

Intra-VTA Deltorphin, But Not DPDPE, Induces Place Preference in Ethanol-Drinking Rats: Distinct DOR-1 and DOR-2 Mechanisms Control Ethanol Consumption and Reward

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Background: While there is a growing body of evidence that the delta opioid receptor (DOR) modulates ethanol (EtOH) consumption, development of DOR-based medications is limited in part because there are 2 pharmacologically distinct DOR subtypes (DOR-1 and DOR-2) that can have opposing actions on behavior.

Methods: We studied the behavioral influence of the DOR-1-selective agonist [D-Pen²,D-Pen⁵]-Enkephalin (DPDPE) and the DOR-2-selective agonist deltorphin microinjected into the ventral tegmental area (VTA) on EtOH consumption and conditioned place preference (CPP) and the physiological effects of these 2 DOR agonists on GABAergic synaptic transmission in VTA-containing brain slices from Lewis rats.

Results: Neither deltorphin nor DPDPE induced a significant place preference in EtOH-naïve Lewis rats. However, deltorphin (but not DPDPE) induced a significant CPP in EtOH-drinking rats. In contrast to the previous finding that intra-VTA DOR-1 activity inhibits EtOH consumption and that this inhibition correlates with a DPDPE-induced inhibition of GABA release, here we found no effect of DOR-2 activity on EtOH consumption nor was there a correlation between level of drinking and deltorphin-induced change in GABAergic synaptic transmission.

Conclusions: These data indicate that the therapeutic potential of DOR agonists for alcohol abuse is through a selective action at the DOR-1 form of the receptor.

Key Words: Ethanol, Place Preference, Self-Administration, Delta Opioid Receptor, Reward.

THERE ARE 4 distinct classes of opioid receptors: the mu opioid receptor (MOR), the delta opioid receptor (DOR), the kappa opioid receptor (KOR), and the nociceptin receptor. There is evidence that endogenous opioids exert a strong regulatory action on ethanol (EtOH) consumption in both humans and rodents and that selectively targeting each opioid receptor can either promote or inhibit EtOH consumption. For example, MOR knockout mice drink less EtOH (Roberts et al., 2000), while DOR knockout mice drink more EtOH (Roberts et al., 2001). In addition, KOR antagonists can either promote or reduce EtOH consump-

tion, depending upon the behavioral state of the animal (Mitchell et al., 2005; Walker and Koob, 2008).

The ventral tegmental area (VTA) is important for both opioid reward and regulation of EtOH consumption in rodents. Alcohol-preferring rats self-administer EtOH (McBride et al., 1993) and its primary metabolite acetaldehyde (Rodd-Henricks et al., 2002) directly into the VTA, and VTA cFOS expression increases after exposure to an EtOH-associated context (Hill et al., 2007). Intra-VTA administration of either the nonselective opioid-antagonist naltrexone (Mitchell et al., 2009) or the MOR-selective antagonist CTOP reduces EtOH consumption in rats (Margolis et al., 2008), while intra-VTA administration of the DOR-antagonist TIPP-Ψ increases EtOH consumption (Margolis et al., 2008). The nonselective opioid-antagonist methylnaloxonium also attenuates EtOH place preference in mice (Bechtholt and Cunningham, 2005).

Although there is only 1 gene encoding the DOR (Evans et al., 1992; Simonin et al., 1994), there are 2 pharmacologically distinct classes of DOR ligands: DOR-1 and DOR-2, both of which are blocked by the highly DOR-selective antagonist TIPP-Ψ (Schiller et al., 1993). The metabolically stable enkephalin analog [D-Pen²,D-Pen⁵]-Enkephalin (DPDPE) is a highly selective DOR-1 agonist, while

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[D-Ala²]-Deltorphin II (deltorphin) is a highly selectively DOR-2 agonist (Fang et al., 1994; Horan et al., 1993; Mattia et al., 1991; Vanderah et al., 1994). There is no cross-tolerance between these two DOR agonists (Sofuoglu et al., 1991a,b), and DOR-1 and DOR-2 ligands have different, sometimes opposing, effects on a variety of behaviors (Churchill et al., 1995; Noble et al., 1996; van Rijn and Whistler, 2009; Yu and Bodnar, 1997). We previously reported that DOR-1 activation in the VTA of the rat decreases EtOH consumption (Margolis et al., 2008). However, in mice, systemic administration of the DOR-2 antagonist, naltriben, decreases EtOH consumption (van Rijn and Whistler, 2009), suggesting opposing roles of DOR subtypes on drinking behavior. To study the therapeutic potential of DOR ligands for alcohol abuse and related behavioral disorders, it is essential to differentiate DOR-1 and DOR-2 effects on behavior and synaptic function in brain regions that contribute to reward.

