Mechanisms of 5-HT$_{1A}$ receptor-mediated transmission in dorsal raphe serotonin neurons.

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Key points summary:

In the dorsal raphe nucleus, it is known that serotonin release activates metabotropic 5-HT_{1A} autoreceptors located on serotonin neurons that leads to an inhibition of firing through the activation of G-protein coupled inwardly rectifying potassium channels.

We found that in mouse brain slices evoked serotonin release produced a 5-HT_{1A} receptor mediated inhibitory post-synaptic current (IPSC) that resulted in only a transient pause in firing.

While spillover activation of receptors contributed to evoked IPSCs, serotonin reuptake transporters prevented pooling of serotonin in the extrasynaptic space from activating 5-HT_{1A} IPSCs.

As a result, the decay of 5-HT_{1A}-IPSCs was independent of the intensity of stimulation or the probability of transmitter release.

These results indicate that evoked serotonin transmission in the dorsal raphe nucleus mediated by metabotropic 5-HT_{1A} autoreceptors may occur via point-to-point synapses rather than by paracrine mechanisms.

Abstract

In the dorsal raphe nucleus (DRN), feedback activation by $G\alpha_{i/o}$-coupled 5-HT_{1A} autoreceptors reduces the excitability of serotoninergic neurons, which decreases serotonin release both locally within the DRN as well as in projection regions. Serotonin transmission

within the DRN is thought to occur via transmitter spillover and paracrine activation of extrasynaptic receptors. Here, we tested the volume transmission hypothesis in mouse DRN brain slices by recording 5-HT_{1A} receptor-mediated inhibitory post-synaptic currents (5-HT_{1A} IPSCs) generated by the activation of G-protein coupled inwardly rectifying potassium channels (GIRKs). We found that in the DRN of ePET1-EYFP mice, which selectively express eYFP in serontonergic neurons, the local release of serotonin generated 5-HT_{1A} IPSCs in serotonin neurons that rose and fell within a second. The transient activation of 5-HT_{1A} autoreceptors resulted in brief pauses in neuron firing that did not alter the overall firing rate. The duration of 5-HT_{1A} IPSCs was primarily shaped by receptor deactivation due to clearance via serotonin reuptake transporters. Slowing diffusion with dextran prolonged the rise and reduced the amplitude the IPSCs and the effects were potentiated when uptake was inhibited. By examining the decay kinetics of IPSCs, we found that while spillover may allow for the activation of extrasynaptic receptors, efficient uptake by serotonin reuptake transporters (SERTs) prevented the pooling of serotonin from prolonging the duration of transmission when multiple inputs were active. Together the results suggest that the activation of 5-HT_{1A} receptors in the DRN results from the local release of serotonin rather than the extended diffusion throughout the extracellular space.

Abbreviations:

DRN – Dorsal Raphe Nucleus

5-HT – Serotonin

IPSC – Inhibitory Post-Synaptic Current

sIPSC – Spontaneous Inhibitory Post-Synaptic Current

GIRK – G-protein Coupled, Inwardly Rectifying Potassium Channel

SERT – Serotonin Reuptake Transporter

Introduction

The dorsal raphe nucleus (DRN) is a major source of ascending serotonergic innervation to the forebrain and limbic regions (Jacobs and Azmitia, 1992, Michelsen et al., 2008). Serotonin neurons of the DRN are implicated in multiple behavioral and cognitive functions (Jacobs and Azmitia, 1992, Lucki, 1998), and dysfunction in serotonin signaling is thought to underlie mood disorders and depression (Michelsen et al., 2008). In addition to release in projection regions, vesicular serotonin (5- hydroxytryptamine; 5-HT) release occurs locally in the DRN at somatic (Kaushalya et al., 2008, Colgan et al., 2009), dendritic (de Kock et al., 2006, Colgan et al., 2012), and axonal sites (Bruns et al., 2000) where it modulates the activity of DRN neurons (Pineyro and Blier, 1999, Adell et al., 2002, Michelsen et al., 2008, Andrade et al., 2015). Release of serotonin can activate inhibitory $G\alpha_{i/o}$-coupled $5\text{-}HT_{1A}$ autoreceptors that inhibit serotonergic neuron impulse activity through the opening of inwardly rectifying potassium (GIRK) channels (Aghajanian and Lakoski, 1984, De Vivo and Maayani, 1986, Pan et al., 1989, Pan and Williams, 1989, Bayliss et al., 1997, Katayama et al., 1997, Gantz et al., 2015) and inhibition of voltage dependent Ca^{2+} channels (Penington and Kelly, 1990, Penington et al., 1991). By suppressing pacemaker firing, $5\text{-}HT_{1A}$ autoreceptors regulate serotonin levels both locally in the dorsal raphe and in terminal projection regions (Aghajanian and Lakoski, 1984, Pan et al., 1989, Pan and Williams, 1989, Hjorth and Sharp, 1991, Portas et al., 1996, Adell et al., 2002, Michelsen et al., 2008), thereby influencing behaviors such as anxiety and stress (Richardson-Jones et al., 2010).

Ultrastructural studies have found somatodendritic 5-HT_{1A} receptors at both synaptic (Kia et al., 1996) and extrasynaptic sites (Kia et al., 1996, Riad et al., 2000). As 5-HT_{1A} receptors can be located at extrasynaptic sites, it has been thought that serotonergic transmission through these receptors in the DRN occurs by their paracrine activation by low concentrations of transmitter that result from spillover of transmitter out from the synapse (Bunin and Wightman, 1999). In support of this extended form spillover, known as volume transmission, electrochemical studies have found that evoked release in the DRN drives sub-micromolar increases in the concentration of 5-HT throughout the extracellular space (Bunin et al., 1998, Bunin and Wightman, 1998, Jennings, 2013). While the increase in extracellular serotonin has been hypothesized to be sufficient to activate 5-HT_{1A} autoreceptors up to several microns away from release sites (Bunin and Wightman, 1999), the role of volume transmission in gating activation of these autoreceptors in the DRN has yet to be directly addressed.

