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Title Page

5-hydroxytryptamine mediated neurotransmission modulates spontaneous and vagal evoked glutamate release in the nucleus tractus solitary (NTS) - effect of uptake blockade

Patrick S Hosford, Steve W Mifflin and Andrew G Ramage

Department of Integrative Physiology, University of North Texas Health Science Center, Fort Worth, TX 76107 (P.S.H., S.W.M.) and Department of Neuroscience, Physiology and Pharmacology, University College London, Gower Street, London WC1E 6BT U.K. (P.S.H., A.G.R.)

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Running Title Page

Running Title: 5-HT release in the NTS - effect of uptake blockade

Corresponding author: Steve W Mifflin, Department of Integrative Physiology, University of North Texas Health Science Center, 3500 Camp Bowie Blvd. Fort Worth, TX 76107.

E-**mail address:** steve.mifflin@unthsc.edu

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Discussion, 1,696

Abbreviations: ANOVA, analysis of variance; LSD, least significant difference test; aCSF, artificial cerebral spinals fluid; 5-HT, 5-hydroxytryptamine; WAY, WAY-100635, (N-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-N-2-pyridinylcyclohexanecarboxamide); GRAN, granisetron; CIT, citalopram; D-22, decynium-22; TTX, tetrodotoxin; CNQX, 6-Cyano-7-nitroquinoxaline-2,3-dione; 5-HTT, 5-hydroxytryptamine transporter; SERT, serotonin transporter; OCT3, organic cation transporter 3; PMAT, plasma membrane monoamine transporter; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol tetra acetic acid; ATP, adenosine-5'-triphosphate; GTP, guanosine-5'-triphosphate; mEPSCs, miniature excitatory postsynaptic currents; sEPSCs, spontaneous excitatory postsynaptic currents; NTS, nucleus tractus solitarius; ST, solitary tract;

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Abstract

The effect of blockade of either 5-HT/serotonin transporter (5-HTT/SERT) with citalopram or the organic cation transporter 3 (OCT3)/plasma membrane monoamine transporter (PMAT) with decynium-22 on spontaneous and evoked release of 5-HT in the NTS was investigated in the rat brainstem slice treated with gabazine. 5-HT release was measured indirectly by changes in frequency and amplitude of glutamatergic mEPSCs (in the presence of TTX) and evoked EPSCs. Blockade of 5-HT_3 receptors with granisetron reduced, while the 5-HT_3 agonist phenylbiguanide, increased the frequency of mEPSCs. 5-HT decreased mEPSC frequency at low, and increased frequency at high concentrations. This inhibition was blocked by the 5-HT_{1A} antagonist WAY-100635, which was ineffective on its own, while the excitation was reversed by granisetron. Addition of citalopram or decynium-22 caused inhibition, which was prevented by 5-HT_{1A} blockade. Thus in the NTS the spontaneous release of 5-HT is able activate 5-HT_3 receptors but not 5-HT_{1A} 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 decynium-22 caused an increase in amplitude of EPSCs, with a decrease in the paired pulse ratio and increased the number of spontaneous EPSCs after 20Hz stimulation. Thus for the evoked release of 5-HT the low-affinity, high-capacity transporter PMAT but not the 5-HTT/SERT is important in the regulation of changes in 5-HT extracellular concentration.

230 words

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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 physiological state. The central nervous system is crucial to this homeostatic control - 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 (see Andresen and Kunze, 1994). Glutamate is considered to be the major transmitter mediating cardiovascular/visceral afferent input to the NTS (see 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, 2006). 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, 1998) and from the nodose ganglia, the site of vagal afferent cell bodies (Orer et al., 1991; Nosjean et al., 1998). Further the NTS has the densest expression in the brain of the only ionotropic 5-HT receptor, the 5-HT_3 receptor. These receptors are found mainly on vagal afferent terminals (Pratt and Bowery, 1989; Leslie et al., 1989) and blockade of which attenuates vagal afferent excitation of NTS neurons (Ramage and Mifflin, 1998; Jeggo et al., 2005). These 5-HT_3 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 TTX and bicuculline in which the frequency of glutamatergic miniature excitatory postsynaptic currents (mEPSCs) was found to be attenuated by blockade of 5-HT_3 receptors (Wan and Browning, 2008). This

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indicates 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 (see Dawes, 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 carried out to investigate the effects of blockade of 5-HT transporter (5-HTT; SERT) with citalopram (Hyttel, 1982) and blockade of the organic cation transporter 3 (OCT3)/plasma membrane monoamine transporter (PMAT) with decynium-22 (D-22; Schömig 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.

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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, decapitated and the brain stem was rapidly removed and completely immersed in ice-cold (<4°C) high-sucrose artificial cerebrospinal fluid (aCSF) that contained (in mM) 3 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 2 $MgSO_4$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 D-glucose, and 206 sucrose, pH 7.4 when continuously bubbled with 95% O_2-5% CO_2. The brain stem was mounted in a vibrating microtome (VT1200S, Leica Microsystems, Bannockburn, IL, U.S.A) and coronal or horizontal slices (300μm thickness) were cut with a sapphire knife (Delaware Diamond Knives, Wilmington, DE, U.S.A). Once cut, slices were transferred to an incubation chamber containing (in mM) 126 NaCl, 26 $NaHCO_3$, 3 KCl, 2 $CaCl_2$, 2 $MgSO_4$ and 10 D-glucose. The incubation chamber was continually gassed with 95% O_2 – 5%CO_2 to maintain solution pH and re-circulate the aCSF. Slices were incubated at room temperature for at least 90 min 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 aCSF at room temperature (2-3 ml min^{-1}). 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, U.S.A.). Neurons were visualized using an upright microscope (Olympus BX50) fitted with near-infrared differential

