Published in final edited form as:

Psychopharmacology (Berl). 2013 August ; 228(3): 389–400. doi:10.1007/s00213-013-3041-9.

μ-Opioid receptors in the stimulation of mesolimbic dopamine activity by ethanol and morphine in Long-Evans rats: a delayed effect of ethanol

John P. Valenta, Martin O. Job, Regina A. Mangieri, Christina J. Schier, Elaina C. Howard, and Rueben A. Gonzales

College of Pharmacy, Department of Pharmacology, The University of Texas at Austin, Austin, TX 78712, USA

Abstract

Rationale—Naltrexone, a non-selective opioid antagonist, decreases the euphoria and positive subjective responses to alcohol in heavy drinkers. It has been proposed that the μ-opioid receptor plays a role in ethanol reinforcement through modulation of ethanol-stimulated mesolimbic dopamine release.

Objective—To investigate the ability of naltrexone and β-funaltrexamine, an irreversible μ-opioid specific antagonist, to inhibit ethanol-stimulated and morphine-stimulated mesolimbic dopamine release and to determine whether opioid receptors on mesolimbic neurons contribute to these mechanisms.

Methods—Ethanol-naïve male Long Evans rats were given opioid receptor antagonists either intravenously, subcutaneously, or intracranially into the ventral tegmental area (VTA), followed by intravenous administration of ethanol or morphine. We measured extracellular dopamine *in vivo* using microdialysis probes inserted into the nucleus accumbens shell (n=114).

Results—Administration of naltrexone (intravenously) and β-funaltrexamine (subcutaneously), as well as intracranial injection of naltrexone into the VTA did not prevent the initiation of dopamine release by intravenous ethanol administration, but prevented it from being as prolonged. In contrast, morphine-stimulated mesolimbic dopamine release was effectively suppressed.

Conclusions—Our results provide novel evidence that there are two distinct mechanisms that mediate ethanol-stimulated mesolimbic dopamine release (an initial phase and a delayed phase), and that opioid receptor activation is required to maintain the delayed-phase dopamine release. Moreover, μ-opioid receptors account for this delayed-phase dopamine response, and the VTA is potentially the site of action of this mechanism. We conclude that μ-opioid receptors play different roles in the mechanisms of stimulation of mesolimbic dopamine activity by ethanol and morphine.

Keywords

microdialysis; naltrexone; nucleus accumbens shell; ventral tegmental area; morphine; fused silica microinjection; alcohol; addiction biology; BFNA; β-fna

Corresponding author: Rueben Gonzales The University of Texas at Austin College of Pharmacy 2409 University Ave, Stop A1900 Austin, TX 78712-1113 rgonzales@austin.utexas.edu Phone: (512)-471-5192 Fax: (512)-475-6088.

Both John Valenta and Martin Job contributed equally to this work and should be considered co-first authors.

Conflict of Interest: The authors declare no conflicts of interest.

Introduction

The mesolimbic dopaminergic system, which includes dopaminergic neurons that project from the ventral tegmental area (VTA) to the nucleus accumbens (NAcc), is thought to play a role in ethanol reinforcement (for review, see Gonzales *et al.* (2004)). Endogenous opioidergic systems are also thought to play a role in ethanol reinforcement (for review see Herz (1997) and Méndez and Morales-Mulia (2008)). For example, naltrexone, a non-selective opioid antagonist, is FDA approved for clinical use in the management of alcoholism (Spanagel and Vengeliene 2012). More specifically, the μ -opioid receptor appears to be involved in the regulation of alcohol consumption (O'Brien *et al.* 1996; Becker *et al.* 2002; Hall *et al.* 2001; Myers *et al.* 1999; Lasek *et al.* 2007; Roberts *et al.* 2000; Hyytiä and Kiianmaa 2001) and β -funaltrexamine, a selective μ -opioid antagonist (Ward *et al.* 1982), is effective in suppressing ethanol intake in rats (Stromberg *et al.* 1998; Krishnan-Sarin *et al.* 1998).

It has been proposed that blockade of the μ -opioid receptor suppresses ethanol reinforcement by attenuating ethanol-stimulated mesolimbic dopamine activity (Herz 1997). A reduction of ethanol intake during an operant self-administration procedure was observed following naltrexone pretreatment and was correlated with decreased ethanol-evoked dopamine in the NAcc of ethanol-experienced rats (Gonzales and Weiss 1998). However, the extent of this effect that was mediated by the μ -opioid receptor, and the specific brain regions involved, are unknown.

Our hypothesis is that blockade of μ -opioid receptors attenuates ethanol-stimulated dopamine release in the NAcc shell, and that this mechanism is mediated by opioid receptors in the VTA. To test this hypothesis, we prepared male Long Evans rats for intravenous drug administration and intracerebral microdialysis from the NAcc shell. We first examined the effect of naltrexone or β -funaltrexamine pretreatments on ethanol-evoked increases in dopamine release. In subsequent experiments, rats were also prepared with a microinjection guide cannula aimed at the VTA to evaluate the effect of microinjections of naltrexone into the VTA on ethanol-stimulated or morphine-stimulated dopamine release. Doses of opioid antagonists that were effective at suppressing morphine-stimulated dopamine release could not prevent ethanol-stimulated dopamine release, but prevented it from being as prolonged.

Methods and Materials

Rodents

We used male Long-Evans rats (Charles River Laboratories, Wilmington, MA), weighing 300–600 grams on the day of dialysis ($n = 114$). The rats were dual-housed at 25°C on a 12 hr light/dark schedule with *ad libitum* access to food and water in an AAALAC-accredited facility. Rats were handled for at least four days prior to surgery. All procedures were carried out in compliance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin.

Surgery

We implanted a jugular catheter and used stereotaxic surgery to implant up to two guide cannulae; one microdialysis probe guide cannula (21 gauge, Plastics One, Roanoke, VA) aimed at the nucleus accumbens shell (probe tip coordinates in mm relative to bregma: anterior-posterior (AP) +2.2, medial-lateral (ML) +0.9, dorsal-ventral (DV) –8.3) and an additional microinjection guide cannula (22 gauge, Plastics One, Roanoke, VA) aimed at the VTA (fused silica tip coordinates –5.25 AP, +0.65 ML, –8.7 DV), for microinjection experiments (on the same side as the microdialysis guide cannula). The catheter, made from

Silastic® tubing (0.30 mm inner diameter (ID), 0.64 mm outer diameter (OD), Fisher Scientific, Hampton, NH), was passed under the skin and mounted adjacent to the guide cannula on top of the head using dental cement. Rats were allowed to recover for at least three days in single-housing before experiments started. Further details regarding the surgery can be found in (Howard *et al.* 2008).

