REGION-SPECIFIC CONTRIBUTION OF THE VENTRAL TEGMENTAL AREA TO HEROIN-INDUCED CONDITIONED IMMUNOMODULATION

Lee Wade Hutson

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Approved by:

Donald T. Lysle

Todd E. Thiele

Charlotte Boettiger

ABSTRACT

Lee Wade Hutson: Region-Specific Contribution of the VTA to Heroin-Induced Conditioned Immunomodulation
(Under the direction of Donald T. Lysle)

Dopamine receptor stimulation is critical for heroin-conditioned immunomodulation; however, it is unclear whether the ventral tegmental area (VTA) contributes to this phenomenon. Hence, rats received repeated pairings of heroin with placement into a distinct environmental context. At test, they were re-exposed to the previously heroin-paired environment followed by systemic lipopolysaccharide treatment to induce an immune response. Bilateral GABA agonist-induced neural inactivation of the anterior, but not the posterior VTA, prior to context re-exposure inhibited the ability of the heroin-paired environment to suppress peripheral nitric oxide and tumor necrosis factor-α expression, suggesting a role for the anterior VTA in heroin-conditioned immunomodulation.

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CHAPTER I: INTRODUCTION

It was long believed that the nervous and immune system functioned independently of one another; however, this idea was challenged by Ader and Cohen in 1975 when a Pavlovian associative learning model was used to demonstrate for the first time that a previously neutral stimulus can acquire immune altering effects after pairing with an immunomodulatory drug. In this study, Ader and Cohen paired the immunosuppressive drug cyclophosphamide with saccharin. Results revealed no evidence of an immunomodulatory effect from exposure to saccharin among control animals, however an immunosuppressive effect was observed among conditioned animals when exposed to saccharin, indicating that saccharin had acquired immune altering properties similar to those of cyclophosphamide. Subsequent studies conducted by Ader and colleagues further demonstrated conditioned immunomodulatory effects on the autoimmune disease systemic lupus erythematosus (Ader and Cohen 1982), humoral immunity (Ader, Cohen et al. 1982; Ader, Kelly et al. 1993), and cell-mediated immunity (Ader and Cohen 1992), which established psychoneuroimmunology as a new field of study.

Building upon the work of Ader and Cohen, others began to investigate the neural circuitry involved in conditioned immunomodulation. Brain regions of interest were identified based on previous research that mapped out a basic neural circuit of the

conditioned taste aversion model (Pacheco-Lopez, Niemi et al. 2005), which was used to model conditioned immunosuppression by pairing saccharin with cyclosporine A, an immunologic drug. Excitotoxic lesions were performed in brain after the acquisition phase. Results revealed that disruption of insular cortex and ventromedial hypothalamus, but not the amygdala, signaling prevented the expression of conditioned immunosuppression of lymphocyte proliferation, IL-2, and interferon-γ (Pacheco-Lopez, Niemi et al. 2005).

In addition to findings indicating that the effects of immunologic drugs are capable of producing conditioned immunosuppression when paired with previously neutral stimuli, research suggests that opiates directly affect the immune system's ability to defend against infection (Luttgens 1949; Hussey and Katz 1950; Bussiere, Adler et al. 1993; Friedman and Eisenstein 2004). In particular, opioid administration has detrimental health consequences in addition to the possible development of addictive behaviors and dependence. Clinical studies have revealed abnormalities in basic immune parameters in heroin users, including a decrease in circulating lymphocytes, natural killer cell activity, cytokine production, and antibody-dependent cellular cytotoxicity (Nair, Navaratnam et al. 1986; Govitrapong, Suttitum et al. 1998; Olson, Zabetian et al. 2005; Yardeni, Beilin et al. 2008). Several immune parameters that are critical for innate immune responses are altered by opioid use, such as the expression of inducible nitric oxide synthase (iNOS) (Lysle and How 2000) and the production of the proinflammatory cytokines, tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) (Chao, Molitor et al. 1993; Pacifici, di Carlo et al. 2000). Thus, these studies suggest that chronic opioid administration results in an impaired ability to defend against infectious disease (Theodorou and Haber 2005).

Interestingly, opioid-induced immunosuppression is mediated by the central nervous system (Shavit, Depaulis et al. 1986; Fecho, Maslonek et al. 1996; Lysle, Hoffman et al. 1996), and based on the previously described studies on conditioned immunomodulation, the immunosuppressive effects of exogenous opioid administration cannot exclusively be attributed to acute pharmacological effects on physiology. Early research from our laboratory has shown that the immune altering effects of opioids, including those of morphine and heroin, can be conditioned to environmental stimuli by pairing opioid administration with exposure to a distinct environmental context. As a result, a morphine-paired context (CS) can acquire immune altering effects. For example, following conditioning sessions during which morphine injections were paired with a distinct context, rats exhibited significant reductions in mitogenic responsiveness of lymphocytes, natural killer cell activity, and interleukin-2 production when re-exposed to the distinct context in a drug free state, demonstrating for the first time morphine-conditioned immunosuppression (Coussons, Dykstra et al. 1992). It was later discovered that dopamine and glutamate are involved in conditioned immunomodulation (Hsueh, Kuo et al. 1999; Kuo, Chen et al. 2001), which led to our laboratory investigating the contributions of specific neurotransmitters to morphine- and heroin-conditioned immunosuppression. Findings from our laboratory demonstrated that dopamine receptor activity was necessary for the expression of conditioned immune alterations. Furthermore, administration of a D_1-like receptor antagonist prior to re-exposure to the conditioned stimulus prevented the suppression of natural killer cell activity (Saurer, Ijames et al. 2008). Similar immunomodulatory effects have also been demonstrated with heroin (Fecho and Lysle 2000; Lysle and Ijames 2002; Saurer, Ijames et al. 2008).

