

Association of time-dependent changes in mu opioid receptor mRNA, but not BDNF, TrkB, or MeCP2 mRNA and protein expression in the rat nucleus accumbens with incubation of heroin craving

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Abstract

Rationale and objectives Responding to heroin cues progressively increases after cessation of heroin self-administration (incubation of heroin craving). We investigated whether this incubation is associated with time-dependent changes in brain-derived neurotrophic factor (BDNF) and methyl-CpG binding protein 2 (MeCP2) signaling and mu opioid receptor (MOR) expression in nucleus accumbens (NAc), dorsal striatum (DS), and medial prefrontal cortex (mPFC). We also investigated the effect of the preferential MOR antagonist naloxone on cue-induced heroin seeking during abstinence.

Methods We trained rats to self-administer heroin or saline for 9–10 days and then dissected the NAc, DS, and mPFC at different abstinence days and measured mRNA and protein levels of BDNF, TrkB, and MeCP2, as well as MOR mRNA (*Oprm1*). In other groups, we assessed cue-induced heroin seeking in extinction tests after 1, 11, and 30 abstinence days, and naloxone's (0–1.0 mg/kg) effect on extinction responding after 1 and 15 days.

Results Cue-induced heroin seeking progressively increased or incubated during abstinence. This incubation was not associated with changes in BDNF, TrkB, or MeCP2 mRNA or protein levels in NAc, DS, or mPFC; additionally, no

molecular changes were observed after extinction tests on day 11. In NAc, but not DS or mPFC, MOR mRNA decreased on abstinence day 1 and returned to basal levels over time. Naloxone significantly decreased cue-induced heroin seeking after 15 abstinence days but not 1 day.

Conclusions Results suggest a role of MOR in incubation of heroin craving. As previous studies implicated NAc BDNF in incubation of cocaine craving, our data suggest that different mechanisms contribute to incubation of heroin versus cocaine craving.

Keywords BDNF · Extinction · MeCP2 · μ opioid receptor · Heroin self-administration · Relapse · Withdrawal · Incubation of drug craving · Naloxone

Introduction

Relapse to heroin use in humans can occur after prolonged abstinence and is often triggered by exposure to heroin-associated cues that provoke drug craving (Wikler 1973; O'Brien et al. 1986). Using extinction and reinstatement procedures in rats, we and others reported time-dependent increases in cue-induced cocaine (Neisewander et al. 2000; Grimm et al. 2001) and heroin (Shalev et al. 2001; Zhou et al. 2009) seeking after forced abstinence (termed herein “abstinence”) from the drugs. This phenomenon was termed incubation of drug craving (Grimm et al. 2001). Studies on the neurobiological mechanisms of incubation of drug craving have focused primarily on cocaine (Lu et al. 2005; Hollander and Carelli 2007; Conrad et al. 2008; Koya et al. 2009; Wolf and Ferrario 2010; Pickens et al. 2011).

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Recent studies have begun to explore mechanisms of incubation of heroin craving. Kuntz et al. (2008, 2009) assessed changes in mRNA expression of several immediate early genes (*Arc*, *EGR1*, *EGR2*, *Fos*, and *Homer1b/c*) in nucleus accumbens (NAc) and medial prefrontal cortex (mPFC), as well as other genes (brain-derived neurotrophic factor (*Bdnf*), *Calb1*, *Dusp5*, *Dusp6*, *Npy*, and *Rgs2*) in mPFC following extinction tests for cue-induced heroin seeking on abstinence days 1 and 14. They found that time-dependent increases in cue-induced heroin seeking were associated with time-dependent increases in *Bdnf*, *Dusp5*, and *Calb1* mRNA levels in mPFC. Recently, we found that incubation of heroin craving is associated with time-dependent increases in ventral tegmental area (VTA) and NAc glial cell-line derived neurotrophic factor (*Gdnf*) mRNA (Airavaara et al. 2011). However, interfering with endogenous GDNF function by chronic delivery of anti-GDNF antibodies into VTA or NAc had no effect on this incubation. These studies illustrate that critical causal mechanisms of incubation of heroin craving are yet to be identified.

Here, we first assessed expression changes in BDNF, TrkB (the BDNF receptor), and methyl-CpG binding protein 2 (MeCP2) signaling in NAc, dorsal striatum (DS), and mPFC after 1, 11, and 30 days of abstinence from heroin. We chose the NAc, because previous studies indicate that activation of BDNF-TrkB signaling in this brain area potentiates incubation of cocaine craving, conditioned responding to cocaine-associated cues, and cocaine reward (Grimm et al. 2003; Bahi et al. 2008; Graham et al. 2007, 2009; Ghitza et al. 2010). We chose the DS, because a recent study indicates a role of both BDNF and MeCP2 signaling in this brain area in escalation of cocaine self-administration (Im et al. 2010). We chose the mPFC because previous studies indicate that activation of BDNF-TrkB signaling in this brain area inhibits cocaine reward, as well as cue- and cocaine-priming-induced reinstatement of cocaine seeking (Berglind et al. 2007; McGinty et al. 2010; Sadri-Vakili et al. 2010). We also investigated BDNF and MeCP2 signaling in mPFC and NAc because exposure to psychostimulant drugs decreases MeCP2-BDNF promoter IV interaction in mPFC (Sadri-Vakili et al. 2010) and increases MeCP2 phosphorylation in NAc (Deng et al. 2010).

We found no evidence for changes in BDNF and MeCP2 signaling in NAc, DS, and mPFC during abstinence from heroin self-administration. Therefore, we used the same brain tissues to assess time-dependent changes in the mRNA levels of the mu opioid receptor (MOR). Activation of MOR in NAc plays a critical role in heroin self-administration (Vaccarino et al. 1985; Koob 1992). Additionally, studies using systemic injections of the preferential MOR antagonists naloxone and naltrexone (Goldstein and Naidu 1989) have

demonstrated a role of MOR in cue-induced reinstatement of alcohol, amphetamine, and nicotine seeking, as well as incubation of sucrose craving (Liu and Weiss 2002; Burattini et al. 2006; Grimm et al. 2007; Burattini et al. 2008; Liu et al. 2009). However, the role of MOR in incubation of heroin craving or cue-induced heroin seeking is yet to be determined. We found that in NAc, but not mPFC or DS, MOR mRNA levels decreased on abstinence day 1 and returned to basal levels on days 11 and 30. Therefore, in a follow-up study to determine a functional role of the MOR in incubation of heroin craving, we assessed the effect of systemic injections of naloxone on cue-induced heroin seeking in extinction tests performed on abstinence days 1 and 15.

