DORSAL HIPPOCAMPAL ASTROCYTE SIGNALING REGULATES HEROIN-CONDITIONED IMMUNOMODULATION BUT NOT HEROIN-CONDITIONED PLACE PREFERENCE

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ABSTRACT

Jacqueline E. Paniccia: Dorsal hippocampal astrocyte signaling regulates heroin-conditioned immunomodulation but not heroin-conditioned place preference
(Under the direction of Donald T. Lysle)

Repeated context-heroin pairings result in Pavlovian associations that manifest as heroin-conditioned appetitive responses or peripheral immunomodulation upon re-exposure to heroin-conditioned stimuli. The dorsal hippocampus (DH) is a critical neural substrate governing these context-heroin associations. Within the DH, there appears to be divergent mechanisms mediating heroin-conditioned Pavlovian responses. Evidence suggests that DH interleukin-1 signaling regulates heroin-conditioned immunomodulation but not heroin-conditioned place preference (CPP). The present study sought to further investigate the role of DH neuroimmune signaling in heroin-conditioned Pavlovian responses. Astroglial activity has been implicated in both drug addiction and mechanisms of learning and memory. As such, we employed chemogenetic tools to examine the involvement of DH astrocytes in the expression of both heroin-conditioned immunomodulatory and appetitive responses. Interestingly, chemogenetic stimulation of DH astroglial G_i-signaling disrupted heroin-conditioned immunomodulation but did not alter heroin-CPP. These data provide further evidence that differential DH mechanisms regulate heroin-conditioned Pavlovian responses.

To my parents:
Thank you for all your support and continuing to be my #1 fans.

ACKNOWLEDGEMENTS

Many thanks to the Lysle Laboratory, Christina L. Lebonville, Meghan E. Jones, Shveta V. Parekh, and to my advisor, Donald T. Lysle, whose guidance, support, and editing were instrumental in completing these experiments and writing this thesis.

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AAV	adeno-associated virus
ANOVA	analysis of variance
cAMP	cyclic adenosine monophosphate
CNO	clozapine-*N*-oxide
CS	conditioned stimulus
CPP	conditioned place preference
ΔΔCT	Comparative CT
DH	dorsal hippocampus
DMSO	dimethyl sulfoxide
DREADD	designer receptors exclusively activated by designer drugs
GFAP	glial fibrillary acidic protein
IHC	immunohistochemistry
IL-1	interleukin-1
IL-1β	interleukin-1β
IL-1R1	IL-1 receptor type 1
IL-1RA	interleukin-1 receptor antagonist
iNOS	inducible nitric oxide synthase
LPS	lipopolysaccharide
mRNA	messenger RNA
NAc	nucleus accumbens
NF-κB	nuclear factor-κB
NO	nitric oxide

PB	phosphate buffer
Rpl13a	60S ribosomal protein L13a
RT-qPCR	reverse transcription quantitative real-time PCR

CHAPTER 1: DORSAL HIPPOCAMPAL ASTROCYTE SIGNALING REGULATES HEROIN-CONDITIONED IMMUNOMODULATION BUT NOT HEROIN-CONDITIONED PLACE PREFERENCE [1]

Introduction

Repeated pairings between environmental stimuli and the subjective and physiological effects of heroin result in robust associative learning. The consequent stimulus control over physiology and behavior is integral to heroin addiction, and has detrimental health consequences that represent a growing public health concern. Heroin-associated contextual stimuli can act as conditioned stimuli (CS) that trigger Pavlovian appetitive conditioned responses, including conditioned place preference (CPP) (Tzschentke, 1998). Additionally, drug-paired contextual stimuli can act as discriminative stimuli or occasion setters that signal drug availability and thus engender drug-seeking behavior in instrumental paradigms (Crombag et al., 2008; Fuchs et al., 2008). Regardless of the specific role of the contextual stimulus, the hippocampus is essential for context-drug associative learning (Kutlu and Gould, 2016). In particular, the dorsal hippocampus (DH) plays a critical role in drug-induced CPP (Corrigall and Linseman, 1988; Meyers et al., 2003; Xia et al., 2017) as well as context-induced drug-seeking behaviors (Fuchs et al., 2007; Fuchs et al., 2005; Ge et al., 2017; Xie et al., 2010).

In addition to heroin-conditioned appetitive responses, heroin-associated contextual

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stimuli can elicit the immunomodulatory effects induced by opioids (Lysle and Ijames, 2002). Heroin and other opioids negatively alter host immunity (McCarthy et al., 2001; Wang et al., 2011). Following repeated context-heroin pairings, exposure to the heroin-paired CS is sufficient to evoke heroin-conditioned suppression of lipopolysaccharide (LPS)-induced peripheral immune parameters (Lysle and Ijames, 2002). We have characterized heroin-conditioned immunomodulation as classically conditioned response that follows the principles of learning (Szczytkowski and Lysle, 2007), and found it is mediated through DH-dependent processes. GABA agonist-induced DH inactivation during CS exposure significantly disrupts heroin-conditioned suppression of LPS-induced peripheral indices of nitric oxide (NO) production (Szczytkowski et al., 2013). Thus, the DH is an essential component of the neural circuitry governing the retrieval or utilization of the context-heroin association that controls host immunity.