Drugs

Morphine sulphate pentahydrate (NIDA drug supply program) was dissolved in saline (1.0 mg/ml, 1.0 ml/kg). Naltrexone hydrochloride (Sigma-Aldrich, St. Louis, MO) was dissolved in saline for systemic infusion (0.03, 0.1, 0.3 or 1.0 mg/ml, and 1.0 ml/kg) or in artificial cerebrospinal fluid (ACSF; 149 mM NaCl, 2.8 mM KCl, 1.2 mM MgCl₂, 1.2 mM CaCl₂, 0.25 mM ascorbic acid, 5.4 mM D-glucose) for intracranial injection (265 ng/μl). β-funaltrexamine hydrochloride (Tocris Bioscience, Ellisville, MO or NIDA drug supply program) was dissolved in deionized water and appropriate NaCl was added to the solution to make it 0.9% (w/v) with a concentration of 20 mg/ml (1.0 ml/kg). A 10% (w/v) solution of ethanol (1 g/kg, 10 ml/kg) in saline was made from 95% ethanol (Aaper Alcohol and Chemical Co., Shelbyville, KY). All vehicle infusions were matched in volume with the pretreatment/treatment volumes.

Microinjection

Microinjection needles (28 gauge, Plastics One, Roanoke, VA) extended 4.5 mm past the guide cannula and were fitted with a length of fused silica (40 μm ID, 150 μm OD, Polymicro Technologies, Phoenix, AZ) that extended 1 mm beyond the tip of the needle. One continuous length of fused silica extended to the syringe needle (1.5–2 meters in total length) and was connected to the syringe needle with Tygon® tubing. The flowing microinjector was inserted into an awake, freely moving animal 45 min prior to the ethanol or morphine infusion and a CMA Microdialysis syringe pump (Stockholm, Sweden) was used to deliver the solution at 0.2 μl/min (for a total of 2.4 μg). The flow was stopped just prior to the ethanol or morphine infusion by disconnecting the fused silica from the syringe needle. This technique allowed the microinjection to be successfully carried out with the microinjector staying implanted throughout the experiment and without manipulating the animal (which causes dopamine release, see Figure 1c).

Microdialysis

For each rat we used a laboratory-constructed probe (1.5 mm active area, 13 or 18 kilodalton MW cut-off) perfused with ACSF. The rats were anesthetized only for probe implantation; otherwise, rats were conscious and freely moving. The dialysis chamber was a 16"×16"×12" clear plexiglass chamber with an Instech swivel (Plymouth Meeting, PA) connecting the microdialysis probe to a fused silica transfer line. The transfer line was connected to a 1 ml Exmire syringe. The syringe was operated by a CMA Microdialysis syringe pump. We implanted the probe flowing at 2 μl/min, followed by an overnight recovery (with the flow rate at 0.2 μl/min). The next day, we set the flow rate to 2.0–2.3 μl/min and waited at least 2 hours before collecting baseline samples. These pump settings gave an average measured flow rate of 1.97 ± 0.02 μl/min (range = 1.6–2.4). Only rats with a steady flow rate throughout the experiment were included in the analysis; if any sample for a given rat varied more than 10% from the average flow rate for that rat, the rat was excluded from the analysis. Samples were taken at 5 min intervals, except during one naltrexone experiment in which samples were taken at 3 min intervals.

In general, experiments were designed to test the effect of two opioid receptor antagonists on ethanol-stimulated mesolimbic dopamine release. Morphine-stimulated dopamine release experiments were used to determine doses of antagonists which led to effective μ-opioid

receptor blockade. In systemic naltrexone experiments, drug (or saline) “pretreatment” was administered as an intravenous (i.v.) bolus 20 min prior to the i.v. ethanol, saline, or morphine bolus infusion (“treatment”). In β -funaltrexamine experiments, drug (or saline) “pretreatment” was administered subcutaneously 20–25 hr before i.v. saline infusion (“treatment”), and then (at least 1.5 hours later) either ethanol or morphine was administered as an i.v. bolus (“treatment”). In microinjection experiments, naltrexone or ACSF was administered intracranially (“pretreatment”) prior to the i.v. ethanol or morphine bolus infusion (“treatment”). See Table 1 for a summary of experiments performed.

Calcium Dependency

Upon completion of the experiments, the perfusate was switched to calcium-free ACSF. A sample was taken after 1 hr to verify that dialysate dopamine was due to calcium-dependent exocytotic release. Only rats with a minimum of 50% calcium-dependent dopamine release were included in the final analysis.

High- Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC)

We used HPLC to measure dialysate dopamine concentrations and GC to measure dialysate ethanol concentrations. Details regarding the GC and HPLC methodology can be found in the supplement.

Histology

After the dialysis experiment, the rats were overdosed with sodium pentobarbital (150 mg/kg) and perfused intracardially with saline followed by 10% formalin. The brains were extracted and placed in 10% formalin overnight. The brains were sliced, stained with cresyl violet, and examined under a microscope to confirm probe placement using (Paxinos and Watson 1998) and microinjector placement using (Paxinos and Watson 2007).

Data Analysis

Each dopamine concentration was converted to percent basal values using two points to define the baseline. Statistical analyses were done using mixed model repeated-measures ANOVA, with type I error set to $P < 0.05$ as described in (Carrillo and Gonzales 2011), using two baseline points and five post-ethanol (or morphine) infusion points. We performed *post hoc* contrasts comparing individual time points with baseline values within groups when significance was detected for time, and comparing individual time points between treatment groups when significance was detected between groups or for a treatment $\times$ time interaction (a Bonferroni corrected alpha was used). Values are reported as mean $\pm$ SEM. In all analyses using ANOVA we monitored for inequality of variances using Levene's test. There was a single homogeneity of variance violation for the 25-min time point in the analysis of β -funaltrexamine pretreatment on ethanol-stimulated dopamine release.

Results

Basal dialysate dopamine concentrations and effects of saline or naltrexone infusion

To examine whether saline infusion (pretreatment) altered dialysate dopamine concentrations, we combined data from the saline pretreatment experiments that were controls for ethanol infusion ($n=11$) with those that were controls for the morphine experiments ($n = 4$). Saline infusion did not significantly alter the baseline dialysate concentration (Figure 1a, open circles). The basal concentration of dopamine was 0.9 ± 0.2 nM.

To determine the effect of naltrexone infusion (pretreatment) on dialysate dopamine concentrations we combined data from the animals in the two systemic 5-min naltrexone experiments (one with morphine, one with ethanol). In the first experiment, several doses of naltrexone (0, 0.03, 0.1, and 0.3 mg/kg) were given 20 min before infusion of a single dose of morphine. In the second experiment, several doses of naltrexone (0, 0.3 or 1.0 mg/kg) were given 20 min before infusion of saline or ethanol. Interestingly, the lowest dose of naltrexone tested (0.03 mg/kg) produced a transient 20% stimulation of dialysate dopamine above baseline (Figure 1a, closed circles, $F_{5,230}=3.00$, $P < 0.05$, naltrexone $\times$ time interaction). After 20 min, the dopamine concentration stimulated by infusion of 0.03 mg/kg naltrexone was not significantly different from baseline. Doses higher than 0.03 mg/kg naltrexone did not significantly affect dialysate dopamine compared with saline infusion for the 20 min period post-infusion. Baseline dialysate dopamine levels (nM) before naltrexone infusion were as follows: 0.03 mg/kg ($n = 4$) = 3.2 ± 0.1 ; 0.1 mg/kg ($n = 5$) = 1.3 ± 0.2 ; 0.3 mg/kg ($n = 17$) = 1.3 ± 0.2 ; 1 mg/kg ($n = 10$) = 1.1 ± 0.2 .

