

Correction: 21 May 2009



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## Supporting Online Material for

### **Phasic Firing in Dopaminergic Neurons Is Sufficient for Behavioral Conditioning**

Hsing-Chen Tsai, Feng Zhang, Antoine Adamantidis, Garret D. Stuber, Antonello Bonci, Luis de Lecea, Karl Deisseroth\*

\*To whom correspondence should be addressed. E-mail: [deissero@stanford.edu](mailto:deissero@stanford.edu)

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#### **This PDF file includes:**

Materials and Methods  
Figs. S1 to S7  
References

**Correction:** Several minor typographical errors were corrected, and slight text edits were made to improve consistency and clarity.

## **Materials and Methods**

### **Subjects**

Tyrosine hydroxylase (TH)::IRES-Cre transgenic mice (EM:00254) were obtained from the European Mouse Mutant Archive and mated with C57BL/7 wildtype mice (*I*). All experimental subjects have been backcrossed at least 5 generations. Unless specified otherwise, mice were housed four to five per cage in a colony maintained with a standard 12 hr light/dark cycle and given food and water *ad libitum*. Experimental protocols were approved by Stanford University IACUC and meet guidelines of the National Institutes of Health guide for the Care and Use of Laboratory Animals.

### **Virus preparation**

To construct Cre-inducible recombinant AAV vectors (2-4), the DNA cassette carrying two pairs of incompatible lox sites (loxP and lox2722) was synthesized and the ChR2-EYFP transgene was inserted between the loxP and lox2722 sites in the reverse orientation. The resulting double-floxed reverse ChR2-EYFP cassette was cloned into a modified version of the pAAV2-MCS vector carrying the EF-1 $\alpha$  promoter and the Woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) to enhance expression. The recombinant AAV vectors were serotyped with AAV5 coat proteins and packaged by the viral vector core at the University of North Carolina. The final viral concentration was  $2 \times 10^{12}$  genome copies (gc)/mL.

### **Stereotactic AAV injection and cannula implantation**

Mice aged 6 to 8 weeks were anesthetized using a ketamine/xylazine mixture (ketamine, 80 mg/kg; xylazine, 15-20 mg/kg) diluted in phosphate-buffered saline (PBS, pH 7.4). 1 $\mu$ l of purified double-floxed AAV:ChR2-EYFP virus was injected unilaterally in the right VTA (from bregma: anterior-posterior, -3.44mm; lateral, 0.48mm; and dorsal-ventral, 4.4mm). Cannula guides for fiber insertion were also implanted over the same stereotactic coordinate as mentioned above (Fig. S4). For each animal, the cannula was secured using dental cement. All mice were singly housed after surgery and recovered for at least 12 days before behavioral and electrophysiological experiments.

## **Immunohistochemistry**

To determine the specificity of ChR2-EYFP expression in DA neurons, TH::IRES-Cre mice transduced with the double-floxed AAV:ChR2-EYFP virus were anesthetized with ketamine/xylazine and perfused transcardially with PBS followed by 4% paraformaldehyde (PFA) dissolved in PBS. The brains were removed and post-fixed in PBS containing 4% PFA overnight at 4 °C, and subsequently immersed in a cryoprotectant consisting of PBS containing 30% sucrose until settling (~48 hr at 4 °C). 45 µm coronal brain sections were collected and washed in PBS, treated with 0.3% Triton X-100 (PBST), and immersed in a blocking solution consisting of 3% normal donkey serum dissolved in PBS. Localization of DA cell bodies and fibers was confirmed by labeling with chicken anti-tyrosine hydroxylase antibody (1:500). Neuronal cell bodies were identified using the monoclonal anti-NeuN antibody (1:500). The staining procedure was same as described previously (5). Transduction efficiency was quantified using a confocal microscope by comparing the EYFP cells with TH immunoreactive cells.

