Mice were removed from colony and allowed to acclimate for 1 h in a Med Associates sound-attenuating chamber. After the acclimation, mice were anesthetized with isoflurane and then decapitated. Brains were removed quickly and transferred to a 1°C–4°C oxygenated (95% O2/5% CO2) ACSF. After brain slice preparation, slices recovered for 1 h in a submerged holding chamber (25°C) containing oxygenated (95% O2/5% CO2) ACSF. After recovery in the holding chamber, slices were transferred to the recording chamber where they were continuously perfused with oxygenated and heated (25°C) ACSF at a rate of 2 ml/min. For optical whole-cell recordings, light stimulation was produced from a Lamba XL light source (Sutter Instruments) shone through a bandpass filter (Semrock, 475 nm wavelength, 20 nm bandwidth). The blue light was guided from the light source through the objective lens using a double lamp-housing adapter (Olympus), resulting in illumination of the slice. Light stimuli were −2 ms in duration and occurred every 10–20 s. oEPSCs of 100–1000 pA were recorded while voltage-clamped at −70 mV in the presence of picrotoxin (25 μM). Although we did not quantify the levels of viral expression for comparison between the BLA and the PBN, we adjusted initial oEPSCs from each of the inputs such that all baseline oEPSCs were between 100 and 650 pA at the beginning of each experiment. For large starting oEPSCs, neutral density filters and microscope apertures were used to adjust light intensity such that all experiments were recorded on baseline oEPSCs within the defined amplitude range. Evoked oEPSCs were of a short, invariant latency, consistent with monosynaptic excitation. In data analysis, oEPSCs were normalized to the amplitude of the baseline oEPSCs recorded. After achieving whole-cell configuration, cells were allowed to equilibrate for a minimum of 5 min before baseline was started. Postsynaptic parameters were monitored continuously throughout the duration of the experiments. Data are represented as an average of the peak amplitude of 3 sweeps. For voltage-clamp recordings, electrodes of 2.5–5.0 MΩ were filled with (in mS) as follows: 117 Cs gluconate, 20 HEPES, 0.4 EGTA, 5 TEA, 2 MgCl2, 4 Na2ATP, 0.3 Na3GTP (pH 7.2–7.4, osmolarity 290–295). Input resistance, holding current, and series resistance are monitored continuously. Experiments with input resistances that change by ≥20% are excluded from data analysis. All whole-cell data were recorded with Clampex version 10.2 and analyzed with pClamp version 10.2 (Molecular Devices). For whole-cell voltage-clamp recordings, the optical interface was automated in Clampex and included an automated camera (Molecular Devices) controlled light pulse duration and frequency. For optical field potential recordings, light stimuli were ~2 ms in duration and occurred every 20 s. All field potential data were collected using Clampex 10.2 (Molecular Devices).

**Analysis of field potential recordings.** All field potential recordings were analyzed via Clampfit 10.2 (Molecular Devices) as previously described (Shields et al., 2009). All field recordings contain a 20 min baseline before agonist application, with the exception of the atipamezole preincubated experiments for which atipamezole was present during baseline, and all data points are normalized to the baseline 5 min before agonist application. Plotted time courses for field experiments are represented as 1 min averages.

