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Interleukin-1 signaling in the basolateral amygdala is necessary for heroin-conditioned immunosuppression

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Abstract

Heroin administration suppresses the production of inducible nitric oxide (NO), as indicated by changes in splenic inducible nitric oxide synthase (iNOS) and plasma nitrate/nitrite. Since NO is a measure of host defense against infection and disease, this provides evidence that heroin can increase susceptibility to pathogens by directly interacting with the immune system. Previous research in our laboratory has demonstrated that these immunosuppressive effects of heroin can also be conditioned to environmental stimuli by repeatedly pairing heroin administration with a unique environmental context. Re-exposure to a previously drug-paired context elicits immunosuppressive effects similar to heroin administration alone. In addition, our laboratory has reported that the basolateral amygdala (BLA) and medial nucleus accumbens shell (mNAcS) are critical neural substrates that mediate this conditioned effect. However, our understanding of the contributing mechanisms within these brain regions is limited. It is known that the cytokine interleukin-1 (IL-1) plays an important role in learning and memory. In fact, our laboratory has demonstrated that inhibition of IL-1β expression in the dorsal hippocampus (DH) prior to reexposure to a heroin-paired context prevents the suppression of measures of NO production. Therefore, the present studies sought to further investigate the role of IL-1 in heroin-conditioned immunosuppression. Blockade of IL-1 signaling in the BLA, but not in the caudate putamen or mNAcS, using IL-1 receptor antagonist (IL-1Ra) attenuated heroin-conditioned immunosuppression of NO production as measured by plasma nitrate/nitrite and iNOS mRNA expression in spleen tissue. Taken together, these findings suggest that IL-1 signaling in the BLA is necessary for the expression of heroin-conditioned immunosuppression of NO production and may be a target for interventions that normalize immune function in heroin users and patient populations exposed to opiate regimens.

Keywords

Basolateral amygdala; Heroin; IL-1; Learning; IL-1 receptor antagonist; Nitric oxide; Immune conditioning; opioid

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1. Introduction

A high incidence of infection has long been observed among opiate users (Bussiere et al., 1993; Friedman and Eisenstein, 2004; Hussey and Katz, 1950; Luttgens, 1949). Non-sterile intravenous drug use, including needle sharing, is one possible mechanism for this effect; however, opiates also produce abnormalities in defensive immune parameters. Consistent with the latter, heroin users exhibit decreases in circulating lymphocytes, natural killer (NK) cell activity, cytokine production, and antibody-dependent cellular cytotoxicity (Govitrapong et al., 1998; Nair et al., 1986; Yardeni et al., 2008). Similarly, rats treated with morphine, the centrally active metabolite of heroin, exhibit lower rates of lymphocyte proliferation, splenic NK cell activity, blood lymphocyte proliferation, and proinflammatory cytokine production (Fecho et al., 1993a; Fecho et al., 1993b, 1996a,b; Lysle et al., 1993; Saurer et al., 2006a,b). Rats treated with heroin also show decreased measures of nitric oxide (NO), a vital component of pathogen resistance (Lysle and How, 2000). NO, an important part of the functioning immune system, is released by multiple immune cell types and also regulates immune function (Bogdan, 2001, Lewis et al., 2010, MacMicking et al., 1995, Uehara et al., 2015, Nathan and Shiloh, 2000). Thus, opiates might increase risk of infection through modulation of these important immune measures.

Interestingly, the immunosuppressive effects of opiates have been shown to be able to be conditioned to environmental stimuli. Our laboratory has demonstrated that repeated pairing of morphine or heroin with exposure to a distinct environmental context endows the context with conditioned immunosuppressive effects through associative learning mechanisms (Coussons-Read et al., 1994a,b; Coussons et al., 1992; Lysle and Ijames, 2002; Szczytkowski and Lysle, 2007). Furthermore, a mesolimbic neural circuit has been shown to mediate opiate-conditioned immunosuppression, and this circuitry includes the basolateral amygdala (BLA), medial nucleus accumbens shell (mNAcS), dorsal hippocampus (DH), and ventral tegmental area (Hutson et al., 2014; Saurer et al., 2008; Szczytkowski et al., 2011, 2013; Szczytkowski and Lysle, 2008, 2010). Despite the insights gained from these studies, our understanding of the central mechanisms mediating heroin-conditioned immunosuppression within these brain regions is limited.

Central IL-1 signaling has been shown to be particularly important in neural plasticity, learning, and memory. For example, expression of the IL-1 isomer, IL-1β, in the rat hippocampus is increased following long-term potentiation (LTP) induction, and maintenance of LTP is disrupted by administration of IL-1receptor antagonist (IL-1Ra) (Schneider et al., 1998). Furthermore, IL-1 receptor type 1 (IL-1R1) knockout mice display impairments in hippocampal-dependent memory and disrupted induction of LTP (Avital et al., 2003). Similarly, animals treated in two separate tasks with IL-1Ra following either repeated foot shock and exposure to a spatial learning task displayed impaired fear conditioning and performance in the Morris water maze, respectively (Yirmiya et al., 2002). Finally, our laboratory has demonstrated that inhibition of IL-1β mRNA expression in the DH attenuates the immunosuppressive effects of a previously heroin-paired context. Specifically, genetic knockdown of IL-1β in the DH prior to reexposure to a previously heroin-paired context attenuated heroin-conditioned immunosuppression of peripheral NO

production, as measured indirectly by quantifying levels of NO metabolites nitrate and nitrite in blood plasma and splenic mRNA expression of the NO synthesizing enzyme, inducible nitric oxide synthase (iNOS), (Szczytkowski et al., 2013). Thus, there is substantial evidence for the role of IL-1 signaling in learning and memory and specifically in heroin-conditioned immunosuppression to a context.

