2007

# Conditioned effects of heroin on nitric oxide expression are susceptible to extinction and latent inhibition

Jennifer L. Szczytkowski-Thomson
*Messiah University*, jthomson@messiah.edu

Donald Lysle

**Conditioned effects of heroin on the expression of inducible nitric oxide synthase in the rat are susceptible to extinction and latent inhibition**

Abstract

Rationale

The administration of heroin has been shown to inhibit the induction of nitric oxide, a molecule known to play a critical role in immune function. Previous research has shown that this alteration can be conditioned to environmental stimuli that have been associated with drug administration. However, it remains unknown whether the conditioned effects of heroin on nitric oxide formation follow accepted principles of learning.

Objective

This study sought to determine whether manipulations that induce extinction and latent inhibition, two learning paradigms known to reduce the expression of conditioned responses, would alter heroin's conditioned effects on the expression of inducible nitric oxide synthase (iNOS).

Materials and methods

The conditioning procedure involved repeated pairing of heroin administration with placement into a standard conditioning chamber. Rats were repeatedly exposed to the chambers without heroin reinforcement to determine whether the conditioned response would extinguish. To induce latent inhibition, rats received repeated exposure to the chamber before the start of conditioning to inhibit the acquisition of the conditioned response. Ten days after the final

conditioning session, all rats were injected with lipopolysaccharide (LPS) to induce iNOS expression. Spleen and liver tissue were removed to determine iNOS expression using reverse transcriptase polymerase chain reaction. Blood was collected to determine the concentration of nitrite/nitrate.

Results

The results showed that both extinction and latent inhibition reduced the conditioned effects of heroin on the production of nitric oxide.

Conclusion

This study provides the first evidence that the conditioned effects of heroin on nitric oxide production follow accepted principles of learning.

Introduction

The high prevalence of opportunistic infections among heroin users has long suggested that heroin use may alter resistance to infectious disease (Luttgens 1949; Hussey and Katz 1950; Louria et al. 1967; Garten et al. 2004). Although there has been speculation that this high rate of infection may be due to non-sterile intravenous administration of drug, there is increasing evidence suggesting that opiates directly alter immune status (Brown et al. 1974; McDonough et al. 1980; Nair et al. 1986; Donahoe et al. 1986; Novick et al. 1989; Oschorn et al. 1990; Govitrapong et al. 1998; Sharp et al. 2001). Studies in our laboratory have shown that heroin induces alterations in a number of immunological parameters including alterations in natural killer cell activity, T- and B-lymphocyte proliferation, and nitric oxide production (Fecho et al.

2000; Lysle and How 2000). The reduction in nitric oxide production induced by heroin administration is dose-dependent and appears to be mediated through opioid receptors, as these alterations are reversed upon administration of the opioid antagonist, naltrexone (Lysle and How 2000). The production of nitric oxide by immune cells, particularly macrophages, provides a substantial degree of microbial resistance (Green et al. 1990; Nathan and Hibbs 1991; Vincendeau et al. 1992; Rossi et al. 1999). Mice lacking the gene for inducible nitric oxide synthase show an increased susceptibility to parasitic and bacterial infections (MacMicking et al. 1995; Wei et al. 1995; Lindgren et al. 2004). The production of large quantities of nitric oxide by macrophages also provides resistance to viral infections and displays tumor cytotoxicity (Karupiah et al. 1993; Chang et al. 2003; Hrabak et al. 2006). In addition to its role as an anti-microbial agent, nitric oxide also serves many immunoregulatory functions. There is evidence that nitric oxide suppresses the formation of antibodies to tetanus toxoid and sheep red blood cells after Salmonella typhimurium immunization (Al-Ramadi et al. 1992; Eisenstein et al. 1994). Numerous studies have also shown an anti-proliferative effect of nitric oxide on lymphocytes (Albina and Henry 1991). Thus, nitric oxide plays a critical role in immune processes and may be involved in the altered susceptibility to infection evident in heroin users.

Although it is now apparent that heroin impairs immune status, there has been limited research on how conditioned drug cues may also induce changes in immune function. Classical or Pavlovian conditioning is a well-characterized learning phenomenon in which a formerly neutral stimulus [the conditioned stimulus (CS), such the conditioning chamber] will begin to elicit a response [the conditioned response (CR)] after repeated pairing with a biologically relevant stimulus [the unconditioned stimulus (US), such as heroin] that had elicited a response upon its

first presentation (Pavlov 1927). Several investigators have shown that many of the physiological and behavioral responses to drugs of abuse may be conditioned to previously drug-paired stimuli. For example, environmental stimuli that had previously been paired with morphine administration can elicit such morphine-like effects as hyperthermia when presented in the absence of morphine (Miksic et al. 1975; Eikelboom and Stewart 1979; Schwarz and Cunningham 1990). In line with these studies, our laboratory has recently provided new data indicating that heroin's effects on nitric oxide expression may be conditioned to environmental stimuli. In those investigations, rats received subcutaneous injections of heroin (1 mg/kg) upon placement into a distinctive environment which served as the CS. When rats were subsequently re-exposed to the environment without further heroin administration, the production of nitric oxide was suppressed similar to that seen with heroin administration alone (Lysle and Ijames 2002). These data provided the first evidence that heroin-induced alterations in nitric oxide expression may be conditioned to environmental stimuli.

