PRESYNAPTIC 5-HT<sub>1B</sub> RECEPTOR-MEDIATED SEROTONERGIC INHIBITION OF GLUTAMATE TRANSMISSION IN THE BED NUCLEUS OF THE STRIA TERMINALIS

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Abstract—Activation of neurons in the bed nucleus of the stria terminals (BNST) plays a critical role in stress and anxiety-related behaviors. Previously, we have shown that serotonin (5-HT) can directly modulate BNST neuronal excitability by an action at postsynaptic receptors. In this study we built upon that work to examine the effects of 5-HT on excitatory neurotransmission in an in vitro rat BNST slice preparation. Bath application of 5-HT reversibly reduced the amplitude of evoked excitatory postsynaptic currents (eEPSCs). These effects were mimicked by the 5-HT<sub>1B</sub> receptor agonist, sumatriptan, and by the 5-HT<sub>1B</sub> receptor selective agonist, CP93129. Conversely, the effects of 5-HT and sumatriptan could be blocked by the 5-HT<sub>1B</sub> receptor-selective antagonist, GR55562. In contrast, the 5-HT<sub>1A</sub> receptor agonist 8-OH DPAT or antagonist WAY 100635 could not mimic or block the effect of 5-HT on eEPSCs. Together, these data suggest that the 5-HT-induced attenuation of eEPSCs was mediated by 5-HT<sub>1B</sub> receptor activation. Moreover, sumatriptan had no effect on the amplitude of the postsynaptic current elicited by pressure applied AMPA, suggesting a possible presynaptic locus for the 5-HT<sub>1B</sub> receptor. Furthermore, 5-HT, sumatriptan and CP93129 all increased the paired pulse ratio of eEPSCs while they concomitantly decreased the amplitude of eEPSCs, suggesting that these agonists act to reduce glutamate release probability at presynaptic locus. Consistent with this observation, sumatriptan decreased the frequency of miniature EPSCs, but had no effect on their amplitude. Taken together, these results suggest that 5-HT suppresses glutamatergic neurotransmission in the BNST by activating presynaptic 5-HT<sub>1B</sub> receptors to decrease glutamate release from presynaptic terminals. This study illustrates a new pathway by which the activity of BNST neurons can be indirectly modulated by 5-HT, and suggests a potential new target for the development of novel treatments for depression and anxiety disorders. © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: serotonin, excitatory postsynaptic currents, presynaptic receptor, patch clamp recording, anxiety disorders.

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Abbreviations: ACSF, artificial cerebrospinal fluid; BLA, basolateral amygdala; BNST, the bed nucleus of the stria terminals; BNST<sub>L</sub>, anterolateral BNST; CRF, corticotrophin-releasing factor; 5-CT, 5-Carboxamidotryptamine; DRN, dorsal Raphe nucleus; eEPSCs, evoked excitatory postsynaptic currents; EPSCs, excitatory postsynaptic currents; IL<sub>L</sub>, infralimbic cortex; IPSCs, inhibitory postsynaptic currents; mEPSCs, miniature excitatory postsynaptic currents; NMDA, N-methyl-D-aspartic acid; PbN, parabrachial nucleus; PFC, prefrontal cortex; PPR, paired pulse ratio; PVN, paraventricular nucleus of the hypothalamus; SSRIs, selective serotonin reuptake inhibitors; ST, stria terminals; TTX, tetrodotoxin; VTA, ventral tegmental area.

Majority of BNST neurons are inhibited by activation of postsynaptic 5-HT$_{1A}$ receptors (Levita et al., 2004). Significantly, we have shown that local infusion of a 5-HT$_{1A}$ agonist, 5-carboxamidotryptamine (5-CT), into the BNST attenuates the acoustic startle response in rats, suggesting that 5-HT$_{1A}$ receptor-mediated inhibition of BNST neurons has an anxiolytic action.

In addition to a direct modulation through activation of postsynaptic 5-HT receptors, the excitability of BNST neurons could also be modulated indirectly by affecting synaptic transmission in this nucleus. The BNST receives substantial glutamatergic input from the prefrontal cortex (PFC), basolateral amygdala (BLA), and hippocampus (Weller and Smith, 1982; Dong et al., 2001a; Walker et al., 2003; Massi et al., 2008), and recent studies have shown that this input can be modulated by several neurotransmitters and neuromodulators including glutamate itself, nor-epinephrine, dopamine, CRF, and cannabinoids (Egli et al., 2005; Grueter and Winder, 2005; Muly et al., 2007; Kash et al., 2008; Massi et al., 2008; McCelligott and Winder, 2008, 2009). However, no study to date has examined the effects of 5-HT on synaptic transmission in the BNST. In the present study, we directly addressed this issue by using patch clamp recordings from an in vitro BNST slice preparation to examine the effect of 5-HT on glutamatergic neurotransmission and identify the underlying 5-HT receptor subtype(s) mediating the effect.