Within the DH, we have discovered a distinct role of the neuroimmune system in governing heroin-conditioned immunomodulation. The role of the pro-inflammatory cytokine interleukin-1β (IL-1β) is well established in hippocampal-dependent memory processes (Goshen et al., 2007; Jones et al., 2015), and there is evidence to suggest its involvement in the development and maintenance of long-term potentiation (Donzis and Tronson, 2014; Yirmiya and Goshen, 2011). Furthermore, signaling through the active IL-1 receptor, IL-1 receptor type 1 (IL-1R1), is vital in hippocampal-dependent learning (Ben Menachem-Zidon et al., 2011). We have determined that both expression of DH IL-1β and signaling through IL-1R1 is required during presentation of heroin-paired cues for heroin-conditioned immunomodulation to occur (Paniccia et al., 2018; Szczytkowski et al., 2013). Interestingly, this involvement of IL-1 signaling in heroin-conditioned responses does not extend to heroin-conditioned appetitive behaviors (Paniccia et al., 2018).

The neuroimmune system is a vastly complex network involving multiple cell types and signaling molecules. Relevant to our model, astrocyte activity has been implicated in both mechanisms of learning and memory (Ben Achour and Pascual, 2010; Jones et al., 2018b; Ota et al., 2013), and substance use disorders (Lacagnina et al., 2018; Miguel-Hidalgo, 2009; Scofield and Kalivas, 2014). Astrocytes can directly alter neuronal function and synaptic plasticity through the release of gliotransmitters (Haydon and Carmignoto, 2006) and cytokines (Lacagnina et al., 2018; Santello and Volterra, 2012). Interestingly, astroglia have been shown to support hippocampal-dependent learning and memory through the expression of IL-1β (Jones et al., 2018a) and IL-1R1 (Ben Menachem-Zidon et al., 2011). While a mechanistic link between astrocyte activity and subsequent IL-1β release has not yet been confirmed, astrocytes may be a critical cell population involved in mediating heroin-conditioned immunomodulation. Moreover, the role of hippocampal astroglia in heroin-conditioned appetitive responses is presently unknown. Thus, the current study is aimed at extending our knowledge of neuroimmune regulation of heroin-conditioned Pavlovian responses, and examining the role of astrocyte activity in heroin-condition immunomodulation and heroin-CPP. We employed chemogenetic techniques to evaluate the importance of DH astroglial signaling during exposure to heroin-associated contextual stimuli. An adeno-associated viral construct was used to selectively target DH astroglia and express G_i-coupled designer receptors exclusively activated by designer drugs (DREADDs) in this cell population. DREADDs are mutated muscarinic receptors that no longer respond to endogenous ligands and instead are activated by clozapine-N-oxide (CNO) (Roth, 2016). CNO-induced stimulation of astroglial G_i-signaling will attenuate induction of cyclic adenosine monophosphate (cAMP) (Jones et al., 2018b) and have distinct functional outcomes for cellular activity. Overall, the present study investigated the involvement of hippocampal

astroglial G_i-signaling, in two Pavlovian procedures: heroin-conditioned immunomodulation and heroin-CPP.

Materials and Methods

Animals. Adult, male Lewis rats (~225-250 g) were purchased from Charles River Laboratories (Kingston, NY). All rats were individually housed on a 12-hour reversed light-dark cycle. Animals were handled regularly prior to and throughout experimental procedures. Animals received ad libitum home cage access to food and water. All procedures were conducted in compliance with regulations by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

Drug Administration. Heroin (diacetylmorphine, National Institute on Drug Abuse Drug Supply Program, Bethesda, MD) was dissolved in 0.9% sterile saline. Heroin was stored at 4°C until use at room temperature. In all experiments, heroin was administered subcutaneously at a dose of 1 mg/kg. This dose was selected based on prior research showing that it induces conditioning and alters endotoxin-induced indices of NO production (Lysle and How, 2000; Lysle and Ijames, 2002; Szczytkowski and Lysle, 2007). Clozapine-N-oxide (CNO; Sigma, St. Louis, MO or the National Institutes of Health, Bethesda, MD) was dissolved in a vehicle of 0.9% sterile saline with 0.5% dimethyl sulfoxide (DMSO). In both experiments, CNO (3 mg/kg) or vehicle was administered subcutaneously. Lipopolysaccharide (LPS; derived from E. coli, serotype O55:B5, Sigma) was dissolved in 0.9% sterile, pyrogen-free saline. In Experiments 1, LPS (1 mg/kg) was administered subcutaneously. This LPS dose produces sickness behavior and induces measures of NO production.

Surgical Procedures. Animals were fully anesthetized with a 1 mg/kg intraperitoneal injection of ketamine hydrochloride (100 mg/mL) mixed with xylazine (100 mg/mL) in a 9:1 (vol:vol) ratio. An astroglial Gi-coupled DREADD virus (AAV8-GFAP-hM4D(Gi)-mCherry)

was infused into the DH. The DREADD construct was packaged into an adeno-associated virus (AAV) by the University of North Carolina at Chapel Hill Vector Core (Chapel Hill, North Carolina). Injectors (33 Gauge, Plastics One) were directed bilaterally at the DH (AP -3.4 mm, ML ± 3.1 mm, DV -3.2 mm, relative to bregma, 15° angle laterally, (Paxinos and Watson, 2006)). Purified viruses were obtained pre-dialyzed (350 mM NaCl, 5% D-sorbitol in PBS) and were microinjected at a viral titer of 2.0 x 1012 particles/mL (Experiment 1) or 9.8 x 1012 particles/mL (Experiment 2). Virus infusions of 0.7 μL per hemisphere were delivered bilaterally at a rate of 0.05-0.1 μL/min. At the end of the infusion, injectors were left in place for 10-15 min to allow for diffusion away from the injection site. Following virus infusion surgeries, animals remained in their home cage for three weeks to allow for post-operative recovery and astroglial DREADD expression.