The development and persistence of the CR is dependent on several factors, and certain experimental manipulations may produce a weakening or inhibition of this response. Two of the most widely studied experimental paradigms leading to reduced expression of the CR are extinction and latent inhibition. Extinction is evident when after conditioning has taken place, repeated exposure to the CS without the US decreases the CR. Latent inhibition is a process by which repeated non-reinforced exposure to a stimulus before conditioning will inhibit the formation of a CR to that stimulus (Lubow and Moore 1959). Extinction and latent inhibition have been studied extensively within models of conditioning to test hypotheses concerning retroactive and proactive stimulus interference, respectively (Pineno and Miller 2005). The

susceptibility of conditioned, heroin-induced immune alterations to the effects of extinction and latent inhibition demonstrate that this conditioning paradigm is a true form of associative learning and adheres to accepted principles of learning. Furthermore, given the important health consequences that may result from conditioned immune alterations, the assessment will identify procedures that influence the CR.

Given this information, it was hypothesized that both pre- and post-exposure to the drug-paired environment (i.e., the CS) should reduce the conditioned effects of heroin on nitric oxide production. To test this hypothesis, the present study evaluated the effects of two behavioral manipulations, pre- and post-exposure to the CS, on the conditioned suppression of inducible nitric oxide synthase (iNOS). Previous research in our laboratory has shown that heroin induces a dose-dependent reduction in lipopolysaccharide (LPS)-induced iNOS mRNA expression in the spleen and liver. The findings reported here are important because they indicate that previously heroin-associated environmental stimuli are not only capable of inducing alterations in nitric oxide expression but that these effects may be modified by manipulations of the relationship between the environment and drug delivery. These results further implicate a role for associative learning processes in the conditioned effects of heroin on nitric oxide and suggest that these effects may be mediated, at least in part, by centrally located neural substrates of learning.

## 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. The animals were allowed access to food and water ad libitum throughout the experiment. All animals were given a 2-week habituation period before the start of experimental manipulations and were handled regularly during this time. During the course of the experiment, all animals were removed from their cages and weighed on a daily basis. 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". In all experiments, n = 4 rats per group.

Drug administration

Heroin (diacetylmorphine) was obtained from NIDA (Bethesda, MD) and dissolved in 0.9% sterile saline. Animals received a subcutaneous injection of heroin at 1 mg/kg immediately before placement in the conditioning chamber on each of the five conditioning trial days. This dose was selected based on prior experiments in our laboratory showing that a 1 mg/kg dose of heroin alters LPS-induced iNOS mRNA expression and induces conditioning (Lysle and How 2000; Lysle and Ijames 2002). A subsequent study used 3.0 or 0.3 mg/kg heroin doses. The 0.3 and 3.0 mg/kg doses were included to investigate the effect of altering the dose of heroin administered during conditioning on the expression of extinction and latent inhibition. This study also included a control group that was injected with saline-vehicle during conditioning.

Conditioning procedures

To condition heroin's effects on iNOS expression, all animals received five 60-min training sessions in which they received a subcutaneous injection of heroin upon placement into a

standard conditioning chamber. Training sessions were separated by 48 h. The conditioning chambers (BRS/LVE, Laurel, MD, USA) were contained in a room separate from the animal colony and contained a metal grid floor design and cedar bedding to create an environment distinct from that of the home cage. All conditioning took place during the dark phase of the light cycle, and the conditioning chambers were kept dark. The test day took place 10 days after the final conditioning session. Animals were placed back into the conditioning chambers without administration of heroin. After 60 min, the animals were removed from the chambers and given a subcutaneous injection of LPS (1,000 μg/kg) to induce iNOS production. Six hours after LPS administration, all animals were killed, and samples of spleen, liver, and blood were collected for analysis. The 6-h time point was selected based on previous research in our laboratory showing maximal iNOS induction at 6 h after LPS administration (Lysle and How 2000).

Extinction

This study tests whether post-conditioning exposure to a heroin-paired environment without further drug administration would attenuate the conditioned effects of heroin on nitric oxide production. In this experiment, rats were assigned to one of five groups. A schematic representation of these treatment groups is shown in Table 1. Four of the groups underwent conditioning, during which, rats received an injection of heroin (1 mg/kg) upon placement into the conditioning chamber. Two of the conditioned groups [extinction (Ext) and extinction/control (Ext/Ctl)] were then subjected to the extinction procedure, whereas the other two groups remained in their home cages [conditioned (Cond) and conditioned/control (Cond/Ctl)]. The extinction procedure consisted of ten consecutive days of exposure to the conditioning chambers for 1 h a day without administration of heroin. The test day took place on the day after the

completion of the extinction procedure just described. On the test day, two groups [one group that had received extinction (Ext) and one group that had not (Cond)] were re-exposed to the conditioning chambers, whereas the other groups remained in their home cages. Re-exposed animals were placed in the chambers for 1 h without heroin and received an injection of LPS (1,000 μg/kg) upon removal. All remaining animals also received an LPS injection at this time. Extinction control (Ext/Ctl) and conditioning control (Cond/Ctl) groups were used to control for any ancillary effects of the extinction and conditioning procedures, respectively. These groups underwent the conditioning protocol but were not re-exposed to the chamber on test day. The fifth group (HC) received no conditioning and remained in the home cage throughout the duration of the experiment, serving as a general control.

