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Both synthesis and reuptake are critical for replenishing the releasable serotonin pool in *Drosophila*

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Abstract

The two main sources of serotonin available for release are expected to be newly synthesized serotonin and serotonin recycled after reuptake by the serotonin transporter (SERT). However, their relative importance for maintaining release and the time course of regulation are unknown. We studied serotonin signaling in the ventral nerve cord of the larval *Drosophila* central nervous system. Fast-scan cyclic voltammetry at implanted microelectrodes was used to detect serotonin elicited by channelrhodopsin2-mediated depolarization. The effects of reuptake were probed by incubating in cocaine, which is selective for the serotonin transporter in *Drosophila*. *p*-chlorophenylalanine (PCPA), an inhibitor of tryptophan hydroxylase2, was used to investigate the effects of synthesis. Stimulations were repeated at various intervals to assess the time course of recovery of the releasable pool. Reuptake is important for the rapid replenishment of the releasable pool, on the 1 minute time scale. Synthesis is critical to the longer-term replenishment (10 min) of the releasable pool, especially when reuptake is also inhibited. Concurrent synthesis and reuptake inhibition decreased both serotonin tissue content measured by immunohistochemistry (by 50%) and the initial amount of evoked serotonin (by 65%). Decreases in evoked serotonin are rescued by inhibiting action potential propagation with tetrodotoxin, implicating endogenous activity in the depletion. These results show synthesis is necessary to replenish part of the releasable serotonin pool that is depleted after reuptake inhibition, suggesting that regulation of synthesis may modulate the effects of serotonin reuptake inhibitors.

Keywords

FSCV; *Drosophila*; tryptophan; SERT; cocaine; SSRI

Introduction

The serotonin transporter (SERT) plays a key role in regulating serotonin signaling by clearing serotonin from the extracellular space and recycling it to the intracellular space where it can be repackaged into vesicles (Murphy *et al.* 2004). Polymorphisms conferring decreased SERT activity have been associated with anxiety and the development of depression under stressful conditions (Murphy *et al.* 2004, Lasky-Su *et al.* 2005, Li and He 2007). Therefore, SERT is a molecular target for many neuropsychiatric disorders and

selective serotonin reuptake inhibitors (SSRIs) are commonly used to treat the approximately 5–10 % of Americans diagnosed with depression per year (Bauer *et al.* 2007). The time between SSRI treatment onset and effect is delayed and 30–40 % of people with depression do not respond to SSRI treatment (Smits *et al.* 2007). A better understanding of the physiology of serotonergic regulation might help explain these effects.

In the central nervous system, serotonin is synthesized by a two-step enzymatic process beginning with the rate-limiting enzyme, tryptophan hydroxylase 2 (Tph2), which converts tryptophan into 5-hydroxytryptophan (Zhang *et al.* 2004, Coleman and Neckameyer 2005). After packaging into vesicles, serotonin is released via exocytosis into the extracellular space. Serotonergic neurons are spontaneously active at 0.5 to 1 Hz in the invertebrate lobster (Ma *et al.* 1992) and 0.6 Hz in mammals, with a subpopulation undergoing burst firing (Chu *et al.* 2004). Serotonin synthesis may also play a role in mental illnesses as polymorphisms conferring decreased Tph2 expression have been associated with suicidal behavior (Li and He 2007), but this remains controversial (Haghighi *et al.* 2008).

Serotonin neurobiology has been studied extensively in mammalian models, particularly in mice. For example, SERT knockout mice have been used to probe the function of SERT and these mice have increased extracellular concentrations of serotonin (Bengel *et al.* 1998, Mathews *et al.* 2004). Other studies have shown Tph2 activity correlates with serotonin release (Gartside *et al.* 1992) and Tph2 polymorphic variants can decrease serotonin concentrations (Zhang *et al.* 2004). Therefore, both Tph2 and SERT activity are expected to regulate neuronal serotonin concentrations. However, the relative importance of these roles as well as the time course of replenishment of the functional serotonin pool is not well understood.

In this study, we address this question by evaluating the roles of synthesis and reuptake in maintaining the size of the releasable serotonin pool in *Drosophila*. *Drosophila* larvae were chosen as a model system because they boast a fully developed network of serotonergic neurons in 5 days, within a small, easily accessible and relatively simple central nervous system (Yuan *et al.* 2005, Hamasaka and Nassel 2006). An advantage of *Drosophila* is that genetic mutants can be quickly and easily made, facilitating large scale genetic screens. However, before screens can be performed to assess the effects of mutations on serotonin regulation, an understanding of the normal regulation of serotonin release is needed. We have developed a method to monitor endogenous neurotransmitter changes in *Drosophila* at a microelectrode after channelrhodopsin-2 (ChR2) mediated stimulation (Borue *et al.* 2009, Vickrey *et al.* 2009). Measurements of real-time neurotransmitter dynamics are challenging because of the small size of the *Drosophila* nervous system and the only other method is to measure clearance of exogenously applied neurotransmitter (Makos *et al.* 2009). Our method of stimulating release allows measurements of the releasable pool, the amount of serotonin available for exocytosis. Synthesis was pharmacologically inhibited by PCPA. Cocaine was chosen to inhibit reuptake, instead of a traditional SSRI, because *Drosophila* SERT (dSERT) has slightly different pharmacology than in mammals. Cocaine has greater affinity and is more specific for dSERT than SSRIs such as fluoxetine (Porzgen *et al.* 2001). We found that both synthesis and reuptake are needed to maintain serotonergic release, with reuptake being more important for the short-term replenishment of the releasable pool and synthesis more important over longer periods.

Materials and Methods

Fly stocks

Flies containing Channelrhodopsin-2 (ChR2) under the control of a GAL4 binding upstream activator sequence (a gift from Christian Schroll, Universitat Wurzburg) were crossed to

flies expressing Tph2-GAL4 (a gift from Jaeson Kim, Korea Advanced Institute of Science and Technology) to generate a homozygous line. Because ChR2 was only expressed in cells where GAL4 was present, ChR2 was only expressed in serotonergic neurons that express tryptophan hydroxylase 2 (Tph2). More details about fly crosses are given in the supplemental methods. Larvae were fed on yeast supplemented with 10 mM all-trans retinal (Sigma-Aldrich, St. Louis) for 2–3 days prior to the initiation of experiments while protected from light.

Immunohistochemistry

To estimate neuronal serotonin levels, we performed immunohistochemistry for the neurotransmitter. Larval ventral nerve cords (VNCs) were dissected and imaged as previously described (Chen and Condron 2008). See supplemental methods for details. Neuronal serotonin content was quantified using imageJ. Mean gray levels (MGLs), including neuronal cell bodies, were measured in a rectangle that covers the middle two-thirds of the imaged neuropil region. This area showed the least amount of staining variability between VNCs but also tended to experience the smallest reductions in staining, which may minimize the impact of drug treatments. VNCs incubated in drugs were processed at the same time as control VNCs and were normalized as a percent MGL in accompanying buffer controls.

Instrumentation and electrochemistry

Carbon-fiber microelectrodes were made in house from single T-650 carbon fibers (7 μm diameter, Cytec Engineering Materials, West Patterson, NJ), as previously described (Swamy and Venton 2007). A Dagan ChemClamp potentiostat was used to collect electrochemistry data (Dagan, Minneapolis, MN; custom-modified). Data acquisition software and hardware were the same as described by Heien *et al.* (2003). To detect serotonin, the voltage was ramped from 0.2 V to 1.0 V, then to -0.1 V and back to 0.2 V at 1,000 V/s with a repetition rate of 10 Hz (Jackson *et al.* 1995). A silver-silver chloride wire was used as a reference electrode. Electrodes were calibrated with 1 μM serotonin after use *in situ*.

