Increased Number and Activity of a Lateral Subpopulation of Hypothalamic Orexin/Hypocretin Neurons Underlies the Expression of an Addicted State in Rats

Supplemental Information

Supplemental Methods and Materials

Animals

Male Sprague Dawley rats (~300g, 6-8 weeks upon arrival; Charles River, Raleigh, NC and Kingston, NY) were used for all experiments. Rats were single-housed under a reverse 12:12 h light cycle in a temperature- and humidity-controlled animal facility with ad libitum access to food and water. For all behavioral experiments, animals were tested at a similar time each day during their dark (active) cycle.

Drugs

Cocaine HCl (National Institutes of Drug Abuse, Research Triangle Park, NC) was dissolved in 0.9% sterile saline. As previously reported (1), SB-334867 [1-(2-methylbenzoxazol-6-yl)- 3-[1,5]naphthyridin-4-yl urea hydrochloride; provided by NIDA] was suspended in 2% dimethylsulfoxide and 10% 2-hydroxypropyl-b-cyclodextrin (Sigma) in sterile water; 0, 10 or 30 mg/kg was given in a volume of 4 mL/kg (i.p.) at 30 min prior to testing. SB has 50-fold selectivity for OX1Rs over orexin-2 receptors and 100-fold selectivity over approximately 50 other molecular targets (2, 3).

Intravenous catheter and stereotaxic surgery

One week after their arrival, animals were anaesthetized with isoflurane (1-3% with a flow rate of 2L/min) and received an analgesic (rimadyl, 5mg/kg, s.c.). Chronic indwelling catheters were inserted into the right jugular vein and exited the body via a port between the scapulae. Animals

received prophylactic i.v. cefazolin (10mg) and heparin (10U) daily starting 1 day after surgery and continuing throughout self-administration training. Following catheter implantation, a subset of animals that were to receive morpholino antisense infusions were transferred to a stereotaxic instrument (Kopf Instruments). Guide cannulae (22 gauge; Plastics One) were implanted to a depth of 2mm above the LH orexin field (A/P: -3.0, M/L: 2.6; D/V: 7.5 from skull surface at 6° angle) or the medial orexin field (A/P: -3.0, M/L: 1.6; D/V: 6.5 from skull surface at 6° angle) and were permanently fastened to the skull using acrylic cement. Obturators were inserted into each cannulae to prevent blockage. Rats were allowed to recover for 1 wk after surgery prior to beginning self-administration training.

Demand curve fitting

Demand curves were fit to data derived from the within-session BE paradigm via our lab's focused-fitting approach (4, 5). Briefly, each animal's brain cocaine concentration was calculated to determine relative stability during a session. Curves were fit to data points that occurred up to two points following the maximum price at which the subject maintained its preferred level of drug consumption (*P*max), as determined by a <25% decrease in brain cocaine concentration, with the omission of the first ('loading') data point. Demand curves were fit using standard regression techniques, where the values α and Q_0 in the exponential demand equation (4-6) were manipulated to minimize the residual sum of squares, i.e. the square of the difference between the logarithm of the experimentally measured demand and the logarithm of the demand predicted by the exponential demand equation was found for each price and then summed across all prices. α is an index of demand elasticity, an inverse measure of motivation for drug; calculated Pmax is the price at which the point slope of the demand curve is equal to -1, representing the maximum effort that the animal is willing to expend to defend preferred brain cocaine concentration; and Q_0 estimates consumption at null effort (4, 6). Consistent with our previous studies (4, 5), demand curves fit the data with R^2 values >0.85 in all cases.

Rats that failed to restabilize on the threshold procedure following ShA (n=2), LgA (n=1) or IntA (n=1) were excluded from further testing.

Test of compulsive responding

Compulsive responding for drug was assessed using a punished responding procedure as previously described (5). A relatively high cocaine infusion dose (0.38mg;2.6sec) was held constant throughout the session; shock was omitted for the first two 10min bins, however, beginning in the third bin, footshocks were delivered during each cocaine infusion and these increased in amplitude every 10min on a tenth-$\log_{10}$ scale: 0.13; 0.16; 0.20; 0.25; 0.32; 0.40; 0.50; 0.63; 0.79 milliamps. Shock resistance was characterized as the maximum cumulative charge in millicoulombs (mC) an animal self-administered in any one bin.