**Optical whole-cell recordings.** Whole-cell voltage-clamp recordings were performed as previously reported (Grueter and Winder, 2005; Grueter et al., 2006; Kash and Winder, 2006; Silberman et al., 2013). Briefly, after brain slice preparation, slices recovered for 1 h in a submerged holding chamber (25°C) containing oxygenated (95% O2/5% CO2) ACSF. After recovery in the holding chamber, slices were transferred to the recording chamber where they were continuously perfused with oxygenated and heated (25°C) ACSF at a rate of 2 ml/min. For optical whole-cell recordings, light stimulation was produced from a Lamba XL light source (Sutter Instruments) shone through a bandpass filter (Semrock, 475 nm wavelength, 20 nm bandwidth). The blue light was guided from the light source through the objective lens using a double lamp-housing adapter (Olympus), resulting in illumination of the slice. Light stimuli were −2 ms in duration and occurred every 10–20 s. oEPSCs of 100–1000 pA were recorded while voltage-clamped at −70 mV in the presence of picrotoxin (25 μM). Although we did not quantify the levels of viral expression for comparison between the BLA and the PBN, we adjusted initial oEPSCs from each of the inputs such that all baseline oEPSCs were between 100 and 650 pA at the beginning of each experiment. For large starting oEPSCs, neutral density filters and microscope apertures were used to adjust light intensity such that all experiments were recorded on baseline oEPSCs within the defined amplitude range. Evoked oEPSCs were of a short, invariant latency, consistent with monosynaptic excitation. In data analysis, oEPSCs were normalized to the amplitude of the baseline oEPSCs recorded. After achieving whole-cell configuration, cells were allowed to equilibrate for a minimum of 5 min before baseline was started. Postsynaptic parameters were monitored continuously throughout the duration of the experiments. Data are represented as an average of the peak amplitude of 3 sweeps. For voltage-clamp recordings, electrodes of 2.5–5.0 MΩ were filled with (in mS) as follows: 117 Cs gluconate, 20 HEPES, 0.4 EGTA, 5 TEA, 2 MgCl2, 4 Na2ATP, 0.3 Na3GTP (pH 7.2–7.4, osmolarity 290–295). Input resistance, holding current, and series resistance are monitored continuously. Experiments with input resistances that change by ≥20% are excluded from data analysis. All whole-cell data were recorded with Clampex version 10.2 and analyzed with pClamp version 10.2 (Molecular Devices). For whole-cell voltage-clamp recordings, the optical interface was automated in Clampex and included an automated camera (Molecular Devices) controlled light pulse duration and frequency. For optical field potential recordings, light stimuli were ~2 ms in duration and occurred every 20 s. All field potential data were collected using Clampex 10.2 (Molecular Devices).
lish whether the cell was PBN-responsive. Approximately 1 of 10 cells was either PBN-unresponsive or the response generated by the light stimulation was too small to be appropriately interpreted and classified. The membrane potentials of the cells that we recorded from in the BNST rested between −70 mV and −85 mV before any current injection. Once it was determined that a BNST cell was PBN-responsive, we injected current into the cell until its membrane potential was between −45 mV and −60 mV. Within these membrane potentials, the cell would either fire or a feedforward IPSP would be generated. If the cell fired within these membrane potentials, we classified it as a PBN-excited cell; if an IPSP was observed, we classified the cell as a PBN-inhibited cell. For cell profile recordings, current was injected into cells until their membrane potential was between −50 mV and −60 mV. Progressive 10 pA current steps current were then delivered to the cell from which we determined input resistance. By visual inspection of the responses to hyperpolarizing current injection, we determined whether a cell exhibited Ih current. From the responses to depolarizing steps, we determined whether a cell demonstrated fast- or slow-rise action potentials. Fast-rise action potentials had an action potential rise time of ~25 ms, whereas slow-rise action potentials had a rise time of ~40 ms.

For drug application experiments, cell type was determined by injecting current until the cell membrane potential rested between −45 and −60 mV and then cell type was determined by visual inspection as described above. For PBN-inhibited cells, current was injected into the cell so that the IPSP was observed during drug-application recordings. A 5 min baseline of the IPSP was taken before drug application. For PBN-excited cells, no current was injected into the cell. A 5 min baseline of the EPSP observed at the resting membrane potential of the cell was taken before drug application.

All whole-cell data were recorded with Clampex version 10.2 and analyzed with pClamp version 10.2 (Molecular Devices). Recordings contained a 5 to 10 min baseline recording before drug application. All data points were normalized to the first 5 min of baseline. Plotted time courses for whole-cell experiments are represented as 30 s averages.

Statistics. Experiments comparing a difference to baseline were analyzed using a Student’s t test. Experiments comparing baseline to two consecutive drug treatments were compared using a repeated-measures one-way ANOVA and a Tukey’s multiple-comparisons test for pairwise comparisons of baseline versus guanfacine-treated, baseline versus atipamezole-treated, and guanfacine- and atipamezole-treated. The significance of the presence of Ih currents in PBN-inhibited and atipamezole-treated, and guanfacine-treated versus atipamezole-treated.

comparisons of baseline versus guanfacine-treated, baseline versus atipamezole-treated. one-way ANOVA and a Tukey’s multiple-comparisons test for pairwise comparisons of baseline versus guanfacine-treated, baseline versus atipamezole-treated, and guanfacine- and atipamezole-treated. The significance of the presence of Ih currents in PBN-inhibited and atipamezole-treated, and guanfacine-treated versus atipamezole-treated.

Results
PBN input to the dorsal anterior BNST elicits EPSCs that drive two classes of postsynaptic responses

We used an optogenetic strategy to activate PBN or BLA inputs to the BNST through stereotoxic injection of one of these regions with AAV5-CalMKIIα-ChR2:YFP (see Figs. 1A, D2 and 4F, inset, right). After a minimum of 5 weeks, mice expressed ChR2-YFP at the injection site (Figs. 1D2 and 4F, inset, right), as well as in axons within the BNST (Figs. 1D1 and 4F, inset, left).