EXPERIMENTAL PROCEDURES

Animals

Male Sprague–Dawley rats (5–7 weeks old, Charles River, Raleigh, NC, USA) were used in this experiment. Animals were housed 4–5 per cage and had access to food and water ad libitum. All experimental protocols strictly conform to National Institutes of Health guidelines for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of Emory University. Cares were taken to minimize the stress and suffering of rats before sacrificing.

Slice preparation

Slices containing the anterolateral BNST (BNST$_{AL}$) were obtained as previously described (Rainnie, 1999a; Muly et al., 2007). Briefly, under isoflurane anesthesia (Fisher Scientific, Hanoverpark, IL, USA), animals were decapitated and the brains rapidly removed and immersed in a cold (4 °C) 95–5% oxygen/carbon dioxide oxygenated “cutting solution” with the following composition (in mM): NaCl (130), NaHCO$_3$ (30), KCl (3.50), KH$_2$PO$_4$ (1.10), MgCl$_2$ (1.0), glucose (10), supplemented with kynurenic acid (2.0). Slices containing the BNST$_{AL}$ were cut at a thickness of 350 M using a Leica VTS-1000 vibratome (Leica Microsystems Inc., Bannockburn, IL, USA). Slices were kept in oxygenated “cutting solution” at room temperature for 1 h before transferring to regular artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl (130), NaHCO$_3$ (30), KCl (3.50), KH$_2$PO$_4$ (1.10), MgCl$_2$ (1.30), CaCl$_2$ (2.50), and glucose (10). Slices were kept in the regular ACSF for at least 30 min before recording.

Patch clamp recording

Individual slices were transferred to a recording chamber mounted on the fixed stage of a Leica DMLFS microscope (Leica Microsystems Inc., Bannockburn, IL, USA), where they were maintained fully submerged and continuously perfused with oxygenated 32 °C ACSF at a flow rate of 1–2 ml/min. Individual BNST neurons were identified by using differential interference contrast (DIC) optics and infrared (IR) illumination with an IR sensitive CCD camera (Orcia ER, Hamamatsu, Tokyo Japan). All cells recorded were confined to the BNST$_{AL}$ as previously reported (Levita et al., 2004; Hammack et al., 2007). Patch pipettes were pulled from borosilicate glass and had a resistance of 4–6 MΩ. The patch recording solution had the following composition (in mM): 130 K-glucuronate, 2 KCl, 10 HEPES, 3 MgCl$_2$, 2 K-ATP, 0.2 NaGTP, and 5 phosphocreatine, titrated to pH 7.3 with KOH, and 290 mOsm. Data acquisition and analysis were performed using a MultiClamp 700B amplifier in conjunction with pClamp 10.0 software and a DigitData 1320A AD/DA interface ( Molecular Devices, Burlingame, CA, USA). Whole cell patch clamp recordings were obtained and whole cell currents were filtered at 2 kHz and digitized at 10–20 kHz. The membrane potential was held at −60 mV for all neurons if not specified. Only those BNST neurons which had a stable membrane potential more negative than −55 mV and an action potential that was not present more than 2 Hz were used. Access resistance was monitored throughout the experiments and neurons showing more than a 15% change of access resistance were discarded.

Recording of evoked EPSCs

Excitatory postsynaptic currents (EPSCs) onto BNST neurons were evoked as previously described (Muly et al., 2007). In brief, a concentric bipolar stimulation electrode (FHC, Bowdoinham, ME, USA) was placed on the afferent fibers of the stria terminals (ST). Evoked EPSCs (eEPSCs) were recorded in the presence of the GABA$_A$ receptor antagonist SR 95531 (5 M) to block GABA$_A$ receptor-mediated inhibitory postsynaptic currents (IPSCs). One train of five single square wave pulses (150 μs, 0.2 Hz) was delivered every 2 min throughout the experiment to induce EPSCs. For analysis, the peak eEPSC amplitude was calculated as the mean response to each series of five stimulations. The mean of three stable eEPSCs obtained immediately before drug treatment was considered as baseline eEPSCs. All eEPSCs values were normalized to the baseline amplitude and expressed as the percentage of baseline.