While 5-HT_{1A}-receptors in the DRN *in vivo* are thought to tonically modulate firing rates (Fornal et al., 1996, Mundey et al., 1996, Hajos et al., 2001, Haddjeri et al., 2004), electrophysiological studies using brain slices have found in contrast that stimulation evokes only 5-HT_{1A} receptor-mediated hyperpolarizations in DRN neurons that cause only brief pauses in neuron firing (Williams et al., 1988, Pan et al., 1989, Morikawa et al., 2000, Levitt et al., 2013). To examine the mechanisms that underlie the synaptic activation of 5-HT_{1A} receptors we recorded from identified serotonergic neurons in the dorsal raphe using ePET1-eYFP mice. Through recording of 5-HT_{1A}-receptor mediated IPSCs, we found that evoked serotonin release generated 5-HT_{1A}-receptor mediated currents that rose and fell in a second. This resulted in transient pauses in firing without affecting overall firing rates. While spillover likely allowed for the activation of some extrasynaptic receptors, serotonin reuptake transporters prevented transmitter pooling from adjacent release sites from extending the duration of transmission. These findings suggest that serotonergic transmission in the DRN occurs by the local activation of 5-HT_{1A} autoreceptors near the sites of transmitter release with limited cross talk between synapses.

Methods

Ethical Approval. All procedures were performed in accordance with United States law on the use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of Case Western Reserve University, Cleveland, OH.

Slice preparation and visualization. Mice were deeply anesthetized with isoflurane and coronal brain slices (220 μM) containing the dorsal raphe nuclei (DRN) were obtained from 4 – 8 week old male and female ePET1-eYFP mice (Scott et al., 2005). ePET1-eYFP mice were donated by Dr. Evan Deneris at Case Western Reserve University. Brain slices were cut using a vibratome (Leica) in ice-cold cutting solution containing (in mM) 75 NaCl, 2.5 KCl, 6 $MgCl_2$, 0.1 $CaCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 2.5 $_{D}$-glucose, and 50 sucrose continuously bubbled with 95% O_2 and 5% CO_2. After cutting, slices were transferred to artificial CSF (ACSF) containing (in mM) 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 1.2 NaH_2PO_4, 21.4 $NaHCO_3$, and 11.1 $_{D}$-glucose bubbled with 95% O_2 and 5% CO_2 and incubated at 35° C for at least 45 minutes prior to use. MK-801 (10 μM) was included during the incubation to block NMDA receptors. After incubation, slices were transferred to a recording chamber and constantly perfused at 2 ml/min with oxygenated ACSF warmed to 34 ± 2° C. Slices were visualized with a BX51WI microscope (Olympus) with custom-built infrared gradient contrast optics. eYFP was visualized by fluorescence using a custom built cyan LED (505 nm, Luxeon Star) with a 515 nm long pass dicroic mirror and a 535/30m emission filter (Chroma).

Immunohistochemistry. 8-week old ePet-EYFP mice were deeply anesthetized with avertin (44 mM tribromoethanol, 2.5% tert-amyl alcohol) 20 ml/kg. Mice were transcardially perfused with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4), followed by a 20 minute perfusion with 4% paraformaldehyde (Electron Microscopy Sciences) in PBS. Brains were post-fixed for 2 hours in 4% paraformaldehyde. Fixed brains were sectioned coronally in 20-μm sections using a freezing microtome (American Optical Corp.) and mounted on Superfrost plus slides (Fisher), then air-dried. Sections were permeabilized with 0.3% Triton X-100 in PBS (PBS-T) for 15 minutes and blocked in 5% normal goat serum for 1 hour at room temperature. Slides were incubated with 1:1000 rabbit anti-GFP antibodies (Invitrogen) in PBS-T overnight at 4° C. Slides were incubated with 1:500 goat anti-rabbit alexa fluor 488 (Invitrogen) in PBS-T for 2 hours at room temperature. Slides were coverslipped using prolong gold (Invitrogen). Fluorescent images were obtained using an Olympus BX51 compound microscope (Olympus) with a 10x UPlanFl, NA 0.3 lens with a SPOT RT color digital camera (Diagnostic Instruments, Sterling Heights, MI). All images were processed using ImageJ.

Electrophysiology. Whole-cell voltage-clamp recordings (V_h = - 60 mV) were made using an Axopatch 200B amplifier (Molecular Devices). Patch pipettes (1.5 – 2.5 MΩ) were pulled from borosilicate glass (World Precision Instruments). The pipette intracellular solution contained (in mM): 115 K-methylsulphate, 20 NaCl, 1.5 $MgCl_2$, 10 K-HEPES, 10 BAPTA-tetrapotassium, 2 ATP, 0.3 GTP, and 6 sodium phosphocreatine, pH 7.4, 275 mOSM. Data were acquired using an ITC-18 interface (Instrutech) and Axograph X (Axograph Scientific) at 10 kHz and filtered to 2 kHz. Series resistance was not compensated and recordings were discarded if the access resistance rose above 15 MΩ. In the case of experiments with reduced extracellular calcium, $MgCl_2$ was substituted to maintain a constant divalent ion concentration. All drugs were applied by bath perfusion. Recordings were made in the dorsomedial portion of the DRN. Serotonergic neurons within the DRN were fluorescently identified by eYFP expression driven by the PET1 enhancer (Scott et al., 2005). In some experiments, dextran (35,000 – 50,000 M.W.) was used to slow diffusion via macro-molecular overcrowding. Dextran was added to ACSF (5% weight by volume) and bath perfused after achieving stable recordings. Bath temperature was monitored to ensure that it did not change by more than 1° C.

Cell attached recordings were made with internal solution containing (in mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 1.2 NaH_2PO_4. Data were acquired in voltage-clamp (0 mV) at 10 kHz and filtered to 5 kHz. Seals between the recording electrode and neuron ranged from 5 – 12 MΩ. Phenylephrine (3 μM) was included in the bath solution to mimic *in vivo* excitatory drive (Vandermaelen and Aghajanian, 1983).

Stimulation and iontophoresis. Serotonin release was evoked with an extracellular, ACSF-filled monopolar glass electrode. The stimulating electrode was placed 50-100 μm away from the cell body of the recorded neuron. A single stimulation pulse (20 – 60 μA, 0.5 ms) was used to drive serotonin release. To prevent glutamate- and GABA-mediated synaptic responses, recordings were made in the presence of DNQX (10 μM), MK801 (10 μM), picrotoxin (100 μM) and CGP55845 (200 nM). BAPTA (10 mM) in the internal pipette solution was used to prevent intracellular calcium signaling. In traces illustrating electric stimulation, the stimulation artifact was blanked for presentation. For the iontophoretic application, serotonin (100 mM) was ejected as a cation (160 nA) for 2000 ms using an Iontophoresis Generator (Dagan) from thin-walled iontophoretic electrodes placed ~ 30 μm from the cell. A negative retention current of 15-30 nA was applied to prevent leakage of serotonin.

Materials. Picrotoxin was from Abcam. DNQX, MK-801, CGP 55845, SB 216641 hydrochloride, WAY 100635 maleate, citalopram hydrobromide, R-(-)-phenylephrine hydrochloride, forskolin, DPCPX, and serotonin hydrochloride were from Tocris Bioscience. K-methylsulphate was from Acros Organics. BAPTA was from Invitrogen. D-glucose and HEPES sodium salt were from Sigma-Aldrich. Dextran (35,000 – 50,000 M.W.) was from MP Biomedicals. All other chemicals were from Fisher Scientific.