Heroin-conditioned Immunomodulation. The heroin-conditioning paradigm employed here has been described previously (Szczytkowski et al., 2011; Szczytkowski et al., 2013). Briefly, all animals received five 1-h pairings of heroin with a conditioning chamber (conditioned stimulus, CS). The conditioning chambers (BRS/LVE, Laurel, MD; H 26.7 cm × D 24.1 cm × W 30.5 cm) were located in a room separate from the vivarium. The chambers contained metal grid flooring and cedar bedding to create an environment with different olfactory, tactile, and visual characteristics relative to the home cage. The chambers were enclosed within sound- and light-attenuating chambers (H 36.8 cm x D 34.3 cm x W 50.8 cm) with a house fan to mask background noise. Heroin-conditioning sessions took place during the dark phase of the light cycle and were separated by 48 h. Following the last conditioning session, animals remained undisturbed in their home cage for 6 days. Animals were randomly assigned to four groups according to a 2 (CS or home cage) x 2 (drug or vehicle) between-subjects design. In Experiment 1, animals received either an injection of CNO or vehicle. Thirty minutes after drug

treatment, the animals were re-exposed to the heroin-paired context (CS) for 1 h in the absence of heroin or remained in their home cage. Immediately after the CS exposure or equivalent home cage stay, the animals were injected with LPS and placed into their home cages until tissue collection, 6 h later.

Heroin-conditioned Place Preference. The conditioned place preference (CPP) apparatus was located in a room separate from the vivarium. A three-chambered apparatus was used, with the two large chambers containing distinct olfactory, visual, and tactile cues from home cage, as well as each other. Animals were habituated to the CPP apparatus. During habituation to the CPP apparatus, baseline test, and each subsequent CPP test, animals were given free access to all three chambers for 15 min in a heroin-free state. Behavior within the apparatus during test sessions was video recorded using a Sony Handycam (HDR-CX455, 9.2 megapixels). The time spent in each side of the apparatus was scored manually by an experimenter blind to treatment assignment. Twenty-four hours after habituation, a pre-conditioning baseline CPP test was conducted to determine unconditioned side preferences. Using a biased conditioning procedure, heroin was paired with the initially non-preferred side of the apparatus. Saline-conditioned controls were included to test for unconditioned drift in side preference that might occur with repeated exposure to the apparatus.

Assignment to heroin- and saline-conditioned groups, as well as to the order of heroin and saline conditioning sessions, was counterbalanced based on unconditioned side preferences. Animals received a heroin or saline injection and were confined to one side for 30 min. The next day animals were injected with the opposite treatment and confined to the opposite side for 30 min. Conditioning continued as an alternating regimen across a total of 10 daily sessions. Animals then received a CPP test. After heroin CPP was confirmed, as indicated by significantly increased time spent on the heroin-paired side during the CPP test relative to the baseline test,

animals were assigned to treatment groups, counterbalanced based by initial and post-training preferences. In Experiment 2, animals received two CNO test sessions, 24 h apart, with CNO or vehicle administered 30 min prior to Test 1, and the opposite treatment administered prior to Test 2. There were no statistical differences between these two tests, thus data across CNO test days were combined to increase power. Data are presented for both experiments as time (sec) spent in the heroin-paired side during CNO test and as change in time spent in the heroin-paired side during CNO test relative to baseline Additionally, CPP score is reported and is defined as the time spent in the heroin-paired side minus that in the saline-paired side.

Tissue Collection and Histology. Animals were sacrificed via cervical dislocation (Experiments 1) or transcardial perfusion (Experiment 2). In studies examining the effects of heroin-conditioned immunomodulation (Experiments 1), samples of spleen and blood plasma were collected 6 h following LPS injection to assess indices of NO production. Spleen tissue for RNA extraction was divided into ~100 mg samples which were stored in RNAlater (Ambion, ThermoFisher Scientific, Waltham, MA). Brain tissue from both experiments was post-fixed in 4% paraformaldehyde for 48 h, cryoprotected in 30% sucrose in 0.1 M phosphate buffer (PB; pH = 7.4), and stored at 4°C until sectioned. All brain tissue was frozen and sectioned into 40 µm coronal slices via cryostat (CM3050 S, Leica, Buffalo Grove, IL) or freezing microtome (SM 2000R, Leica). To ensure DREADD specificity, sections were labeled using standard immunohistochemistry (IHC) methods as described below. All tissue sections were analyzed by an experimenter blind to treatment group.

Immunohistochemistry. To verify cell-type specificity of GFAP-hM4Di(Gi)-mCherry expression in Experiments 1 and 2, sections were washed three times for 10 min in 0.1 M PB (pH = 7.4) and incubated in 5% Normal Goat Serum (NGS; Vector Laboratories, Burlingame, CA) and 0.5% Triton-X100 for 60 min at room temperature. Tissue was then incubated overnight

at 4°C in 5% NGS, 0.5% Triton-X100, and primary antibody, mouse anti-GFAP (1:1000, ThermoFisher Scientific, Waltham, MA, Cat# MS-1376P) or mouse anti-NeuN (1:1000, Millipore, Burlington, MA, Cat# MAB377). The next day, tissue was washed three times for 10 min in 0.1 M PB (pH = 7.4) and then incubated at room temperature in 5% NGS, 0.5% Triton-X100, and secondary antibody for 2 h. Secondary antibodies used for visualization were conjugated with Alexa-Fluor dyes (Alexa-488, 1:1000, Invitrogen, ThermoFisher Scientific, Cat#A-11001). Tissue was then washed three times for 10 min in 0.1 M PB (pH = 7.4), mounted onto SuperFrost Plus slides (ThermoFisher Scientific), and coverslipped using Vectashield HardSet mounting medium (Vector Laboratories). Slides were stored at 4□C until time of analysis. Specificity of each primary antibody was verified in control experiments.