Emerging evidence suggests that a limbic neural circuit mediates the expression of heroin-induced conditioned immune alterations, and this circuit likely includes the ventral tegmental area (VTA). In support of this, either GABA agonist-induced neural inactivation of, or dopamine D_1-like receptor antagonism in, the basolateral amygdala (BLA) blocks heroin-induced conditioned immunosuppression (Szczytkowski and Lysle 2008; Szczytkowski and Lysle 2010). Moreover, unilateral dopamine D_1-like receptor antagonism in the BLA coupled with contralateral NMDA glutamate receptor antagonism in the nucleus accumbens shell (NAc shell) significantly attenuates the expression of heroin-conditioned immunosuppression. In contrast, ipsilateral manipulation of the same brain regions fails to disrupt heroin-conditioned immunomodulation (Szczytkowski, Fuchs et al. 2011). These findings suggest that dopamine in the BLA, via the stimulation of D_1-like receptors, is necessary for obligatory intrahemispheric interactions between the BLA and the NAc in the control of heroin-conditioned immune alterations.

The critical source of dopamine involved in heroin-conditioned immunomodulation has not been investigated even though the VTA is the likely candidate. Opioid administration results in the disinhibition of dopaminergic projection neurons and the release of dopamine at terminal regions via the stimulation of μ receptors on VTA GABAergic inter- and projection neurons. Similarly, heroin-associated stimuli increase the activity of VTA neurons (Kiyatkin and Rebec 2001). Recently, investigations have focused on elucidating the function of various subregions of the VTA. Studies suggest that the VTA is a heterogeneous structure with distinct subregions differentially affecting drug-induced behaviors. For example, μ opioid receptor antagonism in the anterior, but not posterior, VTA prevents the acquisition of cocaine-induced conditioned place preference (Soderman and Unterwald 2008). Furthermore,

rats self-administer the $GABA_A$ antagonist picrotoxin into the anterior, but not the posterior, VTA (Ikemoto, Kohl et al. 1997). Furthermore, a study utilizing retrograde tracing techniques determined that dopaminergic efferents from the VTA to BLA originate in the anterior VTA (Ford, Mark et al. 2006), whereas a large proportion of dopaminergic projections from the posterior VTA terminate in the NAc (Ikemoto 2007). Based on these findings and the importance of dopamine signaling in the BLA in conditioned immunosuppression (Szczytkowski and Lysle 2008; Szczytkowski and Lysle 2010; Szczytkowski, Fuchs et al. 2011), we hypothesized that the functional integrity of the anterior, but not posterior, VTA is necessary for the expression of heroin-conditioned immunomodulation.

The present study selectively targeted the anterior or posterior VTA in order to evaluate the distinct contributions of these VTA subregions to the expression of heroin-induced conditioned immunomodulation. Rats underwent a conditioning procedure which consisted of repeated pairings of heroin administration with placement into a distinct environment. Following the conditioning regimen, rats received microinfusions of saline vehicle or a cocktail of the $GABA_B$/$GABA_A$ agonists, baclofen/muscimol into the anterior or posterior VTA in order to temporarily inactivate these VTA subregions. Rats were then re-exposed to the previously heroin-paired environment in a drug free state. Six hours following re-exposure, rats received a subcutaneous injection of lipopolysaccharide (LPS) to induce an immune response. In order to assess CS-induced alterations in immune status, the effects of these manipulations were examined on the expression of the proinflammatory mediators, iNOS and TNF-α, in the spleen and/or plasma.

CHAPTER II: MATERIALS AND METHODS

Animals

Male Lewis rats, weighing 225–250 g, were purchased from Charles River Laboratories (Raleigh, NC, USA). Upon arrival, animals were housed individually in plastic cages 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 for the time spent in the conditioning chambers when food and water were not available. All animals were given a 2-week habituation period before the start of experimental manipulations and were handled regularly during this time. All procedures described were approved by the IACUC of the University of North Carolina at Chapel Hill and conformed to National Institutes of Health (NIH) Guidelines on the Care and Use of Laboratory Animals.