Statistics and Analysis. All data are shown as mean ± SEM. Statistical significance ($p < 0.05$) was assessed by paired and unpaired Student's t-tests, ANOVAs, and Pearson's correlations as noted (InStat 3.0). Decay kinetics of 5-HT_{1A} IPSCs were fit with a single exponential using a Simplex algorithm optimized by the sum of squared errors in Axograph X (Axograph Scientific). Firing rates in cell-attached experiments were calculated from the average inter-spike intervals.

Results

Evoked 5-HT_{1A}-IPSCs mediate a transient pause in DRN serotonergic neuron firing.

Voltage clamp recordings were made from $eYFP^+$ serotonergic neurons located in the dorsomedial DRN in brain slices obtained from ePET1-eYFP mice (Fig. 1A). A single stimulation was used to elicit 5-HT_{1A} receptor mediated inhibitory post-synaptic currents (IPSCs). In all neurons tested, IPSCs were abolished by the 5-HT_{1A} receptor antagonist, WAY 100635, (200 nM, 97 ± 1% reduction in amplitude, n = 5, $p < 0.01$, Student's paired t-test; Fig. 1B), and IPSCs were reliably observed in >95% of serotonin neurons (30/31 serotonergic neurons; 85 ± 10 pA, n = 30, Fig. 1B). IPSCs were eliminated by TTX (200 nM, 98 ± 1% reduction, n = 3, $p < 0.001$, Student's paired t-test), calcium-free ACSF (n = 3, 97 ± 2% reduction, $p < 0.001$, Student's paired t-test), and barium (200 μM, 81 ± 1% reduction, n = 4 $p < 0.01$, Student's paired t-test)(Fig. 1C). Thus, recorded IPSCs resulted from vesicular serotonin release acting on 5-HT_{1A} receptors to mediate a potassium conductance, likely via G-protein coupled, inwardly rectifying potassium (GIRK) channels (Pan et al., 1989). Evoked IPSCs rose and fell within approximately 1 s. IPSCs activated in 168 ± 7 ms (10-90% rise time, n = 30) following a lag of 75 ± 3 ms (10% onset, n =

30), and decayed with a time constant of 0.44 ± 0.3 sec (n = 30). This resulted in an overall duration of 0.58 ± 0.4 sec measured at half maximal amplitude (n = 30, Fig. 1D).

To examine the consequence of evoked serotonin release on excitability, cell-attached recordings of DRN firing rates were made from serotonin neurons. In vivo, DRN neurons fire in a regular pattern at 0.5 - 3 Hz. To mimic the excitatory noradrenergic tone that facilitates firing (Baraban and Aghajanian, 1981, Haddjeri et al., 2004), phenylephrine (3 μM) was added to the bath to maintain regular pacemaker firing (Vandermaelen and Aghajanian, 1983). Single stimulation evoked serotonin release induced a 2.56 ± 0.41 second pause in firing (n = 10) that was blocked by the 5-HT_{1A} receptor antagonist WAY 100635 (200 nM; n = 5; Fig. 1E). Evoked serotonin release did not cause a lasting depression of firing, as basal and post-pause firing rates were similar (n = 5; p = 0.33; Fig. 1E, F). Baseline-firing rates did not correlate with the duration of the evoked pause (Fig. 1G). Thus, serotonin release is capable of affecting DRN activity by generating transient pauses in serotonin neuron firing.

Receptor deactivation and not receptor desensitization terminated evoked 5-HT_{1A}-receptor signaling.

At many synapses, receptor desensitization can speed the decay of receptor-mediated currents to shorten the duration of signaling (Trussell et al., 1993). To determine whether desensitization of 5-HT_{1A} receptors regulates the duration of the IPSC, a paired-pulse protocol with an interstimulus interval of 4 s was used (Fig. 2A). Evoking IPSCs four seconds apart resulted in a 73 ± 4% reduction in the amplitude of the second IPSC (n = 7; p < 0.001). The paired-pulse depression was partially attenuated in the presence of the 5-HT_{1B} antagonist, SB 216641 (1 μM; 58 ± 8% reduction in amplitude of the second IPSC; n = 4; p = 0.02 vs. 1st IPSC; p = 0.04 vs. reduction in control; Fig. 2C), confirming that presynaptic activation by 5-HT_{1B} receptors by the first stimulation inhibited the subsequent release of serotonin (Morikawa et al., 2000). SB 216641 (1 μM) had no effect on the amplitude of unpaired IPSCs (p = 0.78 amplitude, n = 14), indicating that pre-synaptic 5-HT_{1B}-receptors were not tonically active in slices (Morikawa et al., 2000). To examine whether some depression was post-synaptic in origin, 5-HT was exogenously applied using paired iontophoretic applications (Fig. 2B). The amplitude and decay kinetics of the two resulting currents generated 4 s apart were similar (amplitude: 1st iontophoretic current: 113 ± 14 pA, 2nd iontophoretic current: 123 ± 16 pA, n = 5; p = 0.69, Fig. 2C; τ_{Decay}: 1st iontophoretic current: 0.57 ± 0.06 s, 2nd iontophoretic current: 0.61 ± 0.06 s, n = 5;

p = 0.78), suggesting that the paired-pulse depression of IPSCs primarily occurred due to pre-synaptic mechanisms. When serotonin was iontophoretically applied during the decay phase of the evoked 5-HT_{1A} IPSC (1 second following stimulation) (Fig 2D), the amplitude and kinetics of the resulting current were also identical to currents that were not preceded by an IPSC (amplitude, during IPSC: 120 ± 21 pA, without IPSC: 124 ± 21 pA, n = 5, p = 0.89; τ_{Decay}: during IPSC: 0.80 ± 0.22 s, without IPSC: 0.78 ± 0.21 s, n = 5, p = 0.96; Fig. 2E). The fact that exogenously serotonin evokes similar currents when applied on its own or during the decay of IPSCs suggests that limited desensitization of 5-HT_{1A} receptors occurred during the decay phase of the IPSC. Although iontophoresis and stimulation may recruit different receptor pools, these results suggest that receptor deactivation not desensitization controls the duration of serotonin transmission in the raphe.

Clearance via serotonin reuptake transporters limited serotonin spillover and was the primary mechanism driving 5-HT_{1A}-receptor deactivation.

