5HT$_3$ Agonists Activate Catecholamine Neurons in The Solitary Tract Nucleus of the brainstem

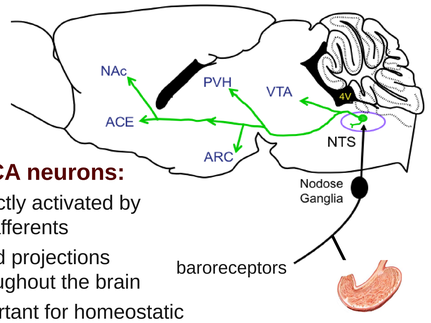
Brandon L. Roberts, Ran Ji Cui, Huan Zhao, Xiaojun Li, Mingyan Zhu, Suzanne M. Appleyard

Programs in Neuroscience, Department of VCAPP, Washington State University, Pullman, WA

Introduction

The solitary tract nucleus (NTS) broadly impacts homeostatic regulation as it is the primary site through which visceral afferent information concerning cardiovascular, respiratory and gastrointestinal systems enters the brain (Andresen & Kunze, 1994; Saper, 2002). The A2/ C2 group of catecholamine (CA) neurons lie within the NTS and are ideally situated to co-ordinate afferent signaling to multiple brain regions through their extensive projections, including to the hypothalamus, amygdala, nucleus accumbens and other brainstem nuclei (Petrov et al, 1993; Riche et al, 1990; Travagli et al, 2006; Ueta et al, 2000; Wang et al, 1992). Release of norepinephrine and epinephrine at these target nuclei can affect a broad number of behaviors, including stress, anxiety, reward, food intake and cardiovascular function (Cole & Sawchenko, 2002; Leibowitz et al, 1988; Smith & Aston-Jones, 2008) and A2/ C2 catecholamine neurons have been implicated in the regulation of these functions (Itoh & Bunag, 1993; Kubo et al, 1990; Olson, et al., 2006; Rinaman, 2011; Simon et al, 1985).

Serotonin (5-HT) is a biogenic amine synthesized both in the CNS and the enteric nervous system. Serotonin is most commonly associated with influencing mood and anxiety, but has also been shown to be important for the control of a broad range of functions, including food intake and cardiovascular function. Depletion of brain serotonin produces hyperphagia and weight gain and drugs that increase serotonin levels decrease food intake (for review see Lam et al., 2011). Serotonin receptors are expressed throughout the NTS, including 5-HT$_3$ receptors localized on incoming sensory afferent terminals (Leslie et al., 1990; Pratt and Bowery, 1989). The NTS receives inputs from the raphe and serotoninergic terminals make contacts with CA neurons (Pickel et al., 1984). However, the cellular effects of serotonin on NTS-CA neurons are not well understood. Hindbrain 5-HT$_3$ receptors participate in the control of meal size and CCK-induced satiation (Hayes & Covasa, 2006) and 5-HT$_3$ antagonists are used clinically to alleviate nausea (Marty, 1989). Furthermore, activation of 5-HT$_3$ receptors modulates glutamate release in the NTS (Wan & Browning, 2008; Takenaka et al., 2011). Given the proposed role of NTS-CA neurons in the control of food intake and other autonomic functions (Itoh & Bunag, 1993; Kubo et al, 1990; Rinaman, 2011; Simon et al, 1985), the goal of these studies were to determine what effects serotonin has on NTS-CA neurons, which receptor mediates these effects and what the underlying cellular mechanisms are.



NTS-CA neurons:

1. Directly activated by ST afferents
2. Send projections throughout the brain
3. Important for homeostatic regulation

Materials and Methods

NTS Slice. Horizontal medullary slices from the TH-EGFP mice (8-30 weeks) were cut to preserve medial NTS (mNTS) with the solitary tract (ST). This allowed us to isolate electrical stimuli to ST by placing a small (100µm) concentric bipolar electrode 1-3mm from the mNTS. Whole cell recordings were made using an external bath solution containing: (mM) 125 NaCl, 3 KCl, 1.2 KH2PO4, 1.2 MgSO4, NaHCO3, 10 Dextrose, 2 CaCl2, bubbled with 95% O2 / 5% CO2 30-34°C; pH=7.3, 300-310 mOsm. Inernal recording solution contained: (mM) 10 NaCl, 125 KCl, 11 EGTA, 1 CaCl2, 2 MgCl2, 10 HEPES, pH=7.3, 295-300 mOsm. Neurons were recorded from NTS within 200 µm rostral or caudal from obex and medial to the ST. Patch electrodes, 3-5 MΩ, were guided to neurons using both fluorescence (FITC) and differential interference contrast (DIC) optics (Olympus BX51). Voltage clamp and current clamp recordings were made with an Axopatch 700B and pClamp 10 software (Axon Instruments). Only neurons not exceeding holding currents of 50 pA at VH= -60 mV for the 10 minute control period (input resistance > 120 MΩ) were studied further. Stimulation intensities were 2x threshold.

