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Evidence for the Nucleus Accumbens as a Neural Substrate of Heroin-Induced Immune Alterations

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ABSTRACT

Administration of opioid drugs such as heroin produces several immunosuppressive effects, including decreases in natural killer (NK) cell activity, lymphocyte proliferative responses, and nitric oxide production. Interestingly, opioids have been shown to alter many immune parameters indirectly by modulating the immunoregulatory actions of the central nervous system. Recently, it has been demonstrated that morphine inhibits NK cell activity through a neural pathway that requires the activation of dopamine D_1 receptors in the nucleus accumbens shell. The present study examined whether the nucleus accumbens also mediates the effects of heroin, a more commonly abused opioid, on several parameters of immune status in Lewis rats. The results showed that bilateral administration of the dopamine D_1 receptor antagonist *R*-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride (SCH-23390; 0.015 and 0.15 μg/side) into the nucleus accumbens shell blocked decreases in splenic NK activity produced by heroin (3 mg/kg s.c.) but did not attenuate the suppression of splenocyte proliferative responses to concanavalin-A or lipopolysaccharide (LPS). A subsequent experiment was performed to evaluate the effect of D_1 receptor antagonism on LPS-induced expression of inducible nitric-oxide synthase (iNOS) in vivo. These results showed that intra-accumbens SCH-23390 administration prevented heroin-induced reductions of iNOS mRNA expression in spleen, liver, and lung tissues and attenuated the suppression of nitric oxide levels in plasma. Collectively, these findings indicate that nucleus accumbens dopamine D_1 receptors are critically involved in heroin-induced immune alterations.

Opioid drug use is associated with adverse health consequences. Heroin abusers display abnormalities in basic immune parameters and have high rates of viral, bacterial, and fungal infections (Louria et al., 1967; Govitrapong et al., 1998). Although the high incidence of infectious diseases among abusers is commonly attributed to increased pathogen exposure or insalubrious behaviors, opioids exert potent immunomodulatory effects that may further enhance infection susceptibility. Controlled studies in humans demonstrate that morphine produces immunosuppressive effects in healthy, nondrug-using subjects (Yeager et al., 1995), and clinical reports indicate that higher doses of opioid analgesics are associated with greater frequencies of infectious complications among burn victims and patients that underwent cardiac surgery (El Solh et al., 2006; Schwacha et al., 2006). Thus, the use of opioids, whether for therapeutic or recreational purposes, may directly influence resistance to infectious diseases.

In animal models, morphine has been shown to suppress a variety of immune parameters, but the mechanisms underlying these effects are not fully understood (Lysle et al., 1993; Lockwood et al., 1994). Opioids modulate immunity in a highly complex manner by interacting with opioid receptors expressed by multiple cell types throughout the body. For example, in vitro studies show that morphine can inhibit macrophage phagocytosis and interferon-γ production through direct interactions with immunocyte opioid receptors (Peterson et al., 1987; Szabo et al., 1993). In addition,

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ABBREVIATIONS: NK, natural killer; iNOS, inducible nitric-oxide synthase; LPS, lipopolysaccharide; SCH-23390 *R*-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride; Con-A, concanavalin A; RT-PCR, reverse transcriptase-polymerase chain reaction; ANOVA, analysis of variance; SKF38393, 2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1*H*-3-benzazepine; CNS, central nervous system.

morphine administration in vivo has been shown to inhibit NK activity and lymphocyte proliferative responses indirectly by acting within the brain to disrupt the immunoregulatory functions of the nervous system (Weber and Pert, 1989; Hernandez et al., 1993). Neural modulation of immunity by morphine is a well documented phenomenon that involves both neuroendocrine and autonomic efferent pathways (Fuchs and Pruett, 1993; Fecho et al., 1996).

Our laboratory has recently begun examining the role of central dopamine receptors in opioid-induced immune alterations. Similar to virtually every drug of abuse, opioids stimulate dopamine transmission in the nucleus accumbens (Pontieri et al., 1995), and it is widely recognized that such alterations in dopamine signaling modulate the motivational and reinforcing properties of opioids. Interestingly, at least some of morphine's immunomodulatory effects are also dependent on dopamine transmission. Specifically, the activation of dopamine D_1 receptors in the nucleus accumbens shell is necessary for morphine to suppress splenic NK cell activity and sufficient to inhibit NK responses in the absence of morphine (Saurer et al., 2006a). Thus, nucleus accumbens dopamine receptors seem to play a critical role in neuroimmune modulation by morphine.

Heroin administration has also been shown to inhibit immune parameters such as NK activity, lymphocyte proliferation, and nitric oxide production (Fecho and Lysle, 2000; Lysle and How, 2000). Although heroin is more commonly abused than morphine, only a limited number of studies have examined the mechanisms of heroin's immunomodulatory effects. A common assumption is that the biological effects of these drugs are the same, as heroin (diacetylmorphine) is readily metabolized to morphine. Although similar in many respects, there are several key pharmacological differences between heroin and morphine. For example, heroin produces different subjective psychological effects in humans (Martin and Fraser, 1961), has a more rapid onset of action (Oldendorf et al., 1972), and is more potent in producing behavioral effects (Kaiko et al., 1981). Furthermore, because the unique heroin metabolite 6-monoacetylmorphine has been shown to produce analgesic effects by engaging different opioid receptor populations than morphine (Rady et al., 1991; Rossi et al., 1996), it is possible that heroin may also elicit immune alterations via different receptor mechanisms.