Materials and methods

Methods overview

In experiments 1 and 2, we trained rats to self-administer heroin over 10 days for 6 h/days. Some rats were then tested for cue-induced heroin seeking in extinction tests during abstinence days 1, 11, and 30 while others were killed at the same time but were never tested in extinction for cue-induced heroin seeking. Different rats were used for the behavioral and molecular experiments, because we investigated time-dependent basal heroin-induced neuroadaptations in NAc, DS and mPFC during abstinence rather than cue-induced acute molecular changes. We measured mRNAs and protein levels of BDNF, BDNF's preferential subtype TrkB (Chao and Hempstead 1995; Reichardt 2006) (phosphorylated and total), and MeCP2. We also measured mRNA levels of MOR in NAc, DS, and mPFC. Due to a lack of commercial (or non-commercial) antibodies selective for MOR protein, we were unable to measure MOR-1 protein levels in NAc, DS, and mPFC using Western Blot.

In addition to the evaluation of the basal levels of the molecules of interest in the NAc, DS, and mPFC on abstinence days 1, 11, and 30, we also assessed acute molecular changes in the different signaling molecules in NAc and DS in the group of rats tested for cue-induced heroin seeking in extinction tests on abstinence day 11. After the 30-min extinction test, the heroin-trained rats were immediately decapitated and their brains removed for subsequent molecular analyses. These analyses were ran in parallel with the group of rats that were trained for heroin-self-administration for 10 days, killed on abstinence day 11 but were never re-exposed to the self-administration chambers.

In experiment 3, we trained rats to self-administer heroin for 9 days (9 h/day) and then tested for cue-induced heroin seeking in extinction tests on abstinence days 1 and 15.

Prior to the extinction tests, rats were divided into three groups according to their mean number of infusions and active lever presses during heroin self-administration training. Two groups received either 0.3 or 1.0 mg/kg of naloxone, respectively, while a third group received saline vehicle injections. All injections were given 10 min prior to the extinction tests.

Subjects

Male Sprague–Dawley rats (Charles River, Raleigh, NC), weighing 350–400 g prior to surgery, were used. The rats were group-housed (two per cage) prior to surgery and individually housed after surgery. The rats were maintained in the animal facility under a reversed 12:12-h light/dark cycle with food and water freely available. Procedures followed the guidelines outlined in the Guide for the Care and Use of Laboratory Animals (eighth edition; <http://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-Use-of-Laboratory-Animals.pdf>). In experiments 1 and 2, out of the 109 rats, 3 were excluded due to catheter problems and 10 due to failure to acquire stable heroin self-administration. In experiment 3, out of the 54 rats, 11 were excluded due to catheter problems and 4 due to failure to acquire stable self-administration.

Intravenous surgery

Rats were anesthetized with sodium pentobarbital and chloral hydrate (60 and 25 mg/kg, i.p., respectively), and silastic catheters were inserted into the jugular vein, as described previously (Lu et al. 2007; Bossert et al. 2009). The catheters were attached to a modified 22-gauge cannula and mounted to the rats' skulls with dental cement. Buprenorphine (0.1 mg/kg, subcutaneous (s.c.)) was given after surgery to relieve pain and rats were allowed to recover for 5–7 days before heroin self-administration training. During the recovery and training phases, catheters were flushed every 24–48 h with gentamicin (5 mg/ml, Butler Schein, Dublin, OH) and sterile saline.

Apparatus

The rats were trained in self-administration chambers, which were located inside sound-attenuating cabinets and were controlled by a Med Associates (Georgia, VT) system. Each chamber had two levers located 9 cm above the floor. Presses on one active and retractable lever activated the infusion pump; presses on the other inactive and stationary lever had no consequences. The rat's catheter was connected via a modified cannula (Plastics One, Roanoke, VA) to a liquid swivel (Instech, Plymouth Meeting, PA) via polyethylene-50 tubing that was protected by a metal spring.

Behavioral procedures

Training phase

The training procedure is similar to that used in our previous studies on incubation of cocaine and heroin craving (Lu et al. 2004, 2005; Koya et al. 2009; Airavaara et al. 2011). On the first day of training, the rats were brought to the self-administration room where they were chronically housed in the experimental chambers. In experiments 1 and 2, rats were trained to self-administer heroin (diacetylmorphine HCl; NIDA, Baltimore, MD) or sterile saline (a control condition) during six 1-h sessions per day (the sessions were separated by 5 min) over 10 days under a fixed-ratio-1 with 20-s timeout reinforcement schedule. Heroin was dissolved in saline and self-administered at a dose of 0.075 mg kg⁻¹ infusion⁻¹ over 3.5 s (0.10 ml/infusion). The heroin unit dose is based on our previous work (Airavaara et al. 2011). In experiment 3, rats were trained to self-administer heroin for 9 days under the same experimental conditions, except that the daily training comprised of three 3-h sessions/day (the sessions were separated by 1 h) and the heroin unit dose was 0.1 mg/kg⁻¹ infusion⁻¹ (Shalev et al. 2001). In all experiments, active lever responses led to the delivery of a heroin infusion and a 5-s tone-light compound cue (2,900 Hz, 20 dB above background; 7.5 W white light located 4 cm above the active lever). The onset of each infusion was followed by a 20-s timeout period, during which lever presses were recorded but did not result in any infusions or compound cue delivery. The self-administration sessions started at the onset of the dark cycle and began with the insertion of the active lever and the illumination of a red house light that remained on for the duration of the session. At the end of each 1-h (experiments 1 and 2) or 3-h (experiment 3) session, the house light was turned off, and the active lever was retracted. At the end of the training phase, the groups to be tested on different days were matched for their drug intake and active lever presses during training.

Abstinence phase

At the end of the training phase, the rats were brought back to the animal colony room and handled three times per week during the abstinence phase. In experiments 1 and 2, the rats to be tested were then brought to the self-administration chambers on the morning of the extinction tests, which were conducted 1, 11, or 29–33 days (hereafter termed day 30; experiment 1) or 1 and 15 days (experiment 3) after the last heroin self-administration session. Half of the heroin-trained

rats underwent extinction tests; the other half and the saline control rats were not re-exposed to the training chambers.