TH immunohistochemistry. Mice were anesthetized (2% tribromoethanol) and then perfused with 4% paraformaldehyde. After cryoprotection of the brainstem tissue with 20% sucrose, sequential sections were prepared using a cryostat. Sections were processed for immunofluorescence using standard techniques (Appleyard et al., 2005). Mouse monoclonal anti-TH (Millipore, Billerica, MA) was used at a final dilution of 1:1000 (v/v). After rinsing, sections were incubated in biotinylated horse anti-mouse/rabbit immunoglobulin-G Cy3 (1:200) (Jackson Immuno-research Laboratories, West Grove, PA). Sections were mounted in rostral-caudal order and high resolution confocal images were acquired using an Olympus IX81 DSU spinning disk confocal microscope. Co-localization was then counted throughout the extent of the NTS and DMNV, including the co-localization in specific NTS sub nuclei.

Statistics. All data are presented as means ± SEM. Differences in drug effects were tested by repeated measured ANOVA, using turkey's post-hoc analysis. Differences were considered statistically significant for p-values < 0.05 unless otherwise stated.

Mice. TH-EGFP mice were on a C57Bl/6J background. Transgenic mice were housed on a 14hr light/ 10hr dark cycle at ambient temperature in the Department of Comparative Medicine Mouse Facility. Mouse chow (PM Feed Inc. St Louis, MO) and water were provided *ad libitum*. Genotyping and breeding of mice were as described previously (Appleyard et el., 2007). All animal procedures were conducted with the approval of the Institutional Animal Care and Use Committee at WSU in accordance with the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals (PHS Policy) and the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Guide).

Drugs. All drugs were obtained from Tocris

5-HT increases the frequency of sEPSCs in TH-EGFP neurons

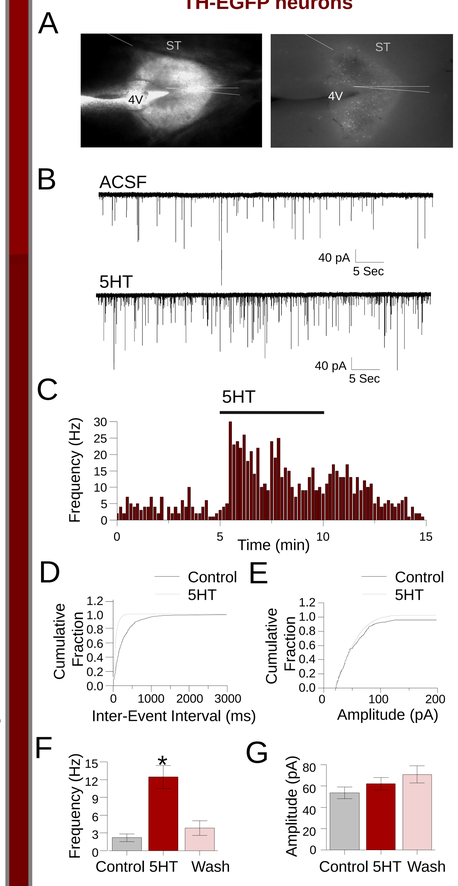


Figure 1 (**A**) Visualization of NTS brain slice taken from a TH-EGFP mouse using DIC and fluorescence. (**B**) Representative trace of 5-HT increasing spontaneous glutamate release in TH-EGFP neurons. (**C**) Average frequency binned into 10 second periods from a cell treated with 5HT. Cumulative fraction of inter-event interval (**D**) and amplitudes **(E)** during baseline and 5-HT treatment in a representative cell. Average effect of 5-HT on frequency (**F**) and amplitude **(G)** of sEPSCs in TH-EGFP neurons (n= 6; * p< 0.05).

5-HT effect mediated by the 5-HT$_3$ receptor

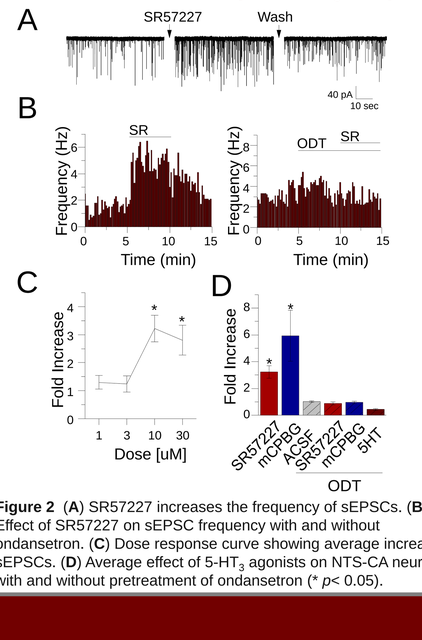


Figure 2 (**A**) SR57227 increases the frequency of sEPSCs. (**B**) Effect of SR57227 on sEPSC frequency with and without ondansetron. (**C**) Dose response curve showing average increase of sEPSCs. (**D**) Average effect of 5-HT$_3$ agonists on NTS-CA neurons with and without pretreatment of ondansetron (* p< 0.05).