The goals of the present study were two-fold. First, based on our prior investigations indicating the importance of the nucleus accumbens in the immunological effects of morphine (Saurer et al., 2006a), we sought to determine whether the nucleus accumbens was also involved in heroin's suppressive effects on splenic NK activity and mitogen-induced lymphocyte proliferation. Second, to further explore the mechanisms of heroin's effects in the context of an in vivo immune response, the present study assessed the role of nucleus accumbens D_1 receptors in heroin-induced alterations of inducible nitric-oxide synthase (iNOS) expression. The iNOS enzyme has a critical role in disease resistance and possesses potent antimicrobial effects owing to the generation of nitric oxide. Under normal conditions, iNOS is expressed at low levels, but its expression is markedly up-regulated in response to infection or immune challenge. In the present study, iNOS production was induced with lipopolysaccharide (LPS), the major immunogenic component of Gram-negative bacteria. Importantly, this model permits the evaluation of heroin's effects on a critical immune response in vivo.

Materials and Methods

Animals. Adult male Lewis rats weighing approximately 200 to 250 g were purchased from Charles River Laboratories (Raleigh, NC). Upon arrival, animals were individually housed and maintained on a reverse 12-h light/dark cycle. Animals were habituated to handling and the colony room environment for 2 weeks before any experimental manipulation. Food and water were available ad libitum throughout the experiment.

Drugs. Heroin (diacetylmorphine sulfate; National Institute on Drug Abuse, Bethesda, MD) and SCH-23390 (Sigma-Aldrich, St. Louis, MO) were dissolved in sterile 0.9% saline. For all experiments, heroin or saline injections were administered in a 1.0 ml/kg volume.

Surgery. Stereotaxic surgeries were performed under anesthesia induced with a 0.2-ml intramuscular injection of a 1:1 (v/v) mixture of ketamine hydrochloride (100 mg/ml) and xylazine hydrochloride (20 mg/ml). Animals receiving SCH-23390 microinjections into the nucleus accumbens shell were implanted with bilateral 26-gauge guide cannulae (Plastics One, Roanoke, VA) aimed at the following coordinates: AP, +1.7; ML, ±0.8, and DV, −5.4 (coordinates expressed as millimeters from bregma). Animals were given a 1-week recovery period before testing.

Drug Administration. The optimal dose of heroin for producing immune alterations was first established by conducting a dose-response analysis. In the heroin dose-response experiment, rats were assigned to one of five groups (n = 4/group). Each group received a single subcutaneous injection of heroin at a dose of 0, 1, 3, 5, or 10 mg/kg. The range of heroin doses used in the present study was selected based on previously published findings showing that heroin significantly inhibits NK activity and lymphocyte mitogenic responses at 1 mg/kg, with much more pronounced effects at 10 mg/kg (Fecho and Lysle, 2000).

A subsequent experiment was performed to evaluate the involvement of nucleus accumbens dopamine D_1 receptors in heroin's effects. For this manipulation, animals were assigned to one of six groups (n = 5–6/group) in which they received a bilateral microinjection of SCH-23390 (0, 0.015, or 0.15 μg/side) into the nucleus accumbens shell immediately before injection with saline or heroin (3 mg/kg s.c.). The doses of SCH-23390 used in the current study were selected based on our previous work that showed that morphine-induced suppression of NK activity is partially attenuated at a dose of 0.015 μg/side and fully blocked at a dose of 0.15 μg/side (Saurer et al., 2006a). In addition, because the goal of the microinjection studies was to determine whether SCH-23390 administration would block the effects of heroin, we selected the 3-mg/kg dose of heroin, because this was the lowest dose that produced the most prominent and consistent effects across all immune parameters examined in the dose-response experiment. Microinjections of SCH-23390 were delivered in a 0.5-μl volume per side via a bilateral 33-gauge injector that protruded 2 mm beyond the tip of the guide cannula (final coordinates, AP, +1.7; ML, ± 0.8; and DV, −7.8). Microinjections were performed over a 30-s period using a microsyringe pump (Harvard Apparatus Inc., Holliston, MA), and the injector was left in place for 1 min to allow diffusion of the drug away from the injection site.

To evaluate the role of nucleus accumbens D_1 receptors in the effects of heroin on lipopolysaccharide (LPS)-induced iNOS production, animals were assigned to one of six groups (n = 3–4), in which they received bilateral microinjections of SCH-23390 (0, 0.015, or 0.15 μg/side) into the nucleus accumbens shell as described above. Immediately after microinjection, rats were administered heroin (3 mg/kg s.c.) or saline in conjunction with a 1-mg/kg injection of LPS (serotype 055:B5; Difco, Detroit, MI). Six hours after the injection of LPS, animals were sacrificed by cervical dislocation. Blood samples were taken from the abdominal aorta for the determination of

plasma nitrite/nitrate levels, and spleen, liver, and lung tissues were collected for RNA extraction. This experiment was replicated using the same design, and the data were combined for analysis (n = 6–7/group).

