



Supporting Online Material for

Cholinergic Interneurons Control Local Circuit Activity and Cocaine Conditioning

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MATERIALS AND METHODS

Subjects

BAC transgenic Choline Acetyltransferase (ChAT)::Cre mice were obtained from GENSAT (stock name: Tg(Chat-cre) 24Gsat/Mmcd) (**1**) and mated with C57BL6 mice from Charles River. Experimental mice were either heterozygous for Cre (+/-) or else control littermates (-/-). Mice were group housed in a colony maintained on a reversed 12 hr light/dark cycle and given food and water *ad libitum*. Experimental protocols were approved by Stanford University IACUC to meet guidelines of the National Institutes of Health guide for the Care and Use of Laboratory Animals.

Virus production

As described previously (2, 3), Cre-inducible recombinant AAV vectors were based on a DNA cassette carrying two pairs of incompatible lox sites (loxP and lox2722) with the opsin (either ChR2(H134R) or eNpHR3.0) inserted between the lox sites in the reverse orientation. This double-floxed reverse opsin cassette was cloned into a version of the pAAV2-MCS vector carrying the EF-1 α promoter and the Woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) to enhance expression. Full maps of the Cre-inducible ChR2 AAV construct, as well as the eNpHR3.0 transgene, are available at http://www.stanford.edu/group/dlab/optogenetics/sequence_info.html. 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 3×10^{12} particles/mL for the ChR2 virus and 1.5×10^{12} particles/mL for the eNpHR3.0 virus.

Stereotactic virus injection, cannula/patchcord implantation, and light delivery

Mice were anesthetized with ketamine/xylazine (60 μ l/mouse of a mixture of 80mg/ml ketamine and 12mg/ml xylazine), and then placed in a stereotactic head apparatus. Surgeries were performed on 4-6 week old mice for physiology experiments and 8-12 week old mice for behavior experiments. Ophthalmic ointment was applied to prevent the eyes from drying. A midline scalp incision was made followed by a craniotomy, and

then virus was injected with a 10 μ l syringe and a 34 gauge metal needle. The injection volume and flow rate (1 μ l at 0.15 μ l/min) were controlled by an injection pump. Each NAc received two injections (injection 1: AP 1.15mm, ML .8mm, DV -4.8mm; injection 2: AP 1.15mm, ML .8mm, DV -4.2mm). The virus injection and fiber position were chosen so that virtually the entire shell was stimulated. Given the small size of the shell in mice, it is not possible to limit the virus spread and the light entirely to the medial shell, and the medial part of the core was included (medial to the anterior commissure). After injection the needle was left in place for 5 additional minutes and then very slowly withdrawn. For behavioral experiments mice were injected bilaterally, and then bilateral cannulas with a center-to-center distance of 1.5 mm were placed above the injection sites (AP 1.15mm, DV 3.8mm). To manipulate neuronal activity during behavior, light) was bilaterally delivered through two 300 μ m diameter optic fibers (0.37 N.A.) that were inserted through the cannulae to allow the fiber to project 200-300 μ m past the end of the cannulae.

Acute brain slice physiology

Coronal cerebral brain slices were prepared from adult mice with virus previously injected (>2 weeks prior to slicing), using standard techniques in strict accordance with a protocol approved by the Animal Care and Use Committee at Stanford University. Coronal slices 250 μ m thick were cut with a vibratome using a sapphire blade in ice cold N-methyl-D-glucamine (NMDG)-based cutting solution containing 135 mM NMDG, 1 mM KCl, 1.2 mM KH_2PO_4 , 20 mM choline bicarbonate, 10 mM glucose, 1.5 mM MgCl_2 and 0.5 mM CaCl_2 . Slices were maintained thereafter in artificial cerebral spinal fluid (ACSF) containing 119 mM NaCl, 2.5 mM KCl, 2.5 mM CaCl_2 , 1.3 mM MgCl_2 , 1 mM NaH_2PO_4 , 26.2 mM NaHCO_3 and 11 mM glucose. Slices were maintained in ACSF at 37°C for 30 minutes, and thereafter at room temperature. ACSF was bubbled constantly with 95% O_2 /5% CO_2 and heated to 34°C for all experiments. Neurons were visualized on an upright microscope (Leica DM-LFSA) equipped with both DIC optics and a filter set for visualizing eYFP using a $\times 40$ water-immersion objective and a charge-coupled device (CCD) camera. Whole-cell recordings were made from neurons using the electrode solution containing 120 mM potassium gluconate, 20 mM HEPES,