Preparation of ventral nerve cords (VNCs) and data collection

VNC preparation and data collection were performed as described (Borue *et al.* 2009). Ventral nerve cords (VNCs) from wandering, 5 day-old larval *Drosophila* were used because they are the most mature larvae and have a fully developed serotonergic system (Sykes and Condron 2005). Five day old larval VNCs were dissected in modified Schneider's insect media (15.2 mM MgSO_4 , 21 mM KCl, 3.3 mM KH_2PO_4 , 53 mM NaCl, 5.8 mM NaH_2PO_4 , 5.4 mM CaCl_2 , 11.1 mM Glucose, 5.3 mM Trehalose, pH = 6.2). 4-Chloro-DL-phenylalanine (PCPA) and cocaine hydrochloride were purchased from Sigma-Aldrich. Tetrodotoxin (TTX) was obtained from Alomone Labs (Stock #T-550, Jerusalem, Israel) and resuspended in doubly distilled H_2O to obtain a 1 mM stock concentration. Frozen aliquots were diluted to the working concentration (0.5 μM) in buffer at the time of the experiment. VNCs were incubated in buffer or drug for 25–30 min prior to the initiation of experiments, a time that is sufficient for drugs to diffuse into the nerve cord (Borue *et al.* 2009). An electrode was implanted in the neuropil using a micromanipulator and allowed to equilibrate for 5 min before data collected. After the collection of at least 30 seconds of baseline data, VNCs were exposed to 10 s of intense blue light to stimulate release. This stimulation length produces close to maximal serotonin release (Borue *et al.* 2009). The light source was a 10W halogen microscope bulb with a standard fluorescein excitation filter (450 – 490 nm) that was manually-switched.

Data analysis

Electrochemical data was analyzed using Tar Heel CV software (gift of Mark Wightman). For a detailed description of serotonin detection in the fly using FSCV, see Borue et al. (2009). Cyclic voltammograms were used to verify serotonin was detected. The current at the maximum serotonin voltage was converted to serotonin concentration using post calibration data for each electrode and concentration changes plotted vs time. The initial stimulated peak height was used to normalize data for multiple stimulation assays, where stimulations were performed with the start of the 10 s stimulation occurring every 1, 2, 5, or 10 minutes. A series of *in vitro* experiments performed in a flow cell showed that electrode sensitivity to serotonin decreased slightly over the course of multiple exposures (Supplemental figure 1). A linear correction coefficient was applied to all data from multiple stimulation experiments to correct for changes in electrode sensitivity. This correction does not change the interpretation of data because it does not change the relationships between categories of VNCs. Statistical analyses of pooled data including two-tailed Student t tests were conducted using Excel. One and two way ANOVAs was performed using GraphPad Prism software (San Diego, CA). Means are given as \pm standard error of the mean (SEM).

Results

Neuronal serotonin content after pharmacological manipulations

We explored the effects of pharmacological manipulations on neuronal serotonin content as measured by immunohistochemistry, the most common method for studying neurotransmitters in *Drosophila*. While immunohistochemistry provides only an estimate of neuronal serotonin tissue levels, due to antibody-mediated amplification of fixed serotonin, these rough numbers can be used to show the relative importance of each of the drugs on tissue content (Mize *et al.* 1988). A control VNC, incubated in buffer, shows normal serotonergic morphology and serotonin tissue content (Fig. 1a). The VNC is a segmented structure that contains four serotonergic neurons per segment, two on each side of the midline (Chen and Condron 2008). The serotonergic cell bodies are visible as pairs of brightly stained circles on either side of the neuropil while the projections appear as two strips of brightly stained material overlying the cell bodies. The imaging was optimized to show residual serotonin staining in drug-exposed VNCs and therefore the control VNC appears slightly over-exposed.

The effect of spontaneous firing as well as synthesis and reuptake inhibition on tissue content was tested using pharmacological agents. Action potential propagation was inhibited using 0.5 μ M tetrodotoxin (TTX) to test the effects of spontaneous neuronal activity on tissue content. A representative image after TTX incubation (Fig. 1b) shows similar staining brightness to the buffer control. Reuptake was inhibited with cocaine, which has a 6-fold greater affinity for SERT than the dopamine transporter in the fly (Porzgen *et al.* 2001). The example images show the staining is dimmer after 10 μ M cocaine incubation (Fig. 1c). Concurrent application of TTX with cocaine ameliorates the decreases in staining intensity (Fig. 1d). Synthesis was blocked by p-chlorophenylalanine (PCPA), which inhibits the neuronal serotonin synthesis enzyme Tryptophan hydroxylase 2 (Tph2) (Coleman and Neckameyer 2005). Similar to cocaine, 100 μ M PCPA incubation decreased serotonin content, particularly in the projections, with the most proximal and distal abdominal segments being most affected (Fig. 1e). Slightly brighter serotonin staining is observed with PCPA and TTX (Fig. 1f) compared to PCPA only. With both cocaine and PCPA incubation (Fig. 1g), the staining intensity is much dimmer than control or with either drug alone, indicating less tissue content. The addition of TTX with cocaine and PCPA increases the brightness (Fig. 1h).

Average tissue content is plotted as a percentage of control in Fig. 1i. A one-way ANOVA, comparing buffer, cocaine, PCPA and cocaine + PCPA, showed a significant effect of drug treatment on serotonin content ($p < 0.001$). Bonferonni post-tests were used for pairwise comparisons (see Supp. Table 1 for p values). Cocaine significantly decreased tissue content by about 30 % ($p < 0.001$) and PCPA significantly decreased tissue content by about 40 % ($p < 0.001$). With both PCPA and cocaine, serotonin content decreases significantly to 50% of control ($p < 0.001$). Tissue content is significantly smaller for the combination of PCPA and cocaine than just cocaine ($p < 0.01$) but not compared to PCPA ($p > 0.05$).

For TTX results, Student t-tests were used to compare the tissue content after drug treatments with and without TTX. Serotonin tissue levels after TTX incubation were not significantly different from buffer (Fig 1i, $p = 0.25$). Significantly higher tissue contents were observed with cocaine + TTX than with just cocaine ($p = 0.002$). PCPA + TTX was not significantly greater than PCPA incubation alone ($p = 0.16$). Serotonin content increased to ~80% of control when TTX is administered in addition to PCPA and cocaine, a significant increase over PCPA + cocaine ($p < 0.0001$).

Characterization of stimulated serotonin release in the fly

The immunohistochemistry results show that synthesis and reuptake inhibition decreased serotonin content, but these studies do not reveal how much serotonin is functionally available for release. We utilized a novel combination of methods to elicit and detect real-time serotonin release in *Drosophila* (Borue *et al.* 2009). Release was mediated by channelrhodopsin-2 (ChR2), a blue light-activated, cation-selective ion channel that was expressed under the control of the serotonergic neuron-specific driver, Tph2-GAL4 (Zhang *et al.* 2004). Fast-scan cyclic voltammetry (FSCV) at an implanted carbon-fiber microelectrode was used to detect serotonin in the extracellular space in real time.