In order to expand upon this line of research, the present study explored the contribution of IL-1 in two other brain regions whose functional integrity is necessary for opiate-conditioned immunosuppression: the BLA and mNAcS (Saurer et al., 2009; Szczytkowski et al., 2011; Szczytkowski and Lysle, 2008). The BLA and mNAcS contain a high density of IL-1 receptors (Ericsson et al., 1995; Yabuuchi et al., 1994), therefore they may be brain regions where IL-1 exerts actions as a neuromodulator. In support of this, intracerebroventricular IL-1β administration modulates neuronal firing in the BLA (Yu and Shinnick-Gallagher, 1994). To manipulate IL-1 receptors in the present study, IL-1Ra was infused into the BLA or mNAcS prior to re-exposure to the previously heroin-paired context or the home cage as a control. Samples were collected for the assessment of spleen iNOS expression and plasma nitrite levels after systemic lipopolysaccharide-induced (LPS) immune challenge. We hypothesized that exposure to the heroin-paired context would depress LPS-induced NO production, and this response would be rescued by IL-1Ra administration.

2. Materials and methods

2.1. Animals

Male Lewis rats, weighing 225–250 g (N = 76), were purchased from Charles River Laboratories (Kingston, NY, USA). Upon arrival, animals were housed individually in a colony room with a reversed light-dark (12-h) cycle maintained through artificial illumination. Animals were allowed access to food and water *ad libitum* throughout the experiment except during the time spent in the conditioning chambers. All animals were given a 2-week habituation period before the start of experimental manipulations and were handled regularly during that time. All animal procedures followed the Guide for the Care and Use of Laboratory Animals (National Research Council (U.S.). Committee for the Update of the Guide for the Care and Use of Laboratory Animals. et al., 2011) and were approved by the University of North Carolina at Chapel Hill's Institutional Animal Care and Use Committee (IACUC).

2.2. Drug administration

Heroin (diacetylmorphine) from The National Institute on Drug Abuse (NIDA) Drug Supply Program (DSP; Bethesda, MD, USA) was dissolved in 0.9% sterile saline and administered subcutaneously at a dose of 1.0 mg/kg. This dose was selected based on prior experiments in our laboratory showing that it induces conditioning and alters LPS-induced iNOS mRNA expression in spleen tissue (Lysle and How, 2000; Lysle and Ijames, 2002; Szczytkowski and Lysle, 2007). Lipopolysaccharide (LPS; derived from *E. coli*, serotype 055:B5) was dissolved in sterile, pyrogen-free saline to a dose of 1.0 mg/kg. This serotype has been used at this dose previously in our laboratory to induce measures of NO and it also produces

sickness behavior. It must be noted that different activity would likely be seen in other serotypes at the same dose even from within the same species (Caroff et al., 2002). Human recombinant interleukin-1 receptor antagonist (IL-1Ra; Genscript, Piscataway, NJ, USA; Catalog No: Z00367) was reconstituted in 0.9% sterile saline to a final concentration of 2.5 μg/μL, aliquoted, and then stored at −20 °C.

2.3. Surgical procedures

Animals were fully anesthetized with 1 mL/kg intraperitoneal injections of 9:1 (vol:vol) ketamine hydrochloride (100 mg/mL) mixed with xylazine (100 mg/mL), were shaved on the top of the head, and were placed into the stereotaxic apparatus. Twenty-six-gauge guide cannulae (Plastics One, Roanoke, VA, USA) were directed bilaterally towards the BLA (AP −2.5 mm, ML ± 5.0 mm, DV −6.6 mm, relative to bregma), mNAcS (AP +1.7 mm, ML ± 0.8 mm, DV −5.4 mm, relative to bregma), or posterior caudate-putamen (pCPu; anatomical control region; AP −2.5 mm, ML ± 5.0 mm, DV −4.0 mm, relative to bregma). Cannulae were secured to the skull with screws and dental acrylic. Animals were given a 2-week post-surgical recovery period before the start of conditioning trials.

2.4. Conditioning procedure

All animals received five conditioning sessions in standard conditioning chambers (BRS/LVE, Laurel, MD, USA). Chambers were fitted with a metal grid floor and cedar bedding beneath the floor to create an environment distinct from the home cage in olfactory and tactile cues. A white noise generator (50 dB, 50–60 Hz) was used to mask background noise. All conditioning took place during the dark phase of the light cycle in an experimental room away from the colony room. The conditioning chambers were kept dark to minimize effects on circadian rhythms. Immediately before each conditioning session, the animals received a subcutaneous injection of heroin (1 mg/kg) followed by placement into the conditioning chamber for 60 min. Conditioning sessions were separated by 48 h.

