Supplemental information: Borue, Condron, Venton, J. Neurochem.

Supplemental Methods

Fly crossing

UAS-ChR2; Tph2(clone 1)-gal4 homozygotes were generated using reciprocal balancer lines to track cross progeny. The genotype of the newly created parent line was confirmed by out-crossing. Flies from the line were out-crossed to flies homozygous for Cha-gal4. Progeny were fed all-trans retinal, and exposed to blue light at 4 days old. All larvae contracted in the presence of blue light confirming that the parent line was homozygous for ChR2. A separate set of flies were crossed to UAS-GFP. Progeny were fixed and stained at 4 days old. All progeny stained for GFP in the Tph2-gal4 expression pattern indicating that the parent line was homozygous for Tph2-gal4.

Immunohistochemistry

VNCs were incubated in buffer for 30 minutes prior to fixation with 4% paraformaldehyde for 1 h at room temperature. VNCs were then incubated in phosphate buffered saline with 1% tween (PBT) overnight at room temperature with 1:666 anti-serotonin (rabbit polyclonal, ImmunoStar). Alexafluor goat anti-rabbit 568 (Molecular Probes) secondary antibody was subsequently applied at 1:1000 in PBT overnight at room temperature. VNCs were mounted onto slides in 90% glycerol/2.5% 1,4-diazabicyclo[2.2.2]octane and allowed to clear overnight prior to imaging with a confocal microscope.

Supplemental table 1: One way ANOVA of drug effects on neuronal serotonin content.

Comparison	t	P value
Buffer vs Coc	6.972	P < 0.001
Buffer vs PCPA	5.702	P < 0.001
Buffer vs PCPA + Coc	9.105	P < 0.001
Coc vs PCPA	1.627	P > 0.05
Coc vs PCPA + Coc	3.858	P < 0.01
PCPA vs PCPA + Coc	2.034	P > 0.05

One way ANOVA of data shown in figure 1. There is a significant ($p < 0.0001$, $F_3=29.0$) overall difference in the serotonin content between groups. Table shows Bonferroni post test comparisons. Significant values are highlighted.

Supplemental table 2: One way ANOVA of drug effect during initial stimulation.

Peak height comparisons

Comparison	t value	P value
Buffer vs TTX	3.944	P < 0.01
Buffer vs Coc	1.825	P > 0.05
Buffer vs PCPA	2.269	P > 0.05
Buffer vs PCPA + Coc	7.61	P < 0.001
Buffer vs PCPA + Coc +TTX	0.304	P > 0.05
Coc vs PCPA	0.508	P > 0.05
Coc vs PCPA + Coc	5.451	P < 0.001
Coc vs PCPA + Coc + TTX	1.461	P > 0.05
PCPA vs PCPA + Coc	4.766	P < 0.001
PCPA vs PCPA + Coc + TTX	1.805	P > 0.05
PCPA + coc vs PCPA+Coc+TTX	5.700	P < 0.001
TTX vs Coc	4.866	P < 0.001
TTX vs PCPA	5.098	P < 0.001
TTX vs PCPA + Coc	9.144	P < 0.001
TTX vs PCPA + Coc + TTX	2.616	P > 0.05

t_{50} comparisons

Comparison	t value	P value
Buffer vs TTX	0.838	P > 0.05
Buffer vs Coc	12.72	P < 0.001
Buffer vs PCPA	0.324	P > 0.05
Buffer vs PCPA + Coc	13.42	P < 0.001
Buffer vs PCPA + Coc +TTX	8.448	P < 0.001
Coc vs PCPA	9.983	P < 0.001
Coc vs PCPA + Coc	3.674	P < 0.01
Coc vs PCPA + Coc + TTX	0.451	P > 0.05
PCPA vs PCPA + Coc	11.57	P < 0.001
PCPA vs PCPA + Coc + TTX	7.293	P < 0.001
PCPA +coc vs PCPA+Coc+TTX	3.426	P > 0.05
TTX vs Coc	8.419	P < 0.001
TTX vs PCPA	0.482	P > 0.05
TTX vs PCPA + Coc	10.31	P < 0.001
TTX vs PCPA + Coc + TTX	6.392	P < 0.001

One way ANOVA of data shown in figure 4. One way ANOVA shows an overall significant effect of drug on means for both peak height ($p < 0.0001$, $F_5=18.9$) and t_{50} ($p < 0.0001$, $F_5=69.5$). Tables show Bonferroni post test comparisons. Significant values are highlighted.