Clearance of monoamines in the extracellular space is achieved by a combination of diffusion and uptake by transporters. The relative contributions of each vary widely across monoamine synapses (Courtney and Ford, 2014). In the DRN, it has been hypothesized that due to the limited role of uptake, diffusion of serotonin may extend the duration of serotonin transmission by allowing activation of distal extra synaptic 5-HT_{1A} receptors through spillover transmission (Bunin and Wightman, 1999, Jennings, 2013). To determine the role of serotonin reuptake transporters (SERTs) in regulating 5-HT_{1A} activation, the selective serotonin reuptake inhibitor (SSRI) citalopram (Celexa, 200 nM) was bath applied while recording IPSCs. Citalopram significantly reduced the amplitude of 5-HT_{1A} IPSCs (39 ± 8% inhibition, n = 5, p < 0.05, Fig. 3A-C). This was likely due to citalopram-induced tonic activity of pre-synaptic 5-HT_{1B} receptors (Morikawa et al., 2000), as combining the 5-HT_{1B} antagonist, SB216641 (1 μM), with citalopram instead led to a stable increase in IPSC amplitude (ctrl: 134 ± 16 pA; citalopram + SB216641: 210 ± 33 pA, n = 5, p = 0.03, Fig. 3A-C). Bath application of citalopram (200 nM) in both the absence and presence of SB 216641 (1 μM) greatly prolonged the duration of 5-HT_{1A} IPSCs. Compared to controls, IPSCs recorded in citalopram had delayed peak times (citalopram: 134 ± 22% increase vs. controls, n = 5, p = 0.003; citalopram + SB: 189 ± 29% increase, n = 5, p = 0.047; p = 0.292 citalopram vs. citalopram + SB, Fig. 3D) and were significantly slower to decay (τ_{Decay}; citalopram: 963 ± 120% increase vs. controls, n = 5, p = 0.001; citalopram + SB: 830 ±

250% increase, n = 5, p = 0.005; p = 0.6334 citalopram vs. citalopram + SB, Fig. 3D). As a result, citalopram induced a 5-fold increase IPSC duration (half width) with or without SB 216641 (p = 0.98), indicating that clearance of serotonin clearance by reuptake transporters limits the duration of transmission.

To examine the role of diffusion in receptor activation dextran (5 %, 40 kDa) was applied to slow diffusion via macro-molecular overcrowding. Stable IPSCs were recorded in control ACSF and then dextran (5 %, 40 kDa) was bath applied (Courtney et al., 2014). After 10 minutes of perfusion, dextran reduced the amplitude (54 ± 7% reduction; n = 6; p = 0.02), slowed the rate of rise of 5-HT_{1A} IPSCs (38 ± 14% increase in peak time; n = 6; p = 0.02) (Fig. 4A) but did not alter the decay kinetics of 5-HT_{1A} IPSCs (τ_{Decay}; control: 0.60 ± 0.13 sec, 5% dextran: 0.79 ± 0.08 sec; n = 6; p = 0.31, Student's paired t-test, Fig. 4B). As impairing diffusion limits the escape of serotonin from the synaptic cleft (Min et al., 1998), the reduction in amplitude and delay in time to peak in dextran indicates that spillover of serotonin likely led to the activation of extrasynaptic 5-HT_{1A} receptors which contributed to the peak amplitude of IPSCs (Min et al., 1998, Nielsen et al., 2004, Szabadics et al., 2007, Markwardt et al., 2009, Ford et al., 2010, Courtney and Ford, 2014). The activation of these extrasynaptic receptors by spillover was likely impaired in dextran, which may have resulted in the smaller amplitudes of IPSCs. In addition, dextran also hinders the diffusion-mediated clearance of transmitter, which can prolong the activation of post-synaptic receptors (Min et al., 1998, Markwardt et al., 2009). The lack of a pronounced effect of dextran on IPSC decay suggests that clearance via reuptake, rather than diffusion, is the primary mechanism for receptor deactivation.

Next, we examined the ability of reuptake transporters to limit serotonin diffusion. When in the continuous presence of the selective serotonin reuptake inhibitor (SSRI) citalopram (Celexa, 200 nM) and the pre-synaptic 5-HT_{1B} autoreceptor antagonist SB 216641 (1 μM), bath application of 5% dextran resulted in similar changes in the amplitude (72 ± 6% reduction in 5% dextran, n = 4, p = 0.04)(p = 0.10 vs. change in absence of citalopram) and peak time (44 ± 12% increase in 5% dextran, n=4, p = 0.03; p = 0.75 vs. change in absence of citalopram) of 5-HT_{1A} IPSCs. However, slowing diffusion while reuptake was impaired now significantly extended the duration of IPSCs (τ_{Decay}; control: 3.1 ± 0.6 sec, 5% dextran: 10.8 ± 2.9 sec; n = 4; p = 0.04; p = 0.004 vs. change in absence of citalopram; Fig. 4C - E). Thus when reuptake is inhibited, slowing diffusion now increased the duration of 5-HT_{1A} receptor activation, suggesting that diffusion became the primary clearance mechanism driving receptor deactivation.

Reuptake transporters prevent pooling and crosstalk from prolonging the duration of IPSCs.

Spillover from synaptic sites can result in extrasynaptic pooling and synaptic crosstalk (Otis and Mody, 1992, Isaacson et al., 1993, Balakrishnan et al., 2009, Courtney and Ford, 2014). As pooling allows for the duration of signaling to become dependent on the number of active release sites, greater amounts of transmitter release results in longer durations of receptor activation. While pooling likely accounts for the increases in extracellular levels of serotonin that can be detected by electrochemical approaches (Bunin and Wightman, 1998, Jennings, 2013), it is unclear if it plays a physiological role in the activation of post-synaptic 5-HT_{1A} receptors.

To determine the contribution of transmitter pooling to evoked serotonin transmission, we initially compared the amplitude and duration of IPSCs recorded from serotonin neurons across the DRN (Fig. 5A). We found that across neurons, decay time did not correlate with the amplitude of evoked IPSCs (n = 30, R^2 = 0.017, p = 0.54, Pearson's correlation, Fig. 5B), suggesting that variations in the amount of transmitter release did not alter the duration of 5-HT_{1A} receptor signaling. For a given cell, we next varied the intensity of stimulation used to evoke IPSCs. Decreasing the stimulation intensity reduced the amplitude of events (65 ± 2 % reduction, n = 7; p < 0.001; Student's paired t-test) but again did not alter the rate of decay (n = 7, p = 0.15, Student's paired t-test, Fig. 5C). Likewise, decreasing the probability of transmitter release by lowering the concentration of extracellular calcium from 2.5 mM to 1.0 mM similarly reduced the amplitude of synaptic currents (n = 8, p < 0.001; Student's paired t-test) with no effect on the decay time (n = 8, p = 0.99, Student's paired t-test, Fig. 5D). Because decreases in serotonin release did not result in shorter duration events, these results suggest that the time course of IPSCs under control conditions are not shaped by pooling of serotonin in the extracellular space.