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interference contrast optics and a CCD camera. The resultant image was displayed on a CCTV. 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 ID, 1.2-mm OD, WPI; U.S.A) on a pipette puller (model P-2000, Sutter Instrument, Novato, CA, U.S.A.) and were filled with a solution containing the following (in mM) 128 K Gluconate, 10 KCl, 0.3 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 1 EGTA, 2 ATP and 0.25 GTP. The pH was adjusted to 7.35 with KOH. The pipette resistance ranged from 3 to 5 MΩ. Voltage-clamp recordings were made with an Axopatch 200B (Molecular Devices, Foster City, CA; U.S.A. acquisition rate of 10 kHz, filtered at 2 kHz, and digitized via a Digidata 1320 interface). Data was stored and analyzed on a computer running pClamp10 software (Axon Instruments) or Mini Analysis software (Synaptosoft Inc., Fort Lee, NJ, U.S.A.). 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 min 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 <100pA at V_H = -60mV were taken into further analysis. In all coronal patch-clamp experiments, the bath solution included the sodium channel blocker TTX (1 μM) and the $GABA_A$ receptor antagonist gabazine (25 μM), while for the horizontal sections only contained gabazine (25 μM).

Electrical Stimulation: Horizontal Slice

In these slices evoked excitatory postsynaptic potentials were recorded (EPSCs). 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

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isolated constant current stimulator (DS3, Digitimer, Welwyn Garden City, U.K.). 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 s to evoke submaximal EPSCs. 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 20Hz to evoke an increase in spontaneous EPSC frequency post-stimulus. The amplitude was adjusted to evoke submaximal EPSC inward currents (~100 pA).

Experimental protocol, data analysis and statistics

Frequency and amplitude of mEPSCs were sampled continuously. For the coronal slice experiments these mESPCs was recorded for a control period 10 min in drug free ACSF. To test if the recordings were stable the frequency of mESPCs over the first 200 s was compared with that over the last 200 s of this 10 min period. If the percentage difference in frequency between these two values was ≤ 20% then 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/drugs were then applied and recordings were made over a 10 min window before changing to another drug challenge or to drug free aCSF. The maximum number of drug challenges were 3 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 mESPCs over 200s), which was approximately 5-7 min after application. The drug/s was then washed out with aCSF. This was carried out within the 10 min window. In the phenylbiguanide experiments the 2nd and higher concentration

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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 were 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: Measurement of increases in EPSC activity due electrical stimulation of solitary tract (ST) were taken 1 min after stimulation had ceased, as this is when the maximum increase in activity was observed, and averaged over a 30 s period. Two control stimulations of ST were carried out 10 min apart to determine stability. If stable (change was not greater than 10%) then stimulation was repeated again in presence of either aCSF (control) or citalopram or decynium-22 applied 5 min after the 2nd stimulation and the changes in frequency and amplitude were compared to those of the 2nd control stimulation. All changes are expressed as the mean percentage change from 2nd 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 (ANOVA) with Fisher's least significant difference test (LSD) for post-hoc comparisons. P values <0.05 were considered to be 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-hydroxytrptamine (serotonin) creatinine sulfate, CNQX (6-Cyano-7-nitroquinoxaline-2,3-dione) were obtained from AbcamBiochemicals (Boston, MA, U.S.A), while gabazine

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(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-pyridinylcyclohexanecarboxamide); phenylbiguanide hydrochloride, DMSO (dimethyl sulfoxide) and the chemical ingredients for aCSF from Sigma-Aldrich, St Louis, MO., U.SA. All drugs were dissolved in aCSF except CNQX. For CNQX a 25 mM solution in DMSO was made initially and then this was diluted in aCSF to give a final concentration of 25 μM.

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Results

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

Coronal NTS slices

Effect of applied 5-HT$_3$ receptor ligands on mEPSCs

Application of PBG at concentrations of 1 & 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-HT$_3$ antagonist granisetron (GRN; 1μM) 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 spontaneous frequency of mEPSCs (Fig.1C) reaching a maximum of 36 ±4% between 5 to 7 min 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, while changes in amplitude depend on the properties of postsynaptic cells

Effect of applied 5-HT on mEPSCs

Application of 0.3μM 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). 1μM 5-HT had a mixed effect. In 5 of 11 neurons tested there was a significant decrease in frequency of 62 ± 10% (Fig. 2A and B). In the remaining 6 there was a significant increase in frequency of 403 ± 142% (Fig. 2A and C). Amplitude was unaffected in all cases. Application of 10μM 5-HT significantly increased mEPSC frequency in all cases (n=8) by 1285 ± 276%; Fig. 2A and C),

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and also the amplitude by 21 ± 5% (Fig. 2A). The highest concentration 100 µM (n=5) caused an even larger increase in frequency (2462 ± 543%) and amplitude 52 ± 9%.
In the presence of granisetron (1µM, n=6) 5-HT (10µM) now caused decrease in mEPSC frequency of 30±11 % (Fig. 2B), while the inhibitory action of the lowest concentration (0.3µM) of 5-HT was blocked by the 5-HT_{1A} 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. 3A and B), a similar decrease was observed at ten times higher concentration (5 µM; n=5) of citalopram, 38±6% (Fig. 3B). Similarly blockade of OCT3/PMAT with D-22 1µM (n=4) caused a significant decrease in mEPSC frequency of 33 ± 4% as did the higher concentration of 10µM (n=3; Fig. 3A and C), which caused a decrease in mEPSC frequency of 44 ± 6%. In all cases mEPSC amplitudes remained unchanged (Fig. 3).

Effects of 5-HT_{1A} blockade on mEPSC

Application of aCSF (control; n=11) and WAY-100635 (1µM; n=12) and 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 and 5 µM; n=4) to reduced mEPSC frequency (now only changing by -12 ± 5 and +2 ± 23%, respectively). WAY-100635 pretreatment also blocked the ability of both concentrations of D-22 (1µM; n=6; 10µM; n=4) to decrease mEPSC frequency (Fig. 3A and C). However, at the higher concentration D-22 now caused a small and significant increase in mEPSC frequency (57 ± 39%; Fig. 3A and C). In all cases mEPSC amplitudes remained unchanged.