2.5. Test for expression of heroin-conditioned immunosuppression

Six days following the final conditioning session, animals received bilateral microinfusions of saline vehicle (0.5 μL per hemisphere) or IL-1Ra (1.25 μg/0.5 μL per hemisphere) into the BLA, pCPu, or mNAcS. Injectors extended 2 mm beyond the tip of the guide cannula. Injections were delivered over 2 min, and the injectors sat in place for 1 min after the injection to allow for proper diffusion of fluid away from the infusion site. Thirty minutes later, the rats were re-exposed to either the previously heroin-paired conditioning chamber or their home cage for 60 min. Heroin was not administered. After the 60-min time period, all rats received a subcutaneous injection of LPS, a component of the cell wall of Gram negative bacteria that induces reliable measures of NO production (Lysle and How, 2000). After LPS injection, animals were returned to their home cages and were euthanized via cervical dislocation 6 h later, a time point when near-maximal iNOS induction is observed following LPS administration (Lysle and How, 1999, 2000).

2.6. Histology

Samples of spleen and blood were collected for analysis. Spleen tissue was divided into approximately 100 mg samples which were stored in either RNAlater (Ambion/Thermo Fisher Scientific, Waltham, MA, USA) or protease inhibiting buffer (Pierce™ Protease Inhibitor, Thermo Fisher Scientific, Waltham, MA, USA) for mRNA and protein extraction, respectively. To confirm proper cannula placement, Alcian blue dye was infused via the cannula. Brains were then extracted and flash frozen in a bath of ultra-cold isopentane (Histobath NesLab, Portsmouth, NH, USA; Shandon Lipshaw, Pittsburgh, PA, USA), and stored at −80 °C until further analysis. Coronal sections (40 μm) were taken and stained with cresyl violet for verification of cannula placement. Animals with cannula placement outside of the targeted region are removed from analysis as indicated in the results section.

2.7. RT-qPCR mRNA analysis

Tissue was processed by the UNC Animal Clinical Chemistry and Gene Expression Laboratories according to protocols previously reported (Kim et al., 2002). In brief, spleen tissue was homogenized in RNA lysis buffer (PE Biosystems/Applied Biosystems, Foster City, CA, USA) with Ca^{2+}- and Mg^{2+}-free phosphate buffer using a Fast Prep 120 mixer (QBIOgene, Vista, CA, USA). RNA isolations were purified using the ABI Prism 6700 automated nucleic acid workstation (PE Biosystems/Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. RT-qPCR amplifications were performed in the ABI Prism 7700 Sequence Detector (PE Biosystems/Applied Biosystems, Foster City, CA, USA) in a total volume of 30 μL (10 μL RNA plus 20 μL reaction mixture). RT-qPCR amplification was performed in duplicate with the following cycling parameters: 30 min at 48 °C for the RT reaction and 10 min at 94 °C followed by 40 temperature cycles (15 s at 94 °C and 1 min at 60 °C). Signal intensity was normalized to 18S as a reference gene. In order to verify the stability of 18S, an ANOVA was performed to demonstrate that there were no statistically significant group differences in 18S expression. Normalized values were then normalized against the control group (saline-treated home cage) normalized average Ct for the gene of interest. For graphical representation, delta delta Ct values were linearly transformed. The nucleotide sequences of the qPCR primers and fluorogenic probes used for the iNOS and 18S genes were as follows: iNOS forward: 5′-AGCGGCTCCATGACTCTCA-3′, reverse: 5′-TGCCTGCACC CAAACACCAA-3′, probe: 5′-FTCATGCGGCCTCCTTTGAGCCCTCQ-3′; 18S: forward: 5′-AGAAACGGCTACCACATCCA-3′, reverse: 5′-CTCG AAAGAGTCCTGTATTGT-3′, probe: 5′-FAGGCAGCAGGCGCGCAAAT TACQ-3′[F = 5′-Fluorescein (FAM); Q = Quencher (TAMRA)].

2.8. ELISA protein analysis

Spleen tissue was homogenized using a bead homogenizer (Precellys Evolution, Bertin Instruments, Montigny-le-Bretonneux, France), chilling samples on ice in between each 15 s run. Protein was extracted using cycles of freeze-thaw lysis, centrifugation, and collection of the supernatant. Total protein was quantified by Bradford Assay. Briefly, 10 μL of 1:200 diluted protein, in triplicate, was incubated with 200 μL diluted and filtered Bradford Assay Dye (Bio-Rad Laboratories Inc., Hercules, CA, USA; Catalog No. 500- 0006) for 10 min.

The absorbance of each sample triplicate was measured in a spectrophotometer (Epoch™, BioTek Instruments Inc., Winooski, VT, USA) at 595 nm, averaged, and compared to a concurrently run BSA standard curve to determine μg/μL concentration. Splenic iNOS protein was quantified using a rat iNOS sandwich ELISA kit (LifeSpan BioSciences, Inc., Seattle, WA, USA; Catalog No. LS-F4109). The user manual supplied with the kit was followed with the following parameters: 200 μg of total protein per sample was loaded in duplicate, plates were gently agitated at 55 rpm, and color was allowed to develop for 20 min. The ELISA plate was washed with an automatic plate washer (EL 403, BioTek Instruments Inc., Winooski, VT, USA) as recommended by the kit manual. Each plate was read in a spectrophotometer at 450 nm and compared to a concurrently run standard curve to determine ng iNOS protein per 200 μg of total protein.

