Cellular/Molecular

Serotonin Activates Catecholamine Neurons in the Solitary Tract Nucleus by Increasing Spontaneous Glutamate Inputs

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Serotonin (5-HT) is a critical neurotransmitter in the control of autonomic functions. 5-HT_3 receptors participate in vagal afferent feedback to decrease food intake and regulate cardiovascular reflexes; however, the phenotype of the solitary tract nucleus (NTS) neurons involved is not known. A_2/C_2 catecholamine (CA) neurons in the NTS are directly activated by visceral afferents and are important for the control of food intake and cardiovascular function, making them good candidates to respond to and mediate the effects of serotonin at the level of the NTS. This study examines serotonin's effects on NTS-CA neurons using patch-clamp techniques and transgenic mice expressing an enhanced green fluorescent protein driven by the tyrosine hydroxylase (TH) promoter (TH-EGFP) to identify catecholamine neurons. Serotonin increased the frequency of spontaneous glutamate excitatory postsynaptic currents (sEPSCs) in >90% of NTS-TH-EGFP neurons, an effect blocked by the 5-HT_3 receptor antagonist ondansetron and mimicked by the 5-HT_3 receptor agonists SR5227 and mCPBG. In contrast, 5-HT_3 receptor agonists increased sEPSCs on a minority (<30%) of non-TH neurons. 5-HT_3 receptor agonists increased the frequency, but not the amplitude, of mini-EPSCs, suggesting that their actions are presynaptic. 5-HT_3 receptor agonists increased the firing rate of TH-EGFP neurons, an effect dependent on the increased spontaneous glutamate inputs as it was blocked by the ionotropic glutamate antagonist NBQX, but independent of visceral afferent activation. These results demonstrate a cellular mechanism by which serotonin activates NTS-TH neurons and suggest a pathway by which it can increase catecholamine release in target regions to modulate food intake, motivation, stress, and cardiovascular function.

Introduction

Serotonin (5-HT) is a neurotransmitter that influences a broad range of physiological processes and behaviors, including pain, mood, cardiovascular function, and food intake. Serotonin has extensive effects throughout the CNS; however, an important site of action for the regulation of cardiovascular function and food intake is the nucleus of the solitary tract (NTS) (Merahi et al., 1992; Raul, 2003; Hayes and Covasa, 2006b; Lam et al., 2009). The NTS is the primary site through which visceral afferent information enters the brain and activates second order neurons via glutamatergic synapses (Andresen and Kunze, 1994; Saper, 2002; Berthoud, 2008; Grill and Hayes, 2009). Many types of serotonin receptors are expressed in the NTS, including the 5-HT_3 receptor (5-HT_3R) subtype, which are expressed on vagal afferent terminals (Pratt and Bowery, 1989; Leslie et al., 1990; Merahi et al., 1992; Huang et al., 2004) where they have been shown to modulate glutamate release (Glaum et al., 1992; Wan and Browning, 2008; Takenaka et al., 2011). Activation of 5-HT_3Rs in the NTS contributes both to termination of a meal (Hayes and Covasa, 2006a), severe anorexia (Wu et al., 2012), and cardiovascular reflexes (Jeggo et al., 2005; Jordan, 2005; Weissheimer and Machado, 2007; Ramage and Villalon, 2008), but the phenotype of the NTS neurons regulated by serotonin is not known.

A_2/C_2 catecholamine neurons in the NTS (NTS-CA neurons) are important for the control of many behaviors influenced by serotonin, as interfering with the function of NTS-CA neurons affects food intake, cardiovascular reflexes, and reward (Simon et al., 1985; Kubo et al., 1990; Itoh and Bunag, 1993; Olson et al., 2006; Rinaman, 2011). Ingestion of a meal, gastric distention, and anorexigens (Monnikes et al., 1997; Willing and Berthoud, 1997; Rinaman et al., 1998; Blevins et al., 2008; Williams et al., 2008; Lam et al., 2009) all increase c-fos expression in NTS-CA neurons, as do changes in blood pressure (Chan and Sawchenko, 1998), noxious stimuli (Jin et al., 1994), immune challenge (Lacroix and Rivest, 1997), and opioid withdrawal (Laorden et al., 2000). NTS-CA neurons project to numerous brain regions, including the hypothalamus, amygdala, nucleus accumbens, and brainstem nuclei (Sawchenko and Swanson, 1981; Riche et al., 1990; Wang et al., 1992; Petrov et al., 1993; Ueta et al., 2000; Reyes and Van Bockstaele, 2006; Travagli et al., 2006; Balcita-Pedicino and Rinaman, 2007), and release of catecholamines at these sites affects a broad number of behaviors, including food intake, reward, stress, and cardiovascular function (Leibowitz et al., 1988; Cole and Sawchenko, 2002; Smith and Aston-Jones, 2008).

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Therefore, one potential mechanism by which serotonin could alter these behaviors is by increasing the activity of NTS-CA neurons.

We have previously demonstrated that NTS-CA neurons are second order neurons that receive direct glutamatergic inputs from visceral afferent fibers, resulting in large-amplitude EPSCs that almost always elicit action potentials at afferent firing frequencies <5 Hz (Appleyard et al., 2007). NTS-CA neurons also receive spontaneous (action potential-independent) glutamate inputs whose frequency can be modulated to impact their basal firing rate (Cui et al., 2011). The goal of these studies is to determine what effect serotonin has on NTS-CA neuronal activity and the underlying mechanism(s) involved.