Extinction tests

The extinction tests in the presence of the heroin-associated cues consisted of a single 30-min extinction session on abstinence days 1, 11, and 30 for experiments 1 and 2 or abstinence days 1 and 15 for experiment 3. The experimental conditions were the same as those in the training phase, except that presses on the previously active lever were not reinforced with heroin. Tests started at the onset of the dark cycle and began with the insertion of the active lever and the illumination of the red house light, which remained on for the duration of the session. Active lever responses during testing resulted in contingent presentations of the tone-light cue that was previously paired with heroin infusions, but not heroin.

Experiment 1—time-dependent increases in extinction responding during abstinence from heroin

The purpose of this initial experiment was to verify that time-dependent increases in cue-induced heroin seeking in extinction tests (incubation of heroin craving) are reliably observed at the different abstinence days in which we assessed mRNA and protein expression changes in experiment 2. Three groups of rats ($n=10\text{--}12/\text{group}$) were trained to self-administer heroin for 10 days and were then given a 30-min extinction test on abstinence days 1, 11, or 30; the rats tested on abstinence days 1 and 30 were trained at the same time while the rats tested on abstinence day 11 were trained at a different time. The rats tested on day 11 were rapidly decapitated immediately after the 30-min extinction tests and their brains were extracted for subsequent molecular analyses to assess acute changes in the different signaling molecules (see above and below).

Experiment 2—effect of heroin self-administration on mRNA and protein expression of BDNF, TrkB, MeCP2, and MOR

The purpose of experiment 2 was to determine whether time-dependent increases in cue-induced heroin seeking (incubation of drug craving) during abstinence are associated with time-dependent changes in basal levels of BDNF, TrkB, MeCP2, and MOR in NAc, DS, and mPFC. Six groups of rats ($n=5\text{--}8/\text{group}$) were used in a 2 (training drug, heroin or saline) \times 3 (abstinence day—1, 11, or 30) between-subjects factorial design. The rats were trained to self-administer heroin or saline for 10 days and returned to the animal colony room for their drug-free period. On abstinence day 1, 11, or 30, the rats were rapidly decapitated and their brains were

rapidly extracted. The rats of abstinence days 1 and 30 were trained at the same time while the rats of abstinence day 11 were trained at a different time; the brains of the latter rats were taken at the same time as the rats that underwent the extinction test on abstinence day 11 in experiment 1.

Samples processing for quantitative real-time polymerase chain reaction and Western Blots

Rats were decapitated 1, 11, or 30 days after heroin or saline self-administration, and their brains were removed. Brains were rapidly frozen in $-50\text{ }^{\circ}\text{C}$ isopentane and stored at $-80\text{ }^{\circ}\text{C}$. Bilateral tissue punches of the NAc, DS (approximate AP levels of $+2.4\text{ mm}$ from Bregma (Paxinos and Watson 2005)), and mPFC (approximate AP levels of $+3.7\text{ mm}$ from Bregma) were taken from 1 mm coronal sections cut in a cryostat at $-20\text{ }^{\circ}\text{C}$.

Quantitative RT-PCR assay

Total RNA was isolated from tissue punches obtained from the right side of each brain ($\sim 1\text{ mg}$) using TRIzol Reagent protocol (Invitrogen-Life Technologies, Grand Island, NY) or Agencourt RNAdvance Tissue Kit (Beckman Coulter, Beverly, MA). The RNA integrity numbers were measured using Agilent RNA 600 Nano kit (Agilent Technologies, Waldbronn, Germany) and found to be above eight for all samples. Single-strand cDNAs were synthesized with the Superscript III first strand cDNA synthesis kit according to the manufacturer's protocol (Invitrogen, Life Technologies, Carlsbad, CA).

We measured the long isoforms of BDNF and MeCP2 mRNA because BDNF long isoform confers translational activation by neuronal activity (Lau et al. 2010) and MeCP2 long isoform predominates in the brain and influences BDNF expression (Klein et al. 2007). Fluorescent FAM TaqMan probes were designed using Primer Express 3.0 (Applied Biosystems, Life Technologies, Carlsbad, CA) in the long 3' untranslated regions (3'UTR) of rat BDNF (BDNF_A2, forward primer: GGAGACCCTCCG CAACTGT; reverse primer: GAGCTATGATGT ATCTTAGTGGGTATGAG; MGB probe: TGGTCAGTGGCTGGC) and MeCP2 (MeCP2_A2, forward primer: GTGACAAGCCACCCTGTATTTG; reverse primer: CCTTGTCTGTCCCAACCTTAGATG; MGB probe: ACCAGCTCAAAAAC) and the splicing junctions of the non-truncated rat TrkB between exon 12 and 13 (forward primer: TGGCGAGACATTCCAAGTTTG; reverse primer: AGAGTCATCGTCTGTTGCTGATG; MGB probe: CATGAAAGGCCAGCTT) for quantitative real-time polymerase chain reaction (RT-PCR) analysis.

Rat MOR TaqMan probe was ordered from Applied Biosystems (Rn01430371_m1) and designed to measure

between constitutively spliced exons 3 and 4 of rat *Oprm1* gene in order to detect all isoforms.

Duplex qPCR assays were carried out with technical duplications using the endogenous control Vic-labeled rat GAPDH (ABI cat: 4352338E) with TaqMan® Fast Universal PCR Master Mix in a 7500 Fast TaqMan instrument and with a default thermo-cycling program. Quantification of RT-PCR was carried out according to User Bulletin #2 for ABI Prism 7900 Sequence Detection System. Briefly, ΔC_t values were derived from subtraction of the average C_t value (technical replications) of the target genes (BDNF, MeCP2, TrkB, and MOR) from that of the endogenous control gene for each sample. The $\Delta\Delta C_t$ values for each experimental pair were derived by subtracting average ΔC_t values of test group samples from average ΔC_t values of control group samples, corresponding to the abstinence days (1, 11, and 30). The fold changes were calculated using the $2^{-\Delta\Delta C_t}$ method (Schmittgen and Livak 2008).

