Depressed GABA and glutamate synaptic signaling by 5-HT$_{1A}$ receptors in the nucleus tractus solitarii and their role in cardiorespiratory function

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ABSTRACT

Serotonin (5-HT), and its 5-HT$_{1A}$ receptor (5-HT$_{1AR}$) subtype, is a powerful modulator of the cardiorespiratory system and its sensory reflexes. The nucleus tractus solitarii (nTS) serves as the first central station for visceral afferent integration and is critical for cardiorespiratory reflex responses. However, the physiological and synaptic role of 5-HT$_{1AR}$s in the nTS is relatively unknown. In the present study, we examined the distribution and modulation of 5-HT$_{1AR}$s on cardiorespiratory and synaptic parameters in the nTS. 5-HT$_{1AR}$s were widely distributed to cell bodies within the nTS but not synaptic terminals. In anesthetized rats, activation of 5-HT$_{1AR}$s by microinjection of the 5-HT$_{1AR}$ agonist 8-OH-DPAT into the caudal nTS decreased minute phrenic neural activity via a reduction in phrenic amplitude. In brainstem slices, 8-OH-DPAT decreased the amplitude of glutamatergic tractus solitarii-evoked (TS-) EPSCs, and reduced overall spontaneous excitatory nTS network activity. These effects persisted in the presence of GABA$_{A}$R blockade and were antagonized by co-application of 5-HT$_{1AR}$ blocker WAY-100135. 5-HT$_{1AR}$ blockade alone had no effect on TS-EPSCs but increased excitatory network activity. On the other hand, GABAergic nTS-evoked (nTS-) IPSCs did not change by activation of the 5-HT$_{1AR}$s but spontaneous inhibitory nTS network activity decreased. Blocking 5-HT$_{1AR}$s tended to increase nTS-IPSC and inhibitory network activity. Taken together, 5-HT$_{1AR}$s in the caudal nTS decrease breathing, likely via attenuation of afferent transmission as well as overall nTS network activity.

Keywords: serotonin receptors, autonomic nervous system, patch clamp, EPSC, IPSC
INTRODUCTION

Within the cardiorespiratory reflex axis, the nucleus tractus solitarii (nTS) is vital in its role in integrating and processing visceral afferent signals. It is the initial central site for an effective response to physiological challenges such as low arterial oxygen, blood pressure fluctuations, and other visceral stimuli (Andresen and Kunze, 1994; Kline et al., 2010). The primary excitatory and inhibitory neurotransmitters within the nTS network are glutamate and γ-aminobutyric acid (GABA), respectively (Sapru, 2004). However, numerous neuromodulators may influence their release or postsynaptic receptor function (Kline et al., 2009; Sekizawa et al., 2009).

One neuromodulator within the nTS is serotonin (5-HT), which plays an important role in the cardiorespiratory system (Gillis et al., 1989; Ling et al., 2001). 5-HT within the nTS derives from vagal afferents, raphé neurons and the nTS itself (Steinbusch, 1981; Zhuo et al., 1997), where it may activate one or more 5-HT receptor (5-HTR) subtypes. The medial nTS densely expresses 5-HT$_{1A}$Rs (Liu and Wong-Riley, 2010; Manaker and Verderame, 1990; Thor et al., 1992), one of the 14 identified 5-HTRs. Although not all studies agree (Feldman and Galiano, 1995), 5-HT$_{1A}$Rs in the nTS may produce tachypnea, hypotension or bradycardia (Besenard et al., 2012; Itoh and Bunag, 1991). Likewise, the role of 5-HT$_{1A}$Rs in modulating nTS or dorsal vagal complex synaptic or neuronal activity is unclear as increases, decreases or no change in activity have been reported (Browning and Travagli, 1999; Wang et al., 1997; Feldman, 1995; Takenaka et al., 2011). In other central nuclei, activation of 5-HT$_{1A}$Rs leads to neuronal hyperpolarization and reduced firing rate, and often involves a reduction of glutamatergic and/or GABAergic transmission (Aghajanian and Sanders-Bush, 2002; Ciranna, 2006; Costa et al., 2012; Lalley et
Taken together, while these results suggest 5-HT₁₄Rs modulate cardiorespiratory function via the nTS; the mechanisms by which this may occur remain elusive. In the present study, we sought to determine the distribution and function of 5-HT₁₄Rs in the nTS. Our results show that their activation in the nTS profoundly influences basal respiratory parameters, likely through their modulation of excitatory and inhibitory neurotransmission.

METHODS

Ethical approval and animals. The Animal Care and Use Committee of the University of Missouri approved all experimental protocols in accordance with NIH guidelines (“Guide for the Care and Use of Laboratory Animals”). Male Sprague-Dawley rats (Harlan; n = 58, aged 4 - 8 weeks) were maintained in the vivarium of the Dalton Cardiovascular Research Center accredited by the AAALAC. The animals were held at 22°C and 40% humidity on a 12 hour day/night cycle with water and food available ad libitum.

In vivo preparation and microinjection. Experiments were performed as previously (Mueller et al., 2005; Clark et al., 2011). Briefly, rats (n = 5) were anesthetized with Isoflurane (5%, induction; 2-3% maintenance, in 100% O₂, VetOne) and femoral venous and arterial catheters (PE-10 fused to PE-50, A-M systems) were inserted to enable drug administration and measurement of arterial pressure, respectively. Mean arterial pressure (MAP) and heart rate
(HR) were determined using a PowerLab data acquisition system (ADInstruments). The trachea was cannulated, and rats were mechanically ventilated with O₂-enriched room air. Blood samples were taken to record arterial blood gases (Osmetech, OPTI CCA) and arterial hemoglobin oxygen saturation (HbO₂) was monitored continuously (MouseOx, Starr Life Sciences); PaO₂ levels were maintained at 155.6 ± 1.8 mmHg, PaCO₂ at 52.6 ± 1.8 mmHg, pH at 7.35 ± 0.011, and O₂ saturation at 98.9 ± 0.2%. PaCO₂ was kept at this level to maintain phrenic nerve activity. Rectal temperature was monitored and maintained at ~38°C (Tele-Thermometer, Simpson Electrics). For a measurement of central neural output, a splanchnic and phrenic nerve were isolated via a retroperitoneal or ventral cervical approach, respectively, placed on bipolar Teflon-coated silver electrodes (0.005 – 0.007” A-M Systems), and covered in silicone elastomer (Kwik-Cast, WPI). The recorded phrenic nerve was crushed distally and the contralateral phrenic nerve was cut. Bilateral cervical vagotomy was performed to prevent entrainment of phrenic motor output with the ventilator. Nerve activity was amplified (1000x), filtered (30-3000 Hz, P511, Grass technologies), rectified and integrated using a root mean square converter (time constant: phrenic = 100 ms; splanchnic = 28 ms); sympathetic nerve activity was electronically averaged. Background noise was determined from the signal between bursts of activity. The recorded nerve activity minus noise was defined as splanchnic sympathetic nerve activity (SSNA) and phrenic nerve activity (PhrNA).

