5-Hydroxytryptamine–Mediated Neurotransmission Modulates Spontaneous and Vagal-Evoked Glutamate Release in the Nucleus of the Solitary Tract Effect of Uptake Blockade

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Received November 13, 2013; accepted March 7, 2014

ABSTRACT

The effect of blockade of either 5-hydroxytryptamine (5-HT)/serotonin transporter (SERT) with citalopram or the organic cation transporter 3 (OCT3)/plasma membrane monoamine transporter (PMAT) with decynium-22 (D-22) on spontaneous and evoked release of 5-HT in the nucleus tractus solitarius (NTS) was investigated in rat brainstem slices treated with gabazine. 5-HT release was measured indirectly by changes in the frequency and amplitude of glutamatergic miniature excitatory postsynaptic currents (mEPSCs) [in the presence of tetrodotoxin (TTX)] and evoked EPSCs. Blockade of 5-HT3 receptors with granisetron reduced, whereas the 5-HT1A antagonist phenylbiguanide increased, the frequency of mEPSCs. 5-HT decreased mEPSC frequency at low concentrations and increased frequency at high concentrations. This inhibition was blocked by the 5-HT1A antagonist N-[2-[4-(2-methoxyphenyl)-1-piperazinyl][ethyl]-N-2-pyridinylcyclohexanecarboxamide (WAY-100635), which was ineffective on its own, whereas the excitation was reversed by granisetron. The addition of citalopram or D-22 caused inhibition, which was prevented by 5-HT1A blockade. Thus, in the NTS, the spontaneous release of 5-HT is able to activate 5-HT3 receptors, but not 5-HT1A receptors, as the release in their vicinity is removed by uptake. The ineffectiveness of corticosterone suggests that the low-affinity, high-capacity transporter is PMAT, not OCT3. For evoked 5-HT release, only D-22 caused an increase in the amplitude of EPSCs, with a decrease in the paired pulse ratio, and increased the number of spontaneous EPSCs after 20-Hz stimulation. Thus, for the evoked release of 5-HT, the low-affinity, high-capacity transporter PMAT, but not 5-HT transporter (5-HT)/SERT, is important in the regulation of changes in 5-HT extracellular concentration.

Introduction

Homeostatic mechanisms within the healthy individual maintain mean arterial blood pressure, blood volume, and arterial blood gases within narrow ranges, with appropriate alterations dependent on physiologic state. The central nervous system is crucial to this homeostatic control by monitoring sensory information such as that from arterial baroreceptors and chemoreceptors and those in the cardiopulmonary region. The nucleus tractus solitarius (NTS), located near the dorsal surface of the brainstem, is known to be important in the integration of this visceral sensory information (Andresen and Kunze, 1994). Glutamate is considered the major transmitter mediating cardiovascular and visceral afferent input to the NTS (Talman, 1997; Baude et al., 2009). However, along with some other transmitters, 5-hydroxytryptamine (5-HT; serotonin) has also been implicated in visceral afferent neurotransmission (see Ramage and Villalón, 2008). In this respect, the NTS is rich in 5-HT–containing nerves fibers, which have been shown to originate from the medullary raphé nuclei (Schaffar et al., 1988; Sim and Joseph, 1992; Thor and Helke, 1987) and from the nodose ganglia, the site of vagal afferent cell bodies (Oter et al., 1991; Nosjean et al., 1990). Further, the NTS has the densest expression in the brain of the only ionotropic 5-HT receptor, the 5-HT3 receptor. These receptors are found mainly on vagal afferent terminals (Leslie et al., 1990; Pratt and Bowery, 1989), and their blockade attenuates vagal afferent excitation of NTS neurons (Ramage and Mifflin, 1998; Jeggo et al., 2005). These 5-HT3 receptors cause excitation of NTS neurons via the release of glutamate (Jeggo et al., 2005). This has also been confirmed in the NTS slice in the presence of tetrodotoxin (TTX) and bicuculline, in which the frequency of glutamatergic miniature excitatory postsynaptic currents (mEPSCs) was attenuated by blockade of 5-HT3 receptors (Wan and Browning, 2008). This indicates

This work was funded in part by Biotechnology and Biological Sciences Research Council studentship [Grant BB/F017146/1] (to P.S.H.); and a Bogue Fellowship (to S.W.M.’s laboratory to carry out these experiments).

dx.doi.org/10.1124/jpet.113.211334.