We have previously shown that MOR and DOR-1 activity in the VTA has opposing effects on EtOH consumption. In contrast to the attenuation of EtOH consumption by intra-VTA injection of the MOR-selective antagonist CTOP, intra-VTA microinjection of the DOR-1 agonist DPDPE decreases EtOH consumption through inhibition of GABA release in chronically drinking animals (Margolis et al., 2008). Furthermore, the DPDPE-induced presynaptic inhibition of GABA terminals is inversely correlated with EtOH consumption (Margolis et al., 2008). However, the actions of DOR-2 agonists on GABAergic synaptic transmission in VTA neurons have not been studied. Further, although the evidence indicates that DOR ligands are potentially effective therapeutics for alcohol abuse, the involvement of DOR in

regulating reward and anxiety necessitates consideration of issues related to addiction and abuse liability. Therefore, it is critical to determine whether these compounds are rewarding in EtOH-consuming animals.

Here, we used a conditioned place preference (CPP) paradigm to assess the rewarding effects of the DOR-1 agonist DPDPE and the DOR-2 agonist deltorphin in both EtOH-naïve and EtOH-drinking Lewis rats when administered into the VTA. We also examined the effects of the DOR-2 agonist deltorphin on VTA GABA_A synaptic transmission to determine whether the previously identified relationship between attenuated drinking and inhibition of GABA release in VTA slices in response to a DOR-1 agonist would generalize to a DOR-2 agonist.

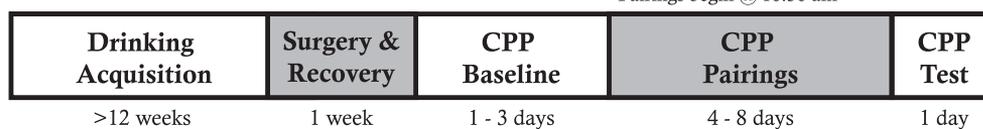
MATERIALS AND METHODS

Animals

Sixty-one male Lewis rats (47 for behavioral experiments and 14 for electrophysiology; Harlan Laboratories, Hayward, CA) weighing between 275 and 300 g on arrival were housed individually in a temperature-controlled colony room (21°C) on a 12-hour reversed light/dark cycle (lights off at 10:00 AM). For pharmacological studies, all animals served as their own controls (within subjects design). Additionally, each animal served in only a single pharmacology experiment (Fig. 1). Animals were all within the same age range at the beginning of each experiment. All experiments were performed during the dark portion of the cycle. Rat chow and water were available ad libitum throughout the experiment. During EtOH self-administration periods (see below), 10% EtOH (v/v; Gold Shield, Hayward, CA) was also available ad libitum. All experimental protocols were approved by the Institutional Animal Care and Use Committee and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH).

Conditioned Place Preference (n = 37):

Lights & Bottles off @ 10:00 am
Pairings begin @ 10:30 am



EtOH consumption (n = 10):



Electrophysiology (n = 14):

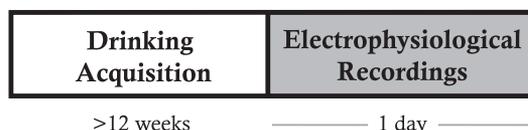


Fig. 1. Experimental timeline for place preference behavior ($n = 37$), ethanol (EtOH)-drinking experiments ($n = 10$), and electrophysiology ($n = 14$). Animals always had access to EtOH and H₂O bottles when not in the conditioned place preference (CPP) apparatus.