Supplemental table 3: Two way ANOVA of drug effects on normalized serotonin release over multiple stimulations.

Stims 1 min apart

Buffer vs Cocaine		
Stim number	**t**	**P value**
Stim 1	0	P > 0.05
Stim 2	4.88	P < 0.001
Stim 3	6.81	P < 0.001
Stim 4	6.52	P < 0.001

Buffer vs PCPA		
Stim number	**t**	**P value**
Stim 1	0	P > 0.05
Stim 2	1.66	P > 0.05
Stim 3	2.11	P < 0.05
Stim 4	3.18	P < 0.01

Cocaine vs PCPA		
Stim number	**t**	**P value**
Stim 1	0	P > 0.05
Stim 2	3.27	P < 0.01
Stim 3	4.66	P < 0.001
Stim 4	3.51	P < 0.01

Stims 10 min apart

Buffer vs Cocaine		
Stim number	**t**	**P value**
Stim 1	0	P > 0.05
Stim 2	2.8	P < 0.05
Stim 3	3.8	P < 0.01
Stim 4	3.89	P < 0.01

Buffer vs PCPA		
Stim number	**t**	**P value**
Stim 1	0	P > 0.05
Stim 2	6.04	P < 0.001
Stim 3	8.44	P < 0.001
Stim 4	9.03	P < 0.001

Cocaine vs PCPA		
Stim number	**t**	**P value**
Stim 1	0	P > 0.05
Stim 2	2.76	P < 0.05
Stim 3	3.96	P < 0.001
Stim 4	4.41	P < 0.001

Two way ANOVA of data shown in figure 5. ANOVA shows a significant ($p < 0.0001$, $F_{3,7}=15.03$) interaction of stimulation number and drug and significant main effects of stimulation number ($p<0.0001$, $F_7=764$) and drug ($p<0.0001$, $F_3=21.3$). Table shows Bonferroni post tests. Significant values are highlighted.

Supplemental table 4: Two way ANOVA comparing the effects different intervals of stimulation.

Cocaine 1 min vs 10 min		
Stim number	**t**	**P value**
Stim 1	0	P > 0.05
Stim 2	5.58	P < 0.001
Stim 3	7.43	P < 0.001
Stim 4	7.01	P < 0.001

PCPA 1 min vs 10 min		
Stim number	**t**	**P value**
Stim 1	0	P > 0.05
Stim 2	0.98	P > 0.05
Stim 3	1.90	P > 0.05
Stim 4	2.14	P > 0.05

Two-way ANOVA of data from figure 5, showing the effect of inter-stimulation time on stimulated release in the presence of cocaine or PCPA. ANOVA shows a significant ($p<0.0001$, $F_{3,1}=27.2$) interaction of stimulation number and group (time between stimulations) for cocaine as well as main effects of stimulation number $p=0.0003$, $F_1=37.0$) and time between stimulations ($p<0.0001$, $F_3=131$). For PCPA, there is no significant interaction between stimulation number and group ($p=0.067$, $F_{3,1}=2.62$) a significant overall effect for stimulation number only ($p<0.0001$, $F_3=193$) but not time between stimulations ($p=.1693$, $F_1=2.16$). Tables show Bonferroni post test comparisons. Significant values are highlighted.

Supplemental table 5: Two way ANOVA of drug effects on serotonin release over multiple stimulations.