In addition to visual inspection of the injection site, the specificity of the ChR2-YFP viral vector injection into the PBN was confirmed by fluorescent immunohistochemical colocalization of ChR2-YFP with CGRP, a high-fidelity marker of PBN inputs within the BNST (Shimada et al., 1985, 1989; Dobolyi et al., 2005). Axosomatic synapses containing CGRP are found in PBN projections to the dorsal BNST (Shimada et al., 1989; Dobolyi et al., 2005). CGRP-expressing neurons that project from the lateral PBN to the extended amygdala have been shown to have functional importance. For example, CGRP-containing neurons that project from the outer external lateral PBN to the central nucleus have been shown to modulate feeding behavior (Carter et al., 2013). Projections from the PBN to the BNST are also thought to be the only source of CGRP within the BNST (Shimada et al., 1985). Therefore, we used CGRP as a marker of PBN terminals in the BNST, although we cannot exclude the possibility that there may be other PBN inputs to the BNST that are not CGRP-containing. Localization of ChR2-YFP and anti-CGRP fluorescent label was seen in the dorsal BNST (Fig. 1D1), indicating that the PBN viral injections resulted in expression of ChR2-YFP in PBN axons in the dBNST. We also performed image analysis with ImageJ (Fiji) software to illustrate a colocalization of ChR2-YFP and CGRP in the BNST, further affirming that some CGRP-expressing PBN terminals in the BNST also express ChR2 (Fig. 1C). Additionally, as has previously been reported, CGRP immunoreactivity was densest in the dorsal anterolateral portions of the BNST (Fig. 1F1) (Dobolyi et al., 2005; Gungor and Pare, 2014). CGRP immunoreactivity was not observed in brains taken from CGRPα knock-out mouse brains (Fig. 1F2).

The PBN has previously been demonstrated to contain vGluT2-containing projection neurons (Niu et al., 2010; Kaur et al., 2013). We confirmed that CGRP immunoreactivity colocalized with vGluT2 in mouse BNST (Fig. 1G). Further, the pattern of ChR2-YFP that we observed in the BNST was overall similar to published CGRP immunoreactivity in the BNST (Dobolyi et al., 2005; Gungor and Pare, 2014). Therefore, we focused our recordings on the anterior dorsal lateral portions of the BNST. Using whole-cell patch-clamp recordings from dorsal anterolateral BNST neurons in acutely prepared brain slices, we confirmed that optical recruitment of these fibers in the BNST using full-field blue LED illumination produced EPSCs (optical EPSCs or oEPSCs). oEPSCs in BNST recorded from PBN-injected mice were observed in the vast majority of cells tested. We could see large oEPSCs ranging up to ~1 nA produced by full-field illumination. oEPSC sizes were reduced for study through the use of neutral density filters and manipulation of the aperture to produce amplitudes for analysis between 100 and 650 pA, therefore keeping all baseline oEPSCs within a defined amplitude range.

We then stimulated the slice once every 10–20 s. One oEPSC was generated from each field stimulation. Evoked oEPSCs were of a very short, relatively invariant latency, consistent with monosynaptic excitation. Representative traces of the dual component EPSCs generated from each light stimulation. Evoked oEPSCs were of a very short, relatively invariant latency, consistent with monosynaptic excitation. Representative traces of the dual component EPSCs generated from each light stimulation.
with full-field illumination producing EPSCs from activation of the PBN projection to the BNST.

To establish the impact of the PBN input on BNST neuronal activity, we optically stimulated this input while recording in current-clamp mode. When activating PBN inputs to the BNST in ex vivo slices prepared from C57BL/6J mice that had been injected with AAV5-CaMKIIα-ChR2-YFP at least 5 weeks prior (Fig. 2A), we observed two overall types of responses to optical stimulation of the PBN input to the BNST. Some PBN afferents within the BNST end in large axosomatic terminal zones that envelop BNST neurons in a manner consistent with “detonator” type synapses, such as the climbing fiber input onto Purkinje neurons in cerebellum (Eccles et al., 1966; Shimada et al., 1989; Dobolyi et al., 2005). Thus, we predicted that we would observe PBN-induced firing in BNST neurons. In 18 of 34 neurons (52.7%) recorded from 14 mice, we indeed observed an EPSP followed by burst firing (Fig. 2B1,B3). In the remaining 16 neurons (41.7%), we observed a large IPSP in response to PBN stimulation (Fig. 2B2,B3). We hypothesized that this IPSP was generated by feedforward recruitment of GABA interneurons in the BNST, as recent evidence indicates a considerable amount of intra-BNST signaling (Poulin et al., 2009; Gungor and Pare, 2014). This was confirmed by blocking the IPSP through application of the ionotropic glutamate receptor antagonist kynurenic acid (4 mM; Fig. 2D1,D2,E) (89.9 ± 9.0%, \( t_{14} = 10.0, p < 0.01, n = 5 \)).