The effect of saline infusion in naltrexone or saline pretreated animals was examined (Figure 1b). Saline pretreatment ($n=4$) or 0.3 mg/kg naltrexone pretreatment ($n=5$) had no effect on dopamine levels after saline infusion. High dose naltrexone (1 mg/kg, $n=5$) pretreatment decreased dopamine levels 15 min after infusion of saline ($F_{6,174} = 3.35$, $P < 0.05$).

To determine the effects of morphine or ethanol infusion on dialysate dopamine concentrations (reported below) we recalculated the baseline as the mean of the two samples just prior to the drug infusion. These baseline dopamine levels (nM) prior to morphine infusion, for naltrexone doses 0.03, 0.1, and 0.3 mg/kg, were 3.8 ± 0.2 ($n=4$), 1.4 ± 0.3 ($n=5$) and 1.4 ± 0.4 ($n=6$), respectively. Baseline dopamine concentrations (nM) for rats pretreated with naltrexone (0.3, or 1.0 mg/kg) prior to ethanol infusion (for the 5-min sample experiment) were 1.3 ± 0.1 ($n=7$), and 1.2 ± 0.2 ($n=7$), respectively.

For the pretreatment analyses, all animals except the 3 min sampling period experiment were included (those animals did not have pre-naltrexone/vehicle samples collected). For the 3-min sampling experiment, baseline dopamine concentrations (nM) for rats pretreated with naltrexone (1.0 mg/kg) prior to ethanol infusion were 1.0 ± 0.2 ($n=8$) while baseline dopamine concentrations were 1.5 ± 0.3 ($n=9$) for the control group.

Systemic naltrexone inhibited morphine-evoked mesolimbic dopamine release

We infused rats with saline ($n = 4$) or different doses of naltrexone (0.03, 0.1 or 0.3 mg/kg i.v., $n = 4, 5$, and 6 , respectively) before infusion of morphine (1 mg/kg i.v.). For rats pretreated with saline, morphine increased dialysate dopamine (about 50% above baseline), and this increase was sustained for 25 min (Figure 2a, open circles). Naltrexone pretreatment significantly attenuated morphine-stimulated dopamine release ($F_{18,89} = 10.1$, $P < 0.05$, naltrexone dose $\times$ time interaction). *Post hoc* analyses revealed that saline controls were different from naltrexone pretreated rats at all time points following morphine infusion ($P < 0.05$).

Systemic naltrexone attenuated ethanol-evoked mesolimbic dopamine release

We infused saline or naltrexone (0.3 or 1.0 mg/kg) 20 min before saline or ethanol (1 g/kg i.v., $n=7$ for each group). In control rats, ethanol infusion increased dopamine (~33% from baseline), and this increase was sustained for at least 25 min (Figure 2b). For naltrexone pretreated rats, ethanol also increased dopamine (~24%), but this increase was not sustained beyond the first 5 min. ANOVA showed a naltrexone dose $\times$ time interaction ($F_{12,174} = 2.46$, $P < 0.05$) and an ethanol treatment $\times$ time interaction ($F_{6,174} = 13.7$, $P < 0.05$). *Post hoc* analysis revealed that naltrexone prevented the increase in dopamine following ethanol

administration from being sustained ($P < 0.05$ at time points 10 min and 15 min post-infusion). The peak dialysate ethanol concentrations were 6.1 ± 0.4 , 6.4 ± 0.6 and 6.1 ± 0.6 mM for saline, naltrexone 0.3 and 1.0 mg/kg, respectively (Figure 2c). ANOVA did not detect a difference in brain ethanol concentrations over time.

To confirm the time course of the delayed inhibitory effect of naltrexone pretreatment, we replicated the experiment using 3-min sampling intervals. For controls ($n = 9$), ethanol increased dopamine (~25% from baseline), and this increase was sustained for at least 15 min (Figure 2d). For naltrexone pretreated rats (1.0 mg/kg i.v., $n = 8$), ethanol also increased dopamine (~17%), but this increase was not sustained beyond the first 6 min. ANOVA detected a naltrexone treatment $\times$ time interaction ($F_{6,89} = 2.57$, $P < 0.05$). *Post hoc* analysis revealed a difference between the naltrexone and control groups at 9 min post-infusion ($P < 0.05$). The peak dialysate ethanol concentrations were 6.7 ± 0.6 and 5.7 ± 0.7 mM for saline and naltrexone, respectively (Figure 2e), and no significant differences in the dialysate ethanol time courses were observed.

β -funaltrexamine inhibited morphine-evoked mesolimbic dopamine release

For clarity, the following sections focus on the ability of β -funaltrexamine to inhibit ethanol- or morphine-stimulated dopamine release. However, the supplemental material includes analyses of dopamine concentrations and effects of β -funaltrexamine pretreatments. In short, neither β -funaltrexamine pretreatment nor saline pretreatment had any effect on dialysate dopamine concentrations, and neither β -funaltrexamine pretreatment nor saline pretreatment had any effect on dopamine during saline treatment.

In these experiments, rats were pretreated subcutaneously with saline (control) or β -funaltrexamine (20 mg/kg) followed by saline (i.v.) 20 – 25 hours later and then (at least 1.5 hours later) by morphine (1 mg/kg i.v.). For control rats, morphine ($n = 6$) increased dopamine release (~200%, Figure 3a) for the entire 25-min experiment. For β -funaltrexamine pretreated rats, the increase in dopamine after morphine was significantly attenuated ($n = 5$). ANOVA detected a β -funaltrexamine treatment $\times$ time interaction ($F_{6,54} = 12.30$, $P < 0.05$). *Post hoc* analysis revealed that controls were different from the β -funaltrexamine-pretreated rats at every time point after morphine infusion ($P < 0.05$ for all comparisons).

β -funaltrexamine attenuated ethanol-evoked mesolimbic dopamine release

In these experiments, rats were pretreated subcutaneously with saline (control) or β -funaltrexamine (20 mg/kg) followed by saline (i.v.) 20 – 25 hours later and then (at least 1.5 hours later) with ethanol (1 g/kg i.v.). For control rats, ethanol increased dopamine (~30–40% of baseline, Figure 3b, $n = 6$ for both groups). For β -funaltrexamine-pretreated rats, the increase in dopamine was significantly attenuated. ANOVA detected a β -funaltrexamine treatment $\times$ time interaction ($F_{6,60} = 2.25$, $P < 0.05$). *Post hoc* analysis revealed that control rats had an increase in dopamine from baseline after ethanol infusion for every time point included in the analysis whereas β -funaltrexamine treated rats showed an increase in dopamine only in the first 10 min after infusion ($P < 0.05$). The peak dialysate ethanol concentrations were 4.5 ± 0.5 and 5.4 ± 0.4 mM for control and β -funaltrexamine pretreated rats, respectively (Figure 3c). ANOVA did not detect a difference in brain ethanol concentrations over time.