Drug Administration

Heroin (diacetylmorphine) was obtained from NIDA (Bethesda, MD, USA) and dissolved in 0.9% sterile saline. Heroin was administered subcutaneously at a dose of 1 mg/kg. This dose was selected based on prior experiments in our laboratory showing that it induces conditioning and alters LPS-induced iNOS and TNF-α mRNA expression in spleen tissue (Lysle and How 2000; Lysle and Ijames 2002; Szczytkowski and Lysle 2007)

Surgical Procedures

Animals were fully anesthetized with 0.35 mL intramuscular injections of 1:1 (vol:vol) ketamine hydrochloride (100 mg/mL) mixed with xylazine (20 mg/mL) and placed into the stereotaxic apparatus. Animals were implanted bilaterally with 26-gauge guide cannulae (Plastics One, Roanoke, VA, USA). The cannulae were angled at 10° and directed towards the anterior VTA (AP −5.0, ML ± 2.2, DV −6.1 mm, relative to bregma) or posterior VTA (AP −6.0, ML ± 2.05, DV −6.3 mm, relative to bregma). Animals were given a 2-week post-surgical recovery period before the start of conditioning trials.

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 design and cedar bedding to create an environment distinct from that of the home cage and to provide both olfactory and tactile cues for conditioning. Artificial noise machines were used to minimize background noise. All conditioning took place during the dark phase of the light cycle in a room separate from the animal colony and the conditioning chambers were kept dark to minimize effects on circadian rhythms. On each conditioning day, a subcutaneous injection of heroin (1 mg/kg) was administered immediately prior to placement into the chamber for 60-min. Training sessions were separated by 48 h.

Test of Heroin-Conditioned Immunomodulation

Six days following the final conditioning session, animals received bilateral microinfusions of saline vehicle (0.3 μL per hemisphere) or baclofen/muscimol (0.3/0.03 nmol per 0.3 μL per hemisphere) into the anterior or posterior VTA. Injectors extended 3 mm beyond the tip

of the guide cannula. Injections were delivered over 1 min, and the injectors were left 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 the previously heroin-paired conditioning chamber or remained in their home cages (home cage control groups) for 60 min. Heroin was not administered on the test day in order to isolate the effect of the CS on immune responses. After the 60-min time period, all rats received a subcutaneous injection of LPS (1000 μg/kg) and were immediately returned into their home cages. LPS, a component of the cell wall of Gram negative bacteria, was used to induce iNOS and TNF-α production. Six hours after LPS administration, all animals were euthanized. The 6-h time point was selected based on previous research in our laboratory showing maximal iNOS induction 6 h following LPS administration (Lysle and How 2000).

Histology

Samples of spleen and blood were collected for analysis. Spleen samples were either stored in an Ambion® RNA Later solution or Roche complete protease inhibitor cocktail solution. To confirm proper cannula placement, Alcian blue dye was infused via the cannula. Brains were then extracted and post-fixed in a 4% paraformaldehyde solution. Following fixation the brains were transferred to a 30% sucrose solution for cryoprotection and then frozen at − 80 °C until further analysis. Coronal sections (40 μm) were taken and stained with cresyl violet for verification of cannula placement. The data of animals with cannula placement outside of the targeted region were removed from subsequent data analyses.

Real-Time qRT-PCR

To determine iNOS and TNF- α expression, real time RT-PCR was performed on tissue samples from the spleen. Total RNA was extracted from a section of each of the tissues using TRI-Reagent (Molecular Research Center, Cincinnati, OH), a modification of the original method described by Chomczynski and Sacchi (1987). RNA was quantified spectrophotometrically (GeneQuant II, Pharmacia-Biotech, Piscataway, NJ, USA). Reverse transcription was performed using Oligo$(dT)_{18}$ primer and Moloney Murine Leukemia Virus-Reverse transcriptase following the protocol of the Advantage RT-for-PCR Kit from Clontech (Palo Alto, CA, USA). Specific products from the PCR reaction were detected with Universal ProbeLibrary Probes (Roche, Indianapolis, IN). PCR amplifications were performed using standard protocols, the LightCycler TaqMan Master Real-Time PCR Kit, and the LightCycler II instrument (Roche, Indianapolis, IN). A master mix containing all reaction components was prepared and then 20 µl of cDNA with master mix was placed in glass capillary tubes designed for use in the LightCycler II system. Primers and probes for immune parameters were as follows: (iNOS) 5′-TGAGGATTACTTCTTCCAGCTCA-3′ and 5′-TGGGTGTCAGAGTCTTGTGC-3′, using probe #25; (TNF-α) 5'-GGGCCTCCAGAACTCCAG-3' and 5'-GAGCCATTTGGGAACTTCT-3', using probe #98. Primers were synthesized by the Nucleic Acids Core Facility (Lineberger Cancer Center, UNC-Chapel Hill). Copy numbers were generated based on an internal standard curve. Amplifications were carried out for 45 cycles and curves showing fluorescence at each cycle were determined by the computer software (Roche). Samples were pre-incubated for 10 min at 95^{o} C to activate the Fast-Start Taq DNA polymerase. The cycle temperatures were 95 and 60 °C and the cycle times were 10 and 30 s for the denaturing and annealing/extending, respectively. Fluorescence level was determined at the end of the

extending phase for each cycle of PCR. A final cooling phase was carried out at 40° C for 30 s. The analysis of the fluorescence in standards and samples over the course of 45 cycles was used to derive the number of copies of the target molecule in each sample. Additionally, assessments of housekeeping gene expression, L13A, were made to verify comparable RNA quality of among samples. L13A primers were 5′-CCCTCCACCCTATGACAAGA-3′ and 5′-GGTACTTCCACCCGACCTC-3′, using probe #74. The data are expressed as copy