Microscopy. In order to verify DREADD DH- and astroglial-specificity, mCherry expression was carefully examined by an experimenter blind to treatment group. DH sections were visualized using confocal microscopy (Zeiss LSM800, Jena, Germany) and representative images for publication were acquired using 1024 x 1024 frame size, 16-bit image resolution, and frame average of 4. Laser lines that excite at 488 nm and 561 nm were used to visualize AlexaFluor-488 and mCherry respectively. Images were deconvolved using Bitplane AutoQuant X3 (10 iterations), and exported to Biplane Imaris Software (Zurich, Switzerland). mCherry was expected to be expressed bilaterally throughout the DH, selectively within the DH, and specifically in DH astrocytes. Animals with non-DH and/or non–astrocyte specific mCherry expression were removed from data analysis.

RNA Extraction and cDNA synthesis. Messenger RNA (mRNA) was extracted to assess measures of NO in the spleen. Spleen tissue was homogenized in 1 mL of cold TriReagent (Molecular Research Center, Cincinnati, OH) using a bead homogenizer (Precellys Instruments, Montigny-le-Bretonneux, France). Tissue was centrifuged, and the homogenate transferred to a

second tube. Next, the samples were shaken and incubated with BCP at room temperature and centrifuged for phase separation. The aqueous layer was thoroughly mixed with isopropanol, incubated at room temperature, and samples were centrifuged to form the RNA pellet. The pellet was then washed three times in 75% ethanol and air dried to remove residual ethanol. The RNA pellet was reconstituted in warm RNase-free water. Absorbance for samples diluted (1:20) in 1xTE (pH = 7.5) was assessed using spectrophotometer (Epoch™, BioTek Instruments Inc., Winooski, VT). Sample mRNA concentrations were read using the Take3 Application and Gen5 Software for Nucleic Acid Quantification (BioTek Instruments Inc.), and A260/280 ratios were assessed to ensure purity.

Sample mRNA input concentration was equalized using PCR-grade water. cDNA was synthesized using the Advantage for RT-PCR Kit (ClonTech, Takara, Mountain View, CA) following the manufacturer's protocol and using the Veriti 96 Well Fast Thermal Cycler (Applied Biosystems, ThermoFisher Scientific). A subset of undiluted cDNA samples were pooled together, and five serial 1:10 dilutions were made to test qPCR reaction efficency. The remaining original sample was then diluted 1:5 in PCR-grade water for qPCR.

qPCR Quantification of Splenic iNOS Gene Expression. qPCR was performed using the TaqMan™ Fast Advanced Master Mix Kit (Applied Biosystems, ThermoFisher Scientific) according to the manufacturer's instructions. Reactions were carried out in triplicate on a 384-well plate, with each individual reaction containing 1.5 µL of cDNA pooled or sample cDNA. In order to assess indices of NO production, levels of splenic inducible nitric oxide synthase (iNOS) gene expression were analyzed. NO is produced by iNOS in response to inflammatory stimuli (Nathan and Shiloh, 2000). Thus, two different genes were analyzed by using the TaqMan™ Gene Expression Assays (FAM): inducible nitric oxide synthase 2 (iNOS/NOS2, Assay ID: Rn00561646_m1, ThermoFisher Scientific) and 60S ribosomal protein L13a (Rpl13a, reference

gene, Assay ID: Rn01475911_g1; ThermoFisher Scientific). A no template control was run to ensure purity of these reactions. Plates were run in the QuantStudioTM 6 Flex RealTime PCR System (Applied Biosystems, ThermoFisher Scientific). Data were collected using the QuantStudioTM RealTime PCR Software with a PCR Run Method as follows: 50°C for 2 min for PCR product contamination degradation, hold at 95°C for 20 sec for polymerase activation, and 45 PCR cycles of 95° C for 1 sec and 60° C for 20 sec with data collection at the end of each cycle. Data were analyzed using the Comparative CT (ΔΔCT) Method. iNOS CT data were normalized to the reference gene (Rpl13a), and then normalized to the overall average of reference normalized values.

Nitrate/nitrite Assay. As NO is degraded quickly, degradation products in plasma can be analyzed in combination with iNOS expression as indices of NO production. Plasma nitrate/nitrite concentrations were assessed using the Griess reagent assay as described previously (Szczytkowski and Lysle, 2007). Briefly, plasma was diluted in dH2O and incubated with nitrate reductase (1.0 U/mL), 0.31 M PB (pH = 7.5), 0.86 mM NADPH (Sigma-Aldrich Inc., Milwaukee, WI), and 0.11 mM flavin adenine dinucleotide in a 96-well plate for 90 min at room temperature in the dark. Following incubation, Griess reagent (1:1 (vol:vol) solution 1% sulfanilamide in 5% phosphoric acid and 0.1% N-(1-napthyl)ethylenediamine dihydrochloride in distilled H2O) was added to the samples and allowed to develop at room temperature. Absorbance was assessed at 550 nm using a spectrophotometer (Epoch™, BioTek Instruments Inc). Reactions were carried out in triplicate. The total micromolar concentration of nitrite was determined for each sample based on a concurrently run standard curve.

Statistical Analysis. Data for each experiment herein was analyzed using 2x2 analysis of variance (ANOVA) in SPSS Statistics (IBM, Armonk, NY). Planned contrasts were made using a two-tailed independent samples t-test with homogeneity of variance determined using Levene's

Test. For Experiments 1, we tested planned comparisons between CS-exposed and corresponding home cage control groups, as well as differences between CS-exposed groups themselves. For analysis of RT-qPCR, ΔΔCT values were analyzed, although the linearly transformed were used to display the data graphically. For Experiments 2, we tested a planned contrast between the heroin-conditioned groups at CNO test for time spent in heroin-paired side, change in time spent in heroin-paired side relative to baseline, and CPP score. Initial verification of acquired CPP was performed using an independent t-test comparing heroin-conditioned to saline-conditioned animals. Statistically significant outliers were detected using Grubb's test and removed from analysis. Alpha was set at p = 0.05.