Basal dialysate dopamine concentrations and effects of microinjection

In these experiments, there were three phases: baseline, then naltrexone (or vehicle) infusion into the VTA, and then intravenous morphine or ethanol infusion. At the beginning of the experiment we took two 5 min dialysate samples as baseline, then placed the flowing

microinjector into its guide cannula, took 9 more samples, stopped flow through the microinjector, infused intravenous ethanol or morphine, then took five more samples.

We measured mesolimbic dopamine in response to placement of the microinjector and infusion of naltrexone or vehicle. The two samples taken before placement of the microinjector determined the baseline for this analysis (1.7 ± 0.3 for the vehicle group, $n = 8$; and 1.2 ± 0.4 for the naltrexone group, $n = 9$). Placement of the microinjector produced a transient spike in accumbal dopamine for the first sample (15% over baseline on average) followed by a gradual increase from baseline to approximately 18% on average just prior to ethanol or morphine infusion (Figure 1c). ANOVA revealed a main effect of time ($F_{10, 150} = 3.59$, $P < 0.05$) over the 45 min microinjection but did not show a main effect of group or an interaction.

The baseline dopamine levels just prior to morphine administration were 1.0 ± 0.5 nM for naltrexone pretreated rodents ($n=4$) and 1.3 ± 0.3 nM for controls ($n=4$). The baseline dopamine levels just prior to ethanol administration were 1.8 ± 0.5 nM for naltrexone pretreated rodents ($n=5$) and 2.3 ± 0.4 nM for controls ($n=7$).

Naltrexone microinjection into the VTA abolished morphine-evoked mesolimbic dopamine release

To verify that the selected dose and timing of naltrexone microinjection effectively blocked VTA opioid receptors, we examined its effect on morphine-stimulated dopamine release. For control rats (ACSF microinjection pretreatment), morphine infusion (1 mg/kg i.v.) led to a robust increase in dopamine release in the NAcc ($n = 4$, Figure 4a). Rats that were pretreated with a naltrexone microinjection into the VTA did not have an increase in dopamine ($n = 4$). ANOVA detected a naltrexone treatment $\times$ time interaction ($F_{6, 36} = 11.10$, $P < 0.05$). *Post hoc* analysis revealed that controls were different from the naltrexone-pretreated rats at every time point after morphine infusion ($P < 0.05$ for all comparisons).

Naltrexone microinjection into the VTA produced a delayed inhibition of ethanol-evoked mesolimbic dopamine release

We next assessed the effect of intra-VTA naltrexone on mesolimbic dopamine after ethanol infusion. For rats pretreated with an intra-VTA microinjection of ACSF, ethanol increased dopamine in the NAcc (~25%, $n = 7$), and this increase was sustained for the entire 25-min experiment (Figure 4b). Naltrexone microinjection into the VTA did not prevent the initial ethanol-evoked increase in dopamine concentration in the NAcc, but this increase was not sustained ($n = 5$). ANOVA detected a naltrexone treatment $\times$ time interaction ($F_{6, 58} = 2.51$, $P < 0.05$). *Post hoc* analysis revealed that dopamine was stimulated above baseline starting at 10 min post-ethanol in controls, whereas in the naltrexone group the dopamine response was significantly above baseline for only the first 5-min sample ($P < 0.05$ for all comparisons). The peak dialysate ethanol concentrations were 4.6 ± 0.4 and 6.5 ± 0.2 mM for control and naltrexone rats, respectively (Figure 4c). There was a significant difference in brain ethanol concentration over time ($F_{1, 10} = 9.59$, $P < 0.05$, naltrexone treatment $\times$ time interaction).

Histology

Probe placements for individual experiments are shown in Supplemental Figures S1, S2, and S3. Only rats that had probes predominantly (> 50%) within the shell of the NAcc were included in the analysis. The microinjector placements are shown in Supplemental Figure S4. Only rats that had the tip of the microinjector within the VTA were included in the analysis.

Calcium dependency

The Ca^{2+} -dependency of dialysate dopamine was ascertained for all rats used in this study ($n=114$). The mean reduction for extracellular dopamine was $81 \pm 1\%$ after perfusion of calcium-free ACSF.

Discussion

Our results provide novel evidence that there are two temporally distinct mechanisms that mediate intravenous ethanol-stimulated mesolimbic dopamine release. The initial phase of ethanol-stimulated dopamine response is not sensitive to opioid receptor blockade. In contrast, μ -opioid receptors modulate a delayed mechanism of dopamine release. Furthermore, our data show for the first time that naltrexone acts, at least in part, in the midbrain to attenuate the delayed mechanism of ethanol-stimulated accumbal dopamine release. These findings provide new insight into the mechanism of action of naltrexone, a drug used for the treatment of alcoholism.

Naltrexone, a non-selective opioid antagonist, did not prevent ethanol-stimulated dopamine release, but reduced the time that the release was stimulated (Figure 2 b and d). The inhibitory mechanism of naltrexone likely involved the μ -opioid receptor because systemic administration of β -funaltrexamine, an irreversible selective μ -opioid antagonist, produced a similar time course of inhibition of dopamine release (Figure 3b). However, experiments using systemic injections of antagonists do not allow conclusions to be made regarding specific sites of action within the brain. Therefore, we investigated the role of opioid receptors in the midbrain, in particular the VTA, in ethanol-stimulated dopamine release. Similar to the systemic injections, microinjections of naltrexone into the VTA produced a delayed inhibition of ethanol-stimulated mesolimbic dopamine release (Figure 4b). This is the first demonstration that naltrexone modulates ethanol-stimulated dopamine release at the level of the midbrain.

Our microinjection technique must be considered for proper interpretation of the results. The microinjection dose of $2.4 \mu\text{g}$ of naltrexone was chosen because this dose has previously been shown to reverse μ -opioid receptor agonist-induced feeding behavior (Echo *et al.* 2002, MacDonald *et al.* 2003) and GABAB receptor agonist-induced feeding behavior (Echo *et al.* 2002). We used a slow infusion rate ($0.2 \mu\text{l}/\text{min}$), and the microinjection needle was fused silica with a small diameter ($150 \mu\text{m}$). This minimized the tissue damage in the VTA as evidenced by the robustness of morphine-stimulated dopamine release, despite infusion of vehicle into the VTA. Furthermore, the proportion of exocytotic dopamine release in the microinjection experiment was comparable to the other experiments, with an average reduction of $87 \pm 3\%$ of dopamine release with the perfusion of calcium-free ACSF. These controls strongly suggest that the microinjection protocol we used allowed us to measure a functional dopamine neuron pool.

