CONTRASTING DISTRIBUTION OF PHYSIOLOGICAL CELL TYPES IN DIFFERENT
REGIONS OF THE BED NUCLEUS OF THE STRA TERMINALIS

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We characterized the electroresponsive and morphological properties of neurons in the bed nucleus of the stria terminalis (BNST). Previously, Rainnie and colleagues distinguished three cell types in the anterolateral region of BNST (BNST-AL): low-threshold bursting cells (LTB; Type-II) and regular spiking neurons that display time-dependent (RS; Type-I) or fast (fIR; Type-III) inward rectification in the hyperpolarizing direction. We report that the same neuronal types exist in the anteromedial (AM) and anteroventral (AV) regions of BNST. In addition, we observed two hitherto unreported cell types: late-firing (LF) cells, only seen in BNST-AL, that display a conspicuous delay to firing, and spontaneously active (SA) neurons, only present in BNST-AV, firing continuously at rest. However, the feature that most clearly distinguished the three BNST regions was the incidence of LTB cells (~40-70%) and the strength of their bursting behavior (both higher in BNST-AM and AV relative to AL). The incidence of RS cells was similar in the three regions (~25%), whereas that of fIR cells was higher in BNST-AL (~25%) than AV or AM (≤8%). Using biocytin, two dominant morphological cell classes were identified but they were not consistently related to particular physiological phenotypes. One neuronal class had highly branched and spiny dendrites; the second had longer but poorly branched and sparsely spiny dendrites. Both often exhibited dendritic varicosities. Since LTB cells prevail in BNST, it will be important to determine what inputs set their firing mode (tonic vs. bursting) and in what behavioral states.

Keywords: bed nucleus of the stria terminalis; anxiety, fear, intrinsic properties, morphology
Despite anatomical similarities (Alheid and Heimer 1988; deOlmos and Heimer 1999; McDonald 2003), dense interconnections (Krettek and Price 1978ab; Dong et al. 2001a), and functional kinship (Walker et al. 2003) between the amygdala and bed nucleus of the stria terminalis (BNST), there is a stark contrast between our understanding of these two structures. For instance, numerous in vitro studies have examined the physiological properties of amygdala neurons, mechanisms of synaptic transmission, neuromodulation, and activity-dependent plasticity (reviewed in Sah et al. 2003; Pape and Pare 2010). In contrast, relatively few reports on these themes are available for the BNST (reviewed in McElligott and Winder 2009; Hammack et al. 2009). As a result, the operations carried out by the BNST remain poorly understood.

The paucity of data on the physiological properties of BNST neurons is particularly problematic. Elsewhere, it was shown that the electroresponsive properties of neurons shape their spontaneous activity and synaptic responses (Llinas 1988). Also, the particular physiological properties of different types of neurons often give rise to distinctive discharge patterns. Consequently, knowing the electroresponsive properties of different neuronal groups can become a tool to identify neurons recorded extracellularly in behaving animals. Therefore, a fundamental step toward understanding BNST is to study the physiological properties of BNST neurons.

So far, the best-characterized BNST neurons are those located in its anterolateral portion (BNST-AL), a region implicated in fear and anxiety (Walker et al. 2003). Rainnie and colleagues reported that there are three main cells types in the dorsal part of BNST-AL (Hammack et al. 2007). When depolarized, the two main types displayed a regular spiking (Type-I) or low-threshold bursting (Type-II) phenotype and both exhibited time-dependent inward rectification in the hyperpolarizing direction. A less common cell type (Type-III) lacked the latter property, instead displaying fast inward rectification in response to hyperpolarization and a regular firing pattern when depolarized. Importantly, this tripartite classification was validated using single-cell reverse
transcriptase polymerase chain reaction, revealing that the three cell types express mRNA transcripts for distinct complements of voltage-gated channels (Hazra et al. 2011) and serotonergic receptors (Hazra et al. 2012).

At present, it is unclear whether similar types of neurons are present in other sectors of BNST. Although there is much disagreement regarding the number and boundaries of BNST nuclei (De Olmos et al. 1985; Ju and Swanson 1989; Moga et al. 1989), it is clear that different BNST regions form contrasting connections. In the anterior portion of BNST (BNST-A) for instance, BNST-AL neurons contribute most BNST outputs to brainstem structures regulating fear expression (Sofroniew et al. 1983; Holstege et al. 1985; Sun and Cassell 1993). In contrast, neurons projecting to the paraventricular hypothalamic nucleus are concentrated in its ventral and medial portions (Prewitt and Herman 1998; Dong et al. 2001b; Dong and Swanson 2006). Therefore, the present study was undertaken to characterize the electroresponsive properties of neurons in different BNST-A regions and to determine whether distinct physiological cell types exhibit contrasting morphological properties.
MATERIALS AND METHODS

Experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with the approval of the Institutional Animal Care and Use Committee of Rutgers University (Newark, NJ). We used adult (60-90 days) male Lewis rats (Charles River Laboratories, New Field, NJ) maintained on a 12 h light/dark cycle. The animals were housed three per cage with ad libitum access to food and water. Prior to the experiments, they were habituated to the animal facility and handling for one week.

Whole-cell patch recording of BNST cells in vitro

Slice preparation

The rats were anesthetized with avertin (300 mg/kg, i.p.), followed by isoflurane. After abolition of all reflexes, they were perfused through the heart with a cold (°C) modified artificial cerebrospinal fluid (aCSF) that contained (in mM): 248 sucrose, 2.5 KCl, 7 MgCl₂, 23 NaHCO₃, 1.2 NaH₂PO₄, 7 glucose. Their brains were then extracted and cut in 300 µm-thick coronal slices with a vibrating microtome while submerged in the same solution as above. After cutting, slices were transferred to an incubating chamber where they were allowed to recover for at least one hour at room temperature in a control aCSF with the following composition (in mM) 124 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 1 MgCl₂, 2 CaCl₂, 10 glucose. (pH 7.2, 300 mOsm). The temperature of the chamber was kept at 34°C for at least 20 min and then returned to room temperature. Slices were then transferred to a recording chamber perfused with oxygenated aCSF at 32°C (7 ml/min).