After drug treatment and sacrifice, Alcian Blue dye was injected via the cannula, and brains were removed and stored in a 4% paraformaldehyde solution for 2 days. Brains were then transferred to a 30% sucrose solution for several days for cryoprotection, frozen, and stored at −80°C for subsequent analysis. Accurate cannula placements were verified by examination of 50-μm coronal tissue sections under a light microscope. Only animals with placements within the targeted region were included in the analysis.

Tissue Collection. For ex vivo assessments of splenic NK activity and mitogen-induced proliferation, rats were sacrificed by cervical dislocation 1 h after heroin or saline injection. The spleen was removed and placed in 7 ml of RPMI 1640 tissue culture medium supplemented with 10 mM HEPES, 2 mM glutamine, and 50 μg/ml gentamicin (supplemented RPMI 1640 medium; Invitrogen, Carlsbad, CA). Each spleen was prepared as a single-cell suspension by gently pressing the tissue between two sterile, frosted microscope slides in supplemented RPMI 1640 medium enriched with 10% fetal bovine serum (complete RPMI 1640 medium; Invitrogen). Splenic leukocytes were counted using a Hemavet 850 cell analyzer (CDC Technologies Inc., Oxford, CT), and cell suspensions were adjusted to 5×10^6 leukocytes/ml by diluting with complete RPMI 1640 medium.

NK Cell Activity Assay. Splenic NK cell activity was assessed using a standard chromium release assay according to previously published procedures (Fecho et al., 1993; Lysle et al., 1993). Adjusted splenocyte suspensions were coincubated with the murine T-cell lymphoma YAC-1. The YAC-1 target cells were labeled by incubation for 70 min with 200 μCi of sodium chromate-51 [^{51}Cr]. YAC-1 cells were then washed three times with complete RPMI 1640 medium to remove exogenous [^{51}Cr]. Splenic leukocytes were used as effectors and were plated in triplicate at 10, 5, 2.5, and 1.25×10^5 cells/well of a 96-well plate. Labeled targets were diluted and plated at 1×10^4 cells/well to give effector/target (E:T) ratios of 100:1, 50:1, 25:1, and 12.5:1. After 5-h incubation at 37°C in a humidified CO_2 incubator, the amount of [^{51}Cr] released into the supernatant was determined using a gamma counter (model 1272 CliniGamma; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Percentage of specific lysis at all E:T ratios was used to calculate lytic units. One lytic unit is defined as the number of splenic leukocytes necessary to lyse 20% of the target cells. Results are reported as the number of lytic units per 10^7 effector cells.

Splenocyte Proliferation Assay. Mitogen stimulation assays were completed following previously published procedures (Fecho et al., 1993; Lysle et al., 1993). Splenic T- and B-lymphocyte proliferation was induced with the mitogens concanavalin-A (Con-A) and LPS (Sigma-Aldrich), respectively. One hundred microliters of the adjusted splenocyte suspensions was pipetted in triplicate into microtiter plate wells containing final concentrations of 0, 0.5, and 5.0 μg/ml Con-A and 0.5 and 5.0 μg/ml LPS to provide background, suboptimal, and optimal mitogen concentrations. All statistical analyses were performed on the results from the 5.0-μg/ml concentrations of Con-A and LPS, because previously published data have established that this concentration elicits optimal proliferation responses under the present parameters (Lysle et al., 1993). Splenocyte cultures were then incubated for 48 h at 37°C in a humidified CO_2 incubator. Each culture well was pulsed with 1 μCi of [^{3}H]thymidine during the last 5 h of the incubation period. Cultures were then harvested onto glass fiber filter paper using an automatic 96-well cell harvester (Tomtec, Orange, CT). The amount of [^{3}H]thymidine incorporated into the DNA of proliferating cells was measured using a liquid scintillation counter (model 1205; PerkinElmer Wallac, Waltham, MA) and is expressed as the mean of the triplicate dpm for the samples from each rat.

