INTRINSIC CONNECTIONS IN THE ANTerior PART OF
THE BED NUCLEUS OF THE STRIA TERMINALIS

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Intrinsic connections in the anterior portion of the bed nucleus of the stria terminalis (BNST-A) were studied using patch recordings and ultra-violet (UV) uncaging of glutamate (GU) in vitro. UV light was delivered at small BNST-A sites in a grid-like pattern while monitoring evoked responses in different BNST-A regions. Three sectors were distinguished in BNST-A, using fiber bundles readily identifiable in trans-illuminated slices: the anterior commissure, dividing BNST-A in dorsal and ventral (BNST-AV) regions, and the intra-BNST component of the stria terminalis, subdividing the dorsal portion in medial (BNST-AM) and lateral (BNST-AL) regions. Overall, GU elicited GABAergic IPSPs more frequently than EPSPs. The incidence of intra-regional connections was higher than inter-regional links. With respect to the latter, asymmetric connections were seen between different parts of BNST-A. Indeed, while reciprocal connections were found between BNST-AL and AM, BNST-AL to AM connections were more frequent than in the opposite direction. Similarly, while GU in BNST-AM or AL often elicited IPSPs in BNST-AV cells, the opposite was rarely seen. Within BNST-AM, connections were polarized with dorsal GU sites eliciting IPSPs in more ventrally located cells more frequently than the opposite. This trend was not seen in other regions of BNST. Consistent with this, most BNST-AM cells had dorsally directed dendrites and ventrally ramified axons whereas this morphological polarization was not seen in other parts of BNST-A. Overall, our results reveal a hitherto unsuspected level of asymmetry in the connections within and between different BNST-A regions, implying a degree of inter-dependence in their activity.

Keywords: bed nucleus of the stria terminalis; glutamate uncaging, fear, anxiety
The bed nucleus of the stria terminalis (BNST) is a poorly understood brain structure that has been implicated in a variety of functions, most relating to negative affects and stress. For instance, the anterior portion of the BNST (BNST-A) was shown to regulate the hypothalamo-pituitary-adrenal (HPA) axis (Radley and Sawchenko 2011; Ulrich-Lai and Herman 2009), to mediate stress-induced relapse to drug seeking (Erb and Stewart 1999), and to generate fear/anxiety responses to diffuse environmental cues (Davis et al. 2010) or predator odors (Fendt et al. 2005).

Although its name suggests otherwise, the BNST is a collection of nuclei. While there is disagreement regarding the number and boundaries of BNST nuclei (Andy and Stephan 1964; De Olmos et al. 1985; Ju and Swanson 1989; Moga et al. 1989), it is clear that different BNST regions form contrasting connections. Within the BNST-A for instance, HPA-regulating neurons are concentrated in its ventral (BNST-AV) and medial (BNST-AM) portions (Dong et al. 2001b; Dong and Swanson 2006a; Prewitt and Herman 1998). In contrast, neurons in the dorsolateral part of the BNST-A (BNST-AL) contribute most BNST outputs to brainstem structures regulating fear expression (Holstege et al. 1985; Moga et al. 1989; Sofroniew et al. 1983; Sun and Cassell 1993).

Similarly, many afferents to the BNST-A form heterogeneous connections with these different regions. For instance, subicular and medial prefrontal inputs target BNST-AM and AV but not BNST-AL while insular axons show the opposite pattern or termination (reviewed in McDonald et al. 1999). Also, monoaminergic inputs are differentially distributed in the BNST-A (reviewed in Krawczyk et al., 2011). Furthermore, the main source of extrinsic input to the BNST, the amygdala, also contributes contrasting projections to different BNST-A regions (Dong et al. 2001a; Krettek and Price 1978b).
While this heterogeneous connectivity suggests a degree of functional specialization within the BNST-A, a seminal series of tracing studies by Swanson and colleagues (Dong and Swanson 2003, 2004, 2006a-c) suggest that different BNST-A regions do not act as independent processing channels, but that they interact via inter-nuclear connections. For instance, they reported that components of BNST-AL, particularly, the oval nucleus, strongly projects to parts of BNST-AV, such as the fusiform nucleus (Dong and Swanson 2004). However, interpretation of these findings is complicated by the fact that the distance between different BNST regions is small relative to the considerable extent of dendritic trees in the BNST (Larriva-Sahd 2006; McDonald 1983). Moreover, this problem is compounded by tracer diffusion from the injection site in the small volume of BNST, particularly along the tract of the pipettes used to inject the tracers.

Another unresolved question relates to the transmitter(s) used by intrinsic BNST axons. Indeed, previous work has revealed that BNST-A contains GABAergic and glutamatergic neurons (Cullinan et al. 1993; Polston et al. 2004; Poulin et al. 2009; Sun and Cassell 1993) with GABAergic cells accounting for the majority of BNST-A cells, and glutamatergic neurons for a minority. Thus, the present study was undertaken to shed light on the organization of intrinsic BNST-A connections using a method that has higher spatial resolution than tract tracing and allows identification of the transmitters involved: glutamate uncaging coupled to patch recordings in vitro.
MATERIALS AND METHODS

Procedures were approved by the Institutional Animal Care and Use Committee of Rutgers University, in compliance with the Guide for the Care and Use of Laboratory Animals (DHHS). Our subjects were male Lewis rats (4-5 weeks old, Charles River Laboratories, New Field, NJ) maintained on a 12 h light/dark cycle. They were housed individually with ad libitum access to food and water. Prior to the experiments, they were habituated to the animal facility and handling for one week.