SR57227 increases the firing rate of TH-EGFP neurons

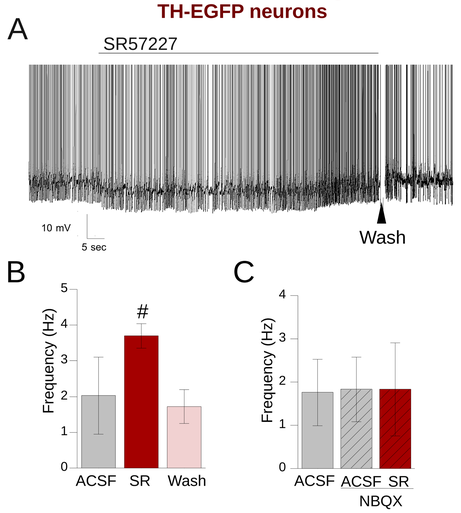


Figure 3 (A) Representative trace showing the effect of SR57227 on action potential firing rate in NTS-CA neurons. (B) Average increase of the action potential firing rate after SR57227 treatment on NTS-CA neurons (n= 7). (C) NBQX, an ionotropic non-NMDA glutamate receptor antagonist, blocks the effect of SR57227 on NTS-CA neurons (n= 6, # p< 0.01).

5HT$_3$ agonists inhibit a minority of TH-EGFP negative neurons

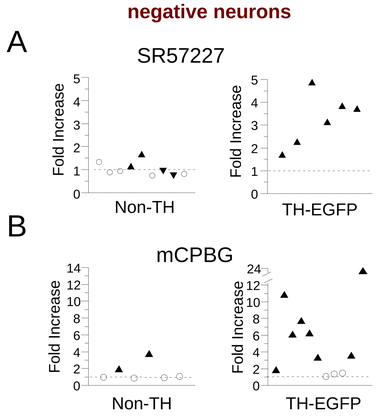


Figure 4 (**A**) SR57227 has a limited effect on the frequency of sEPSCs in TH negative neurons (n= 9), but greatly increases the effect in TH positive neurons (n= 6). (**B**) mCPBG has a very limited effect on the frequency of sEPSCs in TH negative neurons (n= 6), but has a robust effect in TH positive neurons (n= 11) **O**-*not significant;*▲-*significant increase;* ▼- *significant decrease* (KS test; p< 0.05).

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

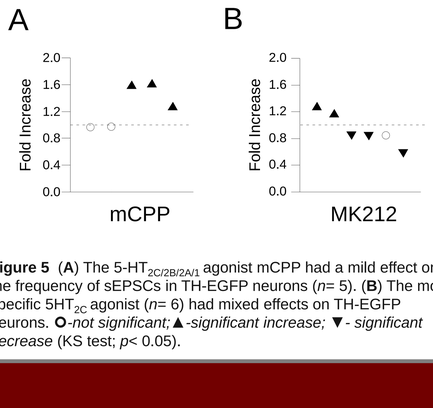


Figure 5 (**A**) The 5-HT$_{2C/2B/2A/1}$ agonist mCPP had a mild effect on the frequency of sEPSCs in TH-EGFP neurons (n= 5). (**B**) The more specific 5HT$_{2C}$ agonist (n= 6) had mixed effects on TH-EGFP neurons. **O**-*not significant;*▲-*significant increase;* ▼- *significant decrease* (KS test; p< 0.05).

5-HT$_3$ agonists decrease the amplitude of ST-EPSCs in TH-EGFP neurons

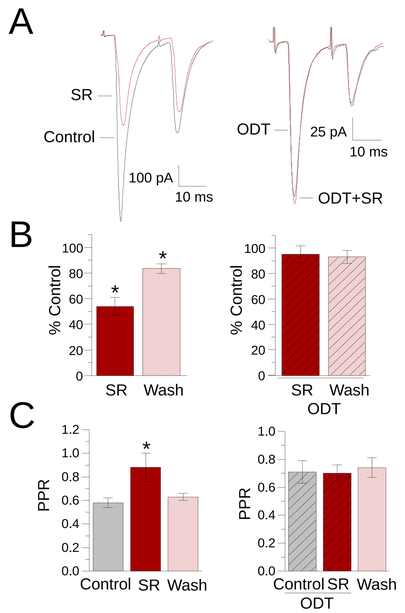


Figure 6 (A) SR57227 significantly decreases the amplitude of ST-EPSCs in TH-EGFP neurons, an effect blocked by pretreatment of ondansetron. (B) Amplitude of ST-EPSCs with respect to control in the presence of SR57227 with and without pretreatment of ondansetron. (C) SR57227 increases the paired pulse ratio (PPR) in TH-EGFP neurons, an effect blocked by ondansetron (n= 12, *p< 0.05).