2.9. Nitrate/nitrite assay

The nitrate/nitrite concentration in plasma samples was assessed using the Greiss reagent assay described previously (Szczytkowski and Lysle, 2007). Briefly, 6 μL of plasma was diluted in 44 μL of dH_2O, and the sample was incubated in the dark for 90 min with 10 μL of nitrate reductase (1.0 U/mL), 20 μL of 0.31 M phosphate buffer (pH 7.5), 10 μL of 0.86 mM NADPH (Sigma-Aldrich Inc., Milwaukee, WI, USA), and 10 μL of 0.11 mM flavin adenine dinucleotide in individual wells of a 96-well plate. Greiss reagent (200 μL, 1:1 (v/v) solution 1% sulfanilamide in 5.0% phosphoric acid and 0.1% N-(1-napthyl) ethyl-enedamine dihydrochloride in distilled water), was added to the samples. The color was allowed to develop for 10 min at room temperature. Absorbance at 550 nm was determined using a spectrophotometer (Epoch™ Microplate Spectrophotometer, BioTek Instruments Inc., Winooski, VT, USA). All reactions were carried out in triplicate. The total micromolar concentration of nitrite was determined for each sample based on a concurrently run standard curve.

2.10. Statistical analysis

Two-way analysis of variance (ANOVA) was performed on data sets using SPSS Statistics (IBM, Armonk, NY, USA). Tukey's Honestly Significant Difference (HSD) post hoc test was used to compare the saline and IL-1Ra treated and contextually re-exposed groups to their respective home cage control groups. Statistically significant outliers were detected using Grubb's test and removed from analysis where noted in the results. For all tests, the significance level was set at $p = 0.05$.

3. Results

3.1. Effects of BLA IL-1 antagonism on heroin-conditioned immunosuppression

Fig. 1 shows cannula placements from experiments targeting the (**A**) BLA, (**B**) pCPu, and (**C**) mNAcS. There were no animals with cannula placement outside of the targeted region upon analysis. One IL-1Ra infusion was not successful, and thus that animal was removed from all analyses.

Fig. 2A shows the effect of intra-BLA IL-1 antagonism on LPS-induced iNOS mRNA expression in the spleen. The ANOVA revealed a statistically significant context by

treatment interaction [$F(1, 17) = 5.403$, $p = 0.033$] and a statistically significant main effect of context [$F(1, 17) = 6.680$, $p = 0.019$], demonstrating heroin context-induced suppression in the LPS-induced increase in splenic iNOS mRNA levels. Tukey's comparisons showed the saline-treated control group was statistically significantly different from the saline-treated group exposed to the heroin-paired context ($p = 0.016$). Thus, the heroin-paired context reduced splenic iNOS mRNA expression. In contrast, the IL-1Ra-treated group that was re-exposed to the heroin-paired context did not differ in splenic iNOS mRNA expression from the IL-Ra-treated home cage control group ($p = 0.998$), indicating that IL-1Ra blocked the effect of the heroin conditioned context on splenic iNOS mRNA.

Fig. 2B shows the effect of intra-BLA IL-1 antagonism on LPS-induced iNOS protein in the spleen. The Grubb's test identified two statistical outliers that were removed from analysis: one animal in the IL-1Ra-treated control group; one animal in the saline-treated context re-exposed group. The ANOVA revealed a statistically significant main effect of context [$F(1, 15) = 7.744$, $p = 0.014$] and a statistically significant main effect of treatment [$F(1, 15) = 6.680$, $p = 0.019$], but no statistically significant interaction [$F(1, 15) = 3.475$, $p = 0.082$]. Tukey's comparisons showed the saline-treated control group was statistically significantly different from the saline-treated group exposed to the heroin-paired context ($p = 0.028$). Thus, the heroin-paired context reduced splenic iNOS protein expression. In contrast, the IL-1Ra-treated group that was re-exposed to the heroin-paired context did not differ in splenic iNOS protein expression from the saline- or IL-Ra-treated home cage control group ($p = 0.907$), indicating that IL-1Ra blocked the effect of the heroin conditioned context on splenic iNOS protein.

Fig. 2C shows the effect of intra-BLA IL-1 antagonism on LPS-induced nitrate/nitrite levels in blood plasma. The ANOVA revealed a statistically significant main effect of context [$F(1, 17) = 9.158$, $p = 0.008$], no statistically significant main effect of treatment [$F(1, 17) = 2.879$, $p = 0.108$], and a statistically significant interaction [$F(1, 17) = 7.360$, $p = 0.015$]. Specifically, Turkey's comparisons showed the saline-treated control group was significantly different from the saline-treated group exposed to the heroin-paired context ($p = 0.005$). Thus, the heroin-paired context reduced plasma nitrate/nitrite levels. In contrast, the IL-1Ra treated group that was re-exposed to the heroin-paired context did not differ in nitrate/nitrite levels from the IL-1Ra-treated home cage control group ($p = 0.996$), indicating that IL-1Ra blocked the effect of the heroin conditioned context on plasma nitrate/nitrite levels. Collectively, these results indicate that IL-1 blockade in the BLA attenuated heroin context-induced suppression of NO production.