Slice preparation

Rats were anesthetized with ketamine, pentobarbital, and xylazine (respectively 80 mg/kg, 60 mg/kg, and 12 mg/kg, i.p.). 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 400 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 7.3, 300 mOsm). The slices were then transferred one at a time to a recording chamber perfused with the latter solution (5 ml/min) plus caged glutamate (4-Methoxy-7-nitroindolinyl-caged-L-glutamate, 1.0 mM; Tocris Bioscience, Bristol, UK). Before the recordings began, the temperature of the chamber was gradually increased to °C.
**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). Biocytin (0.5%) was often added to the intracellular solution to label the recorded cells. 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, a graded series of depolarizing and hyperpolarizing current pulses (20 pA steps, 500 ms in duration) was applied from rest and other pre-pulse potentials. The input resistance (R\textsubscript{in}) of the cells was estimated in the linear portion of current-voltage plots.

**Glutamate uncaging**

To study the intrinsic connectivity of the BNST-A, we used ultra-violet (UV) uncaging of glutamate (GU) at various sites with respect to the recorded cells. UV light pulses (50 ms) were delivered at 0.1 Hz by a LED source (365 nm, 60 mW; CoolLED, Andover, UK) via a 60X immersion objective, yielding UV light spots of ≈150μm in diameter. The microscope rested on a computer-controlled motorized stage, allowing us to move the light spot in a grid-like pattern (50 or 110 μm steps) with respect to the recorded cell (Fig. 1A). At least three UV light pulses were applied at each site while keeping the cells at -90 mV with DC current injection. If a
synaptic response was observed, the pre-stimulus membrane potential of recorded cells was sequentially set to two additional values (-80, and -65 mV), each for at least three light stimuli, and more when response latencies were variable. With this approach, ≈60 min was required to scan the entire BNST-A region in search of sites where UV light application elicited responses in a given postsynaptic cell. PSPs ≤0.2 mV from a membrane potential of -90 mV were excluded.

GU-evoked PSPs could easily be distinguished from spontaneous synaptic events because the latter occurred infrequently and showed no temporal relationship with respect to the light stimulus. Nevertheless, since anterior BNST neurons display spontaneous synaptic events (Dumont et al. 2005, 2008; Gosnell et al. 2011; Guo and Rainnie 2010; Kash et al. 2008) that could be erroneously interpreted as GU-elicited PSPs, the following approach was used to distinguish spontaneous vs. GU-evoked PSPs. For each cell, we estimated the average interval between spontaneous PSPs (inter spontaneous PSP interval, IsPSPI) during the pre-stimulus period. Across all recorded cells, the average was 331 ± 30 ms. We then used the IsPSPI of each cell to determine the duration of a temporal window within which we required PSPs in at least 8 of 9 trials in order to consider them considered as GU-elicited. This “detection window” was set to a quarter of the cell’s IsPSPI. Within this window, the probability of getting 8 or more spontaneous PSPs in 9 traces by chance (i.e. the false positive rate) is then p = 0.000107 (Binomial test). We searched for GU-elicited PSPs in a 300 ms period after stimulus onset by moving our detection window in non-overlapping steps. The average number of non-overlapping detection windows was 4, resulting in a false positive probability of 0.000428 per stimulation site. The average number of stimulus sites per cell was 77, resulting in an average false positive probability of p = 0.0329 per cell. Since we recorded a total of 75 cells, the number of stimulus
positions with PSPs falsely labeled as GU-elicited is equal to \((0.0329 \times 75 = 2.467)\). Given that we observed 277 connections, this represents less than 1% of false positives.

Analyses were performed off-line with the software IGOR (Wavemetrics, Oregon), clampfit (Axon instruments, Foster City, CA), Stimfit (http://www.stimfit.org/) and custom written software using Numpy and Scipy (http://www.scipy.org). Values are expressed as means ± SE. Three complementary statistical approaches were used to assess the significance of the results. A first approach consisted of comparing the proportion of cells showing particular response types (e.g. response vs. no response or EPSP vs. IPSP) with a chi-square test. A second approach compared the proportion of stimulation sites eliciting particular response types across all recorded cells combined with a chi-square test. Third, the results obtained with the latter approach were verified by first computing the proportion of effective stimulation sites for each cell individually, averaging these for different response types, and comparing them with a paired-t-test.

Biocytin revelation for morphological identification of recorded cells

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. Slices were then embedded in agar (3%) and sectioned on a vibrating microtome at a thickness of 100 µm. Sections were washed several times in phosphate buffer (PB, 0.1 M, pH 7.4) and then transferred to a \(\text{H}_2\text{O}_2\) solution (0.5%) in PB for 15 min. After numerous washes in PB, sections were incubated for 12 h in a solution containing 0.5% triton, plus 1% of 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 (DAB) tetrahydrochloride (0.05%, Sigma), 2.5mM nickel ammonium sulfate
(Fisher) and H2O2 (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.

Nomenclature used to designate different BNST subregions

Individual BNST subnuclei cannot be identified with precision in unstained, living slices.
Therefore, we used a simpler subdivision of the 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 Sawnson’s anteroventral, fusiform, parastrial, and
dorsomedial subnuclei plus his subcommisural zone. BNST-AL corresponds to Sawnson’s oval,
juxtacapsular, and anterolateral subnuclei. BNST-AM corresponds to Swanson’s anterodorsal
subnucleus.

RESULTS

Spatial specificity of glutamate uncaging to study intra-BNST connectivity

The usefulness of the GU method to study intrinsic BNST connections depends on
whether it meets the following two criteria. First, that the rise in glutamate concentration
produced by UV light be high and rapid enough to reliably fire neurons located where the light stimulus is applied. Second, that the decay of the glutamate concentration with distance from the UV light stimulus be sufficiently steep such that nearby neurons, not directly exposed to UV light, are not depolarized enough to fire. We first aimed to test whether the GU method meets these criteria in the BNST.

