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ORIGINAL ARTICLE

Acute selective serotonin reuptake inhibitors regulate the dorsal raphe nucleus causing amplification of terminal serotonin release

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Abstract

Selective serotonin reuptake inhibitors (SSRIs) were designed to treat depression by increasing serotonin levels throughout the brain via inhibition of clearance from the extracellular space. Although increases in serotonin levels are observed after acute SSRI exposure, 3–6 weeks of continuous use is required for relief from the symptoms of depression. Thus, it is now believed that plasticity in multiple brain systems that are downstream of serotonergic inputs contributes to the therapeutic efficacy of SSRIs. The onset of antidepressant effects also coincides with desensitization of somatodendritic serotonin autoreceptors in the dorsal raphe nucleus (DRN), suggesting that disrupting inhibitory feedback within the serotonin system may contribute to the therapeutic effects of SSRIs. Previously, we showed that chronic SSRI treatment caused a frequency-dependent facilitation of serotonin signaling that persisted in the absence of uptake inhibition. In this work, we use *in vivo* fast-scan cyclic voltammetry in mice to investigate a similar facilitation after a single treatment of the SSRI citalopram hydrobromide. Acute citalopram hydrobromide treatment resulted in frequency-dependent increases of evoked serotonin release in the substantia nigra *pars reticulata*. These increases were independent of changes in uptake velocity, but required SERT expression. Using microinjections, we show that the frequency-dependent enhancement in release is because of SERT inhibition in the DRN, demonstrating that SSRIs can enhance serotonin release by inhibiting uptake in a location distal to the terminal release site. The novel finding that SERT inhibition can disrupt modulatory mechanisms at the level of the DRN to facilitate serotonin release will help future studies investigate serotonin's role in depression and motivated behavior.

Keywords: 5-HT, FSCV, *in vivo* cyclic voltammetry, serotonin transporter, SERT knockout.

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Selective serotonin reuptake inhibitors (SSRIs) increase extracellular serotonin levels by reducing serotonin transporter (SERT) function. These drugs were developed specifically to treat major depressive disorder, supported by evidence from the monoamine hypothesis linking abnormally low serotonin levels to symptoms of depression (Jacobsen *et al.* 2012). Although SSRIs increase extracellular serotonin levels after a single dose, 3–6 weeks elapse before they exert clinically meaningful antidepressant effects. Because of this time lapse, it is now believed that SSRI efficacy depends on plasticity in serotonergic function and its downstream targets (Blier and de Montigny 1998). This plasticity arises from two key effects of SSRI exposure: increases in extracellular serotonin levels, and reduced spontaneous firing of serotonergic neurons (Pineyro and Blier 1999). The former is a consequence of a functional shift in release and uptake equilibrium because of reductions in SERT function. The latter arises because of increased activation of 5-HT1A

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Abbreviations used: $[5\text{-HT}]_{max}$, maximum evoked concentration of serotonin; μCIT, microinfusion of citalopram hydrobromide; μSAL, microinfusion of saline (vehicle); CIT, citalopram hydrobromide; DRN, dorsal raphe nucleus; FSCV, fast-scan cyclic voltammetry; $SERT^{-/-}$, homozygous SERT knockout; SNpr, substantia nigra *pars reticulata*; SSRI, selective serotonin reuptake inhibitor; $t_{1/2}$, decay time from maximal release amplitude to half-maximal release amplitude; WT, wild-type.

autoreceptors, which inhibit firing of serotonergic neurons (Blier *et al.* 1998). Sustained increases in serotonin levels during chronic SSRI treatment lead to functional desensitization of these autoreceptors, an effect associated with reduced 5-HT1A receptor mRNA (Bortolozzi *et al.* 2012), which leads to reinstatement of normal spontaneous firing (Davidson and Stamford 1995). Studies have shown that this desensitization is a critical component of the antidepressant responses to chronic SSRI treatment in rodent models (Bortolozzi *et al.* 2012; Ferres-Coy *et al.* 2013).

Up to two-thirds of patients initiating SSRI treatment experience treatment-resistant depression, and many experience adverse effects that make continuing treatment undesirable (Trivedi *et al.* 2006). Developing better therapies for depression will require a more complete understanding of serotonin's role in both mood and motivated behavior. While the monoamine hypothesis suggests that increases in serotonin levels correspond to increases in mood, serotonergic activity has also been linked to aversive experiences and punishment (Daw *et al.* 2002). However, recent research shows that the function of serotonin in motivational states and mood is more complex than traditional models suggest. Liu *et al.* (2014) found that selective activation of serotonin-producing neurons in the dorsal raphe nucleus (DRN) of mice elicited appetitive behavioral effects, but further studies by McDevitt *et al.* (2014) suggest that these effects might be mediated solely by glutamatergic projections from these neurons to the VTA. Attempts to describe how serotonergic neurons respond to and encodes for reward and aversion have revealed that both modalities alter single-unit activity of identified serotonergic neurons on multiple timescales (Cohen *et al.* 2015). This suggests an intricate interplay between ambient serotonin levels and bursts of release. Characterizing this interplay is a step toward understanding which aspects of SSRI-mediated plasticity are necessary for mood elevation.

While key studies have correlated activity in serotonergic neurons with appetitive and aversive experiences and behavioral responses, none have established a causal link between activity and serotonin neurotransmission. Dopamine release can be reliably evoked using optogenetic activation of VTA dopamine neurons (Tsai *et al.* 2009), but corresponding evidence is lacking for optically evoked serotonin release in mammalian models. The rapid, multispike burst-firing that drives robust dopamine release is not equivalent to the bursts observed in serotonin neurons *in vivo*, which occur at up to 100 Hz but in trains of only 2–3 spikes (Hajos *et al.* 1995; Gartside *et al.* 2000). Therefore, interpreting the role of serotonin signaling in studies that measure or manipulate neuronal activity is obscured by a lack of information about the magnitude and dynamics of serotonin release. Fast-scan cyclic voltammetry (FSCV) is a chemically selective, time-resolved technique that has been used to characterize the dynamics and regulation of *in vivo* serotonin release measured in the substantia nigra *pars reticulata* (SNpr), a region richly innervated with serotonergic terminals without significant interference from other electroactive neurotransmitters. FSCV has been used to show that serotonin release is under considerably strict regulation as a consequence of a highly sensitive, balanced system of SERT, autoreceptor, and monoamine oxidase activity (Hashemi *et al.* 2012). SERT function is a powerful regulator of serotonin signaling, and when it is disrupted, the consequences can be fatal, as in serotonin syndrome (Boyer and Shannon 2005).