3.2. Effects of posterior caudate putamen IL-1 antagonism on heroin-conditioned immunosuppression

Fig. 3A shows the effects of IL-1 receptor blockade in the pCPu anatomical control region on LPS-induced splenic iNOS mRNA expression. The pCPu is positioned immediately dorsal to the BLA; therefore, this region was targeted to confirm that the effects observed from BLA IL-1 receptor antagonism were not due to diffusion of IL-1Ra into the pCPu through the cannula tract. The ANOVA indicated a statistically significant main effect of context [$F(1,24) = 16.507$, $p < 0.001$] but no main effect of treatment [$F(1,24) = 0.005$, $p =$

0.947] nor context by treatment interaction [$F(1,24) = 0.018$, $p = 0.894$]. Tukey's comparisons showed that the saline- and IL-1Ra-treated groups exposed to the heroin-paired context both exhibited a statistically significant reduction in splenic iNOS mRNA expression compared to the saline- and IL-1Ra-treated home cage control groups (saline, $p = 0.032$; IL-1Ra, $p = 0.048$). Thus, IL-1Ra infused into the pCPu did not block the decrease in LPS-induced splenic iNOS mRNA expression following exposure to a heroin-paired context.

Fig. 3B shows the effects of IL-1 receptor blockade in the pCPu anatomical control region on LPS-induced splenic iNOS protein expression. One statistical outlier in the saline-treated context re-exposed group was identified by the Grubb's test and was removed from analysis. The ANOVA indicated a statistically significant main effect of context [$F(1,23) = 28.408$, $p < 0.001$] but no main effect of treatment [$F(1,23) = 0.678$, $p = 0.419$] nor context by treatment interaction [$F(1,23) = 0.004$, $p = 0.948$]. Tukey's comparisons showed that the saline- and IL-1Ra-treated groups exposed to the heroin-paired context both exhibited a statistically significant reduction in splenic iNOS protein expression compared to the saline- and IL-1Ra-treated home cage control groups (saline, $p = 0.005$; IL-1Ra, $p = 0.005$). Thus, IL-1Ra infused into the pCPu did not block the decrease in LPS-induced splenic iNOS protein expression following exposure to a heroin-paired context.

Fig. 3C illustrates the effect of pCPu IL-1 receptor antagonism on LPS-induced levels of nitrate/nitrite in blood plasma. The Grubb's test identified one statistical outlier in the saline-treated context re-exposed group, which was removed from analysis. The ANOVA revealed a statistically significant main effect of context on plasma nitrate/nitrite levels [$F(1,23) = 22.980$, $p < 0.001$] but no main effect of treatment [$F(1,23) = 1.508$, $p = 0.232$] nor context by treatment interaction [$F(1,23) = 0.366$, $p = 0.551$]. Accordingly, Tukey's comparisons showed that the saline- and IL-1Ra-treated groups exposed to the heroin-paired context both exhibited a reduction in plasma nitrate/nitrite levels compared to the home cage control groups, indicating expression of heroin-conditioned effect in both treatment groups re-exposed to the context (saline, $p = 0.005$; IL-1Ra, $p = 0.029$). Thus, there was no effect of IL-1Ra treatment in the pCPu on the expression of neither splenic iNOS mRNA nor plasma nitrate/nitrite, indicating that IL-1 signaling in this region does not play a role in expression of heroin-conditioned effects on inducible NO. These findings also indicate that the effects of administering IL-1Ra into the BLA are not due to diffusion of IL-1Ra dorsally into the pCPu.

3.3. Effects of medial nucleus accumbens shell IL-1 antagonism on heroin-conditioned immunosuppression

Fig. 4A depicts the effect of mNAcS IL-1 receptor blockade on LPS-induced splenic iNOS mRNA expression. The ANOVA revealed a statistically significant main effect of context [$F(1,22) = 31.93$, $p < 0.001$], but there was no significant main effect of treatment [$F(1,22) = 0.519$, $p = 0.479$] nor context by treatment interaction [$F(1,22) = 0.206$, $p = 0.654$]. Tukey's comparisons showed that the saline- and IL-1Ra-treated groups exposed to the heroin-paired context both exhibited a reduction in splenic iNOS mRNA expression compared to the saline- and IL-1Ra-treated home cage control groups (saline, $p = 0.009$; IL-1Ra, $p = 0.001$). The saline- and IL-1Ra-treated groups exposed to the heroin-paired context both exhibited a

reduction in iNOS mRNA expression compared to groups exposed to the home cage. Thus, IL-1Ra infused into the mNAcS did not block the decrease in LPS-induced splenic iNOS mRNA expression following exposure to a heroin-paired context.

Fig. 4B depicts the effect of mNAcS IL-1 receptor blockade on LPS-induced splenic iNOS protein expression. The ANOVA revealed a statistically significant main effect of context [$F(1,22) = 69.973$, $p < 0.001$], but there was no significant main effect of treatment [$F(1,22) = 3.558$, $p = 0.073$] nor context by treatment interaction [$F(1,22) = 2.051$, $p = 0.166$]. Tukey's comparisons showed that the saline- and IL-1Ra-treated groups exposed to the heroin-paired context both exhibited a reduction in splenic iNOS protein expression compared to the saline- and IL-1Ra-treated home cage control groups (saline, $p = 0.001$; IL-1Ra, $p < 0.001$). The saline- and IL-1Ra-treated groups exposed to the heroin-paired context both exhibited a reduction in iNOS protein expression compared to groups exposed to the home cage. Thus, IL-1Ra infused into the mNAcS did not block the decrease in LPS-induced splenic iNOS protein expression following exposure to a heroin-paired context.

