OPTOGENETIC STIMULATION OF LATERAL HYPOTHALAMIC OREXIN/DYNORPHIN INPUTS IN THE VENTRAL TEGMENTAL AREA POTENTIATES MESOLIMBIC DOPAMINE NEUROTRANSMISSION AND PROMOTES REWARD-RELATED BEHAVIOURS

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ABSTRACT

Reward and reinforcement processes are critical for survival and propagation of genes. While numerous brain systems underlie these processes, a cardinal role is ascribed to mesolimbic dopamine. However, ventral tegmental area (VTA) dopamine neurons receive complex innervation and various neuromodulatory factors, including input from lateral hypothalamic orexin/hypocretin neurons which also express and co-release the neuropeptide, dynorphin ($LH_{ox/dyn}$). Dynorphin in the VTA induces aversive conditioning through the Kappa opioid receptor (KOR) and decreases dopamine when administered intra-VTA. Exogenous application of orexin or orexin 1 receptor (OXR1) antagonists in the VTA bidirectionally modulates dopamine-driven motivation and reward-seeking behaviours, including the attribution of motivational value to primary rewards and associated conditioned stimuli. However, the effect of endogenous stimulation of $LH_{ox/dyn}$-containing projections to the VTA and the potential contribution of co-released dynorphin on mesolimbic dopamine and reward related processes remains uncharacterised. We combined optogenetic, electrochemical, and behavioural approaches to examine this. We found that optical stimulation of $LH_{ox/dyn}$ inputs in the VTA potentiates mesolimbic dopamine neurotransmission in the nucleus accumbens (NAc) core, produces real time and place preference, and increases the incentive value attributed to a Pavlovian food cue. $LH_{ox/dyn}$ potentiation of NAc dopamine release and real time place preference was completely blocked by an OXR1 antagonist. Thus, rewarding effects associated with optical stimulation of $LH_{ox/dyn}$ inputs in the VTA are predominantly driven by orexin rather than dynorphin.

INTRODUCTION

Learning about the availability of resources, such as food, water, and mates, is crucial to species survival. To maximise opportunities to seek and consume rewards, humans and non-human animals alike must learn what signals predict reward availability, often relying on stimuli (cues) that precede or accompany reward delivery. Through Pavlovian conditioning, repeated pairing of a novel neutral stimulus with reward (unconditioned stimulus, US) imbues that neutral stimulus with predictive value, turning it into a conditioned stimulus (CS) than can readily inform goal-directed behaviour. However, this associative process can also attribute the cue with incentive (motivational) value further transforming the CS into an incentive stimulus [1,2]. Incentive stimuli are capable of instigating change in motivational states and directing reward-seeking behaviour independently of the primary reward [3–5].

Dopamine projections from the VTA, a central neurobiological hub of goal-directed, motivated, and reward processing behaviour, are numerous and complex [6]. Mesolimbic dopamine projections from the VTA to the nucleus accumbens (NAc) energize behaviour toward motivationally relevant cues[7,8]. All primary rewards whether natural, such as food, water, mates, or social interaction, or artificial such as all drugs of abuse increase dopamine neurotransmission [9–12]. Early studies have demonstrated that reward predictive cues increased firing of dopamine neurons [13], and thus it was originally thought that activity of dopamine neuronal activity underlies the predictive value of cues. Yet, Flagel and colleagues (2011) demonstrated that dopamine underpins the attribution of incentive value to reward cues, but not the development or expression of predictive value and stimuli associated with

these rewards [14,15]. The mechanisms underlying how dopamine encodes incentive value to motivationally relevant stimuli is not fully elucidated.

A growing body of evidence suggests that mesolimbic dopamine and associated behaviours are modulated by the lateral hypothalamic (LH) peptide, orexin (also known as hypocretin [16]). Orexin A and B are synthesized in the LH and perifornical area of the hypothalamus and project to numerous cortical and subcortical brain structures involved in motivational processes[17–19]. The VTA receives en passant LH orexin-containing fibres ($LH_{ox/dyn}$) with many dense core vesicles also containing the inhibitory neuropeptide, dynorphin [20,21]. Exogenous orexin application to VTA slices increases firing of dopamine neurons projecting to the NAc shell, but not the basolateral amygdala, whereas bath application of dynorphin has the opposite effect, suggesting a distinct role for orexin in promoting VTA-NAc dopamine activity [22]. Moreover, $LH_{ox/dyn}$ neurons respond to reward-related cues and activation of orexin receptor 1 (OXR1) in the VTA augments reward-seeking behaviours [23–27].

Conditioned stimuli imbued with incentive value evoke greater cFos expression in LH neurons, compared to purely predictive conditioned stimuli [28]. Furthermore, systemic injections of the OXR1 antagonist, SB-334867 attenuate cue-, context- or stress-induced reward seeking for various drugs of abuse and natural rewards [23,29–32]. However, it is unknown if $LH_{ox/dyn}$ action in the VTA can influence the incentive value of food cues. In this study, we combined optogenetic, behavioural, and electrochemical methods to systematically test the hypothesis that optical stimulation of $LH_{ox/dyn}$ afferents in the VTA promotes NAc dopamine release, reward-seeking behaviours, and increases the incentive value of food.

METHODS

Subjects

Adult male and female orexin-cre-eGFP mice (post-natal day 60-90) were originally obtained from the Yamanaka lab at the University of Tokyo [33] and bred locally. Mice were group-housed (3-5 per cage) with *ad libitum* access to water and food. Mice were housed in a temperature (21 ± 2ºC) and humidity-controlled (30-40%) room on a 12h reverse light/dark cycle. All testing was conducted during the active (dark) phase of the cycle. All experimental procedures adhered to ethical guidelines established by the Canadian Council for Animal Care and animal use protocols approved by the University of Calgary Animal Care and Use Committee.

Viral Infusion and Optical Fibre Implantation Surgeries

All mice received bilateral infusions of either channelrhodopsin ('ChR2') (AAV2/8-EF1a-DIO-hChR2(H134R)-mCherry; Neurophotonics, Centre de Recherche CERVO, Laval, QC) or control ('mCherry') (AAV2/8-hSyn-DIO-mCherry; Neurophotonics) virus. Mice were anaesthetised with isofluorane gas and secured in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). All measurements were made relative to bregma for viral infusions and optical fibre implantation. Viral injections were performed using a microinjector (Nano-inject II; Drummond Scientific Company, Broomall, PA, USA). Each mouse received 6 infusions into the LH (100 nl per infusion; 23.1 nl/s), 3 in each hemisphere (anterior-posterior (AP) -1.35; mediolateral (ML) ± 0.9; dorsoventral (DV) -5.2, -.5,1, -5.0) for a total of 300 nl per hemisphere. After each infusion, the microinjector was left in place for 3 min to allow diffusion of virus away

from the needle tip. After all infusions were complete in one hemisphere, the microinjector was left in place for an addition 5 min, 500 μm dorsal of the final injection location to allow diffusion of the virus through the brain tissue. All mice received postoperative analgesic (Ketoprofen 5 mg/kg, subcutaneous) and were returned to their home cages and allowed to recover for 6-8 weeks prior to further experimental procedures. The location of the virus expression was examined post hoc.

Mice included in behavioural experiments received a second surgery to implant optical fibres targeted at the VTA, 6 weeks after viral infusions. As above, mice were anaesthetised with isofluorane and secured in a stereotaxic frame (as above). Bilateral optical fibres (Doric Lenses Inc; Quebec City, QC, Canada) were lowered to just dorsal of the VTA (AP -3.5; ML ± 0.5; DV -3.8, -4.0) and secured with Metabond (C & B Metabond, Parkell Inc, Edgewood, NY, USA) and dental cement (Lang Dental Ltd, Rochester, NJ, USA). Mice were returned to their home cage and allowed to recover for 1 week prior to behavioural testing.

Experiment 1: Real-Time Place Preference (RTPP)

Orexin-cre ChR2 (n=8) and Orexin-cre mCherry (n=10) mice implanted with bilateral optical fibres underwent 3 stages of real-time place preference paradigm:

1. *Baseline Session (day 1).* Mice were attached to an extended patch cable and placed in the real-time place preference arena (50cm x 25cm x 50cm; underlit black Plexiglas, [34]). They were allowed to explore the arena for 20 min without receiving any stimulation, in order to record any innate preference for either compartment.

2. *Stimulation Sessions (days 2-4).* Mice were attached to an extended patch cable connected to a laser (473nm, 20Hz, 5mW, 5ms pulses) and placed into the arena again. One compartment of the arena was paired with laser activation (stimulation ON), whereas one compartment was paired with no stimulation (stimulation OFF). The arena compartment paired with stimulation was counter-balanced across mice. Each session lasted 60 min. Mice were tested at the same time every day.
3. *Test Session (day 5).* Mice were again attached to the extended patch cable. They were, again, allowed to explore the arena for 20 min without receiving any stimulation.

Data were collected using EthoVision XT software (Noldus Information Technology, Wageningen, Netherlands). Entry to each compartment of the arena and laser stimulation was signalled by EthoVision XT Software, triggering laser stimulation (473 nm, 20 Hz, 5 mW, 5 ms pulses, 1 s duration). The apparatus was cleaned with Virkon between each mouse. Separate, identical apparatuses were used to test male and female mice.