Because the BNST contains many distinct cell types and we observed that two types of responses were exhibited by BNST neurons in response to stimulation of the PBN input, we examined the intrinsic excitatory properties of these cells to determine whether the two types of responses were indicative of two populations of postsynaptic neurons. We injected hyperpolarizing and depolarizing current in a stepwise fashion through the patch-clamp electrode into both PBN-inhibited cells and PBN-excited cells in the BNST. We noted several features of the cells’ responses to current injection that suggested commonality within these two response groups and distinctions across the groups (Fig. 2C1–C3). First, we saw that 56% (\( n = 9 \) of 16) of PBN-excited cells exhibited \( I_h \) current activity during hyperpolarizing steps, whereas only 20% (\( n = 3 \) of 15) of PBN-inhibited cells exhibited \( I_h \) current (Fig. 2C1 compared with Fig. 2C2). \( \chi^2 \) analysis of the presence of \( I_h \) current in each of the cell types revealed a significant difference between the PBN-inhibited and PBN-excited cells (\( \chi^2 = 4.3, \text{df} = 1, p < 0.05, n = 31 \)). Second, PBN-excited cells had significant differences in their input resistances from PBN-inhibited cells, with the PBN-inhibited cells showing lower input resistances (371.0 MΩ ± 27.3 MΩ, \( t_{23} = 3.1, p < 0.05, n = 13 \)) compared with higher input resistances in PBN-excited cells (491.4 MΩ ± 29.1 MΩ, \( n = 12 \)). Third, we observed that PBN-inhibited cells had a slower rising phase on their action potentials (APs) based on visual inspection, with 15 of 16 PBN-inhibited cells showing slower rising APs, compared with PBN-excited cells where only 1 of 15 firing-type cells had slower rising APs (Fig. 2C3, slow-rise, bottom trace; compared with fast-rise, top trace). These differences between PBN-inhibited and PBN-excited cells indicate the possibility of two distinct populations of cells.

Ultrastructural analysis reveals widespread expression of \( \alpha_{2A} \)-AR within the BNST and expression in asymmetric axosomatic synapses

\( \alpha_{2A} \)-AR stimulation results in depression of excitatory drive in the BNST (Shields et al., 2009). Previous studies in the CeA suggest the possibility that \( \alpha_{2A} \)-ARs presynaptically regulate PBN input to that region (Delaney et al., 2007). To assess the localization of \( \alpha_{2A} \)-ARs in the BNST, we used a genetically modified mouse in which an HA-tag was knocked-in to the N terminus of the \( \alpha_{2A} \)-AR (Fig. 3A1). As antibodies for \( \alpha_{2A} \)-ARs have poor specificity, we used this knock-in mouse so that we were able to visualize...
We hypothesized that these $\alpha_{2A}$-AR-containing terminals making axosomatic asymmetric synapses arose from parabrachial nucleus. To further evaluate this hypothesis, we examined the axon terminals in the ventral BNST. We reasoned that, if our hypothesis was correct, the percentage of labeled axosomatic synapses in the ventral BNST should be lower than in the dorsal BNST in keeping with the lighter parabrachial innervation of this region of the BNST. We examined 195 images taken from the ventral BNST in four animals, representing 3213.4 $\mu$m$^2$. We identified a total of 119 asymmetric synapses in this sample: 80 terminated onto dendritic shafts and 39 onto dendritic spines. Of the axon terminals giving rise to asymmetric synapses onto dendritic shafts, 11.3% were labeled for $\alpha_{2A}$-AR; 7.7% of the terminals making symmetric contacts onto cell bodies, 7% contained label for $\alpha_{2A}$-AR (Fig. 3F). On the other hand, 25% of the terminals making asymmetric synaptic contacts onto cell bodies were labeled for $\alpha_{2A}$-AR (Fig. 3F).

Another possible target for $\alpha_{2A}$-AR containing terminals in the BNST are cell bodies. Accordingly, we examined 34 cell bodies in dorsal anterolateral BNST. We identified all axon terminals making synaptic contacts onto these soma in the single ultrathin section. A total of 60 synaptic contacts were identified, 32 of which were asymmetric and 28 of which were symmetric. Of the
terminals synapsing onto dendritic spines were labeled for α2A-AR. We then examined 43 cell bodies in ventral BNST. We identified all axon terminals making synaptic contacts onto these soma in the single ultrathin section. A total of 58 synaptic contacts were identified, 31 of which were asymmetric and 27 of which were symmetric. Of the terminals making symmetric contacts onto cell bodies, 7.4% contained label for α2A-AR, whereas 12.9% of the terminals making asymmetric synaptic contacts onto cell bodies were labeled for α2A-AR, approximately half the level seen for dorsal BNST (Fig. 3F).

This is consistent with our hypothesis that these α2A-AR containing axosomatic asymmetric synapses arise from the parabrachial nucleus.

We performed χ² analysis on our EM data for both the dorsal and ventral BNST to determine whether the frequency of α2A-AR-containing contacts were greater in asymmetric axosomatic contacts versus symmetric axosomatic contacts. We also performed a χ² analysis to determine whether the frequency of α2A-AR-containing contacts was greater in asymmetric axosomatic contacts versus asymmetric axodendritic contacts. In the dorsal BNST, there was no statistical difference in frequency of symmetric axosomatic α2A-AR-containing contacts versus asymmetric axosomatic α2A-AR-containing contacts (χ² = 3.4, df = 1, not significant, n = 60). There was, however, a significant difference in the frequency of asymmetric axosomatic α2A-AR-containing contacts compared with asymmetric axodendritic α2A-AR-containing α2A-ARs contacts (χ² = 11.3, df = 1, p < 0.01, n = 72). In the ventral BNST, there was no significant difference between the frequency of asymmetric axosomatic α2A-AR-containing contacts compared with either symmetric axosomatic α2A-AR-containing contacts (χ² = 0.5, df = 1, not significant, n = 58) or to axodendritic.