Materials and Methods

NTS slices. Hindbrains of both male and female TH-EGFP mice (6–20 weeks old) were prepared as described previously (Appleyard et al., 2007). All animal procedures were conducted with the approval of the Animal Care and Use Committees at Washington State University (Pullman, WA) and in accordance with the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals (PHS Policy) and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Guide). The hindbrain was removed and placed for 1 min in cold (0–4°C) artificial cerebral spinal fluid composed of (in mM): 125 NaCl, 3 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25 $NaHCO_3$, 10 dextrose, 2 $CaCl_2$, and bubbled with 95%O_2/5%CO_2. The osmolarity was adjusted to 301–305 mOsm using dextrose. The medulla was trimmed to a 2 cm block (rostral-caudal) centered on the obex. A wedge of tissue was removed from the ventral surface to align the solitary tract (ST) with the cutting plane when mounted in a vibrating microtome (Leica VT-1000S). Slices (250 μm thick) cut with a sapphire knife (Delaware Diamond Knives) contained the ST in the same plane as the NTS. Slices were submerged in a perfusion chamber and all recordings were performed at 31–35°C and pH 7.4. Neurons were visualized using an upright microscope (Olympus BX51). Recording electrodes were filled with a solution (in mM): 10 NaCl, 130 K gluconate, 11 EGTA, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 2 NaATP, 0.2 NaGTP, pH 7.3, 297–301 mOsm. Neurons were recorded from NTS within 200 μm rostral or caudal from obex and medial to the ST–medial NTS. Patch electrodes, 3–5 MΩ, were guided to neurons using differential interference contrast (DIC) optics illuminated with infrared light (Olympus B51). Voltage-clamp recordings were made with an Axopatch 700B (Molecular Devices), Digidata 1440A digitizer (Molecular Devices), and pClamp 10 software (Molecular Devices). Only neurons with holding currents not exceeding 100 pA at V_H = −60 mV for the 15 min control period (input resistance >150 MΩ) were studied further. Series resistance was monitored throughout the recordings, and neurons were not included in further analysis if it exceeded 20 MΩ or drifted >25%. Series resistance did not differ between control (ACSF) and treatment. Synaptic currents were evoked with an ultrafine concentric bipolar stimulating electrode (50 μm inner diameter; Frederick Haer Company) placed on the ST 1–3 mm from the recording electrode. Electrical stimuli were delivered from an isolated programmable stimulator (ISO-Flex stimulator with Master-8, A.M.P.I.) triggered to deliver a burst of stimuli (5–50 Hz). Current-clamp recordings were made at resting membrane potentials, and current injections were not used to hold the membrane at set potentials. All membrane potentials reported were corrected for junction potential (14 mV). All drugs were obtained from Tocris Cookson or Sigma.

Statistics. All data are presented as averages ± SEM. Statistical comparisons of drug effects between groups (e.g., non-catecholamine and catecholamine) were made using one-way ANOVA with Tukey's or Bonferroni *post hoc* analysis and Fisher's exact test where appropriate (see Results; Mintab 16, GraphPad). The Kolmogorov–Smirnov test (KS test) was used to determine the significance of the drug effect for individual neurons when analyzing the sEPSC and miniature EPSC (mEPSC) data (mini analysis, Synaptosoft). $p < 0.05$ indicated significant differences.

Results

Serotonin increases frequency of spontaneous glutamate inputs onto TH-EGFP neurons

All TH-EGFP neurons were easily visualized and identified for recordings (Figure *1A*). We have previously shown a >88% co-localization of EGFP with TH in the medial and caudal NTS of these mice (Appleyard et al., 2007; Cui et al., 2011). As reported previously (Appleyard et al., 2007) we found that 90% of the TH-EGFP neurons studied were directly activated by ST afferents, making them second order neurons. Application of 30 μM serotonin increased the frequency of spontaneous EPSCs in all TH-EGFP neurons tested (Fig. 1*B*,*C*,*D*; $p < 0.05$, KS test, $n = 6$). The basal frequency of sEPSCs in TH-EGFP neurons was variable, ranging from 0.2 to 12.5 Hz, as was previously reported (Cui et al., 2011), suggesting that these neurons receive varying levels of basal glutamate tone, at least in the horizontal slice. Bath application of serotonin increased the frequency of sEPSCs from 1.9 ± 0.63 Hz in control (ACSF) to 10.56 ± 2.45 Hz (5-HT) ($n = 6$). The effects of serotonin were reversed after a 5 min wash (ACSF) (Fig. 1*F*, $n = 4$). Serotonin did not significantly change the average amplitude of sEPSCs (Figure 1*E*). On average, serotonin did not significantly effect sEPSC frequency at concentrations of 1 μM (239 ± 78%, $n = 5$), 3 μM (214 ± 96%, $n = 6$), and 10 μM (347 ± 193%, $n = 5$), but significantly increased sEPSC frequency at 30 μM (936 ± 553%, $n = 6$; $p < 0.05$, one-way ANOVA) (Fig. 1*G*). The calculated EC_{50} was 11 μM. Interestingly, serotonin significantly inhibited sEPSC frequency in 2 of 5 neurons ($p < 0.05$, KS test) at 1 μM and 2 of 6 neurons at 3 μM (Fig. 1*G*). No significant inhibition was seen at higher doses.

5-HT_3R agonists mimic the effect of serotonin on sEPSCs in TH-EGFP neurons

To begin to determine which receptor subtype mediates the effects of serotonin, we tested agonists specific for the 5-HT_3R. Application of the 5-HT_3R agonist SR57227 (10 μM) increased the frequency of sEPSCs in 10 of 11 TH-EGFP neurons tested (Fig. 2 *A*,*B*,*D*; $p < 0.05$, KS test). On average, SR57227 increased the frequency of sEPSCs from 3.3 ± 0.7 to 11.5 ± 2.4 Hz, a 331 ± 46% increase over control (Fig. 2 *D*, $n = 11$; $p < 0.05$, one-way ANOVA). This effect was reversed by a 10 min wash (ACSF), with the frequency of sEPSCs decreasing back to 3.6 ± 0.6 Hz or 114 ± 0.2% of control (Fig. 2*A*,*B*). The effects of SR57227 were concentration dependent, with no significant effect seen at the lower doses of 1 μM (120 ± 17%, $n = 4$) and 3 μM (124.6 ± 29.7%, $n = 6$) and a significant increase at both 10 and 30 μM (331 ± 46 and 280 ± 53%, $n = 4$ and 6, respectively; $p < 0.05$, one-way ANOVA) (Fig. 2*C*). SR57227 at 6 μM significantly increased sEPSC frequency compared to ACSF (136 ± 13%, $n = 5$), but the effect was not significantly different from the other doses, meaning it has an intermediate effect. The calculated EC_{50} for SR57227 was ~7 μM.

