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Bi-directional modulation of BNST neurons by 5-HT: Molecular expression and functional properties of excitatory 5-HT receptor subtypes

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Abstract

Activation of neurons in the anterolateral bed nucleus of the stria terminalis (BNST_{ALG}) plays an important role in mediating the behavioral response to stressful and anxiogenic stimuli. Application of 5-HT elicits complex postsynaptic responses in BNST_{ALG} neurons, which includes 1) membrane hyperpolarization (5-HT_{Hyp}), 2) hyperpolarization followed by depolarization (5-HT_{Hyp-Dep}), 3) depolarization (5-HT_{Dep}) or 4) no response (5-HT_{NR}). We have shown that the inhibitory response is mediated by activation of postsynaptic 5-HT_{1A} receptors. Here, we used a combination of *in vitro* whole-cell patch-clamp recording and single cell reverse transcriptase polymerase chain reaction (RT-PCR) to determine the pharmacological properties and molecular profile of 5-HT receptor subtypes mediating the excitatory response to 5-HT in BNST_{ALG} neurons. We show that the depolarizing component of both the 5-HT_{Hyp/Dep} and the 5-HT_{Dep} response was mediated by activation of 5-HT_{2A}, 5-HT_{2C} and/or 5-HT₇ receptors. Single cell RT-PCR data revealed that 5-HT₇ receptors (46%) and 5-HT_{1A} receptors (41%) are the most prevalent receptor subtypes expressed in BNST_{ALG} neurons. Moreover, 5-HT receptor subtypes are differentially expressed in Type I – III BNST_{ALG} neurons. Hence, 5-HT_{2C} receptors are almost exclusively expressed by Type III neurons, whereas 5-HT₇ receptors are expressed by Type I and II neurons, but not Type III neurons. Conversely, 5-HT_{2A} receptors are found predominantly in Type II neurons. Finally, bi-directional modulation of individual neurons occurs only in Type I and II neurons. Significantly the distribution of 5-HT receptor subtypes in BNST_{ALG} neurons predicted the observed expression pattern of 5-HT responses determined pharmacologically. Together, these results suggest that 5-HT can differentially modulate the excitability of Type I – III neurons, and further suggest that bi-directional modulation of BNST_{ALG} neurons occurs primarily through an interplay between 5-HT_{1A} and 5-HT₇ receptors.

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Hence, modulation of 5-HT₇ receptor activity in the BNST_{ALG} may offer a novel avenue for the design of anxiolytic medications.

Keywords

extended amygdala; anxiety disorders; whole cell patch clamp recording; reverse transcriptase polymerase chain reaction

Introduction

Although a large body of evidence suggests that serotonin (5-HT) systems play an important role in modulating behaviors associated with affect, the nature of this function remains perplexing. Indeed, the therapeutic benefit of increasing brain 5-HT with chronic selective serotonin reuptake inhibitors (SSRI) for the treatment of mood disorders is well documented. Paradoxically, however, acute SSRI treatment can also increase behavioral measures of fear and anxiety in both humans and rodents (Pohl et al., 1988, Bagdy et al., 2001, Burghardt et al., 2004). Similarly, 5-HT depletion studies have shown that decreasing brain 5-HT levels can either increase (Hohmann et al., 2007), or decrease (Soderpalm and Engel, 1990, Bechtholt et al., 2007) anxiety-like behavior. In sum, these data suggest that the activation of brain 5-HT systems can be either anxiolytic or anxiogenic depending on the context in which that activation occurs (see (Handley, 1995) for review).

The opposing actions of 5-HT can be explained, in part, by the large number of cognate receptors that mediate the effects of 5-HT. At least 14 subtypes of 5-HT receptors have been identified that are classified into seven major categories (5-HT₁₋₇; for review see (Hoyer et al., 2002)), and are differentially distributed throughout the central nervous system. Moreover, in many brain regions individual neurons can express multiple 5-HT receptors (Pazos et al., 1985), further increasing the complexity of the neuronal response to 5-HT (see (Uphouse, 1997) for review).

One such region, the bed nucleus of the stria terminalis (BNST), is believed to play a critical role in mediating the anxiety-like behavioral response to physiological and psychological stressors (Walker and Davis, 1997, Erb and Stewart, 1999, Leri et al., 2002, Schulz et al., 2002, Fendt et al., 2003, Aston-Jones and Harris, 2004, Hammack et al., 2004, Jasnow et al., 2004, Sullivan et al., 2004). Consistent with this role, lesion or transient inactivation of the BNST reduces many of the behavioral indices of stressor exposure (Brutus et al., 1988, Walker et al., 1997, Erb and Stewart, 1999, Fendt et al., 2003, Aston-Jones and Harris, 2004, Hammack et al., 2004). Moreover, exposure to stressors increases neural activation in the BNST (Duncan et al., 1993, Martinez et al., 1998, Chung et al., 1999, Lino-de-Oliveira et al., 2001), and electrical stimulation of the BNST evokes cardiovascular and behavioral responses similar to those initiated by stressful stimuli (Shaikh et al., 1986, Casada and Dafny, 1991, Dunn and Williams, 1995). Hence, BNST-dependent behaviors may result from a stress-induced increase in the activity of BNST neurons.

Significantly, stressors that activate the BNST also activate central 5-HT systems (Dilts and Boadle-Biber, 1995, Grahn et al., 1999, Funada and Hara, 2001, Lowry, 2002, Summers et al., 2003, Takase et al., 2004), and maladaptive changes in 5-HT release and/or 5-HT receptor function have been proposed to play a major role in the etiology of many stress-related affective disorders, including anxiety disorders (Chaouloff et al., 1993, Lucki, 1996, Deakin, 1998, Ressler and Nemeroff, 2000, Charney, 2003). Critically, many stressors activate a subset of 5-HT neurons in the caudal dorsal raphe nucleus (Grahn et al., 1999, Lowry et al., 2000, Singewald et al., 2003) that preferentially target limbic forebrain regions including the BNST

(Phelix et al., 1992, Commons et al., 2003), suggesting that 5-HT modulation of neural activity in the BNST may play a fundamental role in regulating the behavioral response to stressors.

In both rat and non-human primate, 5-HT fibers preferentially target neurons in the anterolateral cell group of the BNST (BNST_{ALG}; (Phelix et al., 1992, Dong et al., 2001, Freedman and Shi, 2001, Commons et al., 2003)), an area that has been shown to express multiple 5-HT receptors including moderate to high levels of 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, 5-HT₄, and 5-HT₇ receptors (Mengod et al., 1990, Pompeiano et al., 1994, Waeber et al., 1994, Wright et al., 1995, Heidmann et al., 1998, Cornea-Hebert et al., 1999, Xu and Pandey, 2000, Vilaro et al., 2005). The diversity of 5-HT receptor subtypes in this region would suggest a complex response pattern to local 5-HT release.

Recently, we reported that activation of postsynaptic 5-HT receptors could differentially modulate the excitability of BNST_{ALG} neurons (Rainnie, 1999; Levita et al., 2004). Significantly, a substantial number of neurons exhibited both an inhibitory and an excitatory component of their response to 5-HT. The inhibitory response was mediated by activation of postsynaptic 5-HT_{1A} receptors coupled via G_{qi} to inwardly-rectifying potassium channels. Importantly, local infusion of the non-selective 5-HT_{1A,7} agonist 5-carboxyamidotryptamine (5-CT) into the BNST_{ALG} elicits an anxiolytic-like behavioral response (Levita et al., 2004), suggesting that 5-HT_{1A} receptor-mediated inhibition of BNST_{ALG} neurons may modulate anxiety-like behaviors.

However, these same neurons have the capacity to respond to 5-HT with excitation, providing a mechanism by which 5-HT release might differentially elicit anxiolytic or anxiogenic behavior depending on the balance of inhibition and excitation within individual BNST_{ALG} neurons. Significantly, four out of the five 5-HT receptors reportedly expressed in the BNST_{ALG} (5-HT_{2A}, 5-HT_{2C}, 5-HT₄ and 5-HT₇) have been shown to mediate postsynaptic excitatory responses elsewhere in the central nervous system (Araneda and Andrade, 1991, McCormick and Wang, 1991, Stevens et al., 1992, Chapin and Andrade, 2001, Craven et al., 2001, Hoyer et al., 2002, Beique et al., 2004). The present study was designed to fully characterize the 5-HT receptor subtype/s mediating the excitatory response of BNST_{ALG} neurons to 5-HT.

Here, we have used whole-cell patch clamp recording from BNST_{ALG} neurons in conjunction with reverse transcriptase polymerase chain reaction (RT-PCR) to demonstrate that the 5-HT-evoked excitation results from activation of either 5-HT_{2A/2C} receptors, or activation of 5-HT₇ receptors. Moreover, the data suggest that subpopulations of BNST_{ALG} neurons express different combinations of 5-HT receptor subtypes, and the heterogeneous distribution of 5-HT receptor transcripts predicts the heterogeneous 5-HT response profile in BNST_{ALG} neurons.

Experimental Procedures

Subjects

Brain slices for electrophysiology were obtained from male Sprague-Dawley rats 24–40 days old (Charles River, Raleigh, NC). Prior to tissue preparation, rats had unrestricted access to food and water, and were housed four per cage with an alternating 12-hr light-dark cycle (light on at 7:30 am). Effort was taken to minimize the number and suffering of animals, and all procedures were conducted in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals. All procedures were approved by the Institutional Animal Care and Use Committee at Emory University. Because BNST 5-HT responses can be altered by stressor exposure (Hammack et al., 2009), care was taken to minimally handle/stress rats prior to sacrifice.

Tissue Preparation and Patch Clamp Recording

Slices (350 μM) containing the anterolateral BNST cell group were obtained as previously described (Levita et al., 2004). Brains were sectioned in an ice-cold kynurenic-based artificial cerebrospinal fluid (ACSF_{KA}), which contained (in mM): NaCl (130), KCl (3.5), KH₂PO₄ (1.1), MgCl₂ (6.0), CaCl₂ (1.0), NaHCO₃ (30), glucose (10), and kynurenic acid (2). The ACSF_{KA} was adjusted to minimize the excitotoxicity associated with glutamate release during tissue slicing, and included the addition of the glutamate antagonist, kynurenic acid, decreased calcium levels to minimize neurotransmitter release, and increased magnesium levels. Immediately after slicing, slices were hemisected, trimmed, and placed in a holding chamber containing oxygenated ACSF_{KA} at room temperature for 30 min. Slices were then transferred to oxygenated ACSF maintained at room temperature for at least 30 min before recording. For recording, individual slices were transferred to a Warner Series 20 recording chamber (0.5 ml volume) mounted on the fixed stage of a Leica DM-LFS microscope. The slices were maintained fully submerged and continuously perfused with ACSF heated to 32°C, and gassed with a 95%-5% oxygen/carbon dioxide mixture. Individual neurons were identified within the slice using a 40X water immersion objective in conjunction with differential interference microscopy and infra-red illumination (IR-DIC).

Standard whole-cell recordings in current- and voltage-clamp mode were obtained from BNST_{ALG} neurons using techniques described elsewhere (Levita et al., 2004). Patch pipettes were fabricated from borosilicate glass (resistance 4–7 M Ω) and filled with a recording solution of the following composition (in mM): 130 K-Gluconate, 2 KCl, 10 HEPES, 3 MgCl₂, and 5 phosphocreatine, 2 K-ATP, 0.2 NaGTP. Data acquisition and analysis were performed using an Axopatch-1D amplifier (Molecular Devices, Burlingame, CA) in conjunction with pClamp 9.0 software, and a continuous record of each experiment was captured on a chart recorder (Kipp & Zonen, Bohemia, NY). All voltage clamp data were low pass filtered at 1 kHz, and sampled at a frequency determined by the speed of the response that was to be measured.

Drug application

Known concentrations of drugs were applied to the slice dissolved in the ACSF using a continuous gravity fed bath application. Previously, we demonstrated that the response of BNST_{ALG} neurons to 5-HT is unaffected by repeat application of 5-HT (Levita et al., 2004). Consequently, the effects of 5-HT receptor antagonists and second messenger blockers were examined after the initial response to 5-HT had first been determined in control ACSF. The slice was then perfused with the drug of choice for approximately 10–20 min, and the neuronal response to reapplication of 5-HT determined. We typically used 50 μM 5-HT because we have previously shown that this concentration produces stable responses on BNST_{ALG} neurons, and would be expected to activate multiple 5-HT receptor subtypes expressed by a given BNST_{ALG} neuron including those with lower affinity to 5-HT (Rainnie, 1999b, Levita et al., 2004). Most of the experiments described here were conducted using a within-subject design.