In other regions, spillover of transmitter between synapses can be facilitated by blocking uptake (Barbour et al., 1994, Otis et al., 1996, Silver et al., 1996, Overstreet and Westbrook, 2003, Balakrishnan et al., 2009, Courtney and Ford, 2014). As blocking reuptake transporters enabled extended diffusion to prolong the decay of 5-HT_{1A} IPSCs (Fig. 3), we next examined whether blocking reuptake enhanced transmitter crosstalk and pooling. In the presence of citalopram (200 nM) and SB216641 (1 μM), increasing the strength of stimulation

not only increased the amplitude of IPSCs (n = 4, p = 0.01) but also prolonged the time course of decay (80 ± 26% increase, n = 4, p = 0.006; Fig. 5E). Thus efficient uptake of serotonin by transporters limited pooling in the extracellular space from extending the duration of IPSCs. This suggests that the post-synaptic activation of 5-HT_{1A} receptors may result from the local release of serotonin with limited spillover transmission, rather than extended diffusion away from sites of release resulting in transmitter pooling (Fig. 5F).

Spontaneous IPSCs occur with similar kinetics to evoked.

While making long recordings from serotonergic neurons in the DRN, we observed spontaneous IPSCs (sIPSCs) that occurred in 16/38 of neurons (42%; Fig 6A). The kinetics of sIPSCs were similar to evoked 5-HT_{1A} IPSCs (peak time: 346 ± 23 ms; τ_{decay}: 0.37 ± 0.03 sec, n = 27; Fig. 6B, D) and were prolonged in the presence of citalopram (200 nM, n = 10; p < 0.001; Fig. 6C, D). The low frequency of events that could be detected (0.2 ± 0.1 events per 10 minutes, n = 38 neurons) limited quantitative analysis of the underlying mechanisms of individual events. To increase the ability to detect events, phenylephrine (3 μM), forskolin (1 μM), DPCPX (500 nM), and citalopram (50 nM) were included in the bath solution. Activation of adenylyl cyclase by forskolin is known to increase evoked and spontaneous catecholamine release (Beckstead and Williams, 2007, Gantz et al., 2013) and DPCPX was used to prevent adenylyl cyclase from inducing adenosine A1-receptor activity. This pharmacological cocktail increased the frequency of spontaneous events (1.2 ± 0.3 events per 10 minutes, n = 15 neurons) with spontaneous events being observed in 13/15 neurons (87%; Fig. 6E). The apparent increase in spontaneous event frequency may be in part due to increased amplitude of resolvable events (28 ± 3% increase, n = 21 sIPSCs, p < 0.05; Fig. 6F). Bath application of the 5-HT_{1A} antagonist WAY 100635 (200 nM) eliminated sIPSCs (8/8 cells) and sIPSCs were never observed in recordings made in the continuous presence of WAY 100635 (200 nM, 9 cells; Fig 6E), indicating that spontaneous events were driven by 5-HT_{1A} receptor activation. While these observations cannot determine whether evoked and spontaneous IPSCs arose from similar or separate pools of pre-synaptic release sites, the existence of sIPSCs implies that serotonin release from some pre-synaptic sites was sufficient to independently activate post-synaptic receptors without requiring broad stimulation.

Discussion

In the present study, we examined the mechanisms regulating serotonergic transmission by 5-HT_{1A} receptors in the dorsal raphe. We found that stimulation evoked serotonin release generated 5-HT_{1A}-receptor mediated inhibitory currents that rose and fell within a second and paused basal firing without causing long-lasting depressions to firing rates. The transient nature of 5-HT_{1A}-receptor-mediated transmission was primarily driven by transmitter clearance via serotonin reuptake transporters and the ensuing deactivation of 5-HT_{1A} receptors. Reuptake transporters limited the spillover activation of distal, extrasynaptic receptors and prevented serotonin pooling in the extracellular space from prolonging the duration of 5-HT_{1A}-receptor activity via synaptic crosstalk. Taken together, these results suggest that 5-HT_{1A} receptor-mediated serotonin transmission in the DRN occurs through functionally independent synapses despite the observation of a limited amount of spillover.

Local feedback inhibition by inhibitory autoreceptors regulates cellular excitability at multiple monoamine synapses. In midbrain dopamine neurons, evoked and spontaneous vesicular dopamine release activates D2-receptor mediated IPSCs through GIRK channels (Beckstead et al., 2004, Gantz et al., 2013). Like axonal synapses in the striatum (Marcott et al., 2014), somatodendritic transmission in the midbrain is mediated by a high concentration of dopamine (Ford et al., 2009) that occurs in the absence of transmitter pooling and spillover (Ford et al., 2010, Courtney and Ford, 2014). This differs from somatodendritic transmission of noradrenaline in the locus coeruleus where less efficient uptake allows for low concentrations of noradrenaline to pool in the extracellular space when multiple inputs are synchronously active (Courtney and Ford, 2014). The lack of dopamine pooling in the ventral tegmental prevents crosstalk between synaptic sites to maintain independence between release sites (Courtney and Ford, 2014). In this study, we also found that the local release of serotonin in the DRN can activate synaptic 5-HT_{1A} receptors in the absence of pooling. This was likely true for both evoked and spontaneous release due to the similarity in the rise and decay kinetics. Unlike dopamine transmission, however, spillover allowed for the activation of some pool of extrasynaptic receptors. Serotonergic 5-HT_{1A} IPSCs were more variable cell to cell in their duration, and thus time course of inhibition, than was previously observed for midbrain dopamine transmission (Courtney and Ford, 2014). This increased variability may be due to the recruitment of extrasynaptic receptors in serotonin signaling. The lack of pooling at synaptic sites was attributed to efficient uptake by SERT. Like somatodendritic dopamine transmission (Courtney and Ford, 2014), blocking uptake extended the time course of IPSCs such that the

duration of transmission was now dependent upon the amount of serotonin released. How serotonin reuptake transporters both allowed for the spillover activation of extrasynaptic receptors yet prevented pooling and synaptic crosstalk remains unclear. One possibility may be that a non-uniform distribution of SERTs in DRN neurons around different release sites (Colgan et al., 2012) allows serotonin to spillover into the extrasynaptic space yet limits pooling between synapses.