We next tested the effect of another 5-HT_3R agonist, m-chlorophenylbiguanide (mCPBG), on glutamate inputs onto TH-EGFP neurons. mCPBG (30 μM) also increased the frequency of sEPSCs in TH-EGFP neurons from 5.2 ± 0.7 to 30.6 ± 9.6 Hz or 594 ± 191% increase (Fig. 2*D*, $n = 11$; $p < 0.05$, one-way ANOVA). A 10 min wash (ACSF) of mCPBGs partially reversed this effect, with the frequency of sEPSCs reduced to 18.1 ± 6.5 Hz or 411 ± 144% of control ($n = 8$; $p < 0.05$, one-way ANOVA).

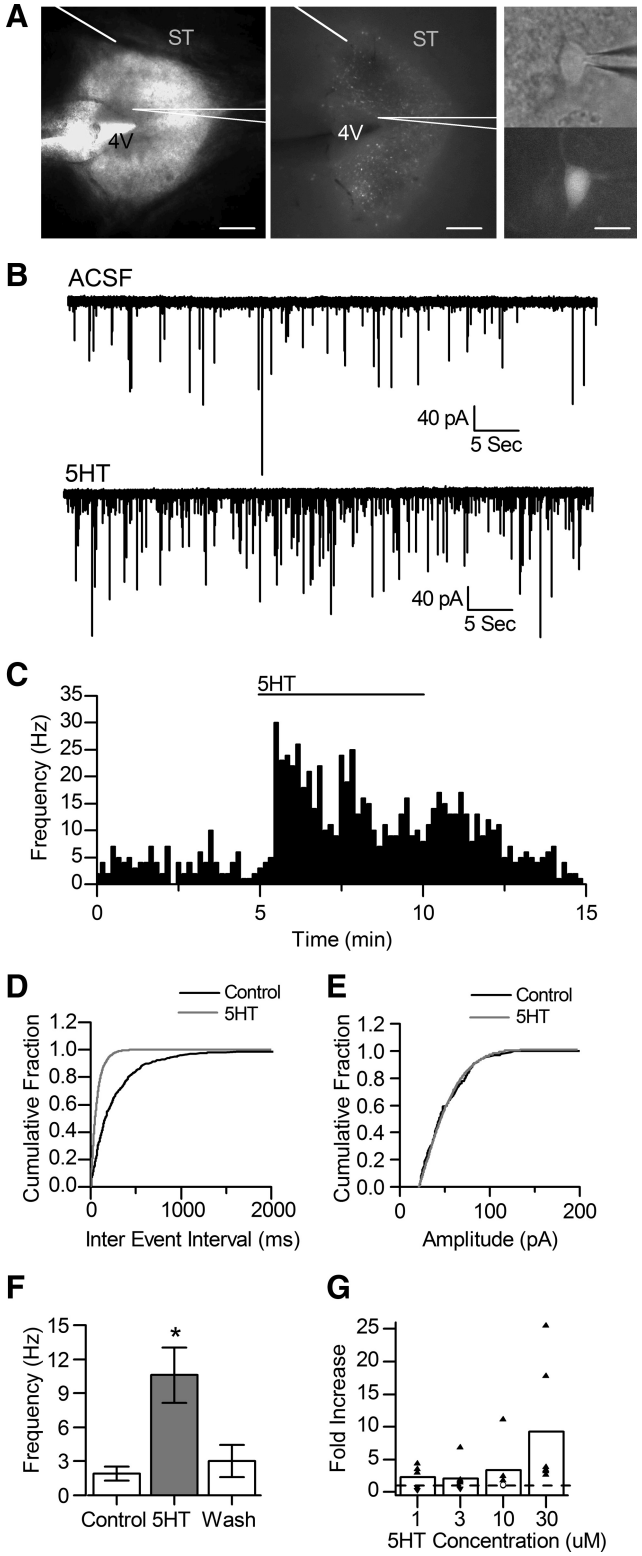

Figure 1. Serotonin increases the frequency of spontaneous glutamate EPSCs in TH-EGFP neurons. ***A***, Visualization of NTS brain slice taken from a TH-EGFP mouse using DIC (left) and fluorescence (right). Scale bars, 1 mM. ***B***, Representative (single) traces from a NTS TH-EGFP neurons during control (ACSF) and following bath application of 30 μM serotonin. $V_m = -60$ mV. ***C***, Average frequency over time, binned into 10 s periods, from a TH-EGFP neuron treated with serotonin. ***D***, ***E***, Cumulative fraction of interevent interval (***D***) and amplitudes (***E***) during baseline (control) and serotonin treatment in a TH-EGFP neuron. ***F***, Average effect of serotonin on frequency of sEPSCs in TH-EGFP neurons ($n = 6$). ***G***, Dose–response curve for serotonin. Error bars indicate SEM; *$p < 0.05$, one-way ANOVA.

5-HT$_3$R antagonists block the effect of serotonin on sEPSCs in TH-EGFP neurons

To confirm the role of the 5-HT$_3$R, we next determined whether the 5-HT$_3$R antagonist ondansetron (ODN, 0.5 μM) blocked the effects of serotonin, SR57227, and mCPBG. ODN alone did not change the frequency of sEPSCs [control = 3.4 ± 0.8 Hz; ODN = 3.4 ± 0.88 Hz; 99.8 ± 8.9% of control (ACSF)] (Fig. 2*B*,*D*, $n = 11$). However, ODN blocked the effects of serotonin to increase sEPSC frequency and actually revealed an inhibitory effect of serotonin on sEPSC frequency, presumably mediated by another serotonin receptor (ODN+ 5-HT = 54.0 ± 8.2% of ODN alone, $n = 5$). ODN also blocked the increase in sEPSC frequency stimulated by the 5-HT$_3$R agonists SR57227 and mCPBG (Fig. 2*D*; SR57227 + ODN = 88.1 ± 10.4% of ODN alone, $n = 7$; and mCPBG + ODN = 101.3 ± 9.0% of ODN alone, $n = 5$; $p < 0.05$, one-way ANOVA).