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Effect of corticosterone

Application of the OCT3 inhibitor corticosterone (1μM, n=5; 3μM, 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 solitary tract (ST) stimulation - single pulse

The effect of transporter inhibitors on synaptic transmission was assessed in 26 further NTS neurons in which EPSCs were evoked by electrical stimulation of the TS in the present of gabazine (25 μM) alone. The mean amplitude of the evoked control EPSC was -90pA± 9pA. Application of CNQX (10mM, n=4) completely abolished TS-evoked inward current.

Citalopram, at the higher concentration (0.5 μM; n=10) failed to alter the amplitude of the first evoked EPSC (-8.1 ± 6.1%), when compared to control (-1.7 ± 1.9). Paired-pulse ratio (PPR) also remained unaltered (0.68 ± 0.06 to 0.66 ± 0.08). D-22 (1μM; n=7) also failed to modify the evoked EPSC (-7.6 ± 5.7%) when compared to control (-1.7 ± 1.9%). Paired-pulse ratio was also unaltered (0.71 ± 0.06 c.f. 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%, when compared to control (-1.7 ± 1.9%). In this case, paired-pulse ratio decreased from 0.75 ± 0.08 to 0.44 ± 0.07 (Fig. 4). Granisetron (3 μM; n=6) significantly decreased the amplitude of the first evoked EPSC by 17.4 ± 5.3%, when compared to control (1.7 ± 2.0%). Paired-pulse ratio significantly increased from 0.64 ± 0.12

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to 0.73 ± 0.12. (Fig. 4). Alterations in paired pulse ratio are suggestive of a presynaptic site of drug action (see discussion).

Trains of 100 pulses at 20 Hz

Spontaneous EPSCs (sEPSCs) were recorded from 17 neurons and the mean frequency and amplitude were 1.6 ± 0.3 Hz and 17.1 ± 1.1pA. Electrical stimulation of the TS (100 pulses at 20Hz) evoked an increase in sEPSC frequency of 106 ± 13% when compared to baseline (Fig.5). This remained unaltered in 2 subsequent stimulations (100 ± 6%). Amplitude was not significantly affected (-2.4 ± 3.85). Citalopram (0.5μM; n=6) failed to alter the TS-evoked increase in sEPSC frequency (99 ± 23%), however D-22 (1μM; n=8) 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).

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Discussion

Spontaneous release

The ability of the selective 5-HT_3 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-HT_3 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-HT_3 receptors with ondansetron did not affect the spontaneous release of glutamate in the NTS (Ciu et al., 2012). This may simply reflect that in these experiments $GABA_A$ 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 and it 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, while at high concentrations caused excitation. This excitation was also associated with very large increases mEPSC frequency. The inhibition by low concentrations was found to be blocked by pretreatment with the 5-HT_{1A} antagonist WAY-100635 (Forster et al., 1995), while 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 only activates

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5-HT_3 receptors, at least in the rat. The affinity for 5-HT at 5-HT_{1A} receptors is approximately 20x higher than at 5-HT_3 receptors; pK_i 8.2 at the r5-HT_{1A} receptor (Watson et al 2000) compared to 6.8 at the r5-HT_3 receptor (Kilpatrick et al., 1989). Comparing the concentrations of 5-HT that cause inhibition (0.3 µM) to those that cause excitation (1µM) 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 constitency 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 over 90% of neurons recorded from responded, supporting the view these receptors are important in visceral afferent processing in the NTS. Interestingly in the murine slice preparation this effect was found to target mainly the A2/2C catecholamine neurons found in the NTS (Ciu 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 decynium-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 overrides the background activation of 5-HT_3 receptors. Surprisingly, 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 decynium-22 there was an observable, but small, increase in mEPSC frequency. It should be noted that this was a very small effect compared to that caused by the

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high concentration of 5-HT added to the slice i.e. a 57 ± 39% increase compared to a 2462 ± 543% increase in mEPSCs frequency. The simplest interpretation of this data would be that there are at least two pathways spontaneously releasing 5-HT, one excitatory utilizing 5-HT_3 receptors and poorly or unregulated by uptake and one inhibitory pathway utilizing 5-HT_{1A} receptors and strongly regulated by uptake. In this respect the failure of WAY-100635 to affect background glutamate release would imply that the 5-HT_{1A} pathway is not spontaneously releasing enough 5-HT to activate 5-HT_{1A} receptors. Only when 5-HT uptake is blocked is there a high enough concentration of 5-HT to activate 5-HT_{1A} receptors. If this is so then ability of uptake blockade to override the background activation of 5-HT_3 receptor mediated glutamate release would be simply be due to a reduction in the total amount of spontaneously released glutamate as detected as mESPCs. However, it is possible that these two 5-HT pathways are controlling glutamate release from the same site, vagal afferent terminals (Wan and Browning, 2008). However, although the NTS does have a high density of 5-HT_{1A} receptors (Pompeiano et al., 1992), no experiments have examined whether 5-HT_{1A} receptors can also be found on these terminals along with 5-HT_3 receptors. However, the data from somatosensory afferents terminating in the spinal cord have shown a small proportion of the 5-HT_{1A} receptors on primary afferent fibers along with 5-HT_3 receptors (Laporté et al., 1995). If this model is correct i.e. both receptors located on vagal afferent terminals then the concurrent activation the 5-HT_{1A} receptor would open K^+ channels causing hyperpolarization and/or inhibit Ca^{2+} channel opening which will reduce transmitter release, thereby opposing the excitatory action of 5-HT_3 receptors opening in Na^+ channels (see Barnes & Sharp, 1999), allowing, at least, at low concentrations of 5-HT the higher affinity 5-HT_{1A} 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 only functioning on the high affinity pathway this may explain the lower potency difference between 5-HT in its

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inhibitory action compared with its excitatory action which is lower than that expected from the binding of 5-HT to 5-HT_{1A} compared to 5-HT_3 receptors, see above. 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-HT_{1A} 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-HT_{1A} receptors causes an initial reduction in the overall release of 5-HT in the cortex until the 5-HT_{1A} receptor desensitizes (see Artigas, 1996). However, there no evidence that in the NTS 5-HT_{1A} receptors are acting, as they do in the dorsal raphé, as somatodendritic autoreceptors so these NTS 5-HT_{1A} receptors should be considered to be postsynaptic (heteroreceptors) i.e. on terminals other than those of raphé projections, presumably similar to those that have been well described in the hippocampus (see Riad et al., 1990; Polter and Li, 2010). Further the present observation that activation of 5-HT_{1A} 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-HT_{1A} receptors in the regulation of vagal afferent excitation of NTS neurons (Oskutyte et al., 2009).