Previously, we showed that 3 weeks of chronic SSRI treatment results in a facilitation of serotonin signaling at higher stimulation frequencies, an effect that was dominated by changes in release, but not uptake (Dankoski *et al.* 2014). This finding suggests that SERT expression and function have profound effects on multiple aspects of neurotransmission. In this work, we present the observation that *in vivo* serotonin release does not vary with frequency as predicted by slice measurements (O'Connor and Kruk 1991; Iravani and Kruk 1997; Bunin and Wightman 1998; Jennings *et al.* 2010). Surprisingly, acute treatment with an SSRI produced frequency-dependent serotonin release. We hypothesized that SERT inhibition facilitated *in vivo* serotonin release in the SNpr via a mechanism unrelated to inhibition of terminal uptake. To test this, we measured the acute effects of the SSRI citalopram hydrobromide (CIT) on serotonin release evoked by stimulations of varying frequency and intensity to enable differentiation between its effects on release versus uptake (Wightman and Zimmerman 1990). While we observed no frequency dependence in drug-naïve mice, acute CIT treatment produced frequency-dependent increases in release. SERT knockout ($SERT^{-/-}$) mice displayed a low level of frequency dependence, but did not demonstrate frequency-dependent facilitation of serotonin release after CIT administration, confirming that CIT's effects on release amplitude are SERT mediated. By microinfusing CIT into the DRN, we demonstrated that SERT inhibition at the level of serotonergic cell bodies was sufficient to drive frequency-dependent effects of CIT; thus, CIT's enhancement of serotonin release can be disassociated from its effects on terminal uptake. Taken together, the findings support the hypothesis that dramatic increases in ambient serotonin levels saturate inhibitory 5-HT1A control over release, leading to a disinhibition of the response to high-frequency stimulations. This newly characterized effect of SSRIs may clarify interpretations of serotonin's role in antidepressant therapies.

Methods

Animals

All experiments were conducted in accordance with protocols approved by the University of North Carolina at Chapel Hill (UNC-CH) Institutional Animal Care and Use Committee

(IACUC protocol no. 12-152.0). Male C57Bl6/J WT (n = 29) and SERT$^{-/-}$ (n = 9, Slc6a4 homozygous knock-out) mice were received from Jackson Laboratories (Bar Harbor, ME, USA) at 5–6 weeks of age and housed in groups of 4 for a minimum of 2 weeks after arrival. Prior to experiments, mice were drug- and test-naïve. Mice were provided with food and water ad libitum and kept on a 12-h light cycle.

Electrochemistry

Carbon-fiber microelectrodes were fabricated as previously described (Cahill *et al.* 1996). Briefly, carbon fibers (7 μm diameter) were aspirated into a glass capillary (external diameter: 0.6, internal diameter: 0.4) and pulled on a vertical capillary puller (Narashige, Tokyo, Japan) so that glass formed a tapered seal with carbon fiber. The exposed length of carbon fiber was trimmed to 150 μm. To enhance sensitivity to serotonin, Nafion (10% in methanol) was applied to microelectrodes in a dip-coating process. Briefly, the tip of each electrode was dipped into Nafion solution then baked at 70°C for 10 min to evaporate solvent. This process was repeated seven times to obtain the desired Nafion thickness. All electrodes were post-calibrated in a flow-injection system to obtain *in vitro* calibration factors, as previously described (Hashemi *et al.* 2009).

Application of waveform, data collection, and electrical stimulation were controlled by Tarheel CV software via an in-house manufactured potentiostat (UEI, UNC-CH Electronics Facility Chapel Hill, NC, USA). Voltammetric measurement of *in vivo* serotonin release has been previously described (Hashemi *et al.* 2009). Briefly, a voltage waveform scanning from −0.1 to 1 V at 1000 V/s was applied at a rate of 10 Hz and held at 0.2 V between scans (Jackson *et al.* 1995).

In vivo experiments

Mice were anesthetized with urethane (2 mg/g body weight, i.p.) prior to stereotaxic surgery. Urethane anesthesia was employed because it has little effect on neurotransmission and cardiovascular and respiratory functions, and it allows anesthesia to be maintained consistently over the 4–8 h time period of the experiment. A stainless steel bipolar stimulating electrode (Plastics One, Roanoke, VA, USA) was implanted into the DRN (AP: −4.1, ML: 0.0, DV: −2.5 to −2.7 mm from Bregma). A carbon-fiber microelectrode was lowered into the SNpr (AP −3.2, ML: +1.5, DV −4.0 to −4.2 mm from Bregma). An Ag/AgCl reference electrode was secured in the contralateral hemisphere. All experiments took place during the light cycle.

Detection of electrically evoked serotonin was optimized by moving the stimulating electrode and carbon-fiber microelectrode in 0.1 mm increments within the given coordinate ranges. Following every experiment, CIT (10 mg/kg, i.p.) was administered to pharmacologically confirm the signal as serotonin. In SERT$^{-/-}$ experiments, where CIT predictably had no effect, high concentrations of evoked serotonin release led to cyclic voltammograms that were easily identifiable as serotonin. The dose of CIT used with i.p. injection was selected to be consistent with previously published studies (Hashemi *et al.* 2009, 2011, 2012; Dankoski *et al.* 2014). In a few experiments, clearance was unaffected by CIT. This was attributed to likely unsuccessful targeting of the SNpr and/or DRN, and these experiments were excluded from statistical analyses. Experiments in which both pre- and post-CIT response to the full range of stimulation frequencies could not be analyzed because of noise or other technical issues were also excluded (total experiments excluded: n = 4).

For microinfusion experiments, a microinjection cannula (custom guide cannula, 10 mm length; Plastics One) was lowered into the DRN (AP: −4.1, ML: 0.0, DV: −2.4 from Bregma), and approximately 5 μL of CIT (12 mM) or vehicle (saline) was ejected into the targeted region through a microinfusion stylus (33 gauge, 11 mm length; Plastics One). The dose and volume of CIT used for microinfusion experiments was selected because it produced the most consistent effects in this acute preparation. After 15 min, the microinjection needle was removed and the stimulating electrode was implanted in the same location, as described above. Because of its close proximity to the cerebral aqueduct, misplacement of the microinjection needle resulted in brain-wide CIT exposure. This was apparent as an immediate and dramatic slowing of serotonin clearance in subsequent recordings in the SNpr, and these experiments were excluded from analyses (n = 6). Uptake response to a systemic injection of CIT at the end of each experiment served as an additional validation of microinjection targeting.