To this end, the spot of UV light (150 µm in diameter and 50 ms in duration) was centered over recorded cells (n = 10) and then gradually displaced away from this site in various directions (steps of 50 µm). In these recordings, the pipette solution included biocytin to allow post-hoc correlation of morphology and responsiveness to uncaged glutamate. A representative example of such an experiment is shown in figure 1B. Red and white dots mark the sites of UV light application that elicited supra- or subthreshold responses, respectively. Application of UV light over the soma (Fig. 1B4) and its immediate vicinity always elicited robust spiking. This is a direct response to uncaged glutamate. However, when the center of the UV spot was moved away from the soma, these direct depolarizing responses eventually became sub-threshold or vanished. These variations (amplitude reduction vs. disappearance) depended on the exact position of the cells’ dendrites with respect to the position of the light spot, with stimuli located ≥150 µm from dendrites never eliciting spiking from rest. For instance, in the case depicted in figure 1B, GU elicited spiking when the UV light was applied over the soma and proximal dendrites (red dots and Fig. 1B2-4) but not when the stimulus was applied over more distal dendrites or at sites ≥150 µm from the soma and dendrites. At some of these sites (Fig. 1B5,6), evidence of GABAergic synaptic connections was obtained. Across all the cells tested in this manner, we did not observe a single case where spiking could be elicited from stimulation sites located ≥200 µm from their somata. Typically, direct responses vanished within 150 µm. Thus,
these results suggest that the GU method has sufficient spatial selectivity to study intrinsic BNST
closures.

Another important consideration when assessing the usefulness of GU to study intrinsic 
BNST connections is whether depolarizing PSPs can be distinguished from direct subthreshold 
responses to uncaged glutamate. Indeed, when UV stimuli are applied near recorded cells, it is 
possible that the evoked depolarizations are not due to synaptically released transmitter, but 
uncaged glutamate. Fortunately, these two types of responses could be readily distinguished. 

Figure 2A1 shows three superimposed direct sub-threshold responses to uncaged glutamate. As 
was typically observed when the light stimulus was applied near the recorded cell, this direct 
response started shortly after the onset of the light pulse, rose gradually for its entire duration, 
and began decaying shortly after its offset (Fig. 2A1).

Compared to direct responses, synaptically evoked PSPs had a longer latency (PSPs, 79.0 
± 4.4; direct, 8.3 ± 0.1 ms), they peaked more rapidly (PSPs, 27.8 ± 1.5 ms; direct, 74.7 ± 0.4 
ms), the onset of their decay phase was not time-locked to the offset of the light stimulus, and 
they sometimes showed conspicuous latency variations. The most direct illustration of the 
distinction between direct and synaptic responses are cases where both phenomena are elicited 
by UV light application at the same site. In the example shown in figure 2A2, the responses 
elicited by multiple consecutive light stimuli are superimposed. All trials started with a direct 
response to uncaged glutamate that showed no latency variations. Superimposed on the decaying 
phase of these direct responses were depolarizing PSPs whose exact latencies and number varied 
from trial to trial. These latency variations, coupled to the differing time course of the two types 
of responses leave no doubt as to their distinct origin. Of course, in the case of GABAergic PSPs,
the distinction was further facilitated by the fact that IPSPs reversed in polarity when the cells were depolarized (see below and Fig. 1B5).

It should be noted that latency variations were typically much smaller than in figure 2A2, as will become clear in subsequent figures. However, since anterior BNST neurons display spontaneous synaptic events (Dumont et al. 2005, 2008; Gosnell et al. 2011; Guo and Rainnie 2010; Kash et al. 2008) that could be erroneously interpreted as GU-elicited PSPs, the following approach was used to distinguish spontaneous vs. GU-evoked PSPs. For each target cell independently, we computed the frequency of spontaneous PSPs and only considered PSPs that largely exceeded the chance-expected (see details in Materials and Methods). With the approach we used, the estimated false-positive rate was around 1%.

Distinguishing GABAergic and glutamatergic PSPs elicited by glutamate uncaging

To identify the transmitters mediating GU-evoked PSPs, we primarily relied on their reversal potentials. That is, PSPs with extrapolated reversal potentials near 0 mV were assumed to be mediated by ionotropic glutamatergic receptors, whereas PSPs with reversal potentials negative to –60 mV were classified as being mediated by GABA-A receptors (Fig. 1B5). In several cases, we verified these inferences by testing whether presumed glutamatergic or GABAergic PSPs were sensitive to drugs that block non-NMDA glutamate receptors (6-cyano-7-nitroquinoxaline-2,3-dione, CNQX, 10 µM; Fig. 2B-C) or GABA-A (picrotoxin, 75 µM; Fig. 2D) responses. In all tested cases (EPSP, n = 5; IPSP, n = 7), the pharmacological experiment confirmed our electrophysiological inference. Here, it should be noted that due to the large rise in glutamate concentration produced by GU, the competitive receptor antagonist CNQX application did not block (only delayed) direct supra-threshold responses to uncaged glutamate
In contrast, EPSPs elicited by synaptically released glutamate were completely abolished (Fig. 2C). The differential sensitivity of direct vs. synaptically mediated glutamatergic responses to CNQX was previously reported in a study relying on local pressure applications of glutamate (Apergis-Schoute et al. 2007).

**Mapping of intrinsic BNST-A connections with glutamate uncaging**

We studied GU-evoked responses in 75 cells where long-term recording stability (≤10% variations in input resistance and ≤ 5 mV in resting potential) allowed extensive mapping of their intra-BNST connections with GU. These include 25 BNST-AL, 28 BNST-AM, and 22 BNST-AV neurons. Consistent with earlier reports on the electroresponsive properties of BNST-A neurons (Guo et al. 2009; Hammack et al. 2007; Hazra et al. 2011, 2012), regular spiking and low-threshold bursting neurons accounted for the vast majority of cells in the three regions examined. However, because no differences in the intrinsic connections were seen between physiological cell types ($\chi^2(X, N = 75) = 7.64, p = 0.27$), the results obtained in the various cell types are pooled below. In all these cells combined, we tested the effects of UV light stimuli at 5739 sites, usually separated by 110 µm. Overall, 5.1% of the stimulation sites elicited a synaptic response. Typical examples of intrinsic BNST-A connections evidenced with GU are shown for individual BNST-AM (Fig. 3A), AL (Fig. 3B), and AV (Fig. 3C) neurons. We first provide a qualitative description of these response patterns; quantitative population analyses will follow.