Fig. 4C represents the effect of mNAcS IL-1 receptor antagonism on LPS-induced levels of nitrate/nitrite in blood plasma. The ANOVA revealed a statistically significant main effect of context [$F(1,22) = 48.266$, $p < 0.001$] and main effect of treatment [$F(1,22) = 6.830$, $p = 0.016$] on plasma nitrate/nitrite levels, but there was not a significant context by treatment interaction [$F(1, 22) = 2.097$, $p = 0.162$]. Tukey's comparisons showed that the saline- and IL-1Ra-treated groups exposed to the heroin-paired context both exhibited a reduction in plasma nitrate/nitrite levels compared to the control groups, indicating expression of the heroin-conditioned effect in both treatment groups re-exposed to the context (saline, $p = 0.006$; IL-1Ra, $p < 0.001$). Together, the results show that IL-1 signaling in the mNAcS does not play a role in the expression of heroin-conditioned effects on inducible NO.

4. Discussion

It is well established that re-exposure to a previously heroin-paired context results in immunosuppressive effects that are similar to heroin administration alone (Hutson et al., 2014; Lysle and Ijames, 2002; Saurer et al., 2009; Szczytkowski et al., 2011, 2013; Szczytkowski and Lysle, 2007, 2008, 2010). The findings from the current studies indicate that IL-1 receptor signaling in the BLA, but not the mNAcS or pCPu, mediates the conditioned effects of a heroin-paired context on splenic iNOS mRNA expression and blood plasma nitrate/nitrite, two measures of NO production. These studies are the first to identify IL-1 signaling in the BLA as an important mediator in the expression of learned associations and, specifically, heroin-conditioned immunosuppression.

The observation that BLA IL-1 is important for the expression of heroin-conditioned immunosuppression expands upon literature indicating the importance of IL-1 signaling in learning and memory. To our knowledge, studies have not examined the role of BLA IL-1 in the expression of heroin-induced learning and memory. However, IL-1 in another region, the hippocampus, has previously been shown to be important for memory consolidation and the maintenance of long-term potentiation (Avital et al., 2003; Goshen et al., 2007; Pugh et al., 2001; Schneider et al., 1998; Yirmiya et al., 2002). Most relevant to the present study, our

laboratory has demonstrated that siRNA-mediated IL-1β gene knockdown in the DH prior to re-exposure to a previously heroin-paired context attenuated the conditioned immunosuppressive effect on splenic iNOS mRNA expression and plasma nitrate/nitrite levels, indicating that hippocampal IL-1β is an important mediator of heroin-conditioned immunosuppression (Szczytkowski et al., 2013). Furthermore, blockade of IL-1 signaling in the DH also prevents the acquisition of heroin-conditioned immunosuppression (Lebonville et al., 2016). Thus, there is substantial evidence that IL-1 signaling in the DH plays a crucial role in learning and memory, and new emerging evidence that this role is substantiated in heroin-conditioned immunosuppression. The current studies are the first to provide evidence that IL-1 signaling in the BLA plays a role in heroin-conditioned immunosuppression as well.

The results show that mNAcS IL-1 receptor antagonism failed to alter heroin-induced conditioned immunosuppression. This was surprising because of the evidence that IL-1 is expressed in this region (Ericsson et al., 1995; Yabuuchi et al., 1994) and that IL-1β immunoreactivity and microglial activation in the mNAcS are robust following a test of morphine conditioned place preference (CPP) (Zhang et al., 2012). Although we do not have evidence that IL-1 expression is induced within our conditioned model, our current results indicate that regardless of whether constitutive or induced expression of mNAcS IL-1 occurs, IL-1 in this region is not necessary for heroin-conditioned immunosuppression. Thus, the present data suggest that specific components of the circuitry required for heroin's conditioned effects involve IL-1 signaling, namely IL-1 within the BLA.

The mechanism by which IL-1 mediates the expression of heroin-conditioned immunosuppression is largely unknown. There is growing evidence that IL-1 is produced by neurons, astrocytes, and microglia (Flannery and Bowie, 2010; Huang et al., 2011; Ringwood and Li, 2008; Yabuuchi et al., 1994; Zhang et al., 2010). One primary source of IL-1β in the brain is glial cells (Davies et al., 1999; Hanisch, 2002; Rothwell et al., 1996; Toda et al., 2002). Microglia may indirectly change neuronal transmission by modulating astrocytic glutamate uptake via the release of astroglial modulators like IL-1 (Jing et al., 2010). Indeed, IL-1 has been identified as a critical mediator of microglia-induced astrocyte activation, as central IL-1 administration causes an upregulation of glial fibrillary acidic protein, which is a marker of astrocyte activation (Balasingam et al., 1994). In support of this assertion, reexposure to a morphine-paired context is sufficient to elicit microglial activation (Schwarz et al., 2011). IL-1 has also been reported to upregulate the proinflammatory cytokines IL-6 and TNF-α (John et al., 2004), both of which have been shown to alter neuronal function (Beattie et al., 2002; McPherson et al., 2011; Santello et al., 2011). Activated microglia may stimulate astrocytic IL-1 receptors directly or upregulate the proinflammatory cytokines IL-6 and TNF-α (Basu et al., 2002; John et al., 2004) to induce astrocytic activation, indicated by an increase in the expression of glial fibrillary acidic protein (Balasingam et al., 1994; Lee et al., 2010). Through these mechanisms, IL-1 can dose-dependently attenuate astrocytic glutamate uptake and thus increase extracellular glutamate levels (Jing et al., 2010). Consistent with subsequent changes in neurotransmission, bath application of IL-1β alters electrophysiological activity in various brain regions. For example, *in vitro* IL-1β application produces electrophysiological changes in various brain regions, such as decreased inhibitory transmission in the hippocampus