Evoked release

The present observation that the amplitudes of evoked (solitary tract 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-HT_3 antagonist ondansetron (Wan and Browning, 2008).

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Again these data can be interpreted (Mennerick and Zorumski, 1995; see Zucker and Regehr, 2002) that these 5-HT_3 receptors are located presynaptic to the recording site and that vagal afferent excitation causes the release of 5-HT in the NTS. The failure of citalopram to affect either the amplitude of evoked EPSC or the paired pulse ratio supports the above view that the high-affinity low-capacity transporter (5-HTT) is not involved in the regulation of this glutamate-releasing 5-HT_3 receptor pathway, even when driven. However, the ability of decynium-22 to increase the amplitude of evoked EPSC and decrease the paired pulse interval does indicate that OCT3/PMAT plays a role in the regulation of 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-HT_3 pathway (see above). Again this increase was observed for a train of stimuli at 20Hz. 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. Further, 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-HT_{1A} pathway in the evoked release of glutamate, consistent with *in vivo* data that 5-HT_{1A} receptors are not involved in the regulation of vagal afferent excitation of NTS neurones (Oskutyte et al., 2009). Even if they were involved, the absence of a detected 5-HT_{1A}-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-HT_{1A}-mediated inhibition of spontaneous glutamate release. The site/s

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at which vagal afferent activation causes this release of 5-HT remains 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 raphé. However the site must be in close juxtaposition to vagal afferent terminals.

The present data demonstrates 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-HT_3 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-HT_3-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-HT_{1A} 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 rising high enough to activate 5-HT_{1A} receptors. The question arises why is only one pathway regulated by both types of uptake and other, the so called excitatory 5-HT_3 pathway when stimulated, by only the low-affinity, high-capacity transporter? It should be noted that the regulation of this latter 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 physiological 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-HT_{1A} pathway plays a role in the regulation of low level release of glutamate by vagal afferents i.e. in normal

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physiological regulation. Whereas the excitatory 5-HT_3 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/pathological change in the cardio-respiratory/alimentary system that needs to be regulated. If the 5-HT, as suggested above, 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 only be removed effectively by the low-affinity, high-capacity uptake transporter. However as there is yet no evidence that these 5-HT_{1A} 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).

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Authorship contributions

Participated in research design: Hosford, Mifflin and Ramage

Conducted experiments: Hosford

Performed data analysis: Hosford and Mifflin

Wrote or contributed to the writing of the manuscript: Hosford, Mifflin and Ramage

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Legends for Figures

Fig. 1. Rat coronal brain slice pretreated with TTX and gabazine showing the effects of activation and inhibition of 5-HT_3 receptors on patch clamp recordings of spontaneous mEPSC from NTS neurons. Panel A shows representative experimental traces (5 sweeps superimposed) of mEPSC activity showing the effect of two concentrations of PBG (1 and 10 μm) and the effect of pretreatment with GRN (1μM) on the low concentration PBG excitation from separate experiments. Panel B and C show frequency histograms of mEPSCs in separate experiments in which (B) the 5-HT_3 agonist phenylbiguanide (PBG) and (C) antagonist granisetron (GRN) were applied alone via the bath.

Fig. 2. Rat coronal brainstem slice pretreated with TTX and gabazine showing the effects of 5-HT on patch clamp recordings of mEPSC from NTS neurons. Panel A shows representative experimental traces (5 sweeps superimposed) of mEPSC activity from separate experiment showing the concentration (0.3 - 100 μM) related effect of 5-HT. It should be noted that for concentration of 1μM the data has been split into two groups, into those in which 5-HT caused inhibition (5 out of 11 neurons) and those in which 5-HT caused excitation (6 out 11 neurons) similar for Panels B and C. Panel B is a histogram of the mean % change (Δ) in mEPSCS frequency showing the inhibitory action of the two lower concentrations of 5-HT (0.3 and 1 μm) and plus a high concentration (10 μm) in the presence of the 5-HT_3 antagonist granisetron. Panel C is a histogram of the mean % change (Δ) in mEPSCS frequency showing the excitatory effects of the lowest concentration of 5-HT in the presence of the 5-HT_{1A} antagonist WAY-100635 (WAY) along with increasing concentrations of 5-HT alone. The vertical bars in Panels B and C show the S.E.M. * P<

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0.05, **P<0.01, ***P<0.001 compared to control (note the control is the same in Panel B and C) using one-way analysis of variance with Fisher's least significant difference test.

Fig. 3. Rat coronal brainstem slice pretreated with TTX and gabazine showing the effects of 5-HT uptake blockade on patch clamp recordings of spontaneous mEPSC from NTS neurons. Panel A shows representative experimental traces overlaid 5 times of mEPSC activity from separate experiment showing the effect of citalopram (CIT) and decynium-22 (D-22) alone and in presence of the 5-HT_{1A} antagonist WAY-100635 (WAY). Panel B is a frequency histogram of the % change (Δ) in mEPSC frequency showing the mean effects of WAY-100635, the high affinity low capacity uptake inhibitor (5-HTT) citalopram 0.5 μm and at 5 μm and both concentrations in the presence of WAY-100635. Panel C is similar but shows the effects of two concentrations of the low affinity high capacity uptake inhibitor (OCT3/PMAT) decynium-22 alone and in presence of WAY-100635. The verticals bars in Panels B and C show the S.E.M. * P< 0.05, **P<0.01, compared to control (note the control is the same in Panel B and C) using one-way analysis of variance with Fisher's least significant difference test.