Latent Inhibition

This study tests whether pre-exposure to the conditioning chamber would create a latent inhibition of the conditioned effects of heroin on iNOS expression. As with the previous experiment, rats in this experiment were assigned to one of five groups. A schematic representation of the treatment groups is shown in Table 1. Four of the groups underwent conditioning, during which, rats received an injection of heroin (1 mg/kg) upon placement into the conditioning chamber. Two of the groups that would receive conditioning [latent inhibition (LI) and latent inhibition/control (LI/Ctl)] underwent the latent inhibition procedure before the start of conditioning, whereas the other two groups remained in the home cages. The latent inhibition procedure consisted of ten consecutive days of exposure to the conditioning chambers before the first conditioning session. These pre-exposures lasted for 1 h, and no drug was given at this time. On the day after the final pre-exposure, the rats were subjected to the conditioning

procedures described above. On the test day, two groups [one that had undergone pre-exposure (LI) and one group that had not (Cond)] were re-exposed to the conditioning chambers, whereas the other animals remained in the home cage. Re-exposed animals received an injection of LPS (1,000 μg/kg) upon removal from the chambers. All remaining animals also received an LPS injection at this time. Latent inhibition control (LI/Ctl) and conditioning control (Cond/Ctl) groups were used to control for any ancillary effects of the pre-exposure and conditioning procedures, respectively. These groups underwent the conditioning protocol, but were not re-exposed to the chamber on test day. A fifth group (HC) received no conditioning and remained in the home cage throughout the duration of the experiment, serving as a general control.

Dose dependency

In this set of experiments, we sought to determine the effects that altering the dose of heroin given during conditioning might have on the expression of extinction or latent inhibition. During the conditioning phase of the experiment, animals received five sessions in which an injection of 0, 0.3, or 3.0 mg/kg heroin was given upon placement into the conditioning chambers. For each experiment, rats were assigned to one of six groups. For the extinction experiment, one group of rats from each of the three conditioning doses of heroin was subjected to the extinction procedure. After the last conditioning session, the extinction groups were given ten consecutive days of 1 h exposures to the conditioning chambers. For the latent inhibition experiment, one group of rats from each of the three conditioning doses of heroin was subjected to the latent inhibition procedure. For ten consecutive days before the first conditioning session, the latent inhibition groups were pre-exposed to the conditioning chambers for 1 h each day. There was no drug given during the extinction or the latent inhibition sessions. On test day, all animals were

re-exposed to the conditioning chambers. Upon removal from the chambers, all animals received an injection of LPS (1,000 μg/kg).

Real-Time RT-PCR

To determine iNOS expression, real-time reverse transcriptase polymerase chain reaction (RT-PCR) was performed on tissue samples from the spleen and liver. 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). For the RT-PCR, reverse transcription is 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).

PCR amplifications were performed using the Fast Start™ DNA Master SYBR Green I real-time PCR kit (Roche) and the LightCycler instrument (Roche). A master mix containing all reaction components was prepared for all reactions, with each reaction using a 20-ml mix placed in glass capillary tubes specifically designed for use in the LightCycler system. The PCR primer set for iNOS, 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′ and 5′-GGGTGTCAGA-GTCTTGTGCCTTTGG-3′ was synthesized by the Nucleic Acids Core Facility (Lineberger Cancer Center, UNC-Chapel Hill). Copy numbers were generated from an external standard curve. Amplifications were carried out for 40 cycles, and curves showing fluorescence at each cycle were determined by the computer software (Roche). Samples were pre-incubated for 10 min at 95°C to activate the Fast-Start Taq DNA polymerase. The cycle temperatures were 95, 60,

and 72°C for the denaturing, annealing, and extending, respectively. The cycle times were 15, 5, and 25 s for the denaturing, annealing, and extending, respectively. Fluorescence level was determined at the end of the extending phase for each cycle of PCR. The analysis of the fluorescence level in standards and samples over the course of 40 cycles was used to derive the number of copies of the target molecule in each sample. Additionally, assessments of housekeeping gene expression, cyclophilin, were made to assure comparable quality of RNA among samples. The sequence of the cyclophilin primers was 5′-CCAAGACTGAGTGGCT-3′ and 5′-AGATTACAGGGTATTGCG-3′. The data are expressed as a copy number of iNOS (per 10 ng cDNA) based on the standard curve using the Lightcycler software (Roche).

Furthermore, to confirm the nature of amplification product, a melt curve analysis was conducted after the final PCR cycle. This analysis involved denaturing the products by slowly heating them to 95°C, during which, fluorescence is continuously measured.