A related detail for consideration of our microinjection experiment is the extent of diffusion of naltrexone during the experiment. We observed a spike in dopamine release with placement of the microinjector (see Figure 1c), and therefore, we chose a 45 min continuous infusion protocol in order to let the rodent behavior and dopamine levels stabilize after insertion of the microinjection needle and to allow diffusion to occur prior to the experimental manipulation. Therefore, we must consider the possibility that naltrexone is reaching structures adjacent to the VTA. Evidence suggests that naltrexone may have diffused approximately 1 mm away from the site of injection. Previous work by Myers *et al.* (1966) suggests that the microinjection diffusion may be approximated with large molecular weight dyes. A pilot experiment was done to examine the extent of diffusion of cresyl violet into the VTA using our microinjection protocol. Diffusion was limited to less than a 1 mm

radius in all directions (Figure 5). Substantial insight into drug diffusion after long-term microinjection can be gained from a study by Sendelbeck and Urquhart (1985) which used radioactive methotrexate and antipyrine infused at a rate of 1 μ L/hr for 7 days, to measure the diffusion of the drugs in a rabbit brain. At a distance of 1 mm, the maximal radioactivity found was about 10% of the radioactivity at the injection site, and at 2 mm, the maximal radioactivity was about 1%. Additionally, an immunohistochemistry study by Freund *et al.* (2010) showed a visible gradient of tetrodotoxin with the diffusion limited to a maximum of about 1.5 mm from the site of diffusion at 1 hr. It is likely that naltrexone diffusion follows a similar gradient, with the concentration of naltrexone dropping below levels necessary to effectively block opioid receptors within that radius. A pilot animal in our microinjection experiment with a naltrexone microinjection placed 1 mm dorsal to the VTA showed normal, uninhibited morphine-stimulated dopamine release, indicating that the naltrexone concentration at that distance was too low to effectively block opioid receptors. Given that a 1 mm radius of diffusion would include areas outside the VTA, we cannot completely rule out the possibility that opioid receptors outside of the VTA may contribute to the delayed mechanism of ethanol-stimulated accumbal dopamine release. However, we can firmly conclude that the opioid receptors in the VTA are not involved in the initial mechanism of ethanol-stimulated dopamine release.

There are multiple opioid receptors (μ , δ , and κ), and only activation of μ - or δ -opioid receptors enhances mesolimbic dopamine release (Herz 1997). In order to characterize the specific contribution of the μ -opioid receptor in ethanol-stimulated dopamine release, we used morphine as a positive control. Morphine has a significantly higher (> 100 fold) affinity for μ -relative to δ -opioid receptor (Raynor *et al.* 1994). β -funaltrexamine suppresses systemic morphine-evoked dopamine increase in the NAcc (Di Chiara and Imperato 1998), whereas naltrindole, a highly selective δ -opioid antagonist, does not (Borg and Taylor 1997). Thus, morphine-evoked increase in mesolimbic dopamine is predominantly due to activation of the μ -opioid receptor, and inhibition of the morphine-stimulated dopamine response by naltrexone is also due to action on the μ -opioid receptor. The present results confirm that systemic injection of naltrexone or β -funaltrexamine both inhibited morphine-stimulated mesolimbic dopamine release. Furthermore, intracranial injection of naltrexone showed a similar inhibition, providing evidence that morphine-stimulated dopamine release is likely due to action in the midbrain. In contrast, these same pretreatments did not affect the *initial* rise in accumbal dopamine after ethanol treatment, indicating that this phase does not depend on the μ -opioid receptor. Conversely, our results do implicate μ -opioid receptors in modulating a *delayed* phase of ethanol-stimulated dopamine release. There is evidence that β -funaltrexamine also binds to the μ - δ opioid receptor complex (Rothman *et al.* 1988; Rothman *et al.* 1991), and at present, we cannot rule out the possibility that μ - δ opioid receptor complexes are also involved in the delayed phase of ethanol-stimulated dopamine release.

Previous studies have suggested that opioid receptors modulate ethanol-stimulated dopamine release in rats. Tanda and Di Chiara (1998) reported that systemic naloxonazine, a selective μ_1 -opioid receptor antagonist, blunted ethanol-stimulated dopamine release using 20 min sampling periods. Ramchandani *et al.* (2011) reported that a variant of the human μ -opioid receptor modulates ethanol-stimulated dopamine release in a transgenic mouse model. However, these previous studies lacked the temporal resolution necessary to investigate the initial mechanism of ethanol-stimulated dopamine release. Our data support and extend these studies by using a higher resolution time course of microdialysis sampling to show that the inhibitory effects of both naltrexone and β -funaltrexamine are not immediate, but delayed.

The mechanism of ethanol-evoked mesolimbic dopamine is thought to include ethanol-mediated activation of endogenous opioid peptide release (Rasmussen *et al.* 1998; Marinelli *et al.* 2005; Jarjour *et al.* 2009, Mendez *et al.* 2010) and activation of opioid receptors (Xiao *et al.* 2007). However, naltrexone did not inhibit ethanol-stimulated dopamine neuron firing suggesting the existence of an opioid receptor independent mechanism *in vitro* (Theile *et al.* 2011), and the present data confirms that an opioid receptor-independent mechanism for ethanol-stimulated dopamine release is also present *in vivo*.

Several microdialysis studies have reported acute ethanol-enhanced opioid peptide release in several brain regions of the rat, including the NAcc (Marinelli *et al.* 2005) and VTA (Jarjour *et al.* 2009). Although those studies detected opioid peptides in the first post-ethanol sample, they used 30 min sampling, so we cannot accurately pinpoint the initiation of peptide release. Other studies detected significant β -endorphin release in the NAcc, but significance was not reached in the first 30-min sample (Olive *et al.* 2001; Marinelli *et al.* 2003). Our data suggests that higher temporal resolution sampling may reveal a delayed release of opioid peptides. More detailed studies are needed to firmly establish whether the time course of ethanol-stimulated opioid release matches that of the inhibition of ethanol-stimulated dopamine release by naltrexone or β - funaltrexamine.

Taken together, the present results show that intravenous ethanol stimulates an opioid-independent phase of dopamine release before an opioid-dependent phase. This raises the interesting question of the potential mechanisms of the initial opioid-independent stimulation of mesolimbic dopamine. Glutamatergic input from the pedunculopontine tegmentum (PPTg) appears to be very influential on dopamine neuron burst firing, with modulatory control of the PPTg coming from the laterodorsal tegmentum (Sesack and Grace 2010). Modulatory afferents to the VTA include serotonin from the dorsal raphe and norepinephrine from the locus coeruleus. Several neurotransmitter antagonists have already shown robust inhibition of ethanol-stimulated dopamine release, including 5HT₃ receptor antagonists (Carboni *et al.* 1989), nicotinic receptor antagonists (Blomqvist *et al.* 1993), and glycine receptor antagonists (Molander and Soderpalm 2005). However, these studies all used long sampling periods (20 min or more), making it impossible to distinguish between the early and late phases of dopamine release. More work needs to be done using high-temporal resolution microdialysis in order to investigate the origins of the early phase of dopamine release.