Electrophysiology

Under visual guidance with differential interference contrast and infrared video-microscopy, we obtained whole-cell patch recordings of BNST neurons using pipettes (7-10 MΩ) pulled from
borosilicate glass capillaries and filled with a solution containing (in mM): 130 K-gluconate, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 10 KCl, 2 MgCl2, 2 ATP-Mg, and 0.2 GTP-tris(hydroxy-methyl)aminomethane (pH 7.2, 280 mOsm). The liquid junction potential was 10 mV with this solution and the membrane potential was corrected accordingly. Current-clamp recordings were obtained with an Axoclamp 2B amplifier and digitized at 10 kHz with a Digidata 1200 interface (Axon Instruments, Foster City, CA).

To characterize the electroresponsive properties of recorded cells, graded series of depolarizing and hyperpolarizing current pulses (0.01 nA, 500 ms in duration) were applied from rest and other pre-pulse potentials, as pre-hyperpolarization of different magnitudes can greatly affect spike latency due to the interaction between A-Type and T-Type currents (Molineux et al., 2005). The input resistance ($R_{in}$) of the cells was estimated in the linear portion of current-voltage plots.

To study the morphological properties of recorded neurons, 0.5% biocytin was added to the pipette solution in a subset of experiments. No special current injection protocol had to be used to label BNST cells with biocytin. It diffused into the cells as we studied their electroresponsive properties. At the conclusion of the recordings, the slices were removed from the chamber and fixed for 1 to 3 days in 0.1 M phosphate buffer saline (pH 7.4) containing 4% paraformaldehyde.

**Biocytin visualization**

Slices were embedded in agar (3%) and re-sectioned with a vibrating microtome at 100 µm. Sections were washed several times in phosphate buffer (PB, 0.1 M, pH 7.4) and then transferred to a H$_2$O$_2$ solution (0.5%) in PB for 15 min. After numerous washes in PB, sections were incubated for 12 h at 20°C in a solution containing 0.5% triton, 1% solutions A and B of an ABC kit (Vector, Burlingame, CA) in PB. The next day, they were washed in PB (5 x 10 min). Biocytin was
visualized by incubating the sections in a 0.1 M PB solution that contained diaminobenzidine
tetrahydrochloride (0.05%, Sigma), 2.5 mM nickel ammonium sulfate (Fisher) and H$_2$O$_2$ (0.003%)
for 5-10 min. Then, the sections were washed in PB (5 X 10 min), mounted on gelatin-coated slides,
and air-dried. The sections were then counterstained with cresyl violet and coverslipped with
permount for later reconstruction.

All visible processes of the labeled neurons were observed in a microscope using a 40X
objective and photographed. Typically, their processes extended over several sections. To align the
sections, we layered the photographs in Photoshop (Adobe Systems Incorporated, CA) and used
blood vessels or other obvious landmarks present in the various sections to align them. The layers
were then collapsed and the entire neuron drawn.

Nomenclature used to designate different BNST subregions

Individual BNST subnuclei cannot be identified with precision in unstained, living slices.
Therefore, we subdivided BNST-A in three regions, based on the position of major fiber bundles
that can be easily identified in trans-illuminated slices: the anterior commissure, dividing the
BNST-A in dorsal and ventral (BNST-AV) sectors, and the intra-BNST component of the stria
terminalis, subdividing the dorsal portion in medial (BNST-AM) and lateral (BNST-AL) regions.
The correspondence between our subdivisions of the BNST-A and the subnuclei identified by
Swanson and colleagues (Ju and Swanson 1989; Ju et al. 1989) is as follows. BNST-AV
corresponds to Swanson’s anteroventral, fusiform, parastrial, and dorsomedial subnuclei plus his
subcommissural zone. BNST-AL corresponds to Swanson’s oval, juxtacapsular, and anterolateral
subnuclei. BNST-AM corresponds to Swanson’s anterodorsal subnucleus. However, note that in
more recent publications (Dong and Sawnson 2006), Swanson also termed the latter region BNST-
AM.
RESULTS

We studied the electroresponsive properties of neurons in different parts of the BNST-A using visually guided patch recordings in coronal slices kept in vitro (Fig. 1A). We obtained stable recordings from 127 BNST-AL, 87 BNST-AM, and 83 BNST-AV neurons, some of which were morphologically identified with biocytin (n=60). The three cell types observed in BNST-AL by Rainnie and colleagues were also present in BNST-AM and AV, together accounting for >90% of neurons in these regions. However, there were significant regional variations in their electroresponsive properties. In addition, we encountered two previously unknown cell types. Below, we first describe the electroresponsive properties of these various types of neurons (Tables 1-4) and then consider their morphology (Table 5).

Regular spiking (RS) cells (Type-I)

A major cell type observed in all three BNST-A regions displayed a regular spiking (RS) phenotype (Fig. 1B-C; see Table 1 for passive properties and spike characteristics). They correspond to the Type-I cells of Rainnie and colleagues (Hammack et al. 2007). The incidence of RS cells did not vary significantly depending on the BNST-A region ($\chi^2 = 0.44, p = 0.8$; range 23-27% of the cells). In response to depolarizing current pulses, these neurons generated spike trains that displayed frequency adaptation (Fig. 1B-C, top). In response to hyperpolarization, most displayed time- and voltage-dependent inward rectification (Fig. 1B, bottom; Fig. 1D2,3). This phenomenon manifested itself by a depolarizing sag in the voltage response to negative current pulses (Fig. 1B, dashed line in lowest trace). In light of prior observations in BNST-AL (Hammack et al., 2007; Hazra et al., 2011) and other brain regions (Pape 1996), this sag is likely due to the expression of the hyperpolarization-activated mixed cationic current $I_H$. Sag amplitude was generally higher in RS neurons of BNST-AL than AM or AV. Indeed, the proportion of RS cells
with sag amplitudes > 2 mV in the same testing conditions (-0.06 nA current from -65 mV) showed significant regional variations (Fig. 1D; $\chi^2 = 13.1$, $p < 0.0014$). As depicted in the examples of figure 1 and discussed further below, the RS phenotype coincided with marked variations in other properties such as $R_{in}$, amplitude/shape of spike afterhyperpolarizations, and sag amplitude, with no preferential associations between them.