10 mM EGTA, 1 mM MgCl₂, 2 mM Na-ATP, and 0.2 mM Na-GTP (pH 7.3, 290 mOsm/L); in experiments recording IPSCs, KCl was used to replace potassium gluconate. Pipette resistances were 3–5 MΩ, and recordings were made without series resistance compensation. Membrane potentials have been corrected for the error resulting from the liquid junction potentials. The holding potential (V_M) for voltage-clamp experiments was -70 mV. The following agents were added as indicated: 5 μM SR-95531; 5 μM 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(F)quinoxaline (NBQX); 5 μM (*R,S*)-CPP; mecamylamine (10 μM); 5 μM cocaine hydrochloride. The cocaine concentration of 5 μM was carefully chosen by several criteria. First, it was consistent with the choices in previous slice work(4). Second, significantly higher concentrations would result in local anesthetic effects (4). Finally, according to studies of cocaine pharmacokinetics in mice, an i.p. injection of 10mg/kg will yield 4.7 μM of cocaine in the brain after 15 minutes, and 20 mg/kg will yield 9.4 μM, comparable to the levels used in behavioral experiments (5). Photocurrents were evoked using an optical switch with a 300W xenon lamp and either a 470±20 nm or a 580±20 nm bandpass filters; light power at the specimen was 11.52 mW mm⁻² (470 nm) or 10.64 mW mm⁻² (580 nm). Currents filtered at 2 kHz, digitized at 50 kHz, and recorded to disk using pClamp10 software (Axon Instruments). Data are expressed as mean ± standard error of the mean, and statistical significance was determined using the paired or unpaired t-test, as appropriate. For IPSC measurements in MSNs (Fig. 2B-E and Fig. S1A-B), 10 repetitions without light preceded 10 repetitions with light. Each repetition was 5 seconds in length and separated by a 5 second rest period. For testing the cocaine response of ChAT cells in slice (Fig. 4A-C), whole-cell recordings were obtained from the ventral portion of the medial shell, where elevations in spiking were variable as summarized in Fig. 4, but contrasted with typical rundown of spiking in control conditions; exploratory work suggested that ChAT cells in the core and elsewhere in the shell were less responsive to cocaine.

Immunohistochemistry

To determine the specificity of opsin expression in ChAT neurons, mice were anesthetized with beuthanasia and perfused transcardially, first with PBS and then with

4% paraformaldehyde (PFA) dissolved in phosphate-buffered saline (PBS, pH 7.4). The brains were removed and post-fixed in 4% PFA overnight at 4°C, and then equilibrated in 30% sucrose in PBS. 40 µm-thick coronal sections were prepared on a freezing microtome (Leica) and stored in cryoprotectant (25% glycerol and 30% ethylene glycol in PBS) at 4°C. Free-floating sections were washed in PBS and then incubated for 30 min in 0.3% Triton X-100 (Tx100) and 3% normal donkey serum (NDS). Slices were incubated at 4°C overnight with primary antibody in 3% NDS (Goat anti-ChAT 1:200, Millipore). Sections were then washed with PBS and incubated for 2 hr at room temperature with secondary antibodies (Donkey anti-goat conjugated to Cy3 or Cy5, Jackson Laboratories). Slices were then washed, incubated with DAPI (1:50,000) for 20 min, washed again, and mounted on slides with PVA-DABCO. Confocal fluorescence images were acquired on a scanning laser microscope using 5X or 10X air objectives, or a 40X oil immersion objective.

***In vivo* optrode recording**

Simultaneous optical stimulation and extracellular electrical recording were performed as described previously (6). Optrodes consisted of a tungsten electrode (1 MΩ; .005 in; parylene insulation) glued to an optical fiber (300 µm core diameter, 0.37 N.A.), with the tip of the electrode projecting beyond the fiber by 300-500 µm. The electrode was lowered through the NAc in approximately 100 µm increments, and optical responses were recorded at each increment. The optical fiber was coupled to a 473 nm or 560 nm laser. The power density was ~140 mW/mm² at the fiber tip for both wavelengths, which corresponds to a density at the tip of the electrode of about ~7-17mW/mm² for 470nm light and ~10-22 mW/mm² for 560nm light. Signals were amplified and band-pass filtered (300Hz low cut-off, 10 kHz high cut-off) before digitizing and recording to disk. At each site, 5 stimulation repetitions were presented and saved. Each stimulation epoch lasted 10-15 seconds with a recovery period of 80-90 seconds between the onset time of each repetition, and 50 seconds of data were recorded to disk for each repetition.

Conditioned place preference

All behavioral experiments were performed 4-6 weeks after virus injections during the animals' dark (active) cycle. The conditioned place preference (CPP) protocol was similar to those from previous reports of unbiased, balanced place-preference (7). The CPP apparatus consisted of a rectangular chamber with one side compartment measuring 23 cm x 26 cm with black walls and a grating on the floor, a central compartment measuring 23 cm x 11 cm with clear plexiglass walls and a plexiglass floor, and another side compartment measuring 23 cm x 26 cm with white walls and a punched metal floor. Mouse position during each day of testing was monitored using a video-tracking system. Floors were selected such that mice did not display average baseline bias for a particular chamber, and any mouse with a strong initial preference for a chamber was excluded (more than five minute difference spent in the side chambers on day 1). The CPP test consisted of the following. On day 1, each mouse was placed in the central chamber and allowed to freely explore the entire apparatus for 20 minutes (pre-test). Day 2 consisted of conditioning. In the morning, each mouse was confined to one of the side chambers for 20 minutes, and in the afternoon was confined to the other side chamber for the same period of time. For the cocaine CPP experiments, subjects received i.p. cocaine injections (20 mg/kg unless otherwise specified) before placement in one chamber, while subjects received i.p. saline injections of an equivalent volume before placement in the other chamber. (This concentration of cocaine allowed for robust conditioning with a single day of training in control animals, facilitating the optogenetic intervention). Mice received either yellow or bluelight during the 20 minutes in which they explored the compartment that was paired with the cocaine injection, whereas they were connected to a "dummy" fiber that was not emitting light when exploring the other chamber. The intensity of the blue light (470 nm) was chosen to generate power density of 140-200 mW/mm² at the fiber tip, which should correspond to a power density of ~4-7mW/mm² in the middle of the NAc. The intensity of the yellow light (590 nm) was chosen so that there was a power density of 70-140 mW/mm² at the fiber tip, which should correspond to a power density of ~3.5-7mW/mm² in the middle of the NAc. On day 3, exactly as in day 1, mice were placed in the center chamber and allowed to freely explore the entire apparatus for 20 min (post-

test). CPP experiments that did not involve cocaine were performed identically, except that the i.p. injections of cocaine or saline were omitted.