Western blot assay

Samples were homogenized using pestles and microtubes (ISC BioExpress) in 100 μ l of 1× RIPA lysis buffer (Cell Signaling, Danvers, MA) containing inhibitor cocktails for phosphatases (PhosSTOP, Roche Applied Sciences, Indianapolis, IN) and proteases (cOmplete, Ultra tablets, EDTA-free, Roche Applied Sciences) and subsequently sonicated for 10 s. Samples were then reduced with 20 mM DTT at 45 °C for 90 min and centrifuged at room temperature for 10 min. Supernatants were collected and protein concentrations were measured using the Bradford method (Bio-Rad Laboratories, Hercules, CA). Samples were diluted to a final concentration of 1 μ g/ μ l with the lysis buffer and the 4× LDS NuPAGE sample buffer (Invitrogen-Life Technologies). Before electrophoresis, the samples were heated at 70 °C for 10 min and 10 μ g of proteins were loaded onto either a 12 % for BDNF or a 4–12 % NuPAGE neutral polyacrylamide gel (Invitrogen-Life Technologies) for TrkB, pY817 TrkB, and MeCP2. Electrophoresis was carried out in 1× MES buffer for BDNF and in 1× MOPS buffer for TrkB, pY817 TrkB, and MeCP2. After electrophoresis, proteins in the gel were transferred to an Immobilon-FL (Millipore) membrane using NuPAGE transfer buffer (Invitrogen-Life Technologies). β -tubulin serves as endogenous control to normalize for sample loading variability. The following primary antibodies were used: rabbit polyclonal antibody to β -Tubulin (1:10,000; # 926-42211; Li-Cor Biosciences, Lincoln, Nebraska), rabbit polyclonal antibody to BDNF (N-20): sc-546 (1:500; Santa Cruz Biotechnology, Inc.; Santa Cruz, CA), rabbit polyclonal antibody to TrkB (1:750; ab51190; Abcam, Cambridge, MA), rabbit monoclonal antibody to phospho-tyrosine 817

TrkB (pY817TrkB) (1:750; # 2149-1; Epitomics, Burlingame, CA), rabbit polyclonal antibody to MeCP2 (1:500; # 07-013; Millipore, Billerica, MA). The incubation time for all primary antibodies was 1 h. Finally, we assessed the specificity of three commercially available antibodies targeting the N- and C-terminal epitopes of MOR protein (SAB4502048, SAB4300555 and SAB4502047 from Sigma-Aldrich, St. Louis, Mo) for Western blot assay using a positive control of SH-SY5Y neuroblastoma cell line, a negative control of CHO cell line, and NAc, DS, and mPFC tissue lysates. No consistent banding patterns could be observed in our samples under the experimental conditions used (data not shown). Thus, MOR protein expression in NAc, DS, and mPFC could not be measured by Western blot using the Sigma antibodies.

All procedures (e.g., the blocking, primary antibody incubation, washing, secondary antibody incubation, and washing) were carried out according to the Li-Cor Western Blot protocol. After incubation with primary antibodies, blots were washed and incubated for 90 min with anti-rabbit secondary antibodies labeled with IRDyes 680 (Li-Cor Biosciences, # 926-32221). Fluorescence from the fluorophore was assessed using an Odyssey IR fluorescence scanner (Li-Cor Biosciences) and bands were quantified using Odyssey 2.0 software.

Experiment 3—effect of naloxone on extinction responding on abstinence days 1 and 15

The aim of experiment 3 was to assess the effect of the preferential MOR antagonist naloxone (0, 0.3, and 1.0 mg/kg, s.c.) on cue-induced heroin seeking in extinction tests 1 and 15 days after the last heroin self-administration session. Three groups of rats ($n=11$ –16/group) were trained to self-administer heroin for 9 days and were then given a 30-min extinction test on abstinence days 1 and 15. Prior to testing, the rats received injections of either saline (vehicle group, $n=14$) or 0.3 ($n=11$) or 1.0 mg/kg ($n=16$) of naloxone. All injections were given subcutaneously at a volume of 1.0 ml/kg. (–)-naloxone was purchased from Sigma (St. Louis, Mo) and dissolved in sterile saline. The naloxone doses tested are based on previous studies (Li et al. 2011; Grimm et al. 2007).

Statistical analyses

Data were analyzed with the statistical program SPSS (general linear model (GLM)) procedure and significant effects ($p<0.05$) were followed by SPSS post-hoc contrasts within the GLM repeated measures ANOVA module. The dependent variables during the training phase of experiments 1 and 2 were the number of infusions

earned and the number of active and inactive lever presses; the independent variable was the training drug (saline and heroin). In experiment 1, the dependent variables for the extinction test were non-reinforced active lever presses and inactive lever presses and the independent variable was the abstinence day. In experiment 2, the protein and mRNA data were analyzed in a two-way independent ANOVA with training drug (saline and heroin) and abstinence day (days 1, 11, or 30) as the between-subject factors. The level of each protein of interest was normalized to β -tubulin and the normalized value was the dependent variable in the statistical analysis of the different proteins. Fold change (see above) was the dependent measure in the analysis of the different mRNAs. In experiment 3, for the self-administration training data, the dependent variables were the number of infusions earned, the number of active and inactive lever presses performed during the 9 h of the training session, and the independent variable was group (the three experimental groups). For the extinction test, the data were analyzed in three-way mixed ANOVA with lever (active and inactive) and abstinence day (days 1 and 15) as the within-subject factors and naloxone dose (0, 0.3, or 1.0 mg/kg) as the between-subject factor.

Results

Experiments 1 and 2—heroin self-administration training

The heroin-trained rats (total $n=69$) increased their number of infusions earned over time while the saline-trained rats ($n=27$) decreased theirs (Fig. 1a; training drug \times training session ($F(9, 846)=5.3, p<0.05$). Similarly, the number of active lever presses increased over time for the heroin-training rats while the saline-trained rats decreased their responding (Fig. 1b; training drug \times training session \times lever ($F(9, 846)=5.9, p<0.05$). The number of heroin infusions earned during training did not differ between the rats tested for incubation of heroin craving (experiment 1) and those used for the molecular assessment without testing (experiment 2; $p>0.01$).