Low-threshold bursting (LTB) cells (Type-II)

A second major cell type observed in the three BNST-A regions were neurons generating spike doublets or bursts at the onset of depolarizing pulses applied from membrane potentials negative to -70 mV (Fig. 2A2, B2), but single spikes from more depolarized levels (Fig. 2A1, B1). At the end of hyperpolarizing current pulses applied from membrane potentials positive to -70 mV, these low-threshold bursting (LTB) neurons generated spike bursts (Fig. 2A1) or doublets (Fig. 2B1) similar to those seen at the onset of depolarizing current pulses applied from membrane potentials negative to -70 mV. These cells correspond to the Type-II neurons of Rainnie and colleagues (Hammack et al. 2007). The spikes bursts or doublets generated by LTB cells rode on a slower depolarizing potential (arrows in Fig. 2A-B), hereafter termed low-threshold spike, which outlasted the rebound firing. On average, from a membrane potential of –80 mV and with a current injection of 0.04 nA, these low-threshold spikes were $12.4 \pm 0.5$ mV in amplitude and $156.2 \pm 8.0$ ms in duration. Given prior findings in BNST-AL (Hammack et al., 2007; Hazra et al., 2011) and other brain regions (Huguenard 1996), these properties likely reflect the expression of a low-threshold Ca$^{2+}$ conductance ($I_T$).

Not only did the incidence of LTB cells vary significantly depending on the BNST-A region (Fig. 2C1; $\chi^2 = 48.1$, $p < 0.0001$), so did the number of spikes per rebound burst (Fig. 2C2, ANOVA, $F = 4.84$, $p = 0.009$) and the peak instantaneous firing rate reached during these bursts
(Fig. 2C3, ANOVA, F = 4.42, p = 0.01). Bonferroni corrected post-hoc t-tests revealed that the latter two variables were significantly lower amongst BNST-AL than BNST-AV neurons (p’s ≤ 0.012). A similar trend was seen in AL to AM comparisons (p’s ≤ 0.08) and no differences were found between AM and AV neurons. As was seen in RS neurons, sag amplitude was generally higher in LTB neurons of BNST-AL than AM or AV. Figure 2C4 depicts the proportion of LTB cells with sag amplitudes > 2 mV (-0.06 nA current from -65 mV) in the three regions.

In the three BNST-A regions, we also observed neurons identical in most respects to LTB cells with the exception that they did not generate spike bursts, yet did exhibit rebound firing (Fig. 3). As in LTB cells, rebound action potentials rode on a slower depolarizing potential (Fig. 3A, arrow), similar to the low-threshold spikes discussed above. Moreover, in response to juxta-threshold depolarizing current pulses applied from negative to -70 mV, the same cells generated single action potentials that also rode on a conspicuous slow depolarization (Fig. 3B, arrow; 0.04 nA from a membrane potential of -80 mV, 10.8 ± 0.7 mV and 164.0 ± 10.4 ms). Consequently, for the remainder of this study, cells exhibiting these properties are pooled with LTB neurons.

Rare cell types

Together, LTB and RS cells accounted for nearly 80% of BNST-A neurons. However, three other cell types were also encountered, albeit less frequently. We describe them in turn below. The first one corresponds to the Type-III cells of Rainnie and colleagues (Fig. 4A; Hammack et al. 2007). Like RS cells, these neurons exhibited spike frequency adaptation during prolonged depolarizations (Fig 4A, top). However, unlike RS cells, they displayed fast inward rectification in response to hyperpolarizing current pulses (Fig. 4A, inset), hence the acronym fIR. The incidence of fIR neurons varied significantly depending on the BNST-A region (AL, 29%; AM, 8%; AV, 6%; \( \chi^2 = 17.1, p < 0.0002 \)).
Another, even less frequent type of cells were late-firing neurons (LF; Fig. 4B), observed only in BNST-AL (4% of cells). In response to supra-threshold depolarizations, LF neurons displayed a conspicuous delay to firing that was especially pronounced when the current injection was performed from negative to -75 mV (compare Fig. 4B1 and B2 from -65 and -80 mV, respectively). Also characteristic of LF cells was a marked change in the rising phase of voltage responses to depolarizing current pulses as the stimulus intensity was increased (Fig. 4B2, inset). In light of prior findings, this behavior likely reflects the expression of a slowly inactivating potassium current (I_D; Storm 1988). LF cells had the most negative resting potential and lowest R_in of all the cell types identified in this study (see Table 1).

Finally, a small subset of cells stood out because of its highly regular spontaneous activity at rest (hence the designation SA neurons; Fig. 5A). Spontaneous firing rates averaged 4.2 ± 1.1 Hz in whole cell mode at rest (n = 7). It is unlikely that these spontaneous discharges were due to injury because they could be seen in cell-attached mode, albeit at a higher frequency (7.3 ± 1.5 Hz; n = 4). Also inconsistent with the injury hypothesis, SA cells were only encountered in BNST-AV, where they accounted for 8% of the cells. Moreover, action potential duration was markedly lower in SA neurons (0.78 ± 0.05 ms) than in all other BNST-A cell types (Table 1). When depolarized, SA neurons displayed no evidence of spike frequency adaptation (as seen in RS, LTB, and fIR neurons) or acceleration (as seen in LF cells). Instead, they maintained a stable firing rate that increased with the amount of depolarization until challenged by strong currents, in which case spiking started to fail (Fig. 5B-C, top traces and arrows). SA cells did not display evidence of I_H or I_T (Fig. 5B-C).

To summarize the above (Fig. 6A), RS and LTB neurons were predominant in the three BNST-A regions, together accounting for 66.9-91.9% of the cells. In addition, 29% of BNST-AL cells were fIR neurons compared to ≤8% of the cells in BNST-AV and AM. Finally, the last two cell types (LF and SA neurons) were rare and only encountered in one of the three regions: SA
neurons in BNST-AV and LF cells in BNST-AL. The distribution of these various cell types in the three regions is illustrated in Figure 6B. RS and LTB cells were homogenously intermingled in BNST-AM and AL, including its oval nucleus. In contrast, in BNST-AV, RS cells were more concentrated ventrally. Unexpectedly, fIR neurons were concentrated along the internal capsule, in a region corresponding to the juxtacapsular subregion of BNST-AL, although it cannot be ruled out that this concentration overlapped with the oval nucleus. In BNST-AL, outside the juxtacapsular/oval subregions, only one fIR cell was encountered. This is in contrast with BNST-AV and AM where a uniform, albeit low concentration of fIR cells was encountered.