Drugs applied to the tissue were obtained from the following sources: 5-hydroxytryptamine (5-HT, 50 μM); 5-carboxyamidotryptamine (5-CT, 10 μM); 5-methoxytryptamine (50–250 μM); (-)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI; 5–50 μM); *m*-chlorophenylbiguanide (mCPBG, 50 μM); pirenperone (10 μM); and WAY 100635 (200 nM), SB-269970 (1 μM) were all purchased from Sigma-Aldrich (Saint Louis, MO). *m*-chlorophenylpiperazine (mCPP, 5–75 μM); Cisapride (10 μM); CP 93129 (10 μM); BRL 54443 (5–75 μM); LY 344864 (10 μM); -methyl-5-HT (25–250 μM); TCB 2 (10 μM); WAY 161503 (10 μM) from purchased from Tocris Bioscience (Ellisville, MO). MDL100907 (10 nM) was a gift from Aventis Pharmaceuticals and Sumatriptan (50–250 μM) was a gift from Glaxo-Wellcome. All drugs were made as concentrated stock solutions in distilled H₂O, except

MDL100907, which was made in 30% acetic acid, cisapride and BRL 54443, which were made in 50% DMSO, and pirenperone, which was made in 0.1N HCl. The diluted concentration of each vehicle in the bath application was 0.03% acetic acid, 0.05% DMSO, and 0.0001 N HCl. These vehicle solutions did not affect any biophysical properties in BNST_{ALG} neurons. Stock solutions were kept as frozen aliquots at -20°C until required.

Agonist and antagonist concentrations were determined both from pilot studies and from effective concentrations reported in published studies investigating similar responses in other brain regions. Although the selectivity of these agents is concentration-dependent, many of the agents were only reported effective in brain slice preparations at concentrations that likely exceeded the 5-HT receptor subtype selectivity based on receptor binding studies. Most likely this results from penetration issues, diffusional barriers, and the relative lipid solubility of these agents. Hence, higher drug concentrations are often required to produce effective drug levels at the synaptic cleft. In similar studies (Egli and Winder, 2003, Dumont and Williams, 2004), a similar concentration of norepinephrine (100 μM) was used to produce pre- or postsynaptic actions in BNST.

Analysis

The effects of 5-HT application on BNST_{ALG} neurons were determined in current-or voltage-clamp mode. Each response to 5-HT (or 5-HT agonist) was defined as a pure hyperpolarization/outward current (5-HT_{Hyp}), pure depolarization/inward current (5-HT_{Dep}), or mixed response (5-HT_{Hyp-Dep}) based on visual inspection of the response and its calculated reversal potential. For antagonist studies, reductions in the 5-HT response in the presence of antagonist are reported as a percentage of the initial 5-HT response determined before antagonist treatment. All comparisons were made using two-tailed paired t-tests unless otherwise noted. All statistics were performed using GraphPad Prism version 4.02 (GraphPad Software, San Diego, CA).

RT-PCR from isolated BNST sections

For these experiments 8 rats were used to isolate the mRNA from the BNST_{ALG}. Here, 500 μm rat brain slices were prepared as outlined above and the BNST_{ALG} isolated using a dissecting microscope. BNST tissue was then homogenized in Trizol and total RNA was isolated. Both the quality and quantity of each RNA sample was assessed by gel electrophoresis and optical density measurements. PCR conditions were optimized using total RNA isolated from rat BNST_{ALG} so that a PCR product could be detected from (250 pg–1 ng) of total RNA. The isolated RNA was then reverse transcribed using a cocktail containing 5–8 μl diethylpyrocarbonate (DEPC) treated water, RNase inhibitor (40 U/ μl), dithiothreitol (DTT, 0.1 M), and random hexanucleotides (50 ng/ μl) primers. The mixture was rapidly heated to 70°C and then incubated on ice for 2 min. Single strand cDNA was synthesized from the cellular mRNA by addition of 8 μl of an RT master mix consisting of superscript RT III (200 U/ μl), 5X first strand buffer, RNasin (40 U/ μl), DTT (0.1 M), and mixed nucleotide triphosphates (10 mM dNTPs), which was then incubated at 42°C for 50 min. The reaction was terminated by heating the mixture to 70°C for 15 min and then chilling on ice. The resultant cDNA was isolated by adding 1 μl of RNase H (2 U/ μl) and incubating for 20 min at 37°C . The cDNA was amplified using 10X PCR buffer, 3.0 mM MgCl_2 , 10mM dNTPs, 2.5 U of Taq DNA Polymerase (Qiagen) and 100 nM primers. The reactions were duplicated for each cDNA sample for each target gene in order to obtain more convincing results. Specific PCR primers used for each of the 5-HT receptor mRNAs were developed from GenBank sequences with commercially available Oligo software (IDT Tools). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used in all experiments as a positive control. The oligonucleotide primers used for PCR reactions are summarized in Table 1. All reagents were obtained from Invitrogen, unless otherwise stated. Standard PCR was

performed on a PTC-200 Peltier thermal cycler (MJ Research) using the following program: 94 °C for 40 s, 56 °C for 40 s and 72°C for 1min for 40 cycles.

Single-Cell RT-PCR

At the end of each recording session, the cell cytoplasm was aspirated into the patch recording pipette containing ~ 5 µl of RNase-free patch solution under visual control, by applying gentle negative pressure. The contents of the patch pipette were then expelled into an eppendorf tube containing 7 µl of the reverse transcription cocktail (ibid) by applying positive pressure. mRNA was reverse transcribed using 200 U of Superscript RT III and RT was performed in a final volume of 21 µl as described earlier. The cDNA was stored at -20 °C before further processing. The cDNA from each cell was then amplified and screened for the expression of four specific 5-HT receptor mRNAs (5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C} and 5-HT₇) and GAPDH as a positive control marker using a two step multiplex-PCR reaction (Toledo-Rodriguez et al., 2004).

Initially all four genes were simultaneously amplified in a single tube using 2 µl of cDNA from each cell as a template and 100 nM of each of the four primers. The PCR master mix was comprised of 10X PCR Buffer, 3 mM MgCl₂, 10 mM dNTPs, 2.5 U of Taq DNA Polymerase in a final volume of 100 µl. PCR was performed using a 10 min hot start at 95°C followed by a 25 cycle program (94 °C for 40 s, 56°C for 40 s and 72°C for 1 min). Subsequently, 2 µl of the amplified cDNA was used as the template for the second amplification step. Here, each gene was individually amplified in a separate tube and a 40 cycle program using the same PCR master mix and primer concentrations as mentioned above, in a final volume of 20 µl. The products of the second PCR were analyzed by electrophoresis in 1 % agarose gels using ethidium bromide.

Results

Expression of 5-HT receptor mRNA in BNST_{ALG} tissue

Before determining the likely candidates for the 5-HT receptors mediating the excitatory response of BNST_{ALG} neurons to 5-HT, it was first necessary to determine the profile of 5-HT receptor expression in this region. Previous studies had reported moderate to high levels of expression for 5-HT₁, 5-HT_{2A}, 5-HT_{2C}, 5-HT₄, and 5-HT₇ receptors within the BNST as a whole (Mengod et al., 1990, Waeber et al., 1994, To et al., 1995, Waeber and Moskowitz, 1995, Wright et al., 1995, Kia et al., 1996, Heidmann et al., 1998, Sari et al., 1998, Cornea-Hebert et al., 1999); however, these studies did not specifically examine the expression of 5-HT receptor subtypes within the anterolateral region of the BNST. Hence, we micro-dissected the BNST_{ALG} from coronal brain sections, extracted tissue RNA, and then probed the samples for specific 5-HT receptor subtype mRNA using a RT-PCR protocol (n=8 rats). The BNST_{ALG} mRNA expression pattern for multiple 5-HT receptor subtypes was qualitatively similar across all animals, and consistently included transcripts for 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2C}, 5-HT₃, 5-HT₄, 5-HT_{5A}, 5-HT₆, and 5-HT₇ receptors (Fig. 1). These results confirmed the observations of previous *in situ* studies (ibid) and also acted as an expression control for our subsequent single-cell RT-PCR studies (see below). Moreover, these data suggest that 5-HT receptor expression in the BNST_{ALG} is heterogeneous, which supports our electrophysiological data describing a complex response profile to 5-HT (Rainnie, 1999; Levita et al., 2004). In order to determine the 5-HT receptor subtype(s) that mediate the excitatory response of BNST_{ALG} neurons to 5-HT application, we used whole-cell patch-clamp recording and subtype selective antagonists to block the excitatory response to exogenous 5-HT application, and subtype selective agonists to mimic 5-HT induced excitation, followed by single cell RT-PCR screening of the mRNA expression of these receptor subtypes.

Excitation in 5-HT_{Hyp-Dep} neurons

In this study, the response to 5-HT application (50 μ M) was examined in a total of 148 BNST_{ALG} neurons. Consistent with our previous studies (Rainnie, 1999; Levita, *et al* 2004), BNST_{ALG} neurons respond to 5-HT with 5-HT_{Hyp-Dep} (28%), 5-HT_{Dep} (34%), or 5-HT_{Hyp} (16%). On contrast to 5-HT_{Dep}, HT_{Hyp-Dep} has two components, that is, a membrane hyperpolarization immediately followed by a depolarization deflection. The properties of the 5-HT_{Hyp-Dep} and 5-HT_{Dep} responses are summarized in Table 2.

In 5-HT_{Hyp-Dep} neurons, we conducted voltage ramp protocols at the peak of the outward deflection and then again when the inward deflection had reached steady state, the E_{5-HT} was found to shift right significantly from the peak to the steady state ($t(14) = 5.2$, $p < 0.001$, Table 2). At the peak of the outward deflection, the membrane conductance significantly increased by $42 \pm 11\%$ compared to baseline ($t(9) = 3.1$, $p < 0.05$). Importantly, the membrane conductance was $55 \pm 6\%$ greater than baseline when the response reached a steady state level ($t(11) = 4.7$, $p < 0.001$), suggesting that the mixed 5-HT_{Hyp-Dep} response was due to the slow onset of an overlapping inward current, and not due to the gradual decay of an outward current.

Consistent with this hypothesis, in those neurons that exhibited a 5-HT_{Hyp-Dep} response, application of 5-HT (50 μ M) in the presence of the 5-HT_{1A} receptor antagonist, WAY 100635 (200 nM) evoked only a monophasic inward current (Figure 2A) with a reversal potential that was significantly more depolarized than that observed in control ACSF ($t(8) = 7.2$, $p < 0.001$, see Fig. 2B, D). Moreover, application of 5-HT in the presence of the non-selective 5-HT₂ receptor antagonist, pirenperone (10 μ M), evoked only a monophasic outward current in 5-HT_{Hyp-Dep} neurons (Fig. 3A). Importantly, pirenperone application caused a significant increase in the amplitude of the peak outward current ($t(6) = 2.8$, $p < 0.05$; Fig. 3C), and caused a -17.3 ± 3.6 mV leftward shift of the 5-HT reversal potential ($t(6) = 4.8$, $p < 0.01$; Fig. 3B,D). Finally, co-application of WAY 100635 and pirenperone fully blocked the response to 5-HT in these neurons ($n = 3$, Fig. 3A right). These data were consistent with our previous study, in which we showed that the inhibitory component of the 5-HT response in these neurons was mediated by 5-HT_{1A} receptor activation (Levita *et al.*, 2004), and suggested that the 5-HT₂ family of receptors may mediate the excitatory component of the mixed 5-HT_{Hyp-Dep} response. However, pirenperone is not a selective 5-HT₂ receptor antagonist and has affinity for other 5-HT receptor subtypes, including 5-HT₇ receptors. Consequently, we next examined the ability of more selective 5-HT receptor subtype antagonists to block the 5-HT evoked depolarizing component in 5-HT_{Hyp-Dep} BNST_{ALG} neurons.

We first determined whether prior application of the 5-HT_{2A} receptor antagonist MDL 100907 would attenuate the excitatory component of 5-HT_{Hyp-Dep} response. In 8 neurons that exhibited an initial mixed 5-HT_{Hyp-Dep} response, prior application of MDL 100907 (10 μ M) blocked the inward current in 2 neurons, attenuated the 5-HT-induced inward current in 2 neurons ($58 \pm 8\%$ of initial 5-HT current), and had no effect in the remaining 4 neurons, suggesting that 5-HT_{2A} receptors mediate excitation in some 5-HT_{Hyp-Dep} neurons. We subsequently used a similar strategy with the 5-HT_{2C} receptor antagonist RS 102221 (10 μ M). Here, RS 102221 blocked the inward current in 3 of 5 neurons and had no effect in the remaining 2 neurons, suggesting that 5-HT_{2C} receptors also contribute to the 5-HT-induced excitation in some 5-HT_{Hyp-Dep} neurons. Importantly, these antagonists had no observable membrane effects when administered alone. Hence, activation of 5-HT_{2A} and 5-HT_{2C} receptors can mediate an excitatory response in some 5-HT_{Hyp-Dep} neurons. However, since pirenperone also has affinity at 5-HT₇ receptors, we next tested whether a 5-HT₇ receptor antagonist could also block the excitatory response in some 5-HT_{Hyp-Dep} neurons.