Experiment 1—time-dependent increases in extinction responding during abstinence from heroin self-administration

The statistical analysis of total responding demonstrated significant effects of lever ($F(1, 31)=80.1, p<0.05$), abstinence day ($F(2, 31)=8.4, p<0.01$) and an interaction between the two factors ($F(2, 31)=7.7, p<0.01$). Post-hoc

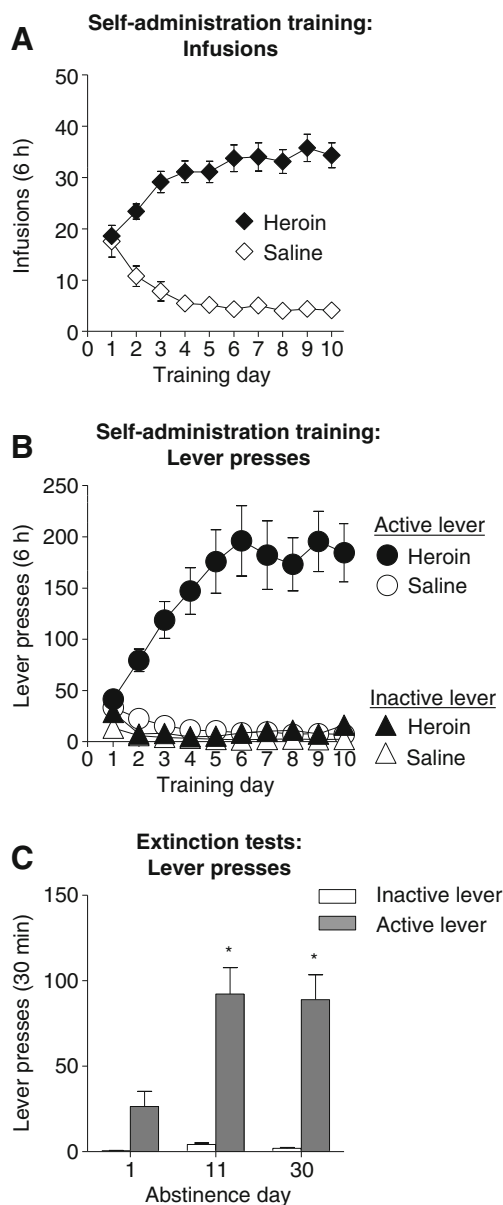


Fig. 1 Heroin self-administration training and time-dependent increases in cue-induced heroin seeking in extinction tests (incubation of heroin craving). **a–b** Training days 1–10—infusions and lever presses. Data are mean \pm SEM number of heroin ($0.075 \text{ mg kg}^{-1} \text{ injection}^{-1}$, $n=69$) or saline ($n=27$) infusions or active and inactive lever presses during the ten 6-h daily self-administration sessions. During training, active lever presses were reinforced under a fixed-ratio-1, 20-s timeout reinforcement schedule while inactive lever presses were never reinforced; heroin injections were paired with a 5-s tone-light cue. **c** Extinction tests—lever presses, data are mean \pm SEM responses on the previously active lever and on the inactive lever during the 30-min extinction sessions, conducted in the presence of the heroin-associated cues but in the absence of heroin ($n=10$ – 12 /group). * $p<0.05$, different from abstinence day 1

analyses demonstrated that responding on the previously active lever in the extinction tests was higher during 11 and 30 days of abstinence than after 1 day (Fig. 1c).

Experiment 2—effect of heroin self-administration on mRNA and protein expression of BDNF, TrkB, MeCP2, and MOR expression

Brain dissection sites and representative blots of protein expression of MeCP2 and BDNF signaling pathways in NAc, DS, and mPFC are shown in Suppl. Fig 1. The levels of mRNA and protein expression of BDNF, TrkB, MeCP2, and MOR expression in NAc, DS, and mPFC, 1, 11, or 30 days after cessation of heroin self-administration are summarized in Table 1.

At the protein level, there was a main effect of abstinence day for BDNF in all three brain areas, TrkB in DS, and

MeCP2 in mPFC ($p < 0.05$). This abstinence day effect is due to higher expression levels on day 11 than on days 1 and 30 (data not shown); this effect likely reflects the fact that the rats from the day 11 groups were trained at a different time point than the rats from the days 1 and 30 groups.

We also measured the protein levels of BDNF and MeCP2 in NAc and DS of the rats that were tested for extinction responding on abstinence day 11 and had their brain extracted immediately after the 30-min test. We compared those results to the BDNF and MeCP2 protein levels of the no-test heroin-trained rats, whose brains were extracted at the same time. Overall, we found no significant differences between the test and no-test rats (Table 2).

Table 1 mRNA and protein expression of BDNF, TrkB, MeCP2, and MOR in nucleus accumbens, dorsal striatum, and medial prefrontal cortex after heroin self-administration