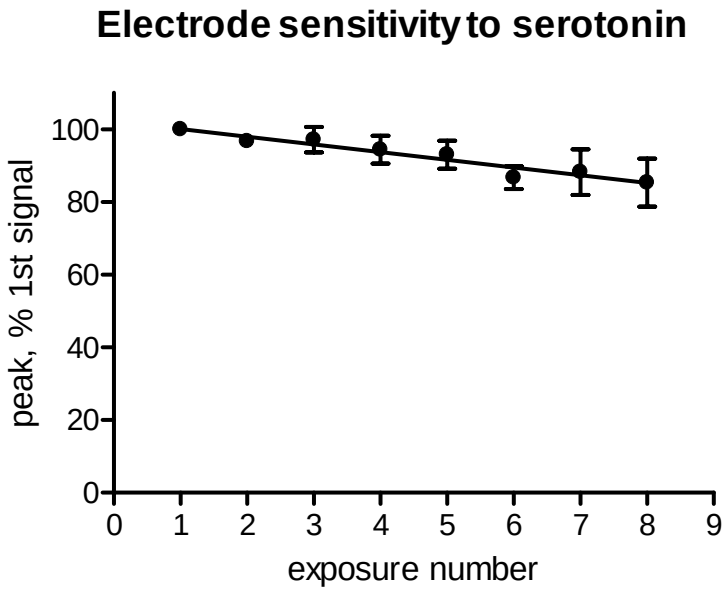
Buffer vs TTX		
Stim number	**t**	**P value**
Stim 1	5.5	P < 0.001
Stim 2	4.4	P < 0.001
Stim 3	3	P < 0.05
Stim 4	2	P > 0.05
Stim 5	1.3	P > 0.05
Stim 6	0.93	P > 0.05
Stim 7	0.33	P > 0.05
Stim 8	0.12	P > 0.05

Buffer vs PCPA + Coc		
Stim number	**t**	**P value**
Stim 1	9.1	P < 0.001
Stim 2	7.7	P < 0.001
Stim 3	6.9	P < 0.001
Stim 4	6.6	P < 0.001
Stim 5	6.2	P < 0.001
Stim 6	5.9	P < 0.001
Stim 7	6	P < 0.001
Stim 8	6	P < 0.001

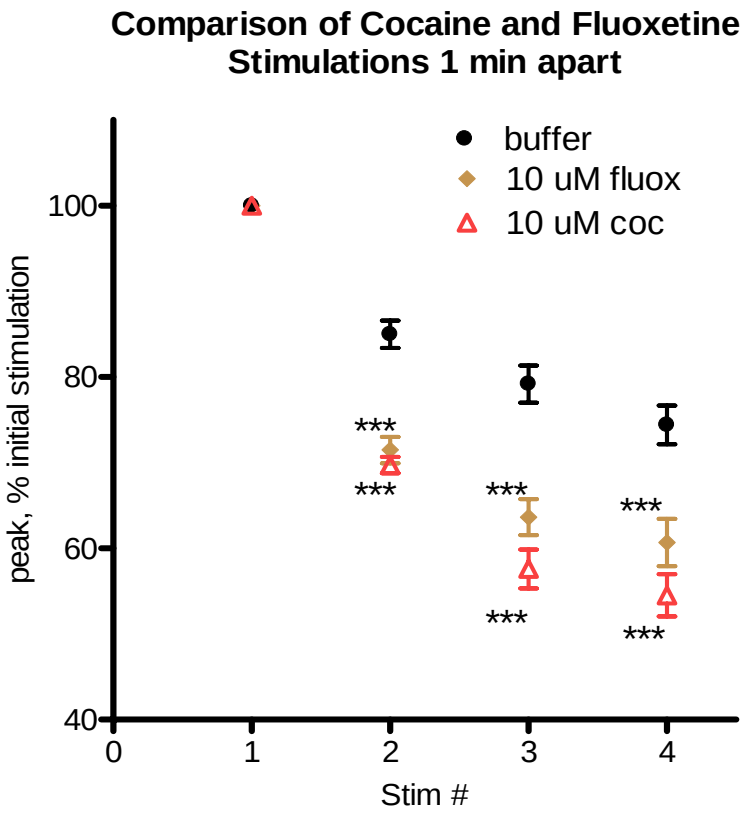
Buffer vs PCPA + Cocaine + TTX		
Stim number	**t**	**P value**
Stim 1	0.07	P > 0.05
Stim 2	0.48	P > 0.05
Stim 3	1.7	P > 0.05
Stim 4	2.3	P > 0.05
Stim 5	2.7	P > 0.05
Stim 6	3	P < 0.05
Stim 7	3.3	P < 0.05
Stim 8	3.5	P < 0.01