For the experiment involving the OXR1 antagonist, SB-334867, male and female orexin-cre mice were infused with ChR2 (n = 10) in LH and implanted with optical fibres targeted at the VTA. Mice underwent the RTPP as above. However, prior to each conditioning session (day 2-4), all mice received an intraperitoneal injection of either SB-334867 (n = 5, 15 mg/kg) (HelloBio Inc., Princeton, NJ, USA; dissolved in vehicle 10% hydroxypropyl-beta-cyclodextrin (HPBCD) and 2% dimethyl sulfoxide (DMSO) in sterile water w/v), or vehicle (n = 5), 15 min prior to being placed into the arena.

Experiment 2: Pavlovian Conditioning

Orexin-Cre ChR2 (n=8) or mCherry (n=10) mice implanted with bilateral optical fibres target at the VTA were used for both RTPP and Pavlovian conditioning in a counter-balanced order, where half first completed experiment 2 followed by experiment 3, and half completed experiment 3 prior to experiment 2.

Food Baseline (2 days). Mice were first given ~20 sucrose pellets (Bio-Serv, Flemington, NJ, USA) in their home cage during the active phase of their circadian cycle in order to overcome neophobia to the sucrose pellets. Next, mice were individually placed into a fresh home cage with a small amount of their bedding in the experimentation room and given access to sucrose pellets for one hour for two days. The amount of sucrose consumed was recorded for each day and compared between groups.

Cue Baseline (3 days). Mice were next attached to an optical fibre patch cable and placed into a sound-attenuated mouse behavioural conditioning chamber (Med Associates, St. Albans, VT, USA; dimensions: 15.24 cm x 13.34 cm x 12.7 cm). The chamber was equipped with a house light, 2 retractable levers, one cue light, and one sonalert (60-65 dB). Mice were allowed to explore the chamber and make responses on the two levers for 1 h for 3 consecutive days, with session duration signalled by house-light illumination. Responses on one lever ('active'; counterbalanced across subjects), resulted in the presentation of a novel 'neutral' stimulus (light + tone) for 8 s. Responses on the other lever ('inactive') was inconsequential but

recorded. Active – Inactive response on day 3 of baseline cue value assessment was compared between groups.

Pretraining (1-3 days). Behavioural chambers were equipped with a magazine, allowing delivery and retrieval of sucrose pellets. Levers were retracted. Mice were trained to retrieve sucrose pellets from the magazine in session where 25 pellets were delivered to the magazine on a variable time schedule (30 s; inter-trial interval [ITI] 10-60 s). Sessions lasted approximately 12 min, and mice remained in the chamber for 1 h to maximize familiarity with sucrose pellets and environment. Sessions were repeated until mice consumed at least 15 sucrose pellets in one session.

Pavlovian Conditioning (7 days). Next, mice underwent daily sessions of Pavlovian Conditioning for 7 consecutive days. Mice were connected to an extended patch cable and laser for each session. Each session consisted of 25 trials in which one sucrose pellet was delivered into the magazine, the cue light was illuminated and the sonalert sounded for 8 s. Laser stimulation (as above) lasted for the 8 s duration of the cue presentation for all mice. All trials were paired (sucrose delivery and cue onset at the same time) on a variable time schedule averaging 90 s, with ITI of 30-150 s. Each session lasted approximately 40 min. Duration of session was signalled by illumination of the house-light.

Conditioned Reinforcement (1 day). 24 h after cessation of the last Pavlovian conditioning session, mice were returned to the behavioural chamber. The chamber was equipped with 2

levers (one active; one inactive in locations matching cue baseline testing), with the food magazine removed. As in cue baseline sessions, mice were given 1 h to respond on both levers for presentation of the compound light-tone cue. Responses on both levers were recorded and compared to each individual mouse's responses in the last session of cue baseline testing.

Food Test (1 day). As in pre-conditioning food baseline testing, mice were placed into a novel home cage with ~20 sucrose pellets in the experimentation room for one hour. The weight of sucrose pellets consumed was recorded and compared to the last day of food baseline testing.

Video Analysis. All Pavlovian conditioning sessions were video recorded. The videos were then scored for approach and orientation (including latency to orient and latency to approach) to the cue by experimenters who were blinded to experimental conditions. Videos were each scored twice to provide inter-rater reliability.

Experiment 3: *In vivo* Fast-Scan Cyclic Voltammetry (FSCV)

Mice (ChR2 n= 8; mCherry n=8) were anaesthetized with intraperitoneal injection of 25% urethane (1.0-1.5 g/kg) dissolved in sterile saline and returned to home cage until they reached the desired plane of anaesthesia. Mice were then secured in a stereotaxic frame inside a Faraday cage, and a single anterior-posterior incision made to expose the skull. The skull was cleaned, and small craniotomies made above the NAc (AP +1.0; ML +1.0) and the VTA (AP -3.5; ML +0.5) and contralateral cortex (AP +1.8; ML -2.0). A chlorinated silver wire (Ag/AgCl)

reference electrode was implanted in the contralateral cortex (DV -0.8) and cemented in place (C&B metabond, as above).

A recording electrode (carbon fibre pulled through a glass pipette, cut to a final exposed length of 100-120 μm length; 7 μm diameter, Goodfellow, Coraopolis, PA, USA) was lowered to the right NAc core (~DV -3.8). Once the electrode was slightly dorsal of the desired location, it was left to equilibrate at 60 Hz for 20 min followed by 10 min at 20 Hz. FSCV data were collected using TarHeel CV (ESA Biosciences Inc., Chelmsford, MA, USA) and High Definition Cyclic Voltammetry Suite (University of North Carolina, Chapel Hill) by applying a 10 Hz triangular waveform (oxidative scans -0.4 V to +1.3 V (vs. Ag/AgCl), reductive scans +1.3 V to -0.4 V, 400 V/s) to the carbon fibre microelectrode. A bipolar stimulating electrode (P1 Technologies [formerly Plastics One], Roanoke, VA, USA), combined with an optical fibre (constructed in house, cut to ~30 μM shorter than the electrode was incrementally lowered, in 100 μm steps, into the VTA. The recording electrode was also incrementally lowered in 100 μm steps. At each increment, a train of current pulses (60 pulses, 4 ms per pulse, 60 Hz) was delivered until phasic DA neurotransmission was reliably observed at the carbon fibre microelectrode. Dopamine was identified by its characteristic oxidation and reduction potentials which occur at approximately +0.6 mV and -0.2 V, respectively (vs. Ag/AgCl).

Experimental data were collected in 120 s files, with stimulation onset occurring 5 s into the recording. 10 recordings of electrical stimulation (60 Hz, 60 pulses), 10 recordings of optical stimulation (laser as above; 473 nm, 20 Hz, 20 mW, 5 ms pulses, 1s duration), and 10 recordings of combined electrical (as above) and optical stimulation (as above) were made in a counter-balanced order across subjects.

Carbon fibre microelectrodes were pre-calibrated to known concentrations of dopamine (250 nM and 1000 nM) as previously described [35] to allow conversion of recorded *in vivo* signals to changes in dopamine concentration using chemometric analysis, principal component regression, and residual analyses using TarHeel and HDCV (as above; [36]). Data were analysed to calculate the peak dopamine and area under the curve for the 10 s period following stimulation onset. Data were compared between electrical, laser, and combined electrical-laser stimulation to determine the effect of $LH_{ox/dyn}$-VTA stimulation on mesolimbic dopamine neurotransmission.

For SB-334867 injections, orexin-cre mice infused with either ChR2 (n = 5; as above) or mCherry virus (n= 4; as above). After expression, we conducted anaesthetised FSCV recording evoked responses to electrical stimulation of the VTA and combined electrical stimulation of the VTA with optical stimulation of $LH_{ox/dyn}$-containing neurons as above. We then gave all mice an intraperitoneal injection of SB-334867 (15 mg/kg). After 15 min, we recorded evoked responses to electrical stimulation and to electrical + optical stimulation and compared the area under the curve and peak dopamine concentration to recordings made prior to administration of the antagonist.

Histology

For behavioural experiments, to check viral transfection, optical fibre placement, and colocalization of orexin and mCherry virus, mice were deeply anesthetized with isoflurane and transcardially perfused with phosphate buffered saline (PBS) and then with 4% paraformaldehyde (PFA). Brains were dissected and post-fixed in 4% PFA at 4°C overnight, then

switched to 30% sucrose. Coronal frozen sections were cut at 30 µm using a cryostat. Sections were heated in citrate buffer (pH=6.0) at 80-85ºC for 40 min to expose antigen. 10% goat and donkey serums were applied to block non-specific binding for 1 h. Sections were then incubated with primary antibody rabbit anti-orexin 1:500 (Phoenix pharmaceuticals, H-003-30) and chicken red fluorescent protein (RFP) 1:2000 (Rockland, 600-901-379) in 1% BSA for 48 h at room temperature followed by incubation with secondary antibody Alexa Fluor 488 donkey anti-rabbit and Alexa Fluor 594 goat anti chicken 1:400 for 1 h on LH sections and for 4-5 h on VTA sections.

For FSCV experiments, to check for electrode placements and viral transfection, mice were transcardially perfused with PBS and then 4% PFA. Brains were dissected and post-fixed in 4% PFA at 4°C overnight, then switched to 30% sucrose. Coronal frozen sections were cut at 30 µm using a cryostat. Sections were blocked in 10% donkey serum for 1 h before incubation in primary antibody rabbit anti-RFP (Rockland, 600-401-379) 1:2000 in 1% BSA for 1 h followed by incubation with secondary antibody Alexa Fluor 594 donkey anti rabbit 1:400 for 1 h.

For all experiments, slices were mounted with Fluoshield (Sigma). All images were obtained on an Olympus virtual slide microscopy VS120-L100-W with a 10x objective (Olympus Canada Inc., Ontario, Canada) and a Leica confocal microscopy TCS SP8 with a 25x objective (Leica Microsystems Inc., Ontario, Canada).