Open field

The open field test was conducted in an open plastic arena (50 cm long x 50 cm wide x 40 cm deep). Mice were individually placed in the center of the chamber and allowed to freely explore for 3 min. Activity in both the center and periphery of the field was measured using an automated video-tracking system (Viewer II, BiObserve). Time in center refers to time the mouse spent in the central 35 x 35 cm area of the open field.

Fear Conditioning

The fear conditioning apparatus consisted of a square conditioning cage (18x18x30 cm) with a grid floor wired to a shock generator and a scrambler, surrounded by an acoustic chamber. The top of the chamber was modified to enable light delivery during training by introducing an opening for the fiber. All mice received continuous yellow light during training but not during testing on the following day (590 nm; same power density as for the CPP experiments). To induce fear conditioning, mice were placed in the cage for 120 seconds; a pure tone (2.9 kHz) was then played for 20 seconds, followed immediately by a 2 second foot-shock (0.5 mA). This procedure was repeated, and 30 seconds after the delivery of the second shock mice were returned to their home cage. Freezing (complete immobility) was quantified for the 30 seconds before the first tone on the conditioning day to assess baseline freezing, as well as the 30 seconds immediately after the final shock on the conditioning day to assess immediate freezing. Contextual and auditory-cued fear conditioning were assessed the day after conditioning. To test contextual fear conditioning, mice were placed in the original conditioning cage, and freezing was measured for 5 min. To test auditory-cued fear conditioning, mice were placed in a different context: a pyramid shaped cage with a plexiglass floor. As a control for the influence of the novel environment, freezing was measured for 2.5 minutes in this new cage, and then the 2.9 kHz tone was played for 2.5 minutes, during which conditioned freezing was measured.

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Optogenetic photoactivation of ChAT interneurons in slice and *in vivo*.

A: Overlay of 15 current traces for the same MSN as in Fig. 2B, with each trace aligned to the light pulse. Some IPSCs are not time locked to the light pulses, whereas many are time locked with latency of ~8ms after light pulse onset.

B: IPSC occurrence as a function of time relative to light pulse for the same neuron. Open bars correspond to the number of IPSCs recorded during light stimulation; grey bars correspond to the number of IPSCs recorded during baseline (before light stimulation) using the same temporal alignment. For this neuron, an asynchronous enhancement in IPSC frequency is evident, in addition to the more prominent synchronous increase.

C: A rescaled presentation of Fig. 2D, displaying population-averaged percentage increase in IPSC frequency as a function of time relative to light pulses during the light-on relative to light-off period ($n=6$). Across the population, an asynchronous enhancement in IPSC frequency is evident, in addition to the more prominent synchronous increase. Pulse parameters for panels A-C: 470 nm, 5 ms pulse duration, 10 Hz.

D: Voltage traces from *in vivo* recordings showing population spikes (presumably generated by ChAT cells expressing ChR2) that track pulsed blue light stimulation at 10Hz (top) but not 100 Hz (bottom; 470 nm light; 10 sec total stimulation duration).

Figure S2. ChAT neuron inhibition disrupts cocaine CPP without affecting CPP in the absence of cocaine.

A: Cocaine CPP, same data as Fig. 4G (left panel) but plotted as difference rather than fold-change. Left: Difference in time in cocaine-conditioned chamber after conditioning versus before conditioning. ($n=10$ ChAT::Cre+, $n=12$ ChAT::Cre-; $p<.01$ for two-tailed t-test; 3 cohorts). Right: Difference in preference for cocaine-conditioned chamber after versus before conditioning, where preference is defined as the difference in time spent

in conditioned chamber versus the unconditioned chamber ($n=10$ ChAT::Cre+, $n=12$ ChAT::Cre-; $p<.01$ for two-tailed t-test; 3 cohorts).

B: CPP without cocaine, same data as Fig. 4G (right panel) and same data presentation as A. (For both panels, $n=9$ ChAT::Cre+, $n=7$ ChAT::Cre-; $p>.05$ for two-tailed t-test; 3 cohorts).

Figure S3. Nicotinic receptor antagonism decreases ChAT interneuron-evoked IPSCs recorded in MSNs.

A: Representative IPSC sweeps from a typical MSN in the acute slice preparation under the conditions of no light, light pulses (470 nm, 10 Hz, 5ms pulse width), and identical light pulses with 10 μ M mecamylamine.

B: Summary graph of IPSCs recorded as in **A** from a population of MSNs before light presentation, with light presentation, and with light and either mecamylamine or vehicle. Light stably increased IPSC frequency from 3.4 \pm 1.3 Hz to 10.1 \pm 1.2 Hz ($p < 0.05$; $n=7$, paired t-test), while mecamylamine reduced this increase to 5.1 \pm 1.8 Hz ($p<0.05$ compared to light-alone within the same cells, paired t-test; $p<.05$ compared to the vehicle control, $n=5$, unpaired t-test).