Drugs and reagents

Racemic citalopram hydrobromide and urethane were from Sigma-Aldrich (St Louis, MO, USA). Nafion (5% in mixed alcohols) was from Sigma-Aldrich and concentrated in a rotovap to replace the solvents with methanol. Replacing mixed alcohol solvents with methanol produces a more uniform layer in the dip-coating process (described above). Intraperitoneal-injected drugs were administered at a volume of 0.01 mL/g body weight.

Data collection

A stainless steel, bipolar stimulating electrode implanted in the DRN was used to apply biphasic 350 μA, 4 ms pulse width stimulation trains, which ranged in frequency from 20 to 60 Hz and ranged in pulse number from 30 to 180 pulses. Where frequency and pulse number are not indicated, a default stimulation of 60 Hz, 60 pulses is used. Frequency range was selected to optimize the accuracy and consistency of $t_{1/2}$ and release rate measurements. Frequency-dependence measurements were collected in the order of descending frequency. Pulse number range was set by the minimum number of pulses necessary to obtain a good signal-to-noise ratio. Pulse number measurements were collected in the order of ascending pulse number.

A minimum of 180 s elapsed between each stimulation train to prevent short-term effects of stimulation (e.g. depletion, baseline instability) from influencing data collection over the experimental period. After CIT administration, no stimulations were applied for 5 min to reduce incidence of subject death (based on observations from prior experiments).

Statistical analysis

The unit of analysis in these data is a single animal pre- and post-drug treatment. Subjects in which both pre- and post-drug data could not be analyzed because of either lack of response to CIT or technical issues were excluded from analyses (10 experiments total). Results are described as average values ± SEM except when representative data is noted. Trends in maximum evoked amplitude

([5-HT]$_{max}$), release rate, and clearance half-life ($t_{1/2}$) were analyzed using a repeated measures ANOVA with Tukey's multiple comparisons *post hoc* analysis.

Release rate and $t_{1/2}$ were obtained from concentration traces evoked by 20–60 Hz stimulations in each subject. Release rate was calculated by fitting lines to the increasing serotonin concentration evoked during the first 2 s of stimulation. Adjustments of the fitting window of up to 0.1 s were made to avoid stimulation artifacts and ensure linear behavior ($r^2 > 0.70$). Release rate analysis assumes that stimulated release follows linear behavior, as predicted by Michaelis–Menten derived kinetic model of neuromodulator release (Wu *et al.* 2001). Decay time ($t_{1/2}$) was calculated in Clampfit (Molecular Devices, LLC, Sunnyvale, CA, USA) as the time (in seconds) that elapse between maximal concentration and half-maximal concentration. One subject was excluded from pre- and post-CIT $t_{1/2}$ analysis because of significant drift after electrical stimulations. This drift did not affect measurements of [5-HT]$_{max}$.

Results

Serotonin release evoked *in vivo* varies with stimulation pulse number, but not frequency

Serotonin release was measured in SNpr during stimulation of the DRN. A representative color plot identifies serotonin evoked by a 325 μA, 60 Hz, 1 s stimulation (Fig. 1a). At the peak potential for serotonin oxidation (0.77 V; white dashed line), the concentration varies with time, showing release and subsequent clearance of evoked serotonin (Fig. 1b).

We previously showed that in contrast to work done in brain slices, serotonin release *in vivo* is not frequency-dependent prior to SSRI administration (Dankoski *et al.* 2014). To better characterize this finding, we compared [5-HT]$_{max}$ evoked by DRN stimulations varying in pulse number and frequency. Varying stimulation frequency