Throughout these recordings we observed spontaneous IPSCs in roughly half of the neurons examined. The similarity in kinetics between these IPSCs and the finding that no events could be detected in the presence of 5-HT_{1A} antagonists suggests that these IPSCs could be the result of spontaneous release of serotonin leading to the activation of 5-HT_{1A} autoreceptors. Similar spontaneous release of dopamine as has been recently described at synapses in the substantia nigra (Gantz et al., 2013). It is unclear if the same pool of serotonergic vesicles mediates both evoked and spontaneous events. At ionotropic synapses, different populations of vesicles have been proposed to underlie spontaneous and evoked release (Ramirez and Kavalali, 2011, Kavalali, 2015). In the presence of phenylephrine which increases DRN firing to 2-3 Hz, the frequency of observable events was only once per ~ 10 min. While many events might be below the level of detection in our experiments, the low frequency of events suggests that these events did not result from background firing of DRN neurons. One possibility may be that these events could arise from dendritic sites, where serotonin release occurs in an action-potential independent manner (Colgan et al., 2012).

Vesicular serotonin release in the DRN occurs at axonal (Bruns et al., 2000), somatic (Kaushalya et al., 2008, Colgan et al., 2009), and dendritic sites (de Kock et al., 2006, Colgan et al., 2012). As evoked serotonin release underlying 5-HT_{1A} IPSCs is modulated by 5-HT_{1B} receptors (Fig. 2) (Morikawa et al., 2000), which are located only on axon terminals (Sari, 2004), axonal release likely contributes to the activation of the synaptic receptors that underlie the IPSC. It is not clear whether these axons originate locally from within the DRN or arise from other brain serotoninergic nuclei (Bang et al., 2012, Andrade et al., 2015). Cell body release sites lack defined pre-synaptic active zones (Colgan et al., 2009) making it unclear the extent to which somatic release participates in evoked IPSCs. While axonal and somatic transmission are dependent on action potentials (Bruns et al., 2000, Colgan et al., 2012), dendritic release is instead impulse-independent relying on local NMDA receptors and L-type calcium channels (de Kock et al., 2006, Colgan et al., 2012). Glutamate receptors were blocked in our experiments, making it less likely that dendritic release sites contributed to evoked 5-HT_{1A} IPSCs.

Recent studies have linked both tonic and transient inhibitory regulation of DRN serotonergic neurons to behaviors such as reward encoding (Ranade and Mainen, 2009, Cohen et al., 2015). During these behaviors, tonic and transient inhibition independently influenced DRN neurons and could be used to identify neuronal subpopulations (Cohen et al., 2015). There is a growing consensus that DRN serotonergic neurons are non-homogeneous, as heterogeneity of these neurons has already been suggested by anatomical, biochemical, and electrophysiological properties (Abrams et al., 2004, Marinelli et al., 2004, Calizo et al., 2011, Vasudeva et al., 2011, Andrade and Haj-Dahmane, 2013). Subpopulations of serotonin neurons, either within the DRN or between various raphe nuclei, are hypothesized to be interconnected, and form complex microcircuits (Bang et al., 2012, Gaspar and Lillesaar, 2012, Altieri et al., 2013). Serotonergic neurons send rich networks of recurrent axon collaterals that can span long distances inside of the DRN, often skipping their nearest neighbors to innervate more distant targets (Altieri et al., 2013). By signaling through independent, 5-HT_{1A}-receptor mediated synapses, serotonergic innervation within the DRN could selectively inhibit targeted microcircuits to dampen terminal serotonin release only in their associated projection regions. Thus, independent synapses with these DRN networks may allow spatial and temporal precision in the encoding of mood and behavior by serotonin throughout the brain.

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Figure Legends:

Figure 1: Electrical stimulation evokes 5-HT$_{1A}$ IPSCs that drive pauses in DRN serotonin neuron firing. *A*. eYFP, driven by the PET1 enhancer, selectively labels serotonin neurons. Shown is eYFP immunoreactivity in a coronal brain slice obtained from ePET1-eYFP mice containing the dorsal raphe nucleus (DRN). AQ labels the cerebral aqueduct and dmDRN labels the dorsomedial dorsal raphe nucleus. Scale bar represents 200 μM. *B.* Left: Averaged 5-HT$_{1A}$ IPSCs from a dorsal raphe neuron evoked by a single stimulation in control conditions (black) and in the presence of the 5-HT$_{1A}$ antagonist WAY 100635 (200 nM; grey). Whole-cell recordings were voltage-clamped to -60 mV. Right: IPSCs could be evoked in 30/31 DRN serotoninergic neurons examined. *C*. Average traces of IPSCs recorded in 200 nM TTX, calcium-free ASCF, and 200 μM barium. *D.* Quantification of the rise and decay kinetics of evoked IPSCs. *E.* Cell-attached recordings made in 3 μM phenylephrine to mimic *in vivo* excitatory drive. Representative traces (left) and quantification (right) demonstrating that evoked serotonin release drives a pause in firing that is absent in the 5-HT$_{1A}$-receptor antagonist WAY 100635 (200 nM). Vertical scale bar represents 25 pA. *F.* Firing rates measured over 5 seconds before stimulation (basal) and over 5 seconds after firing had resumed (post-pause) were identical. *G.* Pearson correlation demonstrating that the duration of the evoked pause was independent of the baseline firing rates (n = 9).

Figure 2: 5-HT$_{1A}$-receptor desensitization does not determine the decay time of IPSCs. *A*. Example trace demonstrating a paired-pulse depression when stimulating at a 4 second interval. *B.* Example trace demonstrating the lack of post-synaptic depression when serotonin was applied by iontophoresis twice at a 4 second interval (Ionto.). *C*. Quantification of amplitude ratio of evoked IPSCs in absence and presence of the 5-HT$_{1B}$ pre-synaptic receptor antagonist SB 216641 (1 μM) or the current generated by the iontophoresis of serotonin at a 4 second interval. *D*. Representative trace demonstrating the lack of receptor desensitization during evoked IPSCs. Serotonin was applied by iontophoresis during the decay of evoked IPSCs (1 second post stimulation; Ionto. 1) and without preceding IPSCs (12 seconds post-stimulation; Ionto. 2). *E*. Quantification demonstrating the similar amplitude (left) and time course (right) of currents generated by serotonin iontophoresis in D.

Figure 3: Serotonin reuptake transporters limit the duration of IPSCs. *A.* Average 5-HT_{1A} IPSCs under control conditions and in the presence of citalopram (200 nM) and citalopram (200 nM) + SB216641 (1 μM), illustrating that blocking serotonin uptake increased the activation of 5-HT_{1A} receptors and led to the tonic activation of 5-HT_{1B} receptors. *B*. Quantification of the change in amplitude of IPSCs induced by citalopram (200 nM) or citalopram (200 nM) + SB216641 (1 μM). *C.* Time course the relative change in IPSC amplitude quantified in B. *D.* Quantification of the change in kinetics in either citalopram or citalopram + SB 216641. In both cases, inhibiting reuptake transporters prolongs the duration of 5-HT_{1A} IPSCs.