Figure S4. Modulation of ChAT interneurons over a range of cocaine-CPP parameters.

A: Dose-response curve for cocaine CPP during eNpHR3.0-mediated inhibition of the ChAT interneurons. Cocaine CPP is significantly decreased in ChAT::Cre+ mice for the standard rewarding dose of 20 mg/kg i.p. ($p < .01$), but not at other concentrations thought to be anxiogenic or insufficient (590 nm light, constant illumination; see Table S2).

B: Stimulation of ChAT neurons with ChR2 does not drive place preference by itself. (470 nm light, 5 ms pulse width, 10 sec of 10Hz stimulation every 30 sec ; $n = 4$, $p > 0.05$ two-tailed t-test).

C: Stimulation of ChAT neurons at 10 Hz with ChR2 does not significantly modulate cocaine place preference for i.p. 10 mg/kg cocaine. (470 nm light, 5 ms pulse width,

constant 10 Hz stimulation during cocaine conditioning; ChAT::Cre+ $n = 6$, ChAT::Cre- $n = 6$; $p > 0.05$ two-tailed t-test).

D: Stimulation of ChAT neurons at 10 Hz with ChR2 does not significantly modulate cocaine place preference for i.p. 20 mg/kg cocaine (470 nm light, 5 ms pulse width, steady 10 Hz stimulation during cocaine conditioning; ChAT::Cre+ $n = 4$, ChAT::Cre- $n = 3$; $p > 0.05$ two-tailed t-test).

Figure S5. Inhibition of ChAT interneurons with eNphR3.0 does not impair contextual or auditory-cued fear conditioning.

A: Percentage time spent freezing was quantified in a standard contextual fear conditioning paradigm. “Baseline” refers to the 30 seconds preceding the first tone-shock pairing. “Immediate” refers to the 30 seconds immediately after the second (and final) tone-shock pairing. “Context” refers to freezing to the same context on the day after the conditioning session. ChAT::Cre+ mice exhibited enhanced immediate and context freezing. ($n = 9$ ChAT::Cre+; $n = 8$ ChAT::Cre-; two-tailed t-test; $p < .05$ comparing ChAT::Cre+ and ChAT::Cre- for immediate and context freezing)

B: Percentage time spent freezing for the auditory-cued fear conditioning paradigm. “Pre-tone” refers to the 2.5 minutes in the new context before the onset of the tone; “Tone” refers to the 2.5 minutes during tone ($n = 9$ ChAT::Cre+; $n = 8$ ChAT::Cre-; two-tailed t-test; $p > .05$ comparing ChAT::Cre+ and ChAT::Cre-).

SUPPLEMENTARY TABLES

ChAT (ChR2-eYFP) (<i>n</i> = 19)		MSN (<i>n</i> = 13)	
V_M (mV)	R_{INPUT} (M Ω)	V_M (mV)	R_{INPUT} (M Ω)
-49.47 \pm 1.07	382.02 \pm 47.30	-65.43 \pm 2.88	223.64 \pm 31.92

TABLE S1:

Membrane voltage (V_M) and input resistance (R_{INPUT}) in brain slices of ChAT neurons expressing ChR2-eYFP and of MSNs that did not express a fluorophore. Both V_M and R_{INPUT} are higher for ChAT neurons than MSNs. ($p = 0.00003$ for V_M ; $p = 0.002$ for R_{INPUT} ; two-tailed t-test; mean \pm S.E.M.)

	mg/kg	0	10	20	40
ChAT::Cre+	<i>N</i>	9	11	10	4
	Conditioned side (min)	8.7	10.3	10.7	10.5
ChAT::Cre-	<i>N</i>	7	12	12	3
	Conditioned side (min)	8.7	8.5	14.2	9.2