Real-Time RT-PCR. To determine iNOS expression, real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was performed. Total RNA was extracted from a section of each spleen, liver, and lung using TRI-Reagent (Molecular Research Center, Cincinnati, OH), and RNA was quantified spectrophotometrically (GeneQuant II; GE Healthcare). For the RT-PCR, 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 (Mountain View, CA). PCR amplifications were performed using the Fast Start DNA Master SYBR Green I real-time PCR kit (Roche Diagnostics, Indianapolis, IN) and the LightCycler instrument (Roche Diagnostics). All reaction components were prepared in a master mix solution, with each reaction using a 20-μl mix placed in glass capillary tubes designed for the LightCycler system. The PCR primer set for iNOS, 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′ and 5′-GGGTGTCAGAGTCTTGTGCCTTTGG-3′, was synthesized by the Nucleic Acids Core Facility (Lineberger Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC). The analysis of fluorescence levels in standards and samples over the course of 40 amplification cycles was used to derive the number of copies of the target molecule in each sample, based on an external standard curve. The cycle temperatures were 95, 60, and 72°C for the denaturing, annealing, and extending, respectively, with cycle times of 15, 5, and 25 s, respectively. Fluorescence level was determined at the end of the extending phase for each cycle of PCR. In addition, assessments of housekeeping gene expression, cyclophilin, were made to ensure 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 the ratio of iNOS mRNA copies (per 10 ng of cDNA) to cyclophilin copy number based on the standard curve using the LightCycler software (Roche Diagnostics). Furthermore, to confirm the nature of amplification product, a melt curve analysis was conducted after the final PCR cycle.

Nitrite Assay. To provide an additional assessment of nitric oxide production, the level of nitrite/nitrate in plasma was determined using the Griess reagent assay. Nitrate and nitrite are formed nonenzymatically when nitric oxide is exposed to oxygen; thus, plasma levels of these products reflect the level of nitric oxide production. To determine total nitrite/nitrate levels, nitrate was first converted to nitrite using nitrate reductase in the presence of NADPH and flavin adenine dinucleotide. Specifically, 6 μl of plasma was mixed with 44 μl of distilled H_2O, 20 μl of 0.31 M phosphate buffer, pH 7.5, 10 μl of 0.86 mM NADPH (Sigma-Aldrich), 10 μl of 0.11 mM flavin adenine dinucleotide (Sigma-Aldrich), and 10 μl of 1.0 U/ml nitrate reductase (Roche Diagnostics) in individual wells of a 96-well plate. Samples were incubated for 1.5 h at room temperature. Then, 200 μl of Griess reagent consisting of a 1:1 (v/v) solution of 1% sulfanilamide in 5.0% phosphoric acid and 0.1% *N*-(1-napthyl)ethyl-enedamine dihydrochloride in distilled water was added to the samples. After incubation for 10 min at room temperature, absorbance was measured at 550 nm using a plate reader (model EL312; BioTek Instruments, Winooski, VT). The total micromolar concentration of nitrite was then determined for each sample based on a standard curve. Recovery of nitrate is greater than 95% in this assay.

Statistics. Results from the heroin-dose effect study were analyzed using one-way analysis of variance. Data analysis for all dopamine antagonist experiments was performed using two-way analysis of variance, with experimental replication entered into the model as a covariate to control for interassay variability in the measured parameters. For the two-way analysis, the first factor was SCH-23390 dosage and the second factor was drug treatment, i.e., heroin or saline. For all data sets, planned comparisons were performed in accordance with a priori hypotheses that SCH-23390 would antagonize the effects of heroin. Planned comparisons in each analysis consisted of pairwise comparisons of means between heroin treatment groups and saline treatment groups at each level of the factor "SCH-23390 dosage." All analyses were performed with the α-level of significance set at $p < 0.05$.

Results

Heroin Dose-Response Analysis. The optimal dose of heroin for producing immune alterations was first established by conducting a dose-response analysis. The results of this experiment are displayed in Fig. 1. Analysis of variance yielded a significant main effect of dose on NK cell activity, indicating that heroin reduced NK activity [$F(4,15) = 16.22$; $p < 0.0001$]. Furthermore, planned comparisons showed that heroin significantly decreased cytolytic activity at every dose examined. Heroin administration also suppressed splenocyte proliferative responses to Con-A and LPS (Fig. 1). There was a significant main effect of heroin on proliferation induced by Con-A [$F(4,14) = 36.51$; $p > 0.0001$]. Planned comparisons further showed that heroin significantly decreased mitogenic responses to Con-A at each dose examined. Analysis of variance also revealed a significant main effect of heroin on LPS-induced proliferation [$F(4,14) = 33.89$; $p < 0.0001$], and this suppressive effect of heroin was evident at each dose tested.

Effect of D_1 Receptor Antagonism on NK Activity. The effect of SCH-23390 administration into the nucleus accumbens shell on heroin-induced decreases in NK activity is shown in Fig. 2. Analysis of variance revealed significant main effects of heroin treatment [$F(1,27) = 18.15$; $p < 0.001$] and SCH-23390 dose [$F(2,27) = 6.02$; $p < 0.01$]. Planned comparisons demonstrated that heroin significantly reduced NK cell activity in the control groups that received saline microinjections [$F(1,27) = 16.48$; $p < 0.001$]. Planned comparisons also revealed that both doses of SCH-23390 blocked the effects of heroin, because there were no significant differences between heroin and saline groups among animals that received SCH-23390.