Prior application of the 5-HT₇ receptor antagonist, SB 269970 (1 μ M) alone had no effect on the membrane properties of BNST_{ALG} neurons, but fully blocked the depolarizing response

to 5-HT or its associated inward current in 5 of the 10 neurons tested. In the remaining 5 neurons, SB 269970 attenuated the 5-HT-induced inward current to $41 \pm 4\%$ of baseline. Significantly, in 3 of 5 neurons that exhibited an attenuated excitatory response to 5-HT in the presence of SB 269970, co-application of SB 269970 and MDL 100907 fully blocked the residual 5-HT response. The inward current in the remaining 2 neurons was not changed by co-application of SB 269970 and MDL 100907. These data are summarized in Fig. 4A. While the number of neurons tested in these studies was not sufficient to determine conclusively the relative ability of the antagonists to block 5-HT excitation in BNST_{ALG} neurons, or whether the pharmacology of these responses differed according to BNST_{ALG} physiological subtype (see below, Hammack et al., 2007), they give clear evidence that the 5-HT-induced excitation in mixed 5-HT_{Hyp-Dep} BNST_{ALG} neurons can be mediated by 5-HT_{2A}, 5-HT_{2C}, and/or 5-HT₇ receptors. Moreover, in some 5-HT_{Hyp-Dep} neurons multiple receptors contribute to the excitatory response to 5-HT. Since 5-HT_{1A} receptors mediate the inhibitory response to 5-HT in these neurons, these data imply that 5-HT_{Hyp-Dep} neurons can express at least 3 functional 5-HT receptor subtypes that mediate their response to 5-HT.

Excitation in 5-HT_{Dep} neurons

As mentioned above, 34% of recorded BNST_{ALG} neurons exhibited a *pure* excitatory response to exogenous 5-HT (5-HT_{Dep}). Hence, we next examined whether the pure excitatory response was mediated by the activation of the same receptor population that mediated excitation in 5-HT_{Hyp-Dep} neurons. In 5-HT_{Dep} neurons, 5-HT induced an inward current, which associated with a significant increase of membrane conductance ($23.4 \pm 1.7\%$, $t(27) = 5.0$, $p < 0.001$), and had a reversal potential closing to that of non-selective cation channels (Table 2).

Similar to the strategy described for 5-HT_{Hyp-Dep} neurons (see above), we first examined whether pretreatment with pirenperone could block 5-HT-induced excitation in 5-HT_{Dep} neurons. In 3/5 neurons tested, 10 μ M pirenperone fully blocked the 5-HT-evoked inward current. In the remaining 2 neurons, pirenperone attenuated the 5-HT-induced inward current by $69 \pm 6\%$.

We next examined the effect of subtype-selective 5-HT receptor antagonists on 5-HT-induced inward current in 5-HT_{Dep} neurons. Prior application of the 5-HT_{2A} antagonist MDL 100907 (10 μ M) fully blocked the 5-HT induced inward current in 4/11 neurons tested, and attenuated the 5-HT evoked current in 6/11 neurons ($57.4 \pm 6.8\%$ of the initial 5-HT current). These data suggest that 5-HT_{2A} receptors mediate at least part of the 5-HT-induced excitatory response in the majority of 5-HT_{Dep} neurons.

We next determined the contribution of 5-HT_{2C} receptor activation to the excitatory response in 5-HT_{Dep} neurons. Pretreatment with the 5-HT_{2C} receptor antagonist RS 102221 (10 μ M) fully blocked the inward current in 2 of 8 neurons tested, attenuated the inward current in 1 neuron (30% of control), and had no noticeable effect in 5 neurons.

In contrast, prior application of the 5-HT₇ antagonist SB 269970 (1 μ M) fully blocked the 5-HT-evoked inward current in 17 of 27 5-HT_{Dep} neurons, and attenuated the inward current to $44 \pm 11\%$ of the amplitude of the initial 5-HT_{Dep} response in 6/27 neurons ($t(5) = 5.3$, $p < 0.01$). Prior application of SB 269970 had no noticeable effect in the remaining 4 neurons. Hence, activation of 5-HT₇ receptors mediated 5-HT-induced excitation in a significant population of 5-HT_{Dep} neurons.

Finally, we examined the effects of co-application of SB 269970 and MDL 100907 in 5-HT_{Dep} neurons that exhibited an attenuated 5-HT response in the presence of SB 269970. Here, co-application of the two antagonists fully blocked the residual 5-HT-induced inward current in all 5-HT_{Dep} neurons tested ($n = 4$). Hence, similar to 5-HT_{Hyp-Dep} BNST_{ALG} neurons, these

data suggest that the 5-HT-induced excitation in 5-HT_{Dep} neurons is predominantly mediated by 5-HT_{2A}, 5-HT_{2C}, and/or 5-HT₇ receptors. Moreover, in some 5-HT_{Dep} neurons multiple receptors contribute to the excitatory response to 5-HT. The relative efficacies of the three 5-HT receptor antagonists in blocking or attenuating the 5-HT_{Dep} response are illustrated in Fig. 4B.

Relative efficacy of 5-HT receptor subtype-selective agonists

As outlined above, both our PCR data and our antagonist studies showed that multiple 5-HT receptors are expressed in the BNST_{ALG}, and more than one receptor subtype can mediate the excitatory response to 5-HT application. Consequently, we next examined the ability of a selection of relatively selective 5-HT receptor agonists to mimic the 5-HT-induced excitation in BNST_{ALG} neurons.

We have previously shown that BNST_{ALG} inhibition by 5-HT was mediated by 5-HT_{1A} receptors, and is always blocked in the presence of a 5-HT_{1A} antagonist (Levita et al., 2004). Consistent with this report, none of the following 5-HT₁ receptor-selective agonists evoked an observable response in any BNST_{ALG} neuron tested: the selective 5-HT_{1B/D} agonist, sumatriptan (10 μ M; $n = 11$), the selective 5-HT_{1B} agonist, CP 93129 (10 μ M, $n = 10$), the selective 5-HT_{1E/F} receptor agonist, BRL54443 (5 – 75 μ M, $n = 4$), or the selective 5-HT_{1F} agonist LY 344864 (10 μ M, $n = 4$).

Conversely, application of the putative broad spectrum 5-HT₂ receptor agonist, α -methyl 5-HT (25–250 μ M) mimicked both the inhibitory and the excitatory response to 5-HT, irrespective of the concentration applied ($n = 7$). Similar results were observed for the 5-HT derivative 5-methoxytryptamine ($n = 3$, 50–250 μ M). The inhibitory action of these agonists were likely mediated by an action at 5-HT_{1A} receptors, and consistent with this interpretation, inhibition was blocked in the presence of the 5-HT_{1A} antagonist WAY 100635. Hence, we next tested the response of BNST_{ALG} neurons to exogenous application of more selective 5-HT₂ receptor subtype agonists. Surprisingly, the selective 5-HT_{2A} agonists, DOI (5 – 50 μ M; $n = 5$), which has been shown to increase *c-fos* expression in BNST_{ALG} neurons (Van de Kar et al., 2001), and TCB 2 (10 μ M, $n = 6$) failed to mimic the excitatory response to 5-HT. Similarly, the potent and selective 5-HT_{2C} agonist, WAY 161503 (10 μ M, $n = 7$) failed to mimic the excitatory response in any BNST_{ALG} neuron tested. In contrast, application of the relatively selective 5-HT_{2B/2C} receptor agonist, mCPP (5–75 μ M), elicited a depolarizing response in 6/14 neurons tested, had no effect in 6/14 neurons, and elicited a hyperpolarizing response in the remaining 2 neurons.

We reasoned that the 5-HT₃ receptor was unlikely to mediate the slow onset and long duration excitatory response due to its rapid activation and desensitization kinetics (Yang et al., 1992, van Hooft and Vijverberg, 1997). As expected, the 5-HT₃ receptor agonist, mCPBG (50 μ M), which can induce a transient inward current in cortical slices (Zhou and Hablitz, 1999), had no effect on either the resting membrane potential or membrane input resistance in 9/10 BNST_{ALG} neuron tested. Similarly, the selective 5-HT₄ receptor agonist, cisapride (10 μ M) (Roychowdhury et al., 1994, Xiang et al., 2005), failed to induce a membrane depolarization in any BNST_{ALG} neuron tested ($n = 12$); however, it did induce a small hyperpolarization in 2 neurons that was accompanied by a decrease of input resistance.

Finally, we examined whether the mixed 5-HT_{1/7} agonist, 5-CT (10 μ M), could mimic the depolarizing response of 5-HT in the presence of the 5-HT_{1A} receptor antagonist WAY 100635 (200 nM). In 50% (9/18) of BNST_{ALG} neurons, 5-CT evoked an inward current that was associated with an $18.9 \pm 4.4\%$ increase in conductance ($t(8) = 3.8$, $p < 0.01$), and a reversal potential of -48.0 ± 3.1 mV (Fig. 5A,B). Moreover, the 5-CT reversal potential did not differ significantly from the E_{5-HT} (-48.9 ± 1.3 mV, $t(33) = 0.31$, $p = 0.76$) for the 5-HT_{Dep} response,

suggesting that 5-CT evoked excitation might be mediated by the same cellular mechanisms as the pure depolarizing response to 5-HT.

Because the effects of 5-CT were not readily reversible on washout with ACSF, we were prevented from blocking the 5-CT-evoked depolarization using a within-subjects design. Hence, we next examined the effects of co-application of WAY 100635 and the 5-HT₇ antagonist SB269970 (1 μM) on the 5-CT induced excitatory response in BNST_{ALG} neurons. In the presence of SB 269970 and WAY 100635, 5-CT failed to induce an inward or outward current in any BNST_{ALG} neuron tested (n = 7, see Fig. 5C; $\chi^2 = 5.459$, df = 1, p < 0.05; Fig. 5D). These results suggest that 5-CT induced an excitatory response in the presence of WAY 100635 that was mediated by 5-HT₇ receptors. Significantly, in 3 of 4 BNST_{ALG} neurons that exhibited an initial 5-HT_{Hyp-Dep} response, subsequent application of 5-CT in the presence of WAY 100635 induced an inward current (5.3 ± 0.9 pA) that was accompanied by an $14.2 \pm 6.3\%$ increase in membrane conductance (baseline 4.03 ± 1.20 nS, 5-CT 4.74 ± 1.54 nS), which had a reversal potential of -52.5 ± 4.1 mV. These data confirmed the results of our antagonist study and support the contention that 5-HT₇ receptor activation plays a significant role in the excitatory response to 5-HT in 5-HT_{Hyp-Dep} neurons. However, the lack of response of BNST_{ALG} neurons to several selective 5-HT receptor agonists and the questionable selectivity of some of the commercially available antagonists hindered definitive statements about the true expression pattern of 5-HT receptors in BNST_{ALG}.

Expression of 5-HT receptor mRNA in individual BNST_{ALG} neurons

In an attempt to further clarify the expression pattern of 5-HT receptors in individual BNST_{ALG} neurons we extracted cytosolic mRNA from physiologically identified neurons and then used single-cell RT-PCR to examine their 5-HT receptor mRNA expression profile.

Signal for the mRNA transcripts of at least one 5-HT receptor subtype (Fig 6A) was detected in 67/82 BNST_{ALG} neurons sampled (82%). Of the four 5-HT receptor subtypes initially screened, the percentage of the 82 BNST_{ALG} neurons expressing each receptor was: 5-HT₇ receptor (46%), > 5-HT_{1A} receptor (41%) > 5-HT_{2A} receptor (17%) > 5-HT_{2C} receptor (16%), and transcripts for each 5-HT receptor subtype were expressed either alone (43%) or in combination (39%; Fig 6A, B). For example, of those neurons that express 5-HT₇ mRNA, 33% co-express mRNA for other 5-HT receptor subtypes (5-HT_{1A/7}, 23%; 5-HT_{2A/7} 10%;), whereas only 13% expressed 5-HT₇ receptor mRNA alone. Similarly, while 41% of sampled BNST_{ALG} neurons expressed 5-HT_{1A} mRNA, 28% co-expressed 5-HT_{1A} mRNA with either 5-HT_{2A} and/or 5-HT₇ receptor mRNA and only 13% of these neurons express 5-HT_{1A} mRNA alone. Significantly, 5-HT_{2A} receptor transcripts were almost always detected with other 5-HT receptor subtypes (92% of sample). In contrast, 5-HT_{2C} transcripts were rarely detected with the mRNA for other 5-HT receptor subtypes. Of the 13 BNST_{ALG} neurons in which 5-HT_{2C} mRNA was detected (16%), 12 expressed only 5-HT_{2C} mRNA (see Fig. 6B).