Figure 2 shows serotonin release elicited by 10 s exposure of the VNC to blue light. The peaks in the cyclic voltammogram (CV) measured in a VNC expressing ChR2 (Fig 2a, black line) are located at the same voltages as the 1 μ M serotonin electrode calibration CV (red line), confirming that serotonin was detected. Pharmacological experiments also previously confirmed the identity of the neurotransmitter as serotonin (Borue *et al.* 2009). Fig. 2b shows serotonin concentration changes over time for two consecutive stimulations, performed in the same sample 2 min apart. The peak height decreased for the second stimulation but the time course is comparable. In a control VNC not expressing ChR2, the CV did not show any peaks characteristic of serotonin, indicating serotonin release was not detected (Fig. 2c). The concentration vs. time trace, at the potential for serotonin oxidation (Fig 2d), shows a small fluctuation due to noise, likely from blue light-induced ionic changes that affect the background current.

The data were quantified using two parameters, peak height and time to half-maximal signal decay (t_{50}). Peak height is the maximal detected concentration and t_{50} is the time from the end of the stimulation until the signal has decayed to half the peak height. For VNCs expressing ChR2 dissected in buffer, the peak height for the initial 10 s stimulation was 600 \pm 30 nM and the t_{50} was 4.6 \pm 0.3 s ($n = 35$).

Effects of multiple stimulations

To estimate the rate at which serotonergic neurons can replenish the releasable pool of serotonin, multiple stimulations were performed 1, 2, 5, or 10 minutes apart. As shown in Figures 3a and b, there was no significant difference in average evoked release or t_{50} for the initial stimulation between sample groups. Therefore, to compare the peak heights at different stimulation intervals, we normalized data to the initial peak height for each animal

(Fig. 3c). A two-way ANOVA shows a significant interaction of stimulation number and interval ($p < 0.001$). When stimulations were performed either 1 or 2 minutes apart, a progressive decrease in peak height is evident. The extent of decay was not significantly different for stimulations performed 1 or 2 min apart (Bonferroni post-test, $p > 0.05$). However, when stimulations were performed every 5 or 10 minutes, the signal remained relatively stable and the last stimulation evoked peak concentrations similar to the first (Fig. 3c). Therefore, the replenishment of the releasable pool occurs on a time scale between 2 and 5 min. Because the largest signal decay occurs within the first 4 stimulations, we focused our analysis on these stimulations for pharmacological experiments.

Effect of reuptake inhibition on stimulated release

Cocaine inhibits uptake and increases the time course of serotonergic signaling (Borue *et al.* 2009). Incubation in 10 μM cocaine did not have a significant effect on the initial stimulated peak height but did significantly increase t_{50} ($p < 0.001$, Fig. 4, see Supp. Table 2 for all statistics). The effects of cocaine incubation on stimulated release performed at 1 and 10 minute intervals are shown in Fig. 5. A two-way ANOVA of the data in Fig. 5a showed a significant interaction of stimulation number and drug ($p < 0.001$). The peak height decays significantly more in cocaine-incubated VNCs than in buffer-incubated samples when stimulations are repeated at 1 min intervals (Bonferroni post-test, see Suppl. Table 3 for all p values). On the fourth stimulation, the peak height is $76 \pm 3\%$ of the initial value in buffer but only $54 \pm 3\%$ in cocaine. Cocaine was chosen because it offers a greater selectivity for dSERT than SSRIs like fluoxetine (Porzgen *et al.* 2001). However, similar results were obtained with fluoxetine for closely repeated stimulation experiments (Supp. Fig. 2), showing that interpretation of the cocaine data applies to typical SSRIs as well.

VNCs stimulated every 10 minutes in the presence of cocaine show significantly smaller peak heights for stimulations 2 to 4, decaying by about 20% (Fig. 5b). However, after cocaine, the decay in peak height with 10 min intervals is significantly less than with 1 min intervals (see Supp. Fig. 3 for overlaid data, Two-way ANOVA with Bonferroni post-test, see Supp. Table 4). Therefore, reuptake inhibition has a greater effect on closely repeated stimulations.

Effect of synthesis inhibition on stimulated release

Inhibiting synthesis with PCPA should also reduce the amount of serotonin available for release. However, on the first stimulation, 100 μM PCPA shows no significant difference from buffer (Fig. 4, One way ANOVA with Bonferonni post-test, $p > 0.05$). Similar to the cocaine results, although tissue content is reduced by about one-third after PCPA (Fig. 1 e), the initial stimulation is not significantly reduced.

When stimulations are performed at 1 min intervals in the presence of PCPA, a trend is evident towards smaller release than buffer controls that becomes significant by the 4th stimulation, when the peak height is $64 \pm 3\%$ of the initial value (Fig. 5a, Bonferroni post-test, see Supp Table 3). A significantly smaller peak height than control for stimulations 2 to 4 is also observed with 10 min intervals for PCPA (Fig. 5b). The decay in peak height is very similar for stimulations performed every 1 or 10 minutes with PCPA (Two-way ANOVA with Bonferroni post-test, see Supp. Table 4). Thus, the inter-stimulation time does not play as big of a role when synthesis is inhibited and peak height after four stimulations is similar, regardless of the time between stimulations.

Comparing the PCPA and cocaine data, the signal decays faster for closely repeated stimulations in cocaine than in PCPA (Fig. 5a). The normalized peak heights for the second, third and fourth stimulation are significantly lower in cocaine than in PCPA with 1 min.

intervals (Two-way ANOVA with Bonferroni post-test, see Supp. Table 3). The trends are different when stimulations are repeated 10 min. apart as PCPA peak heights are significantly lower than cocaine for stimulations 2 to 4 (Supp. Table 3). Therefore, cocaine and PCPA have different effects based on the interval of stimulation repetition. During closely repeated stimulations, peak height decreases faster after cocaine while PCPA causes a greater decrease when stimulations are performed 10 minutes apart.

Effects of combined synthesis and reuptake inhibition on stimulated release

To determine if the effects of synthesis and reuptake inhibition are additive, we incubated samples in 100 μ M PCPA and 10 μ M cocaine. Raw data from these VNCs (Supp. Figure 4) shows small peaks at the serotonin oxidation and reduction potentials in the CV, indicating serotonin is released; however, peak height is severely diminished, even on the first stimulation. Pooled data show that VNCs exposed to both drugs exhibited a significant reduction in the initial peak height to about one-third of the buffer value (Fig. 4a). Initial peak height is also significantly lower for PCPA + cocaine than in PCPA or cocaine alone ($p < 0.001$, One-way ANOVA with Bonferroni post-test, Supp. Table 2). The t_{50} for the combination is also significantly greater than buffer or cocaine alone (Fig. 4b, One-way ANOVA with Bonferroni post-test, Supp. Table 2), likely an artifact of the smaller peak height.

Figure 6 shows the effects of concurrent PCPA and cocaine on the peak height of stimulations performed 1 min apart. Because of the large differences in initial peak height, we did not normalize the data. By stimulation 6, the CVs do not display any serotonin specific peaks and the peak height is not significantly different from VNCs that do not express ChR2 (Student t-test, $n = 6-9$, $p = 0.056$). Therefore, when synthesis and reuptake are concurrently inhibited, five stimulations deplete the releasable pool of serotonin to below the limits of detection.

Effect of spontaneous activity on serotonin depletion

To determine the extent to which spontaneous activity plays a role in the depletion of the releasable pool, action potential propagation was inhibited with tetrodotoxin. We predicted that ChR2-mediated release, which occurs as a result of direct depolarization of the neurons, would not be affected by TTX. Indeed, on the initial stimulation, VNCs incubated in 0.5 μ M TTX release significantly more serotonin, 800 ± 60 nM, than those incubated in buffer ($p < 0.01$, Fig. 4a). TTX did not have a significant effect on the t_{50} (Supp. Table 2). The increase in release after TTX implies that endogenous activity depletes approximately one quarter of the releasable serotonin pool. When stimulations were repeated 1 min apart, peak heights were significantly larger than control for TTX until the fourth stimulation (Fig 6, Two-way ANOVA with Bonferroni post-test, See Supp. Table 5). This indicates that the extra serotonin that was not released during endogenous activity was depleted fairly quickly.