## **Acute brain slice preparation and electrophysiology**

Acute VTA horizontal slices (250 µm) were prepared as described previously (6, 7). Dopaminergic ChR2-EYFP neurons were identified via fluorescence. Whole-cell recordings were conducted using a Multiclamp 700B and signals were digitized at 10kHz.

## ***In vivo* optrode recording**

Simultaneous optical stimulation and electrical recording in the VTA of TH::IRES-Cre transgenic mice transduced with double-floxed AAV:ChR2-EYFP was carried out as described previously (8) using an optrode consisting of an extracellular tungsten electrode (1 MΩ, ~125 µm) tightly bundled with an optical fiber (200 µm core diameter, 0.2 N.A.), with the tip of the electrode protruding slightly beyond the fiber end (~0.4 mm) to ensure illumination of the recorded neurons. Recordings were conducted with the optrode initially placed at the boundary of VTA (from bregma: anterior-posterior, -3.44mm; lateral, 0.48mm; and dorsal-ventral, 4.0mm) and gradually lowered in 0.1 mm increments. The optical fiber was coupled to a 473 nm solid-state laser diode with ~20 mW of output from the 200 µm fiber. Single unit recordings were done in mice anesthetized with a ketamine/xylazine mixture as described above. Signals were

recorded and band-pass filtered at 300Hz low/5 kHz high using an 1800 Microelectrode AC Amplifier.

### ***In vivo* fast-scan cyclic voltammetry**

Fast-scan cyclic voltammetry experiments were conducted using methods adapted from (9). Briefly, mice were anesthetized with ketamine/xylazine as described above and placed in a stereotaxic frame. The skull was exposed and holes were drilled above the NAcc (from bregma: anterior-posterior, 1.4 mm; lateral, 1.0 mm) and the VTA (from bregma: anterior-posterior, -3.44mm; lateral, 0.48mm; and dorsal-ventral, 4.4mm). An Ag/AgCl reference electrode was also implanted in the contralateral forebrain. An optical fiber, used for optically stimulating ChR2 expressing DA neurons, was coupled to an extracellular electrophysiological electrode and positioned just dorsal to the VTA. Unit activity was then monitored while the optical fiber and electrophysiological electrode were gradually positioned to where light pulses would induce neuronal firing, thus confirming that the optical fiber was in the VTA and capable of activating DA neurons. A carbon fiber electrode (~100  $\mu\text{m}$  in length) for voltammetric recordings was then lowered (beginning at -3.5 mm and lowered in 0.25 mm intervals) into the NAcc. Voltammetric measurements were made every 100 ms by application of a triangle waveform (-0.4 V to +1.3 V to -0.4 V vs. Ag/AgCl, at 400 V/s) to the carbon fiber electrode, which is capable of detecting the presence of dopamine transients. Data was acquired and analyzed using software written in LabVIEW. Light-evoked dopamine release was identified by the background-subtracted cyclic voltammogram, which was visually compared to voltammograms from the detection of dopamine *in vitro*. Following experiments in anesthetized mice, electrodes were calibrated *in vitro* with 1  $\mu\text{M}$  DA yielding an average calibration factor of 26 nA/ $\mu\text{M}$ , which was used to estimate the concentration of light-evoked dopamine transients *in vivo*.

### **Behavioral tests**

All behavioral tests were conducted at least 12 days post-surgery, during the same circadian period (10:00 – 18:00). The conditioned place preference (CPP) paradigm (10) was used to test the reinforcing properties of phasic DA neuron firing. The CPP apparatus consisted of a rectangular cage with a left chamber measuring 27.5 cm x 21 cm with black walls and a black, smooth plastic floor; a center chamber measuring 15.5 cm x 21 cm with gray walls and a metal