PCPA + Coc vs PCPA + Coc +TTX		
Stim number	**t**	**P value**
Stim 1	9.6	P < 0.001
Stim 2	7	P < 0.001
Stim 3	5	P < 0.001
Stim 4	4.2	P < 0.001
Stim 5	3.3	P < 0.01
Stim 6	2.7	P > 0.05
Stim 7	2.6	P > 0.05
Stim 8	2.3	P > 0.05

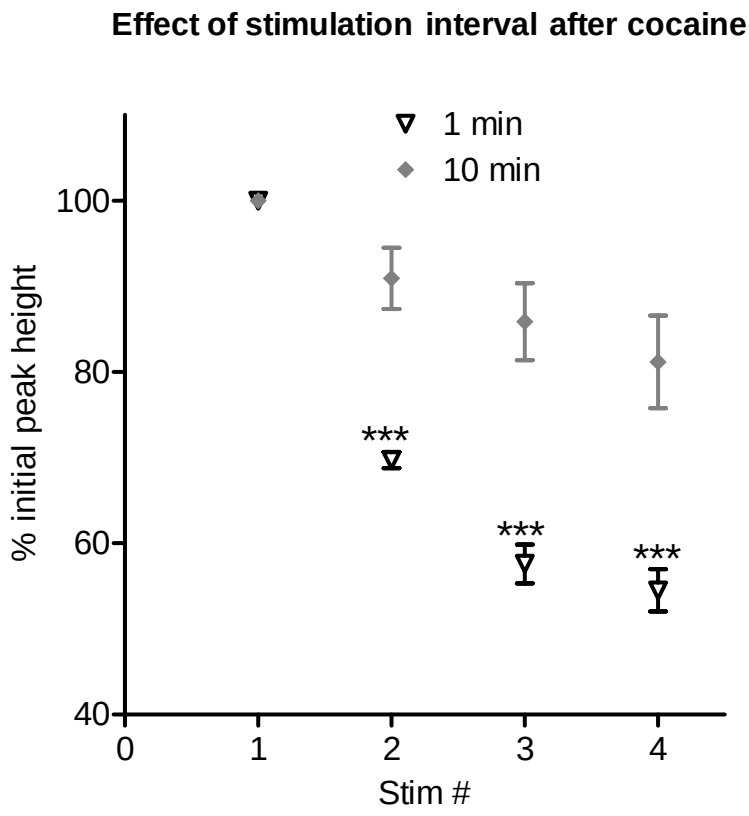
Two-way ANOVA of data shown in figure 6. ANOVA shows a significant interaction of stimulation number and group (drug) ($p < 0.0001$, $F_{3,7}=25$). There are also significant main effects of drug ($p<0.0001$, $F_3=35.3$) and stimulation number ($F_7=241$). Tables show Bonferroni post tests. Significant values are highlighted.



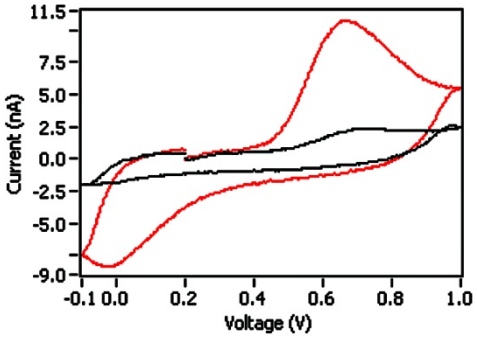
Supplemental Figure 1. Electrode sensitivity to serotonin is influenced by prior exposure to serotonin. Electrodes (n=6) were placed in a flow cell and exposed to 10 s long exposures of 500 nM serotonin every minute. Error bars are SEM. The peak height decreased for repeated exposures and is plotted as normalized data, the percentage of the initial measurement. The decrease is linear (R^2=0.966), so the linear correction was used for all the *Drosophila* data in the paper.