Nitrite/Nitrate Assay

The nitrite/nitrate 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 dH2O, and the sample was incubated in the dark for 90 min with 10 µl of nitrate reductase (1.0 U/ml), 20 µl of 0.31M phosphate buffer (pH 7.5), 10 µl of 0.86mM NADPH (Sigma), and 10 µl of 0.11mM flavin adenine dinucleotide in individual wells of a 96-well plate. Next, 200 ml of Greiss reagent, consisting of a 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, after which, the absorbance was determined using a spectrophotometer set at 550 nm. All reactions were carried out in triplicate. The total micromolar concentration of nitrite was determined for each sample based on a standard curve. Recovery of nitrate is greater than 95% using this assay.

ELISA

To assess TNF-α protein expression, protein was extracted from a section of each homogenized tissue using freeze/thaw lysis in tris-buffer containing antiproteinases. Protein was quantified spectrophotometrically (Bio-Tek, Model EL312 kinetic reader, Winooski, VT, USA) using Bio-Rad protein dye. To account for variability in tissue size, samples were

normalized per unit protein based on the results of the spectrophotometric analysis. Invitrogen (Carlsbad, CA) rat TNF-α ELISA test kits were used to determine the levels of TNF-α protein in each tissue sample. Briefly, samples and standards were added to microtiter wells coated with TNF-α antibody and incubated at room temperature for how long. Wells were washed extensively then incubated with biotinylated antibody. After a second wash, the wells were incubated with Streptavidin-HRP and exposed to a chromagen substrate solution for 30 min. Absorbance at 450 nm was measured with a Bio-Tek (Winooski, VT) Model EL312 kinetic reader. A standard curve was obtained by plotting the absorbance against the concentrations of supplied standards.

Statistical Analysis

Two-way analysis of variance was performed on all data sets. Significant effects were followed-up using Tukey's *post hoc* tests. All analyses were conducted with alpha set at $p < 0.05$. Only significant interactions and main effects are reported.

CHAPTER III: RESULTS

Anterior VTA Inactivation

Figure 1A shows the effect of anterior VTA inactivation on LPS-induced heroin-conditioned iNOS mRNA expression in spleen tissue following exposure to the heroin-paired context or the home cage. The ANOVA of iNOS mRNA copy numbers revealed a significant context by treatment interaction effect [$F(1,16) = 14.882$, $P < 0.005$] and a significant main effect of context [$F(1,16) = 5.975$, $P < 0.05$]. Tukey's post-hoc analyses revealed that saline-treated rats exposed to the heroin-paired context exhibited a reduction in iNOS mRNA expression compared to that in the home cage ($p = 0.001$). Conversely, rats that received intra-VTA microinjections of baclofen/muscimol failed to exhibit differences in iNOS mRNA expression after exposure to the previously heroin-paired environment or the home cage. There were also no differences across groups in housekeeping gene expression (data not shown).

Figure 1B shows the effect of anterior VTA inactivation on LPS-induced plasma nitrate/nitrite levels. The ANOVA of nitrate/nitrite levels revealed a significant context by treatment interaction effect [$F(1,16) = 5.831$, $P < 0.05$]. Tukey's post-hoc analyses revealed that saline-treated rats exposed to the heroin-paired context exhibited a reduction in iNOS mRNA expression compared to that in the home cage ($p = .032$). Conversely, rats that received intra-VTA microinjections of baclofen/muscimol failed to exhibit differences in

iNOS mRNA expression after exposure to the two contexts and compared to the saline-treated home cage control group.