Presynaptic actions of a 5-HT$_3$R agonist on NTS-TH-EGFP neurons

To determine whether the actions of the 5-HT$_3$R agonists are through presynaptic or postsynaptic mechanisms, we examined miniature EPSCs in the presence of 2 μM TTX to block action potentials (APs) (Fig. 3). The voltage was held at −60 mV, the approximate equilibrium potential for chloride, to isolate glutamatergic mEPSCs. In the presence of TTX, 30 μM mCPBG significantly decreased the interevent interval (i.e., increased the frequency of sEPSCs) in all neurons tested ($p < 0.05$, KS test, $n = 5$) with an average frequency of 3.16 ± 0.58 Hz in TTX only to 18.2 ± 8.3 Hz in TTX plus mCPBG ($n = 5$), an average increase of 697 ± 334% (Figure *3A*,*B*,*D*, $p < 0.05$ Student's t test). In contrast, mCPBG had no consistent effect on mEPSC amplitude (Fig. 3*C*,*E*; TTX only = −44.9 ± 3.8 pA; TTX plus mCPBG = −42.3 ± 4.3 pA). TTX did not have a significant effect on baseline EPSC frequency or baseline amplitude (Fig. 3*D*,*E*).

5-HT$_3$R agonists affect less than one third of TH-EGFP negative NTS neurons

To determine whether activation of 5-HT$_3$Rs increased glutamate inputs onto all NTS neurons, we tested the effect of SR57227 (10 μM) on sEPSCs in non-TH-EGFP neurons and found that only 2 of 9 neurons responded with a significant increase ($p < 0.05$, KS test), with SR57227 increasing the sEPSC frequency to 110 and 160% compared to control (Fig. 4*A*). This is a significantly lower response rate than the 10 of 11 TH-EGFP neurons that responded to SR57227 ($p < 0.01$, Fishers exact test). mCPBG also only increased the basal frequency of sEPSCs in 2 of 6 non-EGFP neurons ($p < 0.05$, KS test), compared to 7 of 9 TH-EGFP neurons (Fig. 4*B*).

MK212 and mCPP have mixed effects on sEPSC in TH-EGFP neurons

mCPP, a non-specific serotonin receptor agonist, has been shown to activate c-fos in a subpopulation of TH positive NTS neurons *in vivo* (Lam et al., 2009). We therefore wanted to determine whether mCPP and MK212, a more selective 5-HT$_{2C}$R agonist, would also activate these neurons *in vitro*. mCPP (10 μM) increased the frequency of sEPSCs in 3 of 5 TH-EGFP neurons ($p < 0.05$, KS test). The size of the increase was smaller than the average effect of SR57227 (average mCPP response = 147 ± 11%, $n = 3$; average SR57227 response = 331 ± 46%, $n = 9$). Application of MK212 (10 μM), a 5-HT$_{2C}$R agonist, also slightly increased the frequency of sEPSCs in 2 of 6 TH-EGFP neurons

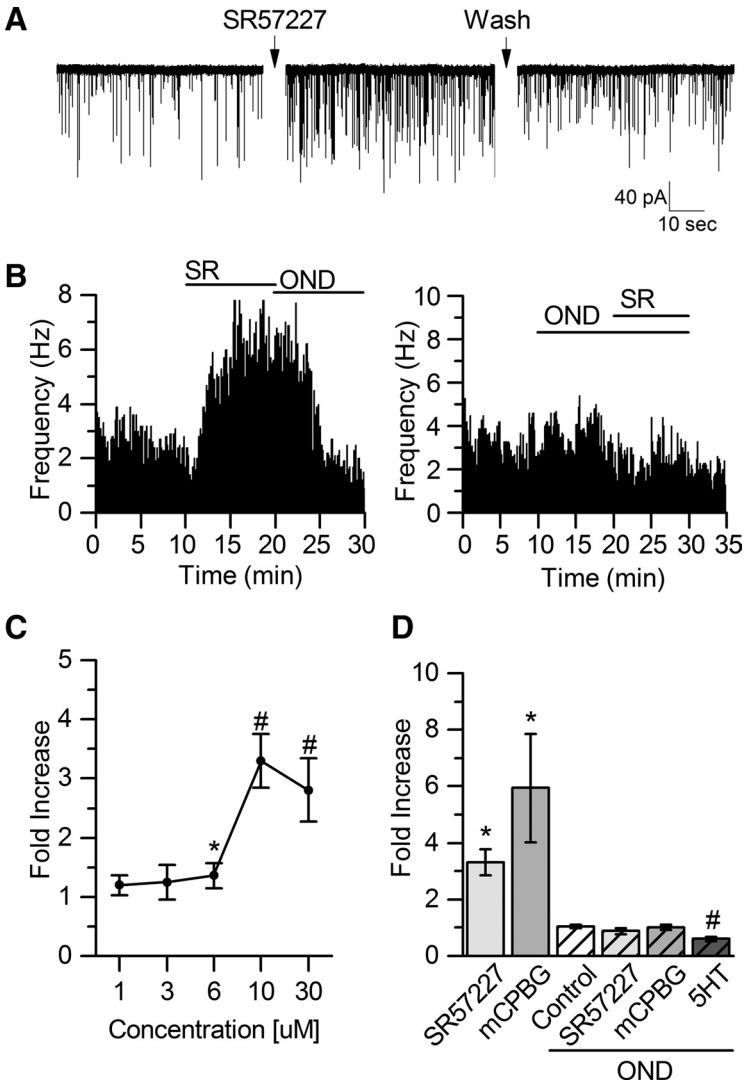

Figure 2. The effects of serotonin on spontaneous glutamate EPSCs in TH-EGFP neurons are mediated by the 5-HT_3 receptor. ***A***, Single representative traces from a NTS TH-EGFP neuron during control (ACSF) and following bath application of the 5-HT_3 receptor agonist SR57227. ***B***, Average frequency over time, binned into 10 s periods, from a TH-EGFP neuron treated with 10 μM SR57227 (SR) with and without pretreatment with the 5-HT_3 receptor antagonist ondansetron (OND) (0.5 μM). ***C***, Concentration response curve showing fold increase in sEPSC frequency following a 10 min exposure to SR57227. *$p < 0.05$ compared to ACSF only; #$p < 0.05$ compared to ACSF, 1 and 3 μM (one-way ANOVA). ***D***, Bar graph showing the average fold change in sEPSC frequency stimulated by 5-HT_3 receptor agonists in NTS TH-EGFP neurons with and without pretreatment of ondansetron. *$p < 0.05$ denotes a significant increase; # $p < 0.05$ denotes a significant decrease in frequency compared to ACSF (one-way ANOVA). Error bars indicate SEM.