grill floor; and a right chamber measuring 27.5 cm x 21 cm with white walls and a punched metal floor. Mouse location within the chamber during each preference test was monitored using a computerized photo-beam system. The apparatus was designed so that mice did not have a consistent bias for a particular chamber, and any mouse with a strong initial preference or avoidance for left or right chamber (by spending more than 75% or less than 25% of test time in either chamber) was discarded to ensure a relatively unbiased CPP. We did this to prevent the unlearned (i.e. unconditioned) preferences for either chamber from confounding the CPP readouts. By doing so, we can avoid complications in measurement due to “ceiling” or “flooring” effect. The CPP test consisted of three phases over 4 days (see Fig. S3 for a schematic of the experimental design). After histology, animals with no Chr2-EYFP expression in the brain due to failed cannula injection were also discarded. On day 1, individual mice were placed in the center chamber and allowed to freely explore the entire apparatus for 15 min (pre-test). Mice were conditioned for two consecutive days. In the first conditioning session (day 2), mice were confined to one of the side chambers for 30min and paired with optical stimulation (1 Hz, 50 Hz, or 0 Hz depending on the experiment). In the second session (day 3), mice were confined to the other side chamber for 30min with a different stimulation parameter. On day 4, similar to day 1, mice were placed in the center chamber and allowed to freely explore the entire apparatus for 15 min (post-test). Both post-test and pre-test were conducted identically.

The stimulation parameters used were:

- Phasic modulation: 25 pulses of 15ms light flashes delivered at 50Hz with a periodicity of 1 minute (see Fig. 3B).
- Tonic modulation: 25 pulses of 15ms light flashes delivered at 1Hz with a periodicity of 1 minute (see Fig. 3B).

The open field test was conducted on different cohorts of mice to measure the effect of optogenetic stimulation on anxiety-like responses and general locomotor ability. The open field test chamber (55cm long x 55cm wide x 40cm deep) is made of PVC plastic. Mice were individually placed in the center of the chamber and allowed to freely explore the arena during a 5-min test session. Both central and peripheral activities were measured using an automated video-tracking system. Percentage of time in center is defined as the percent of total time that

was spent in the central 35 x 35cm area of the open field. Enhanced exploration of the unprotected central portion of this novel open field and reduced thigmotaxis is correlated with reduced anxiety levels. Two cohorts of mice were individually subjected to the test while receiving different light stimulation: 50Hz or 1Hz. A third cohort of mice was subjected to the same test with optic fiber on the head but no light stimulation as a control. Video-tracking hardware and software recorded the movement of each mouse, time spent in the center (sec), average distance of the mouse to the wall (cm), and total distance travelled (cm) during the entire test.

**Fig. S1** Plot of the fidelity of light-evoked spikes during trains of 25 light flashes ( $n = 6$ ). ChR2-EYFP DA neurons were presented with trains of 25 light flashes at 1 Hz and 50 Hz. The number of spikes fired was counted for each bin of 5 spikes. Error bars represent standard error of the mean.

**Fig. S2** Optrode recording of background and light-evoked activity in the VTA in an awake mouse expressing ChR2-EYFP in DA neurons. 15ms duration light flashes (blue bars, not to scale) were delivered at 2 Hz. **(A)** Ten superimposed sweeps showing light-evoked as well as natural spontaneous activity. **(B)** Averaging of the ten sweeps revealing light-evoked spikes.

**Fig. S3** Schematic of the conditioned place preference (CPP) paradigm integrating optogenetic stimulation of ChR2-EYFP DA neurons. The CPP apparatus contains three chambers (left, center, and right; see Materials and Methods for detailed description). On day 1, each mouse was subjected to a 15 min pre-test to determine default preference. On day 2 and 3, each mouse was exposed to 30 minutes of conditioning. To control for any spontaneous preference shifts, mice were divided into two cohorts that received opposite chamber/stimulation pairing. On the first day of conditioning (day 2), mice from the two cohorts were confined in the phasic chamber (left or right chamber paired with 50 Hz stimulation) for 30 minutes. On the second day of conditioning (day 3) each mouse was confined in the opposite chamber and conditioned with the alternate stimulation parameter. On day 4, all mice were subjected to a 15 min post-test, procedurally identical to the pre-test on day 1, to determine the post-conditioning preference.