Paired pulse paradigm

To examine the potential involvement of presynaptic 5-HT receptors, a paired pulse paradigm was employed in which two stimuli were delivered with an inter-stimulus-interval (ISI) of 50 ms. Five pairs of stimuli were delivered with an interval of 5 s between each pair and were averaged to measure the peak amplitude of both eEPSCs. The paired pulse ratio (PPR) was then calculated as the peak amplitude of the second EPSC (P2) divided by the first, EPSC1 (P1). Alterations in the PPR are thought to represent changes in release probability in the presynaptic terminal (Hess et al., 1987; Manabe et al., 1993).

Miniature EPSCs recording

Miniature EPSCs (mEPSCs) were examined in the presence of tetrodotoxin (TTX) (1 μM) and SR 95531 (5 μM). Two sessions of 1 min recording captured before 5-HT application were used as baseline. Another two sessions of 1 min recordings were captured during 5-HT application. All mEPSCs were detected offline using the MiniAnalysis program 6.0 (Synaptosoft Inc., Decatur, GA, USA). The mEPSCs frequency and amplitude were represented as the mean values of two sessions.

Drug application

The following drugs were obtained from Sigma-Aldrich (St. Louis, MO, USA): serotonin, TTX, DNXQ (6,7-dinitroquinoxaline-2,3-dione),...
8-OH-DPAT (8-hydroxy-2-(n-propylamino) tetraline), WAY 100635 (N-[2-[4-((2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyrldyl)cyclohexanecarboxamide), AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate); and (2) Tocris Bioscience (Ellisville, MO, USA): CP 93129 (1,4-Dihydro-3-(1,2,3,6-tetrahydro-4-pyridyl)-SH-pyrol [3,2-b]pyridin-5-one dihydrochloride), GR55562 (3-[3-(Dimethylamino)propyl]-4-hydroxy-N-[4-(4-pyridyl)phenyl]benzamidide dihydrochloride), RS CPP ((RS)-3-(2-Carboxypyridin-4-yl)-propyl-1-phosphonic acid), SR 95531(6-imino-3-(4-methoxyphenyl)-1H)-pyridazinebutanoic acid hydrobromide). Sumatriptan was a generous gift from Glaxo Wellcome. Drugs were made as concentrated stock solutions in distilled H2O, except DNQX, which was made in 100% DMSO. All drugs were applied in the ACSF using a continuous gravity fed bath application unless specifically stated. Pressure-application of AMPA was used to directly elicit postsynaptic AMPA currents. Briefly, a modified patch electrode was filled with ACSF containing AMPA (1 mM) and placed close to the recorded BNST neuron. AMPA receptor-mediated currents were evoked by pressure ejection (20–100 ms, 5–20 psi) using a picospritzer II (Parker Hannin Instrumetation, Cleveland, Oh). The transient pressure application was repeated every minute before and during drug application. After recording four stable baseline AMPA currents, sumatriptan was added to perfusion ACSF and the amplitude of the AMPA currents in the presence of sumatriptan were examined.

Statistics

All data are expressed as the mean±SEM. For the evoked EPSCs, the amplitudes were normalized and expressed as the percentage of the baseline EPSC amplitude. All statistical tests were conducted using Excel 2003 or Graphpad Prism 4.0. A Student’s t-test or paired t-test was used to detect significant differences for unpaired or paired data. A P<0.05 was considered statistically significant for all cases.

RESULTS

eEPSCs in the BNST

Monosynaptic eEPSCs were induced in 90% of BNST neurons by stimulating the ST. The baseline eEPSCs amplitude was set to 60% of maximal eEPSCs amplitude, with amplitudes ranging from 160 to 500 pA. In 10% of BNST neurons stimulation of the ST evoked a polysynaptic EPSC and hence, these cells were not included in our analysis. Consistent with previous reports (Rainnie, 1999a; Egli and Winder, 2003; Egli et al., 2005), the EPSCs evoked at holding potential of −60 mV were nearly completely blocked by prior application of the AMPA/KA receptor antagonist DNQX (20 μM), whereas prior application of the NMDA (N-methyl-D-aspartic acid) receptor antagonist RS-CPP (10 μM) has no or little effect on the eEPSCs (Fig. 1A, B), suggesting that the eEPSCs are mediated primarily by AMPA/KA receptor activation at rest.