Statistics

All statistical analyses were completed using GraphPad Prism 8. All values are expressed as mean ± standard error of the mean (SEM). The alpha risk for the rejection of the null

hypothesis was set to 0.05. All data met criteria for normality unless otherwise specified. All post hoc tests were conducted with Sidak correction for multiple comparisons, unless otherwise stated. The levels of significance are indicated as * and *** to denote $P < 0.05$ and $P < 0.01$, respectively.

RESULTS

EXPERIMENT 1: Endogenous $LH_{ox/dyn}$ release in the VTA promotes place preference

$LH_{ox/dyn}$ neurons are activated in response to a variety of stressors [37] and selective optogenetic stimulation of $LH_{ox/dyn}$ neurons increases the probability of a transition to wakefulness [38]. However, exogenous application of orexin or inhibition of OXR1 in the VTA can promote or inhibit reward related processes, respectively [16]. Therefore, we wanted to test if optogenetic stimulation of $LH_{ox/dyn}$ inputs in the VTA would promote preference or avoidance (Figure 1A-D). We used a real-time place preference (RTPP) paradigm, whereby mice can move freely within the apparatus throughout the session, but entry into one of the compartments is paired with intra-VTA optogenetic stimulation of $LH_{ox/dyn}$ inputs. In the absence of stimulation, there was no significant baseline preference for either compartment displayed by mCherry control or ChR2 expressing mice (mCherry: $t(7) = 1.611$, $p = 0.151$; ChR2: $t(7) = 0.769$, $p = 0.467$; Figure 1C,E). Furthermore, mCherry mice did not develop a significant preference for either compartment of the apparatus over the three stimulation sessions of RTPP (Figure 1C). However, the ChR2 mice demonstrated a significant preference for the compartment paired with intra-VTA $LH_{ox/dyn}$ stimulation over the 3 stimulation days (3 x 2 repeated measures (RM) ANOVA: within subject factors: Day, $F(2,14) = 7.048$; $p = 0.03$;

Stimulation compartment, F (1,2) = 0.185, p = 0.674; between subject factor: Virus, F (1, 2) = 3909, p = 0.001; Virus x Stimulation compartment, F (2,14) = 14.493, p = 0.002; Day x Stimulation compartment x Virus, F (2, 14) = 3.379, p = 0.057; Figure 1E, F). Sidak's post hoc tests confirmed that ChR2 mice spent significantly more time in the stimulation compartment compared to the non-stimulation compartment on days 2 (t (7) = 3.396; p = 0.012) and 3 (t (7) = 4.185, p = 0.004).

To determine if intra-VTA photostimulation of $LH_{ox/dyn}$ can influence associative learning mechanisms, we next examined whether mCherry or ChR2 mice demonstrated a preference for either compartment in the absence of optical stimulation in a subsequent session. While the mCherry mice did not show a significant preference for either compartment, the ChR2 mice spent significantly more time in the compartment previously paired with stimulation (mCherry: t(7) = 0.739, p = 0.484; ChR2: t(7) = 4.603, p = 0.002; Figure 1E). Thus, $LH_{ox/dyn}$ in the VTA can induce rewarding effects upon stimulation as well as form associative memories related to it.

To assess if locomotor activity was influenced by stimulation, we measured distance travelled in the whole apparatus between mCherry and ChR2 mice. The overall distance travelled in each session did not differ between the mCherry (8.6 $\pm$ 1.5 m) and ChR2 (6.0 $\pm$ 2. 6 m) groups (t (14) = 1.730, p = 0.1056). Furthermore, velocity did not differ between mCherry (0.0026 $\pm$ 0.00055 m/s) and ChR2 (0.0017 $\pm$ 0.00055 m/s) mice (t(7) =1.6, p = 0.161). We then examined whether locomotor activity differed between mCherry and ChR2 mice in each compartment. A 2-way ANOVA showed there was no significant difference between the distance travelled in the stimulation ON or stimulation OFF compartments (within subject factor: compartment, F(1,28) = 2.469, p = 0.01274; between subject factor: virus, F(1, 28) =

4.007, p = 0.0551; virus x compartment interaction, F (1,28) = 1.243, p = 0.2744; Figure 1G). Velocity also did not differ between stimulation ON or stimulation OFF chambers (within subject factor: chamber, F (1,28) = 0.5144, p = 0.4792; between subject factor: virus, F (1,28) = 6.391, p = 0.0174; chamber x virus interaction: F (1, 28) = 0.1991, p = 0.6589; Figure 1H). Thus, intra-VTA optical stimulation of $LH_{ox/dyn}$ produced a preference for the compartment paired with stimulation, but this did not influence locomotor activity, suggesting that $LH_{ox/dyn}$ in the VTA can promote contingent learning.

As $LH_{ox/dyn}$-containing neurons corelease the inhibitory neuropeptide dynorphin [20,39] and the fast-acting neurotransmitter glutamate [40], we next examined the role of OXR1 signalling using the OXR1 antagonist SB-334867, in preference for optical stimulation of $LH_{ox/dyn}$ inputs in the VTA. As in Figure 1, we first established that ChR2 mice did not show a significant preference for either compartment prior to stimulation sessions (vehicle: t(4)= 0.562, p = 0.604; SB-334867: t(4)=0.543, p = 0.616; Figure 2A). Next, we compared the percent time spent in each compartment during the stimulation sessions for vehicle and SB-334867-treated mice. The vehicle group developed a significant preference for the compartment paired with $LH_{ox/dyn}$ stimulation in the VTA over the 3 stimulation days (2-way RM ANOVA: day: F(2,8)= 10.768, p = 0.047; compartment: F(1,4)= 34.260, p = 0.04; day x compartment interaction: F(2,8)= 1.520, p = 0.281). Whereas, the SB-334867 group did not develop a significant preference for either compartment over the stimulation sessions (2-way RM ANOVA: day: F(2,8) = 1.323, p = 0.314, compartment: F(1,4)= 0.012, p = 0.917, day x compartment interaction: F(2,8) = 0.285, p = 0.736; Figure 2A,B).

To determine if SB-334867 influenced locomotor activity in either compartment, we measured distance travelled as well as velocity. There was a significant main effect of SB-334867 on distance travelled, but no interaction between drug and compartment suggesting that the influence of SB-334867 on locomotor activity did not have differential effects in the stimulation ON or OFF compartment (2-way RM ANOVA: between subject factor: drug, $F(1,8) = 8.260$, $p = 0.0207$, within subject factor: chamber, $F(1,8) = 0.07155$, $p = 0.7959$, drug x compartment interaction, $F(1,8) = 0.05025$, $p = 0.8283$; Figure 2C). Similarly, administration of SB-334867 had a significant effect on velocity, but this was not different between stimulation ON and OFF compartments (between subject factor: drug, $F(1,8) = 5.672$, $p = 0.0444$, within subject factor: compartment, $F(1,8) = 3.156$, $p = 0.1136$, drug x compartment interaction, $F(1,8) = 3.787$, $p = 0.0875$; Figure 2D).

Lastly, we compared percent time spent in each compartment in the post-stimulation test session. We found that mice injected with vehicle spent significantly more time in the paired compartment compared to the unpaired compartment ($t(4) = 4.071$, $p = 0.002$) ,whereas the mice given SB-334867 showed no significant preference for either compartment ($t(4) = 0.160$, $p = 0.881$; Figure 2A). Thus, inhibition of OXR1 signalling prevented both the development of preference for $LH_{ox/dyn}$ optical stimulation in the VTA as well as its associated memory.

EXPERIMENT 2: Endogenous $LH_{ox/dyn}$ release in the VTA promotes approach to a Pavlovian food cue.

We first determined the baseline food cue evaluation in the absence of optical stimulation. There was no significant difference between the number of responses made for

presentation of the novel compound light-tone food cue between mCherry (7.7 $\pm$ 2.9 presses) and ChR2 mice (5.4 $\pm$ 2.3 presses) ($t(16) = 0.795$, $p = 0.419$). Next, we compared the number of conditioned responses by quantifying approaches to the magazine or food cue during presentation of the food cue across days between mCherry and ChR2 mice as a measure of incentive value attribution. Optical stimulation of $LH_{ox/dyn}$ inputs to the VTA of ChR2 mice showed significantly more conditioned responses compared to that of the mCherry group. (2-way RM ANOVA: Virus: $F(1,9)= 5.774$, $p = 0.04$, Day: $F(2,6)= 1.554$, $p = 0.217$; Virus x Day interaction: $F(1,6)= 0.863$, $p = 0.528$; Figure 3B). We then compared the number of rewards received during optical stimulation of $LH_{ox/dyn}$ inputs to the VTA of mCherry or ChR2 mice. Both groups showed a significant increase in the number of rewards received across days, but no significant difference in the number of rewards received between mCherry or ChR2 mice (2-way RM ANOVA: Virus: $F(1,9) = 0.133$, $p = 0.722$; Day: $F(1,6)= 2.439$, $p <0.05$; Virus x Day interaction: $F(1,9) = 1.747$, $p = 0.198$; Figure 3C). We also compared the effect of optical stimulation on entries into the magazine in the absence of food cue presentation. Both ChR2 and mCherry mice significantly decreased non-CS magazine entries across conditioning days but this did not differ between mCherry and ChR2 mice (2-way RM ANOVA: Virus: $F(1,13)= 1.257$, $p = 0.283$; Day: $F(1,9)= 2.174$, $p = 0.006$; Virus x Day interaction: $F(1,9)= 1.239$, $p = 0.306$; Figure 3D). Taken together, these results indicate that both mCherry and ChR2 mice underwent Pavlovian conditioning in this task, but the optical stimulation of $LH_{ox/dyn}$ inputs to the VTA increased approach to the food cues.