Effect of D_1 Receptor Antagonism on Splenocyte Proliferation. Figure 3 (top) shows the effect of SCH-23390 administration into the nucleus accumbens shell on heroin-induced reductions in proliferative responses to Con-A. The analysis yielded a significant main effect of heroin treatment [$F(1,27) = 27.00$; $p < 0.0001$], but there was not a significant main effect of SCH-23390 dose [$F(2,27) = 0.33$; $p > 0.05$]. Moreover, there was no significant interaction between heroin and SCH-23390 treatment [$F(2,27) = 0.01$; $p > 0.05$]. Planned comparisons further indicated that heroin significantly decreased Con-A induced proliferation at each dose of SCH-23390 tested.

The effect of SCH-23390 administration on heroin-induced decreases mitogenic responses to LPS is shown in the bottom panel of Fig. 3. Analysis of variance revealed a significant main effect of heroin [$F(1,27) = 37.62$; $p < 0.0001$]. There was no effect of SCH-23390 treatment [$F(2,27) = 0.44$; $p > 0.05$], nor was there a significant interaction between SCH-23390 dose and heroin treatment [$F(2,27) = 0.02$; $p > 0.05$]. Planned comparisons indicated that heroin's suppressive effect on LPS-induced proliferation was not blocked by SCH-23390 at any dose, because all heroin-treated groups were significantly decreased relative to the corresponding saline control groups.

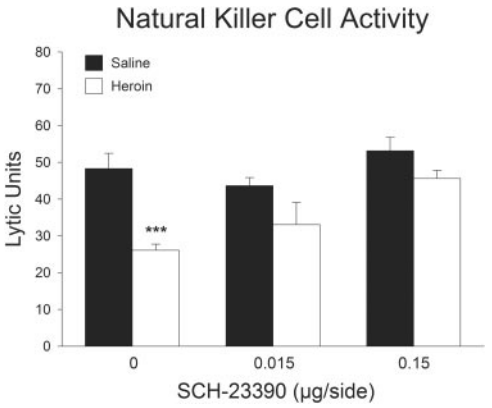

Fig. 2. Effect of D_1 receptor antagonism in the nucleus accumbens on heroin-induced suppression of NK cell activity. Rats received bilateral microinjections of saline or SCH-23390 into the nucleus accumbens shell before a subcutaneous injection of heroin. Data are expressed as lytic units (mean ± S.E.). ***, $p < 0.001$ compared with the saline-treated control group that received an equivalent dose of SCH-23390.

Effect of D_1 Receptor Antagonism on iNOS Expression. A final study was performed to examine whether D_1 receptors in the nucleus accumbens shell also play a role in the effects of heroin on the production of iNOS in vivo. LPS-induced expression of iNOS mRNA in spleen, liver, and lung tissues is shown in Fig. 4. Analysis of variance revealed a significant interaction between SCH-23390 dosage and heroin treatment in the spleen [$F(2,33) = 4.50$; $p < 0.05$], liver [$F(2,33) = 9.74$; $p < 0.001$], and lung [$F(2,33) = 7.91$; $p < 0.01$], indicating that SCH-23390 altered the suppressive effect of heroin in all tissues. Planned comparisons further revealed that among animals that received saline microinjections, heroin treatment significantly reduced iNOS levels compared with saline in the spleen [$F(1,33) = 6.02$; $p < 0.05$], liver [$F(1,33) = 14.07$; $p < 0.001$], and lung [$F(1,33) = 12.11$; $p < 0.01$]. Importantly, planned comparisons also showed that heroin- and saline-treated groups that received the high-

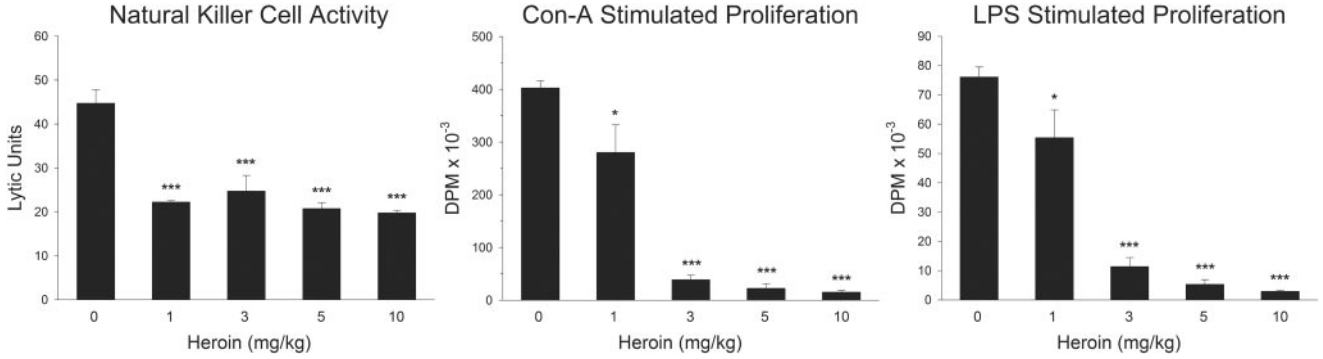

Fig. 1. Effects of heroin dose on splenic immune measures. Data from the cytotoxicity assay are expressed as lytic units (mean ± S.E.). Data from proliferation assays are expressed as disintegrations per minute (mean ± S.E.). *, $p < 0.05$; ***, $p < 0.001$ compared with saline-treated control group.