Extinction and reinstatement testing

Lever pressing was extinguished in daily 2h sessions whereby responses on the active lever did not result in either cocaine infusions or the presentation of light+tone cues. Extinction training lasted at least 7 d and until the last 3 consecutive days had <25 responses on the active lever. Animals were tested for cue-induced reinstatement, whereby light+tone cues were presented without cocaine, once at the start of the session and in response to active lever responses. Between reinstatement tests, animals underwent a minimum of 2 additional extinction sessions and until they recorded 2 consecutive days of <25 responses on the active lever. For cocaine-primed reinstatement, animals were given a priming dose of cocaine (10mg/kg, ip) immediately prior to being placed in the operant chamber and were then tested under extinction conditions. All reinstatement tests were conducted in a within-subjects manner, such that each rat was tested with 2 doses of SB and vehicle for each reinstatement modality. A subgroup of animals was tested for cue-induced reinstatement following mood assays (described below), which corresponded to 16 weeks following IntA/LgA/ShA training and approximately 12 weeks after

the final BE session. In this single test, animals were returned to their original operant chamber and tested for reinstatement under conditions identical to the first cued reinstatement test, but without SB or vehicle pretreatment.

Morpholino antisense injections

Injector cannulae (28G;Plastics One) were lowered through and 2mm below guide cannulae directed at either LH or PF/DMH; animals received a microinfusion of vivo-morpholino orexin antisense (OX-AS) (0.15nmol/0.3µl in 0.5mM phosphate buffer, Gene Tools, 5'-GTATCTTCGGTGCAGTGGTCCAAAT- 3') or control antisense with the reverse sequence to the OX-AS. These compounds have previously been shown not to affect the expression of non-orexin peptides in nearby neurons (7, 8).

Mood assays

Mood assays commenced after 4 weeks of home cage abstinence which followed BE testing. Tests were carried out in a subgroup of animals in the order outlined below and were separated by at least 5 days.

Saccharin preference test: Rats were habituated to bottles over 2 consecutive days. During this period, 2 bottles, one containing water and the other containing a 0.1% saccharin solution, were placed in the rat's home cage, and the location of the bottles were switched every 12h. The following day at noon (test day 1), the 2 bottles were re-introduced to the cage for a total of 2h, with the locations switched after 1h. This procedure was repeated at the same time the following day (test day 2). On both test days, preference for saccharin was calculated by determining the amount of saccharin consumed as a percentage of total liquid intake. Preference scores across both test days were averaged for each animal.

Open field test: Rats were placed in the locomotor chambers described below (*Locomotor testing*) for 15min. The amount of time spent within an 8x8 square matrix in the center of the chamber was determined using Fusion SuperFlex software.

Forced swim test: Rats were placed in a cylindrical Plexiglas tank (38.5 cm high × 30.5 cm diameter; INSTECH) filled with warm water (25°C) for 15 min on the first day and retested for 5 min 24 h later. A digital video camera mounted over the tank recorded behaviors during both tests. The following measures were scored on a video monitor from recordings by an observer blind to the experimental conditions of the animals: (*i*) Swimming: was characterized by swimming around the tank or crossing the cylinder by using the paws or tail without elevating the body out of the water; (*ii*) Climbing: front paw movements against the tank wall bringing part of the body out of the water; and (*iii*) Immobility: the animal was not engaged in neither swimming nor climbing activity. Data from the second test are presented.