Although the specific role of mesolimbic dopamine release remains under debate (Salamone and Correa 2002), data suggests that its release is associated with drug reinforcement (Wise 1996), and the blockade of dopamine receptors can suppress ethanol reinforcement (Rassnick *et al.* 1992). Therefore, the results of our experiment suggest that the limited clinical efficacy of opioid antagonists may be due to their inability to block the initial phase of ethanol-stimulated dopamine release. However, please note that we are using an acute, pharmacological model of ethanol administration and not a self-administration model, limiting the conclusions that could be made from our data in this regard.

Although the blood alcohol concentration (BAC) reached in our acute administration model is 2–3 times higher than those reached in our self-administration model, our acute BAC is pharmacologically relevant (e.g. it is easily reached in chronically exposed animals (Gilpin *et al.* 2009) or during a college binge (Brister *et al.* 2011)). Nonetheless, the corresponding dopamine response of self-administration studies has a different pattern of release, and the effect of opioid antagonists on dopamine release during ethanol self-administration should be investigated.

For the naltrexone microinjection experiment, the dialysate ethanol concentrations were significantly higher in the naltrexone animals during the first and second time points after ethanol infusion. Although this is important to consider during the interpretation of this experiment, our conclusions are justified. The inhibitory effect of naltrexone in the midbrain occurred at 25 min after the ethanol infusion and brain ethanol concentrations were not different between groups at this time point. Furthermore, rodents that received the naltrexone microinjection received more ethanol. Previous work in this laboratory has shown a positive correlation between ethanol concentration and dopamine release (Howard *et al.* 2008). Since this model would have predicted more dopamine release in the animals who received more ethanol, naltrexone-mediated inhibition of dopamine in the animals that showed higher ethanol concentration is evidence in favor of the hypothesis. Therefore, it is unlikely that the different dopamine responses of the control and naltrexone groups are attributable to the differences in ethanol concentrations between these two groups.

In most of the control studies we carried out during the course of these experiments, naltrexone alone did not alter dialysate dopamine concentrations indicating little or no opioid tone is present under normal conditions. However, it is interesting to note that the lowest dose of naltrexone that we tested (0.03 mg/kg, iv) produced a transient stimulation of accumbal dopamine release. The mechanism of this effect is not clear, but the absence of this finding for higher doses of naltrexone strongly suggests that it is not mediated solely by blockade of opioid receptors. On the other hand, we also found that the highest dose of naltrexone we used (1.0 mg/kg, iv) produced a decrease in dialysate dopamine 15–25 min following saline infusion (iv). It is possible that this effect is due to an increase in opioid tone produced by the saline infusion. In both cases, further work will be necessary to fully understand the mechanisms involved.

In conclusion, we have presented data which show that μ -opioid receptors modulate a delayed mechanism of ethanol-stimulated dopamine release in the shell of the NAcc of ethanol-naïve rats, and that opioid receptors in the midbrain are part of this mechanism. In other words, opioid receptor activation is not required for the initiation of mesolimbic dopamine release, but rather, is required for the maintenance of ethanol-stimulated dopamine release. In contrast, naltrexone inhibits morphine-stimulated dopamine release immediately, likely also through action in the midbrain. These findings enhance our understanding of the mechanisms of action of naltrexone on a crucial brain system that is important for reward processing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by a grant from NIH/NIAAA (AA11852). The authors thank the NIDA drug supply program for supplying morphine and β -funaltrexamine. John Valenta, Martin Job, Regina Mangieri, Christina Schier, and Elaina Howard were supported by a training grant T32 AA07471 from NIH/NIAAA. Martin Job and Elaina Howard were also supported by predoctoral fellowships from NIH/NIAAA (F31 AA016741, F31 A016874, respectively). Regina Mangieri was supported by a postdoctoral fellowship from NIH/NIAAA (F32 AA018252). The authors acknowledge the excellent technical assistance of Shannon Zandy, James Reno, Woojung Lee and Jessica Hunter. A portion of the data in this manuscript was previously presented at the Society for Neuroscience meeting (2008, 2011).

References

- Becker A, Grecksch G, Kraus J, Loh HH, Schroeder H, Höllt V. Rewarding effects of ethanol and cocaine in mu opioid receptor-deficient mice. *Naunyn Schmiedeberg's Arch Pharmacol.* 2002; 365:296–302. [PubMed: 11919654]
- Blomqvist O, Engel JA, Nissbrandt H, Söderpalm B. The mesolimbic dopamine-activating properties of ethanol are antagonized by mecamylamine. *Eur J Pharmacol.* 1993; 249(2):207–13. [PubMed: 8287902]
- Borg PJ, Taylor DA. Involvement of mu- and delta-opioid receptors in the effects of systemic and locally perfused morphine on extracellular levels of dopamine, DOPAC and HVA in the nucleus accumbens of the halothane-anaesthetized rat. *Naunyn Schmiedeberg's Arch Pharmacol.* 1997; 355:582–588. [PubMed: 9151296]
- Brister HA, Sher KJ, Fromme K. 21st birthday drinking and associated physical consequences and behavioral risks. *Psychol Addict Behav.* 2011; 4:573–82. [PubMed: 21895347]
- Carboni E, Acquas E, Frau R, Di Chiara G. Differential inhibitory effects of a 5-HT₃ antagonist on drug-induced stimulation of dopamine release. *Eur J Pharmacol.* 1989; 164(3):515–9. [PubMed: 2767122]
- Carrillo J, Gonzales RA. A single exposure to voluntary ethanol self-administration produces adaptations in ethanol consumption and accumbal dopamine signaling. *Alcohol.* 2011; 45:559–566. [PubMed: 21827930]
- Di Chiara G, Imperato A. Opposite effects of mu and kappa opiate agonists on dopamine release in the nucleus accumbens and in the dorsal caudate of freely moving rats. *J Pharmacol Exp Ther.* 1988; 244:1067–80. [PubMed: 2855239]
- Echo JA, Lamonte N, Ackerman TF, Bodnar RJ. Alterations in food intake elicited by GABA and opioid agonists and antagonists administered into the ventral tegmental area region of rats. *Physiol Behav.* 2002; 76(1):107–16. [PubMed: 12175594]
- Freund N, Manns M, Rose J. A method for the evaluation of intracranial tetrodotoxin injections. *J Neurosci Methods.* 2010; 186(1):25–8. [PubMed: 19879897]
- Gilpin NW, Smith AD, Cole M, Weiss F, Koob GF, Richardson HN. Operant behavior and alcohol levels in blood and brain of alcohol-dependent rats. *Alcohol Clin Exp Res.* 2009; 33(12):2113–23. [PubMed: 19740131]
- Gonzales RA, Job MO, Doyon WM. The role of mesolimbic dopamine in the development and maintenance of ethanol reinforcement. *Pharmacol Ther.* 2004; 103:121–146. [PubMed: 15369680]
- Gonzales RA, Weiss F. Suppression of ethanol-reinforced behavior by naltrexone is associated with attenuation of the ethanol-induced increase in dialysate dopamine levels in the nucleus accumbens. *J Neurosci.* 1998; 18:10663–10671. [PubMed: 9852601]
- Hall FS, Sora I, Uhl GR. Ethanol consumption and reward are decreased in mu-opiate receptor knockout mice. *Psychopharmacology (Berl).* 2001; 154:43–49. [PubMed: 11292005]
- Herz A. Endogenous opioid systems and alcohol addiction. *Psychopharmacology (Berl).* 1997; 129:99–111. [PubMed: 9040115]
- Howard EC, Schier CJ, Wetzel JS, Duvauchelle CL, Gonzales RA. The shell of the nucleus accumbens has a higher dopamine response compared with the core after non-contingent intravenous ethanol administration. *Neuroscience.* 2008; 154:1042–1053. [PubMed: 18511209]
- Hyttiä P, Kiianmaa K. Suppression of ethanol responding by centrally administered CTOP and naltrindole in AA and Wistar rats. *Alcohol Clin Exp Res.* 2001; 25:25–33. [PubMed: 11198711]
- Jarjour S, Bai L, Gianoulakis C. Effect of acute ethanol administration on the release of opioid peptides from the midbrain including the ventral tegmental area. *Alcohol Clin Exp Res.* 2009; 33(6):1033–43. [PubMed: 19302084]
- Krishnan-Sarin S, Wand GS, Li XW, Portoghesi PS, Froehlich JC. Effect of mu opioid receptor blockade on alcohol intake in rats bred for high alcohol drinking. *Pharmacol Biochem Behav.* 1998; 59:627–35. [PubMed: 9512064]
- Lasek AW, Janak PH, He L, Whistler JL, Heberlein U. Downregulation of mu opioid receptor by RNA interference in the ventral tegmental area reduces ethanol consumption in mice. *Genes Brain Behav.* 2007; 6:728–735. [PubMed: 17428267]