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. Panel A shows traces of paired evoked EPSCs before, control, and after the addition of the 5-HT_3 antagonist granisetron (GRN; n=6), citalopram (CIT; n=10) and decynium-22 (D-22; n=7 & 4). Panel B shows a histogram of mean % change (Δ) in evoked EPSC amplitude for these drugs. Changes are compared with control using one-way analysis of variance with

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Fisher's least significant difference test. Panel C is histogram of mean paired pulse ratios for these drugs. Changes are compared with control using a Student's paired *t*-test. The verticals bars in Panels B and C show the S.E.M. ns non-significant, * P< 0.05, **P<0.01 compared to control.

Fig. 5. Rat horizontal brainstem slice pretreated with gabazine (25 μM) showing the effects of a train of 100 pulses at 20Hz 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. Panel A shows traces of recordings of sEPSCs overlaid 5 times from the same neuron showing background, the effect of 100 pulses stimulation at 20Hz and again in the presence of decynium (D-22). Panel B below shoes an event histogram of the same experiment. Panels C and D show a histogram of mean % change (Δ) in sEPSC frequency and amplitude respectively. . The verticals bars in Panels C and D show the S.E.M. ns non-significant, ***P<0.001 compared to control using one-way analysis of variance with Fisher's least significant difference test.

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Figure 1

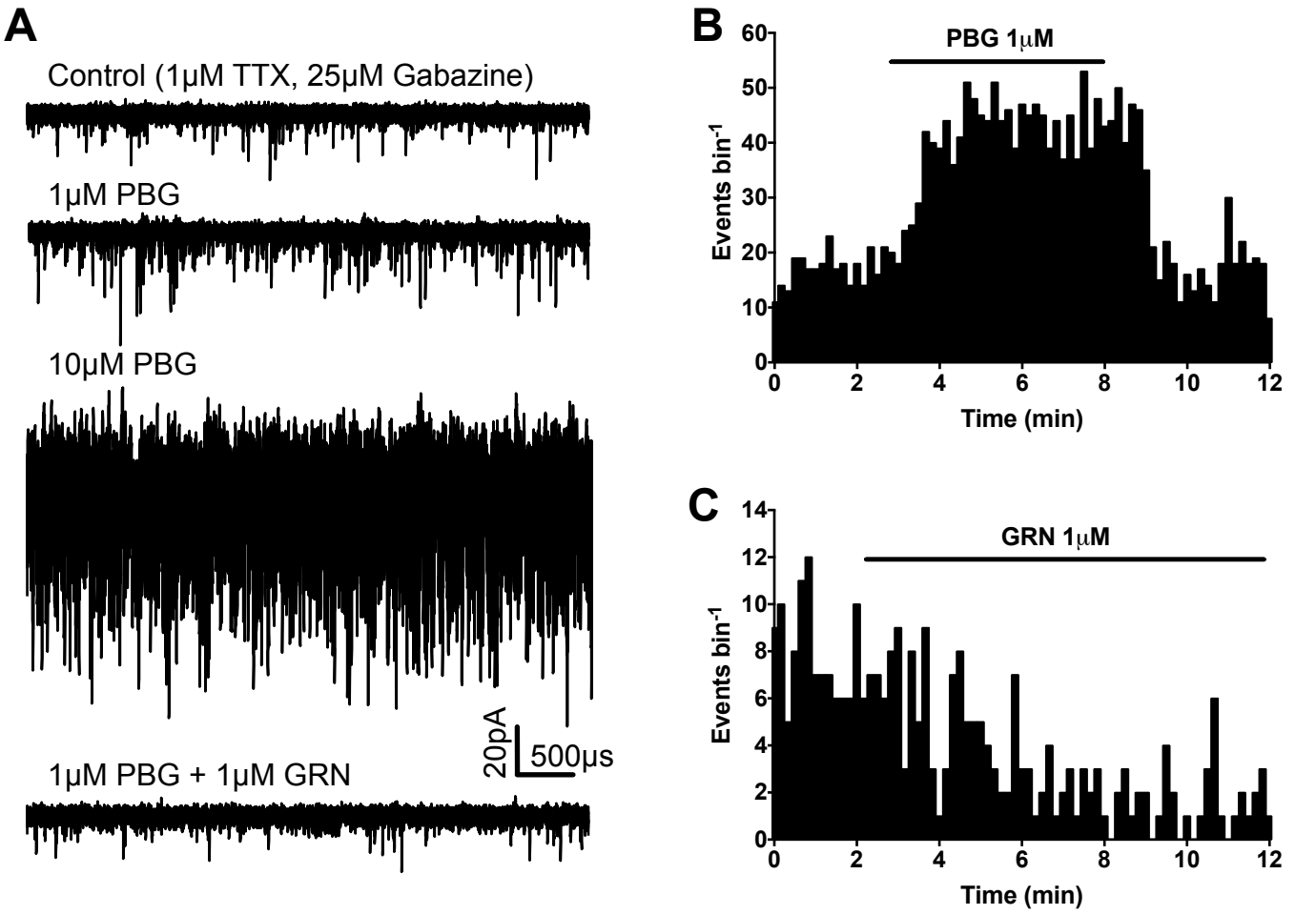

A
Control (1μM TTX, 25μM Gabazine)
1μM PBG
10μM PBG
20pA
500μs
1μM PBG + 1μM GRN
B
PBG 1μM
Events bin-1
Time (min)
C
GRN 1μM
Events bin-1
Time (min)