Comparison of the single-cell 5-HT receptor mRNA expression profile and the observed response to 5-HT in BNST_{ALG} neurons

In the present study, we examined the 5-HT response from a total of 148 BNST_{ALG} neurons. In agreement with our previous study (Levita et al., 2004), BNST_{ALG} neurons responded to the application of 5-HT in any one of four distinct ways: 5-HT_{Hyp-Dep} (28%), 5-HT_{Dep} (35%), 5-HT_{Hyp} (16%), and 5-HT_{NR} (21%). As illustrated in Fig. 8, the 5-HT response profile predicted by BNST_{ALG} 5-HT receptor mRNA expression (5-HT_{Hyp-Dep} 28%, 5-HT_{Dep} 40%, 5-HT_{Hyp} 13%, and 5-HT_{NR} 18%) was not statistically different from the observed 5-HT response profile of BNST_{ALG} neurons, (Chi Square test, $\chi^2 = 1.03$, df = 3, p = 0.80), suggesting that the mRNA expression profile in individual BNST_{ALG} neurons is a true reflection of their relative 5-HT receptor distribution. Consistent with this hypothesis, the relative expression of

5-HT₇ mRNA (46%) was similar to the percentage of excitatory responses induced by 5-CT in BNST_{ALG} neurons (50%) (Chi Square test, $\chi^2 = 0.08$, $df = 1$, $p = 0.78$).

Cell type-specific 5-HT receptor subtype expression

In the study above, we initially sampled mRNA from some cells (~30) without determining the physiological properties of the cell. In the BNST_{ALG}, three physiologically distinct cell types (Type I–III) have been identified based on their differential expression of sub-threshold membrane currents (Hammack et al., 2007). Briefly, Type I neurons were characterized by a depolarizing sag in response to hyperpolarizing current injection that was mediated by the hyperpolarization-activated cation current, I_h and a regular firing pattern in response to depolarization current injection. Type II neurons also expressed I_h , and exhibited a burst-firing pattern that was mediated by activation of the low-threshold calcium current, I_T . Type III neurons did not exhibit characteristics of I_h or I_T expression, but instead exhibited a pronounced inwardly rectifying potassium current, $I_{K(IR)}$. Hence, we next explored the relative expression patterns of 5-HT receptor subtype mRNA in Type I–III neurons. Transcripts for 5-HT receptor subtypes were detected in 7/9 Type I, 37/44 Type II, and 9/12 Type III BNST_{ALG} neurons. The relative expression of each of the 5-HT receptor transcripts are summarized in Fig. 8A. Thus Type I neurons express high levels of 5-HT_{1A} mRNA (56%), Type II neurons express high levels of 5-HT₇ (57%) and 5-HT_{1A} (45%) mRNA, and Type III neurons express high levels of 5-HT_{2C} (58%) mRNA. Significantly, no 5-HT_{2A} and 5-HT₇ mRNA was detected in Type I and Type III neurons, respectively. These results suggest that the three physiological subtypes of BNST_{ALG} neurons not only differ in their physiological properties (Hammack et al., 2007), but also in their 5-HT receptor subtype expression.

Consequently we wanted to determine if the response to 5-HT was cell type specific. Hence, we mapped the 5-HT response profile across the three cell types. The relative distribution of 5-HT responses among 39 Type I, 85 Type II, and 24 Type III neurons is summarized in Fig. 8B. A Chi square analysis revealed a significant difference in the response of the three cell types to 5-HT ($\chi^2 = 15.35$, $df = 6$, $p = 0.018$). To find out the difference between groups, we next compared two groups at a time. No significant difference was observed between the response of Type I and Type II neurons, although a trend was suggested ($\chi^2 = 6.74$, $df = 3$, $p = 0.08$). No difference was found between Type I and Type III neurons ($\chi^2 = 3.58$, $df = 3$, $p = 0.31$). However, the 5-HT response of Type II neurons was significantly different from that of Type III neurons ($\chi^2 = 10.32$, $df = 3$, $p = 0.016$). We then calculated the standardized residuals (R) to determine which response caused the difference between the Type II and Type III neurons. The standardized residual for the 5-HT_{NR} of Type III neurons has a higher magnitude (R=2.22) than the criteria value corresponding to α of 0.05 (1.96), suggesting that Type III neurons had a higher percentage of 5-HT_{NR} than Type II neurons. This analysis was consistent with our observation that Type III neurons had a higher percentage of 5-HT_{NR} (42%) than Type II neurons (14%, Fig. 8).

Discussion

The results outlined above strongly suggest that neuronal activity in the BNST_{ALG} is bidirectionally regulated by 5-HT through complex interactions between multiple 5-HT receptor subtypes. We previously demonstrated that the hyperpolarizing response of BNST_{ALG} neurons to 5-HT was mediated by activation of 5-HT_{1A} receptors coupled to G protein-gated inwardly-rectifying potassium channels, and that *in vivo* activation of BNST_{ALG} 5-HT_{1A} receptors reduced anxiety-like behavior (Levita et al., 2004). In the present report, we used RT-PCR to detect the expression of mRNA transcripts for multiple 5-HT receptor subtypes in BNST_{ALG} tissue and also single BNST_{ALG} neurons. Our PCR data clearly suggest that at least three 5-HT receptor subtypes mediate the excitatory effects of 5-HT on

BNST_{ALG} neurons, including 5-HT_{2A}, 5-HT_{2C}, and/or 5-HT₇ receptors. Moreover, our PCR data confirmed our pharmacological and physiological observations that individual BNST_{ALG} neurons express distinct combinations of 5-HT receptor subtypes. Lastly, we showed that the 5-HT receptor mRNA expression and 5-HT response pattern was cell type specific, suggesting that Type I – III BNST_{ALG} neurons would respond differently to local 5-HT release, which raises the possibility that 5-HT receptor activation may also differentially alter the intrinsic membrane properties of discrete populations of BNST_{ALG} neurons.

5-HT receptor subtypes in the BNST_{ALG}

5-HT neurons in the caudal regions of the dorsal raphe nucleus (DRN) selectively innervate the BNST_{ALG} (Phelix et al., 1992, Commons et al., 2003) where 5-HT fibers have been observed to make contact with BNST neurons (Phelix *et al*, 1992). In response to unpredictable stress, 5-HT levels dramatically increase in caudal DRN projection regions (Amat et al., 1998a, 1998b), and would be expected to increase in the BNST_{ALG}. Hence, the current study was designed to determine the response of BNST_{ALG} neurons to stress-induced 5-HT release. While several studies have demonstrated the presence of multiple 5-HT receptor subtypes within the BNST (Mengod et al., 1990, Waeber et al., 1994, To et al., 1995, Waeber and Moskowitz, 1995, Wright et al., 1995, Kia et al., 1996, Heidmann et al., 1998, Sari et al., 1998, Cornea-Hebert et al., 1999), none have specifically investigated the distribution of these receptors within the BNST_{ALG}. Consistent with these earlier studies we have shown whole tissue mRNA expression for multiple 5-HT receptor subtypes in the BNST_{ALG}, including 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2C}, 5-HT₃, 5-HT₄, 5-HT_{5A}, 5-HT₆, and 5-HT₇ receptors. The cellular localization of these receptors is currently unknown, although it is likely that many of these receptors are somatodendritic, while others, such as the 5-HT_{1B} receptor, may be located presynaptically in axon terminals. However a glial localization for some of these receptor subtypes cannot be ruled out (Fillion et al., 1983, Huang et al., 2004, Patel and Zhou, 2005). Nevertheless, our single-cell RT-PCR data clearly show that at least 4 of these receptors are functionally expressed in the neuronal population of the BNST_{ALG}.

The diversity of 5-HT receptor subtype expression together with the high degree of heterogeneity in BNST neuronal cytoarchitecture (McDonald, 1983, Ju and Swanson, 1989, Larriva-Sahd, 2004), chemoarchitecture (Ju et al., 1989, Walter et al., 1991, Gray and Magnuson, 1992, Day et al., 2004), and physiology (Rainnie, 1999, Egli and Winder, 2003, Hammack et al., 2007), suggest that 5-HT release would induce a complex response profile depending on the neuronal population affected, which may account for the complex relationship between 5-HT receptor activation and anxiety-like behavior (Handley et al., 1993, Handley, 1995, Graeff et al., 1996, Lowry et al., 2005, Lowry et al., 2008).

5-HT receptor subtypes mediating the excitatory response to 5-HT

Previously we have demonstrated that a single receptor population mediates the 5-HT_{Hyp} response in BNST_{ALG} neurons, namely 5-HT_{1A} receptors (Levita et al., 2004). However, the nature of the 5-HT_{Hyp-Dep} response was less clear. Preliminary studies suggested that it resulted from the activation of two separate currents, rather than the activation and passive decay of a 5-HT_{1A} receptor-mediated outward current. This notion was supported by the observation that the reversal potential for 5-HT in 5-HT_{Hyp-Dep} neurons was significantly more depolarized at steady-state as compared to the peak of the response, and that the steady-state reversal potential was always associated with an increase in membrane conductance. These data were consistent with an activation of multiple currents and suggested that in 5-HT_{Hyp-Dep} neurons, the initial membrane hyperpolarization and associated outward current were followed by a slower overlapping membrane depolarization and associated inward current. As expected, when 5-HT was applied in the presence of the 5-HT_{1A} receptor antagonist, WAY 100635, a monophasic inward current was unmasked. The characteristics of the inward current (i.e. amplitude and

reversal potential) were similar to those observed in 5-HT_{Dep} neurons; hence, it was likely that the depolarization response in these two populations of BNST_{ALG} neurons was mediated by the same mechanism(s). Using subtype-selective antagonists, we further demonstrated that the 5-HT-induced membrane depolarization and associated inward current in 5-HT_{Hyp-Dep} and 5-HT_{Dep} neurons were mediated by three 5-HT receptor subtypes, 5-HT_{2A}, 5-HT_{2C}, and 5-HT₇.

5-HT_{2A} receptors—Here we have shown that approximately 17% of BNST_{ALG} neurons express 5-HT_{2A} receptors. Significantly, 5-HT_{2A} receptor transcripts were almost always detected with other 5-HT receptor subtypes, most notably with 5-HT₇ receptors in Type II BNST_{ALG} neurons. Activation of 5-HT_{2A} receptors in the BNST_{ALG} elicits a membrane depolarization that was associated with an inward current, an increase in membrane conductance, and a reversal potential of ~ -48 mV. These data are consistent with the activation of cation channels rather closure of potassium channels, which is consistent with reports from other brain regions which suggest that 5-HT_{2A} receptor activation may be linked through Gq/11 (Hoyer et al., 2002) to activation of a non-selective cation current (Hardy et al., 2005).

Interestingly, in some 5-HT_{Hyp-Dep} and some 5-HT_{Dep} neurons, the 5-HT-induced membrane depolarization or associated inward current was attenuated in the presence of MDL 100907, but not blocked, and the residual 5-HT depolarization was only blocked completely only when MDL 100907 was combined with another subtype selective 5-HT antagonist (see below), suggesting that in subpopulations of BNST_{ALG} neurons the excitatory postsynaptic response to 5-HT was mediated by activation of at least two 5-HT receptor subtypes.

The broad spectrum 5-HT₂ receptor agonists α -methyl 5-HT and 5-methoxytryptamine mimicked both the inhibitory and excitatory response produced by 5-HT; however, the inhibitory response was blocked in the presence of the 5-HT_{1A} antagonist WAY 100635, suggesting that the concentrations applied, these agonists were activating multiple 5-HT receptor subtypes. Interestingly, more selective 5-HT₂ receptor subtype agonists such as DOI, TCB 2 and WAY 161503 failed to mimic the excitatory response to 5-HT. While it is unknown why these agonists did not mimic the excitatory response to 5-HT, these results were particularly surprising given that systemically administered DOI has been shown to produce robust *c-fos* expression in the BNST (Leslie et al., 1993, Van de Kar et al., 2001). However, DOI induced cortical *c-fos* expression is thought to be an indirect process, such that *c-fos* expression was only observed in neurons that do not express 5-HT_{2A} receptors (Mackowiak et al., 1999). Indeed, Scruggs et al (2000) found that DOI-induced activation of the cortex was dependent on activation of 5-HT_{2A} receptors on thalamocortical neurons (Scruggs et al., 2000). Hence, DOI-induced *c-fos* expression in the BNST may be mediated by similar indirect pathways.

5-HT_{2C} receptors—In contrast to the expression of 5-HT_{2A} receptors, the expression of 5-HT_{2C} receptors appeared to be mutually exclusive with the expression of any other 5-HT receptor subtype. Hence, ~ 16 % of BNST_{ALG} neurons express only 5-HT_{2C} transcripts. Significantly, 58% of Type III neurons express 5-HT_{2C} receptor transcripts suggesting that this receptor may be expressed in a unique subpopulation of Type III neurons. Consistent with this hypothesis, 5-HT_{2C} receptors are preferentially expressed by dynorphin containing neurons in the striatal patch compartment (Ward and Dorsa, 1996). Moreover, a recent report by Dumont and colleagues showed that the properties of BNST_{ALG} neurons projecting to the ventral tegmental area were similar to that of Type III neurons (Dumont et al., 2008). If these neurons were to preferentially express 5-HT_{2C} receptors it could provide mechanism by which 5-HT could fine-tune the output of select cell types. However, a larger sample population of Type III neurons will be required before the cell-type selective expression can be confirmed.