Passive properties and spike characteristics in different BNST-A regions

Although the above indicates that, for the most part, the three BNST-A regions are characterized by a similar complement of cell types, it remains possible that they differ in other ways such as passive properties or spike characteristics. Unfortunately, most of these variables did not meet the requirements of parametric ANOVAs (variance homogeneity and normality of distributions). Thus, to address this question, we computed Kruskal-Wallis one-way ANOVAs and corrected the significance level for the number of comparisons (Bonferroni). To minimize the number of comparisons and avoid absurdly stringent significance levels, when two variables were correlated (e.g. $R_\text{in}$ and time constant or spike duration and amplitude), we considered only one of them.

We first compared resting potential ($V_r$), $R_\text{in}$, and spike duration (at half amplitude) of neurons in the three regions (all classes combined; Table 2). A significant region effect was observed for $R_\text{in}$ ($H=48.8, p<0.001$) and spike duration ($H=37.2, p<0.001$). Post-hoc Mann-Whitney tests corrected for multiple comparisons revealed that $R_\text{in}$ was significantly lower in BNST-AL than AV and AM neurons ($p$’s $<0.001$) with no difference between the latter two regions.
Also, spike durations were significantly lower in BNST-AV compared to the other two regions (p<0.001) with no difference between AM and AL.

Since these results could be due to the differing incidence of the various cell types in the three regions, we then repeated these analyses, but separately for the two most frequent cell types (RS, Table 3; LTB, Table 4). Again, we obtained a significant effect of region on $R_{in}$ and spike duration for RS ($R_{in}$, $H=15.6$, p=0.0007; spike duration, $H=9.8$, p=0.007) and LTB ($R_{in}$, $H=21.4$, p=0.00002; spike duration, $H=15.3$ p=0.0005) cells. Post-hoc Mann-Whitney tests yielded nearly identical results in the two cell types. As we observed when comparing regions irrespective of cell types, $R_{in}$ was significantly lower in BNST-AL’s RS and LTB neurons than the corresponding cell classes in BNST-AV and AM (p’s <0.001) with no difference between the latter two regions. Also, spike durations were significantly lower in BNST-AV’s RS and LTB neurons compared to the corresponding cell classes in BNST-AL and AM (p’s<0.001) with no difference between the latter two regions.

Morphological correlates of electroresponsive properties

A total of 60 biocytin-filled neurons were recovered (13 RS, 39 LTB, 6 fIR, and 2 SA) from BNST-AL (n=24), BNST-AM (n=22), and BNST-AV (n=14). Examples of these morphologically identified cells are provided in figures 7-8. In both figures, panel A shows the position and general morphology of the cells depicted in subsequent panels (red, presumed axons; black, soma and dendrites). We identified two main morphological cell types whose prevalence varied depending on the BNST-A region.

The first type of cells had long dendrites that ramified sparingly (Fig. 7A-E, Fig. 8F). They usually exhibited a low density of dendritic spines, typically of the stubby type. These spines were most common proximally (Fig. 7C2, E3, arrowheads); more distal dendrites typically lacked spines.
but usually displayed varicosities (Fig. 7B2, C3). These cells prevailed in BNST-AV (12 of 14), AM (17 of 22), and the ventral part of AL (9 of 11).

The second type of neurons had smaller, but highly branched dendritic trees (Fig. 8B, C). Whereas their proximal dendrites lacked spines, more distal dendritic segments typically displayed a moderate to high density of thin dendritic spines (Fig. 8B2, C2). Most of these cells were recovered from the dorsal part of AL (4 of 6) and along the internal capsule (4 of 7), in regions that correspond to the oval and juxtacapsular regions, respectively.

A previously unreported characteristic, exhibited by both cell types, was the variable emergence site of presumed axons. Axon-like processes could emerge from somata (Fig. 7A, cells c and d; Fig. 8A, cells b, *1, and *2), proximal dendrites (Fig. 7A, cell B; Fig. 8A, cells E and *1) or distal dendrites (Fig. 7A, cells c and d), as far as 200 µm from the soma. In 10% of cells (6 of 60), two or more distinct axon-like branches were seen to emerge from a combination of these sites. Note that in order for a cell to be considered as having more than one putative axon, each had to meet the following criteria. First, its emergence site had to be visible in the microscope by repeatedly changing the focal plane back and forth. Second, these axon-like processes had to emerge from two clearly different parts of the cells. Ambiguous cases were ignored.

Figure 7D illustrates a cell with multiple axon-like processes. This BNST-AM neuron had a putative axon emerging from its soma (Fig. 7D1), two more emerging from dorsally directed dendrites (one of which is visible in Fig. 7D3), and others from ventrally directed dendrites (Fig. 7D4). One of the latter appeared to merge into the anterior commissure. Overall, 28% of BNST-A cells had dendritically-emerging axon-like processes with no significant difference between regions (AL, 29%; AM, 27%; AV, 39%). On average, these axon-like processes emerged 66.5 ± 11.2 µm from the soma (AL, 49.8 ± 10.1 µm; AM, 89.6 ± 28.1 µm; AV, 78.8 ± 26.9 µm).

Importantly, it should be noted that it is unclear whether these axon-like processes truly are
axons or extremely thin dendrites that bear varicosities. Unambiguous determination of their identity will require triple immunofluorescence for biocytin as well as dendritic and axonal markers such as MAP2 and synaptophysin.

The most frequently encountered property were dendritic varicosities, a feature exhibited by 75, 81, and 93% of BNST-AL, AV, and AM neurons, respectively. This property showed no consistent association with the presence or absence of dendritic spines or overall dendritic morphology. For instance, some cells had aspiny distal dendrites with varicosities (Fig. 8B3) and more proximal dendritic segments densely covered with spines, but lacking varicosities (Fig. 8B2). Other cells with dendritic varicosities had uniformly aspiny or sparsely spiny dendrites (Fig. 7B2, C3, E2; Fig. 8D1).