Figure 2

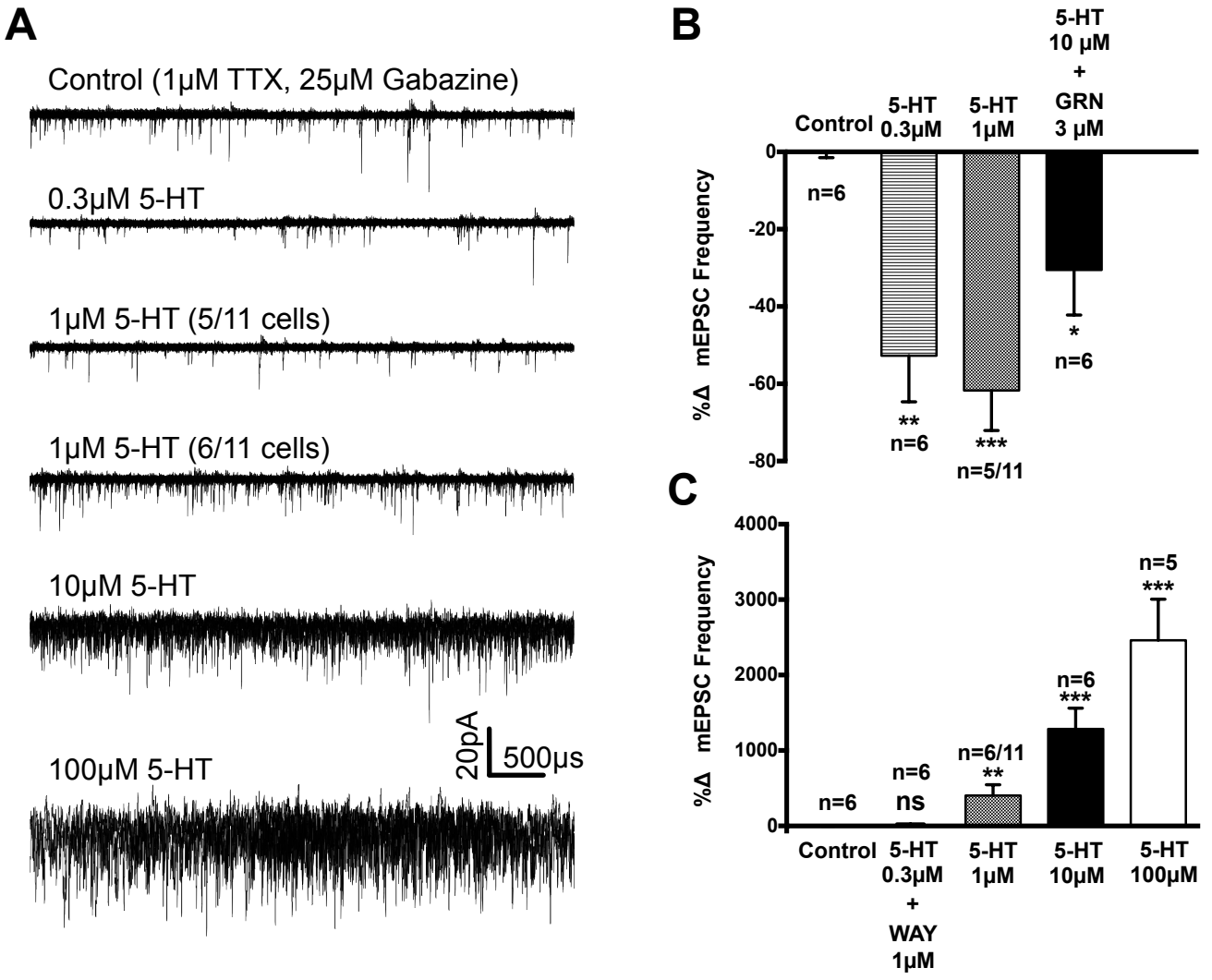

A
Control (1μM TTX, 25μM Gabazine)
0.3μM 5-HT
1μM 5-HT (5/11 cells)
1μM 5-HT (6/11 cells)
10μM 5-HT
100μM 5-HT
20pA
500μs
B
Control
5-HT 0.3μM
5-HT 1μM
5-HT 10 μM + GRN 3 μM
%Δ mEPSC Frequency
0
-20
-40
-60
-80
n=6
**
n=6
***
n=5/11
*
n=6
C
%Δ mEPSC Frequency
4000
3000
2000
1000
0
n=6
n=6
ns
n=6/11
**
n=6
***
n=5
***
Control
5-HT 0.3μM + WAY 1μM
5-HT 1μM
5-HT 10μM
5-HT 100μM