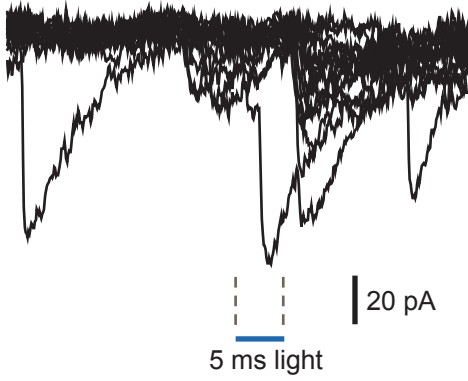
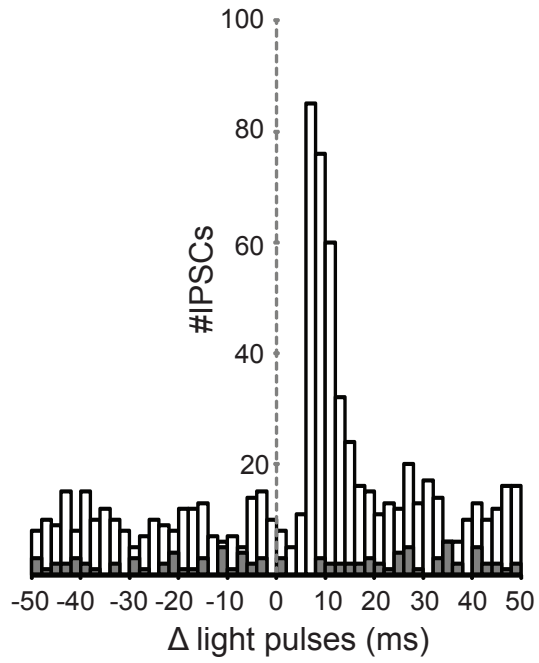
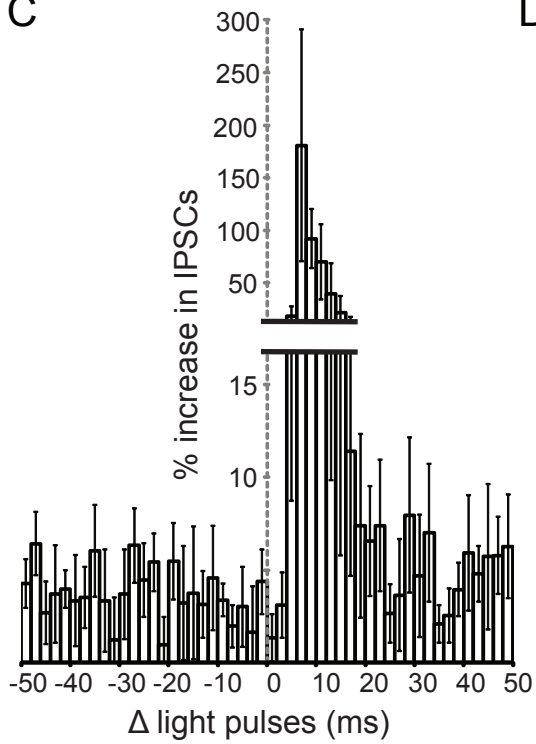
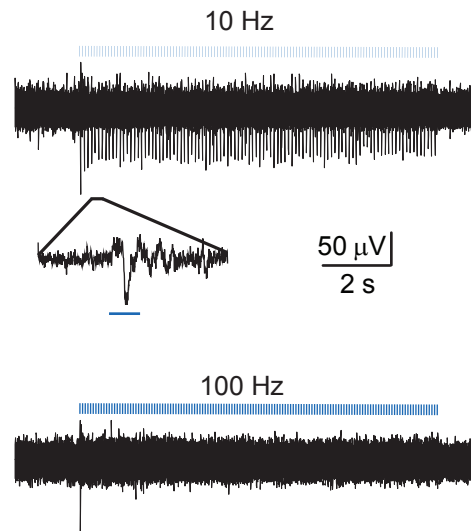
TABLE S2:

Total time spent on the cocaine-paired side on the testing day of the cocaine place preference paradigm (for various cocaine concentrations) when inhibition of the ChAT neurons (with eNphR3.0) was paired with cocaine exposure.

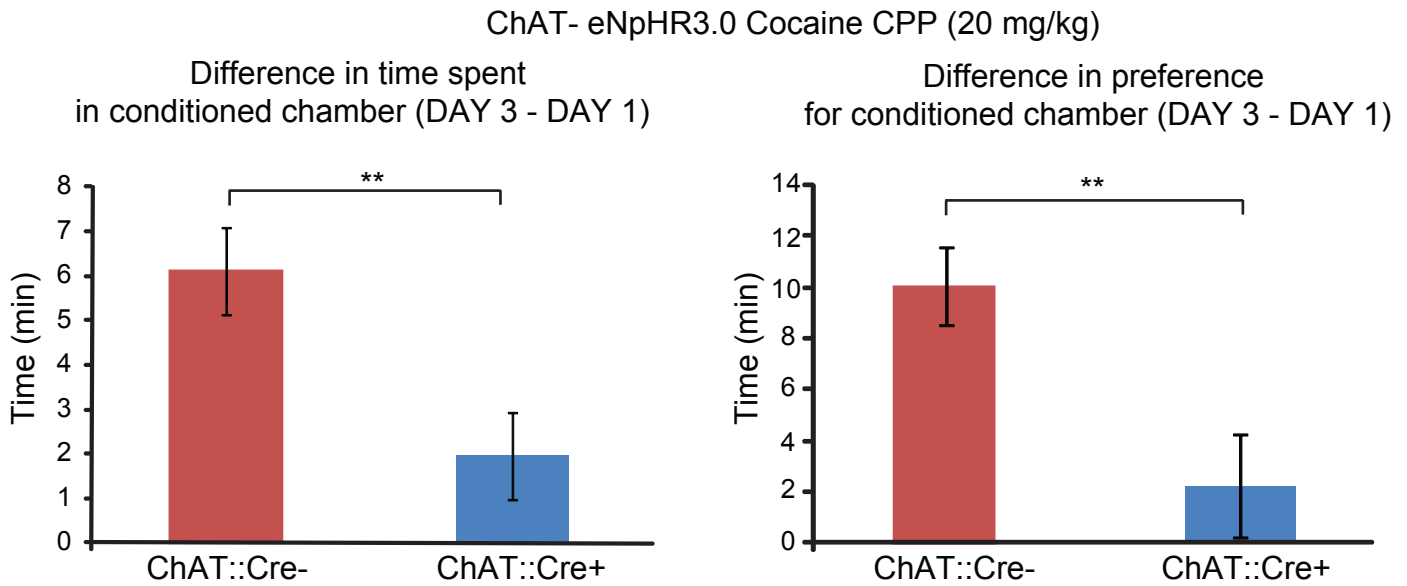
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Figure S1