In figure 3A1-C1, colored circles are used to mark UV stimulation sites that elicited IPSPs (blue), EPSPs (red) or no responses (white). As was typically the case, these three cells responded to a minority of stimulation sites. Also representative of the overall response pattern,
a majority of PSPs elicited with GU were IPSPs. Examples evoked IPSPs (Fig. 3A2-3, B2-3, C2) and EPSPs (Fig. 3C3) are provided. The rise time and duration of evoked PSPs varied within and between cells. This variability probably reflects a number of factors such as electrotonic distances between the activated synapse(s) and soma as well as differences in the number of spikes (and instantaneous firing frequency) of presynaptic neurons recruited by GU. Of course, it is also possible that the number of presynaptic neurons varies between stimulation sites.

The inset in figure 3A1 (lower left) provides a different representation of the results obtained in the same three neurons shown in figure 3. The same representation is used to illustrate the responsiveness of 20 additional neurons in figure 4. We will refer to the results obtained in these cells later on, when describing the general trends identified in this study.

Intrinsic BNST-A connections: population analyses

Consistent with the fact that GABAergic cells represent the main cell type in the BNST-A, most of the intrinsic connections disclosed with GU were inhibitory. Figure 5 shows this in two ways: first (Fig. 5A) by plotting the proportion of cells in which only IPSPs (blue circles), EPSPs (red circles) or both (intersection between the circles) were evoked by GU, and second (Fig. 5B-C) by depicting the proportion of stimulation sites that were effective in eliciting EPSPs or IPSPs across all cells. With the first approach, the prevalence of inhibitory connections was apparent when we considered all BNST-A cells together (Fig. 5A1; $\chi^2(1, N = 75) = 7.18$, $p = 0.0074$) or neurons in different parts of the BNST-A separately (Fig. 5A2-4). However, the differing incidence of inhibitory and excitatory connections was especially marked in the BNST-AL (Fig. 5A2; $\chi^2(1, N = 25) = 4.53$, $p = 0.033$), and progressively less so in the BNST-AM (Fig. 5A3, $p = 0.12$) and the BNST-AV (Fig. 5A4, $p = 0.6$). This result pattern was confirmed using a
different statistical approach whereby the proportion of effective stimulation sites was first
determined for each cell, averaged across cells separately for IPSPs and EPSPs and then
compared with a paired t-test (Fig. 6A). This was used for all BNST-A cells combined (t(74) = 3.87, p = 0.0002) as well as separately for AL (t(24) = 2.77, p = 0.011), AM (t(27) = 2.65, p = 0.013), and AV neurons (t(21) = 1.29, p = 0.212).

The same conclusions emerged from the overall analysis of effective stimulation sites
(Fig. 5B-C). For intra-regional connections (Fig. 5B), that is cases where the stimulation sites
and recorded neurons were in the same BNST-A region, a chi-squared test revealed a significant
dependence between response type (IPSP, EPSP, no response) and BNST region (BNST-AV, BNST-AM and BNST-AV) ($\chi^2(4, N = 2959) = 9.62, p = 0.047$). Post-hoc tests showed that the
proportion of stimulation sites eliciting IPSPs was higher in the BNST-AL than BNST-AV ($\chi^2(2, N = 1979) = 7.56, p = 0.023$), and that the proportions in the BNST-AM are intermediate, and not
significantly different from either the BNST-AL ($p = 0.07$) or BNST-AV ($p = 0.23$).

For inter-regional connections (Fig. 5C), namely instances where the recording and
stimulation sites were located in different BNST-A regions, a more complex picture emerged.
First, irrespective of the type of response observed (IPSPs or EPSPs), the incidence of effective
stimulation sites was much lower than seen in intra-regional connections (intra-regional: 7.41%,
inter-regional: 2.63%, $\chi^2(1, N = 5775) = 84.34, p < 0.0001$). As shown in figure 6B, the same
conclusion was reached using a different statistical approach, namely first determining the
proportion of effective stimulation sites per cell for intra- vs. inter-regional connections,
averaging these values, and then comparing them with a paired t-test ($t(74) = 2.93; p = 0.004$).

Second, IPSPs did not prevail in all inter-regional connections. They did in connections
from and to BNST-AL neurons ($n_{ipsp} = 46, n_{epsp} = 16$; binomial test, $p < 0.0001$), whereas in
connections from and to the BNST-AV, the incidence of inhibitory connections could be equal to
(AV to AM) or even lower than (AM to AV) that of excitatory connections. However, because
the proportion of effective stimulation sites was low in these inter-regional connections, the latter
difference did not reach statistical significance (binomial test, $p = 0.5$).

Properties of GU-evoked PSPs (rise-time, amplitude, duration) did not vary depending on
the BNST-A region where the target cells were recorded or where the light stimuli were applied.
This was true of EPSPs and IPSPs, even with significance levels uncorrected for multiple
comparisons. **Figure 7** therefore shows frequency distributions of IPSP and EPSP properties
using results obtained in the three BNST-A regions combined. See figure legend for
methodological details. It should be noted that for these analyses, compound PSPs were not
included; only well isolated PSPs (presumed single-axon PSPs) that could be measured
unambiguously. However, note that the rise time of compound events, particularly of IPSPs,
were markedly slower than those of isolated PSPs.