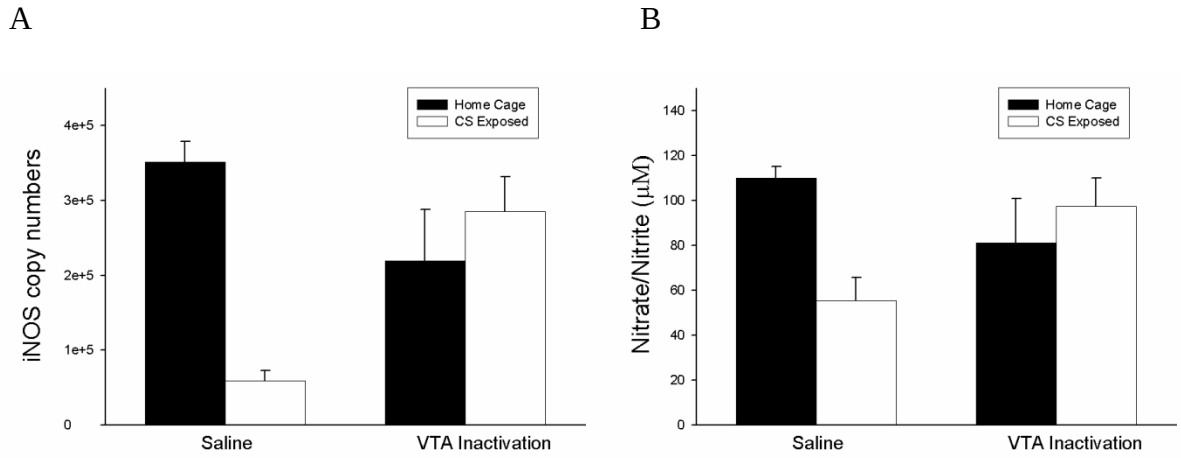

Figure 1: (A) Effects of treatment on LPS-induced expression of iNOS mRNA as determined by real-time RT-PCR. The data are expressed as iNOS copy number per 10ng cDNA based on a standard curve using Roche LightCycler software. (B) Effects of treatment on LPS-induced expression of nitrate/nitrite serum levels as determined by Greiss Reagent Assay. The data are expressed as the mean micromolar concentration of nitrite/nitrate. The error bars represent the standard error of the mean.

Figure 2 shows the effect of anterior VTA inactivation on LPS-induced TNF-α mRNA and protein expression. The ANOVA found a significant context by treatment interaction effect on TNF-α mRNA expression [$F(1,16) = 6.464$ $P < 0.05$] and protein levels [$F(1,15) = 4.578$, $P < 0.05$], as well as a significant main effect of exposure to the context [$F(1,16) = 8.585$, $P < 0.05$] on protein levels. Tukey's post-hoc analyses revealed that saline-treated rats exposed to the heroin-paired context exhibited a reduction in TNF-α mRNA expression ($p = 0.037$) and protein levels ($p = 0.007$) compared to animals re-exposed to home cages. Animals that underwent anterior VTA inactivation prior to CS re-exposure showed no significant difference in LPS-induced TNF-α mRNA expression or protein levels compared to anterior VTA inactivation controls. No differences in housekeeping gene expression were observed across groups (data not shown).

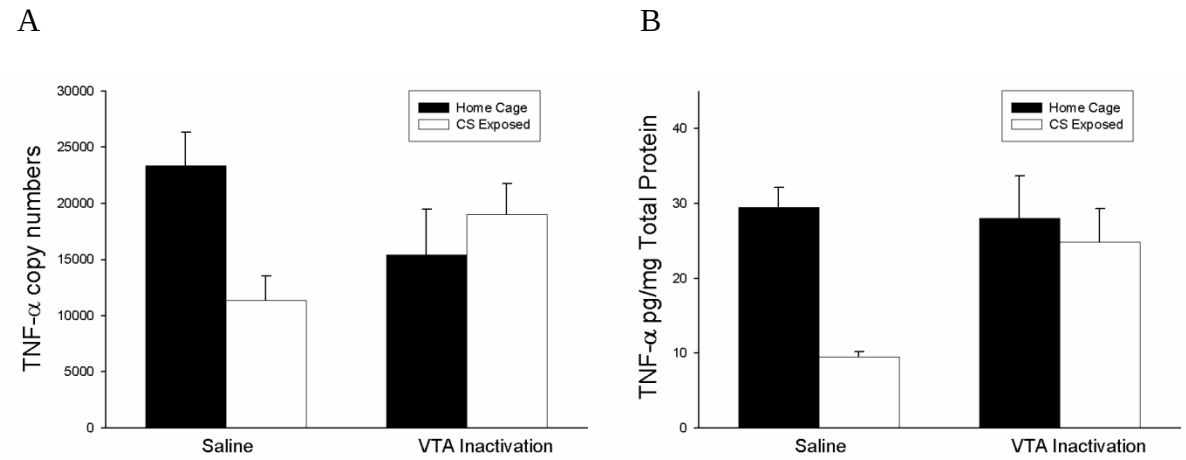

Figure 2: (A) Effects of treatment on LPS-induced expression of TNF-α mRNA determined by real-time RT-PCR. The data are expressed as TNF-α copy number per 10ng cDNA based on a standard curve using Roche LightCycler software. (B) Effects of treatment on LPS-induced production of TNF-α protein determined by ELISA. The data are expressed as picograms of protein per ml. The error bars represent the standard error of the mean.

Posterior VTA Inactivation

Figure 3A shows the effect of posterior VTA inactivation on LPS-induced iNOS mRNA expression. Analysis revealed no significant context by treatment interaction in the posterior VTA; however, the ANOVA revealed a significant main effect of re-exposure to the CS on iNOS mRNA expression [$F(1,15) = 20.327$, $P < 0.0001$].