Nonetheless, activation of 5-HT_{2C} receptors has been demonstrated to mediate a similar membrane depolarization or an inward current to 5-HT in several brain regions, including the pontine reticular formation (Weber et al., 2008), striatum, and subthalamic nuclei (Rick et al., 1995, Hardy et al., 2005, Xiang et al., 2005, Bonsi et al., 2007). Significantly, in several of these studies the depolarization was reported to result from a PLC-dependent reduction in potassium conductance. However, in the present study, the 5-HT_{2B/2C} agonist mCPP induced a membrane depolarization, that was associated with an increase membrane conductance in most neurons, suggesting that 5-HT_{2C} receptors in the BNST_{ALG} may couple through different receptor-effector mechanisms than elsewhere in the brain.

It should be noted that the 5-HT_{2C} receptor antagonist RS102221 also has a low affinity at noradrenergic receptors (pK_i <5 for α₁, <4 for β receptors) (Bonhaus et al., 1997). Dumont and Williams have shown that noradrenergic agonists can excite neurons of the lateral BNST (Dumont and Williams, 2004). Hence, it cannot be discounted that some of the action of this compound may be due to the blockade of an indirect release of norepinephrine within the BNST_{ALG}. However, high concentrations of norepinephrine are required for postsynaptic excitation of BNST_{ALG} neurons (Dumont and Williams, 2004), and the low affinity of RS102221 to noradrenergic receptors suggests that this action of RS102221 is unlikely to block any indirect excitatory actions of norepinephrine.

Consistent with a direct excitatory response to 5-HT_{2C} receptor activation in the BNST, systemic injection of mCPP produced an anxiogenic response in several animal models of anxiety, including the social interaction test, light/dark box and zero maze (Kennett et al., 1989, Shepherd et al., 1994). Moreover, mCPP has also been shown to increase anxiety in humans (Silverstone et al., 1994, Broocks et al., 2001), and increases Fos expression in the BNST_{ALG} (Singewald et al., 2003). Together, these data suggest that activation of 5-HT_{2C} receptors on Type III BNST_{ALG} neurons might be an important locus for mediating the anxiogenic effects of mCPP.

5-HT₇ receptors—It is noteworthy that transcripts for 5-HT₇ receptors were found in 46% of all BNST_{ALG} neurons examined, and yet no Type III neuron examined to date has shown any 5-HT₇ receptor mRNA expression. Moreover, 5-HT₇ receptor expression was often found in association with 5-HT_{1A} receptor expression suggesting that the bidirectional 5-HT_{Hyp-Dep} response in BNST_{ALG} neurons is most likely mediated by activation of these two receptors, respectively. Moreover, because a combination of MDL 100907 and SB269970 was typically required to fully block 5-HT-depolarization, it is likely that many BNST_{ALG} neurons co-express both 5-HT_{2A} and 5-HT₇ receptors.

Consistent with an excitatory response to 5-HT₇ receptor activation, these receptors have been shown to link through G_s (Hoyer et al., 2002), and mediate depolarization in other brain regions, including the paraventricular nucleus of the hypothalamus (Ho et al., 2007), thalamus (Chapin and Andrade, 2001), hippocampus (Tokarski et al., 2003), and cholinergic interneurons of the striatum (Bonsi et al., 2007). As mentioned above, in the BNST_{ALG} the 5-HT-induced depolarization was associated with an inward current, an increase in membrane conductance, and a reversal potential of ~ -48 mV. In the thalamus and dorsal root ganglia the depolarizing response to 5-HT₇ receptor activation has been reported to be mediated by an increase in membrane conductance that resulted from a rightward shift in the voltage dependence of I_h channel activation (Cardenas et al., 1999, Chapin and Andrade, 2001). Future studies will examine the effects of 5-HT on I_h channel activation in BNST_{ALG} neurons.

In vivo studies with 5-HT₇ receptor antagonists, such as SB 269970, have revealed a potential role for 5-HT₇ receptor activation in novel object recognition (Ballaz et al., 2007), stereotypic behavior (Hedlund and Sutcliffe, 2007), as well as anxiogenic and depressive-like behavior

(Wesolowska et al., 2006, Nandam et al., 2007). Moreover, 5-HT₇ knockout mice exhibit impaired contextual fear conditioning (Roberts et al., 2004), and decreased immobility in the Porsolt swim test (Guscott et al., 2005). Both of these effects were consistent with a naturally anxiogenic or depressive effect of 5-HT₇ receptor activation. The high expression of 5-HT₇ receptors in Type I and II BNST_{ALG} neurons suggest cell-specific activation of these receptors likely play a role in modulating these behavioral effects.

Other 5-HT receptor subtypes—Given our previous data (Levita et al., 2004), it was not surprising that receptors of the 5-HT₁ family do not appear to mediate the excitatory response to 5-HT in BNST_{ALG} neurons. Moreover, although we demonstrated the presence of 5-HT₃ receptor transcript in BNST_{ALG} tissue, the response elicited by this ionotropic receptor is rapid and desensitizes rapidly (van Hooft and Vijverberg, 1997), and hence was unlikely to mediate the slow onset depolarizing response to 5-HT. Consistent with this hypothesis the 5-HT₃ receptor agonist mCPBG did not mimic the excitatory response in any neuron tested. We also confirmed that moderate levels of 5-HT₄ receptors are expressed in the BNST_{ALG} (Waeber et al., 1994). However, no effect of the 5-HT₄ agonist, cisapride, was observed in the present study. Because some combination of 5-HT_{2A}, 5-HT_{2C}, and/or 5-HT₇ antagonists were able to block the entire excitatory response to 5-HT in all BNST_{ALG} neurons, it is likely that only these three 5-HT receptor subtypes mediate the slow membrane depolarization in BNST_{ALG} neurons.

Expression of 5-HT receptor subtypes in single neurons

First, no 5-HT receptor mRNA was detected in 18% of the BNST_{ALG} neurons tested, which was similar to the percentage of neurons that failed to respond to 5-HT in our electrophysiological studies (21%). Second, the expression patterns of 5-HT receptor subtypes in single BNST_{ALG} neurons was consistent with the pharmacological response profiles that we observed in our electrophysiological studies, such that some neurons only expressed 5-HT_{1A} mRNA (consistent with 5-HT_{Hyp}), some neurons co-expressed 5-HT_{1A} mRNA with 5-HT_{2A} or 5-HT₇ (consistent with 5-HT_{Hyp-Dep}), and some neurons expressed only 5-HT_{2A}, 5-HT_{2C}, or 5-HT₇ mRNA, or co-expressed 5-HT_{2A/2C}, 5-HT_{2A/7} mRNA (consistent with 5-HT_{Dep}). Third, the distribution of 5-HT responses predicted by the receptor mRNA expression profile did not statistically differ from the observed distribution of 5-HT responses in BNST_{ALG} neurons. Hence, converging evidence suggested that most BNST_{ALG} neurons expressed more than one 5-HT receptor subtype, with 5-HT₇ receptors and 5-HT_{1A} receptors being the most prevalent.

Neither the observed 5-HT response distributions, nor the expression of 5-HT receptor subtypes were uniformly distributed across the three BNST_{ALG} cell types. For example, Type III neurons expressed a higher percentage of 5-HT_{NR}, which corresponded to a higher percentage of cells that did not express any 5-HT receptor subtype. While the observed 5-HT receptor subtype mRNA distribution was consistent with the observed 5-HT response in the Type II neurons, some discrepancy was observed between these two measures in type I and type III neurons (Figure 8). This inconsistency in type I and type III was likely due to the smaller sample size obtained for these BNST_{ALG} cell types. Type II neurons are the most prevalent cell type in BNST_{ALG} (Hammack et al. 2007); hence, more observations were made in this cell type..

While there are limitations to all of the techniques used in this study, the convergence of pharmacological, electrophysiological and molecular data strongly support the conclusion that the complex interactions of multiple 5-HT receptor subtypes, including 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, and 5-HT₇ receptors, modulate BNST_{ALG} activity, and likely play an important role in modulating anxiety-like behavior.

The significance of opposing 5-HT actions

Multiple 5-HT receptor subtypes that have functionally opposing responses have been shown to colocalize on single neurons in other regions of the central nervous system (Roychowdhury et al., 1994, Martin-Ruiz et al., 2001, Zhang et al., 2004). The function of having multiple opposing 5-HT responses in single neurons has been postulated by several investigators. Araneda and Andrade (1991) have suggested that 1) multiple receptors on the same neuron could be targeted by different populations of serotonergic fibers and 2) multiple responses could act to “fine tune” the ability of 5-HT to potentiate excitatory stimulation, perhaps by increasing the signal-to-noise ratio of small and large input (Araneda and Andrade, 1991). Uphouse (1997) proposed that multiple 5-HT receptors on the same neurons increases the variety of signals that can be produced by 5-HT, so that in multiple receptor systems the pattern of the signal, and not just signal strength, are critical in determining the effect of transmission (Uphouse, 1997). Lastly, interactions between the effector systems of different 5-HT receptor subtypes can interact (Uphouse, 1997, Berg et al., 1998) and change the response of each receptor to subsequent agonist application.

We have proposed that the capacity for BNST_{ALG} neurons to respond to 5-HT with both inhibition and excitation might help explain some of the inconsistencies regarding the role of 5-HT in anxiety-like responding (Hammack et al., 2009). Many treatments that increase 5-HT levels have been shown to both increase and decrease anxiety and anxiety-like behaviors (see (Handley et al., 1993, Handley, 1995, Graeff et al., 1996, Lowry et al., 2005, Lowry et al., 2008) for review), and we have suggested that these effects are mediated, in part, by the excitation and/or inhibition of BNST_{ALG} neurons by 5-HT. If BNST_{ALG} activity mediates anxiety-like behavior, then the net effect of 5-HT release within this region will depend on multiple factors, including the extracellular concentration, the affinity of each 5-HT receptor subtype, the balance of receptor subtypes, and the potential interactions between receptor subtypes. Because we have shown that the inhibitory response to 5-HT predominates in naïve animals in most BNST_{ALG} neurons, we have proposed that the normal function of 5-HT release in the BNST_{ALG} is to dampen neuronal activity following exposure to threatening stimuli to prevent an over-activation of this anxiety-related circuitry (Hammack et al., 2009).

However, treatments such as chronic stress or the chronic administration of stress hormones have been shown to decrease 5-HT_{1A} and/or increase 5-HT_{2A} receptors in other brain regions (Kuroda et al., 1992, Ferretti et al., 1995, McKittrick et al., 1995, Crayton et al., 1996, Fernandes et al., 1997, Takao et al., 1997, Lopez et al., 1998, Lanfumey et al., 1999, Lopez et al., 1999, Maines et al., 1999, Katagiri et al., 2001, Ossowska et al., 2001). A reduction in BNST_{ALG} 5-HT_{1A} receptor activity and/or increase 5-HT_{2A} receptor activity would favor an anxiogenic role of BNST_{ALG} 5-HT release, and could disrupt the normally anxiolytic role of 5-HT and produce a behavioral state characterized by pathological anxiety. Further studies are necessary to clarify the specific function of 5-HT receptors in subpopulations of BNST_{ALG} neurons, as well as its influence on the network properties of the BNST_{ALG}.

Summary

In summary, BNST_{ALG} neurons can respond to 5-HT with a complex response pattern. 5-HT_{1A} receptors mediate the inhibitory component of 5-HT_{Hyp-Dep} response, whereas the excitatory component of 5-HT_{Hyp-Dep} and 5-HT_{Dep} response can be mediated by 5-HT_{2A}, 5-HT_{2C}, or 5-HT₇ receptors. The presence of multiple 5-HT receptors on single BNST_{ALG} neurons permits 5-HT to exhibit a much greater variety of signaling than one receptor would allow; furthermore, the net effect on anxiety-like behaviors might depend on the net interaction between the inhibitory and excitatory responses, and exposure to stress or stress hormones or therapeutic pharmacological agents could alter the balance of BNST_{ALG} 5-HT receptors to favor excitation or inhibition, respectively, and this change could underlie the mechanisms by

which chronic stress produces anxiety-like behavioral pathologies, as well as the mechanism for therapeutic action of many anxiolytic drugs.