**Heterogeneous directionality and polarity of intrinsic BNST connections**

There were marked differences in the directionality of intra-regional connections in
different sectors of the BNST-A. In the dorsal but not ventral portions of the BNST-AM,
connections had a preferential directionality, with dorsal GU sites eliciting IPSPs in more
ventrally located cells far more frequently than stimulation sites located ventrally to the recorded
neurons (**Fig. 4A**). Although a few cells exhibited this phenomenon in other BNST regions (**Fig.
4B4, C2, D3,5**), no overall preferential directionality of connections emerged in the BNST-AL
(**Fig. 4B1-3,5-6**), BNST-AV (**Fig. 4D1,24,6**) or the ventral part of BNST-AM (**Fig. 4C1,3,4**).
Another obvious difference between the dorsal (Fig. 4A) and ventral parts (Fig. 4C) of the BNST-AM was the incidence of neurons receiving excitatory inputs. All but one of the BNST-AM neurons in which intrinsic glutamatergic connections were disclosed (12 of 28) were found in the ventral part of the BNST-AM. As in the ventral part of BNST-AM, a high incidence of neurons receiving intrinsic glutamatergic inputs was found in the BNST-AV (12 of 22 or 55%; Fig. 4D), significantly higher than in the BNST-AL where intrinsic glutamatergic inputs were infrequent (7 cells of 25 or 28%; Fisher Exact test, p = 0.045).

Inter-regional connections were also asymmetric (Fig. 5C). Indeed, GU in BNST-AM or AL elicited PSPs in BNST-AV cells (3.66%) much more frequently than in the opposite direction (1.38%, Fisher exact test, p = 0.007). In addition, while reciprocal connections were found between BNST-AL and AM, BNST-AL to AM connections were more frequent than in the opposite direction (% tested stimulated sites: AM to AL, 2.78%; AL to AM, 3.56%; Fisher exact test, p = 0.042).

**Morphological correlates**

To test whether the contrasting directionality of intrinsic connections observed in different BNST-A sectors was dependent on the morphology of BNST-A neurons, we filled 38 neurons with biocytin (AM, n = 12; AL, n = 19; AV, n = 7). Representative examples of biocytin-filled neurons are provided in figure 8A. After filling, the slices were placed in fixative, re-sectioned at 100 μm and the biocytin revealed. The morphology of recorded cells was reconstructed by performing drawings of all labeled elements found in the different sections. Then, based on the matching position of blood vessels and of the cut ends of dendritic and/or
axonal segments, the labeling found in the different sections was aligned. Figure 8B provides examples of such reconstructions.

Consistent with the prevalent dorsoventral connectivity seen in the dorsal portion of the BNST-AM, 11 of 12 cells recovered from BNST-AM (n = 12) had dendrites that extended more in the dorsal (370 ± 157 µm) than the ventral (205 ± 69 µm) direction (t-test, p = 0.047). Moreover, all cells located in the dorsal portion of the BNST-AM (n = 4) contributed ventrally directed axons (Fig. 8B1-2, AM). In contrast, cells in other parts of BNST-A displayed no consistent morphological polarization (Fig. 8B1-2, AL, AV). Of note, whereas the dendritic arbors of BNST-AL (n = 19) and AM (n = 12) neurons were typically confined to the BNST region where their soma was located, BNST-AV neurons often (4 of 7) had dendrites that extended dorsally beyond the anterior commissure and into BNST-AM or AL. This suggests that inter-regional connections targeting BNST-AL or AM neurons typically depended on axons that extended beyond the BNST sector where the parent soma was located. In contrast, for BNST-AV neurons, this was not necessarily the case.

DISCUSSION

It was proposed that the BNST and the central amygdala are part of an anatomical entity termed the extended amygdala (Alheid and Heimer 1988; deOlmos and Heimer 1999). This concept is based on similarities in neuronal morphology and transmitter content (reviewed in McDonald 2003), common inputs from the basolateral amygdala (Krettek and Prince 1978ab; Pare et al. 1995; Savender et al. 1995; Dong et al. 2001a) as well as overlapping projections to a network of motor and autonomic brainstem nuclei thought to generate various aspects of

In contrast to the amygdala however, the physiological organization of the BNST is poorly understood. The BNST is comprised of several subnuclei with much disagreement regarding their exact number and location (Andy and Stephan 1964; De Olmos et al. 1985; Ju and Swanson 1989; Ju et al. 1989; Moga et al. 1989). However, it is commonly accepted that different BNST regions form contrasting connections with the rest of the brain. This suggests a degree of functional specialization within the BNST, raising the question of whether different BNST regions interact with each other or whether they constitute independent processing modules.

The present study was undertaken to address this question, focusing on the intrinsic connections that exist in the anterior part of the BNST. Our study revealed the existence of asymmetric connections within and between different parts of the BNST-A. Because the incidence of inhibitory and excitatory connections varied as a function of the region contributing or receiving these intrinsic connections, our findings raise the possibility that both cooperative and competitive interactions take place within the BNST. Below, we summarize the pattern of intrinsic connections evidenced in the present study and discuss these results in light of earlier findings regarding the anatomy and physiology of BNST-A.

Nature of the synaptic connections evidenced in the present study

Several factors suggest that the vast majority of the synaptic connections evidenced in the present study are monosynaptic and had an intrinsic origin (the pre- and postsynaptic neurons were located within the BNST). All the glutamatergic EPSPs we elicited with the GU method
were ≤6 mV in amplitude (mode of 1 mV). Since all the BNST neurons we recorded had a
resting potential negative to -65 mV, it seems extremely unlikely that such low amplitude EPSPs
could cause enough depolarization to reach spiking threshold (−49.8 ± 0.3 mV) in a neuron not
directly exposed to uncaged glutamate. Indeed, our control experiments (Fig. 1) revealed that
unless the UV light stimulus was applied directly over the recorded soma or the proximal portion
of the dendritic tree, it never elicited spiking. As a result, it seems extremely unlikely that the
responses we observed were polysynaptic. Regarding the intrinsic vs. extrinsic origin of the
connections, the vast majority of the UV light stimuli used to uncage glutamate were applied
entirely within the BNST. While some of the effective stimulation sites straddled BNST
boundaries, they accounted for a minority of the connections we observed. Furthermore, many
of these peripheral stimulation sites were located in the internal capsule, which is largely devoid
of neurons, and the lateral ventricle.