We next assessed whether intra-VTA optical stimulation of $LH_{ox/dyn}$ inputs during Pavlovian conditioning influenced the value of the cue by comparing responses for presentation

of the food cue with the baseline prior to conditioning. While all mice significantly increased responses for the cue after conditioning, there was no significant main effect of virus or a significant responses x virus interaction (2-way RM ANOVA: active - inactive responses: $F(1,17)= 5.420$, $p = 0.0307$; virus: $F(1,16)= 0.062$, $p = 0.806$; active-inactive responses x virus: $F(1,16)= 0.43$, $p = 0.53$; Figure 3E). Furthermore, there was no significant difference in the number of lever presses for the cue between ChR2 and mCherry mice ($t(7) = 0.3292$, $p = 0.7515$; Figure 3F). Lastly, we compared the amount of sucrose consumed in the home cage after conditioning to the baseline food intake. There was no effect of conditioning on homecage sucrose consumption in either mCherry or ChR2 mice (2 -way RM ANOVA: within subject factor: time, $F(1, 16) = 0.1224$, $p = 0.7311$, between subject factor: virus, $F(1,16) = 3.757$, $p = 0.0704$, time x virus interaction, $F(1,16) = 0.7364$, $p = 0.4035$; Figure 3G). Taken together, intra-VTA optical stimulation of $LH_{ox/dyn}$ during Pavlovian conditioning did not change the value of the food cue or consumption of the primary reward.

To determine if the value of the primary reward changed with intra-VTA optical stimulation of $LH_{ox/dyn}$ inputs, sucrose pellets were placed in a fresh cage with a mCherry or ChR2 mouse in the absence of optical stimulation (Figure 3A,G). The amount of sucrose consumed during the baseline and post-conditioning tests did not differ significantly between mCherry (0.029 ± 0.01 KCal/g) and ChR2 mice (0.041 ± 0.014 KCal/g) (2-way RM ANOVA: within subject factor: Time, $F(1,16)= 0.1224$, $p = 0.7311$, between subject factor: Virus, $F(1,16)= 3.757$, $p = 0.0704$, Time x Virus interaction, $F(1,16)= 0.7364$, $p = 0.4035$). Thus, optical stimulation of $LH_{ox/dyn}$ inputs in the VTA during Pavlovian conditioning does not influence the value of the primary reward.

EXPERIMENT 3: Endogenous $LH_{ox/dyn}$ in the VTA release potentiates electrically evoked mesolimbic dopamine neurotransmission

Exogenous application of orexin in the VTA increases dopamine release [25] whereas systemic OXR1 antagonists inhibit cocaine-evoked dopamine [24]. Furthermore, exogenously applied dynorphin decreases dopamine in the NAc [12]. However, it is unknown how optogenetic stimulation of $LH_{ox/dyn}$ inputs in the VTA can influence dopamine in the NAc. We first tested if optical stimulation of intra-VTA of $LH_{ox/dyn}$ inputs alone can evoke dopamine in the NAc core using *in vivo* FSCV. Optical stimulation did not significantly alter NAc dopamine concentration measured as area under the curve (AUC) for the 5 s after stimulus offset in either mCherry (0.30 $\pm$ 0.16 μM) or ChR2 mice (0.076 $\pm$ 0.22 μM) (t (12) = 1.363, p = 0.1979). Thus, intra-VTA optical stimulation of $LH_{ox/dyn}$ inputs alone is insufficient to drive dopamine release in the NAc.

In order to examine whether optical stimulation of $LH_{ox/dyn}$ inputs to the VTA could modulate electrically evoked dopamine neurotransmission (Figure 4A, B), we electrically stimulated dopamine release with or without intra-VTA optical stimulation of $LH_{ox/dyn}$ inputs (Figure 4C, D). In the absence of optical stimulation, evoked stimulation was not significantly different between mCherry (1.42 $\pm$ 0.54 μM) and ChR2 mice (2.28 $\pm$ 0.86 μM) (t(13) = 1.575, p = 0.1393). There was a significant effect of virus on evoked peak dopamine concentration (2-way ANOVA: virus (F(1,13)= 36.198, p = 0.0032)), as well as a significant main effect of optical stimulation (stimulation: F(1,13)= 4.808, p = 0.033; virus x stimulation interaction: F(1,13)= 10.390, p = 0.047). A Sidak's post hoc test indicates no significant difference in evoked peak

dopamine concentration with or without optical stimulation in mCherry mice *(p = 0.1812),* but a significant difference in evoked peak dopamine concentration with optical stimulation in ChR2 mice (*p = 0.0001*; Figure 4E). Similarly, there a was a significant main effect of virus on evoked AUC stimulation type (2-way RM ANOVA: F (1,13) = 7.795, *p* = 0.0153), and a significant main effect of optical stimulation (F(1,13)= 4.48, *p* = 0.0473); virus x stimulation interaction: F(1,13)= 10.38, *p*= 0.0067*;* Figure 4F). Sidak's multiple comparison post hoc tests revealed a significant difference in AUC of evoked dopamine concentration after optical stimulation in ChR2 mice (*p =* 0.001), but not mCherry mice *(p= 0.5391)* (Figure 4F). Taken together, optogenetic stimulation of $LH_{ox/dyn}$ inputs in the VTA significantly potentiated electrically evoked dopamine neurotransmission in ChR2, but not mCherry mice.

To examine whether potentiation of evoked dopamine concentration by intra-VTA optical stimulation of $LH_{ox/dyn}$ inputs was mediated by OXR1 signalling, we measured the effect of the OXR1 antagonist, SB-334867, on evoked dopamine neurotransmission in the presence or absence of optical stimulation of $LH_{ox/dyn}$ inputs to the VTA of ChR2 or mCherry mice (Figure 5A-D). Consistent with that reported in Figure 4, we found that prior to application of SB-334867, optical stimulation of evoked dopamine influenced peak concentration in the NAc of ChR2, but not mCherry mice (3-way RM ANOVA: within subject factors: stimulation, F(1,12)= 1.396, *p* = 0.2603; pre vs post SB-334867: F(1,12) = 0.2061, *p* = 0.6579, between subject factor: virus: F(1,12)= 13.96, *p* = 0.0028; stimulation x virus interaction: F(1,12) = 5.765, *p* =0.0335; stimulation x pre vs post SB-334867: F(1,12)= 3.018, *p* = 0.1079, virus x pre vs post SB-334867 interaction: F(1,12)= 0.00184, *p* = 0.9664, virus x stimulation x pre vs post SB-334867 interaction: F(1,12) = 0.8366, *p* = 0.3784, Figure 5D,E). A Sidak's posthoc test indicated a

significant difference in peak evoked dopamine in ChR2 *(p = 0.0171),* but not mCherry mice *(p =* 0.1941*)* (Figure 4E) prior to SB-334867 administration, but not after (p= 0.1193). After administration of SB-334867, there was no significant effect of optical stimulation on evoked peak dopamine concentration in the NAc of either mCherry or ChR2 mice (2-way RM ANOVA: within subject factor: stimulation, $F(1,6) = 0.19$, $p = 0.6770$, between subject factor: virus, $F(1,6) = 4.98$, $p = 0.0670$, stimulation x virus interaction: $F(1,6) = 1.37$, $p = 0.2861$) . Taken together, potentiation of NAc dopamine by stimulation of $LH_{ox/dyn}$ inputs to the VTA was blocked by administration of SB-334867.

DISCUSSION

Here, we found that endogenous $LH_{ox/dyn}$ release in the VTA does not enhance NAc dopamine on its own, but significantly potentiates evoked mesolimbic dopamine neurotransmission, an effect dependent on OXR1 signaling. Furthermore, optical stimulation of $LH_{ox/dyn}$ in the VTA can produce place preference that is also observed after cessation of optical stimulation, consistent with previous work demonstrating that systemic SB-334867 can inhibit the expression of conditioned place preference (CPP) for food[32] or drugs of abuse[16,23]. During optical stimulation of $LH_{ox/dyn}$ in the VTA, approach to a Pavlovian food cue is enhanced, a key feature of stimuli attributed with incentive value. Thus, $LH_{ox/dyn}$ in the VTA may promote reward-seeking behaviours through potentiating NAc dopamine as well as increasing the incentive value of cues.

Our results extend previous work demonstrating that orexin in the VTA increases striatal dopamine. Inhibition of OXR1 in the VTA disrupts cocaine-potentiated evoked phasic dopamine

release measured with FSCV in the rat caudate putamen [24]. Similarly, intra-VTA application of orexin augments dopamine [41] and potentiates cocaine-induced tonic dopamine release in rat NAc core measured with microdialysis [25]. Intra-VTA optical stimulation of $LH_{ox/dyn}$ inputs alone was not sufficient to augment NAc dopamine but required phasic activation of VTA dopamine neurons to increase dopamine in the NAc core. Consistent with this, intra-VTA orexin can increase phasically evoked (induced by 60 Hz stimulation) dopamine release in the caudate putamen with or without intravenous cocaine [25]. NMDA receptor activation in the VTA is required for phasic dopamine [42,43]. Exogenously applied orexin in the VTA increases NMDA receptor currents and promotes synaptic plasticity of dopamine neurons [44]. Thus, $LH_{ox/dyn}$ in the VTA may require coincident NMDA receptor activation, which likely occurs with 60 Hz stimulation, to potentiate NAc dopamine.