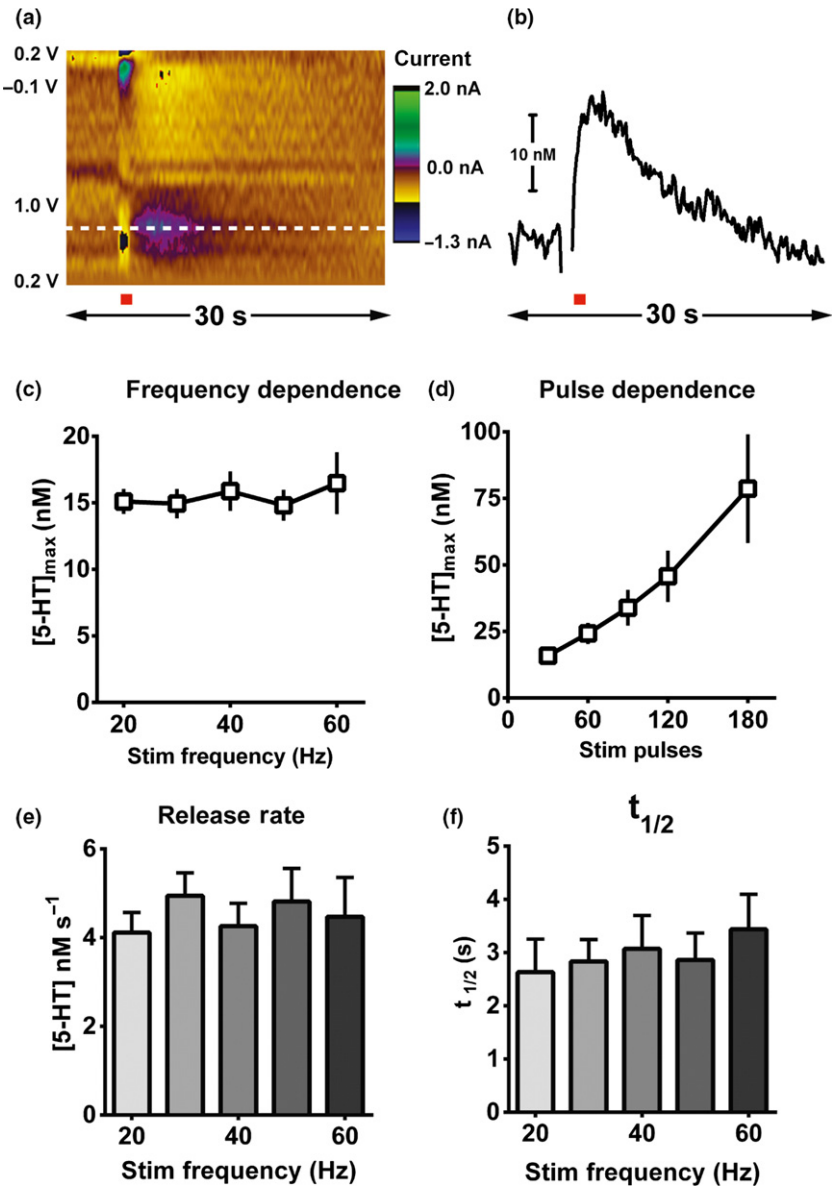

Fig. 1 Serotonin release is not frequency-dependent *in vivo*. (a) Representative color plot showing serotonin release evoked in SNpr by a 60 Hz, 1 s stimulation of dorsal raphe nucleus. Color plot shows time versus waveform voltage, with change in current in pseudocolor. Stimulus duration indicated by red bar. Oxidation potential for serotonin indicated by white dashed line. (b) Concentration trace obtained at serotonin's oxidation potential, scaled using an *in vitro* post-calibration. Stimulus duration indicated by red bar. (c) Average [5-HT]$_{max}$ evoked by 60 pulse stimulation trains applied at 20, 30, 40, 50, and 60 Hz. (d) Average [5-HT]$_{max}$ evoked by stimulation trains of 30, 60, 90, 120, and 180 pulses at 60 Hz. (e) Average rate of change in serotonin concentration evoked by 60 pulse stimulation trains applied at 20, 30, 40, 50, and 60 Hz. (f) Average $t_{1/2}$ for these stimulations. Error bars are ± SEM or + SEM (n = 5–6).

in vivo did not affect $[5\text{-HT}]_{max}$ (Fig. 1c; One-way ANOVA, $F(4,20) = 1.004$, $p = 0.43$); released concentrations at 20 Hz also did not differ significantly from those at 60 Hz (15.11 ± 0.94 nM and 16.49 ± 2.32 nM, respectively). However, $[5\text{-HT}]_{max}$ was significantly affected by varying stimulation pulse number (Fig. 1d; One-way ANOVA, $F(4,16) = 9.374$, $p < 0.001$). Thus, serotonin release evoked *in vivo* could be manipulated by varying stimulation pulse number, but not frequency.

We next examined how stimulation frequency affected $t_{1/2}$ and release rate evoked by varying stimulation frequencies. Contrary to the predictions of simple modeling of neurotransmitter release (Wightman *et al.* 1988; Dreyer and Hounsgaard 2013), release rate did not vary with stimulation frequency (Fig. 1e; One-way ANOVA, $F(4,20) = 0.673$, $p = 0.62$). Release rate driven by the slowest stimulation frequency was not significantly different from release rate driven by the most rapid stimulation frequency (20 Hz: 4.11 ± 0.46 nM/s, 60 Hz: 4.47 ± 0.89 nM/s). As predicted by the model, $t_{1/2}$ also did not vary with frequency (Fig. 1f; One-way ANOVA, $F(4,16) = 1.361$, $p = 0.29$). Clearance following 20 Hz stimulations had similar values to clearance following 60 Hz stimulations (2.63 ± 0.62 s and 3.44 ± 0.66 s, respectively). These results suggest that serotonin neurons might reduce sensitivity to high-frequency stimuli that drive serotonin release.

SERT inhibition enhances serotonin release in a frequency-dependent manner

Next, we examined CIT's effects on neurotransmission dynamics. A duration of 30 min after CIT administration, $[5\text{-HT}]_{max}$ and $t_{1/2}$ had both increased relative to baseline, consistent with previous reports (Hashemi *et al.* 2012; Dankoski *et al.* 2014). Table S1 compares average pre- and post-CIT $[5\text{-HT}]_{max}$, release rate, and $t_{1/2}$ values. In Fig. 2(a) and (b), the effects of CIT are apparent in the serotonergic response to 20 Hz, 60 pulse and 60 Hz, 60 pulse stimulations compared to baseline release. Surprisingly, differences in the magnitude of response to CIT varied with stimulation frequency: after CIT treatment, $[5\text{-HT}]_{max}$