One confound we cannot completely exclude however, is the possibility that uncaged
 glutamate affected axon terminals contributed by neurons located in the BNST or elsewhere.
Indeed, prior studies have revealed that the BNST contains a sub-population of GABAergic axon
terminals expressing NMDA receptors (Gracy and Pickel 1995; Paquet and Smith 2000). Under
this scenario, uncaged glutamate would bind to presynaptic NMDA receptors and cause
sufficient depolarization to trigger GABA release. While this phenomenon cannot be
responsible for the glutamatergic EPSPs we observed, it could account for some of the
GABAergic IPSPs. However, for this effect to occur, the axon terminal expressing NMDA
receptors and its postsynaptic target would have to be located where the light stimulus is applied.
Thus, this effect could only be involved in cases where both a direct response to uncaged
 glutamate and an IPSP were observed. However, such instances were rare in our database
(<7.3% of the connections) and therefore cannot account for the pattern of results we obtained. Finally, while there is clear evidence that axon terminals in the BNST express metabotropic glutamate receptors (mGluRs; Gosnell et al. 2011; Grueter and Winder 2005; Grueter et al. 2006; Muly et al. 2007), it is unlikely that activation of these receptors by uncaged glutamate generated the fast synaptic events we examined because mGluRs are G-protein coupled receptors that exert slow modulatory effects, but do not mediate fast PSPs.

Overall pattern of intrinsic BNST-A connections

**Intra-regional connections.** With respect to intra-regional connections, a marked difference was found between the dorsal part of BNST-AM and the rest of BNST-A. In most of the BNST-A, intra-regional connections displayed no preferential directionality. However, in the dorsal part of BNST-AM, intrinsic connections had a predominant dorsoventral orientation (Fig. 9). Importantly, we found a parallel for this in the morphology of individual BNST-A neurons. Indeed, our reconstructions of biocytin-filled cells revealed that most BNST-AM neurons were morphologically polarized in a way consistent with the directionality of intrinsic connections. That is, their dendrites extended more in the dorsal than in the ventral direction and contributed axons that coursed ventrally. In contrast, neurons recovered from other sectors of the BNST-A showed no consistent orientation of their axons and dendrites.

Although a prior study examined the connectivity of the BNST-AM with *Phaseolus vulgaris*-leucoagglutinin (PHAL) (Dong and Swanson 2006a), it did not comment on the peculiar organization we observed in the dorsal part of the BNST-AM. However, this is likely due to technical limitations inherent to tracing techniques. In order to disclose the type of
organization we observed with GU, one would need to perform extremely small tracer injections, which is nearly impossible.

Another finding that emerged from our study is that the relative incidence of GABAergic and glutamatergic connections varied markedly in the different regions examined. Although GABAergic connections were prevalent overall, in some BNST-A regions glutamatergic connections were nearly as frequent. The incidence of glutamatergic connections was lowest in the BNST-AL and dorsal part of BNST-AM. By contrast, in the BNST-AV and the ventral region of BNST-AM, they accounted for about half the connections (Fig. 8).

The varying incidence of GABAergic and glutamatergic connections in different BNST-A regions is consistent with the results of previous reports that used immunohistochemistry (Esclapez et al. 1993; Hur and Zaborszky 2005; Sun and Cassell 1993) or in situ hybridization (Day et al. 1999; Poulin et al. 2009; Kudo et al., 2012) to study the distribution of neurons that are GABAergic (expressing mRNA for glutamic acid decarboxylase [GAD] 65 and/or 67) and/or glutamatergic (expressing mRNA for the vesicular glutamate transporter 2 – VGLUT2) in the BNST. Considered together, these studies indicate that GABAergic neurons are abundant in all divisions of the BNST-A whereas the incidence of glutamatergic neurons shows marked inter-regional variations. In particular, consistent with our observations, no (or very few) VGLUT2 positive cells were seen in the BNST-AL whereas a significant number was seen in the BNST-AV and AM.

Inter-regional connections. The connections between different BNST-A regions were asymmetric. That is, for all pairs of regions examined, connections were significantly more frequent in one direction than the other (Fig. 9). This was the case of all inter-regional projections involving the BNST-AL: higher incidence from BNST-AL to BNST-AM and AV.
than from the latter two to BNST-AL. Conversely, all inter-regional connections ending in the
BNST-AV were stronger than the reciprocal connections: lower incidence from BNST-AV to
BNST-AM and AL than from the latter two to BNST-AV.

This pattern of connections is consistent with the findings of earlier anterograde (Dong
and Swanson 2004, 2006a) and retrograde (Shin et al. 2008) tracing studies. Indeed, these
studies revealed that components of the BNST-AV receive convergent inputs from the BNST-
AL and AM and that subregions of the BNST-AL and AM are reciprocally connected. However,
it is difficult to compare the relative strength of the connections evidenced here with that seen in
tracing studies because the size of the various PHAL injection sites was not constant.

Nevertheless, the results of Swanson and colleagues appear generally consistent with the notion
that BNST-AL to AM connections (Dong and Swanson 2004) are stronger than in the opposite
direction (Dong and Swanson 2006a). Also consistent with our findings, BNST-AL projections
to BNST-AV (Dong and Swanson 2004) appear denser than in the opposite direction (Dong et al.
2001b).