Nitrite/nitrate assay

The level of nitrite/nitrate in plasma samples was assessed using the Greiss reagent assay. Nitrate and nitrite are formed non-enzymatically when nitric oxide is exposed to oxygen, thus, plasma levels of these products indicate the level of nitric oxide production. Total nitrite/nitrate levels is determined by the conversion of nitrate to nitrite, utilizing nitrate reductase in the presence of NADPH and flavin adenine dinucleotide, and then an assessment using Greiss reagent. Briefly, 6 µl of plasma diluted in 44 µl of dH2O is incubated in the dark for 90 min with 10 µl of nitrate reductase (1.0 U/ml), 20 µl of a 0.31 M phosphate buffer (pH 7.5), 10 µl of 0.86 mM NADPH (Sigma), and 10 µl of a 0.11 mM flavin adenine dinucleotide in individual wells of a 96-well

plate. Then, 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 developed 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 is determined for each sample based on a standard curve. Recovery of nitrate is greater than 95% using this assay.

### Statistical analysis

The data from the initial extinction and latent inhibition studies were analyzed by one-way analysis of variance (ANOVA) followed by planned comparisons to compare all groups to the unmanipulated control. The data from the heroin dose studies were analyzed by two-way ANOVA, with the first factor set as pre- or post- exposure and the second factor as dose. For the extinction experiment, planned comparisons of the extinction group to the conditioned group were conducted at each of the three heroin doses. For the latent inhibition experiment, planned comparisons of the latent inhibition group to the conditioned group were conducted at each of the three heroin doses. All analyses were conducted with the level of significance set at $P < 0.05$.

## Results

### Effects of extinction on conditioned heroin-induced immune alterations

This study tests whether post-conditioning exposure to a heroin-paired environment without further drug administration would attenuate the conditioned effects of heroin on nitric oxide production.

Figure 1 shows the effects of the extinction procedure on LPS-induced expression of iNOS mRNA in the spleen and liver. Analysis of iNOS copy number in spleen and liver tissue revealed a significant main effect of procedure [$F(4,15) = 3.60$, $P < 0.05$; $F(4,15) = 6.96$, $P < 0.01$]. Moreover, there was a significant reduction in iNOS copy numbers for the conditioned (Cond) group as compared to the home cage (HC) group in both the spleen and liver tissue [$F(1,15) = 12.04$, $P < 0.01$; $F(1,15) = 10.18$, $P < 0.01$)]. These data are consistent with our earlier findings indicating that exposure to a previously heroin-paired environment reduces the expression of iNOS mRNA. Most importantly, there were no significant differences between the HC group and the extinction (Ext) group demonstrating that repeated exposure to the drug-paired environment after conditioning reduces the conditioned response. Furthermore, there were no significant differences between the HC group and any of the control groups indicating that these results are specific to the behavioral manipulations and not due to ancillary effects of the conditioning or extinction procedures.

As shown in Table 2, there was also no effect of group on the expression of the housekeeping gene, cyclophilin. This indicates that the alterations in mRNA were specific for iNOS and not the result of an overall reduction in RNA expression or processing.

The data in Fig. 2 show the effects of each procedure on serum nitrite/nitrate levels. The ANOVA revealed a significant main effect of procedure [$F(4,15) = 7.83$, $P < 0.01$] on the levels of nitrite/nitrate in the serum. Planned comparisons showed a significant difference between the HC group and the Cond group [$F(1,15) = 24.21$, $P < 0.001$), further supporting our earlier findings that exposure to a previously heroin-paired environment lowers the levels of nitrite/nitrate in the serum, indicating a reduction in nitric oxide production. There were no significant differences between the HC group and the Ext group indicating that the extinction

procedure attenuated the conditioned response. There were also no differences between the HC group and any of the control groups indicating that the results are specific to the extinction and conditioning procedures. The levels of serum nitrate reported here may result from a variety of sources the individual contributions of which are not distinguished. Whereas iNOS is one source of serum nitrate, there are several other factors that may contribute, including epithelial nitric oxide synthase and dietary sources. The data presented above represent an overall alteration in nitric oxide production.

Effects of latent inhibition on heroin-induced conditioned immune alterations

To test whether a latent inhibition of the conditioned effects of heroin of iNOS could be induced, rats were given exposure to the conditioning chambers before the start of conditioning.

Figure 3 shows the effects of the latent inhibition procedure on LPS-induced iNOS expression in the spleen and liver. Analysis revealed an overall effect of procedure on iNOS copy numbers in the spleen and liver [$F(4,15) = 3.73$, $P < 0.05$; $F(4,15) = 4.72$, $P < 0.05$]. Planned comparisons showed a significant reduction in iNOS levels in both spleen and liver tissue in the Cond group as compared to the HC group [$F(1,15) = 13.91$, $P < 0.005$; $F(1,15) = 16.0$, $P < 0.01$). These findings are also consistent with our earlier reports indicating that exposure to a previously heroin-paired environment will reduce the expression of iNOS mRNA. There were no significant differences between the HC group and the latent inhibition (LI) group indicating that pre-exposure to the drug-paired environment was able to reduce the conditioned effects of heroin on iNOS production. Furthermore, there were no differences between the HC group and any of the control groups.