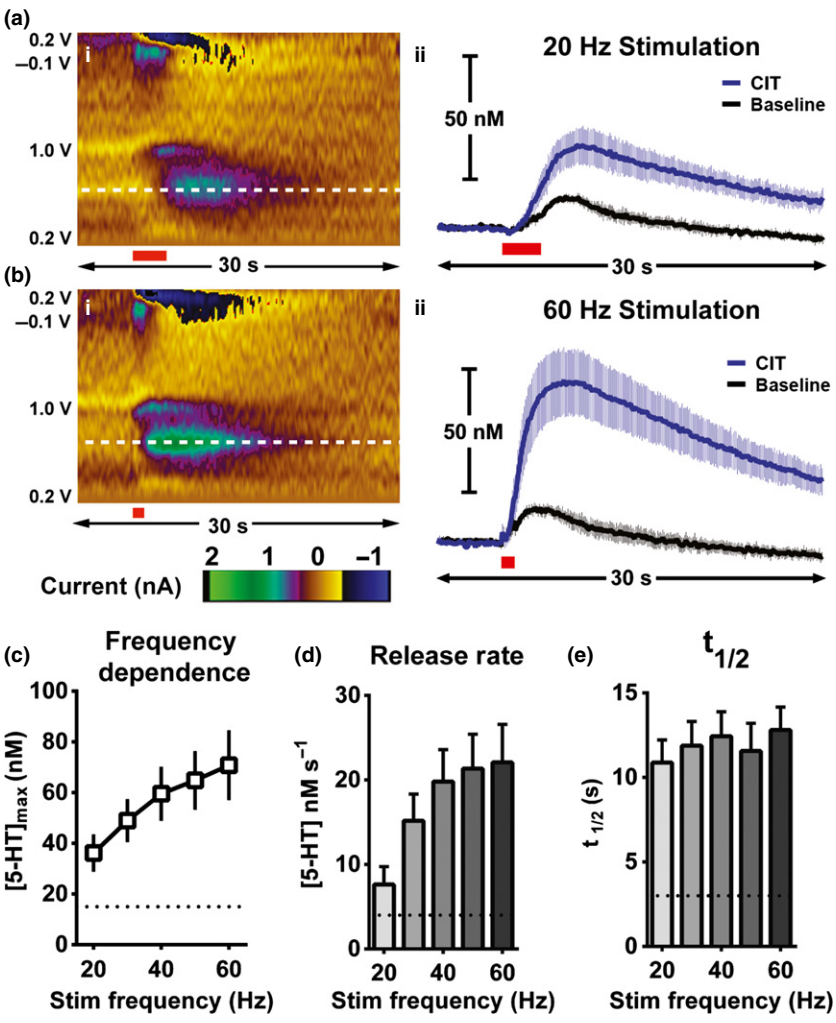

Fig. 2 Citalopram induces frequency-dependent serotonin release. (a) (i) Representative color plot showing serotonin release evoked in SNpr by a 20 Hz, 3 s dorsal raphe nucleus (DRN) stimulation. (ii) Averaged concentration trace showing serotonin release evoked by 20 Hz stimulation at baseline (black line) and 30 min after citalopram hydrobromide (CIT) treatment (blue line) (b) (i) Representative color plot showing serotonin release evoked in SNpr by a 60 Hz, 1 s DRN stimulation. (ii) Averaged concentration trace showing serotonin release by 60 Hz stimulation at baseline (black line) and 30 min after CIT treatment (blue line). (c) Average $[5\text{-HT}]_{max}$ evoked by 60 pulse stimulation trains applied at 20, 30, 40, 50, and 60 Hz, 30 min after CIT treatment. Dotted line indicates approximate $[5\text{-HT}]_{max}$ before CIT for comparison. (d) Rate of release evoked in wild-type mice by 60 pulse stimulations varying in frequency from 20 to 60 Hz following CIT treatment. Dotted line indicates approximate release rate at baseline. (e) $t_{1/2}$ of serotonin evoked by stimulations of varying frequency following CIT treatment. Dotted line indicates approximate $t_{1/2}$ before CIT. Error bars are ± SEM or + SEM ($n = 6$).

significantly varied with stimulation frequency, exhibiting robust frequency dependence (Fig. 2c; One-way ANOVA, $F(4,20) = 16.56$, $p < 0.0001$). To determine which aspects of neurotransmission contributed to CIT-induced frequency dependence, we analyzed release rate and $t_{1/2}$ after acute CIT treatment. The rate of evoked release became significantly frequency-dependent (Fig. 2d; One-way ANOVA, $F(4,20) = 24.18$, $p < 0.0001$), and release rate was significantly different between 20 Hz and 60 Hz stimulations (7.62 ± 2.11 nM/s vs. 22.08 ± 4.50 nM/s, paired t-test, $p < 0.01$). While $t_{1/2}$ was increased following CIT, it did not vary with stimulation frequency (Fig. 2e; One-way ANOVA, $F(4,16) = 1.402$, $p = 0.28$). Uptake after 20 Hz stimulations was not significantly different from uptake after 60 Hz stimulations (10.88 ± 1.35 s vs. 12.80 ± 1.36 s, respectively). Together, these results suggest that CIT treatment altered mechanisms that limit serotonin release, an effect that may be an important component of SSRIs' therapeutic effects.

The ability of uptake inhibitors to enhance serotonin release is SERT-selective

SSRIs slow serotonin clearance by inhibiting its transporter, SERT. To determine whether CIT affects serotonin release by a mechanism not involving SERT, we administered CIT to mice lacking the gene encoding SERT ($SERT^{-/-}$ mice). Baseline serotonin release in $SERT^{-/-}$ mice was frequency-dependent (Fig. 3a, One-way ANOVA, $F(4,32) = 10.91$, $p < 0.05$), and CIT did not increase $[5\text{-HT}]_{max}$ or $t_{1/2}$ relative to baseline in $SERT^{-/-}$ mice (Table S2). When $[5\text{-HT}]_{max}$ in WT+CIT mice was compared to baseline $[5\text{-HT}]_{max}$ in $SERT^{-/-}$ mice, there was a significant interaction between frequency × genotype (Fig. 3a; Two-way repeated measures ANOVA, $F(4,52) = 8.701$, $p < 0.0001$. See also Fig. S1). The effect of genotype on frequency dependence may be as a result of compensation mechanisms that result from genetic deletion of SERT, such as changes in serotonin receptor function and expression (Li 2006; Fox *et al.* 2008; Moya *et al.* 2011), including enhanced 5-HT1A receptor function in the DRN (Soiza-Reilly *et al.* 2015), as well as impaired hypothalamic–pituitary–adrenal axis function (Jiang *et al.* 2009).

CIT significantly increased $t_{1/2}$ in wild-type (WT) mice, but not in $SERT^{-/-}$ mice (Fig. 3a, inset, WT: increased by 9.36 ± 1.96 s, $SERT^{-/-}$: increased by 1.47 ± 2.40 s). Thus, we concluded that the effect of CIT on serotonin release was indeed SERT mediated.

Inhibiting SERT in DRN enhances serotonin release in a frequency-dependent manner

Data presented above shows CIT effects on $[5\text{-HT}]_{max}$ and $t_{1/2}$ after the effects have stabilized. This occurs approximately 30 min after i.p. administration and remains consistent for up to 2 h. However, the onset of these effects follows a pattern

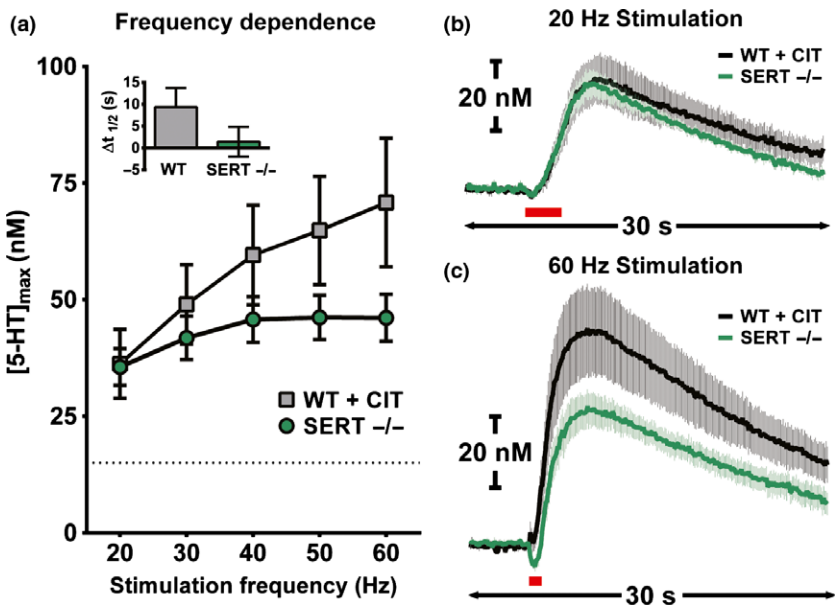

Fig. 3 Homozygous SERT knockout ($SERT^{-/-}$) function mediates frequency dependence of serotonin release. (a) Comparison of average $[5\text{-HT}]_{max}$ evoked by 60 pulse stimulations applied at 20, 30, 40, 50, and 60 Hz in citalopram hydrobromide (CIT)-treated wild-type (WT) mice (gray boxes) and $SERT^{-/-}$ mice (green circles). For comparison, a dotted line indicates approximate baseline $[5\text{-HT}]_{max}$ in WT mice. Inset: Average change in $t_{1/2}$ following CIT treatment in WT (gray) and $SERT^{-/-}$ mice (green). (b) Average $[5\text{-HT}]_{max}$ evoked by 20 Hz, 60 pulse stimulations in CIT-treated WT (black line) and $SERT^{-/-}$ mice (green line). (c) Average serotonin release evoked by 60 Hz, 60 pulse stimulations in CIT-treated WT (black line) and $SERT^{-/-}$ mice (green line).