$LH_{ox/dyn}$ inputs to the VTA contain dynorphin in most dense core vesicles [20]. Orexin and dynorphin have opposing action on firing of VTA dopamine neurons that project to the NAc [22]. Thus, an important question is how endogenous activation of $LH_{ox/dyn}$ inputs to the VTA can influence dopamine output. Potentiation of NAc dopamine by $LH_{ox/dyn}$ inputs in the VTA was blocked by a OXR1 antagonist, suggesting that dynorphin or glutamate from $LH_{ox/dyn}$ were not drivers in this effect. While $LH_{ox/dyn}$ neurons express the fast transmitter glutamate [40] and when optogenetically stimulated can evoke local excitatory postsynaptic currents [45], only about 15% of $LH_{ox/dyn}$ fibers make appositional contacts in the VTA and less than 5% show synaptic specializations onto dopamine neurons [21], even though there are abundant $LH_{ox/dyn}$ fibres throughout the VTA [46]. However, there is a strong presence of orexin-containing dense core vesicles within the VTA [21]. Indeed, orexin in the VTA modulates dopamine neuronal

activity independently of glutamate in a projection-specific manner [22]. Thus, similar to other neuropeptides, orexin is likely released from sites devoid of synaptic specializations away from the active zone [47] to modulate the activity of dopamine neurons and consequent NAc dopamine release.

Optical stimulation of $LH_{ox/dyn}$ inputs in the VTA produces a strong place preference for a context paired with that stimulation. Continuous pairing of the intra-VTA $LH_{ox/dyn}$ stimulation transformed a neutral stimuli (compartment) to a conditioned stimulus, whereby mice spent more time in the paired compartment. $LH_{ox/dyn}$ stimulation in the VTA induced a progressive increase in preference for the paired compartment over the 3 pairing days in an OXR1 dependent manner, suggesting that orexin action in the VTA plays a role in acquisition of this preference. Presumably, dynorphin is also released during $LH_{ox/dyn}$ stimulation, and stimulation of KOR in the VTA typically induces a conditioned place aversion[48] and suppresses NAc dopamine [12]. Inhibition of OXR1 in the VTA increases reward thresholds for electrical self-stimulation, decreases impulsive responding on a 5-choice serial reaction time task, and decreases cocaine self-administration[20]. However, all of these effects are blocked by pretreatment with a KOR antagonist [20], suggesting that orexin in the VTA can negate the aversive effects of dynorphin. Paradoxically, in a CPP paradigm, pretreatment with a KOR agonist in the VTA potentiates preference for cocaine-associated contexts [49,50] and cocaine-evoked NAc dopamine [50]. Thus, it is possible that $LH_{ox/dyn}$ in the VTA could unmask an obscured somatodendritic component of KOR activation in NAc core-projecting dopamine neurons, similar to cocaine, leading to potentiation of place preference or dopamine release.

While outside the scope of the current study, this hypothesis should be tested in future research.

24 hours after cessation of optical stimulation of $LH_{ox/dyn}$ inputs in the VTA, a continued place preference was observed. This suggests a long term encoding of a preference for the environment paired with endogenous orexin release, rather than a transient behavioural influence of the stimulation itself. These data uphold previous findings that intra-VTA OXR1 signaling attenuates CPP to morphine or cocaine[23,41], suggesting that $LH_{ox/dyn}$ in the VTA is sufficient for associating the context with both natural rewards and drugs of abuse. Further, our findings suggest that optical stimulation of $LH_{ox/dyn}$ in the VTA is sufficient to produce reward-related behaviour in the absence of any tangible primary reward. $LH_{ox/dyn}$ neurons respond to a variety of stressful stimuli [37] and optical stimulation of $LH_{ox/dyn}$ in the locus coeruleus is sufficient to enhance threat memory formation [51]. Thus, the target region of $LH_{ox/dyn}$ action is critical for predicting the net effect of OXR1 activation. As such, $LH_{ox/dyn}$ in the VTA is critical for underlying reward-associated behaviours.

Because $LH_{ox/dyn}$ in the VTA was necessary and sufficient for associating the context with preference for stimulation, we wanted to assess if $LH_{ox/dyn}$ in the VTA was involved in the attribution of incentive value to a food reward and/or a Pavlovian food cue. Optical stimulation of $LH_{ox/dyn}$ inputs in the VTA increased conditioned responses to a food cue, suggesting that endogenous orexin release in the VTA modulates incentive value attribution to food cues. However, we did not observe any influence of prior $LH_{ox/dyn}$ stimulation in the VTA on subsequent conditioned reinforcement behaviour, suggesting that these effects did not produce enduring changes to the attribution of incentive value to a food cue in this paradigm.

Furthermore, we did not observe changes in the incentive value of the primary reward, suggesting that $LH_{ox/dyn}$ in the VTA is only implicated in the incentive value attribution to conditioned stimuli. This is an important finding as the role of cues in various behaviours, including aberrant incentive value attribution to drug cues may directly involve orexin signaling and thus provide a putative therapeutic target.

These results complement recent findings by Haight and colleagues [52], whereby inhibition of OXR1 and OXR2 in the paraventricular nucleus of the thalamus (PVT) attenuated sign-tracking, a robust measure of incentive value attribution to a food cue, but did not affect goal-tracking, a measure of purely predictive value of a food cue. They found similar transient effects of OXR1 antagonism in the PVT, with no impact on later reward related behaviour including conditioned reinforcement. Thus, the effects of $LH_{ox/dyn}$ in the VTA or PVT in increase incentive value of stimuli, but only during active orexin signaling in these regions. These and our findings suggest that $LH_{ox/dyn}$ in the PVT or the VTA may play an important role in the contemporaneous expression, but not a lasting encoding of incentive value.

In conclusion, this study demonstrates a significant role for endogenous orexin release in the VTA in the development and expression of RTPP, the attribution of incentive value to a food cue, and the potentiation of mesolimbic dopamine neurotransmission. Given that optical stimulation of $LH_{ox/dyn}$ terminals in the VTA likely release dynorphin in addition to orexin, our results demonstrate that the effects of orexin predominate in mediating reward-seeking behaviour. These findings shift forward our understanding of neural circuitry underpinning reward-related processes and highlight that neuromodulation in distinct target regions may have independent or opposing effects. These findings not only contribute to our knowledge of

general reward processes but, alongside previous work demonstrating a role for orexin in addiction, add support for targeting orexinergic systems as a possible treatment of addiction.

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Author Contributions

C.S.T. designed, performed and analyzed all experiments with supervision of S.L.B. M.R. performed and analyzed behavioural experiments with supervision of S.L.B and C.S.T. M.Q. performed immunohistochemistry experiments. C.S.T. and S.L.B. wrote the manuscript and agree to be accountable for all aspects of the work.

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Figure Legends:

Figure 1. Optogenetic stimulation of $LH_{ox/dyn}$ inputs in the VTA promotes place preference.

A) Schematic of viral strategy. Orexin-cre mice were infused with either channelrhodopsin (AAV2/8-EF1a-DIO-hChR2(H134R)-mCherry; 'ChR2') or control virus (AAV2/8-hSyn-DIO-mCherry; 'mCherry'). (LH: lateral hypothalamus; VTA: ventral tegmental area)

B) Coronal brain sections (modified from [53]) representing i) estimated viral transfection areas in the LH (-1.34 mm in anterior-posterior plane relative to Bregma) and ii) schematic of optical fibre implantation site in the VTA (-3.50 mm in anterior-posterior plane relative to Bregma).

C) Schematic of behavioural apparatus used for all real-time place preference (RTPP) experiments. Blue square in middle panel denotes the chamber associated with 473 nm laser stimulation of $LH_{ox/dyn}$. Compartments were counterbalanced across mice.

D) Immunohistochemical demonstration of fluorophore expression in LH cell bodies genetically encoded fluorophore (green; orexin-cre eGFP) and viral transfection fluorophore (red; ChR2 mCherry); ChRr2 virus infected cells were colocalized with orexin.

E) Time course of RTPP prior to and during optogenetic stimulation of $LH_{ox/dyn}$ inputs in the VTA of mCherry (orange) or ChR2 (blue) mice in the compartment paired with stimulation (filled circles) and the no stimulation compartment (open circles). Over 3 stimulation sessions of RTPP, ChR2 mice developed a significant preference for the compartment paired with $LH_{ox/dyn}$ stimulation whereas mCherry mice did not. In the post-stimulation test session, ChR2 mice preferred the compartment previously paired with stimulation, whereas mCherry mice did not.

F) Representative heat maps of percent time spent in both compartments of the RTPP arena for mCherry and ChR2 mice on stimulation session 3.

G) Photostimulation of $LH_{ox/dyn}$ inputs to the VTA did not alter the distance travelled by mCherry (orange) and ChR2 (blue) mice in either the stimulation ON (filled bars) or stimulation OFF (open bars) compartments of the RTPP arena.

H) Photostimulation of $LH_{ox/dyn}$ inputs to the VTA did not alter velocity of locomotor activity mCherry (orange) and ChR2 (blue) mice in either the stimulation ON (filled bars) or stimulation OFF (open bars) compartments.

Figure 2. OXR1 mediates preference induced by optogenetic stimulation of $LH_{ox/dyn}$ inputs in the VTA.

A) Over 3 stimulation sessions of RTPP, ChR2 mice receiving vehicle injections (blue) developed a preference for the chamber paired with $LH_{ox/dyn}$ stimulation (filled circles) in the VTA, whereas ChR2 mice receiving SB-334867 injections (pink) did not develop a preference for the stimulation ON compartment (filled circles) or the stimulation OFF compartment (open circles) of the RTPP arena. ChR2 mice receiving vehicle injections (blue) also demonstrated a preference for the stimulation ON chamber (filled circles) during the post stimulation test session, whereas ChR2 mice receiving SB-334867 (pink) did not.