Locomotor testing

A subgroup of IntA rats were assessed for general locomotor activity to test whether SB administration was associated with changes in non-specific motor activity, as in our previous publications (1, 9, 10). Rats were placed in locomotor chambers (clear acrylic, 42cm x 42cm x 30cm) equipped with SuperFlex monitors (Omintech Electronics Inc, Columbus, OH) containing a 16 x 16 infrared light beam array for the x/y axis (horizontal activity) and 16 x 16 infrared light beams for the z axis (vertical activity). Activity was recorded by Fusion SuperFlex software. Animals were habituated to the locomotor boxes for 2 h/day for at least 3d, and until the average total distance traveled by each rat was within a range of ±25% of the mean of those days. On test days, total distance traveled, as well as horizontal and vertical beam breaks were recorded over a 2h session. Between test days, animals were again habituated to the boxes

until stable behavior was again observed over 3 consecutive days. All animals received 2 doses of SB and vehicle in a fully counterbalanced order.

Histology

Rats were sacrificed either 1d or 150d following IntA training, or following the final BE test on the sixth day following antisense injections. Rats were anesthetized with ketamine/xylazine and perfused with 0.9% saline followed by 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (pH = 7.4). After an overnight postfix in 4% PFA, brains were cryoprotected in 20% sucrose-azide for at least 24h before being embedded in OCT and sectioned into 40um-thick free-floating sections using a cryostat. For orexin cell counting experiments, tissue was incubated overnight at room temperature in primary antibodies goat anti-orexin-A (1:500; Santa-Cruz Biotechnology, catalog number SC-8070; JCN Antibody database AB_653610) and rabbit anti-Fos (1:1000; Synaptic Systems, catalog number 226003; JCN Antibody database AB_2231974) in 2% normal donkey serum. The next day, the tissue was washed three times in PBST and incubated for 2h in appropriate secondary antibodies coupled to Alexa-Fluor 488/594-conjugated donkey anti-goat/rabbit (Jackson Immunoresearch Laboratories). Sections were then rinsed in PB, mounted onto glass slides and coverslipped using Fluoroshield mounting medium with DAPI (Abcam).

For MCH labeling, tissue underwent three washes in PBST-azide (phosphate buffer saline + 0.03% Triton X-100 + azide), before being incubated overnight at room temperature in primary antibody for rabbit anti-MCH (1:5000; Phoenix Pharm, catalog number H-070-47; JCN Antibody database AB_10013632); biotinylated goat anti-rabbit secondary antibody (1:500; Jackson) and amplified with the avidin biotin complex (ABC; Vector Labs) method (1:500), and visualized with 3, 3′ diaminobenzidine (DAB) with nickel ammonium sulfate, to yield a dark purple somatic stain in MCH neurons. For quantification of orexin in morpholino experiments, tissue was incubated

overnight in the same orexin antibody as above (1:500) followed by 2 h in a donkey anti-goat secondary (1:500; Jackson), amplified with ABC, and visualized with DAB to yield a brown somatic stain in orexin neurons. For both MCH and orexin stains, slices were mounted onto glass slides, washed and dehydrated through alcohol and xylenes, and coverslipped with DPX (Sigma).

Imaging and cell counts

Hypothalamic sections were imaged in tiles with a 16x objective on a Zeiss AxioZoom V16 microscope and then stitched together using Zen Imaging software. The number of cells immunoreactive for orexin-A, as well as the number of orexin-A cells that also expressed Fos, were quantified in three sections taken through the main rostrocaudal extent of the hypothalamic orexin neuron region in each rat. For the orexin-antisense experiment, bilateral accurate injector placement was verified, and then counts were performed in orexin-expressing regions closest to the injector tract in a subset of treated animals to confirm the effectiveness of the antisense approach. For all experiments, the medial and lateral hypothalamic subregions of the orexin cell field were quantified separately by an experimenter blind to experimental groups; the boundary between LH and PF was demarcated by a vertical line 100μm lateral to the lateral-most edge of the fornix.

Statistical analyses

Prism 6.0 (GraphPad Software) was used for all statistical analyses. For each access group and treatment condition, α, Pmax and Q_0 values were calculated as a percentage of baseline values and compared using one-way repeated measures ANOVA with Holm-Sidak post-hoc tests as appropriate. In cases where data were not normally distributed, appropriate non-parametric analyses were used. For the open field test, one animal exhibited behavior that was > 2 SDs above the group average and was excluded from this analysis. A P value of <0.05 was

considered statistically significant. Error bars represent means ± s.e.m. All *n* values represent individual rats.