**Fig. S4** Actual cannula positions in the experimental mice. **(A)** Schematic showing the cannula implantation relative to the VTA. **(B)** Atlas indicating the actual cannula tip positions from the mice used in the CPP experiments. Three cohorts were shown, 50 Hz vs. 1 Hz (blue dots,  $n = 10$ ), 50 Hz vs. No Stim (red dots,  $n = 7$ ), and 1 Hz vs. No Stim (green dots,  $n = 6$ ). Abbreviations used: ml, medial lemniscus; SNC, substantia nigra pars compacta; SNL, substantia nigra pars lateralis; SNR, substantia nigra pars reticularis; VTA, ventral tegmental area. Scale bar: 500  $\mu\text{m}$ .

**Fig. S5** Phasic activation of ChR2-EYFP DA neurons promotes preference for chamber associated with 50 Hz stimulation. For cohorts in **(A)** and **(B)**, the chambers associated with phasic (50 Hz) and tonic (1 Hz) stimulation were switched. (Left panel) Comparison of the total

time spent in each chamber during pre- (white) and post-test (gray). (Right panel) Comparison of the preference scores (time spent in phasic chamber as a fraction of the total time spent in phasic and tonic chambers) for pre- and post-test (A:  $n = 7$ , B:  $n = 6$ ). \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , Student's  $t$ -test.

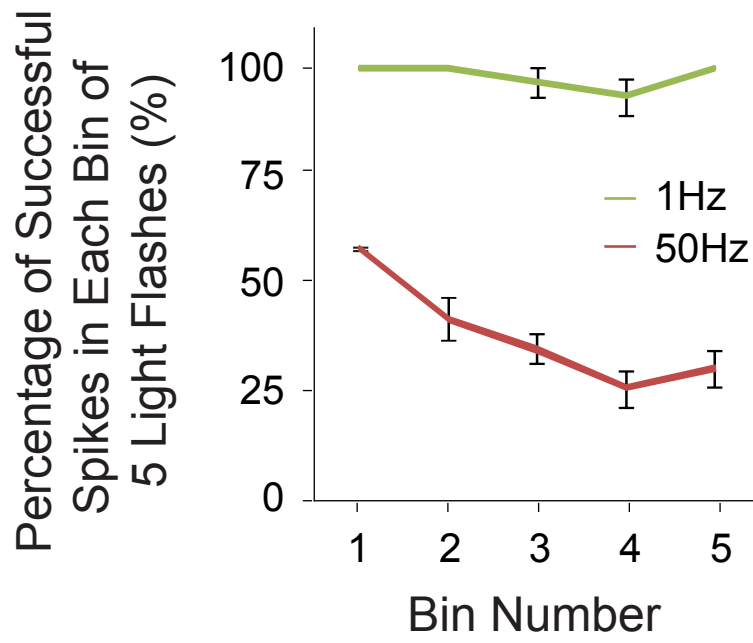
**Fig. S6** Effect of DA neuron photoactivation during open field test. (A) Anxiety-related behavior as measured by the percentage of total time each mouse spent in the defined center region of the open field test chamber (50 Hz,  $n = 5$ ; 1 Hz,  $n = 5$ ; No Stim,  $n = 6$ ). (B) Thigmotaxis as measured by the average distance each mouse spent away from the wall of the open field test chamber. (C) Locomotion as measured by total distance traveled by each mouse during the entire duration of the open field test. No significant difference was observed for all three analyses.

**Fig. S7** Voltammetry trace showing that 1 Hz stimulation induces detectable but very low levels of dopamine release. Dopamine transients were measured in the NAcc during 1Hz VTA photoactivation (25 pulses at 1 Hz, repeated each minute for 30 minutes). Trace shows the average of thirty 1 min sweeps collected over 30 min of continuous recording ( $n = 3$  mice).