that provides additional insights concerning its release-enhancing mechanisms. To characterize CIT onset, 60 Hz, 60 pulse stimulations were applied at 2 min intervals, and changes in $[5\text{-HT}]_{max}$ and $t_{1/2}$ relative to pre-drug release were determined. Fig. 4(a) compares $[5\text{-HT}]_{max}$ and $t_{1/2}$ during the 20 min following i.p. CIT injection (10 mg/kg). Although $t_{1/2}$ is not significantly increased until 14 min after SSRI exposure (14 min time point, $p < 0.01$), $[5\text{-HT}]_{max}$ significantly increases within the first 6 min (6 min time point, $p < 0.01$). Time points that are significantly different from each measurement's baseline are indicated on the graph. Comparison of how the two measurements respond to

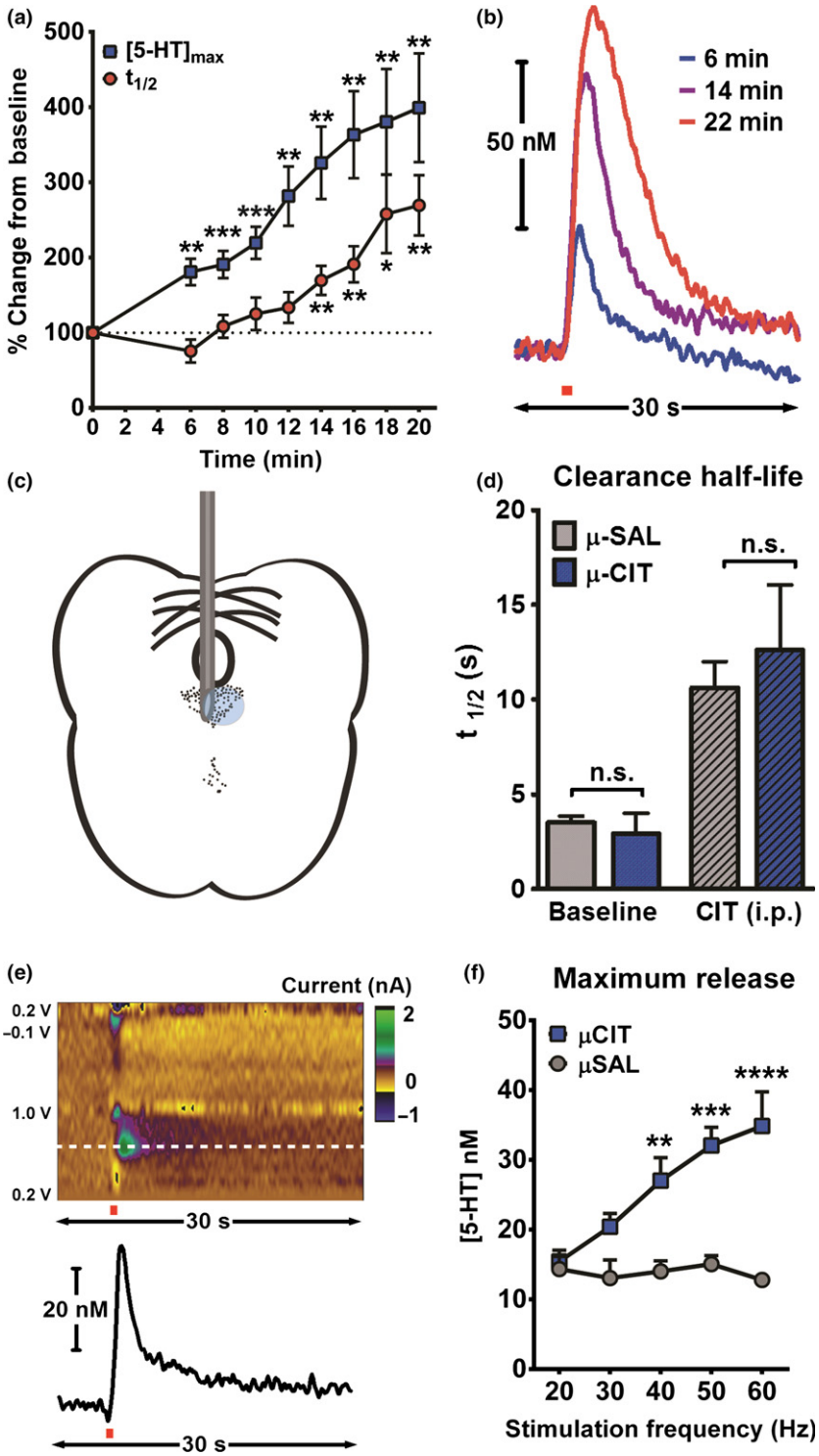

Fig. 4 Enhancement of serotonin signaling by selective serotonin reuptake inhibitors is mediated by dorsal raphe nucleus (DRN). (a) Time course of average percent of $[5\text{-HT}]_{max}$ values (blue squares) and $t_{1/2}$ (red circles) following acute citalopram hydrobromide (CIT) treatment with respect to predrug values. (b) Representative concentration traces showing serotonin release 6, 14, and 22 min after acute CIT treatment. (c) Illustration showing targeting of the DRN for microinfusions (blue shading). (d) Comparison of average $t_{1/2}$ after saline microinfusion (μ-SAL; gray) or CIT microinfusion (μ-CIT; blue) before and after i.p. CIT treatment. (e) Representative color plot (top) and concentration trace (bottom) showing release evoked by 60 Hz, 1 s stimulation after μCIT. (f) Average $[5\text{-HT}]_{max}$ following μSAL (gray circles) and μCIT (blue squares). Error bars are ± SEM or + SEM (n = 4–5). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

CIT over time found a significant interaction between type of measurement x time (Fig. 4a; Two-way ANOVA, $F(8,72) = 2.174$, $p < 0.05$). Representative traces illustrating the separate effects on $[5\text{-HT}]_{max}$ and $t_{1/2}$ can be seen in Fig. 4(b). The apparent disassociation of changes in $[5\text{-HT}]_{max}$ from changes in $t_{1/2}$ led to a hypothesis that CIT's effects on release may be mediated by a different anatomical region than its effects on terminal region uptake. Since frequency-dependent effects of CIT on $[5\text{-HT}]_{max}$ are not observed in brain slices (Jennings *et al.* 2010), where serotonergic terminals are severed from cell bodies, we hypothesized that SERT inhibition in DRN contributed to these effects on electrically evoked release.