Figure 3. Immunolabeling for HA-tagged α2A-AR in mouse BNST. A, Illustrated mouse indicating that the experiments done in Figure 3 used HA-α2A-AR-knockin mice (A1). Immunolabeling directed against the HA tag produced some patches of intracellular labeling in neuronal elements (arrow); however, the bulk of labeling observed appeared to be extracellular (arrowhead), appearing to fill spaces between elements of the neuropil, producing the effect of “outlining” them with reaction product (A2). B–E. When animals were treated with clonidine before death, the “outlining” was less frequent and instead reaction product was observed inside neuronal elements. Dendrites (B, arrow) and preterminal axons (C, arrowheads) were commonly observed. Labeled dendritic spines (C, arrow) and axon terminals (D, E, arrows) were also seen. The labeled axon terminals sometimes made asymmetric synaptic contacts (E).

Scale bar, 500 nm. F, Bar graph showing relative abundance of α2A-ARs in each synapse type in both the dorsal and ventral BNST.
Guanfacine suppresses oEPSCs elicited by PBN fiber recruitment in BNST

Given the likelihood that \( \alpha_{2A} \)-ARs are present on PBN terminals in the BNST, we next assessed whether the \( \alpha_{2A} \)-AR agonist guanfacine differentially affected individual inputs to the BNST using optogenetic strategies outlined in Figure 1. We tested the sensitivity of two different excitatory inputs to the BNST: the BLA and the PBN. We targeted the PBN input to the BNST as described above (Figs. 1 and 4A). Voltage-clamped whole-cell recordings were made in the presence of 25 \( \mu \)M picrotoxin to further isolate oEPSCs. Application of guanfacine (1 \( \mu \)M) depressed oEPSCs produced by activation of PBN afferents within the BNST (Fig. 4B–D) (\( t_{(1,8)} = 9.4, p < 0.01, n = 7 \)). After application of the selective \( \alpha_{2A} \) antagonist atipamezole (1 \( \mu \)M), the amplitude of oEPSCs returned to a level not significantly different from baseline (Fig. 4B, C). We did observe some variability across experiments (Fig. 4C). In some instances, such as the individual experiment (Fig. 4B), there was a clear depression of excitatory transmission by guanfacine that was reversed by atipamezole. In contrast, in other experiments, weaker effects of guanfacine on excitatory transmission were observed (Fig. 4C). Additionally, we performed experiments in which we applied guanfacine (1 \( \mu \)M) to oEPSCs recorded from stimulation of the PBN afferents in the BNST and then allowed for a long washout of guanfacine without subsequent application of atipamezole to the recordings (Fig. 4D). In such experiments, after prolonged washout of guanfacine, the size of oEPSC did not return to baseline levels (Fig. 4D) as was observed in Figure 4B, C (48.3 ± 11.2\%, \( t_{(3)} = 4.3, p < 0.05, n = 3 \)). Therefore, application of the \( \alpha_{2A} \)-AR antagonist ati-
pamezole appears to be necessary for the reversal of the depression of excitatory transmission from the PBN to the BNST by guanfacine.

We also assessed the effect of guanfacine on oEPSCs produced by activation of BLA afferents within the BNST using a similar strategy involving injection of the ChR2-expressing viral vector (AAV5-CaMKIIα-ChR2:YFP) into the BLA 5–12 weeks before brain slice preparation. We observed expression of ChR2-YFP fluorescence at both the BLA injection site (Fig. 4F, inset, right) and in axons within the BNST (Fig. 4F, inset, left). Full-field illumination produced robust oEPSCs that were brought to a range of 100–650 pA through the use of neutral density filters and manipulations of the light aperture. The BLA input to the BNST, in the presence of picrotoxin, showed very little, if any, sensitivity to guanfacine (Fig. 4E, open circles) (32.4 ± 6.3%, t(4) = 5.2, p < 0.01, n = 5). We observed marked rundown in the recordings of the BLA input to the BNST, but the extent of the rundown was the same when ex vivo BNST slices were preincubated in atipamezole (1 μM) (Fig. 4E, closed circles) (42.7 ± 6.2%, t(3) = 6.8, p < 0.01, n = 4) or not (Fig. 4E, open circles). Because of the lack of difference between recordings done in normal ACSF and recordings done with atipamezole preincuba-