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

A



B

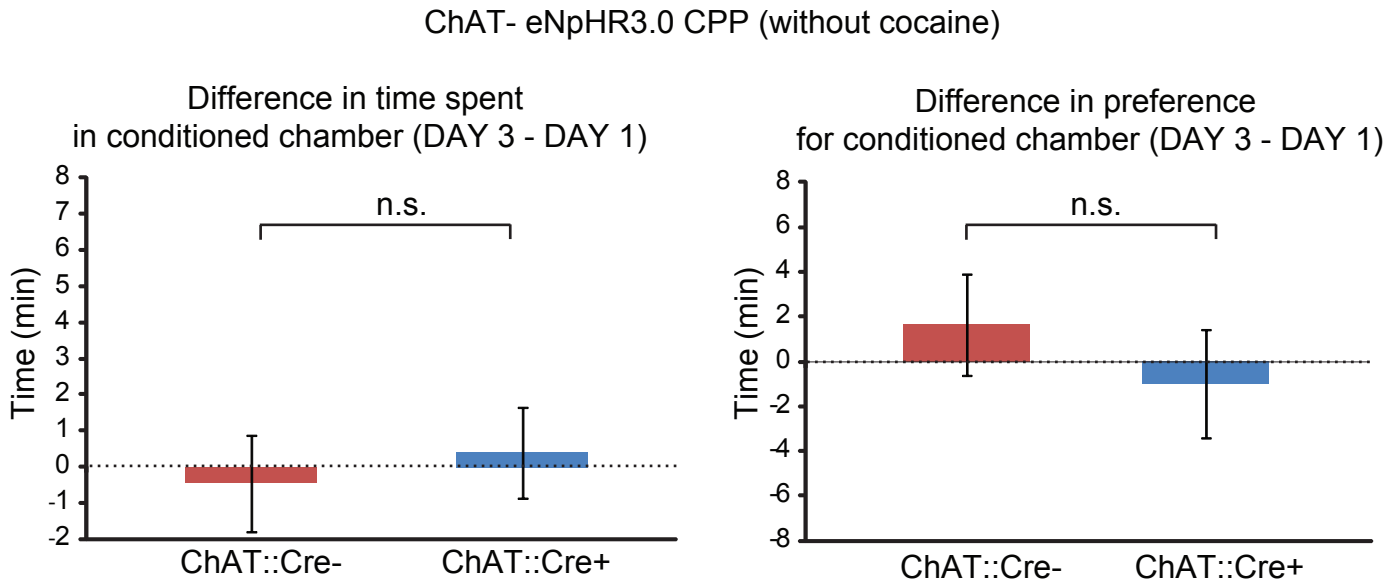