Figure 3

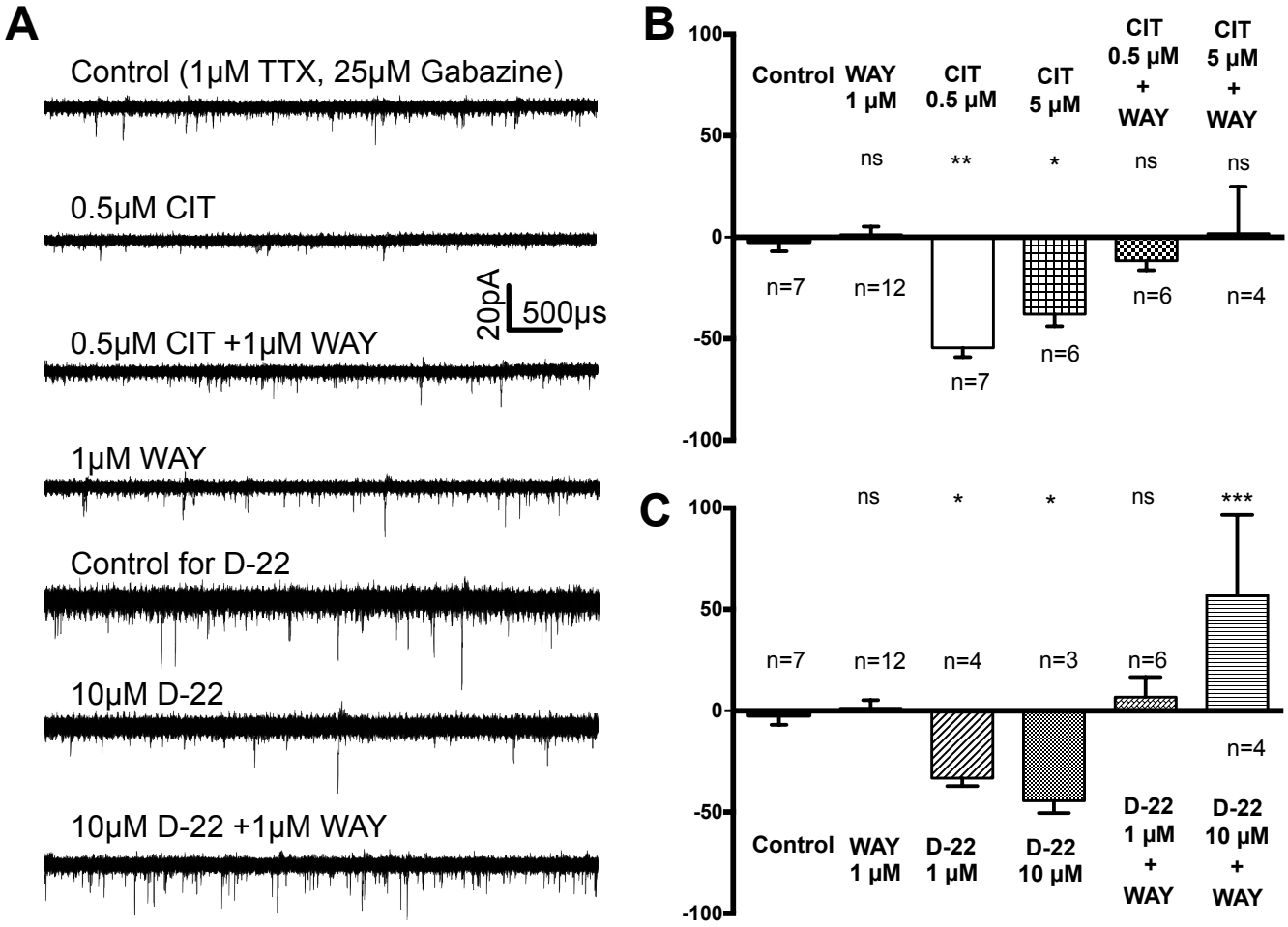

A
Control (1μM TTX, 25μM Gabazine)
0.5μM CIT
20pA
500μs
0.5μM CIT +1μM WAY
1μM WAY
Control for D-22
10μM D-22
10μM D-22 +1μM WAY
B
100
50
0
-50
-100
Control
WAY 1 μM
CIT 0.5 μM
CIT 5 μM
CIT 0.5 μM + WAY
CIT 5 μM + WAY
ns
**
*
ns
ns
n=7
n=12
n=7
n=6
n=6
n=4
C
ns
*
*
ns
***
n=7
n=12
n=4
n=3
n=6
n=4
Control
WAY 1 μM
D-22 1 μM
D-22 10 μM
D-22 1 μM + WAY
D-22 10 μM + WAY