B) Representative heat maps of the percent time spent on day 3 of stimulation sessions in both compartments of the RTPP arena by ChR2 mice receiving either vehicle (left) or SB-334867 (right) injections prior to each stimulation session.

C) Distance travelled of ChR2 mice was reduced in mice administered SB-334867 (pink) compared to vehicle (blue). However, there was no difference in distance travelled in compartments with intra-VTA $LH_{ox/dyn}$ stimulation ON (filled bars) compared to stimulation OFF (open bars).

D) Velocity of ChR2 mice was reduced in mice administered SB-334867 (pink) compared to vehicle (blue). However, there was no difference in velocity in compartments with intra-VTA $LH_{ox/dyn}$ stimulation ON (filled bars) compared to stimulation OFF (open bars).

Figure 3. Optogenetic stimulation of $LH_{ox/dyn}$ inputs in the VTA promotes approach to a Pavlovian food cue.

A) Schematic of behavioural paradigm. Blue square denotes intra-VTA $LH_{ox/dyn}$ stimulation sessions.

B) ChR2 mice (blue) made more conditioned responses throughout conditioning sessions compared to mCherry mice (orange). Conditioned responses were defined as approach to the cue light or entries into the food magazine during the CS presentation. All sessions were video recorded and retrospectively scored by experimenters who were double blinded to experimental conditions.

C) mCherry (orange) and ChR2 (blue) mice consumed significantly more sucrose across conditioning sessions, but there was no difference in the sucrose consumed by ChR2 or mCherry mice.

D) mCherry (orange) and ChR2 (blue) mice made significantly fewer entries into the food magazine when the CS was not illuminated across conditioning sessions but was not different between groups.

E) mCherry (orange) and ChR2 (blue) mice made significantly more active-inactive responses during conditioned reinforcement (CR) sessions (filled bars) compared to pre-conditioning baseline cue value assessment (open bars). There was no difference in the number of active-inactive responses made for the cue between mCherry and ChR2 mice.

F) There was no significant difference in the change from baseline cue value assessment in the number of CS presentations received by mCherry (orange) and ChR2 (blue) mice.

G) mCherry (orange) and ChR2 (blue) mice did not differ in the sucrose consumed per body weight at pre-conditioning baseline (open bars) or post-conditioning (filled bars).

Figure 4. Optogenetic stimulation of $LH_{ox/dyn}$ inputs in the VTA potentiates evoked NAc dopamine release.

A) Schematic of viral injection (LH), electrode and optical fibre placement and stimulation parameters (VTA), and fast-scan cyclic voltammetry (FSCV) recording location in the NAc core.

B) Coronal brain sections (modified from [53]) at 3 different anterior-posterior locations relative to Bregma illustrating the approximate recording location of FSCV data. Approximate electrode tip location was retrospectively determined using the electrode track and recording depths (relative to brain surface).

C) Averaged dopamine traces for mCherry (top left, orange) and ChR2 (top right, blue) groups in response to electrical stimulation. Representative false colour plots of dopamine concentration for mCherry (bottom left) and ChR2 (bottom right) mice following electrical stimulation. In all panels grey box denotes 60Hz 60pulses (1s) electrical stimulation onset and offset. Inset to colour plots, current-voltage plot showing oxidation and reduction peaks for dopamine.

D) Averaged dopamine traces for mCherry (top left, orange) and ChR2 (top right, blue) groups in response to electrical and laser stimulation. Representative false colour plots of dopamine concentration for mCherry (bottom left) and ChR2 (bottom right) mice following electrical and optical stimulation. In all panels blue box denotes optical stimulation (20Hz) and electrical stimulation (60Hz) onset and offset. Inset, current-voltage plot showing oxidation and reduction peaks for dopamine.

E) There was no significant difference in the peak dopamine concentration ([DA]) recorded for electrical and electrical (open bars) and laser (filled bars) stimulation for mCherry mice (orange). However, ChR2 mice (blue) showed significantly greater peak dopamine in response to electrical and laser stimulation (filled bars) compared to electrical stimulation alone (open bars).

F) There was no significant difference in the AUC for dopamine concentration in response to electrical (open bars) and electrical and laser stimulation (filled) for mCherry mice (orange). ChR2 (blue) showed a significant potentiation of dopamine AUC in response to electrical and laser stimulation (filled bars) compared to electrical stimulation alone (open bars).

Figure 5. OXR1 is required for potentiation of evoked NAc dopamine release by optogenetic stimulation of $LH_{ox/dyn}$ inputs in the VTA.

A) Averaged dopamine traces and representative false colour plots in response to electrical stimulation (top) and electrical and laser stimulation (bottom) for mCherry mice before (green) and after administration of SB-334867 (magenta). Insets to colour plots, current voltage plots showing oxidation and reduction peaks for dopamine.

B) Averaged dopamine traces and representative false colour plots in response to electrical stimulation (top) and electrical and laser stimulation (bottom) for ChR2 mice before (green) and after administration of SB-334867 (magenta). Insets to colour plots, current voltage plots showing oxidation and reduction peaks for dopamine.

C) Prior to administration of SB-334867, there was no significant difference in the peak dopamine concentration observed in response to electrical (open bars) or electrical and laser stimulation (filled bars) for mCherry mice (orange). For ChR2 mice (blue), laser stimulation (filled bars) significantly potentiated the peak dopamine concentration observed in response to electrical stimulation (open bars).

D) After administration of SB-334867, there was no significant difference in the peak dopamine concentration observed in response to electrical (open bars) or electrical and laser stimulation (filled bars) for mCherry (orange) or ChR2 mice (blue).