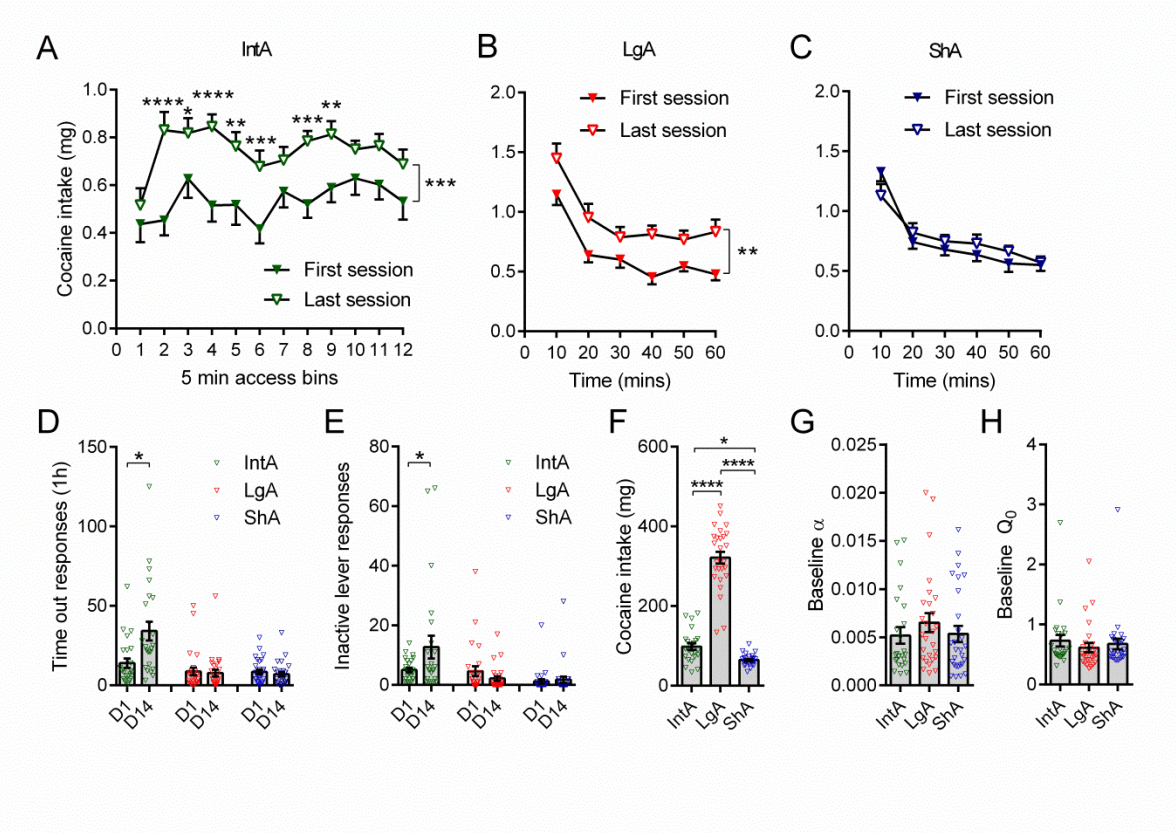

Figure S1. IntA is associated with enhanced cocaine seeking behavior.

(a) IntA animals generally earned more cocaine in each of the 5-min access bins on the final training session compared to their first training session ($F_{11,253}$=1.854, p=0.0459; RM ANOVA). n=24.

(b) A similar trend was observed in LgA animals when comparing intake in 10-min bins in the first hour of the first versus final session ($F_{1,26}$=7.301, p=0.001; RM ANOVA), although this increase was not as profound as that observed in IntA animals. n=27.

(c) There was no escalation of intake in ShA animals in the final session compared to the first session at any time in the self-administration sessions. n=28.