Effect of 5-HT on eEPSCs

Bath application of 5-HT (50 μM) caused a significant reduction in the amplitude of the eEPSCs in BNST neurons (Fig. 1C, D). In all neurons tested, application of 5-HT (50 μM) consistently reduced the amplitude of the eEPSCs to 64.2±3.1% of baseline level (baseline 255±26 pA, 5-HT 164±18 pA; P<0.001, n=10, Fig. 1D). As illustrated in Fig. 1D, the inhibitory effect of 5-HT on the eEPSCs amplitude was reversible and showed complete recovery following 10 min of wash out with control ACSF (baseline 300±42 pA, washout 295±33 pA; 98.5±3.9% of baseline, P=0.5 vs. baseline, P<0.05 vs. 5-HT, n=4). Importantly, the amplitude of eEPSCs did not change during a similar period of continuous perfusion with ACSF, suggesting the 5-HT-induced attenuation of eEPSCs is not due to “run-down” of eEPSCs. Next, we examined the dose-response relationship of the 5-HT-induced modulation of eEPSCs amplitude. The inhibitory effect of 5-HT on eEPSCs was clearly dose-dependent (see Fig. 1E). A low concentration of 5-HT (0.1 μM) evoked only a slight decrease (93.7±4.0% of baseline, n=7) in the amplitude of the eEPSCs, and the 5-HT effect reached a plateau level at 50–100 μM. Fitting a sigmoid curve to the dose-response relationship revealed an EC50 of 4.0 μM for the 5-HT induced suppression of eEPSCs amplitude (Fig. 1E).

Receptor(s) which mediate the 5-HT effect

Multiple subtypes of seven distinct 5-HT receptor families have been identified and cloned (5-HT1a, b, Teitler and Herrick-Davis, 1994; Hoyer et al., 2002). Previous studies had indicated that the G1-coupled 5-HT1A,1B,1D receptors were most often associated with an inhibitory effect on glutamatergic neurotransmission (Fitzgerald and Sanes, 1999; Pickard et al., 1999), whereas the Gq-coupled 5-HT2A receptor subtype has been reported to enhance glutamate transmission (Fitzgerald and Sanes, 1999; Smith et al., 2001; Hasuo et al., 2002; Harsing, 2006). Consequently, we next tested the ability of several 5-HT1 receptor subtype selective agonists and antagonists to mimic and/or block the 5-HT induced attenuation of eEPSCs amplitude. We first tried to mimic the 5-HT response with the mixed 5-HT1B,1D agonist, sumatriptan, which inhibits excitatory transmission in multiple brain regions (Jennings et al., 2004; Jeong et al., 2008). Bath application of sumatriptan caused a dose-dependent reduction in the amplitude of the eEPSCs. Low dose of sumatriptan (1 μM) caused a slight, but non-significant decrease in the amplitude of the eEPSCs (baseline 242±46 pA, sumatriptan 1 μM 233±54 pA; 95.7±5.3% of baseline, n=6, P=0.21). A higher dose of sumatriptan (10 μM) significantly reduced the amplitude of eEPSCs in all neurons tested (baseline 242±27 pA, sumatriptan 10 μM 159±25 pA; 64.6±4.5% of baseline, n=11, P<0.001) (Fig. 2A, C), which is close to the effect induced by 50 μM 5-HT (64.2±3.1% of baseline). Importantly, the effect of sumatriptan was reversible and recovered to 92.5±4.5% of baseline after 10 min of washout with control ACSF (baseline 244±37 pA, washout 229±41 pA; P=0.18 vs. baseline, P<0.01 vs. sumatriptan, n=8).

As sumatriptan is a mixed agonist with high affinity for both 5-HT1B and 5-HT1D receptors (Peroutka and McCarthy, 1989; Lesage et al., 1998), we next examined the effect on the eEPSCs amplitude of exogenous application of the selective 5-HT1D receptor agonist, CP93129 (Matsubara et al., 1991; Chadha et al., 2000). Application of CP93129 (10 μM) mimicked the inhibitory effect of both 5-HT1D, the inhibitory effect of 5-HT on the eEPSCs amplitude was reversible and showed complete recovery following 10 min of wash out with control ACSF (baseline 300±42 pA, washout 295±33 pA; 98.5±3.9% of baseline, P=0.5 vs. baseline, P<0.05 vs. 5-HT, n=4). Importantly, the amplitude of eEPSCs did not change during a similar period of continuous perfusion with ACSF, suggesting the 5-HT-induced attenuation of eEPSCs is not due to “run-down” of eEPSCs. Next, we examined the dose-response relationship of the 5-HT-induced modulation of eEPSCs amplitude. The inhibitory effect of 5-HT on eEPSCs was clearly dose-dependent (see Fig. 1E). A low concentration of 5-HT (0.1 μM) evoked only a slight decrease (93.7±4.0% of baseline, n=7) in the amplitude of the eEPSCs, and the 5-HT effect reached a plateau level at 50–100 μM. Fitting a sigmoid curve to the dose-response relationship revealed an EC50 of 4.0 μM for the 5-HT induced suppression of eEPSCs amplitude (Fig. 1E).
5-HT and sumatriptan on the amplitude of eEPSCs (Fig. 2B). Here, the eEPSCs amplitude was depressed to 61.4 ± 2.0% of baseline levels by CP39129 (baseline 300 ± 10 pA, CP 93129 183 ± 9 pA; n = 10, P < 0.001). In comparison to 5-HT and sumatriptan, the recovery rate from CP93129 application was much slower, showing only partial recovery of the eEPSCs amplitude after 10 min of washout (baseline 322 ± 8 pA, washout 236 ± 11 pA; 74.4 ± 4% of baseline, P < 0.01 vs. baseline, P < 0.01 vs. CP93129, n = 5). These results suggest that activation of 5-HT1B rather than 5-HT1D receptors mediated the inhibitory effect of 5-HT on eEPSCs. To test this hypothesis, we next examined the effect of prior application of the selective 5-HT1B receptor antagonist GR55562 on the modulatory actions of 5-HT and sumatriptan.