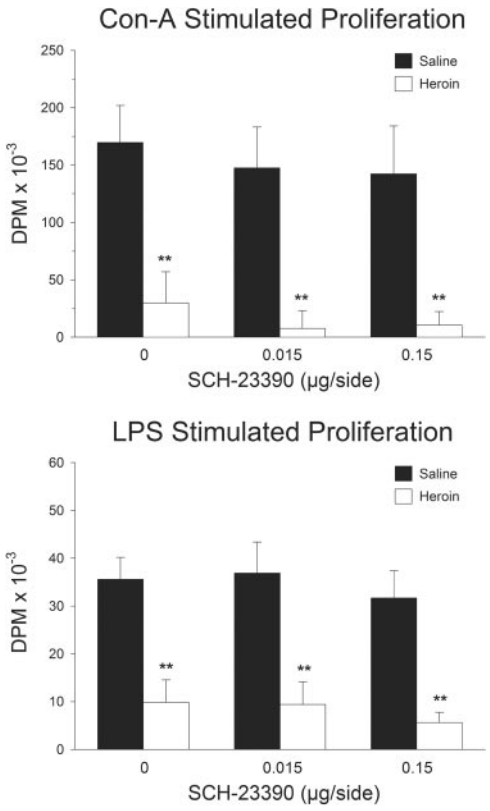

Fig. 3. Effect of D_1 receptor antagonism in the nucleus accumbens on heroin-induced decreases in splenocyte mitogenic responses. Rats received bilateral microinjections of SCH-23390 into the nucleus accumbens shell before a subcutaneous injection of saline or heroin. Data are expressed as disintegrations per minute (mean ± S.E.). **, $p < 0.01$ compared with the saline group that received the same dose of SCH-23390.

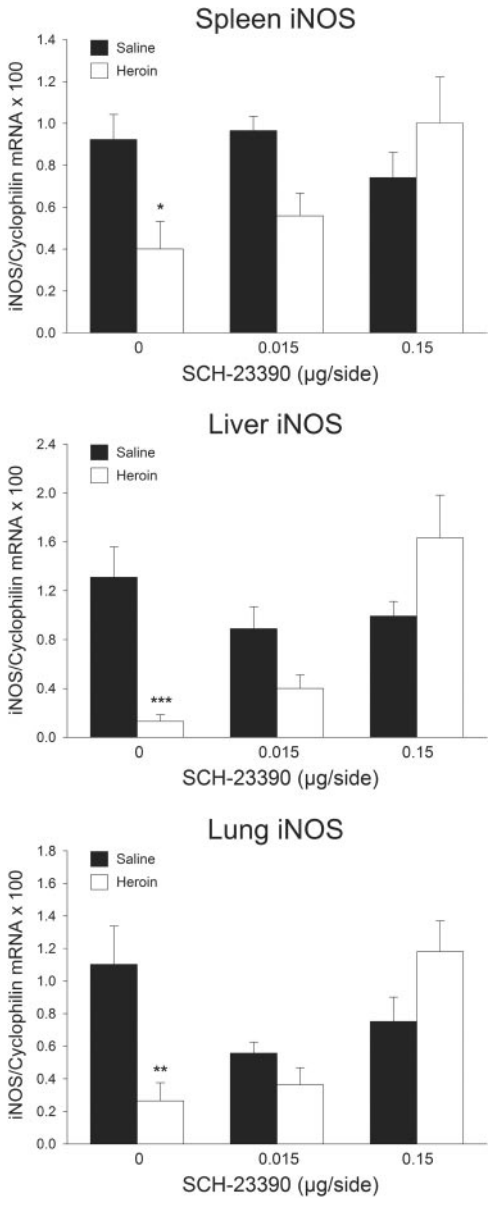

Fig. 4. Effect of D_1 receptor antagonism in the nucleus accumbens on heroin-induced reductions of iNOS mRNA expression. Rats received microinjections of SCH-23390 into the nucleus accumbens shell before subcutaneous injections of saline or heroin in conjunction with LPS administration. Data are expressed as the ratio of iNOS mRNA expression to expression of the housekeeping gene cyclophilin (mean ± S.E.). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with the saline group that received an equivalent dose of SCH-23390.

est dose of SCH-23390 were not significantly different with respect to iNOS expression in the spleen [$F(1,33) = 0.23$; $p > 0.05$], liver [$F(1,33) = 3.96$; $p > 0.05$], or lung [$F(1,33) = 1.37$; $p > 0.05$].