In the present study, inter-regional connections also differed in the relative incidence of
GABAergic and glutamatergic inputs (Fig. 9). Paralleling the intra-regional connections, the
projections of BNST-AL to BNST-AM or AV were almost exclusively GABAergic. Similarly,
return projections from BNST-AM and AV to BNST-AL were also characterized by a scarcity of
glutamatergic connections. In contrast, the connections between BNST-AM and AV included
both glutamatergic and GABAergic projections.

The notion that a proportion of GABAergic and glutamatergic BNST-A neurons
contribute axons that extend beyond the confines of the sub-region where their somata is located
finds support in earlier anatomical and physiological studies. For instance, it was reported that
GABAergic cells in BNST-AM and AV project to the paraventricular hypothalamic nucleus (Herman et al. 2004; Radely et al. 2009; Radley and Sawchenko 2011). Similarly, it was reported that GABAergic BNST-AL neurons project to the central amygdala (Sun and Cassell 1993). Finally, with respect to excitatory outputs, it was shown that the BNST-AV contains glutamatergic neurons that project to the ventral tegmental area (Georges and Aston-Jones 2001, 2002; Jalabert et al. 2009; Kudo et al., 2012).

**Functional implications**

The pattern intrinsic connectivity disclosed in the present study implies that different BNST-A sectors do not act independently. In particular, because the projections of the BNST-AL to BNST-AM and AV are purely inhibitory and stronger than the reciprocating pathways, the BNST-AL is strategically positioned to determine, or at least modulate, activity levels in the rest of the BNST-A. This suggests an arrangement where the BNST-AL, via its inhibitory projections to BNST-AM and AV, acts as a gating mechanism for many BNST-A outputs. When BNST-AL activity is high, this would tend to reduce firing rates in BNST-AM and AV neurons. Conversely, a reduction in BNST-AL activity could cause a positive (or self-reinforcing) feedback effect where disinhibition of BNST-AM from BNST-AL inputs would increase return inhibitory projections from BNST-AM to BNST-AL, resulting in a further disinhibition of the BNST-AM, and so on. In addition to these competitive interactions, our findings raise the possibility that other sectors of BNST-A entertain cooperative relations. Indeed, the high incidence of glutamatergic connections between the BNST-AV and ventral part of BNST-AM suggest that neurons in these two regions may mutually enhance their excitability.

At present, it is difficult to assess how significant the impact of intrinsic BNST-A
connections might be. While the incidence of inter-regional connections was relatively low, it was likely underestimated because many connections, particularly the longer ones, are lost when slices are prepared. In addition, it is likely that intrinsic inputs ending in the distal dendrites of BNST neurons could not be detected due to electrotonic attenuation. Besides, the influence of intrinsic BNST connections will depend on a variety of factors including moment-to-moment variations in the activity of extrinsic afferents as well as modulatory inputs. In any event, the above considerations highlight the difficulty of interpreting lesion and pharmacobehavioral studies. Depending what exact BNST-A sector is lesioned or inactivated, opposite behavioral consequences can emerge.

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AUTHOR CONTRIBUTIONS:
Hjalmar Turesson carried out most of the electrophysiological experiments and analyzed the data.

Olga E Rodríguez-Sierra 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.** Approach used to study intrinsic BNST connections. (A) Glutamate uncaging was used to study the intrinsic connectivity of BNST-A. Patch recordings of BNST-A cells were obtained under visual guidance. Pulses (50 ms) of UV light were delivered (0.1 Hz) at sites of 150 µm in diameter (white circles), uncaging glutamate at the stimulation site. The site of UV light stimulation was systematically moved over the entire BNST-A in a grid-like pattern. Multiple light pulses were applied at each site and from different membrane potentials. (B) Spatial selectivity of glutamate uncaging. A patch recording of a BNST-AL neuron was obtained and the morphology of the cell revealed with biocytin (black, soma and dendrites; red, axon). UV light was applied over, and in the vicinity of, the recorded cell. The center of the UV-light spot was moved in steps of 50 µm. Sites evoking direct supra-threshold responses are indicated by red dots; white circles indicate sub-threshold responses. No supra-threshold responses could be elicited when the center of the UV-light spot was >150 µm from the cell. Examples of direct responses are provided in 1-4. GU at sites 5 and 6 elicited GABA-A IPSPs. Responses elicited from site 5 from different membrane potentials are shown on the bottom left.

**Figure 2.** Distinguishing responses to uncaged glutamate vs. synaptically released transmitters. (A1) Direct sub-threshold response to uncaged glutamate (three superimposed sweeps) from -90
mV. Note slow and invariant rising phase. Downward and upward arrows indicate onset and offset of UV light stimuli, respectively. \textit{(A2)} Case where a direct response and PSPs are triggered from the same stimulation site. Again, note slow and invariant rising phase of the direct response that contrasts with the fast rising phase, variable latency and number of evoked PSPs (four superimposed sweeps). \textit{(B)} Example of direct suprathreshold response to uncaged glutamate and \textit{(C-D)} of PSPs in control aCSF (black), after addition of CNQX \textit{(B-C, gray)} or picrotoxin \textit{(D, gray)}. Direct supra-threshold response to uncaged glutamate resists CNQX \textit{(B)} whereas indirect glutamatergic responses are abolished \textit{(C)}. As shown in \textit{D}, picrotoxin blocked IPSPs elicited by GU.

\textbf{Figure 3.} Examples of response patterns observed in BNST-AM \textit{(A)}, AL \textit{(B)} and AV \textit{(C)} neurons with glutamate uncaging. \textbf{(1)} Photomicrographs of the trans-illuminated slices with position of recorded cells (target cell, white dot) and UV stimulation sites (circles). \textbf{White circles} indicate sites of UV light application that elicited no response. At sites marked by \textbf{blue circles}, UV light elicited IPSPs. At sites marked by \textbf{red circles}, EPSPs were evoked. Examples of IPSPs \textit{(A2-3,B2-3,C2)} and EPSPs \textit{(C3)} are provided at the bottom. Numbers indicate the pre-stimulus membrane potentials (mV) at which the responses were observed. Upward arrows indicate offset of 50-ms UV light stimuli that elicited the responses. \textbf{Inset in A1} (lower left): graphical summary of connections found in the three cells depicted in panels A-C. \textbf{Blue}: inhibitory connections. \textbf{Red}: excitatory connections.