To test whether the DRN mediates CIT effects on $[5\text{-HT}]_{max}$ in terminal regions, we microinfused saline (μSAL) or CIT (μCIT) into the DRN and compared the effects on evoked serotonin in the SNpr (Fig. 4c). To confirm that μCIT did not access the cerebral aqueduct and affect SERT function at the terminal recording site, we compared $t_{1/2}$ of serotonin release evoked by 60 Hz stimulations between μSAL and μCIT before and after a systemic injection of CIT (10 mg/kg, i.p.). We observed no difference in $t_{1/2}$ between μSAL and μCIT at baseline or in response to systemic CIT (Fig. 4f; unpaired *t*-test, pre-i.p. CIT: $p = 0.62$, post-i.p. CIT: $p = 0.57$). If μCIT affected SERT in the recording region, $t_{1/2}$ would not be affected by a systemic injection of CIT (10 mg/kg, i.p.). $t_{1/2}$ was significantly increased by i.p. CIT administration in both μCIT and μSAL subjects, confirming that μCIT did not alter SERT function at the terminal recording site (Fig. 4f; Two-way ANOVA, microinjection treatment: $F(1,4) = 0.22$, $p = 0.66$, i.p. treatment: $F(1,4) = 74.01$, $p = 0.01$, interaction: $F(1,4) = 2.70$, $p = 0.18$). Thus, microinfusions were discretely localized and did not alter SERT function in SNpr.

Next, we compared $[5\text{-HT}]_{max}$ evoked by varying stimulation frequencies. Fig. 4(e) shows a representative color plot and corresponding concentration trace that illustrates the effects of μCIT on serotonin signaling. $[5\text{-HT}]_{max}$ exhibited frequency dependence following μCIT (Fig. 4f; One-way ANOVA, $F(4,12) = 15.23$, $p < 0.01$). In contrast, $[5\text{-HT}]_{max}$ following μSAL was not frequency-dependent (Fig. 4f; One-way ANOVA, $F(4,12) = 1.19$, $p = 0.37$). When we compared $[5\text{-HT}]_{max}$ between μCIT and μSAL, we observed a frequency x treatment interaction (Fig. 4f; Two-way repeated measures ANOVA, $F(4,24) = 13.43$, $p < 0.0001$). *Post hoc* analyses revealed significant differences between $[5\text{-HT}]_{max}$ in μSAL and μCIT groups at 40 Hz (14.02 ± 1.50 vs. 27.03 ± 3.30 nM, $p < 0.01$), 50 Hz (15.08 ± 1.21 vs. 32.07 ± 2.61 nM, $p < 0.001$), and 60 Hz (12.80 ± 0.95 vs. 34.86 ± 4.90 nM, $p < 0.0001$). Thus, SERT inhibition in the DRN is sufficient to induce frequency-dependent enhancement of serotonin release in SNpr, even when local uptake is normal.

Discussion

Frequency-dependent release is well established for dopamine and norepinephrine (Garris and Wightman 1994; Park *et al.* 2009), and it is considered to be a fundamental component of signaling that transmits information about the intensity of inputs driving release. The present work examines the observation that *in vivo* serotonin release in the SNpr does not demonstrate frequency dependence in the same range as other monoamine neurotransmitters. In this study, we investigate the hypothesis that SERT function limits how serotonin release responds to high-frequency stimuli. We first demonstrate that $[5\text{-HT}]_{max}$ can be manipulated by altering stimulation intensity, but not stimulation frequency. Next, we show that acute CIT exposure enhances serotonin release in a frequency-dependent manner. CIT did not affect $[5\text{-HT}]_{max}$ in $SERT^{-/-}$ mice, indicating that this enhancement was indeed SERT mediated. Moreover, $SERT^{-/-}$ mice displayed a low level of baseline frequency dependence, suggesting that genetic loss of the transporter enabled frequency-dependent signaling in these subjects. Finally, we show that CIT's frequency-dependent effects on release, but not its effects on uptake, are mediated by inhibition of SERT in the DRN. Together, these results demonstrate that SERT function in the DRN profoundly limits how terminal serotonin release responds to high-frequency stimuli. They also describe a novel mechanism by which SSRIs can acutely increase serotonin signaling in a manner that retains information about the stimulus driving release. This finding may help us better interpret how serotonin conveys meaningful information about appetitive and aversive experiences and how SSRI therapies treat depressive disorders.

Under drug-naïve conditions, serotonin signaling is under robust regulation from multiple inhibitory elements. The extent of this regulation is illustrated by a nearly 50-fold difference between serotonin concentrations evoked in brain slices versus *in vivo* in the SNpr (Bunin *et al.* 1998; John and Jones 2007; Hashemi *et al.* 2011). In spite of limited release *in vivo*, SERT-mediated uptake rates are comparable between *in vivo* and brain slice preparations (Daws and Toney 2007). Since uptake shapes dynamic neurotransmitter release and maximal evoked concentrations (Wightman *et al.* 1988), these similarities in SERT function predict similarities in frequency-dependent release. Michaelis–Menten derived models predict that concentration will increase with stimulation frequency within a physiological range, based on assumptions that concentration of neurotransmitter released per pulse, density of transporters (V_{max}), and affinity of transporter for its neurotransmitter (K_M) for a given location in the brain remain constant under drug-naïve conditions (Wu *et al.* 2001). In addition to these models, studies of serotonin release in brain slices show frequency-dependent serotonin release (O'Connor and Kruk 1991; Iravani and

Kruk 1997; Bunin and Wightman 1998; Jennings *et al.* 2010). The lack of frequency dependence we report in WT, drug-naïve mice suggests that serotonin release may be regulated by more complex mechanisms than accounted for by previous kinetic models and slice experiments.