ABBREVIATIONS: aCSF, artificial cerebrospinal fluid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; D-22, decynium-22; 5-HT, 5-hydroxytryptamine; 5-HT3, 5-HT transporter; mEPSCs, miniature excitatory postsynaptic currents; NTS, nucleus tractus solitarius (nucleus of the solitary tract); OCT3, organic cation transporter 3; PMAT, plasma membrane monoamine transporter; sEPSCs, spontaneous excitatory postsynaptic currents; SERT, serotonin transporter; SR57227, 1-(6-chloro-2-pyridinyl)-4-piperidinidine hydrochloride; SR-95531, gabazine; ST, solitary tract; TTX, tetrodotoxin; WAY-100635, N-[2-[4-(2-methoxyphenyl)-1-piperazinyl][ethyl]-N-2-pyridinylcyclohexanecarboxamide.
that at least in the slice, 5-HT terminals are continuously releasing 5-HT. As 5-HT neurotransmission is highly regulated by its uptake systems (Daws, 2009), inhibition of these transporters may give a clearer understanding of how these 5-HT pathways regulate the release of glutamate in the NTS. Therefore, the present experiments were performed to investigate the effects of blockade of 5-HT transporter (5-HTT; SERT) with citalopram (Hytteblad, 1982) and blockade of the organic cation transporter 3 (OCT3/plasma membrane monoamine transporter (PMAT) with decynium-22 (D-22) (Schöning et al., 1993; Duan and Wang, 2010) on mEPSCs. In addition, 5-HT regulation of inward excitatory currents evoked by stimulation of the tractus solitarius (TS) was investigated.

Materials and Methods

Animal procedures were conducted according to National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the institutional animal care and use committee.

Brain-Slice Preparation

A total of 75 male Sprague-Dawley rats (75–100g) were used. Rats were deeply anesthetized with isoflurane and decapitated, and the brainstem was rapidly removed and completely immersed in ice-cold (<4°C) high-sucrose artificial cerebrospinal fluid (αCSF) that contained (in mM) 3 KCl, 1 MgCl2, 2 CaCl2, 2 MgSO4, 1.25 NaH2PO4, 26 NaHCO3, 10 d-glucose, and 206 sucrose, pH 7.4 when continuously bubbled with 95% O2–5% CO2. The brainstem was mounted in a vibrating microtome (VT1200S, Leica Microsystems, Bannockburn, IL) and coronal or horizontal slices (300–600μm thickness) were cut with a sapphire knife (Delaware Diamond Knives, Wilmington, DE). Once cut, slices were transferred to an incubation chamber containing (in mM) 126 NaCl, 26 NaHCO3, 3 KCl, 2 CaCl2, 2 MgSO4, and 10 d-glucose. The incubation chamber was continually gassed with 95% O2–5%CO2 to maintain solution pH and recirculate the αCSF. Slices were incubated at room temperature for at least 90 minutes before transfer to a recording chamber.

Electrophysiological Recording

Slices were submerged in a perfusion chamber (RC-26GLP; Warner Instruments, CT) and constantly perfused with oxygenated αCSF at room temperature (2–3 mL/min). The slice was held in place with a slice anchor, which consisted of type 316 stainless steel with Lycra threads, finished with a plastic coating (Warner Instruments, CT). Neurons were visualized using an upright microscope (Olympus BX50) fitted with near-infrared differential interference contrast optics and a cooled camera. The resultant image was displayed on a closed-circuit television. Cells were identified as NTS neurons by location and morphology. Recordings from neurons were made only from within the intermediate NTS. Patch pipettes were pulled from borosilicate glass capillaries with an inner filament (0.90-mm internal diameter, 1.2-mm outer diameter; WPI, Sarasota, FL) on a pipette puller (model P-2000; Sutter Instruments, Novato, CA) and were filled with a solution containing the following (in mM): 128 potassium gluconate, 10 KCl, 0.3 CaCl2, 1 MgCl2, 10 HEPES, 1 EGTA, 2 ATP, and 0.25 GTP. The pH was adjusted to 7.35 with potassium hydroxide. The pipette resistance ranged from 3 to 5 MΩ. Voltage-clamp recordings were made with an Axopatch 200B (Molecular Devices, Foster City, CA) acquisition rate 10 kHz, filtered at 2 kHz, and digitized via a Digidata 1320 interface. Data were stored and analyzed on a computer running pclamp10 software (Molecular Devices, Sunnyvale, CA) or Mini Analysis software (Synaptsoft Inc., Fort Lee, NJ). Cells with a seal resistance of ≥1 GΩ and an access resistance <50 MΩ that changed <15% during recording were considered acceptable. The first 10 minutes of each recording was discarded after the whole-cell access was established, and the holding current reached a steady state. Only recordings with stable holding currents <100 pA at Vh = −60 mV were taken into further analysis. In all coronal patch-clamp experiments, the bath solution included the sodium channel blocker TTX (1 μM) and the GABA<sub>A</sub> receptor antagonist gabazine (25 μM), whereas the horizontal sections contained only gabazine (25 μM).