Figure 3B shows the effect of posterior VTA inactivation on LPS-induced levels of nitrate/nitrite in blood plasma. The ANOVA revealed a significant main effect of re-exposure to the CS on nitrate/nitrite plasma levels [$F(1,15) = 7.284$, $P < 0.05$] and no significant context by treatment interaction.

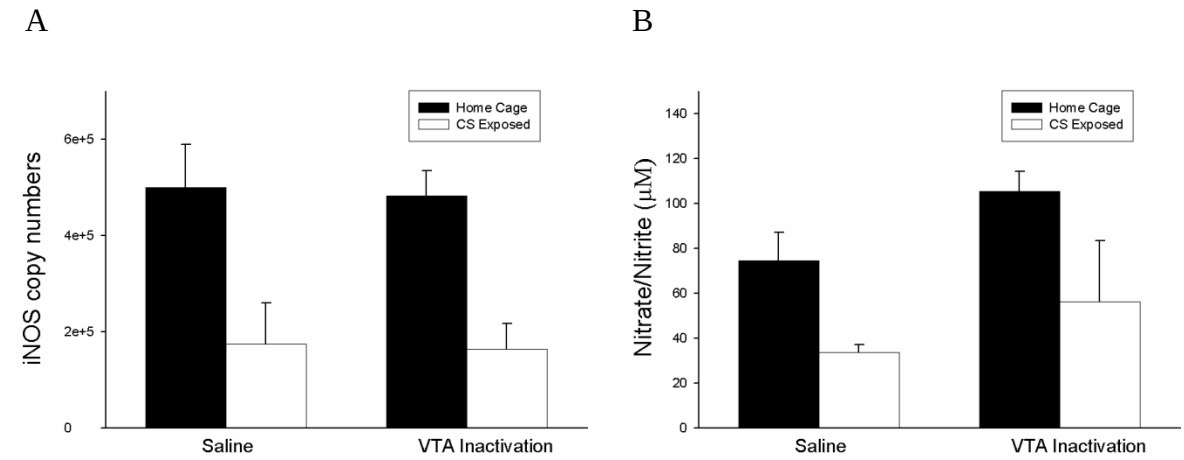

Figure 3: (A) Effects of treatment on LPS-induced expression of iNOS mRNA as determined by real-time RT-PCR. The data are expressed as iNOS copy number per 10ng cDNA based on a standard curve using Roche LightCycler software. (B) Effects of treatment on LPS-induced expression of nitrate serum levels as determined by Greiss Reagent Assay. The data are expressed as the mean micromolar concentration of nitrite/nitrate. The error bars represent the standard error of the mean.

Figure 4A shows the effect of posterior VTA inactivation on LPS-induced TNF-α mRNA expression. The ANOVA of TNF-α mRNA expression revealed a nearly significant main effect of re-exposure to the CS [$F(1,14) = 4.224$, $P = 0.059$]. Most notably, re-exposure to the heroin-paired CS following inactivation of the posterior VTA does not attenuate the immunosuppressive effect of the CS on TNF-α mRNA levels ($p < 0.05$).

Figure 4B shows the effect of posterior VTA inactivation on LPS-induced TNF-α protein levels. The ANOVA of TNF-α protein levels revealed a significant main effect of re-exposure to the CS [$F(1,15) = 14.682$, $P < 0.005$] and drug treatment [$F(1,15) = 10.126$, $P < 0.01$], and there was no significant context by treatment interaction.

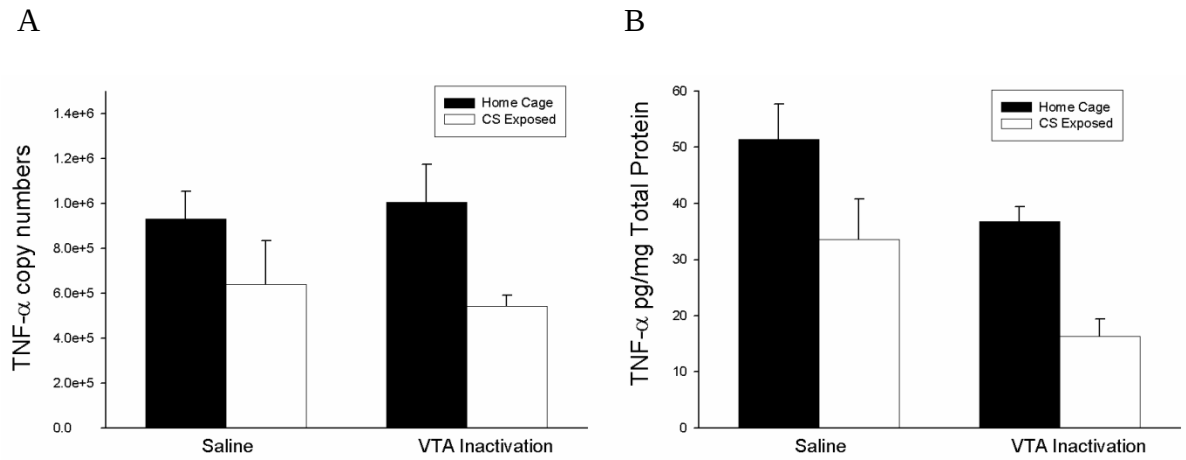

Figure 4: (A) Effects of treatment on LPS-induced expression of TNF-α mRNA determined by real-time RT-PCR. The data are expressed as TNF-α copy number per 10ng cDNA based on a standard curve using Roche LightCycler software. (B) Effects of treatment on LPS-induced production of TNF-α protein determined by ELISA. The data are expressed as pictograms of protein per ml. The error bars represent the standard error of the mean.