Surprisingly, we found no systematic relationship between the morphological and electroresponsive properties of RS and LTB cells. That is, there was as much morphological variability between RS and LTB of neurons as among both of these cell types considered independently. For instance, cells B-E in figure 7 had similar morphological properties including long, poorly ramified dendrites with a low density of dendritic spines, yet two of them were RS cells (Fig. 7B,D) and two were LTB neurons (Fig. 7C,E). Moreover, the RS and LTB phenotypes were also encountered among densely spiny neurons (Fig. 8B, LTB). In fact, the proportion of spiny cells corresponding to RS (30%) and LTB neurons (70%) was nearly identical to that seen among aspiny neurons (RS, 24%; LTB, 76%). Differences in other properties (Table 5) such as soma size, number of primary dendrites, distance to first dendritic branching point, incidence of dendritic varicosities also failed to reach significance between these two prevalent cell types.

Due to their low incidence, our samples of fIR (n = 6) and SA (n =2) neurons are small. With one exception (Fig. 8F), all recovered fIR had densely spiny dendrites and moderately branching dendrites (Fig. 8C). In contrast, both SA cells had aspiny dendrites (Fig. 8, cell labeled
Other approaches to classification

The apparent lack of correlation between the physiological cell types described above and their morphological properties led us to consider other approaches to classification. In particular, for a large array of variables ($V_r$, $R_{in}$, time constant, spike duration, amplitude and threshold, sag amplitude, amplitude and duration of spike after hyperpolarization), we computed frequency distributions and scrutinized them for evidence of discrete clusters (multimodality) we might have missed by adopting the classification of Rainnie and colleagues. We also plotted different combinations of physiological and morphological variables against each other, two or three at a time and in principal component analysis space. However, these various approaches failed to reveal physiological variables that could support a different classification scheme consistent with morphology. Although multidimensional cluster analyses might have identified clusters, these approaches are counter-indicated in the absence of positive evidence that such groupings exist.

DISCUSSION

In many brain regions, a systematic relationship was found between the physiological properties, firing pattern, morphology, connections, and transmitter content of different neuronal types (e.g. thalamus, Steriade and Llinas 1998; striatum, Tepper and Bolam 2004). This knowledge has proven invaluable in interpreting extracellularly recorded unit activity. In contrast, our understanding of BNST is far less advanced. Thus, the present study was undertaken to characterize the electroresponsive and morphological properties of BNST-A neurons. Our experiments revealed that two cell types (RS, LTB) account for the majority of neurons in different
BNST-A regions. Three additional physiological cell types were also identified but their incidence was lower and varied markedly depending on the BNST-A region. Surprisingly, the physiological properties of BNST-A cells showed little correlation with their morphology.

Prior studies on the cellular physiology of BNST-A neurons.

The electroresponsive properties of BNST neurons have received little attention so far. Indeed, most electrophysiological studies have focused on other aspects of BNST physiology such as the influence of various peptides/transmitters (Grueter et al. 2005; Krawczyk et al. 2011a; Li et al. 2012; Lungwitz et al. 2012; McElligott and Winder 2008; Nobis et al. 2011; Puente et al. 2010; Shields et al. 2009), particularly corticotropin releasing factor (Gafford et al. 2012; Ide et al. 2013; Kash and Winder 2006; Oberlander and Henderson 2012; Silberman et al. 2013), mechanisms of addiction and relapse to drug seeking (Conrad et al. 2012; Davis et al. 2008; Dumont and Williams 2004; Dumont et al. 2005, 2008; Grueter et al. 2008; Kash et al. 2008a, 2008b, 2009; Krawczyk et al. 2011b), synaptic plasticity (Weitlauf et al. 2005), and the impact of stress (Conrad et al. 2011).

Although a few studies compared the passive properties of neurons in different BNST-A sectors (e.g. Egli and Winder 2003), most did not examine the temporal dynamics of current-evoked spiking. To our knowledge, a systematic physiological characterization of BNST-A neurons has only been performed in the AL region in general (Hammack et al. 2007; Guo et al. 2009, 2012; Hazra et al. 2011, 2012; Rainnie 1999) and its juxtacapsular sector in particular (Francesconi et al. 2009; Szucs et al. 2010). Three BNST-AL cell types were distinguished, with marked differences in their incidence: LTB cells (Type-II, 55%) and regular spiking neurons that display time-dependent (Type-I, 29%) or near instantaneous (fIR, 16%) inward rectification in the hyperpolarizing direction (Hammack et al. 2007).

This classification of BNST-AL neurons found support in a single-cell RT-PCR study where
the alpha sub-unit expression profile of key ionic channels correlated with the electrophysiological classification (Hazra et al. 2011). Moreover, another study revealed that serotonergic receptor subtypes were differentially expressed in the three cell types. For instance, 5HT-2C receptors were almost exclusively expressed by Type-III neurons whereas 5HT-7 receptors were commonly expressed by Type-I and II neurons but not Type-III cells (Guo et al. 2009; Hazra et al. 2012).

Similarities and differences in the physiological properties of neurons in different parts of BNST-A

The present study corroborates the findings of Rainnie and colleagues regarding the dominant cell types found in BNST-AL and extends them by showing that the same classes of neurons prevail in other BNST-A regions. Within BNST-AL, our results closely match what Rainnie and colleagues reported except for resting potential and $R_{in}$. However, differences in methodology (correction or not for junction potential; how the slices were prepared) or age/strain of the rats are probably responsible. Within BNST-AM and AV, more than 80% of neurons were RS or LTB cells. However, there were significant inter-regional variations in some of their properties. For LTB cells, the number of spikes per rebound burst and the peak instantaneous firing rate reached during these bursts were higher in BNST-AV and AM cells than in BNST-AL neurons. Moreover, LTB cells accounted for a higher proportion of neurons in BNST-AV and AM than in BNST-AL. Also, for RS and LTB cells, the magnitude of the depolarizing sag, presumably due to $I_{H}$, was on average lower in BNST-AV and AM than in BNST-AL. Also noteworthy, the $R_{in}$ of RS and LTB cells was significantly lower in BNST-AL than in the other two regions. Finally, the incidence of fIR neurons was much lower in BNST-AV and AM than in BNST-AL. In the latter region, fIR neurons were concentrated along the internal capsule, in a region that appears to overlap with the juxtacapsular nucleus. However, outside this region, the incidence of fIR neurons was homogeneously low in all three BNST-A sectors, again consistent with the findings of Rainnie and
In addition to the cell classes identified previously, we encountered two hitherto unreported types of neurons, both of which showed little or no evidence of $I_H$ or $I_T$: LF neurons found only in BNST-AL (4% of the cells) and SA neurons only seen in BNST-AM (8% of the cells). In response to supra-threshold depolarizations, LF neurons displayed a delay to firing that was especially pronounced when the current injection was performed from negative to -75 mV. During this delay, the membrane potential depolarized gradually, a behavior that likely reflects the time-dependent inactivation of a slow, A-like potassium current ($I_D$; Storm 1988). Finally, SA neurons spontaneously generated thin (<0.8 ms) spikes at a rate of around 4 Hz from rest. When depolarized, SA neurons displayed no evidence of spike frequency accommodation (as seen in RS, LTB, and fIR neurons) or acceleration (as seen in LF cells). Instead, they maintained a stable firing rate that augmented with depolarization.