Electrical Stimulation: Horizontal Slice

In these slices, mEPSCs were recorded. These synaptic currents could be increased by stimulating the solitary tract (ST) with concentric bipolar stimulating electrodes (50-μm inner diameter; FHC, Bowdoin, ME) placed on the ST approximately 1–3 mm from the recording site. Electrical stimulation was delivered from an isolated constant current stimulator (DS3; Digitimer, Welwyn Garden City, UK). Pulse trains were programmed and triggered via a Master-8 (A.M.P.I, Jerusalem, Israel). Electrical stimulation was delivered as follows: in some experiments, stimuli (0.1–0.5 ms, 20–320 μA, 200 ms apart) were applied every 20 seconds to evoke submaximal mEPSCs. The stimulus interval was altered to allow the first stimulated current to decay completely before the second stimulus. The paired-pulse ratio was calculated as the amplitude of the second current relative to that of the first; alterations in the paired-pulse ratio are suggestive of a presynaptic site of action. In a separate set of experiments, a 100-pulse train was delivered to the ST at 20 Hz to evoke an increase in spontaneous EPSC frequency poststimulus. The amplitude was adjusted to evoke submaximal EPSC inward currents (~100 pA).

Experimental Protocol, Data Analysis, and Statistics

Frequencies and amplitudes of mEPSCs were sampled continuously. For the coronal slice experiments, these mEPSCs was recorded for a control period of 10 minutes in drug-free αCSF. To test whether the recordings were stable, the frequency of mEPSCs over the first 200 seconds was compared with that over the last 200 seconds of this 10-minute period. If the percentage difference in frequency between these two values was ≥20%, the recording was considered stable. The mean of these two values was taken as control. The same conditions applied to the amplitude measurements as well. The test drug or drugs were then applied, and recordings were made over a 10-minute window before changing to another drug challenge or to drug-free αCSF. The maximum number of drug challenges was three per cell. The action of 5-HT or phenylbiguanide alone and in the presence of antagonists was measured once the effect had plateaued (i.e., constant frequency mEPSCs over 200 s), which was approximately 5–7 minutes after application. The drug(s) was then washed out with αCSF. This was carried out within the 10-minute window. In the phenylbiguanide experiments, the second and higher concentration was given after recovery from the lower concentrations. In the 5-HT experiments, all neurons received two challenges of different 5-HT concentrations. In cases where either antagonist or combined agonist/antagonist/uptake inhibitor was used, only one challenge was carried out per neuron; in these cases, the challenge was either the antagonist alone or the combination.

Evoked activity-horizontal slices. Measurements of increases in EPSC activity from electrical stimulation of ST were taken 1 minute after stimulation had ceased, as this is when the maximum increase in activity was observed, and averaged over a 30-second period. Two control stimulations of ST were carried out 10 minutes apart to determine stability. If stable (i.e., change was not greater than 10%), then stimulation was repeated again in presence of either αCSF (control) or citalopram or D-22 applied 5 minutes after the second stimulation, and the changes in frequency and amplitude were compared with those of the second control stimulation. All changes are expressed as the mean percentage change from second control ± S.E.M.

All comparisons between control and test drugs were made using either a Student’s paired t test or one-way analysis of variance with Fisher’s least significant difference test for post hoc comparisons. P values < 0.05 were considered significant.