\textbf{Figure 4.} Plots of intra-BNST connections evidenced with glutamate uncaging in the dorsal \textit{(A)} and ventral \textit{(B)} parts of AM, AL \textit{(C)}, and AV \textit{(D)}. Each of the 20 panels illustrates a different
cell. Note that in dorsally located AM cells, intrinsic inputs prevalently run dorsoventrally. This trend was generally not seen in cells recorded in BNST-AL (B), AV (D) or ventral part of AM (C). While excitatory connections to neurons in BNST-AL and the dorsal part of BNST-AM were rare, they were frequently encountered in AV and ventrally-located BNST-AM cells.

**Figure 5.** Relative incidence of inhibitory and excitatory connections within BNST-A. (A) Proportion of cells that responded with glutamatergic (red) and/or GABAergic (blue) PSPs to glutamate uncaging. From left to right: all recorded cells irrespective of location, AL, AM, and AV cells. (B-C) Proportion of tested stimulation sites eliciting GABAergic (blue) or glutamatergic (red) PSPs when the stimulation and recording sites were in the same (B, Intra-regional) or different (C, Inter-regional) sectors of BNST-A.

**Figure 6.** Properties of intrinsic BNST connections. (A) Proportion of stimulation sites that elicited EPSPs (red) or IPSPs (blue) in BNST neurons. In contrast to figure 5, the proportion of effective sites was computed for each cell separately and then averaged across cells (values are averages ± SEMs). (B) Proportion of effective stimulation sites (EPSPs and IPSPs combined) in intranuclear (black) or internuclear connections (empty bar). As for panel A, the proportion of effective sites was computed for each cell separately and then averaged across cells.

**Figure 7.** Properties of IPSPs and EPSPs elicited by GU in intra- and inter-regional connections in all recorded neurons combined. (A) Frequency distributions of IPSP amplitudes (left), rise times (middle) and durations (right). (B) Frequency distribution of EPSP amplitudes (left), rise times (middle) and durations (right). This analysis only includes connections where individual
PSPs could be resolved; compound events were excluded. All measures were performed from a membrane potential of −90 mV. PSP rise times correspond to time to half of peak amplitude.

**Figure 8.** Morphological correlates of intrinsic connectivity. (A) Photomicrographs showing examples of BNST neurons labeled with biocytin (A1, BNST-AV; A2, BNST-AL). (B) Drawings of eight BNST-A neurons (red, axons; black, somata and dendrites). The neurons labeled 1 in B1 and 2 in B2 are the same cells as shown in A1 and A2, respectively. Note that because all our recordings were performed in the anterior portion of BNST, the rostrocaudal position of recorded cells did not vary much in our experiments (±250 µm).

**Figure 9.** Overall pattern of intrinsic BNST-A connections revealed with GU. Red and blue arrows correspond to glutamatergic and GABAergic connections respectively. For intra-regional connections, the number of blue and red arrows approximates the relative frequency of inhibitory and excitatory connections, respectively. For inter-regional connections, the thickness of the arrows was adjusted to represent the relative incidence of connections.
A spot of UV-light was moved in a grid-like pattern over BNST.
Intra-Regional Connections

<table>
<thead>
<tr>
<th>Region</th>
<th>Excitatory responses</th>
<th>Inhibitory responses</th>
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<tbody>
<tr>
<td>A1 ALL (n = 75)</td>
<td>57%</td>
<td>23%</td>
</tr>
<tr>
<td>A2 AL (n = 25)</td>
<td>68%</td>
<td>16%</td>
</tr>
<tr>
<td>A3 AM (n = 28)</td>
<td>57%</td>
<td>21%</td>
</tr>
<tr>
<td>A4 AV (n = 22)</td>
<td>46%</td>
<td>32%</td>
</tr>
</tbody>
</table>

Inter-regional Connections

<table>
<thead>
<tr>
<th>Region</th>
<th>To AL</th>
<th>To AM</th>
<th>To AV</th>
</tr>
</thead>
<tbody>
<tr>
<td>From:</td>
<td>Excitatory responses</td>
<td>Inhibitory responses</td>
<td></td>
</tr>
<tr>
<td>A1 ALL (n = 75)</td>
<td>2959</td>
<td>1069</td>
<td>980</td>
</tr>
<tr>
<td>A2 AL (n = 860)</td>
<td>230</td>
<td>466</td>
<td>295</td>
</tr>
<tr>
<td>A3 AM (n = 506)</td>
<td>230</td>
<td>466</td>
<td>295</td>
</tr>
<tr>
<td>A4 AV (n = 423)</td>
<td>230</td>
<td>466</td>
<td>295</td>
</tr>
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</table>

B Intra-Regional Connections

C Inter-regional Connections
Effective Stimulation sites (% of tested per cell)

A  

- **EPSPs**
- **IPSPs**

B  

- Effective Stimulation sites (% of tested per cell)

- **Intra-Regional**
- **Inter-Regional**
A  
**IPSPs**

- **Amplitude**
  - $n = 49$
  - $1.37 \pm 0.13$ mV

- **Rise time**
  - $n = 49$
  - $12.4 \pm 0.8$ ms

- **Duration**
  - $n = 49$
  - $52.1 \pm 3.8$ ms

B  
**EPSPs**

- **Amplitude**
  - $n = 53$
  - $1.89 \pm 0.18$ mV

- **Rise time**
  - $n = 53$
  - $9.6 \pm 0.42$ ms

- **Duration**
  - $n = 53$
  - $46.9 \pm 2.6$ ms