Results

Experiment 1: Stimulation of astroglial G_i-signaling in the DH disrupts heroin-conditioned immunomodulation. Experiment 1 examined the role of DH astrocyte signaling in the expression of heroin-conditioned suppression of LPS-induced indices of NO production (see timeline in **Fig. 1A**). DREADD expression, as indicated by mCherry, was observed throughout the DH (**Fig.1B**). Furthermore, hM4Di-mCherry expression was restricted to astrocytes (**Fig. 2**).

CNO-induced stimulation of DH astroglial Gi-signaling attenuated heroin-conditioned splenic iNOS mRNA suppression (**Fig. 1C**). A 2 x 2 ANOVA of splenic Rpl13A mRNA levels revealed no significant differences between the groups ($F(3,18) = 1.48$, $p = 0.252$), validating Rpl13A as a reference gene. A 2 x 2 ANOVA of splenic iNOS mRNA levels revealed significant main effects of CS exposure ($F(1,18) = 30.96$, $p < 0.05$) and CNO treatment ($F(1,18) = 6.05$, $p < 0.05$), but no CS exposure by CNO treatment interaction ($F(1,18) = 2.57$, $p = 0.127$). Planned contrasts revealed that CS exposure significantly reduced splenic iNOS mRNA levels relative to home cage controls in the vehicle-treated ($p < 0.05$). CNO treatment partially attenuated heroin-conditioned suppression of splenic iNOS mRNA expression in that CNO-treated CS-exposed

iNOS mRNA expression was reduced relative to CNO-treated home cage controls ($p < 0.05$), but was higher than vehicle-treated CS-exposed animals ($p < 0.05$). Thus, stimulation of DH astroglial G_i-signaling significantly increased splenic iNOS gene expression, yet does not completely restore mRNA levels to those of control animals.

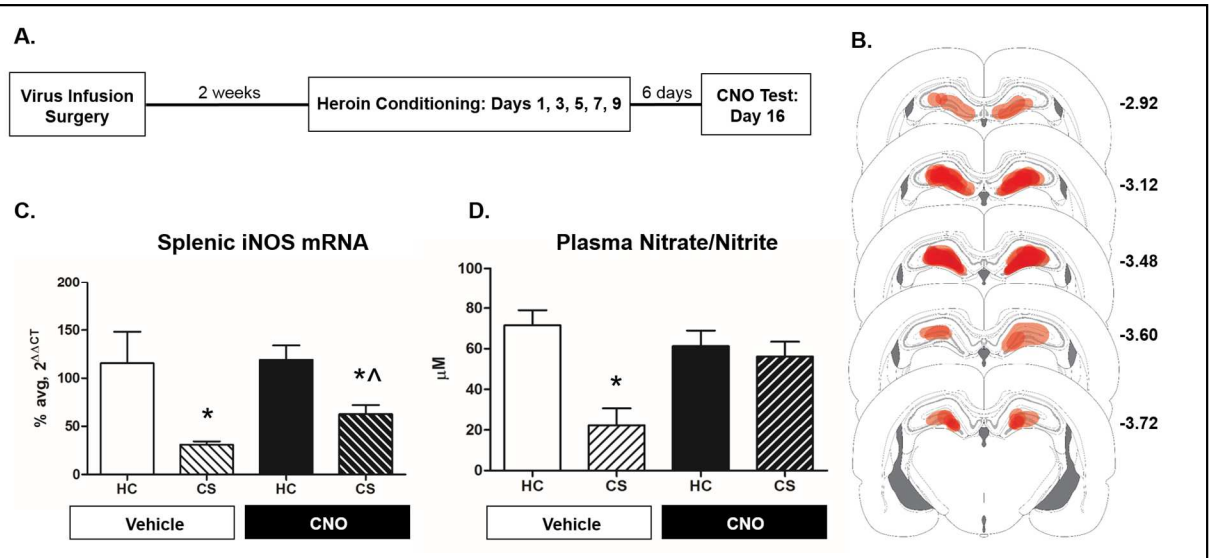

Figure 1: Activation of astroglial Gi-coupled signaling in the DH disrupts heroin-conditioned suppression of peripheral indices of NO production. For Experiment 1, the timeline is depicted (**A**) as well as the spread of GFAP-hM4D(Gi) as indicated by mCherry expression throughout the DH (**B**). Darker red areas are indicative of denser mCherry expression, with coordinates indicating distance from bregma based on Paxinos and Watson (2006). CNO administration significantly attenuated heroin-conditioned LPS-induced splenic iNOS mRNA expression (**C**) and completely blocked heroin-conditioned LPS-induced plasma nitrate/nitrite concentration (**D**). Group sizes were n = 5-6 in the final analysis for splenic iNOS mRNA expression and plasma nitrate/nitrite concentration. * represents statistically significant differences relative to respective home cage control group and ^ denotes statistical significance from CS-exposed counterpart ($p < 0.05$).

In contrast to splenic iNOS mRNA levels, CNO-induced stimulation of DH astroglial Gi-signaling completely inhibited heroin-conditioned suppression of plasma nitrate/nitrite concentration (**Fig. 1D**). A 2 x 2 ANOVA of nitrate/nitrite concentration revealed a significant CNO treatment x CS exposure interaction ($F(1,18) = 8.05$, $p < 0.05$). Planned contrasts revealed that, in vehicle-treated groups, CS exposure reduced plasma nitrate/nitrite concentrations relative to home cage controls ($p < 0.05$), indicating expression of heroin-conditioned immunomodulation. CNO-induced stimulation of G_i-signaling in DH astrocytes restored plasma

nitrate/nitrite concentrations, such that concentrations for the CS-exposed group did not significantly differ from CNO-treated home cage controls ($p = 0.646$) and were higher than vehicle-treated CS-exposed animals ($p < 0.05$).