EtOH Self-Administration

EtOH was self-administered via a 2-bottle continuous access, free-choice paradigm in which 1 bottle contained 10% EtOH (v/v) and the other bottle contained water. Sucrose was never added to the EtOH solution. Animals were weighed daily, and the amount of EtOH and water consumed was measured at the same time each day (10:00 AM). Bottles were identical, and their positions were counterbalanced and rotated daily. Animals were maintained on the 2-bottle choice paradigm for at least 12 weeks until consumption had stabilized (defined as <15% change in drinking over 3 day bins) before surgery and experimentation commenced. Similarly, following surgery, behavioral training did not begin until drinking had returned to presurgical levels (defined as no significant difference between presurgical 3 day bin and final postsurgical 3 day bin). For place preference experiments, animals always had access to EtOH and H₂O bottles when not in the CPP chambers. For EtOH-drinking experiments, baseline drinking was always the 24-hour period prior to microinjection and was always on the same day of the week to control for daily variations in the colony schedule.

VTA Cannulations

Animals were anesthetized and maintained on isoflurane (0.5 l/min) as needed for the duration of surgery. Animals were placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA) and were implanted with bilateral 26-gauge stainless steel chronic guide cannulae (Plastics One, Roanoke, VA) into the VTA (AP, -5.8; ML, ±0.5 to 0.75; DV, -7.0 to -7.5) based on the atlas of Paxinos (Paxinos et al., 1994). The cannulae were implanted at 1.5 to 2 mm above the VTA to prevent trauma to the region during the surgical procedures. Cannulae were secured to the skull with dental cement. At the end of the surgical procedure, animals were treated with penicillin (intramuscularly, 1 mg/kg) and topical antibiotics. A stainless steel dummy cannula (Plastics One) was inserted into each guide cannula and remained in place when the guide cannulae were not in use. All animals were allowed a minimum 1-week recovery period before baseline testing.

For EtOH-consuming animals, surgery did not occur until drinking had stabilized (at least 12 weeks). EtOH bottles were removed from cages 12 hours prior to surgical procedures to minimize interactions with anesthesia. Bottles were replaced immediately following surgery. Drinking and CPP experiments did not commence until drinking had returned to presurgical levels (at least 1 week).

Conditioned Place Preference

Place preference training began after bottles were measured (10:30 AM). Animals were trained in 3-chamber place conditioning boxes (Med Associates, St. Albans, VT) in which 2 chambers (28 × 21 × 21 cm) that differed in color (1 black, 1 white), pattern, light level, and floor texture were separated by a neutral gray chamber (12 × 21 × 21 cm). During the initial baseline period, animals were placed in the neutral chamber and were allowed to freely explore all 3 chambers for a period of 30 minutes. Beam breaks, entries, and time spent in each chamber were automatically recorded using infrared beams. Animals were given a maximum of 3 baseline sessions on sequential days to demonstrate that no chamber bias was present and were excluded from the study if the bias for either conditioning chamber exceeded 250 seconds. During each conditioning session, animals were injected with either drug or saline then immediately confined to 1 of the 2 larger end chambers for 30 minutes. Animals received 2 conditioning sessions (separated by 5 hours) per day for 4 days. Due to timing constraints, 1 group of animals instead received conditioning sessions once daily for 8 days, and these data were combined with 4-day training for analysis.

Animals were counterbalanced such that an equal number of animals received drug injections in the black versus white box. Animals were tested for expression of CPP 1 day after the final conditioning session.

VTA Microinjections

Each injection was made using a 1- μ l syringe (Hamilton, Reno, NV) attached to 20 cm of PE 50 tubing connected to a 33-gauge injection cannula (Plastics One). Microinjections of 0.5 μ l volumes were given at a rate of 0.25 μ l/min using a syringe pump (KD Scientific, Holliston, MA) into each side of the VTA. Injection cannulae extended 1.5 to 2 mm beyond guide cannula to reach a depth of 9.0 mm and were left in place for 1 minute following microinjections to minimize the backflow of drug solution. For CPP experiments, microinjections were made directly prior to placement of animals into 1 of the 2 pairing chambers. For drinking experiments, to best capture deltorphin-induced change in drinking, deltorphin and saline were infused directly prior to lights out (10:00 AM). As each animal served as its own control (within subjects design), animals received the opposite injection on the following week. Microinjections always occurred on the same day of the week to control for daily variations in the colony schedule.