Drugs and Chemicals

Gabazine (SR-95531) hydrobromide, tetrodotoxin (TTX) citrate, adenosine 5′-triphosphate (ATP) disodium salt, granisetron (BRL-43694) monohydrochloride, citalopram hydrobromide, and 5-hydroxytryptamine

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(serotonin) creatinine sulfate, CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) were obtained from Abcam Biochemicals (Boston, MA), whereas gabazine (SR-95531 bromide, 1,1-diethyl-2,2'-cyanine iodide (decynium-22; D22), WAY-100635 maleate [(N-[2-[4-(2-methoxyphenyl)-1-piperazinyl][ethyl]-N-2-pyridinylcyclohexancarboxamide]; phenylbiguanide hydrochloride, dimethylsulfoxide, and the chemical ingredients for aCSF were from Sigma-Aldrich (St. Louis, MO). All drugs were dissolved in aCSF, with the exception of CNQX. For CNQX, a 25 mM solution in dimethylsulfoxide was made initially and then diluted in aCSF to give a final concentration of 25 μM.

Results

mEPSCs were recorded in 72 NTS neurons in the presence of 1 μM TTX and 25 μM gabazine (Fig. 1A). Baseline mEPSC frequency was 2.0 ± 0.6 Hz with mean amplitude 13.5 ± 0.8 pA. mEPSCs could be completely blocked by the application of CNQX (10 μM, n = 3).

Coronal NTS Slices

Effect of Applied 5-HT3 Receptor Ligands on mEPSCs. Application of PBG at concentrations of 1 and 10 μM caused significant increases in frequency (497% ± 112%, n = 6; 1744% ± 614%, n = 4, respectively) and amplitude (18% ± 5% and 42% ± 20%, respectively) of mEPSCs (Fig. 1). In the presence of the 5-HT3 antagonist granisetron (GRN; 1 μM), the ability of PBG (1 μM; n = 4) to increase the frequency (58% ± 14%) and amplitude (−3.5% ± 2%) was significantly attenuated. Granisetron alone (1 μM, n = 6) caused a gradual and significant decline in the spontaneous frequency of mEPSCs (Fig. 1C), reaching a maximum of 36% ± 4% between 5 and 7 minutes, but it had no significant effect on amplitude. It should be noted that changes in frequency of spontaneous mEPSCs are dependent on the probability of vesicle fusion and release and thus can only be altered by presynaptic action/s of a drug, whereas changes in amplitude depend on the properties of postsynaptic cells.

Effect of Applied 5-HT on mEPSCs. The application of 0.3 μM 5-HT decreased the frequency of mEPSCs in all NTS neurons tested (n = 5) by 53% ± 12% with no significant effect on amplitude (Fig. 2); 1 μM 5-HT had a mixed effect. In 5 of 11 neurons tested, there was a significant decrease in the frequency of 62% ± 10% (Fig. 2, A and B). In the remaining six, there was a significant increase in the frequency of 403% ± 142% (Fig. 2, A and C). Amplitude was unaffected in all cases. Application of 10 μM 5-HT significantly increased mEPSC frequency in all cases (n = 8) by 1285% ± 276% (Fig. 2, A and C) and also the amplitude by 21% ± 5% (Fig. 2A). The highest concentration, 100 μM (n = 5), caused an even greater increase in frequency (2462% ± 543%) and amplitude 52% ± 9%.

In the presence of granisetron (1 μM, n = 6), 5-HT (10 μM) now caused a decrease in the mEPSC frequency of 30% ± 11%

Fig. 1. Rat coronal brain slice pretreated with TTX and gabazine showing the effects of activation and inhibition of 5-HT3 receptors on patch-clamp recordings of spontaneous mEPSCs from NTS neurons. (A) Representative experimental traces (five sweeps superimposed) of mEPSC activity showing the effect of two concentrations of phenylbiguanide (PBG) (1 and 10 μm) and the effect of pretreatment with granisetron (GRN) (1 μM) on the low-concentration PBG excitation from separate experiments. (B, C) Frequency histograms of mEPSCs in separate experiments in which the 5-HT3 agonist PBG (B) and antagonist GRN (C) were applied alone via the bath.
(Fig. 2B), whereas the inhibitory action of the lowest concentration (0.3 μM) of 5-HT was blocked by the 5-HT1A antagonist WAY-10035 (n = 8; Fig. 2C).