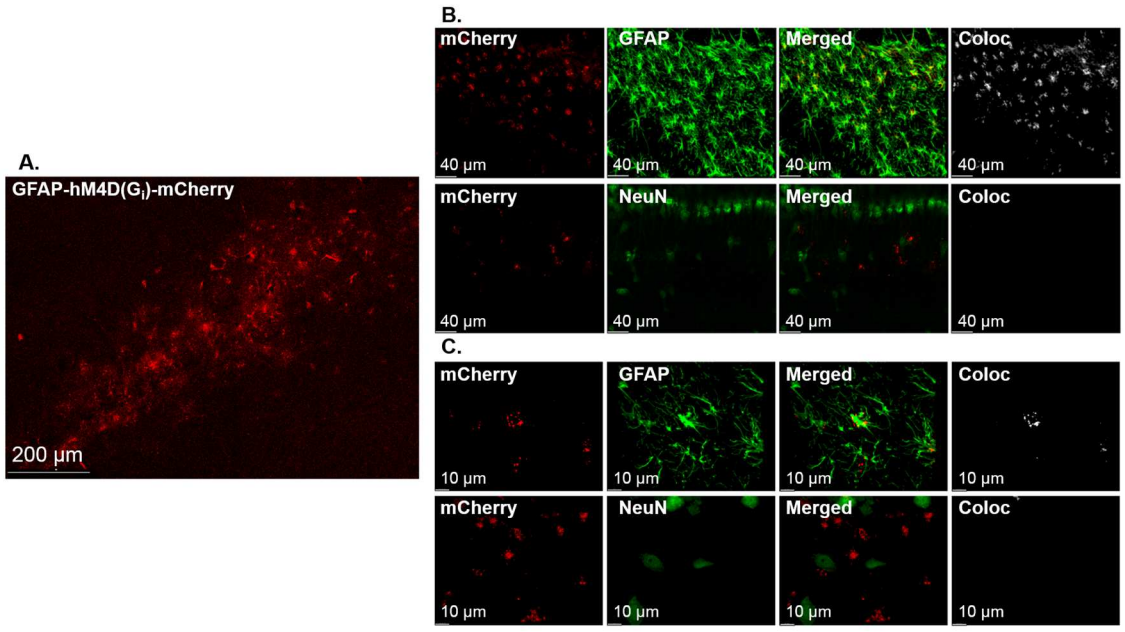

Figure 2: GFAP-hM4Di-mCherry is selectively expressed in DH astrocytes. A representative confocal 10X tile image depicts robust mCherry expression and spread throughout the DH (A). Representative confocal images at 20X demonstrating the mCherry tag is colocalized with astroglial marker GFAP (AlexaFlour-488; top row) but not with neuronal marker NeuN (AlexaFlour-488, bottom row) (B). Representative oil-immersion 63X images demonstrating that mCherry fluorescence is colocalized with astroglial marker, GFAP (Alexa-488; top row), but not with neuronal marker, NeuN (Alexa-488, bottom row) (C). Background signal was subtracted out using Bitplane Imaris Software and Adobe Photoshop.

Experiment 2: Stimulation of astroglial Gi-signaling in the DH does not alter heroin-conditioned place preference. Experiment 2 investigated the role of astrocyte signaling in the expression of heroin-CPP (see timeline in **Fig. 3A**). DREADD expression, as indicated by mCherry, was observed throughout the DH (**Fig.3B**). All animals acquired CPP. Heroin-conditioned animals spent significantly more time in the heroin-paired side than saline-conditioned animals during the CPP test session ($t(12.47) = -3.22$, $p < 0.05$), verifying the effectiveness of the biased conditioning procedure. CPP data were collapsed across experimental CNO test days 1 and 2.

CNO-induced stimulation of DH astroglial G_i-signaling failed to alter heroin-CPP at CNO test relative to controls (**Fig. 3C** and **3D**). Specifically, a 2 x 2 ANOVA for total time spent in the heroin-paired chamber at CNO test revealed a significant main effect of conditioning ($F(1, 26) = 17.63$, $p < 0.05$) with no significant main effect of CNO treatment ($F(1, 26) = 0.44$, $p = 0.511$) nor interaction ($F(1, 26) = 1.37$, $p = 0.253$). Similarly, the 2 x 2 ANOVA for change in time spent in the heroin-paired side relative to baseline indicated a significant main effect of heroin conditioning ($F(1, 24) = 23.28$, $p < 0.05$) with no significant main effect of CNO treatment ($F(1, 24) = 0.12$, $p = 0.729$) nor interaction ($F(1, 24) = 1.88$, $p = 0.183$). Finally, a 2 x 2 ANOVA of CPP scores on CNO test day (data not shown) also revealed a significant main effect of conditioning ($F(1, 26) = 16.18$, $p < 0.05$), with no significant main effect of CNO treatment ($F(1, 26) = 0.12$, $p = 0.736$) nor interaction ($F(1, 26) = 3.91$, $p = 0.059$). Thus, heroin-conditioned animals spent significantly more total time, time relative to baseline, and time relative to saline-paired side in the heroin-paired side independent of CNO treatment. Planned contrasts between the heroin-conditioned groups revealed no differences between these groups regardless of CNO treatment for total time spent in the heroin-paired side ($p = 0.694$), for change in time relative to baseline ($p = 0.444$), and for CPP score ($p = 0.207$).