- MacDonald AF, Billington CJ, Levine AS. Effects of the opioid antagonist naltrexone on feeding induced by DAMGO in the ventral tegmental area and in the nucleus accumbens shell region in the rat. *Am J Physiol Regul Integr Comp Physiol*. 2003; 285(5):R999–R1004. [PubMed: 12907414]
- Marinelli PW, Bai L, Quirion R, Gianoulakis C. A microdialysis profile of Met-enkephalin release in the rat nucleus accumbens following alcohol administration. *Alcohol Clin Exp Res*. 2005; 29:1821–1828. [PubMed: 16269911]
- Marinelli PW, Quirion R, Gianoulakis C. A microdialysis profile of beta-endorphin and catecholamines in the rat nucleus accumbens following alcohol administration. *Psychopharmacology (Berl)*. 2003; 169:60–7. [PubMed: 12759807]
- Méndez M, Morales-Mulia M. Role of mu and delta opioid receptors in alcohol drinking behaviour. *Curr Drug Abuse Rev*. 2008; 1(2):239–52. [PubMed: 19630722]
- Méndez M, Barbosa-Luna IG, Pérez-Luna JM, Cupo A, Oikawa J. Effects of acute ethanol administration on methionine-enkephalin expression and release in regions of the rat brain. *Neuropeptides*. 2010; 44(5):413–20. [PubMed: 20605629]
- Molander A, Soderpalm B. Accumbal strychnine-sensitive glycine receptors: an access point for ethanol to the brain reward system. *Alcohol Clin Exp Res*. 2005; 29:27–37. [PubMed: 15654288]
- Myers RD. Injection of solutions into cerebral tissue: Relation between volume and diffusion. *Physiol Behav*. 1966; 1:171–174.
- Myers RD, Robinson DE. Mu and D2 receptor antisense oligonucleotides injected in nucleus accumbens suppress high alcohol intake in genetic drinking HEP rats. *Alcohol*. 1999; 18:225–233. [PubMed: 10456575]
- O'Brien CP, Volpicelli LA, Volpicelli JR. Naltrexone in the treatment of alcoholism: a clinical review. *Alcohol*. 1996; 13:35–39. [PubMed: 8837932]
- Olive MF, Koenig HN, Nannini MA, Hodge CW. Stimulation of endorphin neurotransmission in the nucleus accumbens by ethanol, cocaine, and amphetamine. *J Neurosci*. 2001; 21:RC184. 1–5. [PubMed: 11717387]
- Paxinos, G.; Watson, C. The rat brain in stereotaxic coordinates. 4th ed.. Academic Press; San Diego: 1998.
- Paxinos, G.; Watson, C. The rat brain in stereotaxic coordinates. 6th ed.. Academic Press; Burlington: 2007.
- Ramchandani VA, Umhau J, Pavon FJ, Ruiz-Velasco V, Margas W, Sun H, Damadzic R, et al. A genetic determinant of the striatal dopamine response to alcohol in men. *Mol Psychiatry*. 2011; 16:809–17. [PubMed: 20479755]
- Rasmussen DD, Bryant CA, Boldt BM, Colasurdo EA, Levin N, Wilkinson CW. Acute alcohol effects on opiomelanocortinergic regulation. *Alcohol Clin Exp Res*. 1998; 22:789–801. [PubMed: 9660303]
- Rassnick S, Pulvirenti L, Koob GF. Oral ethanol self-administration in rats is reduced by the administration of dopamine and glutamate receptor antagonists into the nucleus accumbens. *Psychopharmacology (Berl)*. 1992; 109(1–2):92–8. [PubMed: 1365677]
- Raynor K, Kong H, Chen Y, Yasuda K, Yu L, Bell GI, Reisine T. Pharmacological characterization of the cloned kappa-, delta-, and mu-opioid receptors. *Mol Pharmacol*. 1994; 45:330–334. [PubMed: 8114680]
- Roberts AJ, McDonald JS, Heyser CJ, Kieffer BL, Matthes HW, Koob GF, Gold LH. mu-Opioid receptor knockout mice do not self-administer alcohol. *J Pharmacol Exp Ther*. 2000; 293:1002–1008. [PubMed: 10869404]
- Rothman RB, Bykov V, Mahboubi A, Long JB, Jiang Q, Porreca F, et al. Interaction of beta-funaltrexamine with [3H]cycloFOXY binding in rat brain: further evidence that beta-FNA alkylates the opioid receptor complex. *Synapse*. 1991; 8:86–99. [PubMed: 1652797]
- Rothman RB, Long JB, Bykov V, Jacobson AE, Rice KC, Holaday JW. beta-FNA binds irreversibly to the opiate receptor complex: in vivo and in vitro evidence. *J Pharmacol Exp Ther*. 1988; 247:405–416. [PubMed: 2846819]