## References

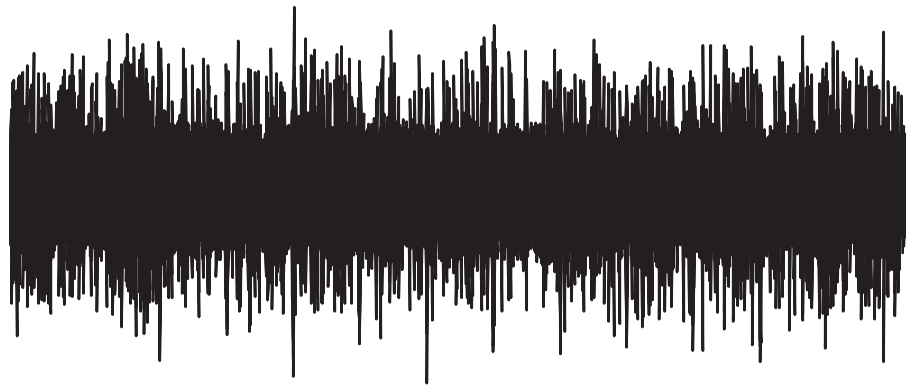
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**Supplementary Figure 1**

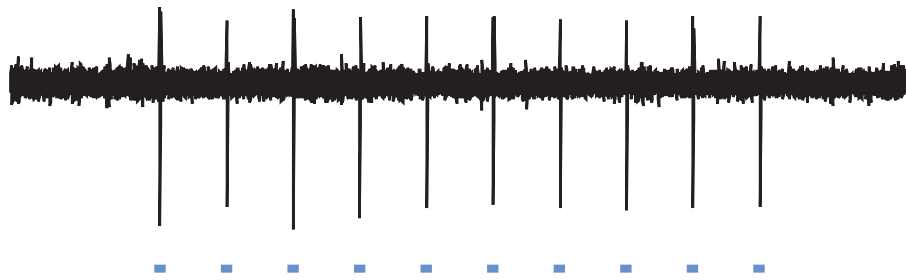
**A**

10 Sweeps of 2Hz Stimulation in an Awake Mouse

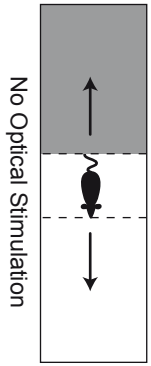


**B**

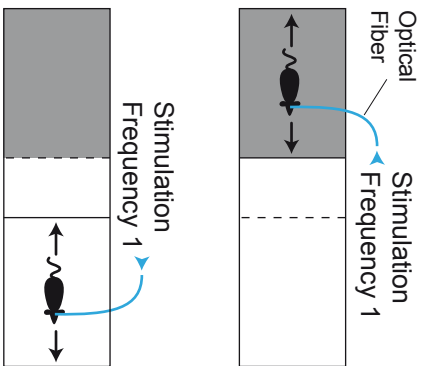
Average Trace of 10 Sweeps from Above



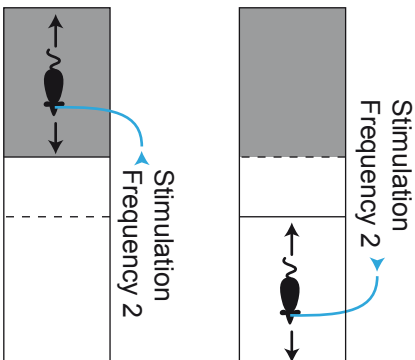
**Day 1**  
Pre-Test  
(15 min)



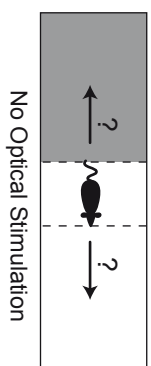
**Day 2**  
Optogenetic Conditioning  
(30 min)