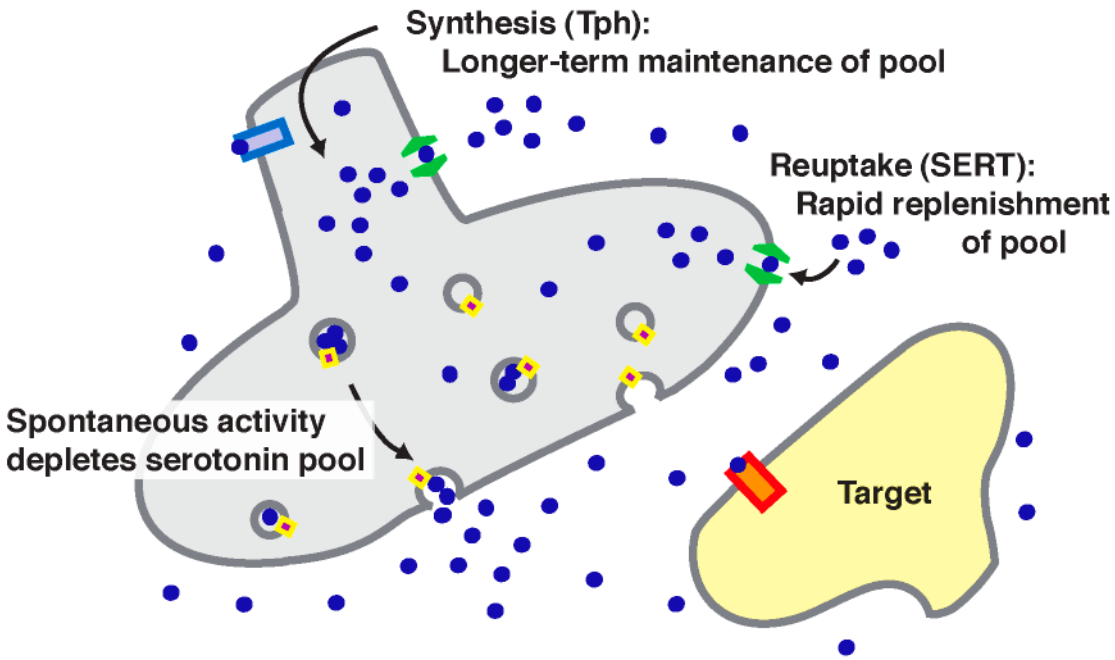
Supplemental Figure 2. Comparison of cocaine and fluoxetine on stimulations repeated 1 min. apart. Samples were incubated in either buffer, 10 µM cocaine or 10 µM fluoxetine (n=6-8). Two-way ANOVA shows a significant interaction between drug and stimulation number ($F_{2,3}$) $p<0.001$). Bonferonni post-tests show no significant differences between the fluoxetine and cocaine data. Stimulations 2, 3, and 4 are significantly less than buffer for both fluoxetine and cocaine (***$p<0.001$).



Supplemental Figure 3. Dependence of serotonin depletion on inter-stimulation time when incubated in cocaine (10 µM). This figure superimposes data from Fig. 5 a and b to allow a visual comparison of cocaine data with different intervals. Peak height was normalized to the initial stimulation. Stimulations were performed 1 (open black triangle) or 10 minutes apart (grey diamond). Data is mean +/- SEM, n = 4-6. Peak heights are significantly different when stimuli are performed 10 as opposed to 1 minutes apart for stimulations 2 to 4 (Two- way ANOVA,*** $p < 0.001$, see Supp. Table 4).



Supplemental Figure 4. Raw data showing effect of 10 µM cocaine and 100 µM PCPA on serotonin release in *Drosophila*. For the first stimulation, the CV from the sample (black line) shows a small oxidation peak for serotonin, confirming that serotonin is released. The electrode calibration (1 µM serotonin) is in red.



Supplemental Figure 5. Diagram of mechanisms regulating the serotonin pool. Reuptake is most important for the short term replenishment (1-2 min. time scale) of the releasable pool. Synthesis is more important for longer term maintenance of the releasable pool. Spontaneous activity depletes the amount of serotonin available for evoked release.