To test whether the depletion of serotonin with PCPA and cocaine was due to spontaneous activity, action potential propagation was concurrently inhibited with TTX. The initial peak height with PCPA, cocaine, and TTX is 610 ± 30 nM, significantly higher than for PCPA + cocaine (Fig 4a, One-way ANOVA, Bonferroni post-test, full statistics in Supp. Table 2) but not significantly different from buffer. The effect of TTX in conjunction with PCPA and cocaine, increasing the concentration 400 nM over cocaine + PCPA, is much larger than the effect of TTX on VNCs that were incubated in only buffer, where the increase was only 200 nM. Serotonin clearance rate in cocaine + PCPA + TTX is consistent with the presence of cocaine (Fig. 4b).

While the initial stimulated concentration is the same for VNCs incubated in buffer and those incubated in the combination of TTX + PCPA + cocaine, the pattern of release for repeated stimulations was different (Fig. 6). With the 3 drug combination, the peak height decreased more rapidly than the buffer control samples with 1 min intervals (peaks 6–8 are significantly lower than buffer for TTX + PCPA + cocaine, Two-way ANOVA with Bonferroni post-test, see Supplemental Table 5). This demonstrates that while TTX rescues the release for the initial stimulation, less serotonin is available for repeated stimulations.

Discussion

In this paper, we use measurements of stimulated serotonin release in *Drosophila* to show that both serotonin synthesis and reuptake determines the size of the releasable pool. The combination of synthesis and reuptake inhibition results in substantial decreases in both neuronal serotonin content and evoked release. While cocaine was used to inhibit reuptake because of its greater selectivity in *Drosophila* for dSERT, similar stimulated release results were obtained for fluoxetine, a classic SSRI. Therefore, the interpretation of these results might be extended to SSRIs, giving insight that rapidly repeated stimulations actually deplete serotonin after acute uptake inhibition and that lowered synthesis enlarges this effect.

The releasable serotonin pool in *Drosophila* is influenced by neuronal activity

To deplete the releasable pool, serotonergic neurons were specifically activated with large, 10 s stimulations. The initial stimulation released a large amount of serotonin, ~600 nM, similar to electrical stimulations in rat substantia nigra slices (Bunin and Wightman 1998, John and Jones 2007). Evoked release was stable when stimulations were performed 5 or more minutes apart, suggesting that the releasable pool is replenished in 2–5 minutes after a large release of serotonin. Similarly, five minute intervals are used in repeated electrical stimulation experiments in rat brain slices to elicit stable serotonin release (Bunin *et al.* 1998).

Serotonergic neurons in mammals and other invertebrates are spontaneously active with firing frequencies about 1 Hz (Ma *et al.* 1992, Chu *et al.* 2004) and spontaneous activity might deplete the releasable pool. Our methodology is uniquely suited to test this hypothesis because ChR2 activation directly depolarizes neurons (Nagel *et al.* 2003); thus, ChR2-mediated stimulation is not dependent on action potential propagation. TTX increased evoked release, implying that serotonergic neurons undergo spontaneous activity in this VNC preparation and that this depletes some of the releasable serotonin pool. The increased release after TTX could result from an increase in total neuronal serotonin content or reallocation of serotonin to the releasable pool. Because neuronal serotonin content was not significantly different from controls after TTX incubation, the immunohistochemistry data suggest a reallocation of the releasable pool, possibly due to accumulation of primed vesicles at release sites. Indeed, peak heights in TTX-incubated VNCs were initially larger but by the 4th stimulation were similar to controls.

Reuptake and synthesis control the size of the releasable pool

While reuptake inhibition and synthesis inhibition have both been shown to regulate release in mammalian models, the relative importance and time course of their effects have not been elucidated for serotonin. Both a releasable pool and a reserve pool of vesicles have been identified in mammals (Ewing *et al.* 1983) and *Drosophila* (Kuromi and Kidokoro 1998), but younger vesicles with newly synthesized neurotransmitter are usually preferentially released (Duncan *et al.* 2003). Mechanisms responsible for maintaining release have been studied for dopamine and long (10 s) stimulations repeated at 20 min intervals deplete

almost all the dopamine readily releasable pool after synthesis inhibition in anesthetized rats (Ewing *et al.* 1983). Subsequent administration of reuptake inhibitors, such as amfonelic acid or cocaine, actually increases dopamine release by activating a reserve pool of vesicles (Ewing *et al.* 1983, Venton *et al.* 2006). Our serotonin results in *Drosophila* with 10 min stimulation intervals also show synthesis inhibition causes at large depletion with repeated stimulation. While reuptake inhibition has less of an effect at 10 min intervals, cocaine does decrease release, implying that, in contrast to dopamine, cocaine does not activate a large serotonin reserve pool. Recycling by SERT still has a small role in maintaining serotonin release on the 10 min time scale.

A different picture emerges with closely repeated stimulations, at 1 or 2 minute intervals. Previous mammalian dopamine studies have found that the release is independent of synthesis and that reuptake plays only a small role in the recovery with 2 min stimulation intervals (Michael *et al.* 1987a, Michael *et al.* 1987b). In contrast, we found that synthesis did play a role in the recovery of serotonin release at 2 min intervals, as evoked release decreased more than control. In addition, cocaine depleted releasable serotonin with closely repeated stimulations significantly more than buffer or PCPA. Therefore, recycled serotonin plays an important part in maintaining the releasable pool and is more important than synthesis on the shorter time scale. These results indicate that the relative importance of synthesis and reuptake may be different for dopamine and serotonin, although more direct comparisons between species are needed.

When both synthesis and uptake inhibition were combined, the result was a large decrease in initial evoked release. Serotonin depletion occurred due to endogenous activity because concurrent TTX administration increased the initial peak to normal levels. Combining synthesis and reuptake inhibition severely depletes the releasable pool, and no serotonin is detectable after 5 stimulations, indicating that during reuptake inhibition synthesis is critical to maintaining release.

Immunohistochemistry has been the traditional method to study neurotransmitters in *Drosophila*, but the functional measure of serotonin release did not always match the tissue content data. For example, PCPA or cocaine alone decreased neuronal content by about a third without significantly decreasing initial peak height. The decreases in staining intensity by acute cocaine are similar to those observed in Tph2 hypomorph flies after chronic fluoxetine feeding (Neckameyer *et al.* 2007). Incubation in cocaine and PCPA decreased both stimulated serotonin release (by 65 %) and serotonin tissue content (by 50 %). Therefore, a large decrease in content may be necessary to affect stimulated release, implying that not all of the measured serotonin content may be available for release. Future studies could test this hypothesis by staining nerve cords after repeated stimulations. However, our studies show that immunohistochemistry estimates of tissue content may not be a good measure of functional serotonin.

In summary, two sources for serotonin to be released are newly synthesized serotonin and serotonin that has been recycled via SERT (Supp. Fig. 5). On a 1–2 min time scale, reuptake is responsible for maintaining about half the releasable pool after 4 stimulations while synthesis maintains about a third. On a longer, 5–10 min time scale, synthesis has the same contribution, a third, while reuptake maintains about a fifth of the pool. Thus, reuptake is important for the rapid replenishment of the releasable pool and synthesis is relatively more important for longer-term maintenance of the releasable pool. Additionally, endogenous activity depletes the releasable pool by about a quarter.