Rats were placed in a stereotaxic apparatus (Kopf Instruments) and the brainstem exposed via a partial occipital craniotomy as previously described (Mueller and Hasser, 2006; Clark et al., 2011). Following completion of surgery, anesthesia was gradually converted from Isoflurane to Inactin (100 mg/kg i.v., 20 mg/kg i.v. supplements as required). Animals were
paralyzed using gallamine (8.3 mg/kg i.v., 1-2 mg/hr i.v. maintenance). Adequate plane of anesthesia was verified regularly by lack of cardiovascular responses to tail pinch (< 5 mmHg increase in MAP).

Cardiorespiratory parameters were allowed to stabilize at least 1 hr, and baseline PhrNA (integrated amplitude and frequency) were constant at least 30 min before starting experimental protocols. Rats received unilateral nTS microinjections of either vehicle control or 8-OH-DPAT (1 and 5 mM, 60 nL, relative to calamus scriptorius (mm): 0.3 rostral, 0.3 lateral, 0.5 ventral; target coordinates were ascertained prior to in vivo experiments to target cardiorespiratory regions and to match in vitro recording sites). Artificial cerebrospinal fluid (aCSF) served as vehicle and was injected prior to 8-OH-DPAT. Subsequently, MAP, HR, O₂ saturation, PhrNA (peak integrated phrenic amplitude and phrenic frequency) and SSNA (mean) were recorded for 45-60 min followed by contralateral aCSF and 8-OH-DPAT microinjections. At the end of the experiment fluorescent retrobeads (LumaFluor Inc., 1:50, in 15-30 nL aCSF) were injected at the nTS target sites to mark microinjection sites. Rats were subsequently euthanized via Inactin overdose and/or euthanasia solution (Beuthanasia-D, 0.1 ml), the brains were removed, fixed in formaldehyde, sectioned in the horizontal plane and subsequently examined.

In vitro brainstem slice preparation, electrophysiology and protocols. As detailed previously (Kline et al., 2010), the brainstem was removed from Isoflurane (VetOne)-anesthetized rats and placed in ice-cold low calcium-high magnesium aCSF (in mM: 124 NaCl, 3 KCl, 1.2 NaH₂PO₄, 1.2 MgSO₄, 25 NaHCO₃, 11 D-glucose, 0.4 L-Ascorbic Acid, 1 CaCl₂ and 2 MgCl₂, saturated with 95%
Horizontal slices (~280 µm) with lengthy segments of the tractus solitarii (TS; Figure 5A) and the nTS were cut using a vibrating microtome (VT 1000S, Leica). Tissue sections were secured via nylon mesh in a superfusion chamber and superfused at ~3 mL/min with standard recording ACSF (in mM: 124 NaCl, 3 KCl, 1.2 NaH2PO4, 1.2 MgSO4, 25 NaHCO3, 11 D-glucose and 2 CaCl2, saturated with 95% O2 - 5%CO2, pH 7.4, ~300 mOsm) at 31 - 33°C.

All recordings were made from nTS cell somas in the caudal medial-commissural nTS, a region that receives cardiorespiratory afferent information (Chitravanshi and Sapru, 1995; Guyenet, 2000). Electrodes (King Precision Glass, type 8250) were pulled with a Flaming/Brown micropipette puller (Sutter Instruments, Model P-97). To record excitatory postsynaptic currents (EPSCs), the following recording solution was used (in mM): 10 NaCl, 130 K+ Gluconate, 11 EGTA, 1 CaCl2, 10 HEPES, 1 MgCl2, 2 Mg-ATP, 0.2 Na-GTP, pH 7.3, ~280 mOsm. The calculated chloride reversal potential with this pipette solution was -58.6 mV. For recordings of inhibitory postsynaptic currents (IPSCs) we used a high chloride-based solution (in mM: 140 CsCl, 5 NaCl, 10 EGTA, 10 HEPES, 1.2 MgSO4, 3 K-ATP, 0.2 Na-GTP, 5 QX314, pH 7.3, ~280 mOsm) (Chen et al., 2009). The calculated reversal potential for IPSC recordings with this high chloride pipette was 2.7 mV. Recording pipettes were guided with a piezoelectric micromanipulator (Burleigh, PCS-6000). Neurons were recorded under voltage clamp configuration using the patch clamp technique. Cells were held at -60 mV. Neurons were rejected if the holding current was more negative than -50 pA upon initial rupture or if series resistance changed more than 20% throughout the experiment. In some experiments the series resistance was compensated. Data
were recorded using a Multiclamp700B amplifier (Molecular Devices), filtered at 2 kHz and sampled at 20 kHz.

Evoked postsynaptic currents were generated with isolated stimulators (A.M.P.I., Master-8 and ISO-Flex) and concentric bipolar stimulating electrodes (F. Haer). Afferent evoked EPSCs were evoked as previously (Kline et al., 2002) by placing a stimulating electrode on the TS containing visceral afferent fibers (Figure 5A). These were defined as TS-EPSCs. Evoked IPSCs were elicited in monosynaptic TS-connected neurons (see below) by placing a second bipolar electrode within the medial nTS. These were defined as nTS-IPSCs. To isolate IPSCs from EPSCs, 10 µM NBQX (non-NMDA receptor blocker; Tocris) and 50 µM AP5 (NMDA receptor blocker; Tocris) was added to the recording aCSF. GABAzine (25 µM SR 95531 HBr, Tocris) was applied at the end of the experiment to confirm IPSC identity. For either evoked postsynaptic current stimulation intensity was increased until a TS-EPSC and/or nTS-IPSC was evoked after which stimulation intensity was set at 1.5x threshold. Subsequently, the role of 5-HT1AR activation or blockade on TS-EPSCs or nTS-IPSCs was examined.

Miniature (m) excitatory and inhibitory postsynaptic currents (mEPSCs and mIPSCs) were recorded without external stimulation and in the presence of 1 µM TTX (Tocris). The lack of AP discharge to current ramps (-20 pA to +50, +100 or +200 pA; 1 s ramp in current clamp mode) at the beginning of the protocol ensured that the events were not due to action potential-driven network activity. mEPSCs were isolated from mIPSCs with GABAzine to block inhibitory currents. mIPSCs were isolated from mEPSCs with NBQX and AP5. Subsequently, the role of 5-HT1AR activation or blockade on mEPSCs or mIPSCs was examined.
Drugs. All receptor pharmacological agents were purchased from Tocris. The role of 5-HT$_{1A}$R activation was tested using the prototypical agonist 8-OH-DPAT. To examine the effects of 5-HT$_{1A}$R activation on cardiorespiratory parameters, 1 and 5 mM 8-OH-DPAT was microinjected in the nTS. To define the role of 5-HT$_{1A}$R activation on GABAergic and glutamatergic transmission, 10 µM 8-OH-DPAT was bath applied to brainstem slices. In the latter, 5-HT$_{1A}$Rs were blocked by use of the specific antagonist WAY100135 (10 µM). To eliminate the influence of the 5-HT$_7$ receptors, the 5-HT$_7$R antagonist SB269970 (5 µM) was used in conjunction with 8-OH-DPAT. All drug treatments were bath applied via a gravity fed reservoir. We typically recorded neurons 1-2 cell layers deep and perfused drugs for 5 minutes to allow penetration into the slice and to compensate for dead space in the perfusion tubing. All other general compounds were purchased from Sigma (St. Louis, MO).