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

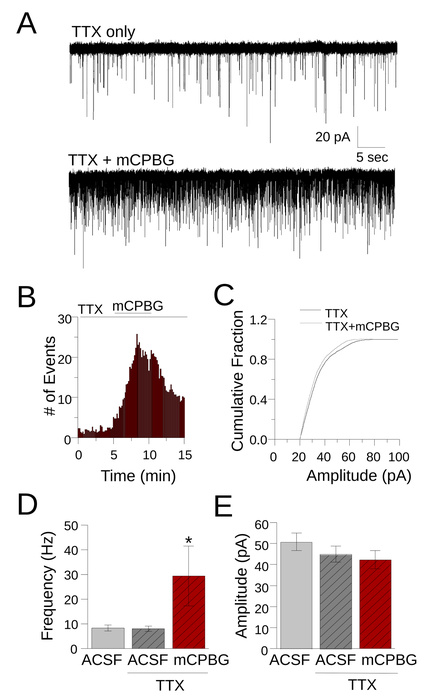


Figure 7: (**A**) Representative trace showing pretreatment of TTX does not block the effect of mCPBG on NTS-CA neurons. (**B**) Number of events broken into 10 second bins showing the effect of mCPBG on sEPSCs after TTX pretreatment. (**C**) Cumulative fraction showing no effect on amplitude. (**D**) Average frequency of mCPBG with TTX pretreatment. (**E**) Average effect of mCPBG on amplitude after TTX pretreatment (n= 5, *p< 0.05).

SR57227 increases cFos activation in NTS TH positive neurons *in vivo*

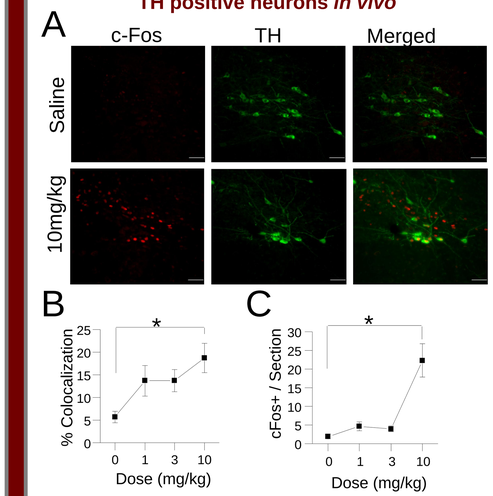
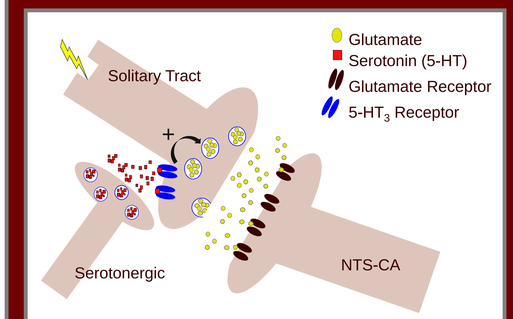


Figure 8 (**A**) Representative images showing cFos and TH staining 90 minutes after saline or 10mg/kg SR57227 i.p injections. (**B**) Percent colocalization of cFos and TH positive neurons. (**C**) Average cFos expression per section (n= 56, * p< 0.05).

Summary

- Serotonin increases the frequency of spontaneous glutamate inputs onto TH-EGFP neurons.
- 5-HT$_3$ agonists mimic the effects of 5-HT and the 5-HT$_3$ antagonist ondansetron blocks 5-HT's effects.
- The 5-HT$_3$ agonist SR57227 increases action potential firing rate in TH-EGFP neurons, an effect blocked by the glutamate receptor antagonist NBQX.
- The 5-HT$_3$ agonist mCPBG increased the frequency, but not amplitude of miniature EPSCs suggesting a presynaptic mechanism.
- 5-HT$_3$ agonists inhibit a minority of EGFP negative neurons showing a preferential effect on TH-EGFP neurons
- Other 5-HT agonists have more subtle and mixed effects on glutamate inputs onto TH-EGFP neurons suggesting 5-HT$_3$ receptors mediate the predominant serotonin drive.
- 5-HT$_3$ agonists decrease the amplitude of solitary tract ST-EPSCs in TH-EGFP neurons, potentially due to depleted presynaptic glutamate similar to the effect of capsaicin.
- SR57227 increases cFos expression in TH positive neurons in the NTS *in vivo*



Acknowledgements

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