(d) IntA animals made significantly more active lever responses during the 1s infusion period (time out responses, which produced no further cocaine) during the 5 min cocaine access epochs in the final training session as compared to the first training session (t_{23}=3.451, p=0.0022; paired samples *t*-test). In LgA and ShA animals, no changes were observed in active

lever responding during infusions and the 20s time out period from the first to final session (p's>0.05; paired samples *t*-tests). IntA: n=24; LgA: n=27; ShA: n=28.

(e) IntA animals made significantly more inactive lever responses in the final training session as compared to the first training session (t_{23}=2.110, p=0.0459; paired samples *t*-test). No such differences were observed in LgA or ShA animals (p's>.05). IntA: n=24; LgA: n=27; ShA: n=28.

(f) Total cocaine consumption across training was significantly lower in IntA and ShA animals (both 1h total access) than in LgA animals (6h total access), revealing that higher cocaine motivation and seeking in IntA subjects occurs independently of amount of drug consumed ($F_{2,76}$=200.3, p<0.0001, ANOVA; IntA vs LgA t_{76}=15.54, p<0.0001; IntA vs ShA t_{76}=2.369, p=0.0204; LgA vs ShA t_{76}=18.61, p<.0001, Holm-Sidak post-hoc comparisons). IntA: n=24; LgA: n=27; ShA: n=28.

(g-h) Prior to IntA, LgA or ShA training, α (g) and Q_0 (h) values were similar among groups. IntA: n=24; LgA: n=27; ShA: n=28.

Error bars are s.e.m. *p<0.05. **p<0.01. ***p<0.001. ****p<0.0001

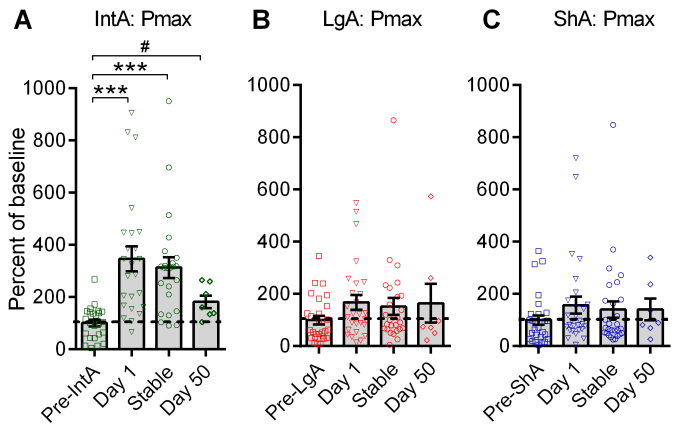

Figure S2. IntA is associated with a persistent increase in Pmax values.

(a) IntA was associated with significantly higher calculated-Pmax values at 1d (t_{23}=4.563, p=0.0001; Holm-Sidak post-hoc test) and ~7d (stable; t_{23}=4.881, p=0.0001; Holm-Sidak post-hoc test); overall RM ANOVA $F_{2,71}$=16.04, p<0.0001. A separate analysis on rats tested at d50 indicated that Pmax values remained significantly elevated at this time point t_6=2.808, p=0.0308. Pre-IntA, Day 1, Stable, n=24; D50 n=7. #p<.05 vs Pre-IntA values.

(b) There was not a significant change in Pmax values in LgA rats, P's>0.05. Pre-IntA, Day 1, Stable, n=27; D50 n=7.

(c) There was not a significant change in Pmax values in ShA rats, P's>0.05. Pre-IntA, Day 1, Stable, n=28; D50 n=7.

Error bars are s.e.m. ***p<.001. #p<.05 vs Pre-IntA values.