This study concentrated on identifying the sources of serotonin that could be released. Serotonin release could also be controlled by autoregulatory feedback. Although a serotonin

autoreceptor has not yet been conclusively identified in the fly, the application of large amounts of exogenous serotonin causes retraction of serotonergic projections (Sykes and Condron, 2005). In addition, a *Drosophila* analog of 5-HT_{1a} receptors regulates circadian rhythms, as the autoreceptor does in mammals (Yuan *et al.* 2005). Drugs such as cocaine or TTX could affect basal levels, leading to differential autoreceptor activation. However, the decrease in staining intensity after cocaine suggests that depletion rather than reallocation of serotonin is responsible for observed decreases in peak height and basal levels of serotonin are expected to be low, 0.1–10 nM (Yoshitake *et al.* 2003, Gardier *et al.* 2003), compared to stimulated release. Future studies could probe autoreceptor function with our technique by monitoring stimulated release after administration of putative serotonin autoreceptor inhibitors.

Serotonin synthesis and SSRIs

While *Drosophila* are not as complex as mammalian models, they have similar release and reuptake machinery that facilitates studies of basic serotonin regulation. Serotonin reuptake inhibitors are thought to improve mood in depressed patients by raising extracellular serotonin concentrations (Murphy *et al.* 2004). Our data show that after acute reuptake inhibition, less serotonin is available for release during closely repeated stimulations and that concurrent synthesis and reuptake inhibition exacerbates this depletion. While other factors may also be important, such as autoreceptor control, the ability to maintain the releasable serotonin pool after uptake inhibition may depend on the upregulation of synthesis. Although acute SSRI treatment can suppress serotonin synthesis (Carlsson and Lindqvist 1978, Stenfors *et al.* 2001, Yamane *et al.* 2001), studies of the effect of chronic SERT inhibition on synthesis have produced mixed results depending on methodology, with both increases (Moret and Briley 1992, Stenfors and Ross 2002) and decreases (Yamane *et al.* 2001, Honig *et al.* 2009) reported. SERT knockout mice and rats exhibit elevated synthesis rates and increased release per stimulation pulse, likely an adaptation to decreased reuptake (Kim *et al.* 2005, Homberg *et al.* 2007).

Serotonin synthesis has been implicated in the pathophysiology of depression. In rodents, the onset of increased synthesis after SSRI administration (Kim *et al.* 2002) correlates with the onset of anti-depressant effect (Shishkina *et al.* 2007). Furthermore, in mice with a 50% decrease in Tph2 expression, a decreased response to antidepressants can be restored by boosting serotonin synthesis with the precursor tryptophan (Cervo *et al.* 2005, Invernizzi 2007). In humans, polymorphisms resulting in decreased expression of Tph2 have been associated with suicidal behavior (Li and He 2007) and low levels of tryptophan have been found in depressed individuals (Cowen *et al.* 1989). SSRI efficacy can be improved by tryptophan administration and patients that respond to SSRIs experience a relapse of depressive symptoms after PCPA or acute tryptophan depletion (Delgado *et al.* 1990). Thus, psychiatric treatments that combine increased synthesis and with reuptake inhibition might be a productive line of study. In the future, large scale screens could be performed to identify genetic elements critical for Tph2 or SERT function and their impact on serotonin release. These studies would be much faster and easier in *Drosophila* than mammals, and would be informative for future development of personalized treatments of psychiatric illness based on genetic screening.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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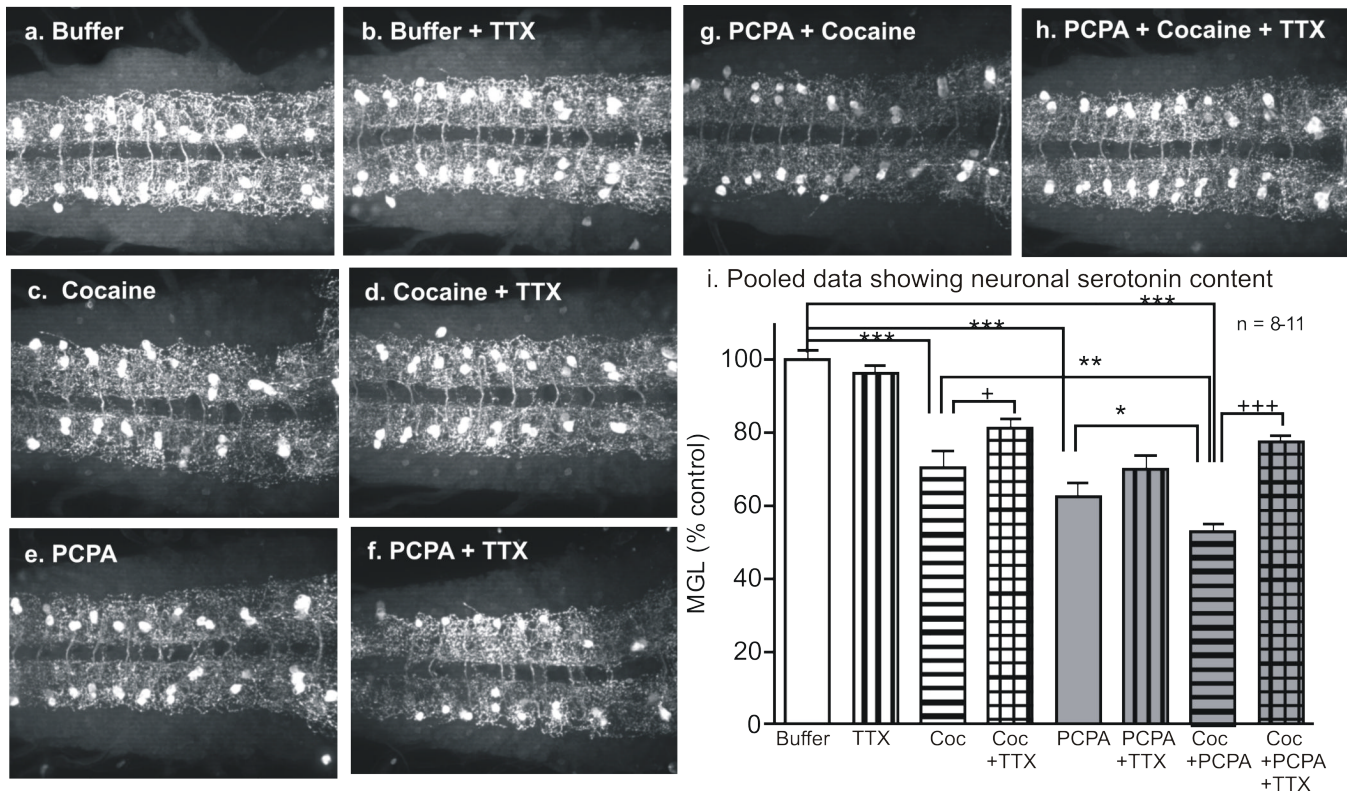


Figure 1.

Effect of reuptake, synthesis, and activity inhibition on neuronal serotonin content.