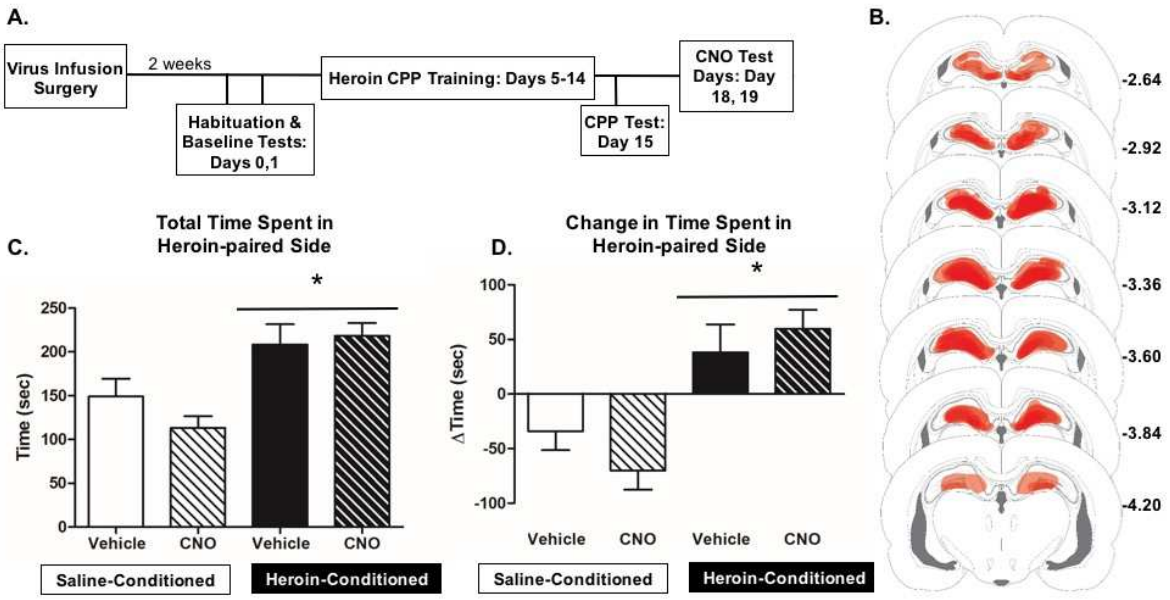

Figure 3: Activation of astroglial G_i-coupled signaling in the DH fails to alter heroin-CPP. For Experiment 2, the experimental timeline is shown (A) as well as the spread of GFAP-hM4D(G_i) as indicated by mCherry expression throughout the DH (B). Darker red areas are indicative of denser mCherry expression with coordinates indicating distance from bregma based on Paxinos and Watson (2006). CNO administration fails to disrupt total time spent in the heroin-paired side (C) or change in time spent in the heroin-paired side relative to a pre-conditioning baseline (D). Group sizes were n = 6 for each saline-conditioned group and n = 8-9 for each heroin-conditioned group in the final analysis of heroin-CPP measures. * represents a main effect of heroin-conditioning, with bar indicating no statistical difference between heroin-conditioned groups ($p < 0.05$).

Discussion

Through associative learning, contextual stimuli can come to elicit heroin-conditioned responses, including CPP and immunomodulation. The DH plays a critical role in contextual learning and memory, and has been implicated in both opioid-conditioned reward (Corrigall and Linseman, 1988) and -conditioned immunomodulation (Szczytkowski et al., 2013). In addition, neuroimmune signaling, in terms of both gliotransmission and cytokine signaling, is essential in learning and memory processes (Ben Achour and Pascual, 2010; Donzis and Tronson, 2014; Santello and Volterra, 2012; Yirmiya and Goshen, 2011) and in some drug-conditioned responses and instrumental behaviors relevant for drug addiction (Haydon et al., 2009;

Lacagnina et al., 2018; Scofield and Kalivas, 2014). Astrocytes, for example, have an established involvement in the IL-1R1 signaling required for some forms of learning and memory (Ben Menachem-Zidon et al., 2011). Findings in the present study significantly extend these lines of research by demonstrating that DH neuroimmune signaling plays a causal and selective role in heroin-conditioned immunomodulation, but not in heroin-CPP. We have demonstrated that IL-1 signaling is necessary for heroin-conditioned immunomodulation but not heroin-conditioned appetitive responses (Paniccia et al., 2018). The present study complements these findings such that undisturbed astroglial signaling during CS exposure is necessary for heroin-conditioned suppression LPS-induced of indices of NO production. Conversely, manipulations of the same signaling pathways failed to disrupt measures of heroin-CPP under the present experimental parameters. Together, our data suggest that divergent mechanisms within the DH govern heroin-conditioned peripheral immunomodulation and heroin-conditioned appetitive behavior.

Findings from our laboratory have furthered our understanding into the role of DH IL-1 signaling in heroin-conditioned Pavlovian responses. We have established sustained, inducible knockdown of DH IL-1β mRNA expression prior to CS exposure disrupts heroin-conditioned suppression of peripheral modulators, including indices of NO production (Szczytkowski et al., 2013). Additionally, signaling of DH IL-1R1 mediates the expression of heroin-conditioned immunomodulation, but antagonism of IL-1R1 does not alter heroin-CPP. Within the hippocampus, both astrocytes and microglia are capable of producing and responding to IL-1β signaling (Friedman, 2001; Hanisch, 2002), indicating either or both of these cell types could facilitate the IL-1 signaling required for heroin-conditioned immunomodulation. While the experiments in the present study strongly suggest astroglia mediate heroin-conditioned immunomodulation, the additional role of DH microglial involvement in this conditioned response should be investigated.