**Effect of Blockade of SERT and OCT3/PMAT on mEPSC.** Application of the SERT inhibitor citalopram (0.5 μM, n = 5) caused a significant decrease in the frequency of mEPSCs of 54% ± 5% (Fig. 3, A and B), and a similar decrease was observed at 10× higher concentration (5 μM; n = 5) of citalopram, 38% ± 6% (Fig. 3B). Likewise, blockade of OCT3/PMAT with 1 μM D-22 (n = 4) caused a significant decrease in mEPSC frequency of 33% ± 4%, as did the higher concentration of 10 μM (n = 3; Fig. 3, A and C), which caused a decrease in mEPSC frequency of 44% ± 6%. In all cases, mEPSC amplitudes remained unchanged (Fig. 3).

**Effects of 5-HT1A Blockade on mEPSC.** Application of aCSF (control, n = 11) and WAY-100635 (1 μM, n = 12) had no significant effect on the spontaneous mEPSC frequency −7.2% ± 2% and 1.2% ± 4%, respectively (Fig. 3). However, pretreatment with WAY-100635 blocked the inhibitory action of either concentration of citalopram (0.5 μM, n = 5; 5 μM, n = 4) to reduced mEPSC frequency (now changing by only −12% ± 5% and +2% ± 23%, respectively). WAY-100635 pretreatment also blocked the ability of both concentrations of D-22 (1 μM, n = 6; 10 μM, n = 4) to decrease mEPSC frequency (Fig. 3, A and C). However, at the higher concentration, D-22 now caused a small and significant increase in mEPSC frequency (57% ± 39%) (Fig. 3, A and C). In all cases, mEPSC amplitudes remained unchanged.

**Effect of Corticosterone**

Application of the OCT3 inhibitor corticosterone (1 μM, n = 5; 3 μM, n = 4) caused no change in mEPSC frequency at both concentrations, −1.5% ± 22% and 10% ± 19%, respectively. mEPSC amplitude remained unchanged.

**Horizontal NTS Slices: TS Stimulation**

**Effect of Transporter Inhibitors on EPSCs Evoked by ST Stimulation: Single Pulse.** The effect of transporter inhibitors on synaptic transmission was assessed in 26 additional NTS neurons in which EPSCs were evoked by electrical stimulation of the TS in the presence of gabazine...
The mean amplitude of the evoked control EPSC was −90 ± 9 pA. Application of CNQX (10 mM, n = 5) completely abolished TS-evoked inward current. Citalopram, at the higher concentration (0.5 mM, n = 10) failed to alter the amplitude of the first evoked EPSC (2.8 ± 6.6%) compared with control (2.1 ± 2.0%). Paired-pulse ratio also remained unaltered (0.71 ± 0.06 versus 0.71 ± 0.08) (see Fig. 4).

The higher concentration (10 μM, n = 4) of D-22 significantly increased the amplitude of the first evoked EPSC by 27.2 ± 6.8% compared with control (−1.7 ± 1.9%). Pairing-pulse ratio was also unaltered (0.71 ± 0.06 versus 0.71 ± 0.08) (see Fig. 4).

Trains of 100 Pulses at 20 Hz. Spontaneous EPSCs (sEPSCs) were recorded from 17 neurons; mean frequency and amplitude were 1.6 ± 0.3 Hz and 17.1 ± 1.1 pA. Electrical stimulation of the TS (100 pulses at 20 Hz) evoked an increase in sEPSC frequency of 106% ± 13% compared with baseline (Fig. 5). This remained unaltered in two subsequent stimulations (100% ± 6%). Amplitude was not significantly affected (−2.4 ± 3.85%). Citalopram (0.5 μM, n = 6) failed to alter the TS-evoked increase in sEPSC frequency (99% ± 23%); however, D-22 (1 μM, n = 7) caused a significant increase in sEPSC frequency (242% ± 20%) (Fig. 5). Neither citalopram nor D-22 affected sEPSC amplitude (8.2% ± 3.4% and 6.5% ± 3.6%, respectively).