Figure 4: Slowing diffusion of serotonin alters the amplitude and kinetics of 5-HT_{1A}-IPSCs. *A.* Average traces of IPSCs recorded before (black) and during (green) the bath application of 5% dextran. *B*. Quantification of the amplitude, peak time, and decay kinetics of IPSCs recorded in A. *C.* Average traces of IPSCs before (black) and during (green) the bath application of 5% dextran recorded in the continuous presence of citalopram (200 nM) and SB 216641 (1 μM). When serotonin reuptake transporters and pre-synaptic autoreceptors were inhibited, slowing diffusion greatly prolonged the decay of 5-HT_{1A} IPSCs. *D.* Quantification of the amplitude, peak time, and decay kinetics of IPSCs recorded in *E*. Relative change in IPSCs in the presence of dextran with and without citalopram (200 nM).

Figure 5: Reuptake transporters prevent transmitter pooling and synaptic crosstalk from prolonging the decay time of 5-HT_{1A} IPSCs. *A.* Example IPSCs recorded from 4 neurons demonstrating the variation in amplitude and duration. *B.* Lack of correlation between decay kinetics and amplitude of 5-HT_{1A} IPSCs in 30 DRN neurons from Fig. 1. *C.* Example IPSCs evoked with different stimulation intensities (low: 10-20 μA; high: 60-80 μA). Inset show the traces normalized and aligned to their peak, illustrating that responses to lower stimuli had similar rates of decay. Right: quantification of amplitudes and decay times between conditions. *D*.

Example IPSCs evoked in 2.5 mM and 1.0 mM extracellular Ca^{2+}. Inset show the traces normalized and aligned to their peak, illustrating that responses in reduced calcium had similar rates of decay. Right: quantification of amplitudes and decay times between conditions. Scale bar (for D and E): 30 pA; 500 ms. n.s. represents $p > 0.05$. *E.* Example traces (left) and quantification (right) of IPSCs in the presence of SB216641 (1 μM) + citalopram (200 nM) evoked with low (10 – 20 μA) and high (60-80 μA) stimulation intensities. Lower trace shows the recordings normalized, illustrating that in the presence of citalopram, larger stimuli prolong the decay time of 5-HT_{1A} IPSCs. *F.* Cartoon schematic of the proposed mechanisms underlying 5-HT_{1A}-receptor-mediated transmission in the DRN. Reuptake transporters limit the extent of transmitter spillover to prevent synaptic crosstalk. This may result in functionally independent sites of transmission.

Figure 6: Spontaneous 5-HT_{1A}–receptor mediated currents have similar kinetics as evoked IPSCs. *A*. Representative trace of evoked and spontaneous IPSCs. *B.* Average spontaneous IPSCs (sIPSC, left) overlaid with average sIPSCs recorded in citalopram (200 nM, center) and average evoked 5-HT_{1A} IPSCs (right). *C.* Quantification of the amplitudes of sIPSCs recorded in control bath and citalopram vs. evoked IPSCs. (ANOVA, $p < 0.001$) *D*. Quantification of the kinetics of the spontaneous IPSCs recorded in control bath and citalopram (200 nM) compared to evoked 5-HT_{1A} IPSCs (ANOVA: peak time $p < 0.001$, τ_{Decay} $p < 0.001$). sIPSCs recorded in control bath had similar kinetics to evoked IPSCs. *E*. Inclusion of phenylephrine (3 μM), forskolin (1 μM), DPCPX (500 nM), and citalopram (50 nM) in the bath increased the frequency of sIPSCs (pharm.). Further addition of WAY 100635 (200 nM) abolished sIPSCs (ANOVA $p < 0.001$). *F*. Histogram demonstrating that sIPSC had significantly greater amplitudes ($p = 0.04$; n = 21 pharm.; n = 27 ctrl.) in the pharmacological bath (from *E*) compared to control bath. * represents $p < 0.05$; *** represents $p < 0.001$; n.s. represents $p > 0.05$