($p < 0.05$, KS test) but caused a significant decrease in the frequency of sEPSCs in 3 of 6 neurons ($p < 0.05$, KS test).

SR57227 increases the action potential firing rate of TH-EGFP neurons

We have reported previously that altering spontaneous glutamate inputs onto NTS-TH-EGFP neurons alters their firing rate (Cui et al., 2011). We therefore wanted to determine whether activation of the 5-HT_3R would increase action potential firing rate of NTS-TH-EGFP neurons, as SR57227 increased the frequency of sEPSCs. Application of 10 μM SR57227 increased the AP firing rate of TH-EGFP neurons from 2.0 ± 1.0 to 3.8 ± 1.6 Hz in 7 of 8 neurons tested ($p < 0.05$, KS test), with an average increase of 254.4 ± 57.2% (Fig. 5*A*, *B*, $n = 7$, $p < 0.05$, Student's *t* test). The average resting membrane potential was −60mV. To test whether this increase in firing was due to the increased frequency of spontaneous glutamate inputs, we tested whether NBQX, an AMPA receptor antagonist, blocked the effect of SR57227 on firing rate. Bath application of NBQX blocked the ability of SR57227 to increase AP firing rate in TH-EGFP neurons [Fig. 5*C*, $n = 6$; control (ACSF), 1.8 ± 0.8 Hz; NBQX, 1.8 ± 0.8 Hz; NBQX + SR, 1.8 ± 1.1 Hz].

5-HT_3R agonists decrease the amplitude of solitary tract-evoked EPSCs

A major source of glutamate in the NTS is the solitary tract afferent fibers. We therefore examined whether 5-HT_3R agonists had any effect on the ability of the incoming visceral afferents in the ST to activate TH-EGFP neurons. Our horizontal brain slice preparation preserves a lengthy segment of the ST in the same plane as the cell bodies of NTS (Fig. 1*A*). This allows placement of the stimulating electrode on the visible ST at a sufficient distance from the recording area to allow activation of the ST with minimal focal activation of local glutamate neurons (Bailey et al., 2008). Brief shocks (100 μs duration) passed through the stimulating electrode evoked EPSCs (ST-EPSCs) in TH-EGFP neurons. As we have described previously, ST-EPSCs in the TH-EGFP neurons had nearly invariant latencies, few failures, and frequency-dependent amplitude depression (Appleyard et al., 2007). As has also been previously reported (Appleyard et al., 2007) some of these ST-EPSCs were compound EPSCs comprised of EPSCs evoked by several individual ST inputs. Bath application of 10 μM SR57227 significantly inhibited the amplitude of the ST-EPSC in all neurons tested (Fig. 6*A*,*B*), from −460.5 ± 75.5 pA in ACSF to −257.8 ± 53.0 pA in SR57227, an effect reversed by ondansetron to −384.3 ± 73.5 pA (Figure 6*A*,*B*) ($p < 0.05$, Student's *t* test) ($n = 12$). The range of inhibition was from 10.7 to 96.4% of control. Pretreatment with 0.5 μM ondansetron also blocked the effects of SR57227 (control = −445.2 ± 116.2 pA vs OND = −450.6 ± 94.9 pA, OND + SR57227 = −425.0 ± 128.4 pA) but had no effect alone on ST-EPSC amplitude (Fig. 6*A*,*B*, $n = 5$). SR57227 also significantly increased the paired-pulse ratio (PPR; EPSC2 amplitude/EPSC1 amplitude when two shocks were applied 20 ms apart), consistent with SR57227 having a presynaptic mechanism of action (PPR in control = 0.58 ± 0.04, SR57227 = 0.88 ± 0.12, wash = 0.63 ± 0.03; Figure 6*A*,*C*, $n = 10$; $p = 0.05$, Student's *t*-test). The effect of SR57227 on PPR was blocked by pretreatment of ondansetron. (Control = 0.70 ± 0.06, OND alone = 0.71 ± 0.08, OND + SR57227 = 0.70 ± 0.06, wash = 0.74 ± 0.07 (Fig. 6*A*,*C*) ($n = 5$). Given that SR57227 increases the firing rate of the TH-EGFP neurons despite the