tion, we think that any differences in amplitude between oEPSCs recorded at baseline and oEPSCs recorded after guanfacine application were the result of rundown of recordings. In the case of these ex vivo optogenetic recordings, we refer to “rundown” as a decrease in the size of the oEPSC over the duration of the recording that appears to be independent of either a drug effect or of cell health. Interestingly, this rundown was observed in the BLA input to the BNST but not the PBN inputs. The 1 μm concentration of atipamezole used was previously sufficient to reverse the effect of α2A-AR agonist actions on electrically evoked EPSCs (Shields et al., 2009). To ensure that we would be capable of observing G_{i/o}-protein coupled receptor modulation of BLA inputs in the BNST even with the rundown of the oEPSC, we examined actions of the GABA_{B} receptor baclofen. The GABA_{B} receptor is virtually ubiquitously expressed on glutamatergic terminals in the CNS, where it depresses glutamate release. Despite the rundown observed in our BLA-to-BNST recordings, we were able to observe robust depression of oEPSCs elicited by BLA fiber activation by baclofen (10 μM) (Fig. 4F) (69.6 ± 9.7%, t(8) = 7.2, p < 0.001, n = 9). Although the level of expression of α2A-ARs on BLA terminals in the BNST is unknown, our electrophysiological data suggest that excitatory transmission from the BLA input to the BNST is insensitive to activation of α2A-ARs (Fig. 4E). These data suggest that guanfacine selectively modulates PBN inputs to the BNST.

Guanfacine depresses PBN-induced current-clamp responses on BNST neurons

As described above, we observed two types of membrane potential responses of BNST neurons to stimulation of the kPBN input: “PBN-excited” and “PBN-inhibited.” We sought to determine whether there were any differences in the sensitivity of each type of response to guanfacine. We again targeted the PBN using the optogenetic strategies described above (Figs. 1 and 5A). For PBN-excited responses, we applied 1 μM guanfacine to the optically evoked EPSCs recorded from the BNST neuron at the cell’s resting membrane potential. We observed that PBN-excited cells that we tested (Fig. 5B) (40.2 ± 6.0%, t(4) = 6.7, p < 0.01, n = 5). Further, this decrease in EPSP size with guanfacine was seen in all PBN-excited cells that we tested (Fig. 5D). In contrast, although the PBN-inhibited cells showed a trend for a decrease in size of IPSP with 1 μM guanfacine application (Fig. 5C) (15.1 ± 7.4%, t(10) = 2.0, not significant, n = 11), the decrease was not significant. Interestingly, we observed that some PBN-inhibited cells show a decrease in IPSP size with guanfacine application, whereas others did not (Fig. 5E). Therefore, the lack of significance of the

Figure 5. Guanfacine reduces EPSPs of PBN-activated cells in the BNST. A, Illustrated mouse indicating that the experiments done in Figure 5 used C57BL/6J mice that were injected with AAV5-CaMKIIα-ChR2:YFP at least 5 weeks prior. B, Guanfacine (1 μM) significantly decreases the size of EPSPs recorded from PBN-activated cells in the BNST (p < 0.05, n = 5). C, Guanfacine (1 μM) has variable effects on IPSPs recorded from the PBN-inhibited cells in the BNST (not significant, p = 11). D, Individual experiments showing the effect of guanfacine (1 μM) on EPSPs recorded from PBN-activated cells in the BNST. EPSPs are reduced by guanfacine across all experiments. E, Individual experiments showing the effect of guanfacine (1 μM) on IPSPs recorded from PBN-activated cells in the BNST. The effect of guanfacine on IPSPs is more variable with IPSPs being reduced in some experiments and unaltered in others.
decrease in the IPSP may be the result of heterogeneity in the sensitivity to guanfacine of the PBN input onto each of these PBN-inhibited cells. Further, because these IPSPs are feedforward, the variability of the IPSPs response to guanfacine likely depends on the sensitivity of both the glutamatergic input and the intervening inhibitory interneuron to \( \alpha_{2A}-\text{AR} \) activation by guanfacine.

**Differential action of guanfacine on optically evoked excitatory responses in Thy1-COP4 mouse BNST**

Analysis of extracellular field potentials can provide a more global analysis of the impact of modulatory receptors on circuit activity. In examining ChR2-YFP expression in Thy1-COP4 transgenic mice, we determined that, although ChR2-YFP was heavily expressed in regions that project to the BNST such as the BLA, cortical regions, and the hippocampus, ChR2-YFP did not colocalize with immunoreactivity from CGRP neuropeptide, a marker of a PBN input to the BNST (Fig. 6A, B1). We performed image analysis using ImageJ (Fiji) software to illustrate that the fluorescent signal from CGRP immunohistochemistry staining that surrounds dBNST cell bodies does not overlap with ChR2-YFP fluorescence in Thy1-COP4 mice. A representative image of this analysis is shown in Figure 6B2.