Immunohistochemistry. Immunohistochemistry was performed as previously (Austgen et al., 2009; Kline et al., 2010). Briefly, deeply anesthetized animals were transcardially perfused with 0.1 M PBS followed by 4% paraformaldehyde (Sigma). After brainstem removal, 30 µm coronal brain sections were cut using a vibratome (VT 1000S, Leica). The sections were rinsed in PBS and then blocked by 10% normal donkey serum (Millipore) in 0.3 % Triton-PBS. Tissue sections were subsequently incubated with primary antibodies against 5-HT$_{1A}$R (guinea pig, 1:500, 550469, BD Pharmingen) and either synaptophysin (mouse, 1:2000; cat #S5768, Sigma), vGLUT2 (vesicular glutamate transporter; rabbit, 1:1000, cat #135403, SYSY) or GAD67 (glutamic acid
decarboxylase; mouse, 1:3000, cat #MAB5406, Chemicon). The following day sections were rinsed and incubated in 0.3 % Triton-PBS including the appropriate Cy-conjugated secondary antibodies from donkey (1:200; Jackson Immuno). Sections were mounted on gelatin coated slides, air dried and coverslipped. Slides were then sealed with nail polish. One section per run was incubated without the primary antibody and served as negative control. No fluorescent staining was present on the negative control. Specificity of the antibody for 5-HT1A R was confirmed via western blot analysis (see below), or previously for synaptophysin (Olucha-Bordonau et al., 2012), vGLUT2 (Berube-Carriere et al., 2009) and GAD67 (Fong et al., 2005).

Immunoreactivity (-IR) was examined with a conventional epifluorescent microscope (BX51, Olympus) equipped with a digital monochrome camera (ORCA-ER, Hamamatsu) and a spinning disc confocal unit (Olympus), or a confocal microscope (FluoView FV 1000, Olympus). Appropriate filter sets and excitation wavelength were used to visualize the different fluorophores. For each fluorophore used, z-stacks (0.5 µM) were taken in the same focal planes. All images were post-processed for clarity using ImageJ (Version 1.45m, NIH) for contrast and brightness, and AutoQuant X (Version X2.2.2, MediaCybernetics) for background subtraction and deconvolution of specified images.

Visceral afferent nerve labeling. In a subset of animals (n = 3), sensory afferents originating from the nodose ganglion were identified in 30 µm horizontal nTS slices through Texas Red dextran fluorescent labeling. Similar to our previous studies, (Austgen et al., 2011; Kline et al., 2002), rats were anesthetized with isoflurane (5% induction, 2–3% maintenance) and the
nodose ganglion was isolated close to the bifurcation of the common carotid artery. Texas Red
dextran (~100 nL, Life Technologies) was pressure microinjected into the ganglion using a glass
capillary and Picospritzer II (2-10 psi, General Valve Cooperation, Cleveland OH). Subsequently,
the neck was sutured and the animal allowed to recover. Postoperative treatment incorporated
Baytril (0.03 ml i.m.; Bayer, Shawnee Mission, KS) and Buprenex (0.02 ml s.q.; Reckitt Benckiser
Pharmaceuticals, Richmond, VA). After 5 days, which is sufficient for the anterograde transport
of the dye to the nTS, rats were transcardially perfused, the nTS isolated and
immunohistochemically processed for 5-HT1ARs as above.

Western blot. As described previously (Kline et al., 2007), frozen nTS tissue was pooled (3 rats),
homogenized in RIPA buffer (1% NP-40, 0.5% DOC, 0.1% SDS, 0.15M NaCl, 50mM Tris/HCl and
2.5mM EDTA), and complemented with protease inhibitors (Complete, mini-EDTA-free tablets;
Roche). Following 2 hours of incubation on ice, samples were centrifuged at 14,000 x G for 30
min at 4°C. Protein concentration of the supernatant was measured by the Micro BCA method
(Pierce, Rockford, IL). Twenty micrograms of protein were separated on 4 - 20% Tris-Glycine gel
(BioRad) and transferred to a PVDF membrane. Primary antibody anti-5-HT1AR (guinea pig,
1:1000, cat #550469, BD Pharmingen), followed by donkey anti-guinea pig secondary antibody
conjugated to HRP (1:10000), was used to immunoblot the membranes. Blots were developed
with Immuno-Star WesternC kid (Bio-Rad) and visualized.
**Data Analysis.** Cardiorespiratory responses to nTS microinjection of 8-OH-DPAT were analyzed at 5 min for 25 seconds and expressed relative to their individual baseline (defined as “1”). There was no significant response to aCSF when microinjected on either side of the nTS, and therefore responses were averaged. The effects of 8-OH-DPAT (1 and 5 mM) were compared to the average responses of aCSF injections analyzed at the 5 min time point. In addition, because the time course of 8-OH-DPAT effects varied among rats, we also determined the maximum change in minute PhrNA (phrenic frequency x amplitude) within the first 10 min, comparing it to aCSF at the same time point. All data are presented as mean ± SEM.

Electrophysiological data were analyzed with pClamp10 (Molecular Devices), MiniAnalysis (Synaptosoft) and Microsoft Excel software. Only nTS cells monosynaptically connected to TS-afferents were analyzed for this study. A direct connection was determined based on a low variability of TS-EPSC onset (i.e. jitter, < 300 µsec.; SD of 20 TS-EPSC latencies from shock artifact, 20 kHz sampling rate) (Accorsi-Mendonca et al., 2011; Doyle and Andresen, 2001; Hisadome et al., 2010; Kline et al., 2002). Reported TS-EPSC properties were an average of 20 events. Spontaneous current (sEPSC, sIPSC, mEPSC and mIPSC) detection was set at 5x the root-mean square noise level and events were manually confirmed. All electrophysiology data are presented as geometric means ± 95% confidence interval (CI) due to the variability of baseline values and to allow description of a general response (Carter et al., 2007; Lando and Zucker, 1994; Sceniak and Maciver, 2008). Responses were also normalized and expressed relative to their individual baseline or vehicle (defined as “1”).
To statistically evaluate the effects of 5-HT$_{1A}$R activation on cardiorespiratory or synaptic parameters, SigmaPlot 12.0 (Systat Software) and GraphPad Prism 6 (GraphPad Software, Inc.) was used. *In vivo* data were analyzed using one-way repeated measures ANOVA followed by Fisher LSD post hoc test where appropriate to identify individual differences among groups. For *in vitro* protocols, the effect of a drug on EPSC amplitude, rise slope, decay slope and group spontaneous and miniature PSCs was compared within treatments using paired $t$-tests, or the Wilcoxon Signed Rank Test if the data were not normally distributed. Spontaneous current amplitude and interevent intervals within an individual cell between baseline and drug condition were tested with Kolmogorov-Smirnov two sample test. Results were considered significantly different at $p$ values $\leq 0.05$.