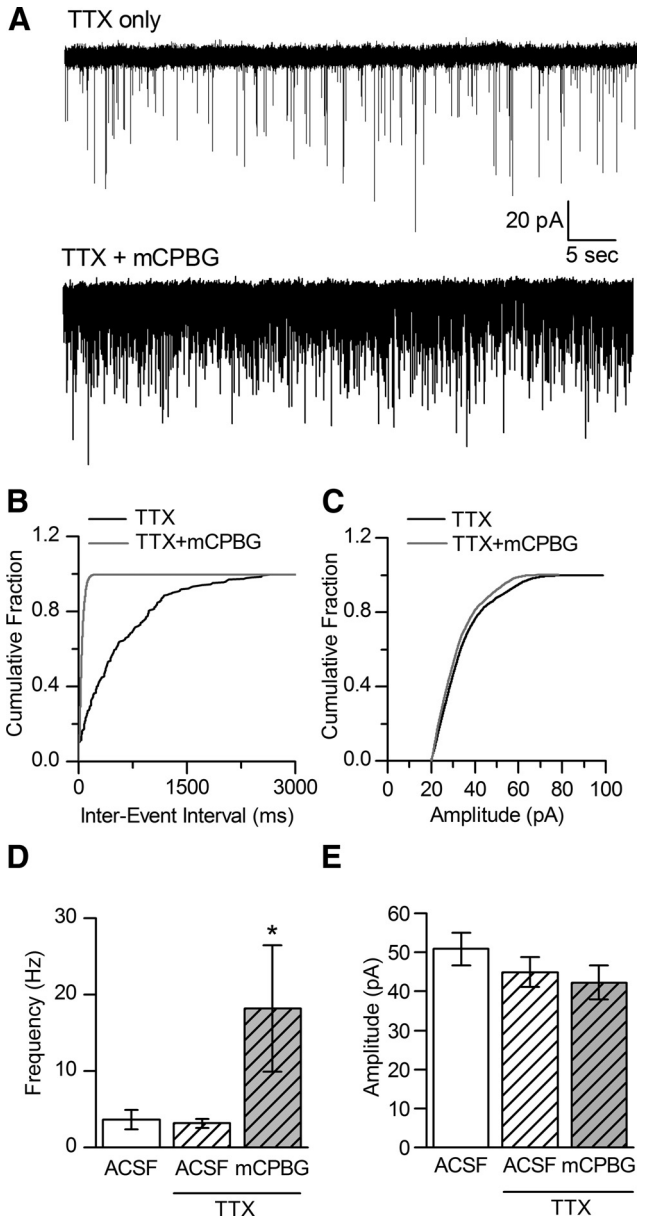

Figure 3. The 5-HT$_3$ receptor agonist mCPBG increase the frequency, but not the amplitude, of miniature EPSCs. ***A***, Representative traces in the presence of TTX during control (ACSF + TTX) and following bath application of the 5-HT$_3$ receptor agonist mCPBG (TTX + mCPBG) in a TH-EGFP neuron. ***B***, ***C***, Cumulative fraction of inter-vent interval (***B***) and amplitudes (***C***) of mEPSCs in control and in 30 μM mCPBG in the presence of TTX from a representative TH-EGFP neuron. ***D***, Graph showing the average frequency of mEPSCs in control conditions, TTX only and TTX + mGPBG. ***E***, Graph showing the average amplitude of mEPSCs in control conditions, TTX only and TTX + mGPBG ($n = 5$) *$p < 0.05$, one-way ANOVA.

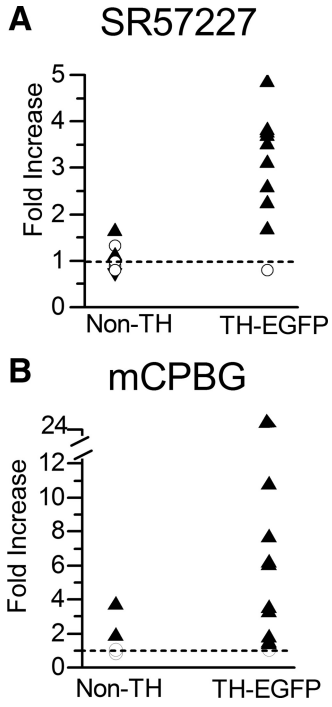

Figure 4. 5-HT$_3$ receptor agonists have a limited effect on the frequency of sEPSCs in TH-negative neurons. Scatter plots showing the fold increase or decrease in sEPSC frequency stimulated by 5-HT$_3$ receptor agonists in both TH-EGFP-negative and TH-EGFP-positive neurons. Each point represents the response in an individual neuron. ***A*** shows the effect of SR57227 in TH negative neurons ($n = 9$) and TH-positive neurons ($n = 11$). ***B*** shows the effect of mCPBG in TH-negative neurons ($n = 6$) and TH positive neurons ($n = 11$). ○ denotes effect was not significant; ▲ denotes a significant increase (KS test; $p < 0.05$); ▼ denotes a significant decrease (KS test; $p < 0.05$).

inhibition of the ST-EPSC inputs, it suggests that the overall effect of SR57227 is to excite NTS-TH neurons.

Discussion

Catecholamine neurons in the NTS are proposed to be important for the control of food intake (Mönnikes et al., 1997; Willing and Berthoud, 1997; Rinaman et al., 1998), reward (Smith and Aston-Jones, 2008; Kenny, 2011), stress responses (Schiltz and Sawchenko, 2007), cardiovascular reflexes (Simon et al., 1985; Kubo et al., 1990; Itoh and Bunag, 1993), and other homeostatic functions (Hollis et al., 2004; Ulrich-Lai and Herman, 2009). Yet little is known about how serotonin, a crucial transmitter in the control of these functions, modulates these neurons. Here we report five key new findings. First, serotonin increases the excitatory glutamate tone onto NTS-TH neurons through activation of 5-HT$_3$Rs. Second, 5-HT$_3$R agonists mimic this effect in a concentration-dependent manner through a presynaptic mechanism to increase glutamate release. Third, this surge in glutamate release increases the firing rate of NTS-TH neurons, an effect blocked by the glutamate receptor antagonist NBQX. Fourth this activation does not require activation of the solitary tract. Fifth, 5-HT$_3$R agonists preferentially modulate NTS-TH neurons compared to non-TH NTS neurons.

Serotonin increases the frequency of glutamate inputs onto NTS-TH neurons through activation of presynaptic 5-HT$_3$Rs

5-HT$_3$Rs are expressed on sensory afferent terminals (Pratt and Bowery, 1989; Leslie et al., 1990; Huang et al., 2004), as well as terminals in close proximity to catecholamine neurons (Pickel et al., 1984). Here we show that serotonin dramatically increases spontaneous glutamate EPSCs onto NTS-TH neurons through activation of 5-HT$_3$Rs, as the effect is completely blocked by the 5-HT$_3$R antagonist ondansetron and mimicked by the 5-HT$_3$R agonists mCPBG and SR57227. The effects of both 5-HT and SR57227 are concentration dependent, with very steep relationships consistent with a cooperative binding relationship (Barnes et al., 1992). This demonstrates for the first time that serotonin increases the basal excitatory glutamate tone onto NTS-CA neurons. Our data support a presynaptic mechanism of action, as 5-HT$_3$R agonists increase the frequency but not the amplitude of mEPCS and have no discernible effect on post-synaptic parame-