CHAPTER IV: DISCUSSION

In the present study, neural activity in the anterior or posterior VTA was inhibited using GABA agonists prior to re-exposure to a heroin-paired environmental context in order to determine if subregions of the VTA play differential roles in the expression of heroin-conditioned immune alterations. Anterior VTA neuronal inactivation disrupted the immunosuppressive effects of the heroin-paired context on LPS-induced TNF-α and iNOS expression. This suggests that the functional integrity of the anterior VTA is necessary for the expression of heroin-induced conditioned immune suppression of proinflammatory mediators. In contrast, posterior VTA inactivation failed to alter heroin-paired context-induced alterations of proinflammatory mediators, suggesting that the posterior VTA does not play a critical role in this phenomenon under the current experimental conditions. These findings expand our understanding of the neural circuitry involved in heroin-conditioned immunomodulation by identifying a source of dopamine that alters the activity of several other brain regions within this circuitry.

Previous research in our laboratory suggests that dopamine regulates multiple elements of the neural circuitry underlying conditioned immunomodulation. In support of this, inactivation of the BLA and blockade of dopamine D_1-like receptors within the BLA prevents the suppressive effect of the heroin-associated context on iNOS induction and on the expression of TNF-α and IL-1β in spleen and liver tissue (Szczytkowski and Lysle 2008;

Szczytkowski and Lysle 2010). Antagonism of dopamine D_1-like receptors in the NAc shell blocks the expression of morphine-induced conditioned immune alterations (Saurer, James et al. 2008). Given that opioid-conditioned immunosuppression is a drug-like conditioned response, D_1-like receptor stimulation in the NAc shell may also be required for immunosuppression in a morphine- or heroin-paired context. According to a recent functional disconnection study from our laboratory (Szczytkowski, Fuchs et al. 2011), the NAc plays a critical role in heron-conditioned immunomodulation as an element of a larger VTA-BLA-NAc circuit. In that study, rats received unilateral administration of the D_1-like dopamine antagonist, SCH23390, in the BLA in combination with unilateral administration of the NMDA or AMPA/kinate antagonist, into the ipsilateral or contralateral NAc on test day. Contralateral manipulation (i.e., disconnection) of the VTA-BLA-NAc circuit inhibited the heroin-conditioned immunosuppression of iNOS and TNF-α in the spleen. Conversely, the ipsilateral manipulation, which left intrahemispheric information processing between the BLA and NAc intact in one hemisphere, failed to alter proinflammatory responses. Given that the VTA is one of the primary sources of dopamine to the BLA (Rosenkranz and Grace 1999), these findings suggest that serial information processing via a VTA-BLA-NAc circuit is necessary for the expression of heroin-conditioned immunomodulation.

Remarkably, the effects of VTA inactivation on conditioned immunomodulation depended on the subregion of the VTA manipulated in the present study, consistent with studies demonstrating similar functional differences between subregions of the VTA (Lee, Guttilla et al. 2007). For example, rats self-administer the $GABA_A$ receptor antagonist picrotoxin into the anterior, but not the posterior, VTA (Ikemoto, Murphy et al. 1997). This effect may be mediated by the disinhibition of dopaminergic neurons in the anterior VTA and

subsequent dopamine release in the NAc because picrotoxin administration into the anterior VTA increases dopamine overflow in the NAc (Ikemoto, Kohl et al. 1997). Furthermore, microinfusions of the $GABA_A$ agonist muscimol into the anterior, but not posterior VTA, significantly reduced cocaine self-administration.

Functional differences between anterior and posterior VTA subregions likely reflect differences in neurochemistry and connectivity between these VTA subregions (Johnson & North 1992; Swanson, 1982). For example, the anterior and posterior VTA contain different proportions of dopaminergic and glutamatergic neurons and GABAergic interneurons, with a higher proportion of GABAergic interneurons and dopaminergic neurons located in the anterior VTA and posterior VTA, respectively (Olson, Zabetian et al. 2005; Yamaguchi, Sheen et al. 2007). Interestingly, VTA efferents to the BLA are localized in the anterior VTA, which was verified using retrograde tracing techniques (Ford, Mark et al. 2006; Ikemoto 2007). Conversely, dopamine neurons from the posterior VTA project to NAc core and medial shell, with a significant amount of overlap in projections between the anterior and posterior VTA. Given the previously discussed importance of dopamine in the BLA in heroin-conditioned immunosuppression, we hypothesized that the anterior, but not posterior, VTA would be important in the expression of heroin-conditioned immunomodulation. The results of the present study support this hypothesis. Our findings also suggest that projections from the posterior VTA, which include projections to the NAc shell and core, are not critical for the expression of the observed heroin-conditioned suppression of proinflammatory mediators. On the other hand, dopaminergic signaling to the NAc originating from the anterior VTA may play a role in heroin-conditioned immunosuppression. Importantly, interactions between the VTA, NAc, and BLA appear to be complex, as dopamine release in