**Morphological correlates of electroresponsive properties**

Three prior Golgi studies described the morphological properties of BNST-A neurons (McDonald 1983; Larriva-Sahd 2004, 2006). For the juxtacapsular nucleus, there is consensus that the majority of neurons are small cells with spiny and often bipolar dendritic trees (McDonald 1983; Larriva-Sahd 2004), consistent with our results. For BNST-AL, there is also agreement that the dominant cell type cell is characterized by an ovoid soma from which emerge 4-5 dendrites that branch several times, are aspiny proximally, moderately to densely spiny more distally, and often exhibit dendritic varicosities (McDonald 1983; Larriva-Sahd 2006). McDonald (1983) likened these cells to the medium spiny neurons found in the central lateral amygdala. However, Larriva-Sahd (2006), focusing on the oval sub-region of BNST-AL, identified 10 additional types of neurons. Although the text of his paper does not comment on more ventrally located-BNST-AL
neurons, his figures 3-5 indicate that the proportion of densely spiny neurons is substantially lower in this sub-region. Instead, similar to what McDonald (1983) reported for BNST-AM neurons, a majority of these cells contribute few dendritic branches that ramify sparingly and exhibit a low spine density.

Overall, these results closely match the properties and distribution of the two classes of biocytin-filled neurons we recovered. However, one feature, not reported previously, was that a proportion of cells appeared to contribute multiple axon-like processes that could emerge from multiple sites. These axon-like processes could emerge from somata or dendrites, in one case 200 µm from the soma. Moreover, several cells contributed two or more axon-like processes emerging from different cellular compartments (soma and dendrite or different dendritic branches). Although novel for BNST, there are many precedents in the literature for dendritically-emerging axons. This was observed in many types of cerebellar, cortical, and hippocampal GABAergic neurons (Palay and Chan-Palay 1974;Amaral 1978;Feldman and Peters 1978;Gulyas et al. 1992) and in dopaminergic cells of the substantia nigra (Juraska et al. 1977;Preston et al. 1981;Tepper et al. 1987). In the latter cell type, action potentials are initiated in dendritically-emerging axons (Hausser et al. 1995), suggesting that in such cells, the main site of synaptic integration is not the soma but the dendritic segment near the point of axonal emergence. However, it is unclear whether the axon-like processes we saw in a proportion of BNST neurons truly are axons or extremely thin dendrites that bear varicosities. Unambiguous determination of their identity will require triple immunofluorescence for biocytin as well as dendritic and axonal markers such as MAP2 and synaptophysin.

Unfortunately, we found little correlation between the morphological and electroresponsive properties of BNST-A neurons. In Type I (RS) and II (LTB) neurons, the only morpho-physiological correlations we found were trivial ones such as an inverse correlation between $R_{in}$ and
soma size. Both physiological cell types could display the morphology of the medium spiny class or the ones with long poorly branched aspiny dendrites.

While these negative results are disappointing, they are consistent with the marked phenotypic heterogeneity among BNST-A neurons. Indeed, BNST-A contains a small group of glutamatergic cells interspersed among a dominant population of GABAergic neurons (Day et al. 1999; Esclapez et al. 1993; Hur and Zaborszky 2005; Kudo et al. 2012; Poulin et al. 2009; Sun and Cassell 1993). Moreover, BNST-A neurons express numerous peptides than can coexist in various combinations (Woodhams et al. 1983). In light of these variations, and given that only two main morphological cell types prevail in BNST-A, one would expect that each morphological cell class includes multiple subsets of neurons with marked phenotypic variations between them.

Thus it appears that in contrast to the thalamus or striatum where physiological properties, firing patterns, and cellular identity are closely related, it will be more challenging to understand BNST-A. A promising approach would be to correlate projection site(s) with physiological and neurochemical properties. Also, given the prevalence of LTB cells in the three regions, it will be important to determine in what behavioral states these neurons fire tonically vs. in bursts. Given that BNST-AL sends strong GABAergic projections to BNST-AM and AV (Turesson et al., 2013), it is likely that these inhibitory inputs play a critical role in setting the firing mode of LTB cells in the other two regions.

Prior studies have revealed that Lewis rats exhibit marked individual variations in the generalization of conditioned fear, response to species-specific stressors, and anxiety levels as assessed with standardized behavioral assays such as the elevated plus maze (EPM; Cohen et al., 2006; Duvarci et al., 2009; Goswami et al., 2010, 2012). Moreover, BNST lesions in Lewis rats were reported to reduce fear generalization, contextual fear, and anxiety in the EPM (Duvarci et al., 2009). A major challenge for future studies will be to determine whether the inter-individual
variations in anxiety seen in this rat strain correlate with differences in the properties or incidence of the various cell types identified in the present study.
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The authors declare that they have no conflict of interest, financial or otherwise.

AUTHOR CONTRIBUTIONS:
Olga E Rodríguez-Sierra carried out most of the electrophysiological experiments and analyzed the data. She also studied the morphological properties of BNST neurons.

Hjalmar Turesson carried out a portion of the electrophysiological experiments and analyzed the data. He also studied the morphological properties of BNST neurons.

Denis Pare designed the experiments, analyzed some of the data, and wrote the manuscript.