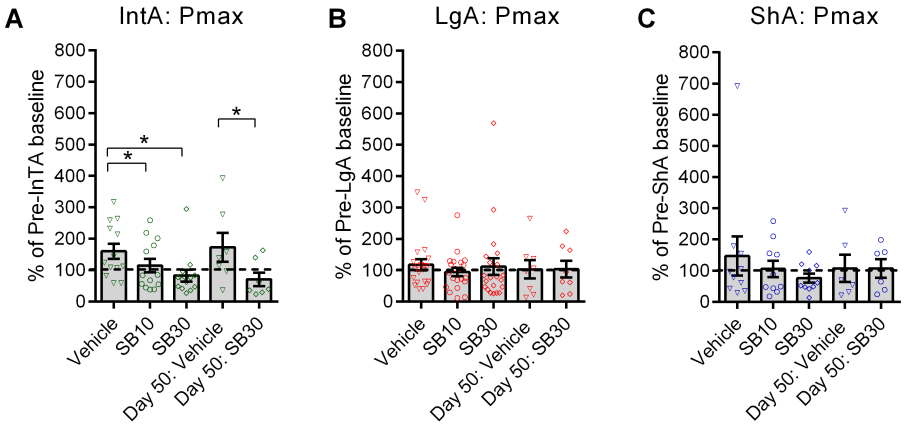

Figure S3. SB (OxR1 antagonist) reverses IntA-induced changes in Pmax.

(a) SB normalized Pmax values to baseline levels in IntA animals at both 10 (t_{12}=2.630, p=0.022) and 30mg/kg (t_{12}=3.131, p=.0173); overall ANOVA $F_{2,38}$=6.651, p=0.0076. SB30 remained effective at normalizing demand even when administered 50d following IntA training (W=-26. p=0.0313). Veh/SB10/SB30: n=13; D50 Veh/D50 SB: n=7.

(b) SB had no effect on Pmax overall in LgA animals (p's>0.05). Veh/SB10/SB30: n=21; D50 Veh/D50 SB: n=8.

(c) SB had no effect on Pmax overall in ShA animals (p's>0.05). Veh/SB10/SB30: n=10; D50 Veh/D50 SB: n=6.

Error bars are s.e.m. *p<0.05.

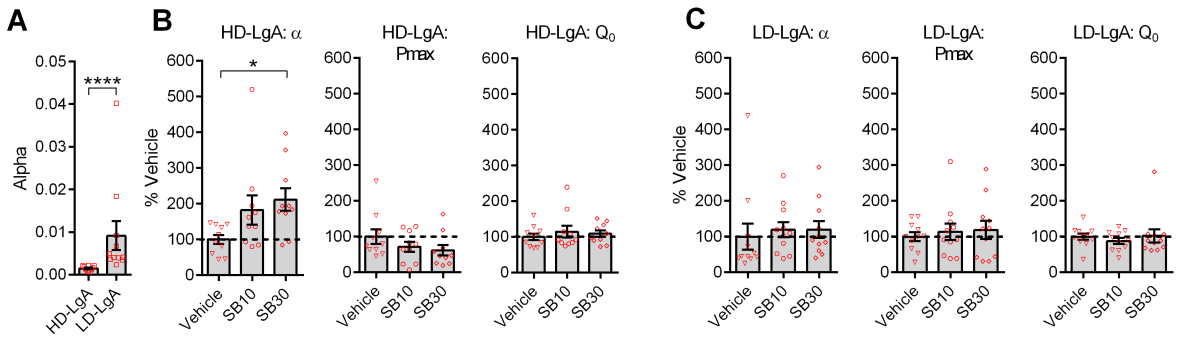

Figure S4. SB reduced demand for cocaine in a subset of high demand (low α) LgA animals.

(a) LgA rats were separated into high- and low-demand (HD and LD, respectively) groups based on a median split of α values under vehicle conditions after LgA; mean α values were significantly lower in the high-demand rats compared to low-demand rats (U=0, p<0.0001; Mann-Whitney test). HD: n=10; LD: n=11.

(b) In high demand (low α) LgA rats, SB30 significantly increased α ($F_{2,29}$=3.943, p=0.0451, ANOVA; t_9=2.683, p=.0495) but had no effect on Pmax or Q_0 values (p>0.05). n=10 for all doses.

(c) SB had no effect on α, Pmax or Q_0 in LD LgA rats (p>0.05). n=11 for all doses.