Abbreviations

5-CT, 5-carboxamidotryptamine
 5-HT, 5-hydroxytryptamine, serotonin
 5-HT_{1A}, serotonin 1A receptor
 5-HT_{1B}, serotonin 1B receptor
 5-HT_{1D}, serotonin 1D receptor
 5-HT_{1E}, serotonin 1E receptor
 5-HT_{1F}, serotonin 1F receptor
 5-HT_{2A}, serotonin 2A receptor
 5-HT_{2B}, serotonin 2B receptor
 5-HT_{2C}, serotonin 2C receptor
 5-HT₃, serotonin 3 receptor
 5-HT₄, serotonin 4 receptor
 5-HT₅, serotonin 5 receptor
 5-HT₆, serotonin 6 receptor
 5-HT₇, serotonin 7 receptor
 5-HT_{Dep}, 5-HT induced depolarization
 5-HT_{Hyp}, 5-HT induced hyperpolarization
 5-HT_{Hyp-Dep}, 5-HT induced hyperpolarization followed by depolarization
 5-HT_{NR}, no response to 5-HT
 ACSF, artificial cerebrospinal fluid
 BNST, bed nucleus of stria terminalis
 BNST_{ALG}, anterolateral bed nucleus of the stria terminalis
 E_{5-CT}, reversal potential of 5-CT response
 E_{5-HT}, reversal potential of 5-HT response
 GAPDH, glyceraldehyde-3-phosphate dehydrogenase
 PCR, polymerase chain reaction
 RT-PCR, reverse transcriptase polymerase chain reaction
 SSRI, selective serotonin reuptake inhibitors

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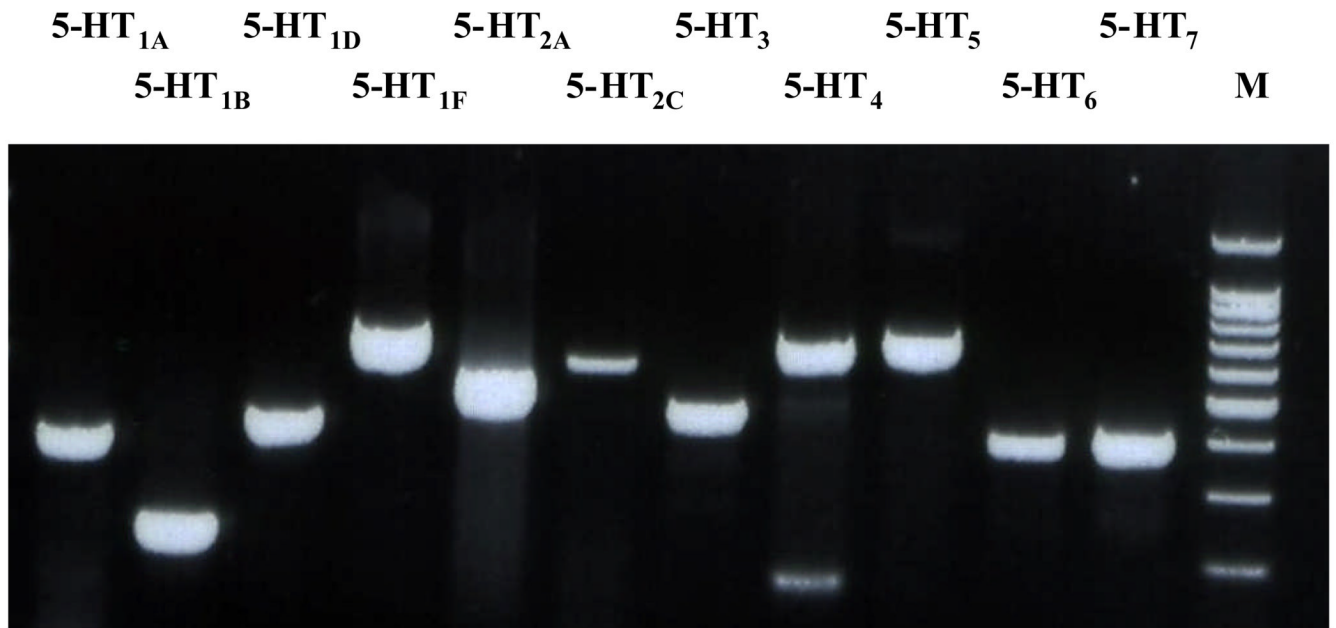


Figure 1. Photograph of an agarose gel showing the expression of 5-HT receptor subtypes mRNA in the BNST_{ALG} tissue. Whole BNST_{ALG} tissue was dissected from 500 μ m BNST slices. M denotes a molecular weight marker.

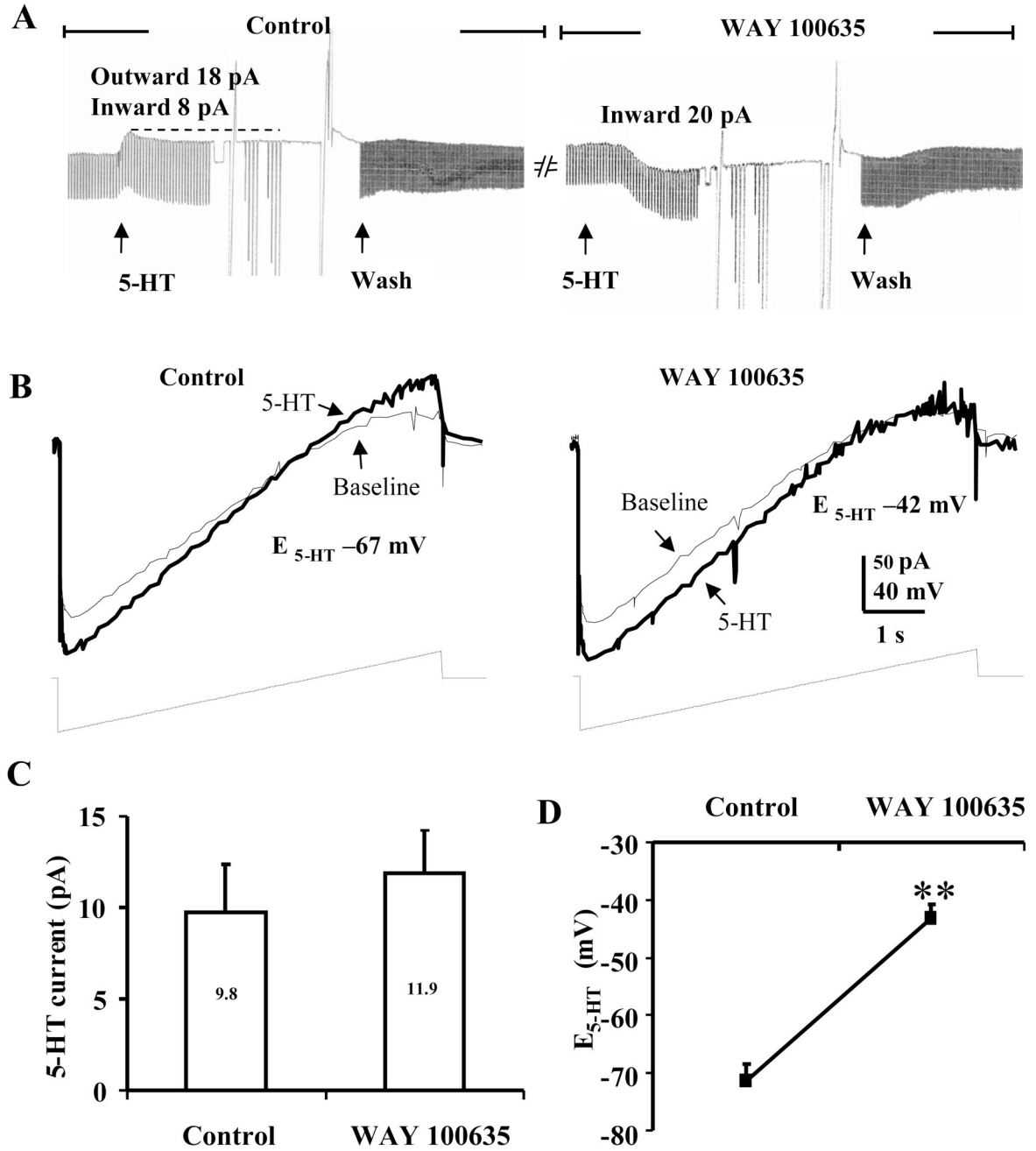


Figure 2. Monophasic inward current to 5-HT unmasked by the 5-HT_{1A} antagonist, WAY 100635 in BNST_{ALG} neurons that initially exhibited a 5-HT_{Hyp-Dep} response to 5-HT. **A**, Application of 5-HT_{1A} antagonist WAY 100635 (200 nM) unmasked an inward current. **B**, Ramp traces showing the shift of E_{5-HT} to more depolarized level of -42 mV when 5-HT was applied in the presence of WAY 100635, which suggested the activation of a non-selective cation current. **C**, Histograms showing the inward currents elicited by 5-HT in the control condition (9.8 ± 2.6 pA, $n = 9$) and in the presence of WAY 100635 (11.9 ± 2.3 pA, $n = 9$). **D**, In the presence of WAY 100635, E_{5-HT} was significantly shifted from the control at -71.4 ± 2.9 mV to -43.2 ± 2.4 mV, $n=9$. **, paired t-test, $p < 0.01$.

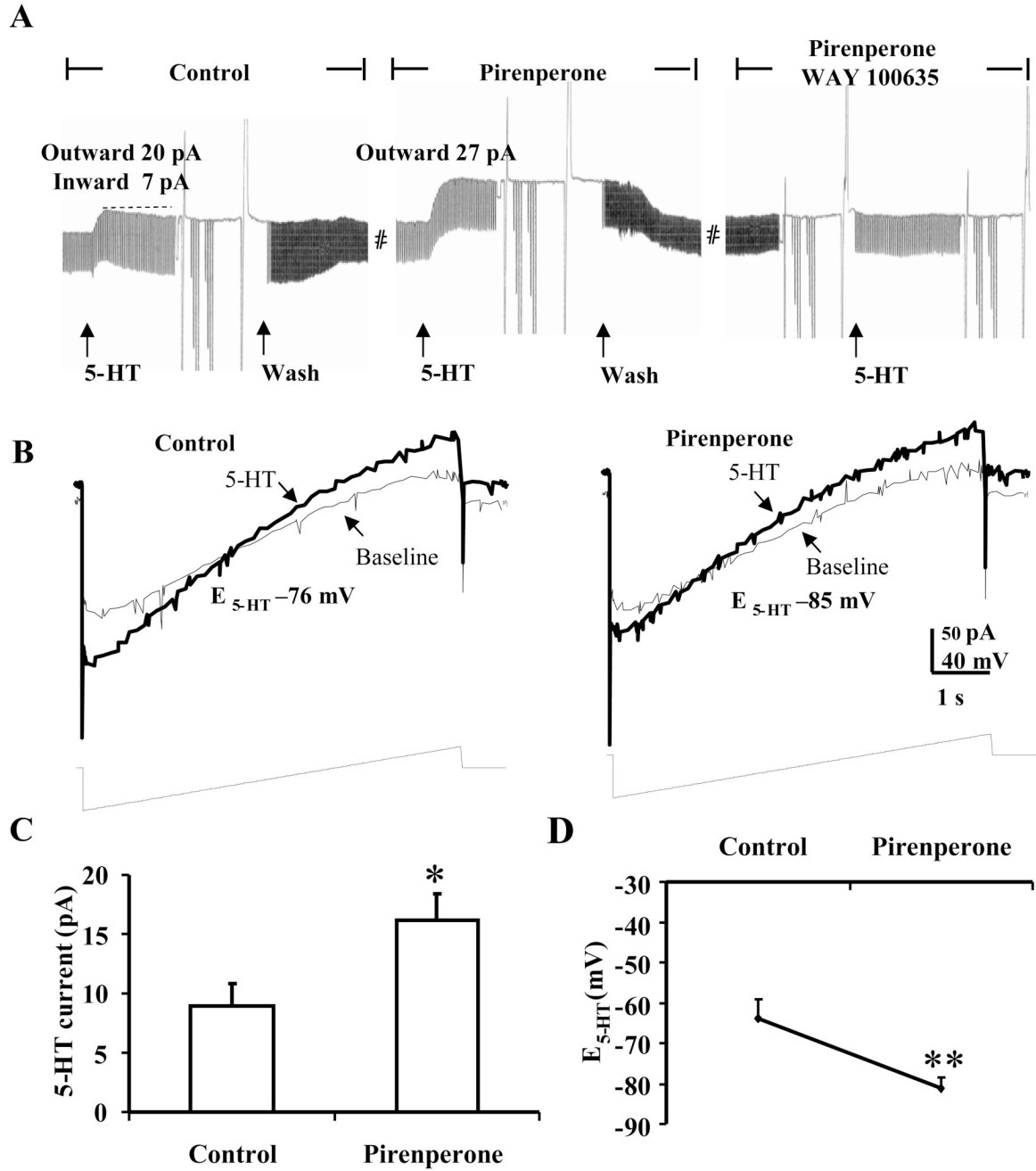


Figure 3. Monophasic outward current to 5-HT in the presence of the nonselective 5-HT₂ antagonist pirenperone in BNST_{ALG} neurons that initially exhibited a 5-HT_{Hyp-Dep} response to 5-HT. A, In a BNST_{ALG} neuron exhibited initial 5-HT_{Hyp-Dep} response, 5-HT (50 μM) induced a monophasic outward current in the presence of pirenperone (10 μM); B, Ramp traces showing the shift of E_{5-HT} to more hyperpolarized level of -85 mV when 5-HT was applied in the presence of pirenperone, suggesting the activation of a potassium current; C, Histograms showing pirenperone pre-application increased the amplitude of 5-HT-induced outward currents in BNST_{ALG} neurons (ACSF 9.0 ± 1.9; pirenperone 16.1 ± 2.2 pA, n=7); D, E_{5-HT}

was shifted to the more hyperpolarized level in the presence of pirenperone (ACSF -63.8 ± 4.8 mV, pirenperone -81.1 ± 2.7 mV; $n=7$. *, ** paired t-test, $p<0.05$ and 0.01 respectively.