Effect of D_1 Receptor Antagonism on Plasma Nitrite/Nitrate Levels. SCH-23390 administration into the nucleus accumbens shell also attenuated the effects of heroin on LPS-induced nitric oxide levels in the plasma as determined by the Greiss reagent assay (Fig. 5). Analysis of variance revealed a significant interaction between SCH-23390 dosage and heroin treatment [$F(2,33) = 7.03$; $p < 0.01$], indicating that the effect of heroin differed as a function of SCH-23390 dosage. Planned comparisons demonstrated that heroin significantly reduced plasma nitrite/nitrate levels compared with saline treatment among animals that received saline microinjections [$F(1,33) = 50.80$; $p < 0.0001$]. SCH-23390 microinjections did not completely block heroin's effect however, because planned comparisons showed significant differences between heroin and saline groups at the highest dose of SCH-23390 [$F(1,33) = 5.29$; $p < 0.05$].

Discussion

Although there is substantial evidence that opioids disrupt parameters of immune status by interacting with μ-opioid receptors in the central nervous system, the neural mechanisms that underlie these effects are poorly understood. Alterations in dopamine signaling produced by opioid exposure may be particularly relevant to opioid-induced immune dysfunction given reports that mesoaccumbens dopamine neurons modulate the immune response (Deleplanque et al., 1994; Devoino et al., 1997). Previous work from our laboratory has demonstrated that morphine-induced suppression of NK cell activity is mediated by dopamine D_1 type receptors located in the nucleus accumbens shell, whereas dopamine D_2 type receptors do not seem to be involved (Saurer et al., 2006a). The present study supports and extends our previous work by showing that D_1 receptors in the nucleus accumbens shell also mediate the inhibitory effects of heroin on splenic NK activity and LPS-induced nitric oxide production.

Before the examination of dopamine receptor mechanisms

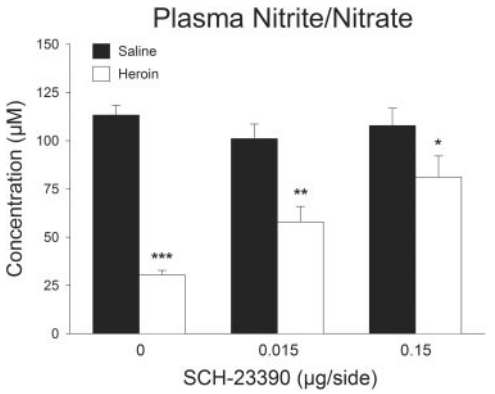

Fig. 5. Effect of D_1 receptor antagonism in the nucleus accumbens on heroin-induced reductions of plasma nitrite/nitrate. Data are expressed as the plasma nitrite/nitrate concentration in micromolar (mean ± S.E.). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with the saline group that received an equivalent dose of SCH-23390.

in the present study, a dose-response analysis was conducted to determine the lowest dose of heroin needed to induce an optimal suppression of functional immune measures, i.e., NK activity and splenocyte proliferative responses to the T-cell mitogen Con-A, and the B-cell mitogen LPS. The heroin doses used in the present study (1, 3, 5, and 10 mg/kg) were selected based on a previous report that heroin significantly inhibits these parameters at doses as low as 1 mg/kg, with more pronounced effects at 10 mg/kg (Fecho and Lysle, 2000). The present results reveal that heroin induces a maximal suppression of NK activity at doses of 1 mg/kg or higher, whereas doses of 3 mg/kg or higher produce the most prominent effects on lymphocyte proliferative responses. Therefore, heroin was administered at a dose of 3 mg/kg in the subsequent manipulations involving SCH-23390 microinjections.

The present findings indicate the effects of heroin on immune status involve both dopamine-dependent and dopamine-independent neural mechanisms. These results are consistent with previous reports that the centrally mediated effects of opioids on different immune parameters involve distinct neuroimmune pathways. For example, microinjection of morphine into the periaqueductal gray matter inhibits splenic NK activity but does not affect lymphocyte proliferation (Weber and Pert, 1989; Lysle et al., 1996). Furthermore, administration of β-adrenergic blockers selectively prevents reductions in splenic lymphocyte proliferation after morphine treatment (Fecho et al., 1993), whereas neuropeptide Y_1 receptor antagonism selectively blocks the suppression of NK activity (Saurer et al., 2006b). The current results provide additional evidence that distinct neural pathways are involved in these effects by showing that SCH-23390 administration abolishes the suppressive effect of heroin on NK activity but does not prevent decreases in lymphocyte proliferative responses to Con-A or LPS. These results corroborate our previous findings that the stimulation of dopamine receptors with the selective D_1 agonist SKF38393 inhibits splenic NK activity (Saurer et al., 2006a) without affecting lymphocyte proliferation (unpublished observations).