(Wang et al., 2000; Zeise et al., 1997, 1992), amygdala (Bajo et al., 2015; Yu and Shinnick-Gallagher, 1994), and hypothalamus (Feleder et al., 1998). In addition, IL-1β alters excitatory transmission in the brain (Miller and Fahey, 1994), and, more specifically, in the hippocampus (Bellinger et al., 1993; Coogan and O'Connor, 1997; Cunningham et al., 1996; Luk et al., 1999), and amygdala (Yu and Shinnick-Gallagher, 1994). Thus, although IL-1 is critical for the expression of the conditioned effect, it may be indirectly exerting its effects by altering the expression of other cytokines which ultimately modulate neuronal activity. Taken together, the present results indicate that IL-1 can robustly alter neural activity in the BLA that is critical for the expression of heroin conditioned effects.

The mechanism by which these central processes affected by IL-1 signaling changes lead to peripheral immune effects is unclear. Another lingering question is whether BLA IL-1 is involved in associative memory expression processes or whether it is involved in the efferent communication between the brain and peripheral immune organs. The central amygdala is involved in regulating descending efferent responses from the amygdala (Sah et al., 2003) and thus may provide clues toward IL-1's role in the BLA. Previous evidence showing that IL-1 in the DH is involved in both acquisition and expression of heroin-conditioned immunosuppression, and yet is not involved in the mechanism by which one injection of heroin suppresses the immune system (Lebonville et al., 2016), suggests that the role of IL-1 may lie in the realm of memory, rather than efferent processes. It is not known, however, how BLA IL-1 might functionally differ from IL-1 in the DH. Another possibility is that IL-1Ra disrupts state-dependent recall, rather than memory expression *per se*. These questions should be thoroughly explored in future studies.

A few limitations surround the use of human recombinant IL-1Ra in this study. Firstly, IL-1Ra itself does not allow us to determine the relative roles of IL-1 isoforms, IL-1α and IL-1β, since they both act through IL-1R1. Our previous evidence from studies investigating IL-1 signaling in the DH suggests that the IL-1β isoform may play more of a role in heroin-conditioned immunosuppression, but it has not been determined if the important role of this isoform reprises in the BLA. Furthermore, it has been suggested that, although use of human recombinant IL-1Ra is fairly common-place, that this foreign protein may elicit an immune response. We do not believe that a response of this kind would be responsible for the effects seen in the BLA as this same manipulation within the mNAcS did not modulate heroin-conditioned immunosuppression. However, specificity of this effect to action through the IL-1R1 receptor, rather than to an ancillary antigenic response, should be addressed with additional studies.

The neural mechanisms involved in other forms of immune conditioning studies such as those using cyclosporine A paired with a tastant, have shown that lesioning of the amygdala does not alter the expression of taste-conditioned immunosuppression (Pacheco-Lopez et al., 2005). Thus, the amygdala is not involved in this form of immune conditioning let alone the IL-1 signaling in this area. Interestingly, this suggests that different types of unconditioned stimuli or different types of conditioned stimuli engage distinct neural circuitry, or perhaps taste versus context conditioning have a differential reliance on contextual memory. The comparisons are interesting but complex. For example, it was also demonstrated that the insular cortex and ventromedial hypothalamus were required throughout taste conditioned

immunosuppression or only during expression, respectively (Pacheco-Lopez et al., 2005) but it is unknown whether these regions are also required for contextual immune conditioning using heroin and whether IL-1 plays a critical role in those regions.

The negative health consequences associated with opioid use have been well characterized. Due to the prevalence of opioid use, such as heroin and medicinal opioids, it is important to understand the mechanisms that mediate the conditioned effects of opioids on immune function. While we do not directly test the downstream effects of NO modulation on pathogen resistance and survival, it is likely that these, and other opiate-conditioned immune effects, contribute to the health consequences of opiate users. These studies provide insight into a novel mechanism mediating heroin-conditioned immunosuppression and raise new questions that could direct future studies aimed at elucidating other important factors involved in this effect. It is possible that these findings could provide insight into IL-1-mediated conditioned responding in other models of associative learning, potentially leading to novel treatments aimed at both drug and non-drug disorders.