the NAc is proposed to be regulated by BLA glutamatergic efferents (Jones, Day et al. 2010). Consistent with this, temporary neural inactivation of the BLA results in decreased discriminative stimulus-induced evoked NAc dopamine release. In addition, BLA inactivation has no effect on NAc dopamine release following VTA stimulation, indicating that the BLA does not modulate dopamine release indirectly through the VTA (Howland, Taepavarapruk et al. 2002). Taken together, these previous findings suggest that dopaminergic signaling from the anterior VTA may result in dopamine release at the terminal level in the NAc. Future studies will need to test this hypothesis by examining the effects of dopamine antagonism in the NAc on conditioned immunosuppression.

The present study enhances our understanding of the neural circuit involved in the conditioned effects of heroin on immune function by demonstrating that the anterior VTA is necessary for the heroin-conditioned suppression of iNOS induction and expression of TNF-α in spleen tissue. Previous investigations into the neural circuitry involved in associative learning have conclusively revealed the importance of the BLA in the expression of conditioned responses. However, results previously discussed indicating that the amygdala is not involved in the expression of cyclosporine A-conditioned immunosuppression of lymphocyte proliferation, IL-2, and interferon-γ conflict with well-established evidence supporting the involvement of the amygdala in conditioned responding. Methodological differences may explain the inconsistent results. It has been suggested that the route of administration of a CS may have an effect on the outcome of the experimental manipulation. For example, in one study investigators sought to determine if either passive intraoral administration of saccharin or voluntary saccharin administration via a bottle followed by the pairing of LiCl affected the expression of CTA. Results of the study indicated that when

conditioning is performed using a bottle, the amygdala is not involved. However, when experimenters conditioned animals using intraoral administration of the CS, the amygdala was found to be required for CTA learning (Schafe, Thiele et al. 1998). It was suggested that the lack of continuity with previous findings may be due to intraoral and bottle administration of the CS facilitating different types of learning, Pavlovian and instrumental, which activate different neural pathways. This could possibly explain the study by Pacheco-Lopez and colleagues (2005) suggesting that the amygdala isn't involved in the expression of conditioned immunomodulation, as they used the bottle self-administration method to deliver the CS. It is noteworthy that, of the two administration methods, intraoral administration is most analogous to the contextual conditioning in the current study insofar that there is no response requirement in either procedure, and the findings agree with previous research in our laboratory regarding the involvement of the amygdala in heroin-conditioned immunomodulation. Furthermore, Lopez and colleagues used an immunosuppressive drug as the unconditioned stimulus compared to the use of heroin in the current study. It is known that heroin readily passes through the blood-brain barrier and activates pathways throughout the brain; however, cyclosporine A does not cross the blood-brain barrier. Therefore, the results cannot be directly compared to the current study due to the use of an immunosuppressive drug as a CS that is limited to the peripheral nervous system compared to the use of centrally and peripherally acting opiate as a CS.

Future studies will examine the contribution of dopamine in brain regions involved in context-reward associations, such as the hippocampus. We have shown that inactivation or inhibition of IL-1β cytokine expression in the dorsal hippocampus (DH) prevents heroin-conditioned immunosuppression of LPS-induced nitric oxide production (Szczytkowski,

Lebonville et al. 2013), and it's possible that the interactions between the DH and the VTA are bidirectional. In support of this, D_1-like dopamine receptor stimulation in the DH is required for the drug context-induced reinstatement of cocaine-seeking behavior (Xie and Fuchs, in prep). Furthermore, a bi-synaptic functional circuit conveys information from the CA3 region of the dorsal hippocampus to the anterior VTA (Luo, Tahsili-Fahadan et al. 2011) via the lateral septum (LS). As a result, GABA agonist-induced inactivation of the LS attenuates excitatory and inhibitory neuronal firing in the VTA following CA3 stimulation. In addition, inactivation of the CA3, as well as the LS, impairs drug context-induced reinstatement of cocaine-seeking behavior. Luo and colleagues propose that activation of CA3 glutamatergic pyramidal neurons results in the disinhibition of VTA dopamine neurons via this circuit, thus contributing to the expression of context-reward associations.

Based on our prior work, it is evident that immune alterations that result from exposure to drug-associated cues are complex, involving not only the central nervous system but the neuroendocrine system and the sympathetic branch of the autonomic nervous system. Further efforts must be made to elucidate the neural mechanisms that control these effects in order to adequately understand the health consequences associated with opiate use and properly protect and treat susceptible populations.

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