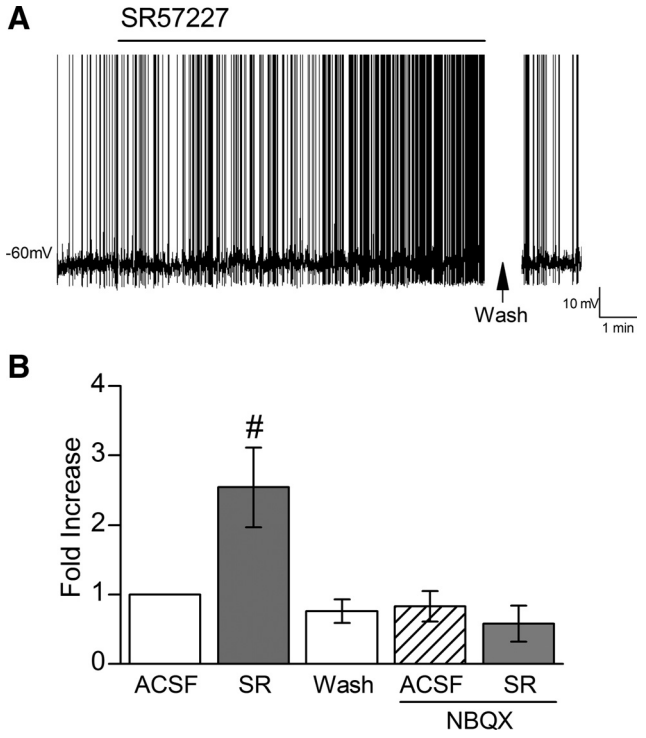

Figure 5. The 5-HT$_3$ receptor agonist SR57227 increases the firing rate of TH-EGFP neurons in a glutamate-dependent manner. ***A***, Representative trace from a current-clamp experiment showing the firing rate of a TH-EGFP neuron. Bath application of 10 μM SR57227 significantly increased the action potential firing rate in all six NTS TH-EGFP neurons tested. ***B***, Average fold increase in action potential firing rate in NTS-EGFP neurons compared to baseline (ACSF/control) following SR57227 (SR) treatment and wash ($n = 6$) and in the presence of the inotropic glutamate antagonist 20 μM NBQX, SR57227 + NBQX and following wash (in NBQX only) in NTS TH-EGFP neurons ($n = 6$). Error bars indicate SEM; $^{\#}p < 0.01$, one-way ANOVA.

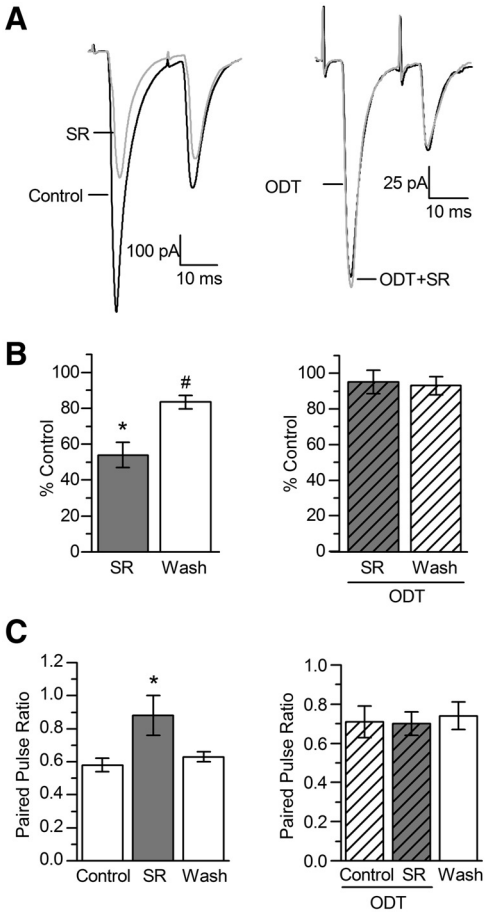

Figure 6. The 5-HT$_3$ receptor agonist SR57227 (SR) (10 μM) significantly decreases the amplitude of ST-EPSCs in TH-EGFP neurons. ***A***, Representative trace of two ST-stimulated EPSCs. ST activation evoked monosynaptic EPSCs in TH-EGFP neurons (Jitter or SD of latency <200 μsec). V_m, -60 mV. SR57227 significantly inhibited the amplitude of the ST stimulated EPSCs. This effect was reversed after a 10 min wash. ***B***, A graph showing the average inhibition of effect of ST-EPCS amplitude by SR57227 and following a 10 min wash in TH-EGFP neurons in control conditions (left) and in the presence of the 5-HT$_3$ receptor antagonist ondansetron (ODT) (0.5 μM). ***C***, A graph showing the paired pulse ratio in ACSF following bath application of SR57227 and following a 10 min wash in control conditions (left) and in the presence of ondansetron (10 μM, right) in TH-EGFP neurons ($n = 12$). Error bars indicate SEM; $^{*}p < 0.05$, one-way ANOVA.

ters. Interestingly, serotonin inhibited sEPSC frequency in the presence of ondansetron, suggesting other 5-HT receptors inhibit glutamate release. 5-HT$_{1D}$Rs are present in the NTS, and their activation inhibits NTS neuronal discharge *in vivo* (Jeggo et al., 2007). We found the 5-HT$_{2C}$R agonist, MK212, inhibits glutamate inputs onto 50% of CA neurons. Low concentrations of serotonin also inhibit inputs onto 50% of CA neurons, potentially due to its higher affinity for 5-HT$_{2C}$Rs and 5-HT$_{1D}$Rs than for 5-HT$_3$Rs (Olivier et al., 1997) and suggesting the intriguing possibility of differential effects of serotonin depending on its concentration and site of release.

5-HT$_3$R activation decreases afferent activation of NTS-TH-EGFP neurons

In contrast to their effect to increase spontaneous glutamate inputs, we found that 5-HT$_3$R agonists decrease the amplitude of ST-EPSCs in NTS-TH neurons. One explanation is that activation of 5-HT$_3$Rs causes such a large increase in spontaneous glutamate release that the readily releasable vesicle pool is depleted; therefore, less glutamate is available during ST stimulation, and the amplitude of the evoked EPSC is decreased. This mechanism is proposed to underlie the effects of the VR1 agonist capsaicin, which also increases sEPSC frequency while attenuating ST-EPSC amplitude in NTS neurons (Doyle et al., 2002; Peters et al., 2010). Multiple lines of evidence support this model. First, 5-HT$_3$Rs are ligand-gated cation channels that are generally excitatory and increase the probability of transmitter release (Funahashi et al., 2004; Derkach et al., 1989; Machu, 2011). Second, activation of 5-HT$_3$Rs depolarizes nodose ganglia neurons (ST afferent cell bodies) (Higashi and Nishi, 1982), increases vagal afferent firing (Niijima, 1981; Blackshaw and Grundy, 1993), and potentiates vagal activation of NTS neurons (Jeggo et al., 2005), suggesting they have excitatory effects on afferents. Third, 5-HT$_3$R antagonists decrease both vagal activation of NTS neurons (Jeggo et al., 2005) and the probability of glutamate release from afferent terminals (Wan and Browning, 2008), consistent with serotonin normally increasing glutamate release. Taken together with our findings, these data suggest that serotonin activates NTS-TH neurons, at least in part by releasing glutamate from ST afferents. Interestingly, we see no effect of the 5-HT$_3$R antagonist in our horizontal slices, suggesting we have lost an endogenous serotonin tone maintained in coronal slices that preserve inputs from raphe nuclei (Wan and Browning, 2008).