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**FIGURE LEGENDS**

**Figure 1.** Regular spiking (RS; Type-I) BNST-A neurons. (A) Trans-illuminated slices as it appeared during our experiments. (B-C) Voltage responses of two different RS neurons (recorded in BNST-AL and AV, respectively) to gradually increasing pulses of positive or negative current applied from -65 mV. Note that the top traces in panels B and C were offset graphically for clarity; the pre-pulse potential was -65 mV, as for the traces just below. (D1-2) Amplitude of voltage responses (y-axis) to negative current pulses (x-axis) for cells shown in C and B, respectively. Voltage responses were measured at the beginning and end of the current pulses (symbols in insets). (D3) Percent RS cells (y-axis) with depolarizing sag > 2 mV (-0.06 nA current from -65 mV) in the three BNST-A regions (x-axis). Abbreviations: AC, anterior commissure; AL, anterolateral; AM, anteromedial; AV, anteroventral; Str, striatum.

**Figure 2.** Low-threshold bursting (LTB; Type-II) BNST-A neurons. (A-B) Voltage responses of two different LTB neurons (recorded in BNST-AM and AL, respectively) to gradually increasing pulses of positive or negative current applied from -65 mV (A1, B1) or -80 mV (A2, B2). Arrows in A and B point to low-threshold spikes. (C) Note that the top traces in panels A1 and B1 were offset graphically for clarity; the pre-pulse potential was -65 mV, as for the traces just below. Graphs plotting the incidence of LTB cells (1), the number of spikes per burst (2), the peak instantaneous intra-burst spike frequency (3), and the proportion of LTB cells with depolarizing sag > 2 mV (-0.06 nA current from -65 mV) (4) in the three BNST regions.

**Figure 3.** Cells generating rebound single spikes. Voltage responses of a BNST-AL neuron to gradually increasing pulses of positive or negative current applied from -65 mV (A) or -80 mV (B). Note that the top two traces in panel A were offset graphically for clarity; the pre-pulse potential
was -65 mV, as for the traces just below.

**Figure 4.** Type-III (fIR, A) and late-firing (LF, B) neurons recorded in BNST-AM and AL, respectively. Voltage responses to gradually increasing pulses of positive or negative current applied from -76 mV (A), -65 mV (B1) or -80 mV (B2). Inset in 4A plots amplitude of voltage response to current pulses (y-axis) as a function of current (x-axis). Inset in B2 shows expanded view of initial voltage response to current injection. Note that the top trace in panel A was offset graphically for clarity; the pre-pulse potential was -76 mV, as for the traces just below. In B1 and B2, the top two traces were also offset graphically for clarity; the pre-pulse potentials were -65 mV and -80 mV, respectively, as for the traces just below.

**Figure 5.** Spontaneously active (SA) neuron recorded in BNST-AV. (A1) Spontaneous firing at rest. (A2) Autocorrelogram of spontaneous activity (inset shows corresponding distribution of interspike intervals). (B-C) Voltage responses to gradually increasing pulses of positive or negative current applied from -65 mV (B), or -80 mV (C). In B and C, the top two traces were also offset graphically for clarity; the pre-pulse potentials were -65 mV and -80 mV, respectively, as for the traces just below. Arrows in B and C point to periods of depolarization when spiking started to fail.

**Figure 6.** Incidence (A) and spatial distribution (B) of the various physiological cell types in different sectors of BNST-A. (A) Incidence: AM, top; AL, middle; AV, bottom. (B1) Distribution of RS (Type-I; red circles) and LTB (Type-II; black circles) neurons. (B2) Distribution of fIR (Type-III; black triangles), LF (thick red circles), and SA (red squares) neurons. Abbreviations: AC, anterior commissure; AL, anterolateral; AM, anteromedial; AV, anteroventral; Str, striatum.
Figure 7. Morphological properties of BNST-A neurons. (A) Scheme showing position and overall morphology of cells depicted in panels B-E (red, axon-like processes, black dendrites and soma). For cell e, only its position is shown because its dendrites overlapped extensively with those of cell d. (B) AL cell (RS, Type-I). Dendritic region enclosed in white dashed rectangle of B1 (lower right) is shown at higher magnification in B2. Note dendritic varicosities in B2. (C) AL neuron (LTB, Type-II). Regions enclosed in dashed rectangles of C1 are shown at higher magnification in C2, 3. Arrowheads point to stubby spines. (D) AM neuron (RS type). (D1) Drawing of the cell. Regions enclosed in dashed rectangle are shown at higher magnification in D2-4. Panels D3 and D4 show axon-like processes emerging from distal dendrites. (E) AM neuron (LTB type). E2-3 show dendritic segments with varicosities (E2) or stubby spines (E3, arrowheads). Abbreviations: AC, anterior commissure; AL, anterolateral; AM, anteromedial; AV, anteroventral; Str, striatum.

Figure 8. Morphological properties of BNST-A neurons. (A) Scheme showing position and overall morphology of cells depicted in panels B-F (red, axon-like processes, black dendrites and soma). For cell f, only its position is shown because its dendrites overlapped with those of cells b and c. Neurons labeled *1 and *2 are not depicted in subsequent panels. (B) AL neuron (LTB, Type-II). (B2) Dendritic segments with high density of spines. (B3) More distal dendritic segment with varicosities. (C) AL neuron (fIR, Type-III). (D) AV neuron (RS type) labeled “d” in panel A. Distal (D1) and proximal (D2) dendritic segments of cell. (E) AV neuron (LTB type). (F) AL neuron (fIR, Type-III). Scale bar in B3 is 15 µm and valid for B2. Scale bar in D1 valid for D2. Abbreviations: AC, anterior commissure; AL, anterolateral; AM, anteromedial; AV, anteroventral; Str, striatum.
Incidence of LTB cells (%)

Peak intra-burst firing rate (Hz)
Spikes per burst

Current (nA)

% cells with Sag

A1
B1
C1

C2

C3

C4

p < 0.0001

p's < 0.012

p = 0.012

p = 0.0011
Incidence (% recorded cells)