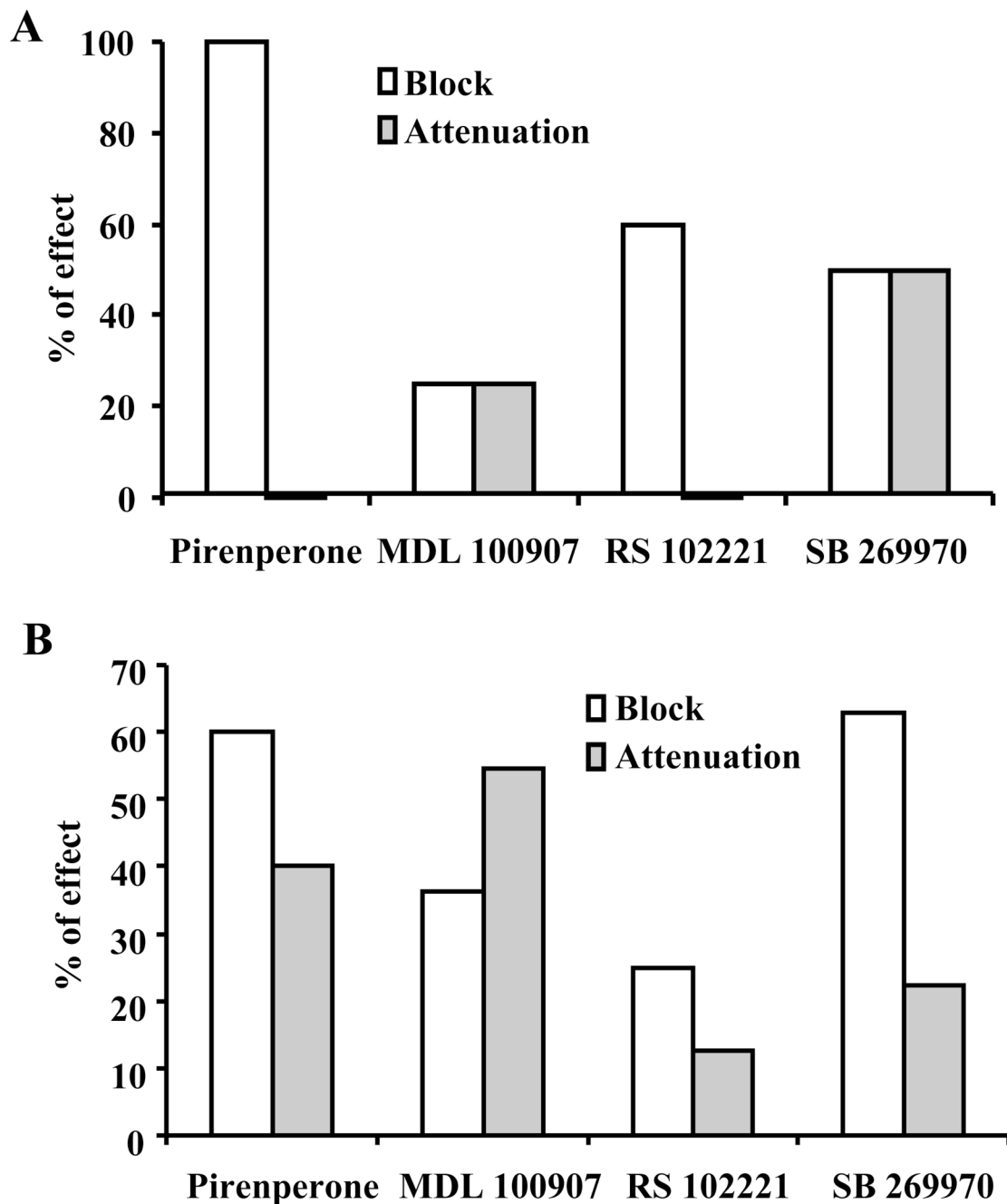


Figure 4.

Blockade of excitatory response to 5-HT by 5-HT_{2A}, 5-HT_{2C} and 5-HT₇ receptor antagonists in BNST_{ALG} neurons. A, in 5-HT_{Hyp-Dep} neurons, the relative efficacies of 5-HT receptor antagonists in blocking or attenuating the excitatory component of 5-HT response; Pirenperone 10 μ M, n=7; MDL 100907, 10 μ M, n=8; RS 102221, 10 μ M, n=5; SB 269970, 1 μ M, n=10. B, in 5-HT_{Dep} neurons, the relative efficacies of 5-HT receptor antagonists in blocking or attenuating the excitatory responses to 5-HT; Pirenperone, 10 μ M, n=5; MDL 100907, 10 μ M, n=11; RS 102221, 10 μ M, n=8; SB 269970, 1 μ M, n=27.

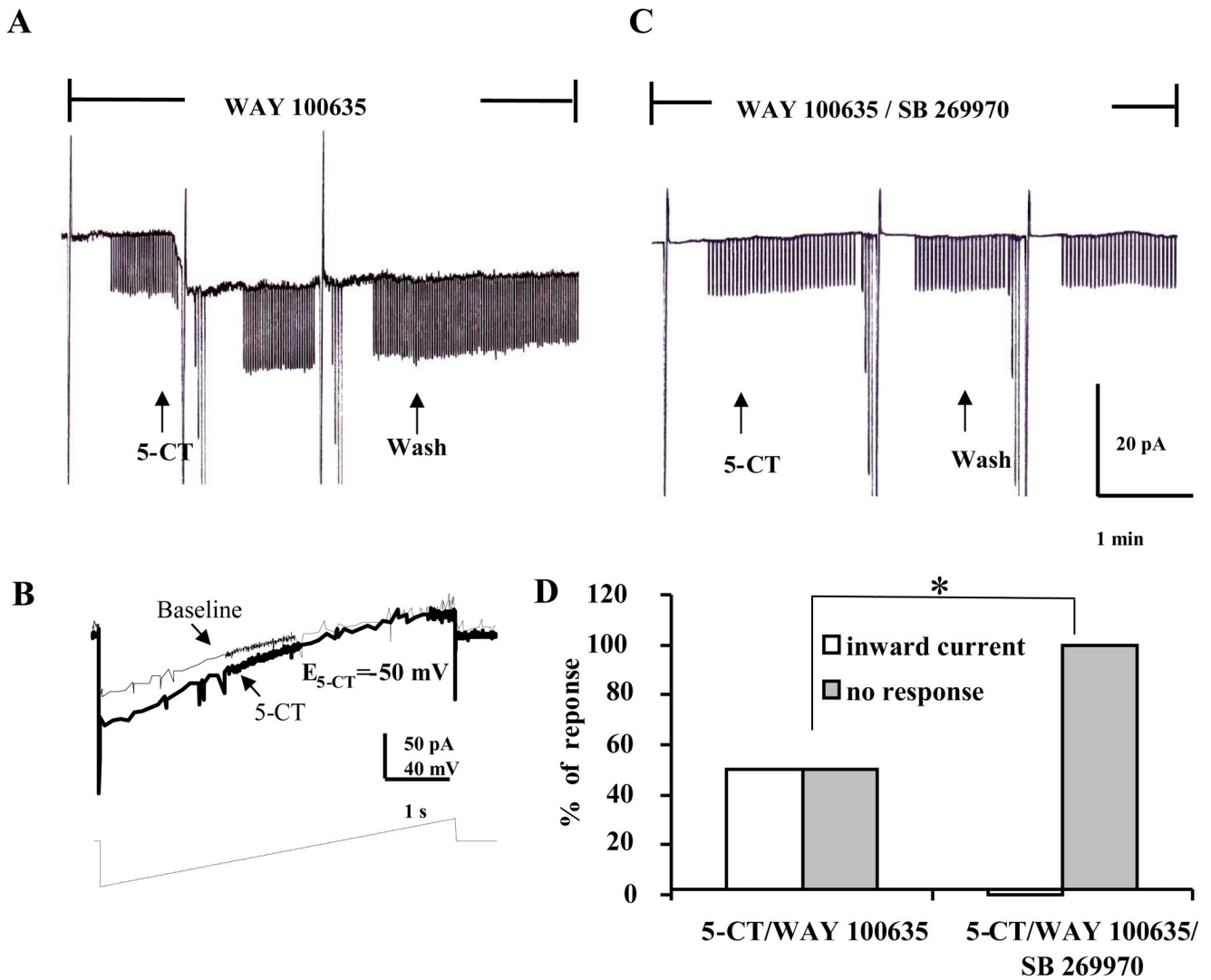


Figure 5.

The 5-CT induced inward current in BNST_{ALG} neurons. A, In the presence of the 5-HT_{1A} antagonist, WAY 100635 (200 nM), 5-CT (10 μM) induced inward current ($8.9 \pm 1.4 \text{ pA}$, $n = 9$), which associated with an increase in membrane conductance (baseline $2.16 \pm 0.30 \text{ nS}$, 5-CT $2.60 \pm 0.40 \text{ nS}$; $t(8) = 3.8$, $p < 0.01$); B, 5-CT induced inward current had a reversal potential of -42 mV , which close to that of non-selective cation channels; C, In the presence of the 5-HT₇ antagonist SB 269970 (1 μM) and WAY 100635 (200 nM), 5-CT failed to induce any change in steady state current; D, Group data showing 5-CT in the presence of WAY 100635 induced an inward current in 50% (9/18) of the BNST_{ALG} neurons tested, but failed to induce any inward current in neurons pretreated with both WAY 100635 and SB 269970 ($n = 7$). *, Chi-Square test, $\chi^2 = 5.459$, $df = 1$, $p < 0.05$.