We prepared *ex vivo* slices from Thy1-COP4 transgenic mice and performed optical field potential recordings in the dBNST (Fig. 6A). We found that full-field optical stimulation in BNST slices from Thy1-COP4 mice produced a synchronized field potential response that was substantially reduced by AMPA receptor antagonists (76.2% from baseline, \( n = 1 \)), which is consistent with N2 responses that we have previously published (Egli et al., 2005; Grueter and Winder, 2005). This indicates only a small amount of expression of ChR2 in postsynaptic BNST neurons in

**Figure 6.** Guanfacine increases field potentials in the dBNST of Thy1-COP4 transgenic mice. **A**, Illustrated mouse indicating that the experiments done in Figure 6 used Thy1-COP4 transgenic mice. **B**, Image of dorsal BNST (63×) showing lack of colocalization of CGRP (red) with ChR2 (green) in Thy1-COP4 mice. NeuN staining is shown in blue (B1). Representative image of ImageJ (Fiji) analysis done of a BNST cell and its surrounding neuropil in a Thy1-COP4 transgenic mouse. ImageJ (Fiji) image analysis does not show colocalization of CGRP (red) with the ChR2 (green). There is also no observed colocalization of CGRP or ChR2 with NeuN (blue) (B2). **C**, Optical field potential (oN) size is increased with guanfacine (1 \( \mu M \)) application in Thy1-COP4 mice (\( p < 0.05, n = 7 \)). Representative trace of oN is shown (inset). **D**, Preincubation of *ex vivo* BNST slices with atipamezole (1 \( \mu M \)) blocks the increase in size of the optical field potentials with subsequent guanfacine (1 \( \mu M \)) application (not significant, \( n = 5 \)).
this mouse line and that the optical field potential elicited is driven by recruitment of glutamatergic afferents. We found that we were able to elicit optical field potentials (oN) that were similar in size to our previously published N2 responses (Fig. 6C, inset) (Egli et al., 2005; Grueter and Winder, 2005).

Surprisingly, when we applied guanfacine (1 μM) to the optical extracellular field potential recordings, we observed an increase in the size of the oN (Fig. 6C) (18.6 ± 6.5%, t(6) = 2.9, p < 0.05, n = 7). This increase in field potential size with guanfacine (1 μM) contrasts with our previous work done using electrical stimulation that shows a decrease in excitatory transmission with guanfacine application (Shields et al., 2009). This increase in optical field potential size with guanfacine application to ex vivo BNST slices of Thy1-COP4 mice was blocked by preincubation of slices with atipamezole (1 μM), further confirming this enhancement is mediated through activation of α2A-ARs (Fig. 6D) (0.1 ± 5.2%, t(4) = 0.02, not significant, n = 5).

Discussion

Here we have optogenetically activated PBN inputs to the BNST for electrophysiological analysis. We find that full-field illumination of ex vivo BNST slices that have been prepared from mice with AAV5-CaMKIIα-Chr2:YFP injected into the PBN at least 5 weeks prior yields a dual component oEPSC that produces cell-type-specific responses. In one cell type, stimulation promotes robust feedforward inhibition. In another, stimulation produces repetitive firing. We found that the α2A-AR agonist guanfacine filters excitatory drive into the BNST by depressing oEPSCs contributing to the depolarizing effects of the BNST input. Using immunoelectron microscopy and a novel knock-in HA-tagged α2A-AR mouse strain, we show that α2A-ARs are present on asymmetric axosomatic synapses in the dorsal BNST, which are consistent with localization to PBN inputs.

α2A-ARs specifically modulate excitatory PBN inputs to the extended amygdala

In contrast to the PBN input to the BSTN, our data suggest that the BLA input to the BNST is largely insensitive to guanfacine. These data are consistent with previous work examining the sensitivity of electrically evoked PBN and BLA inputs to the CeA (Delaney et al., 2007). Similar to our findings in the BNST, this work suggests that the PBN input to the CeA is depressed by α2A-ARs while the BLA is unaffected (Delaney et al., 2007). Although the relative expression of α2A-ARs on the BLA terminals in the BNST is unknown, our study suggests that these terminals are insensitive to α2A-AR activation.

Until the advent of optogenetic approaches, studies in which specific inputs to the BSTN are targeted using electrical stimulation were not possible because of the close proximity of afferent fibers to the BSTN. Through the use of ex vivo slice Chr2 recordings, we are now able to dissect neural circuits in ways that were previously not feasible by targeting individual inputs using optogenetic strategies outlined in this study. Here, we focus on the selective modulation of a PBN input to the dorsal BNST by α2A-ARs because past behavioral work has shown that α2A-AR agonists in the dorsal BNST block stress-induced relapse to drug seeking behavior (Wang et al., 2001). However, recent work has shown that the PBN projects to the CeA and the vBNST with differing densities (Bienkowski and Rinaman, 2013). In future work, it may be interesting to more closely study the impact of the PBN input to these regions on synaptic transmission. Here we demonstrate that optogenetic strategies can be used to explore neuro-modulation of excitatory synapses from selective inputs to the BNST ex vivo.