To ensure that these alterations were specific to iNOS mRNA production and not the result of change in overall RNA, real-time RT-PCR was performed on the housekeeping gene, cyclophilin. Table 3 shows that there was no effect of group on cyclophilin mRNA copy numbers.

Figure 4 shows the effects of the latent inhibition procedure on the concentration of nitrite/nitrate in the serum. Analysis revealed a main effect of procedure on nitrite/nitrate levels [$F(4,15) = 13.8$, $P < 0.001$]. In line with our previous experiments, the Cond group exhibited significantly lower levels of serum nitrite/nitrate than the HC group [$F(1,15) = 41.09$, $P < 0.001$)]. There were no significant differences between the HC group and the LI group, again demonstrating that pre-exposure was able to attenuate the conditioned response. There were also no differences found between the HC group and any of the control groups.

Effect of dose on extinction and latent inhibition

In the following set of experiments, we sought to determine the effects that altering the dose of heroin given during conditioning might have on the expression of extinction or latent inhibition.

Figure 5 shows the effects of heroin conditioning dose on the expression of extinction. The analysis of iNOS copy number revealed a significant main effect of extinction in both the spleen and the liver, respectively [$F(1,18) = 14.7$, $P < 0.01$; $F(1,18) = 5.08$, $P < 0.05$)]. There was also a significant dose by extinction interaction within the spleen [$F(2,18) = 8.72$, $P < 0.005$]. Planned comparisons revealed that both the 3 mg/kg conditioned and the 0.3 mg/kg conditioned groups showed significantly lower iNOS copy numbers in the spleen [$F(1,18) = 15.29$, $P < 0.005$; $F(1,18) = 15.37$, $P < 0.005$)] and liver [$F(1,18) = 5.15$, $P < 0.05$; $F(1,18) = 5.06$, $P < 0.05$)] when

compared to the extinction group at the same dose. These data indicate that the extinction procedure was able to block the conditioned suppression of iNOS in both the spleen and liver when either 0.3 or 3.0 mg/kg of heroin was used during the conditioning phase of the experiment.

Figure 6 shows the effect of heroin conditioning dose on the extinction of LPS-induced nitrite/nitrate production. The assay revealed a significant main effect of extinction [$F(1,18) = 22.38$, $P < 0.001$] on nitrite/nitrate levels in the blood and a dose by extinction interaction [$F(2,18) = 7.72$, $P < 0.01$]. Moreover, there was a significant reduction in nitrite/nitrate levels in both the 3 and 0.3 mg/kg conditioned groups as compared to the extinction groups conditioned at the same heroin dose [$F(1,18) = 22.09$, $P < 0.0005$; $F(1,18) = 14.59$, $P < 0.005$]. Thus, the conditioned alteration in iNOS was attenuated by the extinction procedure at both the high and low heroin doses.

Figure 7 shows the effects of heroin conditioning dose on the expression of latent inhibition. The analysis of iNOS copy number revealed a significant main effect of dose in the spleen and liver, respectively [$F(2,18) = 11.38$, $P < 0.01$; $F(2,18) = 11.12$, $P < 0.01$]. Moreover, the 0.3 mg/kg conditioned group showed significantly lower iNOS copy numbers in the spleen [$F(1,18) = 7.34$, $P < 0.05$] and liver [$F(1,18) = 7.18$, $P < 0.05$] when compared to the 0.3 mg/kg latent inhibition group. The groups treated with 3 mg/kg showed suppressed iNOS levels compared to the saline control groups in both the spleen and liver, respectively [$F(1,18) = 20.07$, $P < 0.001$; $F(1,18) = 19.65$, $P < 0.001$]. These data show that the latent inhibition procedure attenuated the conditioned suppression of iNOS only at the 0.3-mg/kg dose of heroin.

The data in Fig. 8 show the effect of heroin conditioning dose on the induction of latent inhibition of LPS-induced nitrite/nitrate production. The assay revealed a significant main effect

of dose, a main effect of latent inhibition, and a dose by latent inhibition interaction, respectively [$F(2,18) = 7.23$, $P < 0.001$; $F(1,18) = 8.1$, $P < 0.05$; $F(2,18) = 7.86$, $P < 0.005$]. Moreover, there was a significant reduction in nitrite/nitrate levels in the 0.3 mg/kg conditioned group as compared to the 0.3 mg/kg extinction group [$F(1,18) = 22.47$, $P < 0.001$]. In addition, the 3.0 mg/kg groups showed reduced nitrite/nitrate levels as compared to the saline control groups [$F(1,18) = 14.44$, $P < 0.005$). These data reveal that the conditioned suppression of nitric oxide production was attenuated by the extinction procedure only at the 0.3-mg/kg heroin dose.