Group	Saline			Heroin		
	1	11	30	1	11	30
Nucleus accumbens						
mRNA						
MeCP2	0.98±0.05	1.01±0.06	1.02±0.03	0.92±0.08	1.08±0.06	1.25±0.13
BDNF	1.11±0.23	1.12±0.20	1.57±0.55	0.83±0.15	0.78±0.13	1.84±0.37
TrkB	1.07±0.15	0.95±0.10	1.04±0.12	0.93±0.13	0.95±0.10	1.01±0.16
MOR	1.01±0.07	1.04±0.10	1.00±0.70	0.66±0.05*	0.89±0.07	1.06±0.08
Protein						
MeCP2	0.42±0.04	0.50±0.06	0.55±0.08	0.42±0.04	0.45±0.04	0.48±0.06
BDNF	2.10±0.68	0.62±0.09	0.40±0.10	1.58±0.33	0.61±0.17	0.35±0.09
TrkB	0.15±0.02	0.14±0.01	0.13±0.01	0.15±0.01	0.14±0.01	0.14±0.02
pY817TrkB	0.60±0.06	0.66±0.05	0.6±0.03	0.58±0.03	0.60±0.03	0.61±0.05
Dorsal striatum						
mRNA						
MeCP2	1.00±0.02	1.00±0.04	1.00±0.03	0.99±0.03	1.04±0.04	1.11±0.06
BDNF	1.04±0.12	1.02±0.11	1.1±0.23	1.26±0.18	1.17±0.10	0.89±0.10
TrkB	1.01±0.05	1.00±0.04	1.00±0.03	1.06±0.04	1.01±0.08	1.06±0.02
MOR	1.12±0.21	1.08±0.19	1.00±0.04	1.26±0.12	1.14±0.19	1.19±0.2
Protein						
MeCP2	0.63±0.05	0.55±0.05	0.63±0.06	0.57±0.12	0.58±0.06	0.67±0.07
BDNF	3.07±0.75	1.33±0.35	0.58±0.09	2.68±0.61	1.02±0.28	0.70±0.15
TrkB	0.25±0.04	0.27±0.02	0.25±0.01	0.32±0.05	0.28±0.02	0.30±0.04
pY817TrkB	1.71±0.37	1.70±0.18	1.61±0.19	1.90±0.35	1.85±0.22	1.90±0.29
Medial prefrontal cortex						
mRNA						
MeCP2	1.00±0.02	1.00±0.04	1.00±0.03	0.94±0.07	0.96±0.04	1.13±0.06
BDNF	1.01±0.05	1.01±0.05	1.00±0.03	1.11±0.09	0.93±0.6	1.13±0.07
TrkB	1.02±0.11	1.01±0.06	1.03±0.09	1.18±0.15	1.13±0.09	0.90±0.09
MOR	1.16±0.33	1.01±0.05	1.01±0.07	1.31±0.31	1.06±0.11	1.32±0.24
Protein						
MeCP2	0.23±0.03	0.35±0.05	0.30±0.02	0.24±0.03	0.38±0.06	0.29±0.05
BDNF	0.54±0.07	0.77±0.09	0.74±0.05	0.57±0.06	0.85±0.07	0.68±0.09
TrkB	1.93±0.56	2.57±0.51	2.46±0.61	1.68±0.57	2.51±0.40	2.29±0.61
pY817TrkB	3.98±0.86	5.78±0.38	5.25±0.52	4.27±0.84	6.27±0.91	4.44±0.40

Data are presented as mean±SEM. Data for *BDNF_A2*, *MeCP2_A2*, *TrkB_Long*, and *Oprm1* mRNA isoforms are represented as fold changes over the saline group (arbitrary qPCR units) within each abstinence day (mean±SEM). The level of each protein of interest was normalized to β -tubulin (arbitrary unit), $n=5-8$ /group, * $p < 0.05$, different from day 1 saline

Table 2 Comparison of BDNF and MeCP2 protein expression in NAc and DS of the rats tested or not tested for extinction responding on abstinence day 11

	Nucleus accumbens		Dorsal striatum	
	Heroin no-test	Heroin test	Heroin no-test	Heroin test
BDNF	0.08±0.01	0.07±0.01	0.06±0.01	0.06±0.01
MeCP2	0.78±0.06	0.70±0.05	0.40±0.03	0.37±0.03

Data are presented as mean±SEM. The level of each protein of interest was normalized to β -tubulin (arbitrary unit, $n=10$ –11/group)

At the mRNA level in NAc, there was a main effect of abstinence day for MeCP2 ($F(2, 40)=3.2, p<0.05$) and BDNF ($F(2, 40)=4.8, p<0.05$) due to higher expression levels on abstinence day 11, independent of the training drug (saline versus heroin). As in the case with protein levels, this effect is likely due to the fact that the experiments with the rats tested on day 11 were performed at a different time point than the rats tested on day 1 or 30. Additionally, MeCP2 mRNA in the NAc was higher in the heroin-trained group than in the saline-trained group on abstinence day 30 (Table 1). The biological significance of this effect is unknown, because MeCP2 protein levels did not differ between the saline- and heroin-trained rats on day 30 (Table 1).

In contrast to the negative findings described above, we observed time-dependent changes in MOR expression in NAc but not DS or mPFC (Table 1). There were main effects of abstinence day ($F(2, 42)=4.1, p<0.05$) and training drug ($F(1, 42)=6.9, p<0.05$) and an abstinence day×training drug interaction ($F(2, 48)=4.0, p<0.05$). Post-hoc analyses demonstrated that MOR mRNA levels were decreased on abstinence day 1 in the heroin-trained group and progressively increased over time. As mentioned above, MOR protein levels could not be assessed because of lack of a selective antibody.

Experiment 3—effect of acute injections of naloxone on extinction responding on abstinence days 1 and 15

Heroin self-administration training

The rats in the three groups demonstrated reliable heroin self-administration during the training phase (Fig. 2a). The analysis of number of infusions earned demonstrated a main effect of training session ($F(8, 272)=4.1, p<0.05$), but no effects of group or training session×group (Fig. 2a). The analysis of lever responding demonstrated significant effects of lever ($F(1, 33)=34.0, p<0.05$) and lever×training session ($F(8, 264)=2.6, p<0.05$), but no effect of group ($p>0.05$) or interaction between group and the other factors.

Extinction tests

Naloxone injections decreased active lever presses in the extinction tests on abstinence day 15 but not day 1 (Fig. 2b). The analysis demonstrated significant effects of lever ($F(1, 34)=99.4, p<0.05$), abstinence day ($F(1, 34)=63.4, p<0.05$), naloxone dose ($F(2, 34)=4.1, p<0.05$), and lever×abstinence day ($F(1, 34)=42.0, p<0.05$). Post-hoc contrasts demonstrated that active but not inactive lever responding on abstinence day 15 was significantly different between the vehicle group and the 1.0 mg/kg naloxone group ($p<0.05$). Additionally, independent of group, active lever responding on abstinence day 15 was higher than on day 1.

Discussion

We found that cue-induced heroin seeking progressively increased during abstinence from heroin self-administration. These findings confirm previous results on incubation of heroin craving (Doherty and Frantz 2012; Shalev et al. 2001; Zhou et al. 2009). This incubation was not associated with increases in mRNA or protein levels of BDNF, TrkB (phosphorylated or total) or MeCP2 in NAc, DS, or mPFC. Conversely, in the NAc, but not DS or mPFC, MOR mRNA expression decreased during early abstinence below baseline drug-naïve (saline-trained) levels and then returned to drug-naïve levels during late abstinence. Finally, systemic injections of the preferential MOR antagonist naloxone significantly decreased cue-induced heroin seeking after 15 abstinence days but not after 1 day. These data suggest a role of MOR in cue-induced heroin seeking during abstinence.