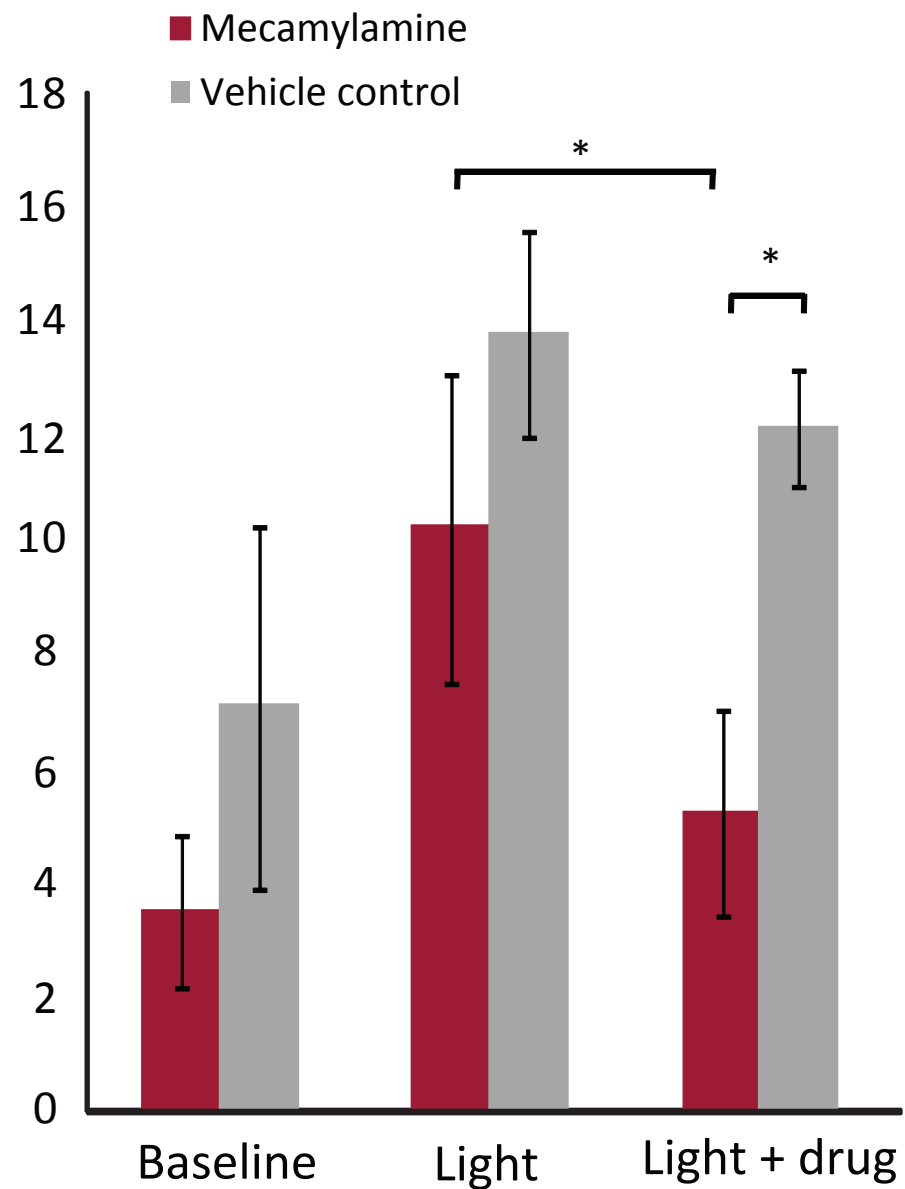
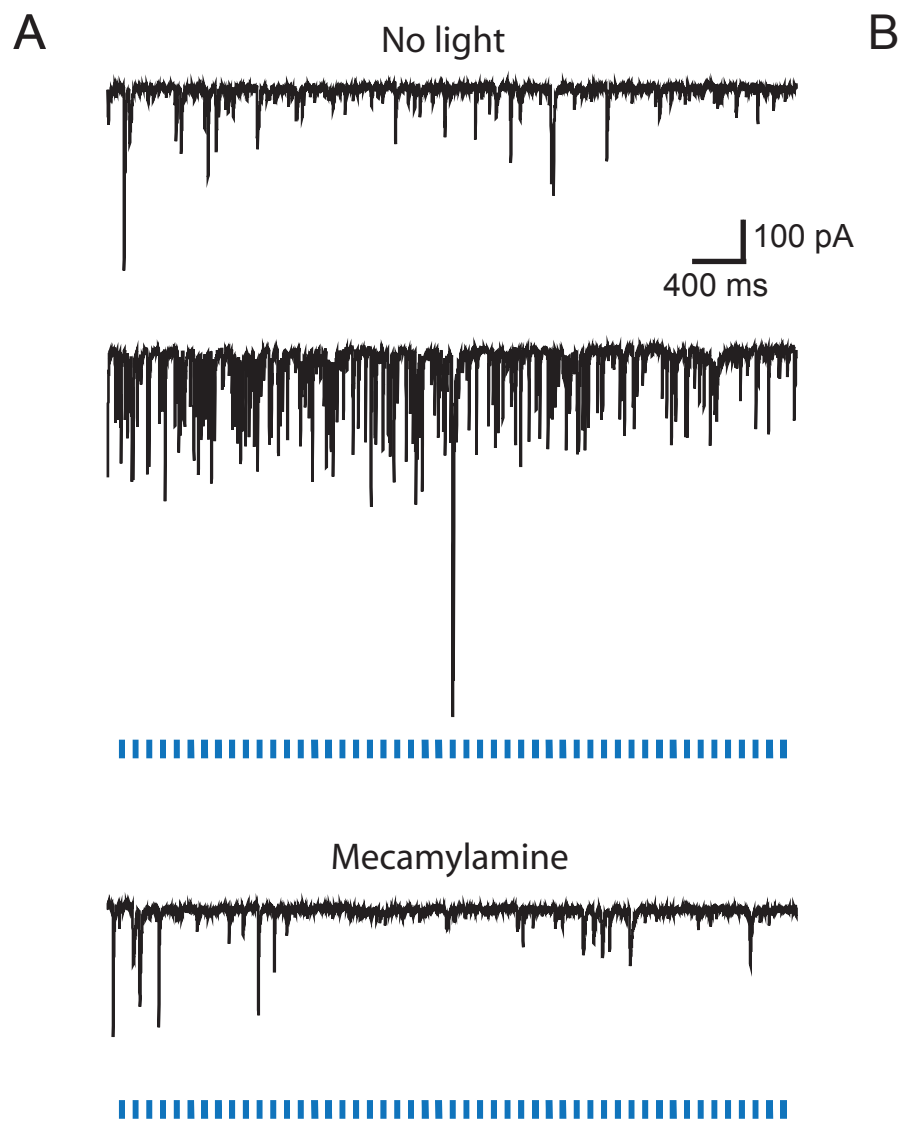