## Discussion

The results presented here provide the first evidence that exposure to a drug-paired environment, either before or after conditioning, can reduce or even eliminate the conditioned effects of heroin on nitric oxide induction. This effect appears to be widespread, as it is found in spleen and liver tissue as well as serum levels of nitrite/nitrate. The use of several control groups further demonstrates that these results are specific to the manipulations and not related to ancillary effects of the conditioning procedures. These results are important because they provide the first evidence that the conditioned effects of heroin on nitric oxide induction follow accepted principles of learning, as they are susceptible to both extinction and latent inhibition. These findings are consistent with prior investigations showing that heroin-induced alterations in immune status may be conditioned to drug-paired stimuli (Lysle and Ijames 2002). In this investigation, animals received subcutaneous injections of heroin upon placement in a distinctive environment. Upon re-exposure to that environment in the absence of drug, the subjects exhibited a reduction in nitric oxide production similar to what is observed with heroin treatment. The present study is consistent with these results in showing that manipulations

involving the animal's experience with the CS will alter the conditioned effects of heroin on iNOS production.

The dose experiments examine the effects of heroin dose on the expression of extinction and latent inhibition. In the extinction experiments, the extinction procedure was able to attenuate the conditioned reduction in iNOS in both the 3.0 and 0.3 mg/kg heroin groups. However, the latent inhibition procedure was only able to lessen the reduction in the 0.3 mg/kg group. This suggests that the high dose of heroin overpowers the pre-exposure parameters used in this experiment. It is possible that extending the number of days on which animals receive pre-exposure may overcome the potency of the higher dose heroin stimulus.

It is well documented in the literature that a drug-paired environment or stimulus may elicit a response similar to that observed upon drug administration. Repeated pairing of drugs that activate reward pathways with environmental stimuli may lead to these stimuli acquiring secondary reinforcing properties and eliciting drug-like responses. There is evidence from both human and animal studies indicating that repeated exposure to drug cues without further drug use (i.e., extinction) decreases these conditioned responses and may, therefore, reduce drug seeking and craving (Childress et al. 1986; Calcagnetti and Schechter 1993). Evidence provided in the current study suggests that exposure to drug-paired stimuli may not only contribute to relapse, but that it may also alter the ability of the subject's immune system to deal with infection. Conditioning of the immune response is a well-documented phenomenon and can be accomplished with a wide variety of immunomodulatory stimuli, including drugs of abuse such as heroin. Considering the increased susceptibility to infection that occurs with opioid use, it is

possible that the conditioned alterations in certain immune parameters that occur in response to exposure to drug-paired cues might also compromise immune function and increase susceptibility to disease. Given the number of animal studies showing decreased drug seeking after extinction, this study sought to determine if it is possible to reduce the conditioned immune response to a drug-paired environment by inducing extinction or latent inhibition. The data presented here indicate that both extinction and latent inhibition attenuate heroin's conditioned effect on iNOS production. Rats that were repeatedly placed in the formerly drug-paired environment without drug administration did not exhibit a reduction in iNOS production when re-exposed to this environment, unlike those animals that had not undergone extinction. Some studies have shown that whereas extinction may reduce the occurrence of conditioned drug-like responses in a controlled environment such as the laboratory, these treatments may have little to no effect on rehabilitation (Dawe et al. 1993; Niaura et al. 1999). This may be due to several factors, including the ability of these cues to be easily reconditioned and the complexity of the cues themselves. Whereas the nature of the CS may be controlled in an experimental setting, there is no way to determine the exact nature of the CS that has become associated with drug delivery in a drug user. Even if all of these cues were identified, it would be extremely difficult to extinguish each one of them. In addition, some studies suggest that extinction is not a mechanism by which the subject unlearns the association between the cue and drug delivery but rather creates a new association in which the cue no longer predicts the availability of the drug (Bouton and Swartzentruber 1991). Data also suggest that as the original association is still intact, it may easily be reconditioned with a single re-pairing of the drug and its cue (Leri and Rizos 2005) or through a variety of other manipulations.

Interestingly, human brain imaging data have shown changes in neural activity within various brain regions after exposure to drug cues in former drug users (Sell et al. 2000). In addition, research has demonstrated an increase in c-Fos expression in the lateral habenula, basolateral amygdala complex, prelimbic cortex, and nucleus accumbens core in rats exposed to drug-associated CS (Miller and Marshall 2005; Zhang et al. 2005). Animal research employing pharmacological manipulations further supports the contribution of these areas to cue-induced drug seeking behavior (See 2005; Rizos et al. 2005; Fuchs et al. 2005). For instance, the basolateral amygdala has been implicated in both the acquisition and extinction of conditioned responses to drug cues (Rizos et al. 2005). Therefore, it may be of interest to investigate whether disruption of this region would be effective in reducing the conditioned effects of heroin on nitric oxide production. More specifically, the modulation of the cholinergic system within the basolateral amygdala has also been shown to attenuate conditioned responses to drug cues (Zarrindast et al. 2005). The cholinergic system is important for learning and memory and has been implicated in the formation of stimulus–drug associations (See et al. 2003). Given that these areas and neurotransmitter systems appear to play a role in the acquisition and expression of the CR to drug cues, it is possible that the conditioned effects of heroin on immune status may also be mediated through activation of some of the same neural circuitry. Regardless of the mechanism, it is important to take into account the profound immune alterations that occur upon exposure to drug-related stimuli and to consider how the various manipulations that reduce these effects might impact the immune system's response to disease. These findings are particularly important because they validate the effects of exposure to a heroin-paired environment on iNOS induction as true conditioning and demonstrate two methods of reducing the CR.