Representative images from VNCs stained for serotonin (white) after 30 minute incubation in buffer or drugs are shown in a–h. (a) VNC incubated in buffer. (b) VNC incubated in 0.5 μ M TTX is similar to buffer. (c) VNC incubated in 10 μ M cocaine is dimmer than buffer, indicating lower serotonin content. (d) 10 μ M Cocaine + 0.5 μ M TTX is brighter than cocaine alone. (e) 100 μ M PCPA is dimmer than buffer. (f) 100 μ M PCPA + 0.5 μ M TTX is similar to PCPA. (g) 100 μ M PCPA + 10 μ M cocaine is dimmer than buffer or either drug alone. (h) 100 μ M PCPA + 10 μ M cocaine + 0.5 μ M TTX is brighter than PCPA + cocaine. (i) Pooled data are quantified as a % of staining intensity in buffer controls. A one-way ANOVA showed a significant effect of drug treatment ($p < 0.001$, full statistics in Supp Table 1). Asterisks indicate significant differences from Bonferonni post-tests (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Plus signs show significant differences comparing the effect of a drug with and without TTX (Student t-tests) (+ $p < 0.05$, +++ $p < 0.001$).

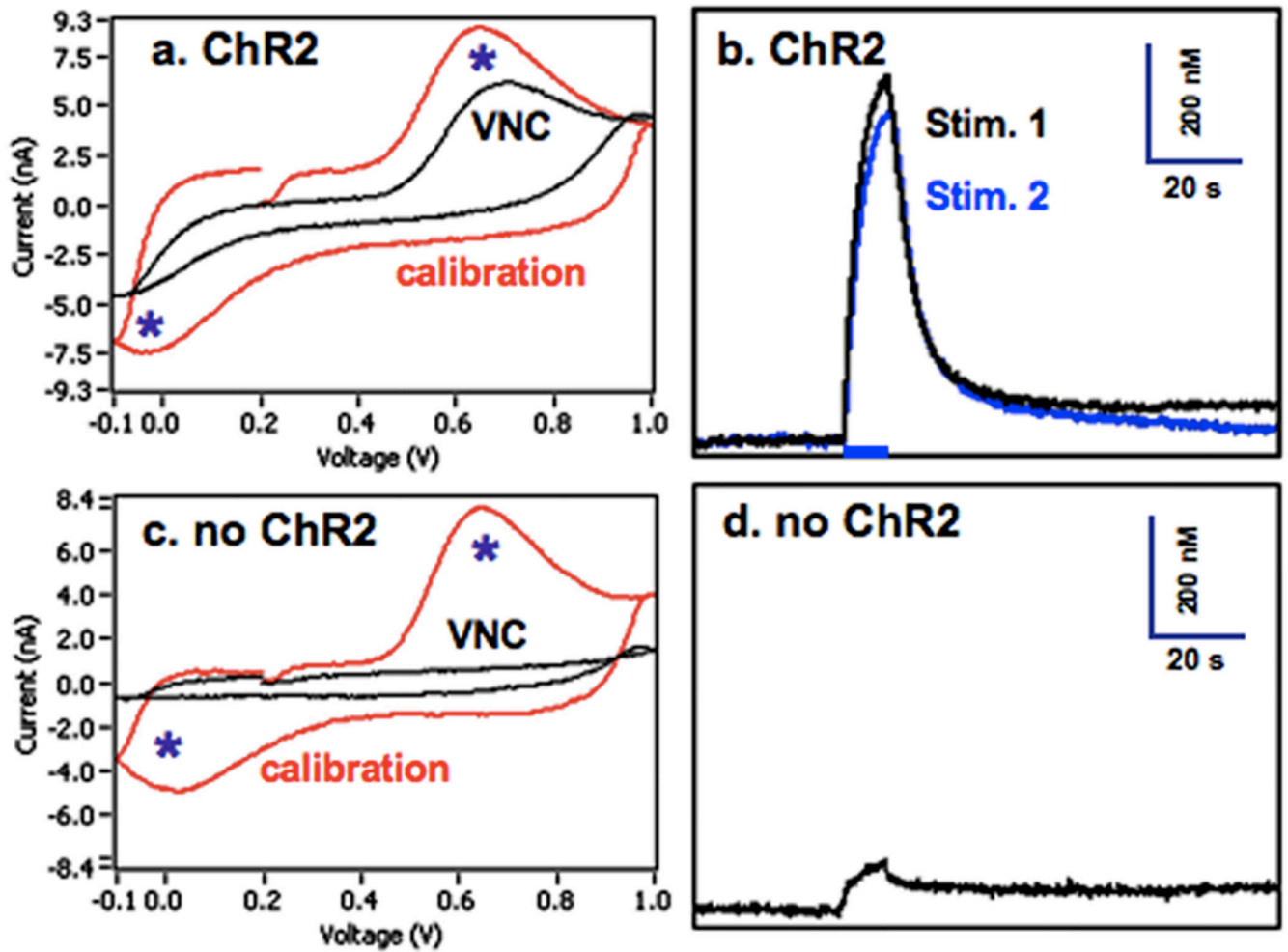
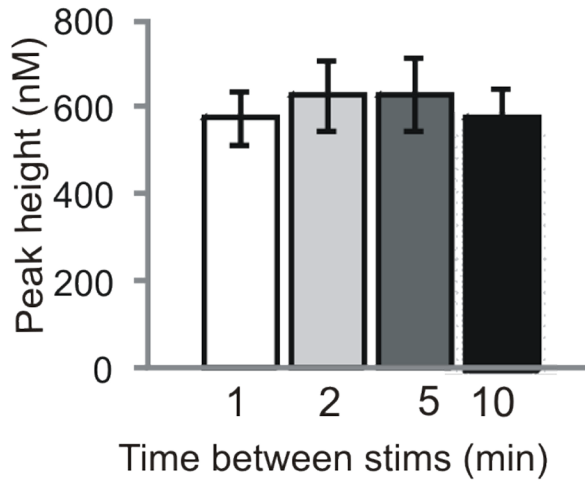
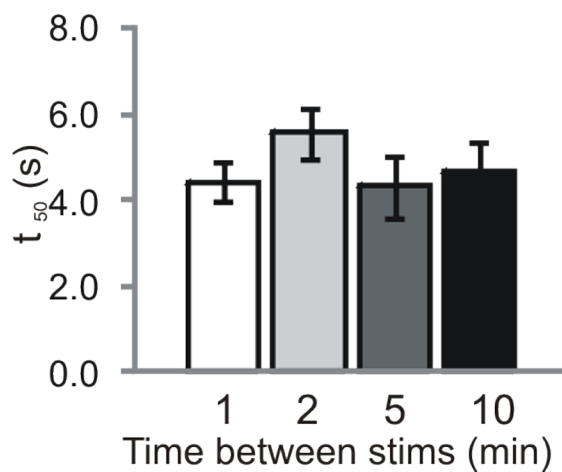
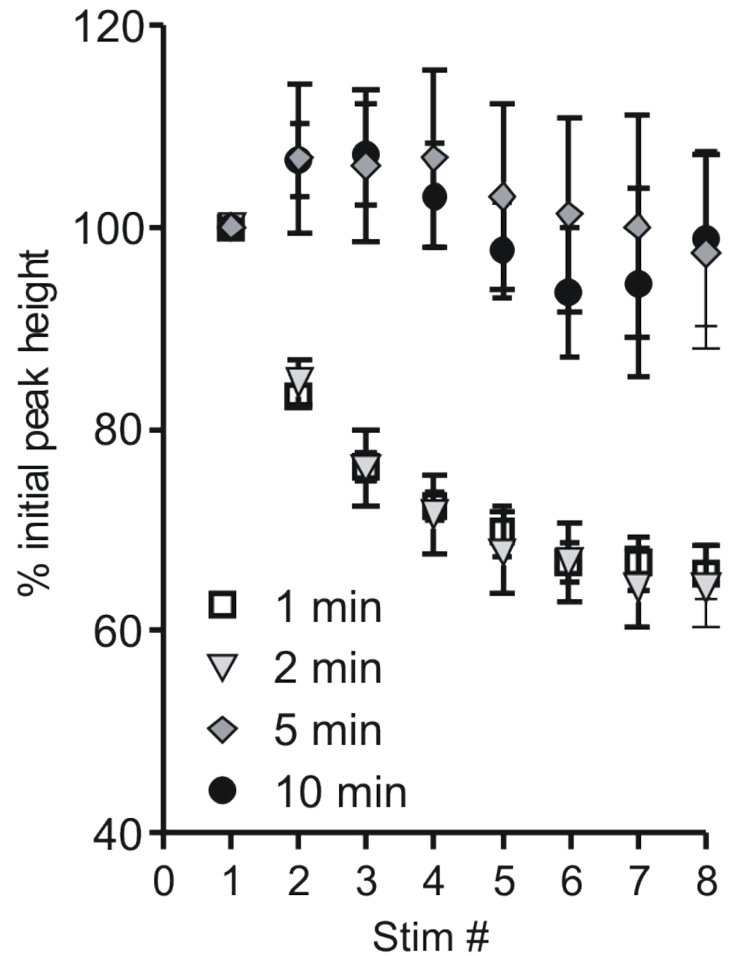