Figure 4

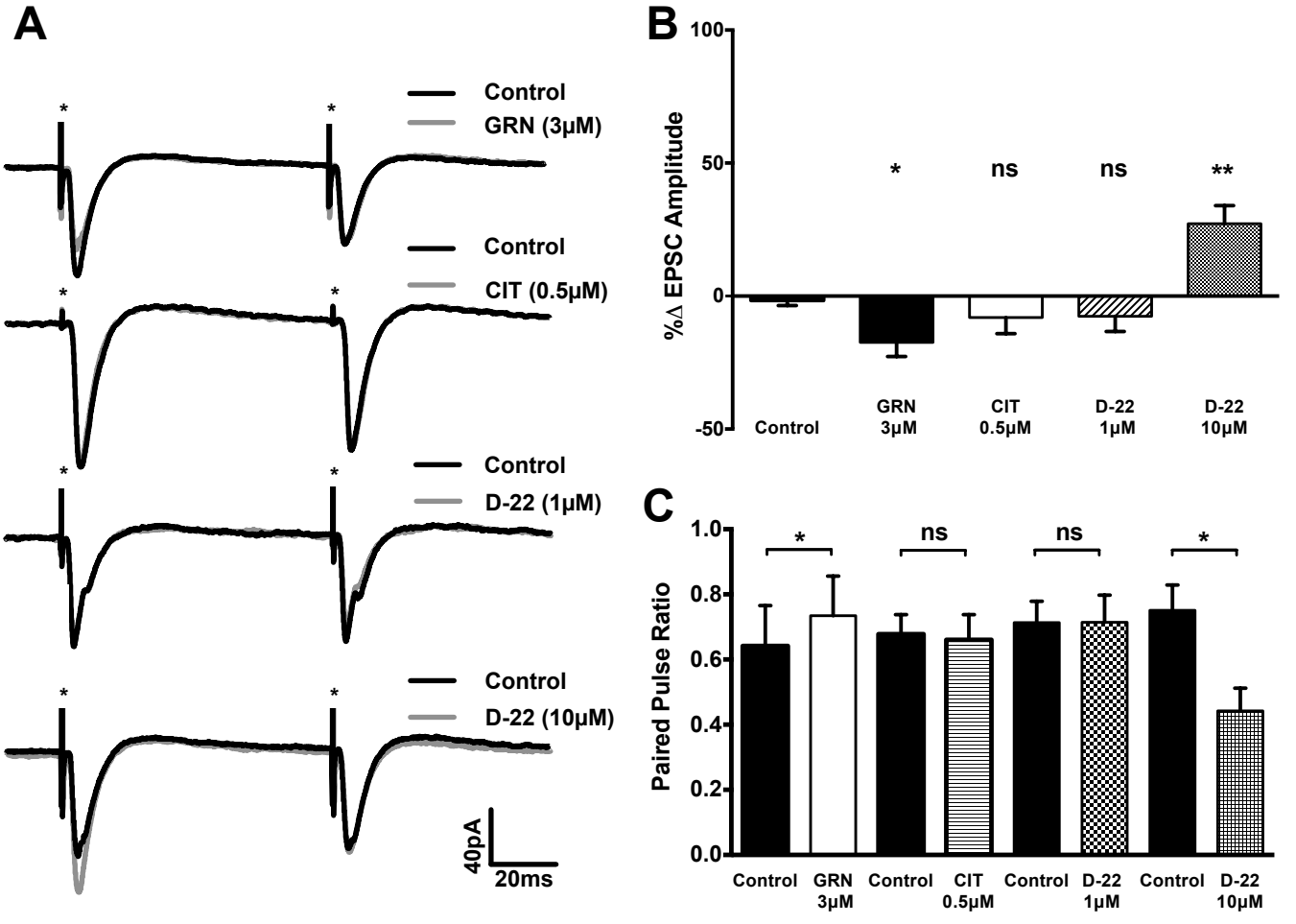
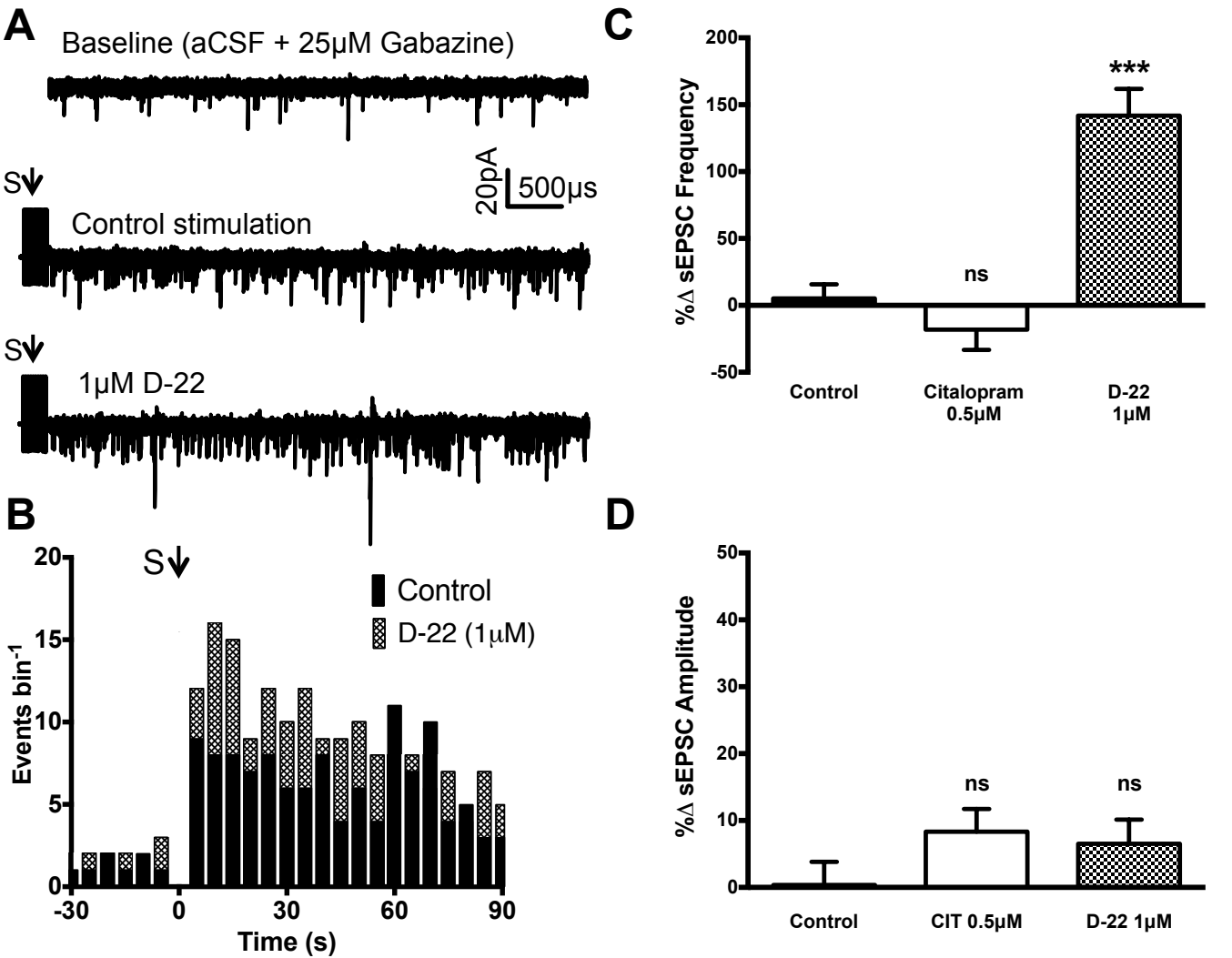

A
Control
GRN (3μM)
Control
CIT (0.5μM)
Control
D-22 (1μM)
Control
D-22 (10μM)
40pA
20ms
B
%Δ EPSC Amplitude
100
50
0
-50
*
ns
ns
**
Control
GRN 3μM
CIT 0.5μM
D-22 1μM
D-22 10μM
C
Paired Pulse Ratio
1.0
0.8
0.6
0.4
0.2
0.0
*
ns
ns
*
Control
GRN 3μM
Control
CIT 0.5μM
Control
D-22 1μM
Control
D-22 10μM

Figure 5


A
Baseline (aCSF + 25μM Gabazine)
S
20pA
500μs
Control stimulation
S
1μM D-22
B
S
Control
D-22 (1μM)
Events bin-1
20
15
10
5
0
-30
0
30
60
90
Time (s)
C
%Δ sEPSC Frequency
200
150
100
50
0
-50
***
ns
Control
Citalopram 0.5μM
D-22 1μM
D
%Δ sEPSC Amplitude
50
40
30
20
10
0
ns
ns
Control
CIT 0.5μM
D-22 1μM