Acknowledgement

This work was supported by United States Public Service Grant DA13371 and Research Scientist Award DA00334 from the National Institute on Drug Abuse. Jennifer Szczytkowski was supported by a National Institutes of Health predoctoral Ruth L. Kirschstein fellowship (DA021467). The authors wish to acknowledge the expert technical assistance of Stephanie Ijames and Timothy Saurer.

References

Albina JE, Henry WL (1991) Suppression of lymphocyte proliferation through the nitric oxide synthesizing pathway. J Surg Res 50:403–409

Al-Ramadi BK, Meissler JJ, Huang D, Eisenstein TK (1992) Immunosuppression induced by nitric oxide and its inhibition by interleukin-4. Eur J Immunol 22:2249–2254

Bouton ME, Swartzentruber D (1991) Sources of relapse after extinction in Pavlovian and instrumental learning. Clin Psychol Rev 11(2):123–140

Brown SM, Stimmel B, Taub RN, Kochwa S, Rosenfield RE (1974) Immunological dysfunction in heroin addicts. Arch Int Med 134:1001–1006

Calcagnetti DJ, Schechter MD (1993) Extinction of cocaine-induced place approach in rats: a validation of the biased conditioning procedure. Brain Res Bull 30:695–700

Chang WW, Su IJ, Lai MD, Chang WT, Huang W, Lei HY (2003) The role of inducible nitric oxide synthase in a murine acute hepatitis B virus (HBV) infection model induced by hydrodynamics-based in vivo transfection of HBV-DNA. J Hepatol 39:834–842

Childress AR, McLellan AT, O'Brien CP (1986) Abstinent opiate abusers exhibit conditioned craving, conditioned withdrawal and reductions in both through extinction. Br J Addict 81:655–660

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocynate-phenol-chlorogorm extraction. Anal Biochem 162:156–159

Dawe S, Powell JH, Richards D, Gossop M, Marks I, Strang J, Gray JA (1993) Does post-withdrawal cue exposure improve outcome in opiate addiction? A controlled trial. Addiction 88:1233–1245

Donahoe RM, Nicholson JKA, Madden JJ, Donahoe F, Shafer DA, Gordon D, Bokos P, Falek A (1986) Coordinate and independent effects of heroin, cocaine and alcohol abuse on T-cell E-rosette formation and antigenic marker expression. Clin Immunol 41:254–264

Eikelboom R, Stewart J (1979) Conditioned temperature effects using morphine as the unconditioned stimulus. Psychopharmacology 61:31–38

Eisenstein TK, Huang D, Meissler JJ, Al-Ramadi B (1994) Macrophage nitric oxide mediates immunosuppression in infectious inflammation. Immunobiology 191:493–502

Fecho K, Nelson CJ, Lysle DT (2000) Phenotypic and functional assessments of immune status in the rat spleen following acute heroin treatment. Immunopharmacology 46:193–207

Fuchs RA, Evans KA, Ledford CC, Parker MP, Case JM, Mehta RH, See RE (2005) The role of the dorsomedial prefrontal cortex, basolateral amygdala, and dorsal hippocampus in contextual reinstatement of cocaine seeking in rats. Neuropsychopharmacology 30:296–309

Garten RJ, Lai S, Zhang J, Liu W, Chen J, Vlahov D, Yu XF (2004) Rapid transmission of hepatitis C virus among young injecting heroin users in Southern China. Int J Epidemiol 33:182–188

Govitrapong P, Suttitum T, Kotchabhakdi N, Uneklabh T (1998) Alterations of immune functions in heroin addicts and heroin withdrawal subjects. J Pharmacol Exp Ther 266:417–423

Green SJ, Meltzer MS, Hibbs JB, Nacy CA (1990) Activated macrophages destroy intracellular Leishmania major amastigotes by an l-arginine-dependent killing mechanism. J Immunol 144:278–283

Hrabak A, Csuka I, Bajor T, Csatary LK (2006) The cytotoxic anti-tumor effect of MTH-68/H, a live attenuated Newcastle disease virus is mediated by the induction of nitric oxide synthesis in rat peritoneal macrophages in vivo. Cancer Lett 231:279–289

Hussey HH, Katz S (1950) Infections resulting from narcotic addiction: report of 102 cases. Am J Med 9:186–193

Karupiah G, Xie QW, Buller RM, Nathan C, Duarte C, MacMicking JD (1993) Inhibition of viral replication by interferon-gamma-induced nitric oxide synthase. Science 261:1445–1448

Leri F, Rizos Z (2005) Reconditioning of drug-related cues: a potential contributor to relapse after drug reexposure. Pharmacol Biochem Behav 80:621–630

Lindgren H, Stenmark S, Chan W, Tarnvik A, Sjostedt A (2004) Distinct roles of reactive nitrogen and oxygen species to control infection with the facultative intracellular bacterium Francisella tularensis. Infect Immun 72:7172–7182

Louria DB, Hensle T, Rose J (1967) The major medical complication of heroin addiction. Ann Int Med 67:1–27