**RESULTS**

*5-HT$_{1A}$-Rs are located somatodendritically in the nTS.*

The presence of 5-HT$_{1A}$Rs in the nTS was examined by immunohistochemistry. The specificity of the 5-HT$_{1A}$R antibody was verified via western blot analysis, where it showed a single band at the expected $\sim 46$ kDa (Figure 1A). 5-HT$_{1A}$R-immunoreactivity (-IR) was found throughout the nTS (Figure 1B) with punctate staining on cell bodies and processes (Figure 1C-F). To examine whether this punctate staining represented 5-HT$_{1A}$R-IR within synaptic terminals, we co-stained sections with the presynaptic marker synaptophysin (Figure 1C); no colocalization was observed. Labeled visceral afferents and terminals from the nodose ganglion were also devoid of 5-HT$_{1A}$R-IR (Figure 1D).
In the nTS the major excitatory and inhibitory transmitters are glutamate and GABA, respectively. Double labeling of 5-HT$_{1A}$Rs with vGLUT2, an antibody targeted towards glutamatergic presynaptic terminals, showed no overlap (Figure 1E), although the punctate staining of vGLUT2 was closely apposed to 5-HT$_{1A}$R-IR. Likewise, the punctate staining of GAD67-IR, which depicts GABAergic terminals, did not colocalize with 5-HT$_{1A}$R (Figure 1F$_1$). However, cell bodies containing GAD67-IR (Fong et al., 2005; Austgen et al., 2009) colabeled with a subset of 5-HT$_{1A}$R-IR (Figure 1F$_2$, arrows). All together, these data suggest that 5-HT$_{1A}$R is somatodendritically located in cells of the nTS.

5-HT$_{1A}$R activation decreases the amplitude and increases the frequency of phrenic nerve discharge.

To explore the functional role of 5-HT$_{1A}$R in the nTS, we microinjected the prototypical 5-HT$_{1A}$R agonist 8-OH-DPAT (1 and 5 mM, 60 nL) into the medial-commissural nTS, the primary central site for cardiorespiratory afferent termination. Histological analysis of the injection sites verified micropipette placement within the caudal nTS (Figure 2C). Baseline cardiorespiratory variables are shown in Table 1. A representative recording of arterial pressure (AP), HR, SSNA and PhrNA in response to 5 mM 8-OH-DPAT is shown in Figure 2A. As shown, microinjection of 8-OH-DPAT produced minimal changes in AP, HR or SSNA. By contrast, 8-OH-DPAT increased PhrNA rate but decreased PhrNA amplitude and minute PhrNA (Figure 2A, B). The changes in cardiorespiratory parameters 5 min (open bar in Figure 2A) after aCSF or 8-OH-DPAT injection were normalized to their baseline pre-injection activity and shown in Figure 2B (baseline = 1).
Neither 1 nor 5 mM 8-OH-DPAT in the nTS significantly altered AP, HR or SSNA compared to aCSF controls \( (p_{\text{AP}} = 0.657; p_{\text{HR}} = 0.171; p_{\text{SSNA}} = 0.323) \). However, both 1 and 5 mM 8-OH-DPAT significantly decreased PhrNA amplitude and significantly increased PhrNA rate (Figure 2B). The combination of a decrease in PhrNA amplitude and increase in PhrNA rate 5 min after 8-OH-DPAT injection resulted in minute PhrNA that was not significantly different from control. However, evaluation of maximum minute PhrNA change within each individual rat (closed bar Figure 2A) revealed significant decreases for both concentrations of 8-OH-DPAT \( (p \leq 0.01; 1 \text{ mM}: 0.78 \pm 0.06 \text{ normalized to baseline } [\text{time point} = 4.4 \pm 1.4 \text{ min}]; 5 \text{ mM}: 0.8 \pm 0.1 \text{ normalized to baseline } [\text{time point} = 3.4 \pm 2.0 \text{ min}]; \text{aCSF}: 1.0 \pm 0.1) \). Overall, our results suggest that activation of 5-HT\(_{1A}\)Rs in the nTS decreases phrenic motor output with no changes in cardiovascular variables (AP, HR) or sympathetic nerve activity.

**Activation of 5-HT\(_{1A}\)R decreases afferent excitatory synaptic transmission.**

5-HT\(_{1A}\)Rs may influence respiratory function through modulation of glutamatergic neurotransmission. To examine the contribution of 5-HT\(_{1A}\)Rs to nTS synaptic signaling, we stimulated the TS (0.5 Hz) to evoke glutamatergic EPSCs in nTS neurons that are monosynaptically connected to visceral afferents. Across the cells tested, TS-EPSCs had a mean amplitude of 69.2 (lower-upper 95\% CI, 58-82) pA, latency of 4.8 (4.4-5.2) ms and jitter of 147.4 (133-163) µsec \( (n = 83, 45 \text{ rats}) \), confirming such cells are monosynaptically connected to TS afferents (Accorsi-Mendonca et al., 2011; Bailey et al., 2006; Hisadome et al., 2010; Kline et al., 2002). As shown in the example from one cell (Figure 3A), perfusion of 10 µM 8-OH-DPAT
reduced the amplitude of TS-EPSCs from its aCSF baseline. This was a significant decrease for the whole sample tested (Figure 3B). TS-EPSC rise time ($n = 9; p = 1.00$) or decay time ($n = 9; p = 0.80$) was not altered with 8-OH-DPAT. Furthermore, failure rate or mean $^2$ variance of EPSC amplitude, indicators of presynaptic alterations, were not altered with 5-HT$_{1A}$R activation ($n = 10; p_{\text{failure}} = 1.00, p_{\text{mean-var}} = 0.92$). The reduction in TS-EPSC amplitude persisted throughout the 5 minute washout period, consistent with prolonged effects of 8-OH-DPAT in other central tissue (Chen et al., 2008).

To examine whether the 5-HT$_{1A}$R-induced decrease in TS-EPSC amplitude resulted from increased GABAergic transmission that would subsequently inhibit glutamate release and/or induce postsynaptic shunting, we examined synaptic currents in the presence of 8-OH-DPAT and GABAzine (GBZ) to eliminate GABA$_A$ receptor-mediated inhibition within the nTS. GBZ alone (25 µM) did not change the amplitude of TS-EPSCs ($n = 7; p = 0.17$). In the presence of GBZ, activation of 5-HT$_{1A}$R by 8-OH-DPAT (10 µM) significantly decreased TS-EPSC amplitude (Table 2). These results suggest that the 5-HT$_{1A}$R-mediated decrease in TS-EPSCs does not occur via an increase in inhibitory GABAergic transmission.