α2-ARs regulate axosomatic synapses in the extended amygdala and in the cerebellum

Our work in the context of other studies suggests similarity in PBN control of extended amygdala neurons and in climbing fiber control of Purkinje cells. First, the PBN input to the BNST shares anatomical features with the climbing fiber input in the cerebellum with heavy axosomatic innervation (Eccles et al., 1966; Shimada et al., 1989). Second, both generate two types of responses upon stimulation: pronounced firing or feedforward inhibition (Mathews et al., 2012). Third, both inputs are selectively regulated relative to other glutamate afferents in the respective regions by α2-ARs (Carey and Regehr, 2009).

We observed that the PBN-initiated current-clamp responses in BNST have differential sensitivity to guanfacine. Both the multiple types of postsynaptic responses in the BNST to PBN stimulation and the variable sensitivity of these responses to guanfacine highlight the complex microcircuitry of the BNST. The BNST is composed of many cell types (Hammack et al., 2007); therefore, it is not surprising that these postsynaptic responses show variable sensitivity to guanfacine, in particular the feedforward PBN-inhibited responses where sensitivity of the intermediary interneuron to guanfacine is also a factor. Our data suggest that guanfacine administration may preferentially filter inhibition of one cell type over activation of another by the PBN. In future studies, determining the nature of these neurons, in particular whether they are projection neurons or interneurons, will begin to provide a more complete picture of how this circuit is regulated. Further, we cannot rule out the possibility of inhibition by our Chr2-expressing viral vector of CGRP-negative projections from the PBN to the BNST that could influence these responses in unforeseen ways.

Evidence for a possible excitatory role of α2A-ARs in the BNST

To our surprise, we observed an increase in the size of an optically induced field potential by guanfacine in Thy1-COP4 mice. These mice have no observed colocalization of the neuropeptide CGRP, a marker of one PBN input to the BNST, with Chr2-YFP, and have very low postsynaptic expression of Chr2 in the BNST, suggesting that α2A-ARs may enhance the actions of another excitatory input to the BNST. As shown in Figure 3, we do observe expression of the α2A-ARs on other presynaptic terminal types. Another possibility is that postsynaptic α2A-ARs in the BNST may increase the excitability of BNST cells. For example, postsynaptic α2A-ARs can increase excitability by modulation of Ih, as has been shown in the prefrontal cortex (Wang et al., 2007). Furthermore, intraperitoneal injections of guanfacine increase c-fos expression in the BNST, suggesting a possible excitatory role for α2A-ARs (Savchenko and Bougher, 2011), although other mechanisms could underlie the increase in c-fos expression with intraperitoneal injection of guanfacine. Finally, it is also possible that guanfacine is decreasing GABAB signaling or increasing glutamatergic signaling from another input.

The PBN input may influence downstream BNST signaling

Because of the behavioral effects of activation of α2A-ARs in the BNST, it seems likely that the depression of the PBN input to the BNST by guanfacine that we observe here may alter downstream signaling of the BNST to brain regions involved in addiction and relapse, such as the VTA (Georges and Aston-Jones, 2001, 2002;
Dumont and Williams, 2004; Silberman et al., 2013) and the NAc (Dong et al., 2001). Alterations in the BNST’s outputs may curtail stress-induced drug cravings or stress-induced reemergence of negative symptoms of withdrawal. Indeed, it has been shown that guanfacine treatment reduces withdrawal-induced anxiety in rats treated with guanfacine (Buffalari et al., 2012). Additionally, it has been shown that injection of an α2A-AR agonist into the BNST will block withdrawal-induced place aversion (Delfs et al., 2000). Therefore, a decrease in excitatory transmission from the PBN to the BNST by α2A-ARs may decrease the aversive withdrawal-like symptoms brought on by stress and help to prevent relapse.

In addition to stress-induced relapse to drug-seeking behaviors, the modulation of BNST signaling by the PBN input may influence anxiety-like behaviors and fear conditioning (Sink et al., 2011, 2013a, b; Gungor and Pare, 2014). The PBN input to other regions of the extended amygdala has been implicated in feeding behavior (Carter et al., 2013) and pain sensitization (Han et al., 2005; Han et al., 2010). The PBN efferents have also been implicated in taste aversion (Mungarndee et al., 2006) and hypercapnic arousal (Kaur et al., 2013). Therefore, the optogenetic strategies outlined in this study for the targeting of PBN inputs to the extended amygdala and other brain regions could be beneficial in better understanding a wide range of conditions, from anxiety disorders, to disorders of energy homeostasis, to pain disorders, to sleep apnea.

In conclusion, our work demonstrates divergent actions of the PBN input on cell responses in the BNST. We demonstrate that depolarizing effects of the PBN appear to be preferentially reduced by the α2A-AR agonist guanfacine. Finally, we show that, in the relative absence of PBN signaling, guanfacine has very different actions on BNST excitability, suggesting a state-dependent aspect to the actions of guanfacine. In future work, it will be important to examine the consequences of specific regulation of the PBN in vivo by guanfacine.

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