Figure 1

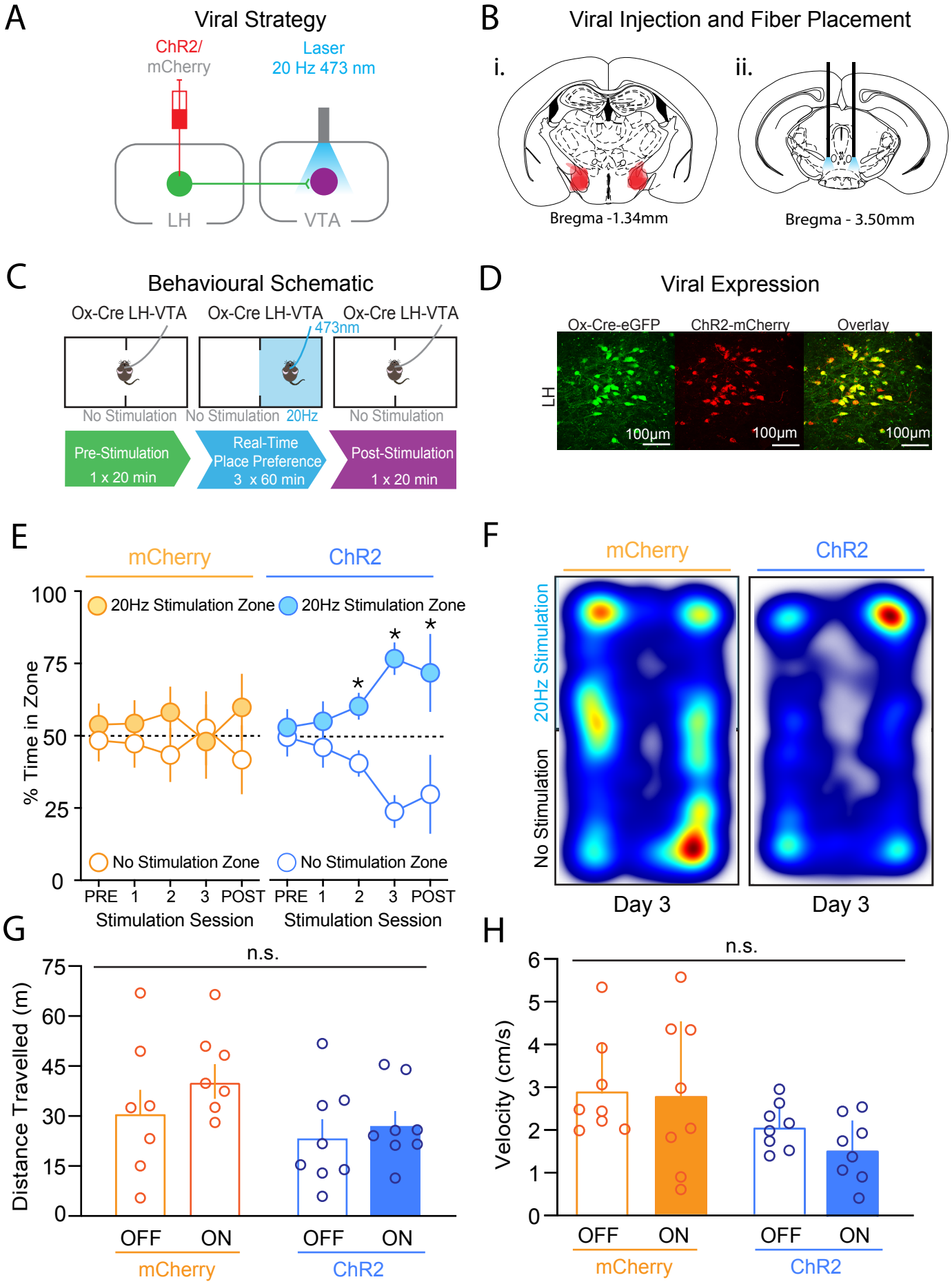

A
Viral Strategy
ChR2/
mCherry
Laser
20 Hz 473 nm
LH
VTA
B
Viral Injection and Fiber Placement
i.
ii.
Bregma -1.34mm
Bregma - 3.50mm
C
Behavioural Schematic
Ox-Cre LH-VTA
Ox-Cre LH-VTA
473nm
Ox-Cre LH-VTA
No Stimulation
No Stimulation 20Hz
No Stimulation
Pre-Stimulation
1 x 20 min
Real-Time
Place Preference
3 x 60 min
Post-Stimulation
1 x 20 min
D
Viral Expression
Ox-Cre-eGFP
ChR2-mCherry
Overlay
LH
100µm
100µm
100µm
E
mCherry
ChR2
20Hz Stimulation Zone
20Hz Stimulation Zone
% Time in Zone
100
75
50
25
0
No Stimulation Zone
No Stimulation Zone
PRE 1 2 3 POST
PRE 1 2 3 POST
Stimulation Session
Stimulation Session
F
mCherry
ChR2
20Hz Stimulation
No Stimulation
Day 3
Day 3
G
n.s.
Distance Travelled (m)
75
60
45
30
15
0
OFF ON
OFF ON
mCherry
ChR2
H
n.s.
Velocity (cm/s)
6
5
4
3
2
1
0
OFF ON
OFF ON
mCherry
ChR2

Figure 2

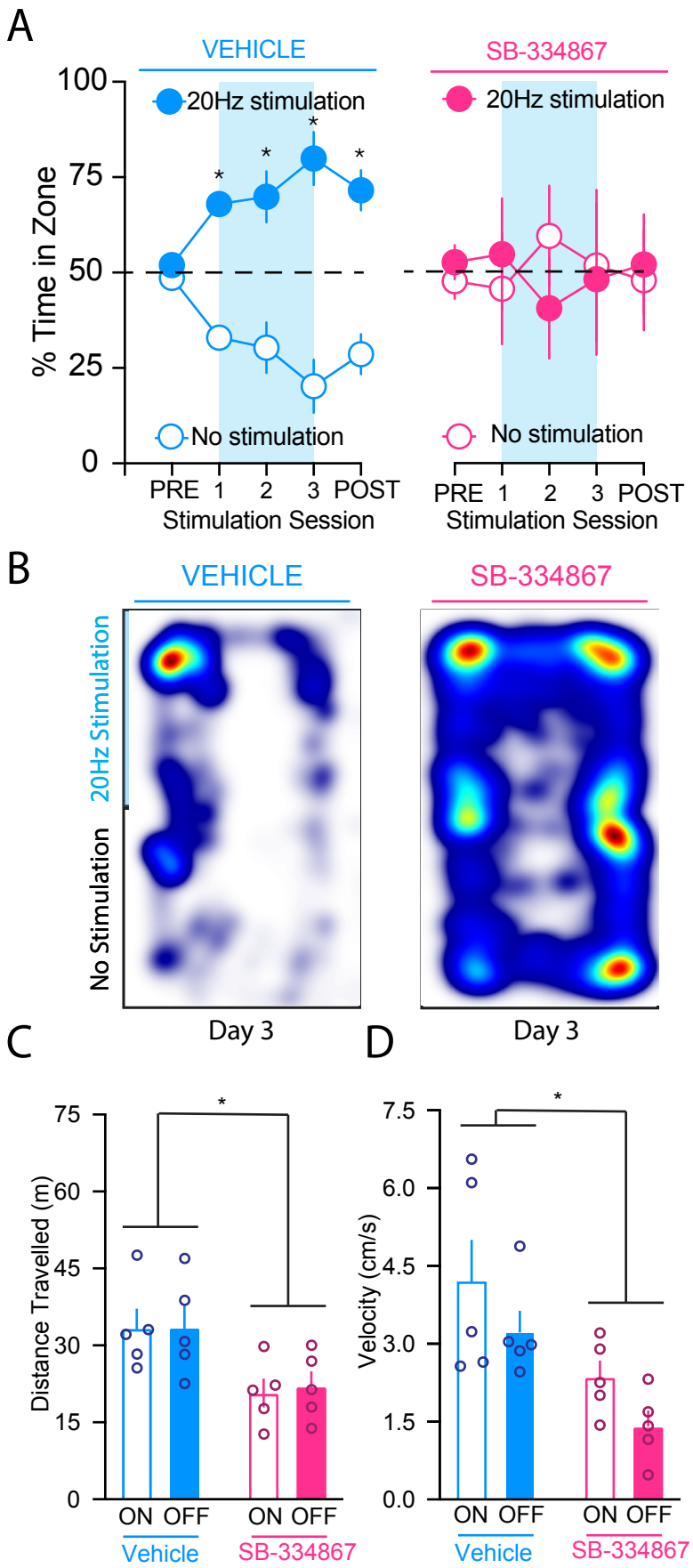

A
VEHICLE
SB-334867
20Hz stimulation
No stimulation
% Time in Zone
100
75
50
25
0
PRE 1 2 3 POST
Stimulation Session
B
VEHICLE
SB-334867
20Hz Stimulation
No Stimulation
Day 3
Day 3
C
D
Distance Travelled (m)
75
60
45
30
15
0
ON OFF ON OFF
Vehicle SB-334867
Velocity (cm/s)
7.5
6.0
4.5
3.0
1.5
0.0
ON OFF ON OFF
Vehicle SB-334867

Figure 3

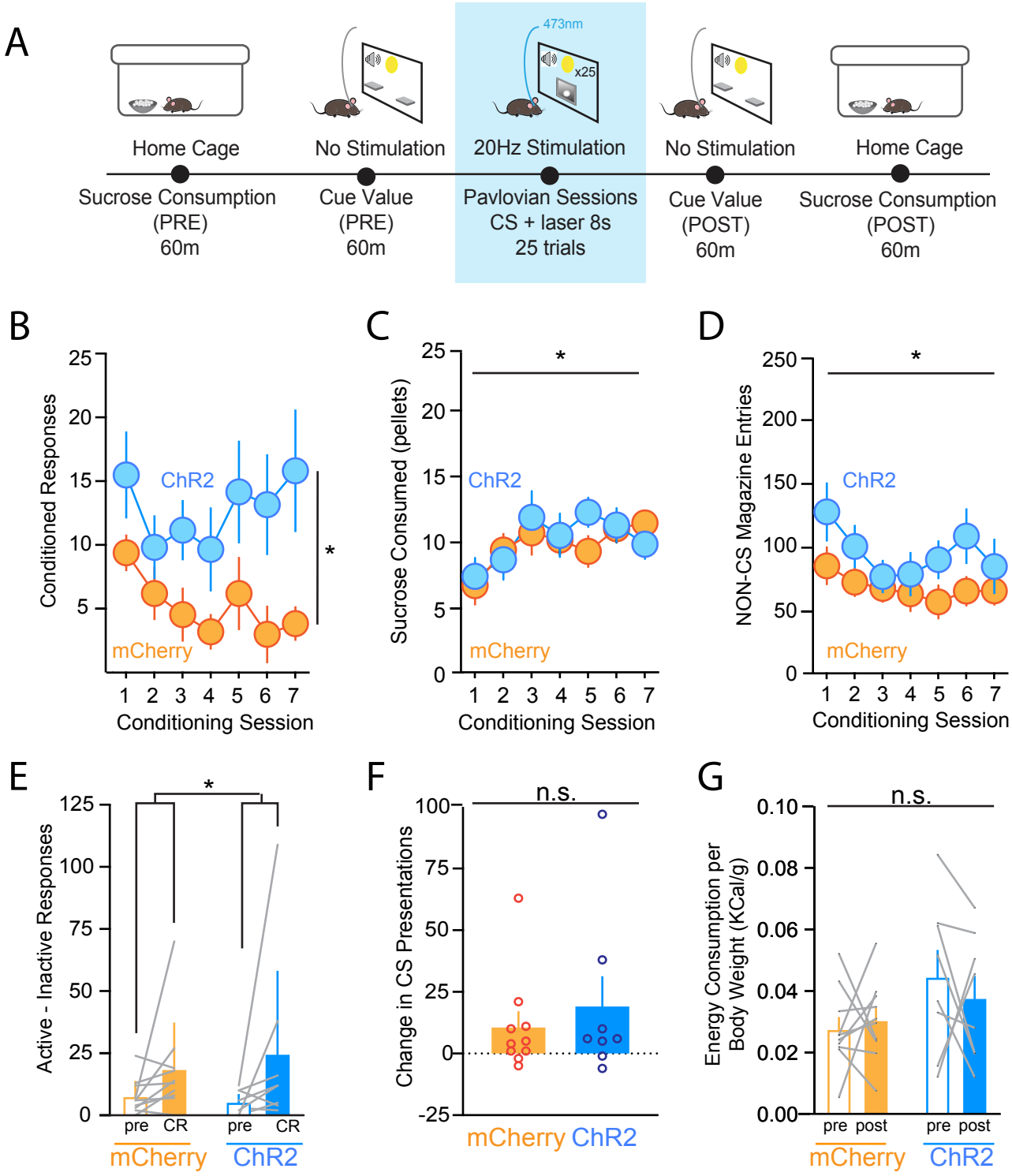

A
473nm
x25
Home Cage
No Stimulation
20Hz Stimulation
No Stimulation
Home Cage
Sucrose Consumption (PRE) 60m
Cue Value (PRE) 60m
Pavlovian Sessions CS + laser 8s 25 trials
Cue Value (POST) 60m
Sucrose Consumption (POST) 60m
B
Conditioned Responses
ChR2
mCherry
Conditioning Session
C
Sucrose Consumed (pellets)
ChR2
mCherry
Conditioning Session
D
NON-CS Magazine Entries
ChR2
mCherry
Conditioning Session
E
Active - Inactive Responses
pre CR pre CR
mCherry ChR2
F
Change in CS Presentations
n.s.
mCherry ChR2
G
Energy Consumption per Body Weight (KCal/g)
n.s.
pre post pre post
mCherry ChR2