Application of GR55562 (10 μM) alone had no effect on the amplitude of eEPSCs (baseline 309 ± 20 pA, GR55562 320 ± 23 pA; 103.9 ± 4.7% of baseline, n = 14, P = 0.5). However, prior application of GR55562 significantly attenuated the inhibitory effect of 5-HT on eEPSCs amplitude (Fig. 2C). In the presence of GR55562 (10 μM), 5-HT (50 μM) reduced the mean eEPSCs amplitude to 80.0 ± 3.5% of baseline (GR55562 358 ± 31 pA, 5-HT in GR55562 287 ± 29 pA, n = 8, P < 0.01) (Fig. 2C), whereas 5-HT (10 μM) only reduced eEPSCs to 91 ± 4% of baseline (GR55562 321 ± 31 pA, 5-HT in GR55562 291 ± 32 pA, P > 0.05, n = 7), which is significantly different from the 5-HT (10 μM) effect in control ACSF (74 ± 4% of baseline, n = 9, P < 0.01) (Fig. 2D). Similarly, the effect of sumatriptan (10 μM) was almost completely abolished by prior application of GR55562. In the presence of GR55562 application of sumatriptan reduced the eEPSCs amplitude to 93.3 ± 2.4% of baseline (GR55562 270 ± 20 pA, sumatriptan in GR55562 251 ± 19 pA; P > 0.05, n = 6) (Fig. 2C, D), which is significantly different from that of sumatriptan application in control ACSF (64.2 ± 3.1%, P < 0.001). Hence, the inhibitory effect of sumatriptan on the eEPSCs amplitude was most likely due to its activity at 5-HT1B rather than 5-HT1D receptors.

Evidence from other brain regions suggested that activation of 5-HT1A receptors can also modulate glutamate release (Koyama et al., 2002; Fink and Gothert, 2007).
Hence, we next examined the effect of application of the selective 5-HT1A receptor agonist, 8-OH DPAT, on the eEPSCs amplitude. At 10 μM, 8-OH DPAT caused a small but non-significant reduction of the eEPSCs amplitude (baseline 283 ± 33 pA, 8-OH DPAT 258 ± 29 pA, 92.2 ± 5.1% of baseline, P = 0.2, n = 6) (Fig. 3A, C). We then tested whether prior application of the selective 5-HT1A receptor antagonist, WAY 100635, could block the inhibitory effect of 5-HT on the amplitude of eEPSCs. Application of WAY 100635 (200 nM) alone had no effect on the amplitude of eEPSCs (baseline 210 ± 22 pA, WAY 100635 204 ± 18 pA, P = 0.42, n = 6). Subsequent application of 5-HT (50 μM) in the presence of WAY 100635 reduced the amplitude of eEPSCs to 67.0 ± 3.3% of baseline (5-HT 135 ± 10 pA, n = 6, P < 0.01) (Fig. 3B, D), which was not significantly different from the 5-HT response in control ACSF (Fig. 1D). These result suggest that 5-HT1A receptor activation is unlikely to contribute to the inhibitory effect of 5-HT on excitatory transmission in the BNST. However, the small residual effect of 5-HT on eEPSCs in the presence of GR55562 might also indicate the contribution of other 5-HT receptor subtype(s).

Presynaptic action of 5-HT

Although 5-HT1B receptor modulation of glutamate transmission has been reported to have a presynaptic locus in many regions of the CNS (Maroteaux et al., 1992; Boschert et al., 1994; Sari et al., 1997; Muramatsu et al., 1998; Riad et al., 2000; Shen and Johnson, 2008), 5-HT1B mRNA is also expressed in BNST tissue (Guo et al., 2009; Hammack et al., 2009). Hence, the 5-HT1B receptor-induced modulation of glutamate transmission could have either a pre- or postsynaptic locus. To determine the site of 5-HT1B receptor action we performed the following experiments.