Figure 1

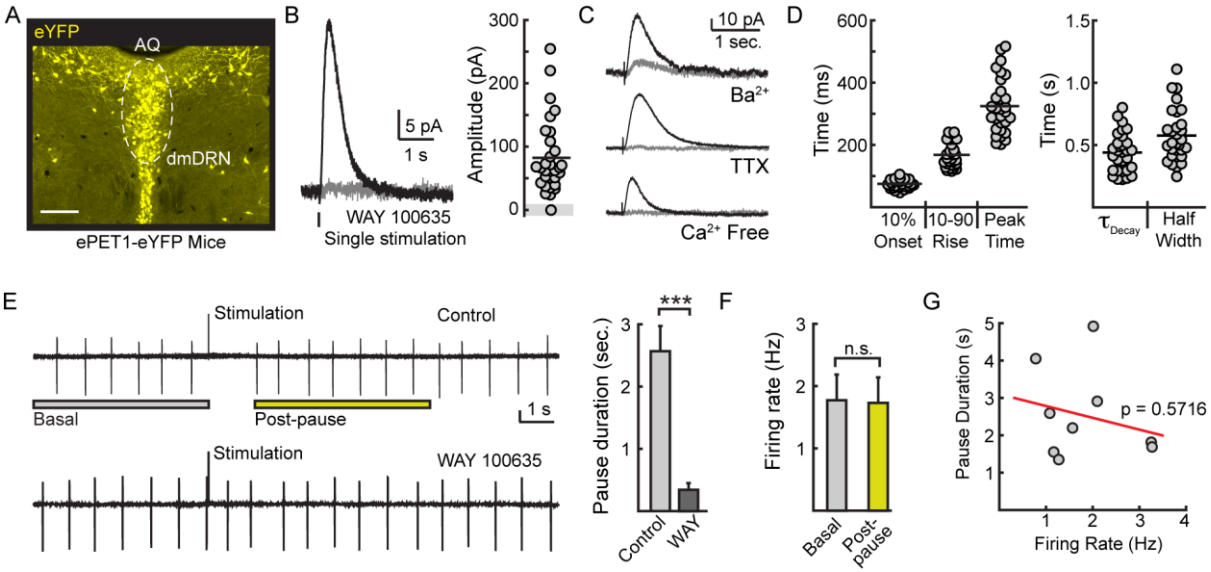

Figure 2

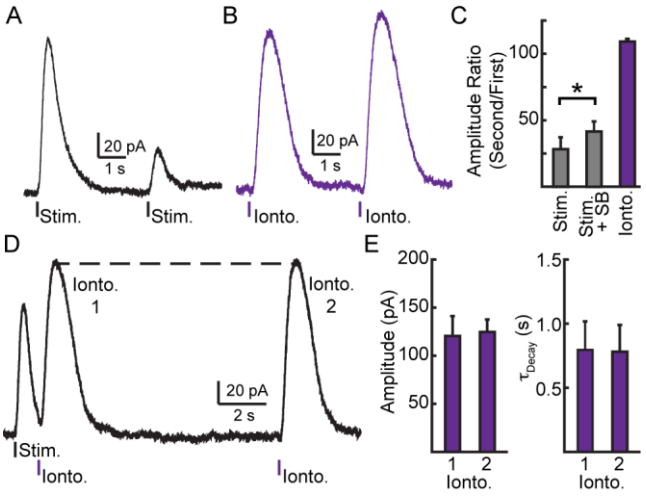

Figure 3

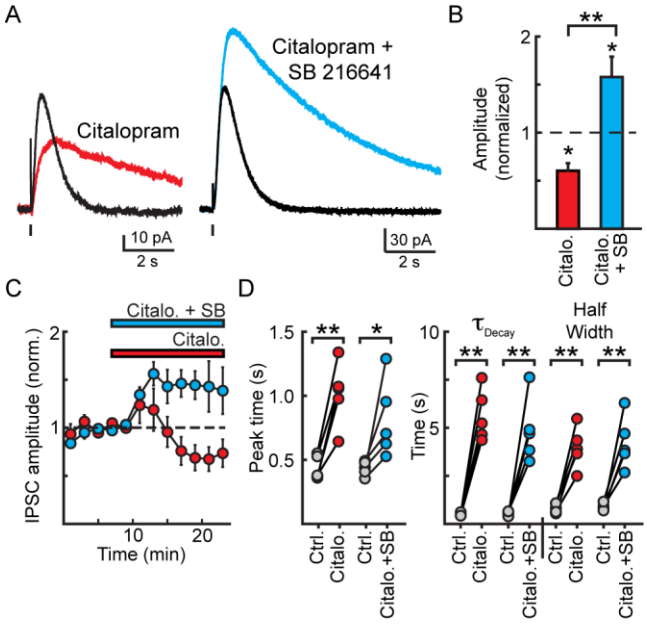

Figure 4

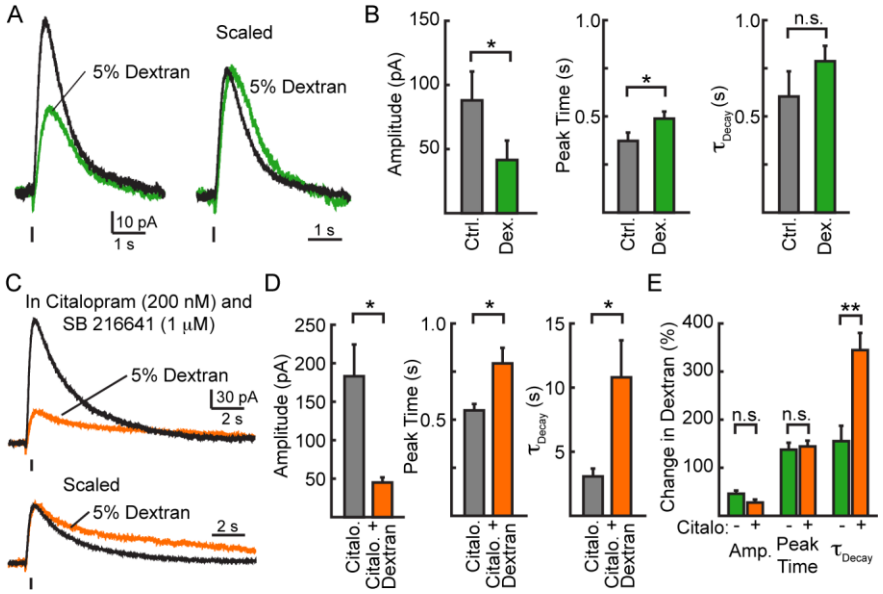

Figure 5

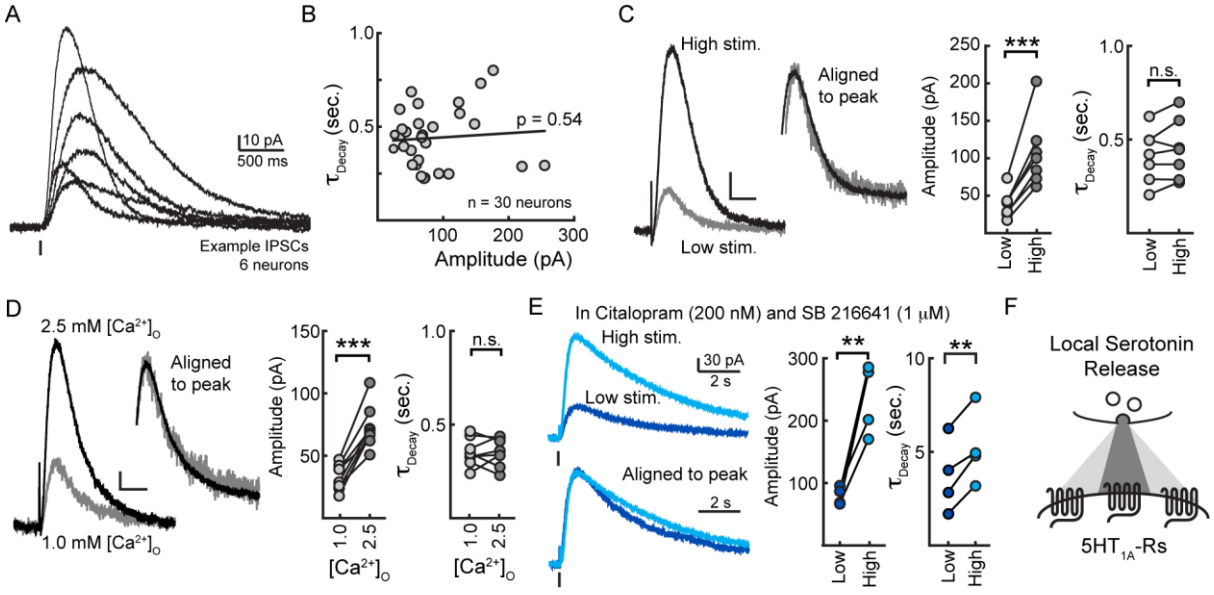

Figure 6