Discussion

Spontaneous Release. The ability of the selective 5-HT3 antagonist granisetron (Sanger and Nelson, 1989) to reduce glutamatergic mEPSCs confirms the observations of Wan and Browning (2008) that the spontaneous release of 5-HT acts via 5-HT3 receptors on vagal afferent terminals to release glutamate in the NTS in rat brainstem slice preparations treated with TTX and gabazine. It should be noted that in vivo experiments, granisetron alone had little effect on spontaneous NTS neuronal activity (Jeggo et al., 2005). In this respect, in the mouse horizontal slice preparation, blockade of 5-HT3 receptors with ondansetron did not affect the spontaneous release of glutamate in the NTS (Cui et al., 2012). This finding may simply reflect that in these experiments, GABA receptors were not blocked and/or rat coronal brainstem slices were used. In addition, it should also be
noted that the pharmacology of murine 5-HT$_3$ receptors differs from that of the rat (Bonhaus et al., 1993). However, activation of 5-HT$_3$ receptors in the present experiments with phenylbiguanide and in the mouse with m-chlorophenylbiguanide or SR57227 caused the expected increases in glutamatergic mEPSCs, which were blocked by granisetron or ondansetron. This was a particularly powerful effect in both species, causing, in some cases, a 2000% increase in mEPSC frequency that was concentration-related. In contrast to PBG, 5-HT in the present experiments caused a concentration-related, biphasic effect on mEPSC frequency. Low concentrations of 5-HT caused inhibition but at high concentrations caused excitation. This excitation was also associated with large increases in mEPSC frequency. The inhibition by low concentrations was blocked by pretreatment with the 5-HT$_1$A antagonist WAY-100635 (Forster et al., 1995), whereas the high-concentration excitation could be reversed to inhibition in the presence of the 5-HT$_3$ antagonist granisetron. WAY-100635 alone had no effect on mEPSCs, indicating that spontaneously released 5-HT activates only 5-HT$_3$ receptors, at least in the rat. The affinity for 5-HT at 5-HT$_{1A}$ receptors is approximately 20× higher than at 5-HT$_3$ receptors (pK$_i$ 8.2 at the r5-HT$_{1A}$ receptor) (Watson et al., 2000) compared with 6.8 at the r5-HT$_3$ receptor (Kilpatrick et al., 1989). Comparing the concentrations of 5-HT that cause inhibition (0.3 μM) with those that cause excitation (1 μM) in the present experiments suggests that this selectivity may be even lower. However, this selectivity would explain why the low concentration of applied 5-HT activated 5-HT$_{1A}$ receptors to cause inhibition of mEPSCs before activating 5-HT$_3$ receptors to produce excitation. In addition, the consistency of the 5-HT$_3$-mediated effect on glutamatergic mEPSCs is similar to that observed in vivo in the rat (Wang et al., 1997; Ramage and Mifflin, 1998; Jeggo et al., 2005), in which more than 90% of neurons recorded responded, supporting the view these receptors are important in visceral afferent processing in the NTS. Interestingly, in the murine slice preparation, this effect targeted mainly the A2/2C catecholamine neurons found in the NTS (Cui et al., 2012).

In the present experiments, blockade of 5-HT reuptake via either the high-affinity, low-capacity transporter (5-HTT or SERT) with citalopram or the low-affinity, high-capacity transporters (OCT3/PMAT) with D-22 caused a decrease in mEPSC frequency rather than the expected increase. This decrease was prevented by pretreatment with WAY-100635, indicating that blockade of 5-HT removal from the extracellular space allows the spontaneously released 5-HT to activate 5-HT$_{1A}$ receptors. Further, this blockade over-rides the background activation of 5-HT$_3$ receptors. It is noteworthy that blockade of either uptake system, even with larger concentrations of the uptake inhibitors in the presence of WAY-100635, failed to produce a 5-HT$_3$-mediated increase in glutamatergic mEPSCs, although with a high concentration of D-22, there was an observable but small increase in mEPSC frequency. It should be noted that this effect was very small compared with that caused by the high concentration of 5-HT