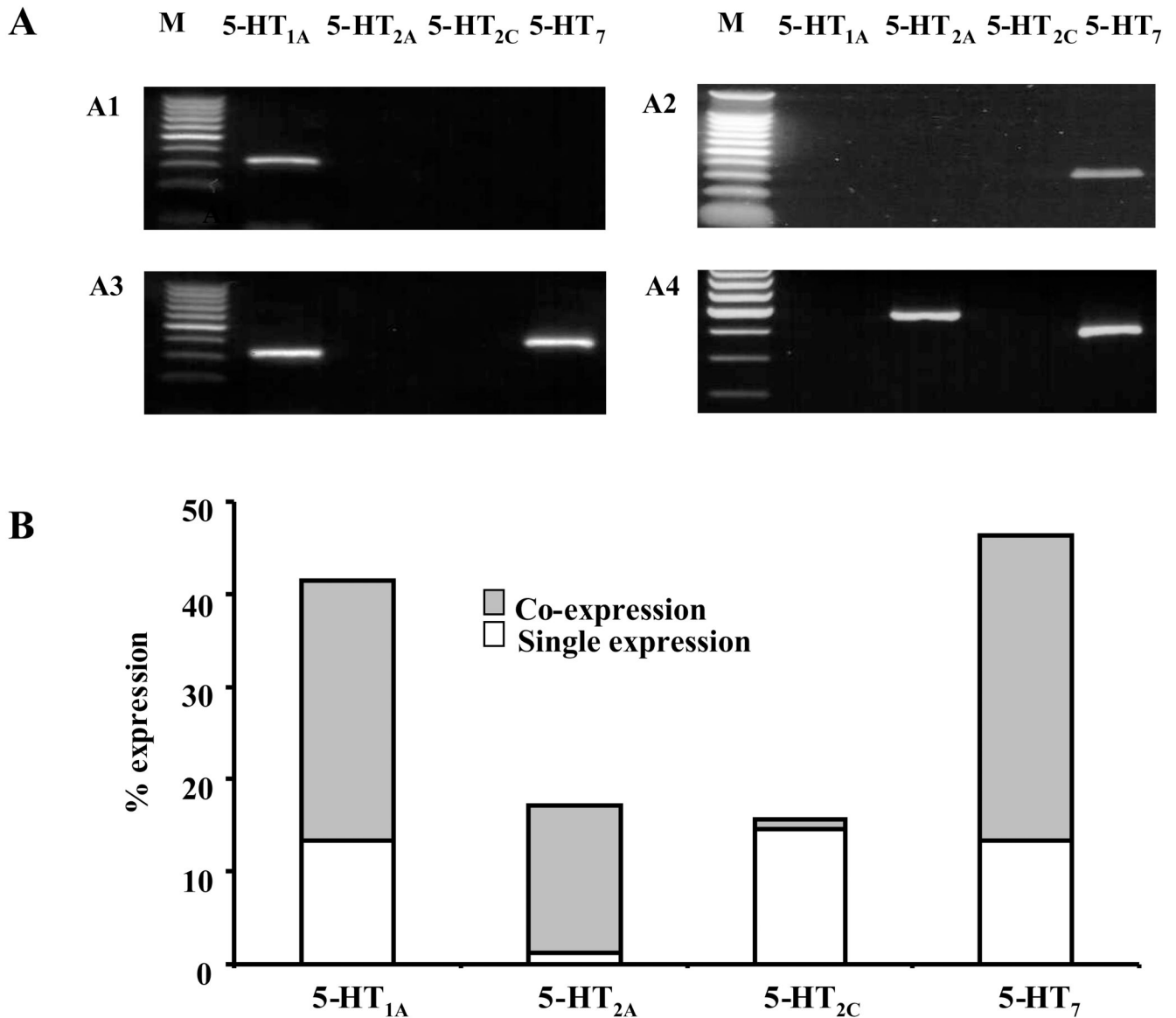


Figure 6. 5-HT receptor mRNA expression in single BNST_{ALG} neurons detected by single cell RT-PCR. A, Representative photographs showing the expression of 5-HT receptor subtype mRNA in single BNST_{ALG} neurons: 5-HT_{1A} mRNA single expression (A1); 5-HT₇ mRNA single expression (A2); 5-HT_{1A}/5-HT₇ mRNA co-expression (A3); 5-HT_{2A}/5-HT₇ mRNA co-expression (A4). B, Summary of 5-HT receptor subtypes mRNA expression in a population of 82 BNST_{ALG} neurons. 5-HT_{2A}, 5-HT_{1A}, 5-HT₇ mRNA tend to be co-expressed with each other whereas 5-HT_{2C} mRNA tend to be expressed alone.

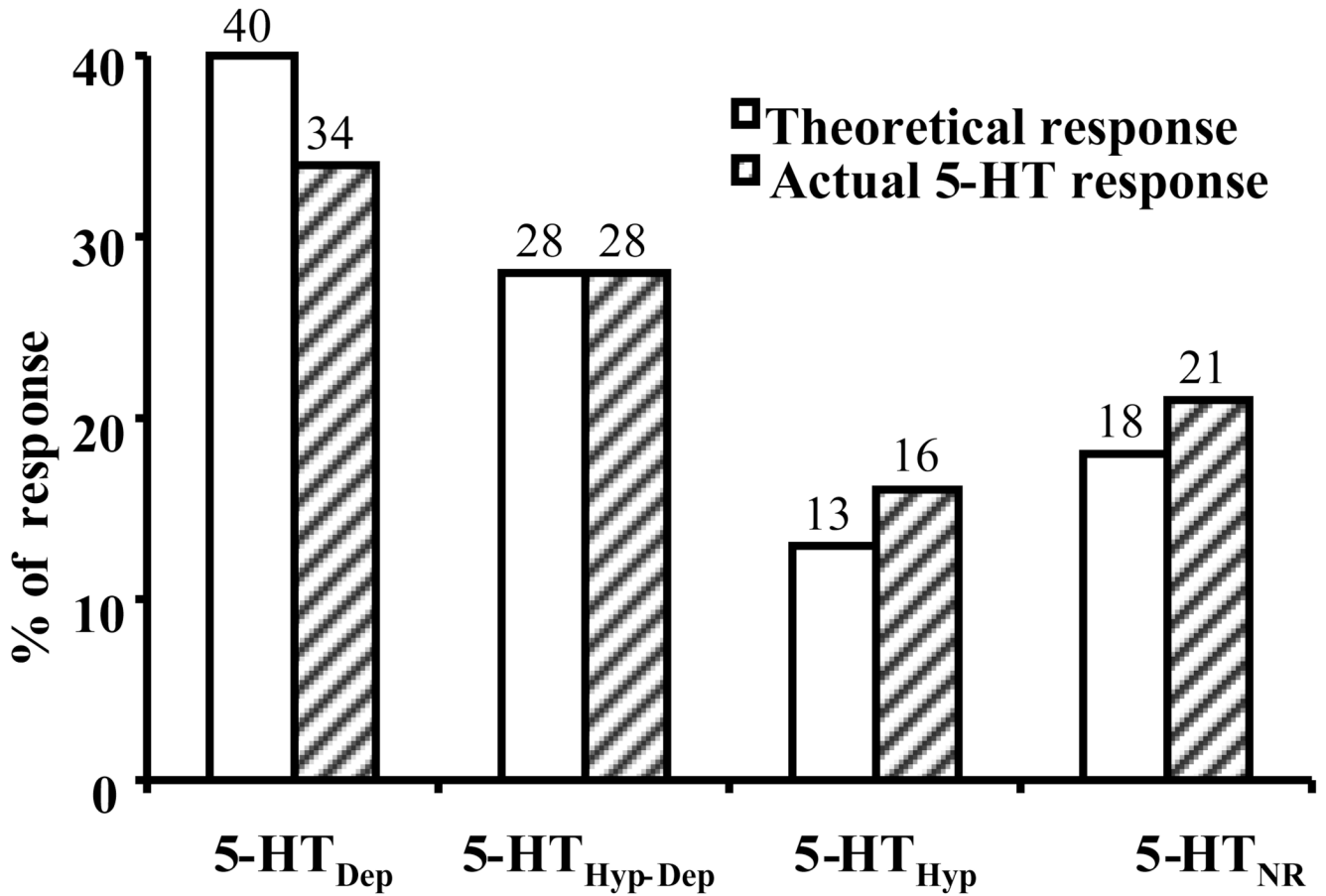


Figure 7.

Comparison of 5-HT receptor mRNA expression and actual 5-HT responses in BNST_{ALG} neurons. Clear bars showed the theoretical percentage of 5-HT response predicted by the expression of 5-HT receptor subtype mRNA in 82 neurons screened. Grey bar is the distribution of actual 5-HT responses from 148 neurons recorded through whole cell patch clamp. There is no statistical difference between theoretical percentages and actual response percentages (Chi Square test, $\chi^2 = 0.08$, $df = 1$, $p = 0.78$).

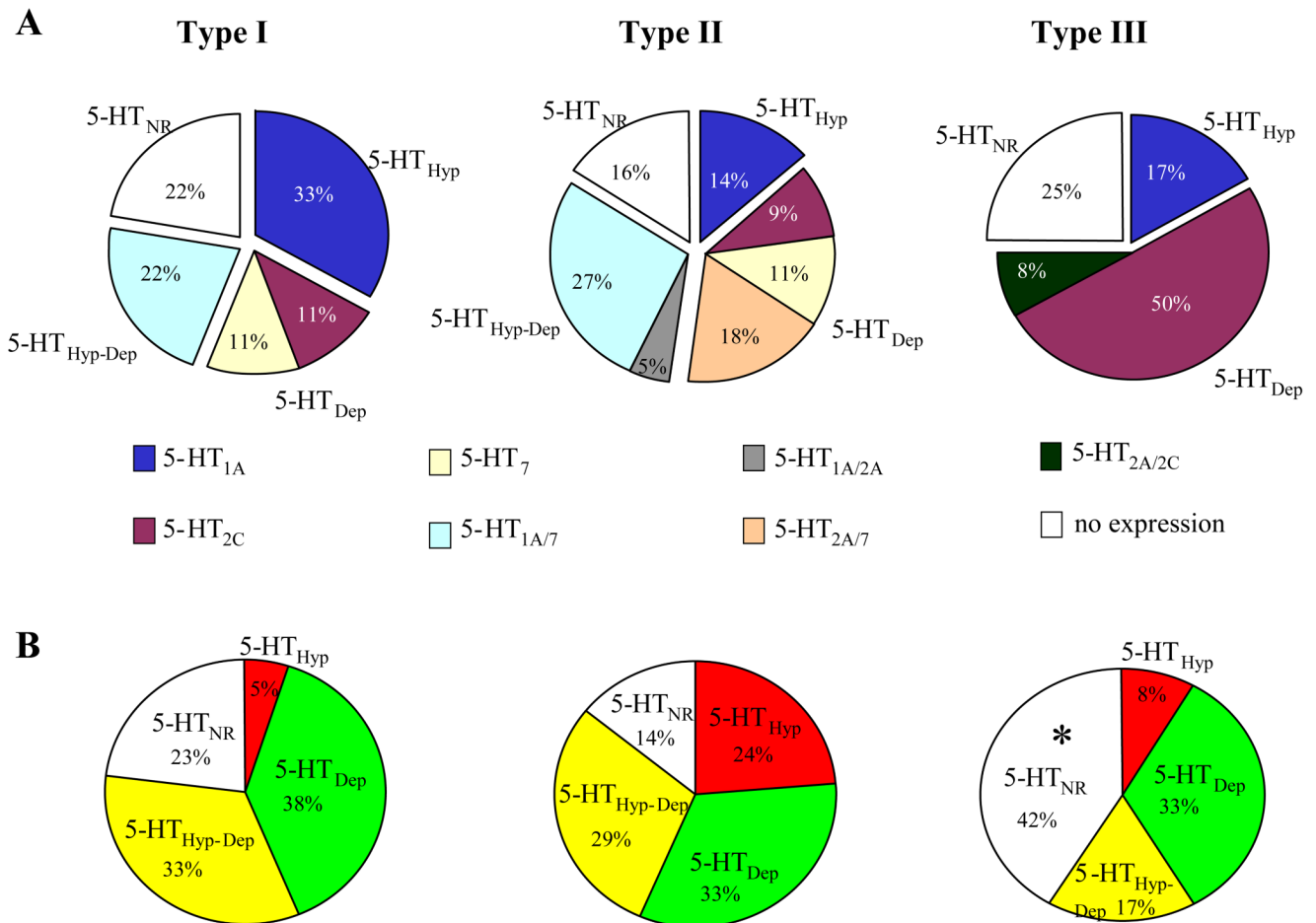


Figure 8. 5-HT receptor mRNA expressions and 5-HT population responses in 3 types of physiology distinct BNST_{ALG} neurons. BNST_{ALG} neurons were divided into 3 cell types (Type I – III) based on their physiological response to depolarizing and hyperpolarizing current injection (Hammack et al., 2007). A, The expression of 5-HT_{1A,2A,2C,7} receptor mRNAs in three types of BNST_{ALG} neurons, Type I, n=9; Type II, n=44; Type III, n=12. B, 5-HT responses examined in 39 type I, 85 type II and 24 type III BNST_{ALG} neurons; Compared to type II, Type III neurons has higher percentage of 5-HT_{NR}. *, Chi Square test followed by post-hoc analysis, R=2.22, p < 0.05.

Table 1

PCR Primers for 5-HT Receptors

Genes	Accession No.	PCR Product size (bp)	Genes	Accession No.	PCR Product size (bp)
GAPDH	M17701	315	5-HT _{2C}	M21410	545
5-HT _{1A}	NM_012585	336	5-HT ₃	U59672	415
5-HT _{1B}	M89954	200	5-HT ₄	AJ011370	610
5-HT _{1D}	M89953	367	5-HT ₅	NM_013148.1	637
5-HT _{1F}	NM_021857	618	5-HT ₆	NM_024365	370
5-HT _{2A}	M30705	465	5-HT ₇	L19654	383

Properties of 5-HT-induced 5-HT_{Hyp-Dep} and 5-HT_{Dep} responses in the BNST_{ALG}neurons

Table 2

5-HT response	membrane response (mV)	membrane current (pA)	reversal potential (mV)	Conductance (nS)	
				Baseline	5-HT
5-HT _{Hyp-Dep} peak	-5.79 ± 0.63 (n=26)	-17.0 ± 2.2 (n=26)	-79.1 ± 1.3 (n=15)	3.38 ± 0.57 (n=10)	4.67 ± 0.89* (n=10)
5-HT _{Hyp-Dep} Steady state	2.83 ± 0.24# (n=26)	9.0 ± 1.5# (n=26)	-66.5 ± 1.7 (n=15)	3.15 ± 0.42 (n=12)	4.97 ± 0.78** (n=12)
5-HT _{Dep}	4.0 ± 0.7 (n=19)	13.2 ± 1.8 (n=28)	-48.5 ± 1.4 (n=32)	2.83 ± 0.94 (n=28)	3.52 ± 1.02** (n=28)

5-HT (50 μM) induced membrane potential or current changes were recorded in current clamp or voltage clamp mode respectively.

*, ** p<0.05, 0.01 respectively, paired t-test.

#, these values are determined as relative to the peak of the 5-HT_{Hyp-Dep}.