Hippocampal astrocytes are capable of expressing IL-1R1, and IL-1β administration triggers receptor upregulation of this receptor (Friedman, 2001). IL-1β action at astroglial IL-1R1 evokes nuclear factor-κB (NF-κB) signaling cascades (Srinivasan et al., 2004) and thus elicits the transcription of pro-inflammatory factors, including IL-1β and other cytokines, serving as a potential positive feedback loop for IL-1β expression. Presently, we establish a role for hippocampal astrocyte activity in mediating heroin-conditioned immunomodulation. The same chemogenetic stimulation of astroglial G_i-signaling used herein attenuates cAMP induction in DREADD-positive astrocytes (Jones et al., 2018b). As converging evidence suggests that activity of NF-κB is modulated by cAMP induction (Gerlo et al., 2011), it is possible astroglial G_i-signaling attenuates IL-1β production in hippocampal astrocytes. Future experiments should be aimed at testing the relationship between astrocyte activity and subsequent IL-1 signaling in heroin-conditioned immunomodulation.

The current study strongly suggests that DH astroglial signaling is a critical component in the expression of heroin-conditioned immunomodulation, but not heroin-CPP. The absence of effects on heroin-CPP were surprising given the established role of astroglial activity in addiction (Scofield and Kalivas, 2014). Specifically, prior research has shown that chemogenetic manipulation of astroglial G_q-signaling in the nucleus accumbens (NAc) core ameliorates the ability of cocaine-conditioned stimuli to elicit drug-seeking behaviors (Scofield et al., 2015). Although there is a functional projection from the DH to the NAc core (Peleg-Raibstein and Feldon, 2006), the current study targeted DH astroglial G_i-signaling *in vivo* during exposure to heroin-paired stimuli. While the DH is critical for encoding context-drug associations (Xia et al., 2017), it is the connection from the ventral hippocampus to the NAc shell that drives context-induced heroin-seeking behaviors (Bossert et al., 2016). It is possible that chemogenetic manipulation of ventral hippocampal astroglia would yield downstream consequences for heroin-

conditioned appetitive responses. The current data suggest astroglial involvement varies across conditioned appetitive behaviors as a function of evoked signaling pathway, target brain region, animal model, and drug of abuse.

The neuroimmune system is both impacted by opioid administration and serves as a key regulator of opioid-induced responses. Opioids produce alterations in hippocampal GFAP and IL-1β protein expression that are attenuated through anti-inflammatory compounds, including ibudilast (Hutchinson et al., 2009). At the same time, ibudilast administration reduces opioid withdrawal and simultaneously increases antinociception (Hutchinson et al., 2009). These findings indicate the neuroimme system differentially regulates opioid-induced responses depending on the type of response in question. Consistent with this, the current study establishes a divergence in mechanism governing heroin-conditioned responses.

The data demonstrating astroglial G_i-signaling disrupts heroin-conditioned immunomodulation are in line with recent findings demonstrating that modulation of DH astroglial signaling directly alters hippocampal-dependent mechanisms of learning and memory (Adamsky et al., 2018). Notably, chemogenetic stimulation of astroglial G_i-signaling did not fully restore LPS-induced NO measures. It is possible that astrocytes are not the only cellular component involved in the expression of heroin-conditioned immunomodulation. Consistent with this, astroglial-mediated neuronal alterations improve hippocampal-dependent memory, while neuronal activation alone impairs it (Adamsky et al., 2018). We have previously demonstrated hippocampal neuronal involvement in heroin-conditioned immunomodulation (Szczytkowski et al., 2013). Given the current findings that astrocyte activity mediates heroin-conditioned immunomodulation, the possibility of astrocyte-neuron interplay and the specific mechanisms involved, will merit further investigation.

In the current set of experiments we employed a 2 x 2 statistical design in which all animals received intra-DH infusions of AAV8-GFAP-hM4D(G_i)-mCherry. Thus, DREADD expression was present in all animals and transfection alone could not account for group differences in heroin-conditioned immunomodulation or heroin-CPP. Furthermore, all animals were thoroughly examined for site- and cell-type-specific expression which did not differ across groups. Although there have been recent concerns of CNO effects irrespective of DREADD expression (Gomez et al., 2017), other groups report no effect of CNO administration alone during experiments involving astroglial chemogenetic techniques (Adamsky et al., 2018; Bull et al., 2014; Scofield et al., 2015). While we do not presently report use of a control DREADD, CNO did not alter any of the current measures relative to vehicle in home cage controls. Thus, effects on reported measures were likely induced by astroglial G_i-signaling pathway manipulation, specifically. Importantly, we have recently demonstrated CNO attenuates LPS-induced cAMP expression in mCherry-positive DH astrocytes using the same viral construct (Jones et al., 2018b). This confirms CNO exerts its effects through the stimulation of G_i-signaling cascades and the inhibition of downstream cAMP within DH astrocytes.

In summary, the present study suggests that divergent mechanisms within the DH regulate Pavlovian heroin-conditioned responses. The current findings suggest that astrocyte signaling in the DH regulate conditioned immunomodulatory, but not conditioned appetitive, effects of heroin. The immunomodulatory effects of heroin can exacerbate infectious and other disease progression in addicts (Ninković and Roy, 2013; Wang et al., 2011). Since immunomodulation can become conditioned to environmental stimuli over the course of chronic heroin use, the detrimental health effects of heroin may persist in heroin-associated environments even after cessation of drug use. This suggests that interference with specific neuroimmune

substrates that maintain heroin-conditioned immunomodulation may be a promising therapeutic target for harm reduction in heroin use disorders.

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