First, we examined the effect of sumatriptan on direct postsynaptic AMPA currents evoked by pressure-application of AMPA at a holding potential of −60 mV (Fig. 4A). As summarized above, the eEPSCs were mediated mainly by AMPA/KA receptors but not NMDA receptors at rest, thus we used AMPA instead of glutamate to avoid the excitotoxicity associated with NMDA receptor activation. If 5-HT1B receptor activation reduced eEPSCs through a postsynaptic mechanism, sumatriptan application should also decrease the amplitude of pressure application-induced AMPA currents.
Pressure ejected AMPA (1 mM) evoked an inward current with a mean amplitude of 219 ± 100 pA (n = 5). Bath application of sumatriptan (10 μM) had no effect on postsynaptic AMPA currents (220 ± 20 pA, 100.4 ± 0.4% of baseline, n = 5, P = 0.4; Fig. 4B), suggesting that postsynaptic 5-HT1B receptors are unlikely to contribute to the 5-HT induced modulation of eEPSCs.

Second, we used a paired pulse paradigm in which two stimuli were delivered with an inter-stimulus-interval of 50 ms (see Methods). Here, an alteration of PPR is used as a measure of a change in release probability from presynaptic terminals. Application of 5-HT (50 μM) reduced amplitude of eEPSCs and concomitantly caused a significant increase in the PPR compared to baseline (baseline 1.35 ± 0.11; 5-HT 1.71 ± 0.16, P < 0.05, n = 8, see Fig. 5A, B). Similarly, application of sumatriptan (10 μM; baseline 1.51 ± 0.08, sumatriptan 1.82 ± 0.09, P < 0.01, n = 9, Fig. 5C), or CP93129 (baseline 1.33 ± 0.17; CP93129 10 μM 1.59 ± 0.26; P < 0.05, n = 8, Fig. 5D) also caused a significant increase in PPR. Together the increased PPR and decreased eEPSCs amplitude suggest that activation of 5-HT1B receptors located on presynaptic terminals function to inhibit glutamate release.

To confirm a presynaptic site of action, we examined the effects of sumatriptan application on the frequency and amplitude of mEPSCs recorded in the presence of TTX (1 μM) and the GABAA receptor antagonist SR95531 (5 μM). As shown in Fig. 6A, sumatriptan (10 μM) decreased the frequency of mEPSCs observed in BNST neurons. A cumulative fraction plot showed an increase of inter-event-interval during sumatriptan application (Fig. 6B right). In all neurons tested sumatriptan consistently reduced the frequency of mEPSCs to an average of 63.7 ± 3.7% of baseline (baseline 5.2 ± 1.2 Hz, sumatriptan 3.5 ± 0.9 Hz, n = 11, P < 0.01), but had no effect on the amplitude of mEPSCs (baseline 12.3 ± 1.2 pA, sumatriptan 12.0 ± 1.2 pA, 97.5 ± 2.3% of baseline; n = 11, P = 0.41) (Fig. 6C). The data are consistent with the sumatriptan-induced increase in PPR, and add further support for a presynaptic site of action for 5-HT1B receptor activation.
DISCUSSION

In this study, we have shown that 5-HT acts to attenuate excitatory neurotransmission in the BNST, an effect that was reversible, could be mimicked by 5-HT1B receptor agonists, and blocked by the selective 5-HT1B receptor antagonist, GR55562. Furthermore, we provide evidence that 5-HT1B receptor activation had no effect on postsynaptic AMPA currents, but increased the PPR of eEPSCs, and decreased the frequency of mEPSCs, suggesting that 5-HT acts on presynaptic 5-HT1B receptors to decrease glutamate release from presynaptic terminals.

Glutamatergic input onto BNST neurons

Tract tracing studies have shown that the ST represents the major afferent input to the BNSTAL from key components of the limbic circuit including the BLA, PFC, and hippocampus (Weller and Smith, 1982; Dong et al., 2001a; Walker et al., 2003; Massi et al., 2008). In this study, EPSCs were evoked by stimulating the ST and thus most likely represent the response to excitatory input originating from these upstream regions. Importantly, in almost all neurons examined stimulation of the ST evoked a monosynaptic EPSC as previously reported (Muly et al., 2007), suggesting that this represents a direct excitatory input onto BNST neurons. Previous studies showed AMPA/KA and NMDA receptors mediate the EPSPs/EPSCs in the BNST (Dumont et al., 2005, 2008; Egli et al., 2005; Massi et al., 2008) and the AMPA/NMDA ratio shows a dynamic change in cocaine self-administration (Dumont et al., 2005) or morphine dependent rats (Dumont et al., 2008). However, the near total blockade of eEPSCs by DNXQ and the little effect of RS-CPP on eEPSCs at the resting membrane potential of BNSTAL neurons suggest that at rest AMPA/KA receptor activation mediates the eEPSCs, which consistent with the AMPA receptor dependency showed by Winder and colleagues (Egli and Winder, 2003; Egli et al., 2005).