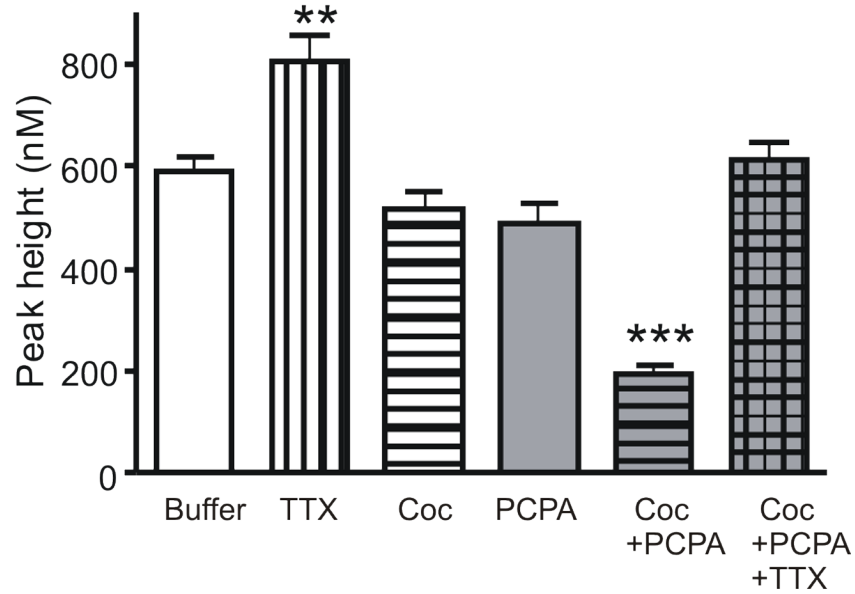
Figure 2.

Raw data of evoked serotonin release. (a–b) VNC expressing ChR2 in serotonin neurons. (a) The background-subtracted CV from the sample (black line) verifies serotonin detection because the CV is similar to that obtained during electrode calibration with 1 μ M serotonin (red line). (b) Concentration vs time traces for two, 10 s stimulations performed 2 min. apart. The peak from the second stimulation (blue) is smaller than the first (black). The duration of the 10 s stimulation is marked by a blue line underneath the trace. (c–d) Control VNC lacking ChR2 expression. (c) The sample CV (black line) does not show any serotonin specific peaks (as in the calibration, red line). (d) Concentration vs time trace shows a small fluctuation in current (about 10 % normal release) resulting not from serotonin but likely from other ionic and electrical changes.

a. Peak height for initial stimulation**b. t_{50} for initial stimulation****c. Repeated stimulations****Figure 3.**

Serotonin release during stimulations repeated at 1, 2, 5 or 10 min intervals. Pooled data are mean \pm SEM, $n = 6-9$. (a) The peak height for the initial stimulation is not significantly different for the different groups (Two-way ANOVA with Bonferonni post-test, $p > 0.05$). (b) t_{50} , the time to half decay for the initial stimulation, is also not different for the different groups (Two-way ANOVA with Bonferonni post-test, $p > 0.05$). (c) Normalized peak height vs stimulation number. Peak height decays when stimulations are performed 1 (squares) or 2 minutes (triangles) apart but is stable when stimulations are performed 5 (diamonds) or 10 min apart (circles).

a. Peak height for initial stimulation



b. t_{50} initial stimulation

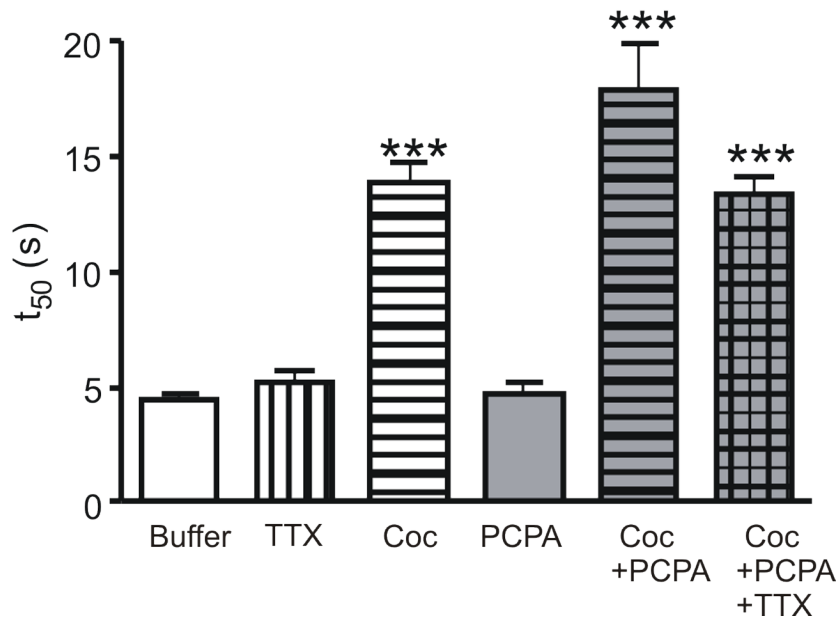
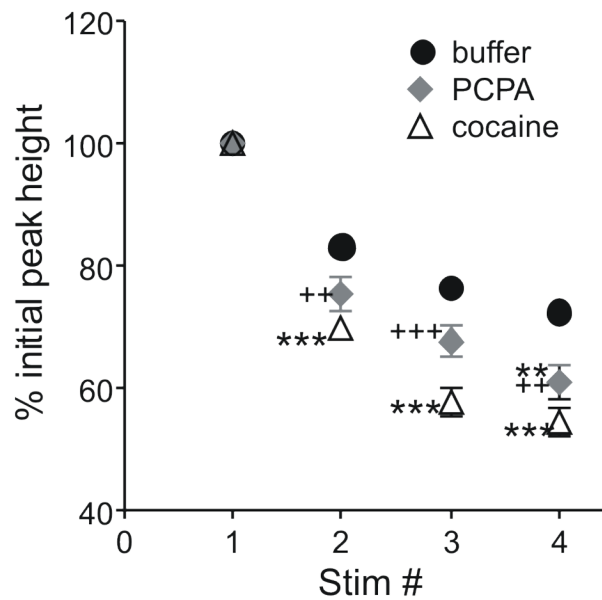