Figure 4

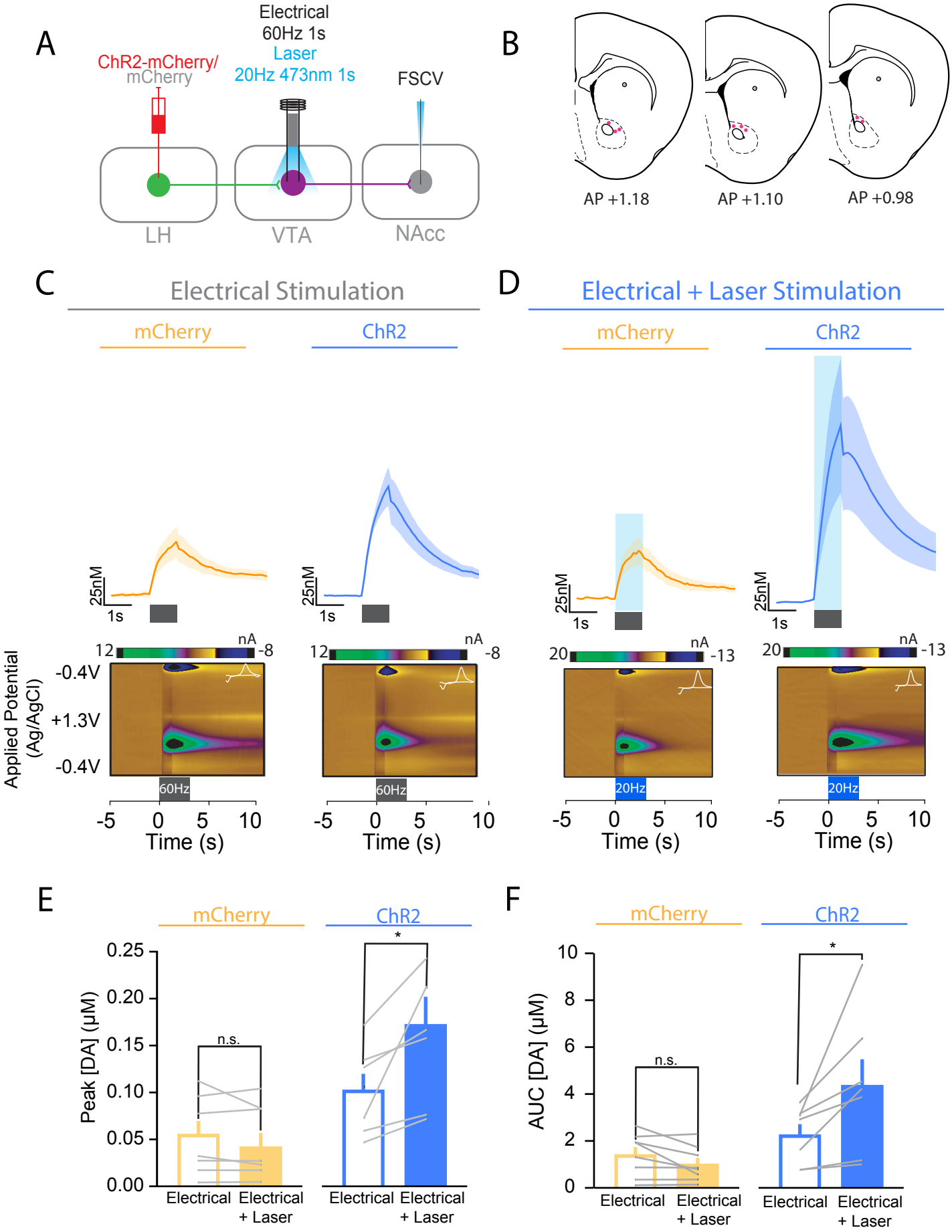
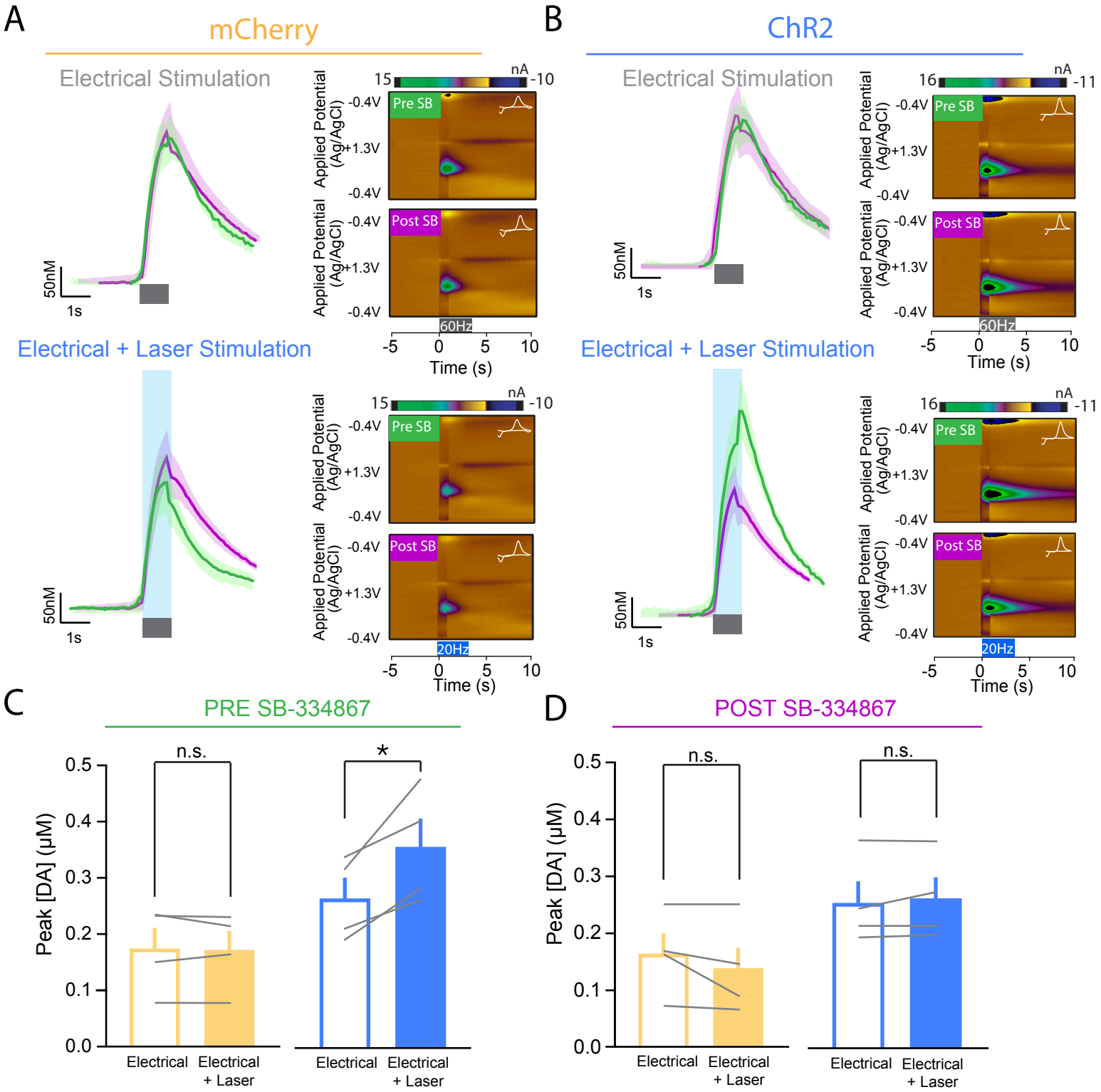

A
ChR2-mCherry/
mCherry
Electrical
60Hz 1s
Laser
20Hz 473nm 1s
FSCV
LH
VTA
NAcc
B
AP +1.18
AP +1.10
AP +0.98
C
Electrical Stimulation
mCherry
ChR2
25nM
1s
nA
12
-8
-0.4V
+1.3V
-0.4V
Applied Potential
(Ag/AgCl)
60Hz
-5
0
5
10
Time (s)
D
Electrical + Laser Stimulation
mCherry
ChR2
20
-13
20Hz
E
mCherry
ChR2
Peak [DA] (μM)
0.25
0.20
0.15
0.10
0.05
0.00
n.s.
*
Electrical
Electrical
+ Laser
F
AUC [DA] (μM)
10
8
6
4
2
0

Figure 5


A
mCherry
Electrical Stimulation
Electrical + Laser Stimulation
B
ChR2
Electrical Stimulation
Electrical + Laser Stimulation
Pre SB
Post SB
Applied Potential (Ag/AgCl)
-0.4V
+1.3V
-0.4V
nA
15
-10
16
-11
60Hz
20Hz
Time (s)
-5
0
5
10
50nM
1s
C
PRE SB-334867
D
POST SB-334867
Peak [DA] (μM)
0.0
0.1
0.2
0.3
0.4
0.5
n.s.
*
Electrical
Electrical + Laser