Presynaptic action of 5-HT1B in modulating glutamate release

Inhibition of excitatory synaptic transmission can result from either pre-and/or post-synaptic mechanisms. In this study, converging evidence suggest that the inhibitory effect of 5-HT on eEPSCs was mediated by a presynaptic mechanism, most likely by decreasing the probability of glutamate release from presynaptic terminals. Furthermore, we report that 5-HT1B receptor agonists and antagonist mimicked or blocked 5-HT effect on eEPSCs, an effect that was not mimicked by 5-HT1A receptor agonist or antagonist. This result is consistent with previous studies from other brain regions showing a 5-HT1B receptor-mediated decrease in excitatory neurotransmission (Singer et al., 1996; Muramatsu et al., 1998; Pickard et al., 1999). Noticeably, although GR55562 effectively blocked the 5-HT effect on eEPSCs, the small residual effect of 5-HT suggest that the contribution of another 5-HT receptor subtype could not be excluded. Nevertheless, this study provided the first evidence for a 5-HT1B receptor-mediated presynaptic inhibition of glutamate release in the BNST.

Consistent with our results, binding studies have shown a high level of 5-HT1B receptor binding sites in the BNST (Bonaventure et al., 1997; Cloez-Tayarani et al., 1997, 1998). 5-HT1B receptors are widely distributed in the CNS, however they are mainly expressed on axon terminals (Maroteaux et al., 1992; Boschert et al., 1994; Sari et al., 1997; Manrique et al., 1999; Riad et al., 2000). In many regions, 5-HT1B receptors are thought to act as terminal...
autoreceptors to regulate 5-HT release (Sari et al., 1997). However, the results outlined above further suggest that in the BNST some of these 5-HT1B binding sites are located on excitatory axon terminals. Indeed, in situ hybridization studies have shown that 5-HT1B receptor mRNA is highly expressed in each of the regions sending major excitatory inputs to the BNSTAL, including the BLA, PFC, and hippocampus, (Cloez-Tayarani et al., 1997). It is possible that in projection neurons from these regions mature 5-HT1B receptor protein is transported to the axon terminals that innervate the BNSTAL. Moreover, our own RT-PCR study has shown 5-HT1B mRNA expression in the BNST (Guo et al., 2009; Hammack et al., 2009). As the majority of BNST neurons are GABAergic (McDonald, 1983; Cullinan et al., 1993; Sun and Cassell, 1993), 5-HT1B receptor activation may also function to regulate intrinsic or extrinsic release of GABA from axon terminals of BNST neurons.

**Functional significance of 5-HT1B receptor-mediated modulation of glutamatergic transmission in the BNST**

Accumulating evidence indicates that the BNST plays an important role in the adaptive response to stress (Vyas et al., 2003; Sullivan et al., 2004a; Pego et al., 2008), anxiety-like behaviors (Walker et al., 2003; Hammack et al., 2004) and drug-seeking behaviors (Aston-Jones and Harris, 2004; Smith and Aston-Jones, 2008). As mentioned

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**Fig. 6.** Sumatriptan decreased the frequency of mEPSCs. Spontaneous mEPSCs in BNST were recorded in the presence of TTX (1 μM) and GABA antagonist SR 95531 (5 μM). (A) Traces showing mEPSCs recorded from BNST neurons, and sumatriptan application reduced the frequency of mEPSCs (right). (B) Cumulative plots showed the increase of inter-event interval of mEPSCs. (C) Group data indicated sumatriptan reduced the frequency but have no effect on the amplitude of mEPSCs. n=11, **, paired t-test P<0.01.
above, the BNST receives excitatory inputs mainly from key structures of limbic circuit and then relays these inputs to multiple subcortical structures including the PVN, the ventral tegmental area (VTA), the parabrachial nuclei (PbN), the DRN, as well as the nucleus of the solitary tract (NTS) (Holstege et al., 1985; Berk, 1987; Peyron et al., 1997; Dong et al., 2001b; Georges and Aston-Jones, 2002; Walker et al., 2003; Dong and Swanson, 2004; Choi et al., 2007). Each of these structures is intimately involved in regulating the autonomic and endocrine response of an organism to environmental pressure (see; Herman et al., 2003; Walker et al., 2003; Lowry et al., 2008 for reviews). Hence, the BNST may serve as a gate-control in these important affective circuits. Recently, Massi and colleagues reported that presynaptic CB1 receptors attenuate glutamate release from prefrontal cortical afferents making synapses onto BNST neurons that project to the VTA. These authors suggest that CB1 modulation of glutamate release in the BNST may play a critical role in modulating reward pathways. We propose that serotonergic modulation of presynaptic glutamate release may serve a similar function in regulating the behavioral response not only to appetitive stimuli but also aversive environmental stimuli.