Error bars are s.e.m. *P<0.05. ****P<0.0001

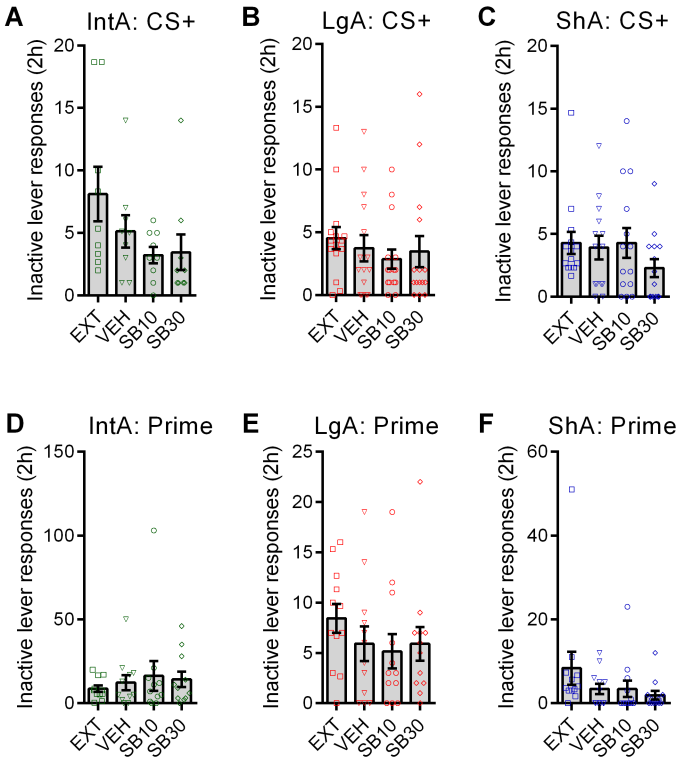

Figure S5. SB did not affect responding on the inactive lever during cued or primed reinstatement tests.

(a-c) Across all groups, there was no effect of SB on inactive lever responding during cued reinstatement testing (RM ANOVA; $p>0.05$). IntA: n=9; LgA: n=15; ShA: n=14 for all doses.

(d-f) Across all groups, there was no effect of SB on inactive lever responding during primed reinstatement testing (RM ANOVA; $p>0.05$). IntA: n=11; LgA: n=12; ShA: n=12 for all doses.

Error bars are s.e.m.

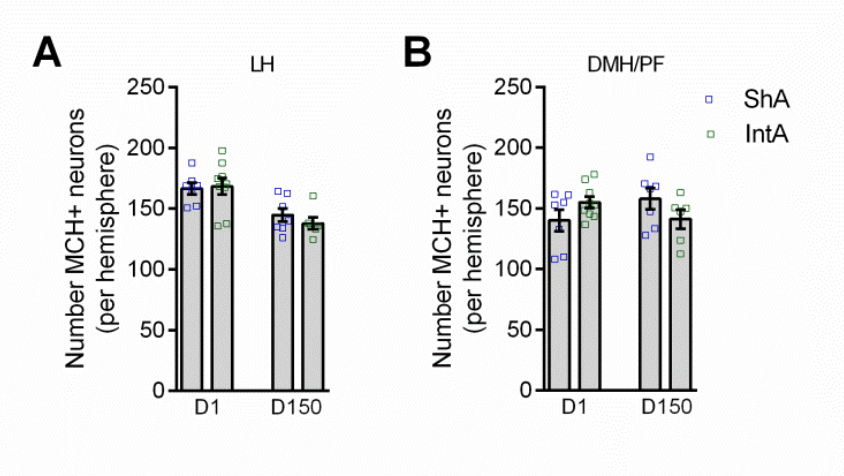

Figure S6. No difference in number of MCH neurons in IntA rats.

- **(a)** There was no difference in the numbers of LH MCH neurons in IntA vs ShA rats at either day 1 or day 150, p's>0.05.
- **(b)** There was no difference in the numbers of DMH/PF MCH neurons in IntA vs ShA rats at either day 1 or day 150, p's>0.05.