While 8-OH-DPAT is a specific 5-HT$_{1A}$R agonist, it may exhibit some affinity for the 5-HT$_2$R (Lovenberg et al., 1993). To exclude a potential 5-HT$_2$R contribution, we examined the effect of 8-OH-DPAT (10 µM) on TS-EPSCs in the presence of the 5-HT$_2$R blocker SB269970 (5 µM). SB269970 alone did not change TS-EPSCs amplitude ($n = 9; p = 0.15$). Adding 8-OH-DPAT to the SB269970 perfusion solution significantly decreased TS-EPSC amplitude (Table 2). These
results show that 8-OH-DPAT decreases glutamatergic EPSCs even in the presence of 5-HT\textsubscript{7}R blockade suggesting its actions occur through 5-HT\textsubscript{1A}Rs.

**5-HT\textsubscript{1A}-activation reduces excitatory network activity independent of GABA\textsubscript{A}R and 5-HT\textsubscript{7}R.**

Recordings of spontaneous (s)EPSCs in the absence of stimulation allows evaluation of the nTS network activity within the available circuitry of the slice (Fortin and Champagnat, 1993). As shown in the representative cell (Figure 3C), 5-HT\textsubscript{1A}R activation with 10 μM 8-OH-DPAT significantly reduced sEPSC event frequency compared to aCSF baseline. Overall, 5-HT\textsubscript{1A}R activation significantly shifted the cumulative fraction of sEPSC intervals to the right (Figure 3D; \(p < 0.05\), Kolmogorov-Smirnov two sample test) and decreased mean sEPSC frequency (Figure 3E, \(n = 9\)). By contrast, 5-HT\textsubscript{1A}R activation did not change the cumulative fraction of sEPSC amplitudes (not shown) nor their mean amplitude (aCSF baseline, 22.4 (19-27) pA vs. 8-OH-DPAT, 23.1 (18-29) pA; \(n = 11\); \(p = 1.00\)).

Similar to TS-EPSCs, the 5-HT\textsubscript{1A}R mediated decrease in spontaneous currents was not influenced by GABAergic or 5-HT\textsubscript{7}R inhibition. Individually, GBZ did not alter sEPSC frequency (\(n = 7\); \(p = 0.47\)) or amplitude (\(n = 7\); \(p = 0.25\)). In the presence of GBZ, 5-HT\textsubscript{1A}R activation by 8-OH-DPAT significantly decreased sEPSC frequency but did not change sEPSC amplitude (Table 2). Furthermore, 5-HT\textsubscript{7}R blockade alone did not alter sEPSC frequency (\(n = 13\); \(p = 0.27\)) nor amplitude (\(n = 13\); \(p = 0.38\)). Adding 8-OH-DPAT during SB269970 perfusion significantly decreased sEPSC frequency but not amplitude (Table 2). Such data show that 8-OH-DPAT
decreases sEPSCs in the presence of GABAergic and 5-HT$_7$R blockade suggesting its actions occur through 5-HT$_{1A}$Rs.

5-HT$_{1A}$R-activation does not alter mEPSCs.

sEPSCs derive from action potential-dependent and independent glutamate release (Fortin and Champagnat, 1993). To examine the effects of 5-HT$_{1A}$R activation on EPSCs that occur locally at the synapse and independent of action potentials (i.e., mEPSCs), we added TTX (1 µM; a voltage-gated Na$^+$-channel blocker) and GBZ to the perfusion solution. This treatment did not change EPSCs amplitude ($n=11$; $p=0.32$), but decreased EPSC event frequency in 5 out of 11 cells (3 did not change and 3 increased). The total sample, however, did not reach significance ($n=11$; $p=0.52$). The addition of 8-OH-DPAT did not change mEPSC amplitude or frequency (Table 2). These results suggest 5-HT$_{1A}$R-activation alters synaptic transmission via action potential-dependent mechanisms.

Blocking 5-HT$_{1A}$Rs increases excitatory network activity and ablates the effect of 8-OH-DPAT.

We examined the tonic role of 5-HT$_{1A}$Rs on TS- and sEPSCs by blocking the receptor with 10 µM WAY100135, a selective 5-HT$_{1A}$R antagonist (Cliffe et al., 1993). Blocking 5-HT$_{1A}$Rs did not alter evoked TS-EPSC amplitude (Figure 4A). Moreover, subsequent application of 8-OH-DPAT in the presence of WAY100135 did not change TS-EPSC amplitude (Table 2). In contrast to TS-EPSCs, WAY100135 significantly increased sEPSC frequency (Figure 4B) but not amplitude
(aCSF baseline, 21.6 (18-25) pA vs. WAY100135, 21.3 (18-25) pA; n = 9; p = 0.73). Concurrent 8-38 OH-DPAT and 5-HT\textsubscript{1A}R block did not change sEPSC frequency or amplitude (Table 2). Taken together, these data suggest that 5-HT\textsubscript{1A}Rs are constitutively active within the nTS network, but not at the sensory afferent-nTS synapse.

**Activation of 5-HT\textsubscript{1A}R decreases GABAergic network but not evoked activity.**

5-HT\textsubscript{1A}Rs decrease inhibitory transmission in other brainstem nuclei, including synaptic signaling in the nucleus ambiguus originating from the nTS (Chen et al., 2008). Whether 5-HT\textsubscript{1A}Rs alter evoked or spontaneous inhibitory postsynaptic currents (IPSCs) in the nTS is unknown. In the following protocols, IPSCs were recorded at -60 mV using a high chloride intracellular recording solution (calculated E\textsubscript{Cl}\textsuperscript{-}, 2.7 mV) and pharmacologically isolated. Sequential stimulation of the TS and nTS (Figure 5A) elicited monosynaptic EPSCs and IPSCs, respectively (Figure 5B top) (Chen and Bonham, 2005). Blocking glutamatergic AMPA (10 µM NBQX) and NMDA (50 µM AP5) receptors (AMPAR and NMDAR, respectively) eliminated the TS-EPSCs and isolated nTS-evoked and spontaneous IPSCs (Figure 5B middle). Overall, nTS-IPSC amplitudes were not altered by glutamate receptor blockade (aCSF baseline, 73.9 (48-113) pA vs. NBQX + AP5, 62.2 (32-122) pA; n = 10; p = 0.91). By contrast, spontaneous events, which initially comprised of a mixture of sEPSCs and sIPSCs under these recording conditions, significantly decreased in frequency during NMDAR and AMPAR blockade (aCSF baseline, 3.9 (2.3-6.6) Hz vs. NBQX + AP5, 0.9 (0.6-1.3) Hz, n = 19; p < 0.001). This illustrates the prevalence of excitatory nTS network activity. Subsequent application of GABA\textsuperscript{zine} eliminated all evoked and
spontaneous IPSCs, verifying that the nTS evoked- and spontaneous IPSCs were GABAergic (Figure 5B bottom).

Compared to baseline (during NBQX + AP5 perfusion), concurrent activation of 5-HT_{1A}Rs decreased the amplitude of nTS-IPSCs in 5 out of 8 cells. However, this did not reach statistical significance in either the normalized or raw current values (Figure 5C). In contrast, 5-HT_{1A}R activation significantly decreased sIPSC frequency (Figure 5D) but not amplitude (baseline, 55.0 (39-77) pA vs. 8-OH-DPAT, 51.2 (38-69) pA; n = 9; p = 0.65). These data suggest a general decrease of inhibitory network activity with activation of 5-HT_{1A}R.