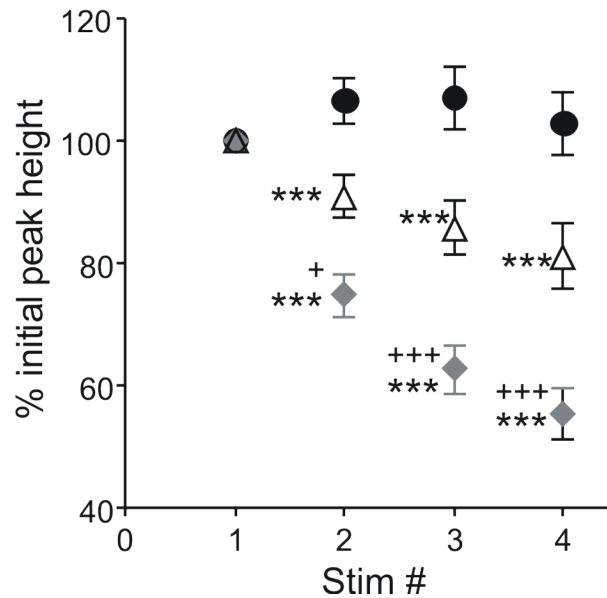
Figure 4.

Effect of PCPA, cocaine and TTX on initial stimulation. Data are mean \pm SEM (n=12–26). (a) Peak height for initial 10 s stimulation. A one-way ANOVA shows a significant effect of drug ($p < 0.001$). Incubation in both 100 μ M PCPA and 10 μ M cocaine significantly decreases peak height while incubation in TTX alone significantly increases peak height (Bonferonni post-test, see Supp. Table 2 for all p values for this figure). (b) t_{50} for initial stimulation. A one-way ANOVA shows a significant effect of drug ($p < 0.001$). t_{50} is significantly for cocaine but not PCPA or TTX alone (One-way ANOVA with Bonferonni post-test). ** $p < 0.01$, *** $p < 0.001$

a. Stimulations 1 min apart



b. Stimulations 10 min apart

**Figure 5.**

Effect of cocaine and PCPA on repeated stimulations. Reuptake was inhibited by 10 μ M cocaine (triangles) or synthesis by 100 μ M PCPA (diamonds). Control samples incubated in buffer are black circles. Normalized peak height (mean \pm SEM, $n = 4-6$) plotted for stimulations performed every (a) 1 minute ($n = 6-9$) or (b) 10 minutes. Two-way ANOVA for each graph showed significant interaction of stimulation number and drug ($p < 0.001$). Asterisks indicate significant deviations from buffer while plus signs next to PCPA values indicate a significant difference from cocaine. See Supp. Table 4 for all statistical values. * or + $p < 0.05$, ** or ++ $p < 0.01$, *** or +++ $p < 0.001$.

Stimulations 1 min. Apart

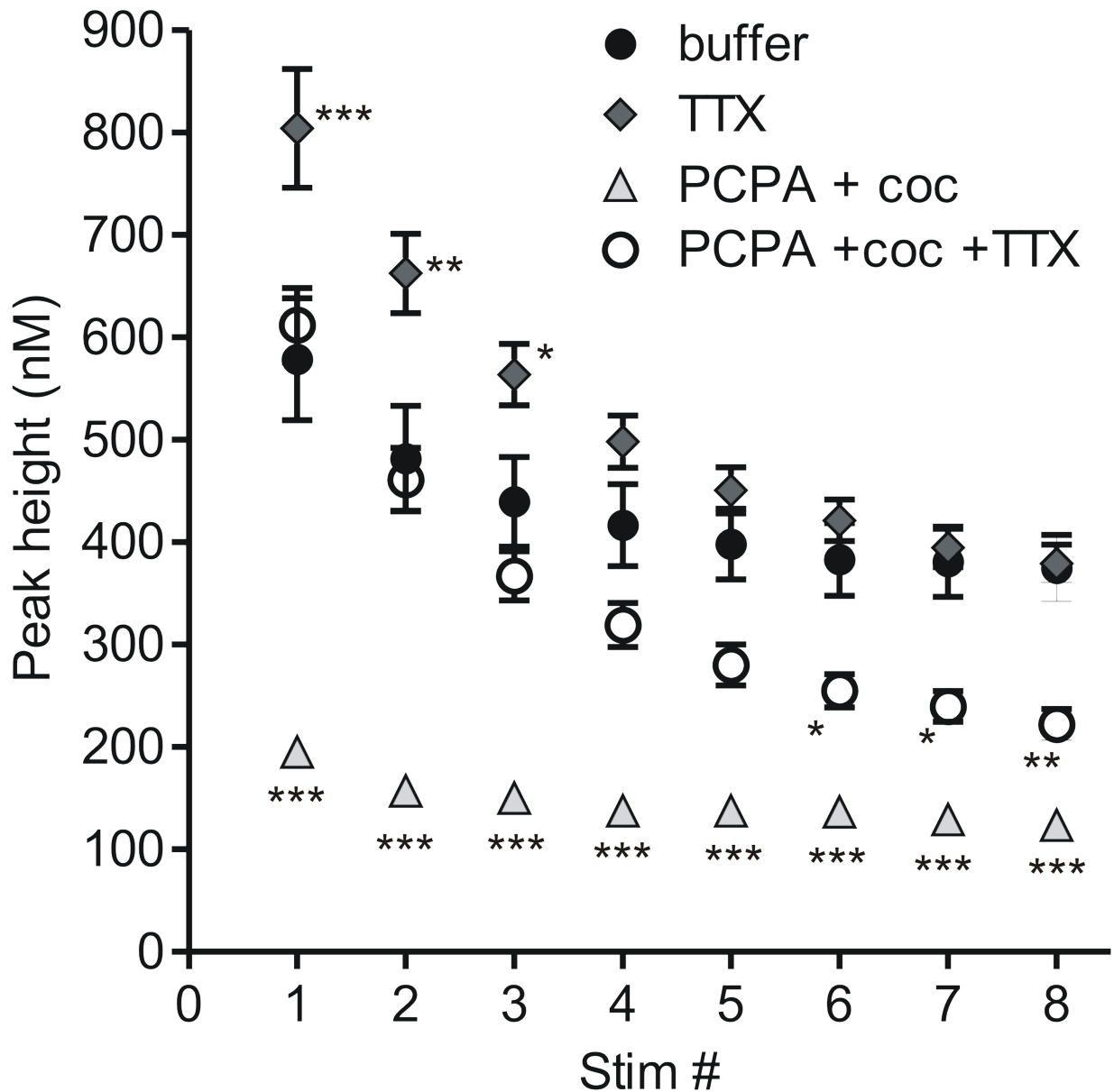


Figure 6.

Effect of TTX on depletion of evoked serotonin release. Stimulations were performed 1 min apart ($n = 8-10$). Because of the large differences in initial peak height, signals were not normalized. Two-way ANOVA showed a significant interaction between drug and stimulation number ($p < 0.001$). Incubation in $0.5 \mu\text{M}$ TTX (diamonds) produces larger initial stimulations than buffer controls (black circles). Incubation in $100 \mu\text{M}$ PCPA + $10 \mu\text{M}$ cocaine (triangles) results in less release. With TTX + PCPA +cocaine (open circles), peak height is similar to buffer controls for the first few stimulations but then becomes significantly smaller. Significant deviations from buffer are denoted by asterisks (See Suppl. Table 5 for all statistics. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).