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**Fig. 4.** Rat horizontal brainstem slice pretreated with gabazine (25 μM) showing the effects of 5-HT uptake blockade on patch-clamp recordings of evoked EPSCs from NTS neurons by electrical stimulation (0.1–0.5 ms, 20–320 μA) of the solitary tract and the paired-pulse ratio. (A) Traces of paired evoked EPSCs in control and before and after the addition of the 5-HT$_3$ antagonist granisetron (GRN; n = 6), citalopram (CIT; n = 10), and D-22 (n = 7 and 4). (B) Histogram of mean % change (Δ) in evoked EPSC amplitude for these drugs. Changes are compared with control using one-way analysis of variance with Fisher’s least significant difference test. (C) Histogram of mean paired-pulse ratios for these drugs. Changes are compared with control using a Student’s paired t test. Vertical bars (B and C) show the S.E.M. ns, nonsignificant, *P < 0.05, **P < 0.01 compared with control.
added to the slice (i.e., a 57% ± 39% increase) compared with a 2462% ± 543% increase in mEPSCs frequency. The simplest interpretation of these data would be that there are at least two pathways spontaneously releasing 5-HT, one excitatory using 5-HT3 receptors and poorly or unregulated by uptake and one inhibitory pathway using 5-HT1A receptors and strongly regulated by uptake. In this respect, the failure of WAY-100635 to affect background glutamate release would imply that the 5-HT1A pathway is not spontaneously releasing enough 5-HT to activate 5-HT1A receptors. Only when 5-HT uptake is blocked is there a high enough concentration of 5-HT to activate 5-HT1A receptors. If this is the case, then the concurrent activation of the 5-HT1A receptor would open K+ channels, causing hyperpolarization, and/or inhibit Ca2+ channel opening, which would reduce transmitter release, thereby opposing the excitatory action of 5-HT3 receptors opening in Na+ channels (Barnes and Sharp, 1999), allowing at least at low concentrations of 5-HT the higher-affinity 5-HT1A receptor effects to predominate, as seen with low concentration of 5-HT, added to slices even when uptake is functioning. Further, as uptake is mainly functioning only on the high-affinity pathway, this may explain the lower potency difference between 5-HT in its inhibitory action compared with its excitatory action, which is lower than that expected from the binding of 5-HT to 5-HT1A compared with 5-HT3 receptors (see preceding discussion). Finally, these data also indicate that both types of uptake blockade surprisingly cause a similar rise in the background extracellular concentration of 5-HT, at least when 5-HT is spontaneously released.

The ability of 5-HT reuptake blockade to cause activation of 5-HT1A receptors is reminiscent of one of the explanations for the delay in onset of the therapeutic effect of 5-HT uptake inhibitors in the treatment of depression. That is, although 5-HT uptake is blocked immediately, the activation of 5-HT1A

Fig. 5. Rat horizontal brainstem slice pretreated with gabazine (25 μM) showing the effects of a train of 100 pulses at 20 Hz of electrical stimulation (S; 0.1–0.5 ms, 20–320 μA) of the solitary tract on patch clamp recordings of spontaneous (s) EPSCs from NTS neurons. (A) Traces of recordings of sEPSCs overlaid five times from the same neuron showing background, the effect of 100 pulses stimulation at 20 Hz, and again in the presence of decynium (D-22). (B) An event histogram of the same experiment. (C and D) Histogram of mean % change (Δ) in sEPSC frequency and amplitude, respectively. The vertical bars in (C and D) S.E.M. ns, nonsignificant. ***P < 0.001 compared with control using one-way analysis of variance with Fisher’s least significant difference test.
receptors causes an initial reduction in the overall release of 5-HT in the cortex until the 5-HT1A receptor desensitizes (Artigas et al., 1996). However, there is no evidence that in the NTS 5-HT1A receptors are acting, as they do in the dorsal raphe as somatodendritic autoreceptors, so these NTS 5-HT1A receptors should be considered to be postsynaptic (heteroreceptors), that is, on terminals other than those of raphe projections, presumably similar to those that have been well described in the hippocampus (Riad et al., 2000; Polter and Li, 2010). Furthermore, the present observation that activation of 5-HT1A receptors failed to decrease the amplitude of the mEPSC indicates that these receptors are located presynaptic to the recorded NTS neurons. It should be noted that in vivo experiments have so far failed to determine a role for 5-HT1A receptors in the regulation of vagal afferent excitation of NTS neurons (Oskutyte et al., 2009).