**Activation of 5-HT_{1A}R alters inhibitory postsynaptic receptor function.**

mIPSCs were isolated by the addition of TTX (1 µM), AP5 and NBQX to the aCSF. As expected, TTX, AP5 and NBQX significantly decreased the frequency of postsynaptic currents from aCSF baseline, which contains action potential-dependent and -independent EPSCs and IPSCs (aCSF baseline, 2.6 (1.0-6.6) Hz vs. TTX + AP5 + NBQX, 0.9 (0.6-1.6) Hz; n = 8; p < 0.001). The amplitude of the postsynaptic currents did not change (n = 8; p = 0.47). Such data suggest a decrease in excitatory and AP-dependent network activity.

Compared to TTX-AP5-NBQX baseline, 8-OH-DPAT did not alter mIPSC frequency (Table 3). However, 5-HT_{1A}R activation significantly decreased mIPSC amplitude without affecting mIPSC rise time or decay time. This suggests that 5-HT_{1A}R-activation affects action potential-independent mechanisms at the synapse for inhibitory transmission in the nTS.
Blockade of 5-HT$_{1A}$R and inhibitory network activity.

5-HT$_{1A}$Rs constitutively modulate EPSCs within the nTS (see above). To determine if the same holds true for inhibitory transmission, we blocked 5-HT$_{1A}$R by perfusing WAY100135 while recording inhibitory currents. Evoked nTS-IPSC amplitude increased in 4 of 6 cells during 5-HT$_{1A}$R blockade when compared to baseline condition; however, as a whole it did not reach statistical significance (Figure 6A). sIPSCs frequency also increased by 5-HT$_{1A}$R activation in 8 of 10 cells (Figure 6B) but did not reach statistical significance in the mean data. The sIPSC amplitude did not change (baseline, 38.1 (28-53) pA vs. WAY100135, 42.2 (32-56) pA; n = 10; $p = 0.23$).

**DISCUSSION**

In the present study, we demonstrate 5-HT$_{1A}$Rs are located throughout the nTS, primarily at postsynaptic neurons and apposed by glutamatergic and GABAergic terminals. 5-HT$_{1A}$R-activation *in vivo* overall decreased central respiratory output but did not alter cardiovascular parameters. Synaptically, activation of 5-HT$_{1A}$Rs reduced both excitatory glutamatergic and inhibitory GABAergic synaptic transmission, whereas 5-HT$_{1A}$R-inhibition of glutamatergic transmission was tonic at rest. These results provide insight into the functional role of this serotonin receptor subtype in the nTS and respiratory system.
Previous studies reported that \textit{in vivo} activation of 5-HT$_{1A}$Rs in the nTS excites, inhibits or does not alter neuronal activity (Oskutyte et al., 2009; Wang et al., 1997). These varying responses may result from differences in 5-HT$_{1A}$R distribution, including disparity in its localization within the axon, dendrite, soma, synaptic or extrasynaptic regions, as well as differential activation of cellular phenotypes including glutamatergic or GABAergic cells. In the present study, we show via immunohistochemistry that 5-HT$_{1A}$Rs were localized primarily within cell bodies and fibers throughout the nTS. Similar distributions in the nTS have been observed by others in the rat, including its protein (Liu and Wong-Riley, 2010; Manaker and Verderame, 1990; Thor et al., 1992) and mRNA (Pompeiano et al., 1992), as well as within humans (Spurney et al., 1997). We advance these studies by providing evidence that such 5-HT$_{1A}$R labeling is apposed to, but not co-labeled with, presynaptic markers; including those for presynaptic terminals in general (synaptophysin), glutamatergic terminals (vGLUT2) and GABAergic terminals (GAD67). Moreover, labeled visceral afferents and terminals were also devoid of 5-HT$_{1A}$R staining. Such results suggest 5-HT$_{1A}$Rs are not localized to presynaptic terminals nor sensory afferents, but rather within cells of the nTS.

We further provide functional evidence that unilateral 5-HT$_{1A}$R activation within the medial-commissural nTS alters respiratory but not cardiovascular parameters. Specifically, the most prominent effect of 5-HT$_{1A}$R activation was to decrease phrenic amplitude resulting in an overall reduction in minute nerve activity. Consistent with our \textit{in vitro} data, this likely occurred via 5-HT$_{1A}$R-reduction of one or more active respiratory-related circuits such as nTS projections to the retrotrapezoid nucleus, rostral ventrolateral medulla, or rostral ventral respiratory group (Alheid et al., 2011; Kline et al., 2010). Activation of 5-HT$_{1A}$Rs also increased phrenic rate similar
to previous studies in which 8-OH-DPAT administered into the fourth ventricle or the dorsal motor nucleus of the vagus increases diaphragm muscular activity or phrenic rate (Besnard et al., 2012; Sporton et al., 1991). These effects may also indicate reduced glutamatergic excitatory transmission with 5-HT$_{1A}$R activation and are consistent with increased phrenic rate following kynurenic acid injections into the caudal nTS (Costa-Silva et al., 2010). Of note, unilateral injection of 8-OH-DPAT decreased phrenic minute nerve activity by approximately 20%; we anticipate that this response would have been even greater had the agonist been administered bilaterally. Although not likely because arterial pressure did not change, it is possible that responses would have been even greater in animals with afferent denervation to eliminate compensatory responses. Taken together, the data suggest that 5-HT, acting at 5-HT$_{1A}$Rs in the nTS, can have a significant impact on respiratory regulation.

5-HT$_{1A}$R activation decreased afferent evoked TS-EPSC amplitude and the overall frequency of spontaneous excitatory network activity. This network 5-HT$_{1A}$R modulation is tonic at rest, as evidenced by the increase in its activity following receptor blockade. In addition, it is independent of GABA as shown by the prevailing effect of 8-OH-DPAT with co-application of GABAzine. 5-HT$_{1A}$R-induced changes likely result from actions at the postsynaptic complex as evidenced by unaltered TS-EPSC failure rate and mean variance of its amplitude, as well as mEPSC frequency (indicators of presynaptic alterations). Such results are consistent with its postsynaptic immunolocalization (present study and Riad et al. (2000)). While TS-EPSC amplitude was reduced by 5-HT$_{1A}$R activation, the lack of changes in s/mEPSC amplitude suggest a need to summate smaller 5-HT$_{1A}$R-mediated events in order to observe larger end responses, such as those occurring during synchronized TS-EPSCs. The 5-HT$_{1A}$R-mediated
decrease in sEPSC frequency then likely originates from reduced activity of cells that synapse onto the recorded cell. However, while our results are consistent with studies showing a decrease of glutamatergic transmission with 5-HT₁A R activation in other central neurons (Ciranna, 2006; Costa et al., 2012; McCall and Clement, 1994), including a decrease in nTS firing rate (Feldman, 1995), they are not consistent with a recent nTS study demonstrating 1 μM 8-OH-DPAT did not alter synaptic transmission in Wistar rat slices (Takenaka et al., 2011). Such disparate results may be due to technical differences, rat strain, drug dosage, or perhaps the influence of GABAergic transmission. Taken together, we demonstrate 5-HT₁A R restrains glutamatergic neurotransmission in the nTS to alter cardiorespiratory parameters.