Role of MOR in incubation of heroin craving

We found that NAc MOR mRNA levels were decreased on abstinence day 1 and returned to baseline levels on days 11 and 30. The reduced expression of NAc MOR expression during early abstinence may lead to decreased sensitivity to heroin-associated cues and low cue responding in the extinction tests on day 1. Our subsequent data from experiment 3, demonstrating that systemic injections of the preferential MOR antagonist naloxone (Goldstein and Naidu 1989) decreased “incubated” cue-induced heroin seeking after 15 abstinence days but not 1 day suggest a role of NAc MOR in incubation of heroin craving. However, this conclusion must await future studies because of several missing pieces of empirical data and unresolved methodological and conceptual issues that are discussed below.

One critical piece of empirical data is a demonstration that local blockade of core or shell NAc MOR by highly selective MOR antagonists like CTAP or CTOP (Pelton et al. 1986) or

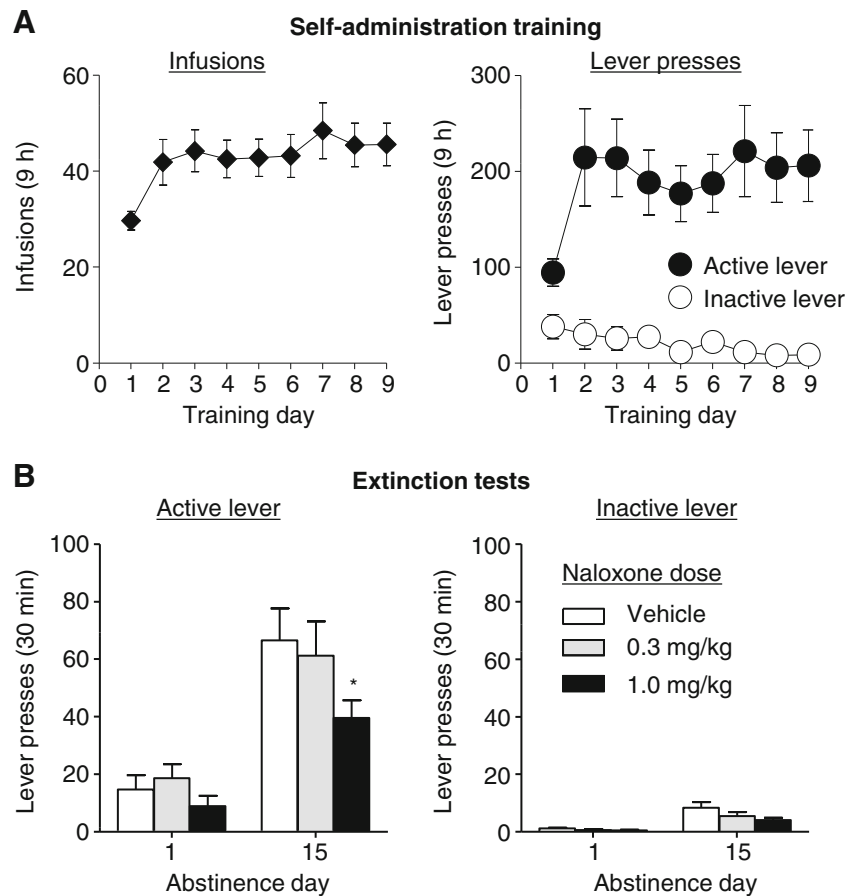


Fig. 2 Effect of systemic injections of the preferential MOR antagonist naloxone on cue-induced heroin seeking in extinction tests after 1 or 15 abstinence days. **A** Training days 1–9—infusions and lever presses. Data are mean±SEM number of heroin ($0.1 \text{ mg kg}^{-1} \text{ injection}^{-1}$, $n=39$) infusions, and active and inactive lever presses during the nine 9-h daily self-administration sessions. During training, active lever presses were reinforced under a fixed-ratio-1, 20-s timeout reinforcement schedule while inactive lever

presses were never reinforced; heroin injections were paired with a 5-s tone-light cue. **B** Extinction tests—lever presses, mean±SEM responses on the previously active lever and on the inactive lever during the 30-min extinction sessions, conducted in the presence of the heroin-associated cues but in the absence of heroin. Prior to the extinction tests, the rats were injected with saline (vehicle) or naloxone (0.3 or 1.0 mg/kg, s.c.), $n=11\text{--}16/\text{group}$. * $p<0.05$, different from vehicle for active lever responding on abstinence day 15

β -funaltrexamine (Takemori et al. 1981) would mimic the systemic effect of naloxone on cue-induced heroin seeking on day 15. Another important data piece is the demonstration that the time-dependent changes in MOR mRNA levels are followed by time-dependent changes in protein levels, which we could not verify because of lack of selective antibodies for MOR protein in the Western blot assay. This demonstration is important, because previous studies reported dissociation between mRNA and protein levels in studies on relapse to heroin and cocaine seeking. For example, we previously reported time-dependent changes in VTA and NAc GDNF mRNA but not protein expression (Airavaara et al. 2011). Additionally, Sutton et al. (2003) reported dissociations in the mRNA and protein expression of NAc glutamate receptors after extinction of cocaine self-administration. There is also evidence from many studies that psychostimulant exposure triggers differential regulation of mRNAs and their proteins (e.g., glutamate receptors) (Wolf 2003; Self 2004).

A methodological issue to consider is that our experimental conditions in experiments 2 (MOR mRNA) and 3 (systemic naloxone) were not identical (see “Methods overview”). However, it is unlikely that naloxone would have had different effects on extinction responding during early and late abstinence if rats were trained for 6 h/day for 10 days instead of 9 h/day for 9 days in experiment 3. This is because previous studies with other drug and non-drug rewards have demonstrated a reliable effect of naloxone or naltrexone on cue-induced reward seeking under different experimental conditions (Liu and Weiss 2002; Burattini et al. 2006; Grimm et al. 2007; Burattini et al. 2008; Liu et al. 2009). Another methodological issue is that the preferential MOR antagonist naloxone also binds at lower affinity to delta and kappa opioid receptors (Goldstein and Naidu 1989). Thus, in the absence of data demonstrating that selective kappa and delta opioid receptors do not decrease extinction responding on abstinence day 15, the role of these

receptors in incubation of heroin craving cannot be ruled out. In this regard, however, the role of the delta receptor can probably be ruled out. Under experimental conditions similar to those used in experiment 3, we found that systemic injections of the selective delta receptor antagonist naltrindole (Portoghese et al. 1990) (0, 1.0, or 3.0 mg/kg, $n=6-7$ per dose) had no effect on abstinence day 15 extinction responding (mean \pm SEM active lever presses/30 min of 75.2 \pm 22.6, 75.2 \pm 17.5, and 88.1 \pm 9.4, respectively, unpublished data).