In contrast to current knowledge regarding opioid effects on NK cytolytic activity and lymphocyte proliferative responses, the physiological mechanisms by which opioids modulate nitric oxide production in response to endotoxin (LPS) exposure are poorly understood. Some studies have shown that opioids can modulate LPS-induced nitric oxide production in vitro (Iuvone et al., 1995; Singhal et al., 1998), whereas others demonstrate the involvement of central opioid mechanisms (Gomez-Flores et al., 1999). Further complicating efforts to elucidate precise mechanisms are reports that morphine may augment (Martucci et al., 2007) or inhibit (Iuvone et al., 1995) the generation of nitric oxide in response to LPS. Such discrepancies are probably because of numerous factors, including, but not limited to, the dose of morphine, the duration of exposure, the cell types or tissues under investigation, the time point at which nitric oxide is measured, and the temporal relationship between opioid exposure and LPS stimulation. Moreover, because immunomodulation involves complex interactions between many physiological systems, removal of immune cells from the organism for in vitro assays may preclude the influence of endocrine and autonomic systems that regulate immune functions under normal physiological conditions. Therefore, it is imperative to validate in vitro and ex vivo findings with in vivo models. The results of studies using in vivo models of LPS-induced endotoxemia in the rat demonstrate that heroin administration reduces protein and mRNA levels of iNOS in several organs and decreases plasma nitrite/nitrate levels (Lysle and How, 2000; Lanier et al., 2002). The present experiments extend these findings and provide the first evidence that these effects of heroin are mediated by the CNS by showing that reductions of LPS-induced iNOS production in spleen, liver, and lung tissues are dependent on the activation of dopamine D_1 receptors in the nucleus accumbens. In addition to identifying the nucleus accumbens as a critical neural substrate of the effects of heroin on in vivo immune responses, these findings are important because they suggest that the dopaminergic input to the nucleus accumbens per se may have a fundamental role in immune regulation.

Although the results of the current study implicate the CNS in modulating heroin-induced alterations of iNOS production, it is possible that heroin may have produced concomitant local elevations of nitric oxide in peripheral cells that were not detectable at the tissue level. As mentioned above, several investigators have shown that morphine augments macrophage-derived nitric oxide production under basal and LPS-stimulated conditions in vitro (Singhal et al., 1998; Martucci et al., 2007). However, because heroin metabolites act at multiple receptor sites throughout the body, any changes in iNOS production that are observed in the in vivo milieu should reflect the overall influence of heroin, despite potentially contrasting central and peripheral influences, or discordant effects on individual cell types. Although speculative, one possibility is that the centrally mediated inhibitory effects of heroin on iNOS production may override any peripheral or direct stimulatory effects. It is interesting to note that animals that received the highest dose of SCH-23390 in combination with heroin actually showed a trend toward increased iNOS expression compared with the controls (Fig. 4). Because SCH-23390 treatment alone did not increase iNOS levels, it is possible that blockade of the central pathways responsible for heroin's inhibitory influence may have unmasked a more subtle peripheral stimulatory effect. An important issue for future studies will be to tease apart the relative contributions of central versus peripheral influences of heroin on iNOS production in vivo.

An interesting question raised by the current findings is how alterations in central dopamine receptor activity translate to changes in peripheral immune function. Although the efferent pathway from the nucleus accumbens to peripheral immune compartments has not been specifically identified, there is accumulating evidence that the sympathetic nervous system may be involved. For example, neural control of splenic NK cell activity is mediated primarily by the sympathetic nervous system via the splenic nerve (Irwin et al., 1990; Katafuchi et al., 1993), and prior investigations have demonstrated that neuropeptide Y, a sympathetic transmitter in the peripheral nervous system, mediates reductions in NK cell activity produced by either systemic morphine or intra-accumbens D_1 agonist administration (Saurer et al., 2006b). The nucleus accumbens shell has direct connections with several hypothalamic nuclei such as the lateral hypothalamus, which are known to modulate splenic NK activity (Heimer et al., 1991; Wenner et al., 1996). Thus, one possibility is that dopamine transmission within the nucleus accumbens modulates splenic NK activity through interactions with hypothalamic autonomic regulatory centers. Although less is known about the central mechanisms that modulate iNOS expression in peripheral tissues such as the spleen, liver, and lung, the sympathetic nervous system may likewise play a major role. Interestingly, stress-induced suppression splenic cytokine responses to LPS were shown to be mediated by the sympathetic nervous system (Meltzer et al., 2004). Given the many similarities between the effects of stress and exogenous opioids on immune function, it is tempting to speculate that similar mechanisms mediate heroin's effect on nitric oxide production. Indeed, several stressor-induced immune alterations are actually mediated by endogenous opioids (Shavit et al., 1984; Tseng et al., 2005), and both exogenous opioids and acute stressors induce marked increases in nucleus accumbens dopamine levels (Rougé-Pont et al., 1993; Pontieri et al., 1995). However, further research is necessary to evaluate whether elevations in accumbens dopamine modulate stress-induced immune alterations in a manner similar to heroin.

Overall, the present study provides substantial evidence for the involvement of the dopaminergic input to the nucleus accumbens in heroin-induced immune alterations. In addition, the present findings are the first to demonstrate that the effects of heroin on nitric oxide production are modulated by the CNS. Given the importance of nitric oxide in resistance to infectious diseases and immune regulation, the current findings suggest that heroin-induced alterations in dopamine signaling may be directly related to the immunological abnormalities and increased infection susceptibility among heroin users. Furthermore, as elevations in dopamine transmission are a common feature among various immunomodulatory stimuli, these findings have may have important implications regarding endogenous physiological mechanisms of neuroimmune regulation.

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