Decreased brain 5-HT levels are thought to be one of the main causes of depression and anxiety disorders (Ressler and Nemeroff, 2000). Serotonergic afferents in the BNST originate from neurons located in the caudal DRN (Parent et al., 1981; Phelix et al., 1992a; Commons et al., 2003). We and others have shown that 5-HT fibers in the BNST make contact with BNST neurons including those that contain the stress hormone CRF (Phelix et al., a;b; Hammack et al., 2009). Hence, release of 5-HT within the BNST could have a significant impact on the overt behavioral response to sensory stimuli. Previously we have shown that local inhibition of the BNSTAL following infusion of a 5-HT1A receptor agonist has an anxiolytic action (Levita et al., 2004). Here we extended this study to show that 5-HT can also reduce the excitability of the BNSTAL by activating heterosynaptic 5-HT1B receptors located on glutamatergic afferents. We propose that the anxiolytic action of 5-HT in the anterolateral BNST would represent the combined action of reducing intrinsic neuronal excitability as well as reducing the excitatory drive onto these same neurons (see Fig. 7 for a schematic diagram). By extrapolation, therefore, changes in the expression of 5-HT1B receptor expression may result in a significant alteration in the behavioral response to affective stimuli.

Indeed, alterations in 5-HT1B receptor expression has been observed in patients with bipolar disorder and schizophrenia (Lopez-Figueroa et al., 2004), and substance abuse-disorder and major depression appear to be associated with a 5-HT1B receptor polymorphism (Huang et al., 2003). Moreover, emerging preclinical behavioral studies indicate that the 5-HT1A receptor plays a role in the modulation of aggression, anxiety, stress sensitivity and drug reinforcement (Clark and Neumaier, 2001; Przegalinski et al., 2004; Tatarczynska et al., 2004; Olivier and van Oschot, 2005; Svenningsson et al., 2006; Muraki et al., 2008). The results of the current study suggest that modulation of 5-HT1B receptor activation in the BNSTAL may represent a novel target for the development of treatment strategies for stress and anxiety-like behaviors. The effects of BNST 5-HT1B receptor activation in anxiety models need to be examined in future studies.

We have proposed that the BNSTAL and the DRN are reciprocally linked in a closed-loop feedback system (Hammack et al., 2009), whereby stress activation of the BNST CRF neurons would ultimately result in downstream excitation of DRN 5-HT neurons, followed by feedback release of 5-HT in BNST. Release of 5-HT into the BNST would attenuate the glutamatergic drive onto BNST neurons via activation of presynaptic 5-HT1B receptors, and also move these same neurons away from action potential generation through postsynaptic 5-HT1A receptor activation. Concurrent activation of 5-HT1A and 5-HT1B receptors would then act to attenuate anxiety-like behavior.

SSRIs are one of the most widely prescribed antidepressant and anxiolytic treatments, which act to increase extracellular levels of 5-HT by inhibiting its reuptake into presynaptic terminals. As a region with substantial 5-HT projections and a high expression of the 5-HT transporter protein, extracellular 5-HT levels would be expected to increase in the BNST during SSRIs treatment. Hence, some of the therapeutic actions of the SSRIs may result, in part, from increasing levels of 5-HT in the BNST effectively reducing the activity of BNST neurons through a synergistic activation of pre- and postsynaptic receptors. However, following prolonged periods of stress 5-HT receptor expression in the BNST is altered in favor of an excitatory response to 5-HT, due to a downregulation of 5-HT1A.
receptor mRNA and an upregulation of 5-HT<sub>7</sub> receptor mRNA (Hammack et al., 2009). Hence, the effects of traditional SSRIs may be compromised and the development of novel drugs that selectively target defined 5-HT receptor populations in the BNST<sub>AL</sub> may represent a new approach for anxiolytic pharmacotherapy.

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Boschert U, Amara DA, Segu L, Hen R (1994) The mouse 5-HT<sub>2A</sub> receptor mRNA and an upregulation of 5-HT<sub>7</sub> receptor mRNA (Hammack et al., 2009). Hence, the effects of traditional SSRIs may be compromised and the development of novel drugs that selectively target defined 5-HT receptor populations in the BNST<sub>AL</sub> may represent a new approach for anxiolytic pharmacotherapy.