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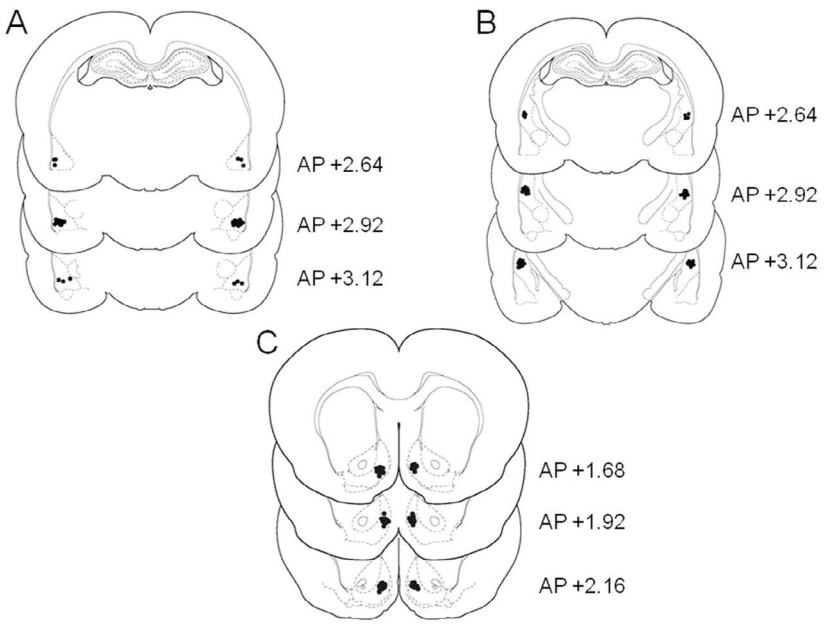

Fig. 1.

Illustration of cannula placement. Symbols represent the ventral most point of the injection cannula tracts in the (A) BLA, (B) pCPu, and (C) mNAcS. Numbers represent distance from bregma based on the rat brain atlas of Paxinos and Watson (2009).

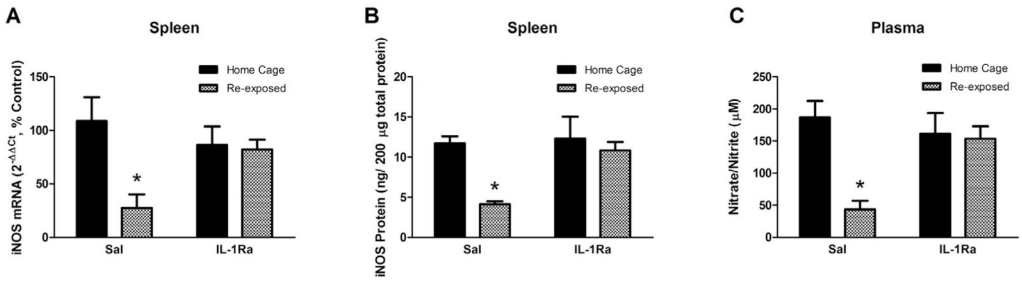

Fig. 2.

Effects of BLA IL-1 antagonism on LPS-induced expression of splenic iNOS mRNA (A) and protein (B) and plasma nitrate/nitrite (C). For mRNA data, mean displayed is % of saline-treated home cage control. Sample sizes range from n = 4–6 per group. Symbols (*) represent statistically significant difference relative to respective home cage control group ($p < 0.05$).

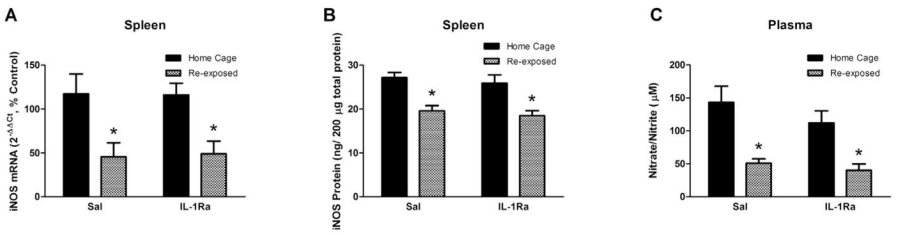

Fig. 3.

Effects of pCPu IL-1 antagonism on LPS-induced expression of splenic iNOS mRNA (A) and protein (B) and plasma nitrate/nitrite (C). For mRNA data, mean displayed is % of saline-treated home cage control. Sample sizes range from n = 6–7 per group. Symbols (*) represent statistically significant difference relative to respective home cage control group ($p < 0.05$).

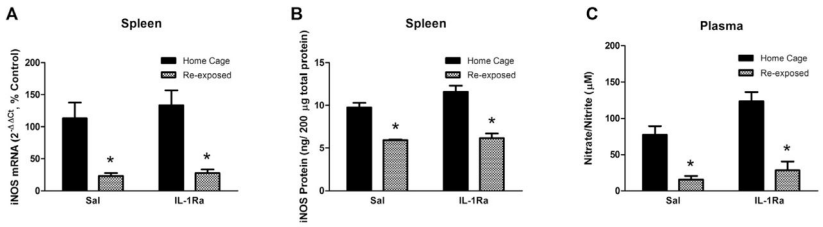

Fig. 4.

Effects of mNAcS IL-1 antagonism on LPS-induced expression of splenic iNOS mRNA (A) and protein (B) and plasma nitrate/nitrite (C). For mRNA data, mean displayed is % of saline-treated home cage control. Sample sizes range from n = 6–7 per group. Symbols (*) represent statistically significant difference relative to respective home cage control group ($p < 0.05$).