**Evoked Release.** Our observation that the amplitudes of evoked (ST stimulation) EPSCs are reduced and the pulse-paired ratio is increased in the presence of granisetron is similar to that previously observed with the 5-HT3 antagonist ondansetron (Wan and Browning, 2008). Again, these data can be interpreted as follows (Mennerick and Zorumski, 1995; see Zucker and Regehr, 2002): these 5-HT3 receptors are located presynaptic to the recording site, and vagal afferent excitation causes the release of 5-HT in the NTS. The failure of citalopram to affect either the amplitude of evoked EPSCs or the paired-pulse ratio supports the view that the high-affinity low-capacity transporter (5-HTT) is not involved in the regulation of the increase in the extracellular concentration of 5-HT when it is increased by vagal afferent stimulation, but not the background concentration of 5-HT caused by its spontaneous release by this 5-HT3 pathway (see preceding discussion). Again, this increase was observed for a train of stimuli at 20 Hz. In this respect, vagal afferent stimulation at this frequency causes a consistent detectable 5-HT release in vivo in the NTS (Hosford et al., 2011). These data support the view that OCT3/PMAT, but not 5-HTT, is involved in the regulation of the increase in the extracellular concentration of 5-HT in response to vagal afferent stimulation. Furthermore, the failure of the selective OCT3 blocker corticosterone (Engel and Wang, 2005) to have any effect on mEPSCs frequency suggests that PMAT rather than OCT3 is the transporter involved. However, the failure to see an effect with citalopram is surprising, although consistent with in vivo experiments (Hosford et al., 2012). There was no detected involvement of the 5-HT1A pathway in the evoked release of glutamate, consistent with in vivo data that 5-HT1A receptors are not involved in the regulation of vagal afferent excitation of NTS neurons (Oskutyte et al., 2009). Even if they were involved, the absence of a detected 5-HT1A-mediated effect in the presence of either uptake inhibitor would not be surprising because a large increase in the extracellular level of 5-HT, as shown by adding high concentrations 5-HT to the slice, would easily override the 5-HT1A-mediated inhibition of spontaneous glutamate release. The site or sites at which vagal afferent activation causes this release of 5-HT remain to be determined.

Release could be directly from these afferents themselves, possibly a subpopulation, and/or from 5-HT-containing nerve terminals originating from the medullary raphe. However, the site must be in close juxtaposition to vagal afferent terminals.

The present data demonstrate that the monoamine low-affinity, high-capacity uptake transporter, probably PMAT rather than OCT3, and not the high-affinity, low-capacity transporter 5-HTT (SERT) is involved in the regulation of the rise in extracellular concentration of 5-HT in the NTS in response vagal afferent stimulation. This rise in 5-HT activates 5-HT3 receptors, which in turn causes the release of glutamate, confirming in vivo observations (Jeggo et al., 2005). The data from Wan and Browning (2008) indicate that this 5-HT3-dependent glutamate release comes from vagal afferent terminals. However, it has been suggested that glia may also provide either an additional or alternate source (Jeggo et al., 2005). Nevertheless, when studying spontaneous release of 5-HT within the NTS in brainstem slices, a 5-HT1A receptor-mediated pathway is uncovered when either type of uptake is blocked. Furthermore, both types of uptake within this pathway are equally effective in regulating the extracellular concentration and preventing it from rising high enough to activate 5-HT1A receptors. The question arises of why only one pathway is regulated only by both types of uptake and other, the so-called excitatory 5-HT3 pathway, when stimulated by the low-affinity, high-capacity transporter. It should be noted that the regulation of this pathway by low-affinity, high-capacity transporter would imply that a large concentration of 5-HT is released by vagal afferent activation. These differences may reflect different physiologic roles of these two putative 5-HT pathways in the regulation vagal afferent input into the NTS. It is speculated that the tightly regulated inhibitory 5-HT1A pathway plays a role in the regulation of low-level release of glutamate by vagal afferents (i.e., in normal physiologic regulation). Whereas the excitatory 5-HT3 pathway, which would cause a large potentiation of the amount of glutamate released by these afferents, may be required when there has been some profound or pathologic change in the cardiorespiratory/alimentary system that needs to be regulated. If the 5-HT, as suggested already, is also released by vagal afferents, then this would be a feed-forward system and presumably the large amounts of 5-HT being released by the high activity of vagal afferents can be removed effectively only by the low-affinity, high-capacity uptake transporter. However, as there is yet no evidence that these 5-HT1A receptors are involved in the vagal afferent activation of NTS neurons, a possible alternative is that they may be involved in other NTS functions not related to visceral afferent processing (Oskutyte et al., 2009).

**Acknowledgments**

The authors thank Dr. Talvinder Sihra for many enlightening discussions.

**Authorship Contributions**

**Participated in research design:** Hosford, Mifflin, Ramage.

**Conducted experiments:** Hosford.

**Performed data analysis:** Hosford, Mifflin.

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**References**


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