An important inconsistency between slice and *in vivo* serotonin measurements is highlighted in our comparison of serotonin release in WT versus SERT$^{-/-}$ mice. In brain slices, WT mice exhibit frequency-dependent serotonin release. Frequency dependence is attenuated in SERT$^{-/-}$ mice and after bath application of CIT in WT mice (Jennings *et al.* 2010). This finding aligns well with the predictions of a Michaelis–Menten derived model: since uptake shapes net evoked serotonin concentration, lack of uptake leads to similarities in net concentration evoked by every frequency. In contrast, the results of these *in vivo* studies found that genetic deletion of SERT causes frequency dependence where it was not previously present. One critical difference between the two experimental preparations is that *in vivo* preparations preserve intact circuitry between somatodendritic and axonal locations. Thus, *in vivo*, regulation at the level of serotonergic cell bodies contributes to terminal serotonin release, while slice preparations are able to measure regulation at the terminal in isolation from somatodendritic effects. We hypothesize that the same mechanisms promoting frequency-dependent release in CIT-treated WT mice *in vivo* promote frequency dependence in SERT$^{-/-}$ mice *in vivo*. The difference in robustness of the observed frequency dependence between the two groups may be as a result of altered serotonergic function in SERT$^{-/-}$ mice that compensates for lack of SERT; notably, enhanced 5-HT1A feedback to the DRN has been shown in SERT$^{-/-}$ mice (Soiza-Reilly *et al.* 2015), which may compensate for decreased function of 5-HT1A receptors within the DRN (Li 2006).

The mechanism underlying the efficaciousness of SSRIs as antidepressant therapies has remained somewhat inscrutable because of methodological inability to resolve their net effects on serotonin signaling. Microdialysis experiments, which examine changes in basal extracellular serotonin, have shown that acute CIT exposure increases ambient levels approximately 400% in the DRN and nearly 200% in forebrain terminal regions (Invernizzi *et al.* 1992). These increases in extracellular concentration require *increases* in serotonergic neuronal firing, but paradoxically, acute CIT exposure also induces 5-HT1A receptor-mediated *decreases* in spontaneous firing of serotonergic neurons (Perry and Fuller 1992; Gartside *et al.* 1995; Hajos *et al.* 1995). The 5-HT1A receptor, serotonin's autoreceptor, is known to rapidly internalize following acute exposure to both serotonin and CIT (Riad *et al.* 2001, 2004). This internalization may be driven by an acute CIT-induced increase in extracellular serotonin levels that saturates available 5-HT1A receptors. In contrast, continually elevated serotonin levels during chronic SERT inhibition may drive more persistent 5-HT1A desensitization and down-regulation of 5-HT1A mRNA in the DRN. It is this persistent down-regulation of autoreceptors that is believed to underlie the therapeutic efficacy of SSRIs (Blier *et al.* 1998; Bortolozzi *et al.* 2012). The present observation of enhanced release after acute CIT treatment helps reconcile the results of previous microdialysis and electrophysiological studies. We hypothesize that acute CIT causes basal serotonin concentrations to saturate 5-HT1A receptors in the DRN, leaving serotonergic neurons unable to up-regulate the inhibitory response to phasic inputs, such as high-frequency electrical stimulations. This results in a disinhibition of the response to stimulation that enables a frequency-dependent facilitation of terminal release. Thus, although basal serotonergic firing rates decrease, activity-dependent increases in extracellular serotonin levels throughout the brain can be driven by the DRN's increased responsiveness to phasic release-driving stimuli.

Acute SSRI exposure profoundly amplifies serotonin signaling in an input-coherent manner, but it remains unclear how amplifying serotonin release influences behavior. Recently, studies that selectively activate serotonin neurons using optogenetics in behaving animals have explored serotonin's role in motivated behavior. Liu *et al.* (2014) found that activating DRN serotonergic neurons is sufficient to drive real-time place preference and operant responding, suggesting that increased serotonin release can drive hedonic and motivated behaviors. However, work by McDevitt *et al.* (2014) indicates that glutamate co-released from serotonergic terminals is required for these DRN-driven behaviors, with serotonin playing a less critical role. Although serotonin neurons have been observed burst-firing at 100 Hz in trains of 2–3 spikes (Jacobs 1991; Hajos *et al.* 1995; Jacobs and Fornal 1999), and firing in response to optical stimuli can follow stimulation frequencies up to 50 Hz in channelrhodopsin-expressing serotonin neurons (Liu *et al.* 2014; McDevitt *et al.* 2014), our finding implies that neuronal response to high-frequency stimuli may not accurately predict the magnitude of serotonin release. In this study, higher frequency electrical stimulations did not result in increased serotonin release in drug-naïve mice. More work in this area is needed to determine to what extent optogenetic stimulation of serotonergic neurons evokes serotonin release, and whether this release is physiologically sufficient to drive appetitive or aversive behaviors.

In this study, we demonstrate a novel mechanism by which acute SSRIs can amplify serotonin release. Importantly, this mechanism requires intact circuitry between serotonergic cell bodies and their terminal release sites, which is uniquely enabled by *in vivo* serotonin measurements. The amplification of serotonin release is frequency-dependent, retaining information about the rate of stimulation, and is qualitatively similar to results observed in animals treated with SSRI for chronic periods (Dankoski *et al.* 2014). This similarity

between acute and chronic responses to SSRIs complicates how we interpret the role of serotonin in the antidepressant response. We hypothesize that during initial exposure to SSRIs, frequency-dependent burst-firing may be masked by dramatic increases in ambient serotonin levels. As extracellular serotonin levels return to pre-treatment levels over the chronic treatment period, facilitation of burst-firing may register as larger, clearer signals throughout the brain. A recent study by Cohen *et al.* (2015) in optically identified serotonergic neurons underscored the importance of both tonic and phasic firing modes of serotonin neurons. Our work concurs that a better understanding of these modes, and SERT's role in regulating them, may lead to more effective and expedient methods of treating depressive disorders.

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All experiments were conducted in compliance with the ARRIVE guidelines.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1. Comparison of frequency dependence in WT+CIT, $SERT^{-/-}$, and $SERT^{-/-}$ + CIT groups.

Table S1. Effects of acute CIT on $[5\text{-HT}]_{max}$, release rate, and $t_{1/2}$ in WT mice.

Table S2. Effects of acute CIT on $[5\text{-HT}]_{max}$ and $t_{1/2}$ in $SERT^{-/-}$ mice.

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