5HT₁A R could attenuate TS-EPSC amplitude via a decrease in postsynaptic AMPA currents or reduction of AMPARs on the membrane. It has been shown that 5HT₁A R-activation diminishes AMPA currents via decreased GluR1 subunit phosphorylation and inhibition of Ca²⁺/calmodulin-dependent kinase II (Cai et al., 2002). Conversely, blockade of 5HT₁A Rs increases AMPAR phosphorylation, potentially increasing AMPA currents (Schiapparelli et al., 2005). Although long-term 5-HT₁A R modulation may alter AMPAR membrane expression, it is unlikely that postsynaptic AMPAR expression is changed during our brief five minute exposure of 8-OH-DPAT as mEPSCs were unaltered. Whether such mechanisms occur in nTS neurons requires further investigation.

While glutamate is the primary excitatory neurotransmitter in the nTS, it is counterbalanced by the inhibitory neurotransmitter GABA. We show that 5-HT₁A R activation also decreased GABAzone-sensitive nTS-evoked IPSCs in the majority of cells, and significantly
reduced inhibitory nTS network activity (sIPSCs). Moreover, activation of the 5-HT$_{1A}$R reduced spontaneous excitatory and inhibitory currents by a similar magnitude (~30%). However, because the frequency of basal sEPSCs is greater than that of sIPSCs [4.8 Hz (Table 2) vs. 0.9 Hz (Table 3), respectively] the net effect of 5-HT$_{1A}$R activation to reduce excitatory events may predominate. Of note, while there was some tonic influence of 5-HT$_{1A}$Rs on IPSCs, it was relatively weak and as a whole this effect did not reach statistical significance. Such results are consistent with reports that 5-HT$_{1A}$R activation inhibits sIPSCs but its blockade alone had no effect (Chen et al., 2012; Chen et al., 2008; Lee et al., 2008). Thus, the inhibitory influence of 5-HT$_{1A}$Rs may be greater on glutamatergic than GABAergic transmission, altering its influence on activity in the nTS circuit, and thus respiration. However, the importance of nTS-derived GABA and the influence of 5-HT$_{1A}$R has been observed by others. For instance, 5-HT$_{1A}$R activation decreased nTS-derived IPSCs within the dorsal motor nucleus of the vagus (Browning and Travagli, 1999) as well as nTS-derived IPSCs within the nucleus ambiguus (Chen et al., 2012).

8-OH DPAT is a prototypical 5-HT$_{1A}$R receptor agonist, but may also bind to the 5-HT$_{7}$R, a relatively new member of the 5-HT receptor family. However, the observed effects of 8-OH-DPAT in this study are likely due to 5-HT$_{1A}$R since the decrease in EPSCs was abolished by application of the specific 5-HT$_{1A}$R antagonist WAY100635, and prevailed during blockade of the 5-HT$_{7}$R receptor using the established antagonist SB269960. While there may be some pharmacological overlap between both receptor subtypes, their primary pathways have been shown to differ as activation of 5HT$_{1A}$Rs typically inhibits adenylyl cyclase to decrease cAMP production, whereas 5HT$_{7}$-receptors activation causes the opposite effect (Millan et al., 2008).
Likewise, 5HT$_1$A Rs inhibit synaptic transmission in hippocampal neurons whereas 5HT$_7$-receptors produce an increase in transmission (Costa et al., 2012).

In summary, we demonstrate 5-HT$_1$A Rs reduce glutamatergic and GABAergic neurotransmission within the nTS. These effects are tonic at rest. However, the tonic activation is greater for spontaneous EPSCs than that of IPSCs. 5HT is implicated in modulation of the cardiovascular, respiratory and gastrointestinal systems under a variety of conditions. For instance, direct evidence has shown that 5-HT$_1$A Rs in the nTS reduce bronchopulmonary C-fiber-induced apneas (Zhuang et al., 2012), whereas indirectly they have been suggested to induce bradycardia, depressor responses and sympathoinhibition (Itoh and Bunag, 1991; Feldman and Galiano, 1995). Moreover, 5-HT has been implicated in sudden infant death syndrome and sleep apnea (Hilaire et al., 1993; Kinney, 2005). Children that succumb to SIDS have lower 5-HT$_1$A Rs in the nTS (Machaalani et al., 2009). In the intermittent hypocapnic hypoxia piglet, a SIDS model, 5-HT$_1$A R immunoreactivity is reduced in the nTS (Say et al., 2007). These data demonstrate a powerful physiological role for 5-HT$_1$A Rs in the nTS. Moreover, these results advance our understanding of the serotonin/5-HT$_1$A R-glutamate and GABA systems and their interactions and provide potential mechanisms for the role of serotonin in vital autonomic and respiratory systems.

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**Figure 1: Localization of 5-HT$_{1A}$R within the nTS.**

**A.** Immunoblot verifying antibody specificity for 50 µg of protein from the nTS. A single band for 5-HT$_{1A}$R was confirmed at the appropriate size of ~46 kDa.  

**B.** Coronal section of the medial/commissural nTS (enclosed area) labeled with 5-HT$_{1A}$R antibody. Scale 200 µm. TS = tractus solitarii; CC = central canal.  

**C.** nTS neurons colabeled for 5-HT$_{1A}$R and synaptophysin. Note that both stainings are clearly separated indicating that 5-HT$_{1A}$R are localized somatodenritic within the nTS. Scale 25 µm.  

**D.** Neuron expressing 5-HT$_{1A}$R immunoreactivity with attached fluorescent Texas-Red dextran labeled visceral afferent terminals. Note, afferent terminals or fibers (arrow heads) do not colabel with 5-HT$_{1A}$R. The image has been background subtracted and deconvolved for clarity. Scale 20 µm.  

**E.** Section showing absent colocalization of 5-HT$_{1A}$R and vGLUT2, a marker for glutamatergic terminals. Scale 20 µm.  

**F.** 5-HT$_{1A}$R immunoreactivity in close apposition to GAD67-identified GABAergic cell terminals (F1), and colocalization with GABAergic cell bodies (F2, arrows). Note the absent colocalization of 5-HT$_{1A}$R and GAD67-negative cells (arrow heads). Scale 20 µm.  