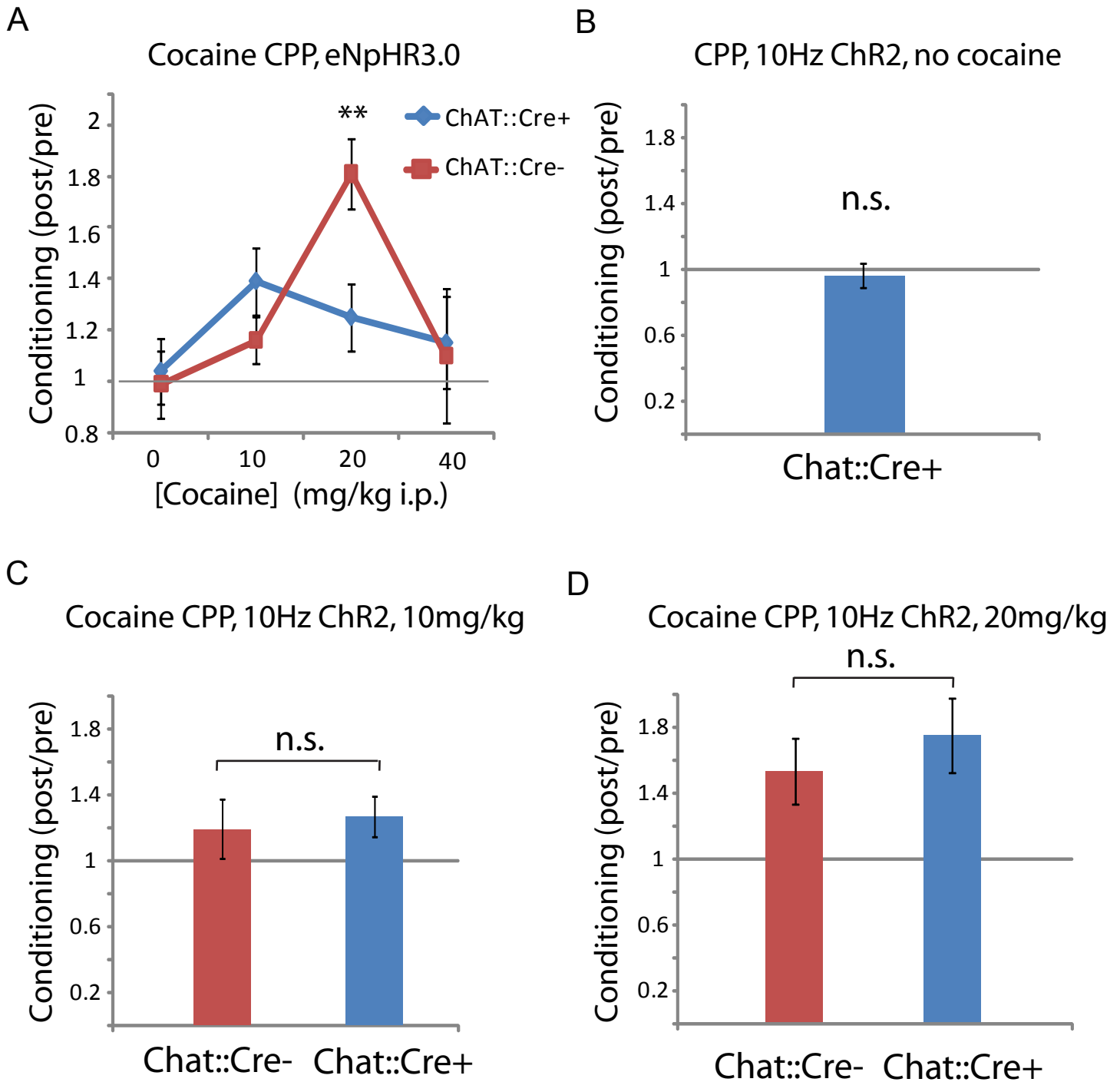
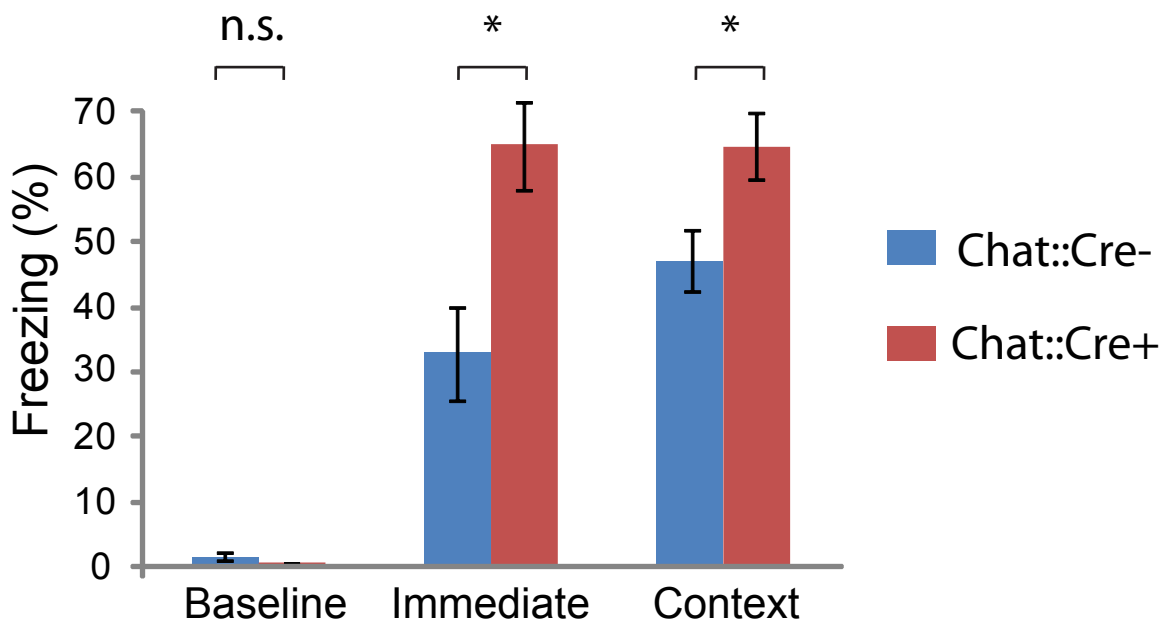


Figure S3





A



B