Serotonin increases firing rate of NTS-TH neurons indirectly by increasing excitatory glutamate inputs

Transmitters and hormones can dynamically adjust the firing threshold of neurons by altering spontaneous glutamate inputs

(Lee et al., 2010; Sutton et al., 2006), including NTS-TH neurons (Cui et al., 2011). SR57227 increased the firing rate of NTS-TH neurons in an AMPAR-dependent manner; suggesting that activation of 5-HT_3Rs increases NTS-TH neuronal firing indirectly by increasing glutamate tone. Furthermore, serotonin increases glutamate release independently of afferent activation, indicating that serotonin can activate NTS-TH neurons even in the absence vagal feedback.

Serotonin preferentially effects catecholamine over noncatecholamine neurons in the NTS

In contrast to the almost universal responsiveness of NTS-TH neurons to serotonin, less than one-third of non-TH NTS neurons respond to 5-HT_3R agonists, and the size of the responses was considerably smaller than that in catecholamine neurons. This identifies TH neurons as an important subpopulation of NTS neurons that are activated by serotonin. 5-HT_3Rs in the NTS contribute to the control of meal size (Hayes and Covasa, 2006b) and modulate cardiovascular reflexes (Merahi et al., 1992; Sévoz-Couche et al., 2003; Jeggo et al., 2005), suggesting that they have an important action on homeostatic functions. NTS 5-HT_3Rs are also critical for the severe anorexia induced by ablation of NPY neurons in the arcuate nucleus of the hypothalamus (ARC) (Wu et al., 2012). A glutamatergic projection from the NTS to the parabrachial nucleus (PB) is similarly required for this anorexia. As norepinephrine (NE) is released locally in the NTS (Al-Khrasani et al., 2003; Arakawa et al., 1991), it is possible that activation of 5-HT_3Rs increases NE release, which then activates NTS glutamate neurons projecting to the PB. Alternatively, some of the NTS-TH neurons or the 30% of non-TH neurons that respond to 5-HT_3R activation could be glutamatergic. The degree of the anorexia seen with activation of 5-HT_3Rs in the NTS and the use of 5-HT_3R antagonists to treat nausea clinically (Machu, 2011) suggest that their effects on food intake may be protective through eliciting nausea to non-nutritive/poisonous food.

Physiological implications for serotonin activating NTS-TH neurons

NTS-CA neurons make extensive projections to many nuclei, including the hypothalamus, nucleus accumbens, and the dorsal motor nucleus of the vagus nerve or DMNV (Cunningham and Sawchenko, 1988; Wang et al., 1992; Rogers et al., 2003; Reyes and Van Bockstaele, 2006; Rinaman, 2011). Our finding that serotonin activates >90% of NTS-TH neurons predicts that serotonin would cause an increase in catecholamine release at most of these sites. NTS-CA neurons participate in neuronal circuits that widely influence homeostasis and behaviors including food intake, reward, anxiety, stress, and cardiovascular reflexes (Lacroix and Rivest, 1997; Chan and Sawchenko, 1998; Van Bockstaele et al., 2001; Laorden et al., 2002; Krout et al., 2005). Therefore, our results elucidate a mechanism by which serotonin could influence catecholamine modulation of these behaviors and functions.

Afferent terminals in the NTS that directly activate NTS-TH neurons are responsive to several signals that modulate food intake and other homeostatic functions, including cholecystokinin or CCK (Appleyard et al., 2007), ghrelin (Cui et al., 2011) and opioids (Cui et al., 2012). This suggests a model in which the ST afferent terminals themselves integrate humoral signals, resulting in different amounts of glutamate release and activation of NTS-TH neurons. Such an integrative capacity has already been proposed for the vagus (Browning and Travagli, 2010; Dockray, 2009).

Endogenous source of serotonin

Serotonin-positive nerve terminals are found throughout the medial NTS (Steinbush, 1981), including in close proximity with NTS-CA neurons (Pickel et al., 1984). Neurons in caudal raphe nuclei project to the NTS (Thor and Helke, 1989), and stimulation of caudal raphe nuclei releases serotonin in the NTS (Brodin et al., 1990; Weissheimer and Machado, 2007); furthermore, this projection is required for the anorexia induced by ablation of ARC-NPY (arcuate nucleus-neuropeptide Y) neurons (Wu et al., 2012). Serotonin is synthesized in some afferents (Hery et al., 1986), providing another source of serotonin in the NTS. 5-HT_3Rs are synthesized in nodose ganglia neurons and trafficked to terminals in both the NTS and the gut (Li, 2007); therefore, afferents modulated by serotonin centrally are also likely to respond to serotonin in the gut. The gut is a major source of serotonin, where it regulates GI function and increases vagal afferent feedback (Cirillo et al., 2011). As we found, all NTS-TH neurons receive direct inputs from serotonin-sensitive afferents; another implication of our findings is that NTS-TH neurons are potentially a downstream target of afferents that respond to GI serotonin.

In summary, our data shows that serotonin, through activation of 5-HT_3 receptors, strongly and broadly excite NTS-TH neurons through a presynaptic mechanism to increase glutamate release and action potential generation. This effect is relatively selective to NTS-TH neurons and is independent of vagal afferent activation. These results demonstrate a potential mechanism by which serotonin could activate NTS-CA neurons to increase release of catecholamines at multiple target nuclei and influence behaviors such as food intake, motivation, stress, and cardiovascular function.

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