Lubow RE, Moore AU (1959) Latent inhibition: the effect of nonreinforced pre-exposure to the conditioned stimulus. J Comp Physiol Psychol 52:415–419

Luttgens WF (1949) Endocarditis in “main line” opium addicts. Arch Int Med 83:653–664

Lysle DT, How T (2000) Heroin modulates the expression of inducible nitric oxide synthase. Immunopharmacology 46:181–192

Lysle DT, Ijames SG (2002) Heroin-associated environmental stimuli modulate the expression of inducible nitric oxide synthase in the rat. Psychopharmacology 164:416–422

MacMicking JD, Nathan C, Hom G, Chartrain N, Fletcher DS, Trumbauer M, Stevens K, Xie QW, Sokol K, Hutchinson N, Chen H, Mudgett JS (1995) Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. Cell 81:641–650

McDonough RJ, Madden JJ, Falek A, Shafer DA, Pline M, Gordon D, Bokos P, Kuehnle JC, Mendelson J (1980) Alteration of T and null lymphocyte frequencies in the peripheral blood of human opiate addicts: in vivo evidence for opiate receptor sites on T lymphocytes. J Immunol 125:2539–2543

Miksic S, Smith N, Numan R, Lai H (1975) Acquisition and extinction of a conditioned hyperthermic response to a tone paired with morphine administration. Neuropsychobiology 1:277–283

Miller CA, Marshall JF (2005) Altered Fos expression in neural pathways underlying cue-elicited drug seeking in the rat. Eur J Neurosci 21:1385–1393

Nair MPN, Laing TJ, Schwartz SA (1986) Decreased natural and antibody-dependent cellular cytotoxic activities in intravenous drug users. Clin Immunol 38:68–78

Nathan CF, Hibbs JB Jr (1991) Role of nitric oxide synthase in macrophage antimicrobial activity. Curr Opin Immunol 3:65–70

Niaura R, Abrams DB, Shadel WG, Rohsenow DJ, Monti PM, Sirota AD (1999) Cue exposure treatment for smoking relapse prevention: a controlled clinical trial. Addiction 94:685–695

Novick DM, Oschorn M, Ghali V, Croxson TS, Mercer WD, Chiorazzi N, Kreek MJ (1989) Natural killer cell activity and lymphocyte subsets in parenteral heroin abusers and long-term methadone maintenance patients. J Pharmacol Exp Ther 250:606–610

Oschorn M, Novick DM, Kreek MJ (1990) In vitro studies of the effects of methadone on natural killer cell activity. Isr J Med Sci 26:421–425

Pavlov IP (1927) Conditioned reflexes. Clarendon, London

Pineno O, Miller RR (2005) Primacy and recency effects in extinction and latent inhibition: a selective review with implications for models of learning. Behav Processes 69:223–235

Rizos Z, Ovari J, Leri F (2005) Reconditioning of heroin place preference requires the basolateral amygdala. Pharmacol Biochem Behav 82:300–305

Rossi GR, Cervi LA, Garcia MM, Chiapello LS, Sastre DA, Masih DT (1999) Involvement of nitric oxide in protecting mechanism during experimental cryptococcosis. Clin Immunol 90:256–265

Schwarz KS, Cunningham CL (1990) Conditioned stimulus control of morphine hyperthermia. Psychopharmacology 101:77–84

See RE (2005) Neural substrates of cocaine-cue associations that trigger relapse. Eur J Pharmacol 526:140–146

See RE, McLaughlin J, Fuchs RA (2003) Muscarinic receptor antagonism in the basolateral amygdala blocks acquisition of cocaine-stimulus association in a model of relapse to cocaine-seeking behavior in rats. Neuroscience 117:477–483

Sell LA, Morris JS, Bearn J, Frackowiak RSJ, Friston KJ, Dolan RJ (2000) Neural responses associated with cue evoked emotional states and heroin in opiate addicts. Drug Alcohol Depend 60:207–216

Sharp BM, McAllen K, Gekker G, Shahabi NA, Peterson PK (2001) Immunofluorescence detection of δ opioid receptors (DOR) on human peripheral blood CD4+ T cells and DOR-dependent suppression of HIV-1 expression. J Immunol 167:1097–1102

Vincendeau P, Daulouede S, Veyret B, Darde ML, Bouteille B, Lemesre JL (1992) Nitric oxide-mediated cytostatic activity on Trypanosoma brucei gambiense and Trypanosoma brucei brucei. Exp Parasitol 75:353–360

Wei XQ, Charles IG, Smith A, Ure J, Feng GJ, Huang FP, Xu D, Muller W, Mocado S, Liew FY (1995) Altered immune responses in mice lacking inducible nitric oxide synthase. Nature 375:408–411

Zarrindast MR, Fattahi Z, Rostami P, Rezayof A (2005) Role of the cholinergic system in the basolateral amygdal on morphine-induced conditioned place preference. Pharmacol Biochem Behav 82:1–10

Zhang F, Zhou W, Liu H, Zhu H, Tang S, Lai M, Yang G (2005) Increased c-Fos expression in the medial part of the lateral habenula during cue-evoked heroin-seeking in rats. Neurosci Lett 386:133–137