**Day 3**  
Optogenetic Conditioning  
(30 min)

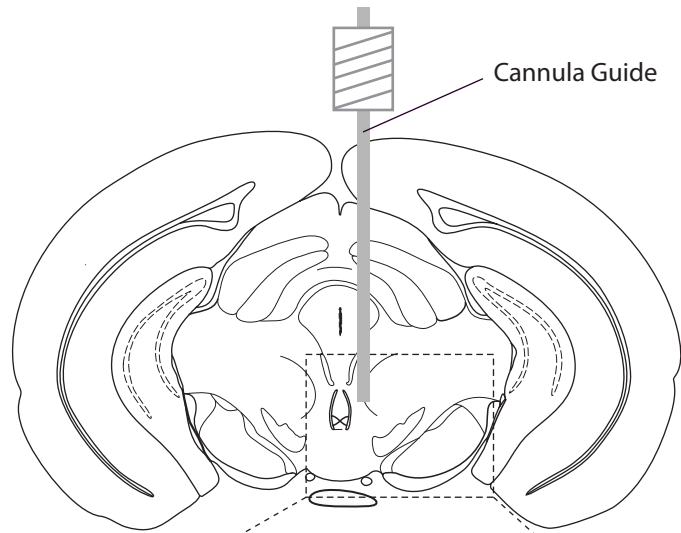


**Day 4**  
Post-Test  
(15 min)

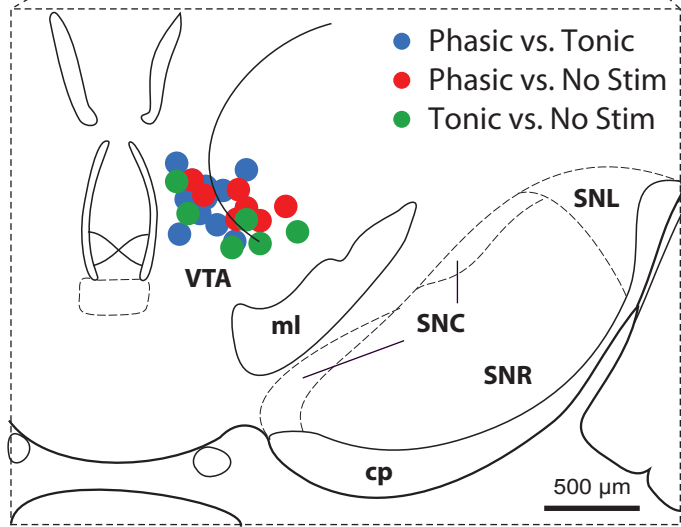


**Supplementary Figure 3**

**A**



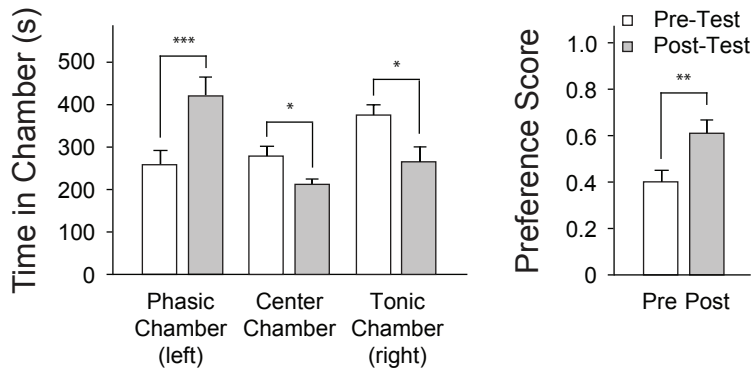
**B**



**Supplementary Figure 4**

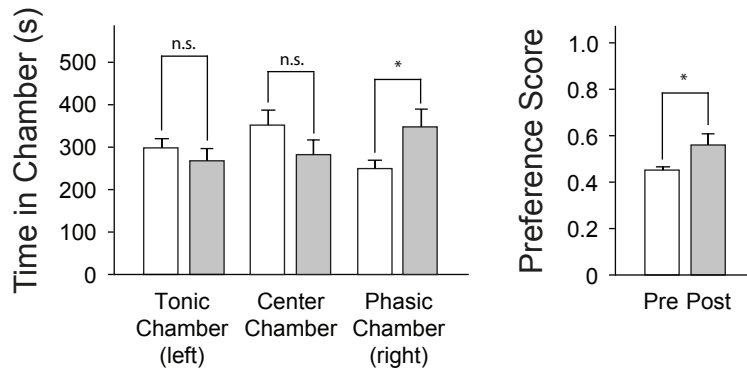