D1: ShA, n=7, IntA, n=9; D150: ShA, n=7, IntA=6. Error bars are s.e.m.

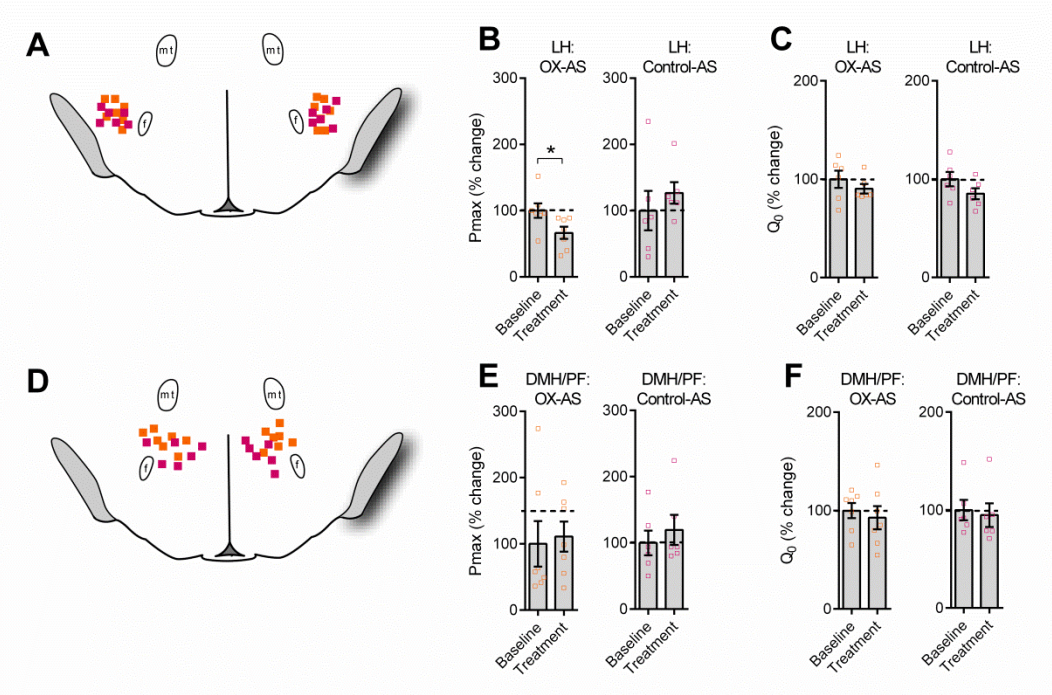

Figure S7. Location of injection tips for OX-AS experiments and effects on Pmax and Q_0.

(a) Location of injection tips directed at the LH orexin cell population. OX-AS injections are depicted in orange. Control-AS injections are depicted in pink. Coronal section, bregma +3.00, adapted from Paxinos and Watson (2006).

(b) Infusions of OX-AS into the LH orexin cell field significantly reduced Pmax values (left; t_6=2.698, p=0.0357). In contrast, infusions of control-AS had no effect on Pmax values (right; p>0.05).

(c) Infusions of OX-AS (left) or control-AS (right) into the LH orexin cell field had no effect on Q_0 values (p's>0.05), indicating that the effects observed of LH orexin-AS on α and Pmax were independent of changes in 'free' intake of cocaine.

(d) Location of injection tips directed at the DMH/PF orexin cell population. OX-AS injections are depicted in orange. Control-AS injections are depicted in pink. Coronal section, bregma +3.00, adapted from Paxinos and Watson (2006).

(e) Infusions of OX-AS (left; p>0.05) or control-AS (right; p>0.05) into the DMH/PF orexin cell field had no effect on Pmax values.

(f) Similarly, infusions of OX-AS (left; p>0.05) or control-AS (right; p>0.05) into the DMH/PF orexin cell field had no effect on Q_0 values.

Ox AS, n=7; Control AS, n=6. Error bars are ±s.e.m.

Supplemental References

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