A conceptual issue to consider is whether the MOR plays a unique role in incubation of cue-induced heroin craving versus a more general role in cue-induced heroin seeking, independent of the abstinence period. Support for a unique role in incubation is the pattern of the behavioral results in experiment 3—the significant effect of naloxone on extinction responding on abstinence day 15 but not day 1. However, we cannot rule out the latter possibility—a general role of MOR in cue-induced heroin seeking—because we did not observe statistically significant interactions of abstinence day \times naloxone dose. Additionally, the non-significant effect of naloxone on day 1 may reflect a floor effect due to low responding during early abstinence.

Finally, our observation of decreased MOR mRNA level in the NAc and the absence of changes in DS 24 h after the last heroin self-administration session is different from the results of Zhou et al. (2006), who found that MOR mRNA levels in the NAc and DS increased 12 h after cessation of non-contingent repeated exposure to escalating morphine doses. These discrepant findings are not surprising because there is evidence that contingent and non-contingent opiate injections cause different brain neuroadaptations (Jacobs et al. 2005; Lecca et al. 2007; Kuntz et al. 2008). Additionally, the effect of opiate exposure on brain MOR expression is highly dependent on the opiate agonist dose and the pattern of drug administration, which is likely the reason for the conflicting literature on this topic (Brodsky et al. 1995; Buzas et al. 1996; Ronnekleiv et al. 1996; Castelli et al. 1997; Sehba et al. 1997; Duttaroy and Yoburn 2000).

Lack of time-dependent changes in BDNF, TrkB, and MeCP2 during abstinence from heroin

The negative findings for protein levels of BDNF (NAc and DS) and MeCP2 (DS) are different from those reported in cocaine studies (Grimm et al. 2003; Im et al. 2010; Pickens et al. 2011). However, several methodological issues should be considered in interpreting our negative data in reference to the previous cocaine results. In the case of BDNF protein, one possibility is the difference in the molecular assay: our negative findings are from a Western blot assay measuring a specific 15 kD band while in the Grimm et al. (2003) study, we used ELISA. However, the possibility that our negative

BDNF findings are due to use of Western blots is relatively unlikely, because Graham et al. (2007) and Im et al. (2010) demonstrated increased BDNF protein in NAc and DS during abstinence from cocaine using Western blot. Additionally, negative data from Western blots for phosphorylated proteins (phosphorylated TrkB in the present work) should be interpreted with caution, because increased phosphorylation in a minority of activated neurons can be diluted by unaltered or even decreased phosphorylation levels in the majority of nonactivated neurons (Marin et al. 2009). Furthermore, our negative MeCP2 data should also be interpreted with caution because due to lack of a commercially available phospho-S421 antibody, we could not measure the phosphorylated state of MeCP2. In this regard, Deng et al. (2010) showed that amphetamine injections selectively increase NAc MeCP2 phosphorylation without changing total MeCP2.

Another potential reason for the absence of changes in BDNF and MeCP2 signaling pathways in our study is the lack of anatomical specificity, because we did not differentiate between the different sub-regions of the NAc (core and shell) and DS (dorsolateral and dorsomedial). This is an important consideration because there is evidence for differential neuroadaptations following drug exposure in the NAc sub-regions (Sutton et al. 2003; Jacobs et al. 2005; Kourrich and Thomas 2009). Additionally, the DS and NAc sub-regions control different drug-related behaviors, including drug relapse (Crombag et al. 2008; Belin et al. 2009). However, it is unlikely that this anatomical resolution issue can explain our negative data, because global changes across the core and shell, as well as the dorsolateral and dorsomedial striatum were observed in BDNF and MeCP2 protein expression, respectively, during abstinence from self-administered cocaine (Grimm et al. 2003; Im et al. 2010).

Another methodological issue concerns the no-test condition of the rats used for subsequent molecular analysis. One interpretation is that we did not observe changes in BDNF and MeCP2 signaling pathways because these alterations may depend on re-exposure to the heroin self-administration context and discrete cues. However, this is unlikely, because we did not observe any differences in BDNF and MeCP2 protein levels in NAc and DS of rats re-exposed or not exposed to the heroin self-administration training context and discrete cues on abstinence day 11 (Table 2). However, based on results of previous studies with heroin (Kuntz-Melcavage et al. 2009) and cocaine (Hearing et al. 2008), it is likely that cue-induced heroin seeking during late abstinence is associated with increases in BDNF mRNA (and protein) expression in mPFC.

It is important to note that our data do not rule out a role of brain BDNF and MeCP2 signaling pathways in incubation of heroin craving, because we only assessed protein and

mRNA levels in NAc, DS, and mPFC. Thus, changes in these signaling pathways may occur in other brain areas. Potential candidates include the VTA and amygdala, where BDNF protein is increased after 30 and 90 days of abstinence from cocaine (Grimm et al. 2003). Additionally, acute cue-driven activation of BDNF signaling during late abstinence (Kuntz-Melcavage et al. 2009) in mPFC or potentially drug-induced long-term BDNF-driven epigenetic changes in this brain area (Sadri-Vakili et al. 2010) may contribute to incubation of heroin craving.

Concluding remarks

The present data suggest a role of NAc MOR in incubation of heroin craving, but this role should be confirmed in future studies. Additionally, the negative data with BDNF, previously implicated in incubation of cocaine craving (Grimm et al. 2003; Lu et al. 2004), and our recent data on the role of VTA GDNF in incubation of cocaine but not heroin craving (Lu et al. 2009; Airavaara et al. 2011) suggest that the mechanisms of incubation of opiate and psychostimulant craving are partially dissociable (Pickens et al. 2011). More generally, these results support the notion that different mechanisms control cue-induced heroin versus cocaine seeking (Rogers et al. 2008; Badiani et al. 2011; Bossert et al. 2011, 2012)

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