**A**

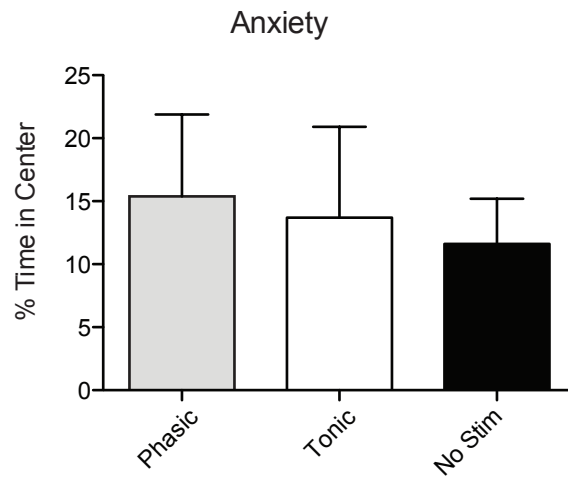
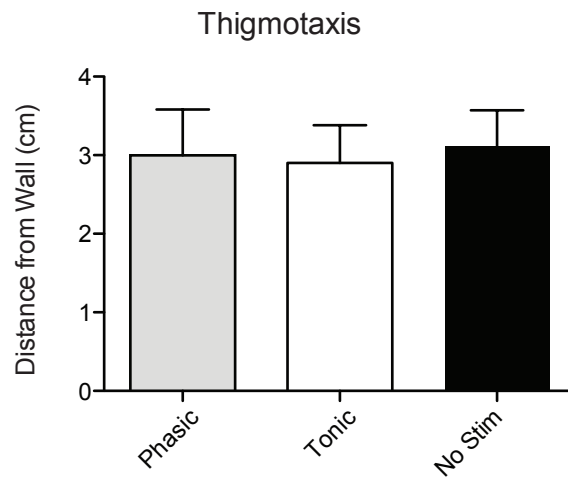
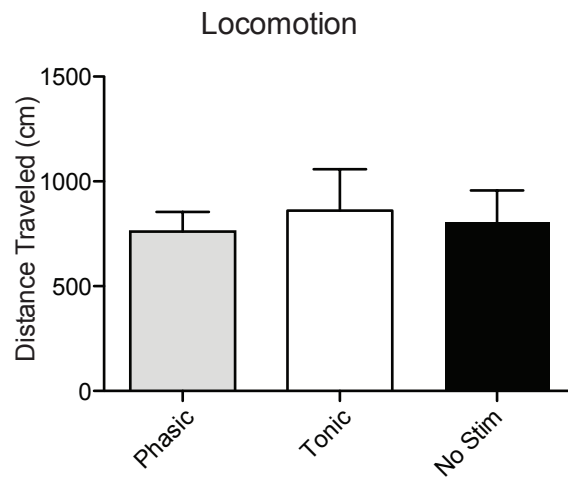
Phasic (Left) vs. Tonic (Right)



**B**

Tonic (Left) vs. Phasic (Right)



**A****B****C**

Voltammetry Recording of Dopamine Transient from Chronic 1Hz Stimulation  
(thirty 1min traces averaged)