**Figure 2: Activation of 5-HT$_{1A}$Rs decreases phrenic motor output.**

**A.** Representative example of pulsatile arterial pressure (AP; red), mean arterial pressure (light red trace superimposed on AP), heart rate (HR; blue), integrated splanchnic sympathetic nerve activity (∫SSNA; purple; mean SSNA in light purple) and integrated Phrenic nerve activity (∫PhrNA, green). Phrenic frequency and minute activity are shown in the bottom panels. Note the decrease in PhrNA amplitude and increase in PhrNA rate after 8-OH-DPAT injection (arrow), resulting in an overall decrease of minute PhrNA. Open box depicts the point of the ‘5 minute’-measurement used for
B (25 sec. average). Closed box indicates time of maximum change in minute PhrNA of this individual example. B. Group data (n = 5) describing the 8-OH-DPAT induced decrease in PhrNA amplitude, increase in rate and the resulting minute nerve activity (measured 5 min after injections; open box in A) when compared to aCSF control. C. Verification of 8-OH-DPAT injection sites. Horizontal slice of the medial-commissural nTS showing an example of a fluorescent bead injection (left) and the schematic summary of all injection sites (right). cnTS = commissural nTS, 4th V = fourth ventricle. * = p ≤ 0.05; ** = p < 0.01 vs. aCSF.

**Figure 3: 5-HT_{1A}-activation decreases afferent synaptic transmission and excitatory nTS network activity.** A. Representative example (average of 20 sweeps) of the reduction in TS-EPSC amplitude induced by the 5-HT_{1A} -agonist 8-OH-DPAT (10 µM). B. Individual data for the response to 8-OH-DPAT displayed as raw values (left) and normalized to baseline (right, baseline defined as “1”). The summary bars show the geometric mean with 95% CI. n = 10; * = p < 0.05. C. Representative example of the reduction in sEPSC event frequency induced by 8-OH-DPAT. D. Cumulative fraction of sEPSC intervals shows a significant shift (n = 11; p < 0.05, Kolmogorov-Smirnov two sample test) to longer intervals in the presence of 8-OH-DPAT. E. Individual data for the sEPSC frequency response to 8-OH-DPAT displayed as raw values (left) and normalized to baseline (right, baseline defined as “1”). Bars show geometric mean with 95% CI; n = 11; *** = p < 0.001.
**Figure 4: Block of 5-HT\textsubscript{1A}R increases excitatory nTS network activity.** Individual responses to 5-HT\textsubscript{1A}R blockade with the specific antagonist WAY100135 (10 µM) are displayed as raw values (left in each panel) and normalized to baseline (right, baseline defined as “1”) for TS-EPSC amplitude (A) and sEPSC event frequency (B). TS-EPSC amplitudes remain unaltered (n = 11; p = 0.97). Spontaneous event frequency, on the other hand, significantly increased indicating a tonic role of 5-HT\textsubscript{1A}R. Bars show geometric mean with 95% CI; n = 9; ** = p < 0.01.

**Figure 5: Activation of 5-HT\textsubscript{1A}Rs decreases inhibitory nTS network activity. A.** Two bipolar stimulating electrodes were used to evoke monosynaptic glutamatergic EPSCs in the recorded neuron from visceral afferents within the solitary tract (TS), and monosynaptic GABAergic IPSCs from the nTS network in the medial portion of the nTS. cnTS = commissural nTS, 4\textsuperscript{th} V = fourth ventricle, stim. = stimulating. **B.** Representative recordings showing TS-EPSCs and nTS-IPSCs upon stimulation. Note, due to our recording conditions glutamatergic and GABAergic currents are downward (negative) deflecting. Application of NBQX and AP5 eliminated all excitatory currents. At the end of an experiment IPSCs were verified by their blockade with GABA\textsuperspine (GBZ) in the recording solution. **C and D.** Individual responses to 5-HT\textsubscript{1A}R activation with 8-OH-DPAT are presented as raw values (left in each panel) and normalized to baseline (right, baseline defined as “1”) for nTS-IPSC amplitudes (C) and sIPSC event frequency (D). While the group nTS-IPSC amplitude did not change (n = 9; p = 0.11), spontaneous event frequency significantly decreased with activation of 5-HT\textsubscript{1A}R (n = 9; * = p < 0.05). Bars show geometric mean with 95% CI.
**Figure 6:** Block of 5-HT\textsubscript{1A}Rs tends to increase inhibitory nTS transmission. Individual responses to 5-HT\textsubscript{1A}R blockade with WAY100135 are displayed as raw values (left in each panel) and normalized to baseline (right, baseline defined as “1”) for nTS-IPSC amplitude (A) and sIPSC event frequency (B). Note, both nTS-IPSC amplitude and sIPSC event frequency remain statistically unaltered, although the majority of cells show a change with WAY100135 treatment towards increased values.

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**TABLE LEGENDS**

**Table 1:** Baseline cardiorespiratory variables. MAP = mean arterial pressure, HR = heart rate, SSNA = splanchnic sympathetic nerve activity, PhrNA = phrenic nerve activity. n = 5.

**Table 2:** Effects of 5-HT\textsubscript{1A}R agonist 8-OH-DPAT in the presence of various blockers. Group values summarizing the continued effect of 8-OH-DPAT during GABA\textsubscript{z}ine (GBZ, GABA\textsubscript{A} receptor blocker) and SB269970 (SB, 5-HT\textsubscript{7} receptor blocker). These effects were ablated by 5-HT\textsubscript{1A}R-antagonist WAY100135 (WAY). mEPSCs were unaltered in the presence of 8-OH-DPAT. Geometric means (lower-upper 95% confidence interval). TS = tractus solitarii, sEPSC = spontaneous EPSC, mEPSC = miniature EPSC.

**Table 3:** Altered inhibitory postsynaptic receptor function with 5-HT\textsubscript{1A}R activation. Group values depicting indicators for changes occurring pre- or postsynaptically. Note, activation of 5-
HT₁ARs significantly decreased mIPSC amplitude, implying a change at the postsynaptic receptor. mIPSC = miniature IPSC. Geometric means (lower-upper 95% confidence interval).
A 5 mM 8-OH-DPAT

1 min

aCSF 1 mM 5 mM 8-OH-DPAT

Phr rate normalized to baseline

Phr amplitude normalized to baseline

min Phr activity normalized to baseline

B

HR [b.p.m.]

AP [mmHg]

SSNA [mV.s]

PnNA [mV.s]

Phr f [min^-1]

min PhrNA [mV.s.min^-1]

5 min maxΔ

C

250 µm

medial nTS

injection site

4th V

4th V

C

R

L

M

p = 0.087

* *
tractus solitarii (TS)
stim. electrode (nTS)
stim. electrode (TS)
recording electrode

A
stim. electrode (TS)
stim. electrode (nTS)
tractus solitarii (TS)
recording electrode

B
TS-stimulation
nTS-stimulation

C
during NBQX & AP5

D
during NBQX & AP5

8-OH-DPATbaseline

normalized to baseline

0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6

0 5 10 15 20 25

0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6

0 5 10 15 20 25
A
in the presence of NBQX & AP5

nTS-IPSC amplitude [pA]

baseline
WAY100135

normalized to baseline

0

100

150

B
in the presence of NBQX & AP5

sIPSC event frequency [Hz]

baseline
WAY100135

normalized to baseline

0

1

2

3

4

5
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