- Salamone JD, Correa M. Motivational views of reinforcement: implications for understanding the behavioral functions of nucleus accumbens dopamine. *Behav Brain Res.* 2002; 137(1–2):3–25. [PubMed: 12445713]
- Sendelbeck SL, Urquhart J. Spatial distribution of dopamine, methotrexate and antipyrine during continuous intracerebral microperfusion. *Brain Res.* 1985; 328(2):251–8. [PubMed: 4039212]
- Sesack SR, Grace AA. Cortico-Basal Ganglia reward network: microcircuitry. *Neuropsychopharmacology.* 2010; 35(1):27–47. [PubMed: 19675534]
- Spanagel R, Vengeliene V. New Pharmacological Treatment strategies for relapse prevention. *Curr Top Behav Neurosci.* 2012 doi:10.1007/7854_2012_205.
- Stromberg MF, Casale M, Volpicelli L, Volpicelli JR, O'Brien CP. A comparison of the effects of the opioid antagonists naltrexone, naltrindole, and beta-funaltrexamine on ethanol consumption in the rat. *Alcohol.* 1998; 15:281–9. [PubMed: 9590512]
- Tanda G, Di Chiara G. A dopamine-mu1 opioid link in the rat ventral tegmentum shared by palatable food (Fonzies): and non-psychostimulant drugs of abuse. *Eur J Neurosci.* 1998; 10:1179–1187. [PubMed: 9753186]
- Theile JW, Morikawa H, Gonzales RA, Morrisett RA. GABAergic transmission modulates ethanol excitation of ventral tegmental area dopamine neurons. *Neuroscience.* 2011; 172:94–103. [PubMed: 20974231]
- Ward SJ, Portoghese PS, Takemori AE. Pharmacological characterization in vivo of the novel opiate, beta-funaltrexamine. *J Pharmacol Exp Ther.* 1982; 220(3):494–8. [PubMed: 6121045]
- Wise RA. Addictive drugs and brain stimulation reward. *Annu Rev Neurosci.* 1996; 19:319–40. [PubMed: 8833446]
- Xiao C, Zhang J, Krnjevic K, Ye JH. Effects of ethanol on midbrain neurons: role of opioid receptors. *Alcohol Clin Exp Res.* 2007; 31:1106–1113. [PubMed: 17577392]

Fig. 1.

Naltrexone or vehicle pretreatment effects. **a)** Naltrexone (NAL) or saline pretreatment effect on dialysate dopamine concentrations. Numbers after NAL in the figure indicate the dose of naltrexone (mg/kg). Naltrexone infusion is at time 0. **b)** The effect of saline infusion in naltrexone or saline pretreated animals. Numbers after NAL in the figure indicate the dose of naltrexone (mg/kg). Saline infusion is at time 0. Naltrexone or saline pretreatment is 20 min prior. **c)** Naltrexone or vehicle microinjections into the ventral tegmental area. Numbers after NAL in the figure indicate the dose of naltrexone (µg). Naltrexone infusion is at time 0. For all figures, asterisks indicate significance from baseline.

Fig. 2.

Intravenous naltrexone effect on morphine- or ethanol- stimulated dopamine release. **a)**

Naltrexone (NAL) effect on morphine-stimulated dopamine (1 mg/kg). **b)** Naltrexone effect on ethanol-stimulated dopamine (1 g/kg). **c)** Dialysate ethanol concentrations from the shell of the nucleus accumbens after intravenous (i.v.) pretreatment with saline or naltrexone and i.v. infusion with ethanol (1 g/kg). The legend is the same as described for fig 2b above. **d)** Naltrexone effect on ethanol-stimulated dopamine (1 g/kg) using 3 min dialysate sampling intervals. **e)** For the 3-minute dialysis experiment, dialysate ethanol concentrations from the shell of the nucleus accumbens after intravenous (i.v.) pretreatment with saline or naltrexone and i.v. infusion with ethanol (1 g/kg). The legend is the same as described for fig 2d above. For all figures, numbers after NAL in the figure indicate the dose of naltrexone (mg/kg).

Morphine or ethanol infusion is at time 0, naltrexone (or vehicle) infusion is 20 min prior, mean $\pm$ SEM is shown for each point, and asterisks indicate difference between control and experimental conditions at those time points.

Fig. 3.

Subcutaneous β -funaltrexamine effect on morphine (1 mg/kg) - or ethanol (1 g/kg) - stimulated dopamine release. **a)** β -funaltrexamine (BFNA) effect on morphine-stimulated dialysate dopamine. **b)** β -funaltrexamine effect on ethanol-stimulated dialysate dopamine. **c)** Dialysate ethanol concentrations for the β -funaltrexamine experiment. For all figures, 20 mg/kg β -funaltrexamine (or saline) was given subcutaneously approximately 24 h before intravenous morphine or ethanol, morphine or ethanol infusion is at time 0, and mean $\pm$ SEM is shown for each point

Fig. 4.

Microinjection of naltrexone into the ventral tegmental area effect on morphine (1 mg/kg) - or ethanol (1 g/kg) - stimulated dopamine release. **a)** Naltrexone (NAL) effect on morphine-stimulated dopamine release. **b)** Naltrexone effect on ethanol-stimulated dopamine release. **c)** Dialysate ethanol concentrations from the shell of the nucleus accumbens after microinjection of naltrexone. Asterisks indicate significant difference between control and experimental conditions at those time points. For all figures, the dose of naltrexone was 2.4 μ g, ethanol or morphine infusion is at time 0, and mean $\pm$ SEM is shown for each point

Fig. 5. Microinjection of cresyl violet into the ventral tegmental area (VTA) was used to estimate the extent of diffusion of naltrexone. Diffusion of cresyl violet was limited to less than 1 mm in any direction. Overlay is from Paxinos and Watson (2007) with the VTA highlighted in yellow (−5.76 mm from bregma)

Table 1

Summary of experimental protocol

Experiment	Group	Day 1 (“pretreatment”)	Day 1 (+20 min, “treatment”)
1	1	saline (i.v.)	morphine (i.v.)
	2	naltrexone (i.v.)	morphine (i.v.)
2	1	saline (i.v.)	saline (i.v.)
	2	saline (i.v.)	ethanol (i.v.)
	3	naltrexone (i.v.)	saline (i.v.)
	4	naltrexone (i.v.)	ethanol (i.v.)
3	1	saline (i.v.)	ethanol (i.v.)
	2	naltrexone (i.v.)	ethanol (i.v.)

Experiment	Group	Day 1 (“pretreatment”)	Day 2 (“treatment”)	Day 2 (+1.5 hours, “treatment”)
4	1	β -funaltrexamine (s.c.)	saline (i.v.)	morphine (i.v.)
	2	saline (s.c.)	saline (i.v.)	morphine (i.v.)
5	1	β -funaltrexamine (s.c.)	saline (i.v.)	ethanol (i.v.)
	2	saline (s.c.)	saline (i.v.)	ethanol (i.v.)

Experiment	Group	Day 1 (“pretreatment”)	Day 1 (+20 min, “treatment”)
6	1	ACSF (i.c.)	morphine (i.v.)
	2	naltrexone (i.c.)	morphine (i.v.)
7	1	ACSF (i.c.)	ethanol (i.v.)
	2	naltrexone (i.c.)	ethanol (i.v.)

Legend: i.v. = intravenous, s.c. = subcutaneous, i.c. = intracranial.