RS (Type-I)  
LTB (Type-II)  
fIR (Type-III)  
LF  
SA

n = 87  
n = 127  
n = 83
| TABLE 1. | Physiological Properties of BNST-A neurons by cell type (values are means ± SEM) |
|---|---|---|---|---|---|---|
| Cell Type | n | Incidence (%) | Rest (mV) | Rin (MΩ) | Time Constant (ms) | Threshold (mV) | Action Potential Amplitude (mV) | Duration (ms) |
| RS (Type-I) | 74 | 24.9 | -70.6 ± 0.8 | 722 ± 45.6 | 31.5 ± 2.6 | -40.8 ± 0.8 | 71.7 ± 1.4 | 1.22 ± 0.35 |
| LTB (Type-II) | 162 | 54.5 | -68.7 ± 0.6 | 686.4 ± 23.2 | 30.4 ± 1.2 | -40.6 ± 0.5 | 71 ± 0.9 | 1.28 ± 0.02 |
| fIR (Type-III) | 49 | 16.5 | -76.9 ± 1.7 | 546.7 ± 27.8 | 38.6 ± 2.7 | -40.1 ± 0.8 | 74.3 ± 1.5 | 1.32 ± 0.05 |
| LF | 5 | 1.7 | -85.8 ± 1.5 | 452 ± 33.5 | 23.3 ± 1.8 | -40.4 ± 1.9 | 71.9 ± 2.9 | 1.37 ± 0.13 |
| SA | 7 | 2.3 | -63 ± 2.8 | 622.6 ± 122.5 | 48.4 ± 7.5 | -45.3 ± 1.8 | 62.9 ± 3.7 | 0.77 ± 0.05 |

| TABLE 2. | Physiological Properties of BNST-A neurons by region (values are means ± SEM) |
|---|---|---|---|---|---|---|---|
| Region | n | Rest (mV) | Rin (MΩ) | Time Constant (ms) | Threshold (mV) | Action Potential Amplitude (mV) | Duration (ms) |
| BNST-AL | 127 | -72.6 ± 0.8 | 539.1 ± 19.5 | 29.5 ± 1.7 | -41.3 ± 0.5 | 73.3 ± 0.9 | 1.31 ± 0.02 |
| BNST-AM | 87 | -69.1 ± 0.9 | 729 ± 38.6 | 32.7 ± 1.6 | -41.1 ± 0.6 | 71.4 ± 1.2 | 1.37 ± 0.03 |
| BNST-AV | 83 | -69.5 ± 0.9 | 799 ± 34.1 | 36.7 ± 1.9 | -39.3 ± 0.7 | 68.9 ± 1.4 | 1.06 ± 0.03 |

| TABLE 3. | Physiological Properties of RS (Type-I) neurons by region (values are means ± SEM) |
|---|---|---|---|---|---|---|
| Region | n | Rest (mV) | Rin (MΩ) | Time Constant (ms) | Threshold (mV) | Action Potential Amplitude (mV) | Duration (ms) |
| BNST-AL | 34 | -70.4 ± 1 | 554.9 ± 51.8 | 27.7 ± 4.8 | -42.3 ± 1 | 73.4 ± 2 | 1.3 ± 0.04 |
| BNST-AM | 21 | -70.7 ± 2 | 876.9 ± 102.3 | 34.6 ± 3.9 | -41.6 ± 1.4 | 67.9 ± 2.6 | 1.27 ± 0.06 |
| BNST-AV | 19 | -71.1 ± 1.8 | 850.1 ± 77.9 | 35 ± 3 | -37.1 ± 1.5 | 67.5 ± 3.7 | 1.01 ± 0.06 |
TABLE 4. *Physiological Properties of LTB (Type-II) neurons by region* (values are means ± SEM)

<table>
<thead>
<tr>
<th>Region</th>
<th>n</th>
<th>Rest (mV)</th>
<th>Rin (MΩ)</th>
<th>Time Constant (ms)</th>
<th>Threshold (mV)</th>
<th>Action Potential Amplitude (mV)</th>
<th>Duration (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNST-AL</td>
<td>51</td>
<td>-67.8 ± 1</td>
<td>567.3 ± 31.1</td>
<td>29.4 ± 1.6</td>
<td>-41.8 ± 0.9</td>
<td>70.9 ± 1.7</td>
<td>1.33 ± 0.05</td>
</tr>
<tr>
<td>BNST-AM</td>
<td>59</td>
<td>-67.7 ± 1</td>
<td>690.5 ± 40.9</td>
<td>31.6 ± 1.9</td>
<td>-40.8 ± 0.7</td>
<td>71.2 ± 1.5</td>
<td>1.38 ± 0.05</td>
</tr>
<tr>
<td>BNST-AV</td>
<td>52</td>
<td>-70.7 ± 1</td>
<td>803.2 ± 41.1</td>
<td>34.2 ± 2.4</td>
<td>-39.3 ± 0.8</td>
<td>68.2 ± 1.6</td>
<td>1.12 ± 0.04</td>
</tr>
</tbody>
</table>

TABLE 5. *Morphological properties of BNST neurons* (values are means ± SEM)

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>n</th>
<th>Soma diameter (μm)</th>
<th>Number of Primary Dendrites</th>
<th>Distance to first dendritic branch from soma (μm)</th>
<th>Length of the inter-varicose axonal segments (μm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>RS (Type-I)</td>
<td>13</td>
<td>19.2 ± 1.1</td>
<td>2.8 ± 0.3</td>
<td>30.1 ± 5.6</td>
<td>4.4 ± 0.3</td>
</tr>
<tr>
<td>LTB (Type-II)</td>
<td>39</td>
<td>19.7 ± 0.7</td>
<td>3.2 ± 0.1</td>
<td>28.3 ± 2.2</td>
<td>5.1 ± 0.3</td>
</tr>
<tr>
<td>fIR (Type-III)</td>
<td>6</td>
<td>19.4 ± 2.2</td>
<td>2.8 ± 0.2</td>
<td>32.5 ± 6.5</td>
<td>4 ± 0.1</td>
</tr>
<tr>
<td>SA</td>
<td>2</td>
<td>19.5 ± 4.5</td>
<td>2.5 ± 0.5</td>
<td>13.2 ± 1.7</td>
<td>5</td>
</tr>
</tbody>
</table>

*For this analysis, we only considered primary dendrites that could be seen to branch at least once.