Slice Preparation and Electrophysiology

Electrophysiological experiments were completed blind to EtOH treatment and consumption levels. Recordings were made throughout the VTA. Rats were anesthetized with isoflurane, and their brains were removed. Horizontal brain slices (200 μ m thick) containing the VTA were prepared using a vibratome (Leica Microsystems, Wetzlar, Germany). Slices were submerged in artificial cerebrospinal fluid solution containing (in mM): 126 NaCl, 2.5 KCl, 1.2 MgCl₂, 1.4 NaH₂PO₄, 2.5 CaCl₂, 25 NaHCO₃, and 11 glucose saturated with 95% O₂-5% CO₂ and allowed to recover at 32°C for at least 1 hour. Individual slices were visualized using a Zeiss Axioskop microscope (Oberkochen, Germany) with differential interference contrast optics and infrared illumination. Whole-cell patch-clamp recordings were made at 32°C using 2.5 to 5 M Ω pipettes containing (in mM): 128 KCl, 20 NaCl, 1 MgCl₂, 1 EGTA, 0.3 CaCl₂, 10 HEPES, 2 MgATP, and 0.3 Na₃GTP (pH 7.2, osmolarity adjusted to 275), plus 0.1% biocytin to label the recorded neuron. Signals were amplified using an Axopatch 1-D amplifier (Molecular Devices, Sunnyvale, CA), filtered at 2 or 5 kHz, and collected at 5 or 20 kHz, respectively, using IGOR Pro (Wavemetric, Lake Oswego, OR). Cells were recorded in voltage-clamp mode ($V = -70$ mV). Series resistance and input resistance were sampled throughout the experiment with 4 mV, 200 ms hyperpolarizing steps. For all experiments, neurons in which there was a change in series resistance of more than 5 M Ω , or 15% of baseline, were excluded from analysis. GABA_A receptor (GABA_AR)-mediated inhibitory postsynaptic currents (IPSCs) were pharmacologically isolated with 6,7-dinitroquinoxaline-2,3 (1*H*,4*H*)-dione (DNQX: 10 μ M), strychnine (1 μ M), and sulpiride (10 μ M). We previously confirmed that this approach isolates GABA_AR signaling with both picrotoxin (Margolis et al., 2008, 2011) and gabazine (Margolis et al., 2011). To measure drug effects on evoked IPSCs, paired electrical pulses (50 ms interval) were delivered once every 10 seconds through stimulating electrodes placed 80 to 250 μ m away from the patched soma. The IPSC amplitude was calculated by comparing a 2-ms period around the peak to a 2-ms interval just before stimulation. Spontaneous events were detected by searching the smoothed first derivative of the data trace for values that exceeded a set threshold, and these events were confirmed visually. Recordings included, but were not limited to, confirmed dopaminergic neurons (Margolis et al., 2008).

Drugs and Doses

EtOH (100%; Gold Shield) was diluted to 10% (v/v) for self-administration. DPDPE (10 mM; Sigma Aldrich, St. Louis, MO) was dissolved in physiological saline. DPDPE dose was chosen based on pharmacological effects on drinking demonstrated in previous studies. Deltorphan II (2.5 mM; Sigma Aldrich) was dissolved in physiological saline or ddH₂O. Deltorphan dose was chosen based on preliminary studies in Sprague–Dawley rats indicating significant CPP at the 2.5 mM concentration. Physiological saline was always injected for conditioning sessions in the nonpaired chamber. For electrophysiology, deltorphan II (1 μ M final concentration; Sigma Aldrich) was applied by bath perfusion (stock solution in H₂O). Stock solution was diluted in artificial cerebrospinal fluid immediately before application.

Perfusions and Histology

At the conclusion of behavioral experiments, animals were anesthetized with pentobarbital and intracardially perfused through the ascending aorta with 0.9% saline followed by 10% formalin. Brains were sectioned coronally around the cannula tracts at 50 μ m, mounted, and stained with cresyl violet or neutral red. Several animals had only a unilateral injection site within the VTA. These were also included in the analysis (Fig. 2).

Data Analysis

For consumption data, drinking was analyzed using 24-hour time points (10:00 AM to 10:00 AM). Baseline drinking was defined as the 24 hours of drinking prior to drug infusion, while infusion drinking

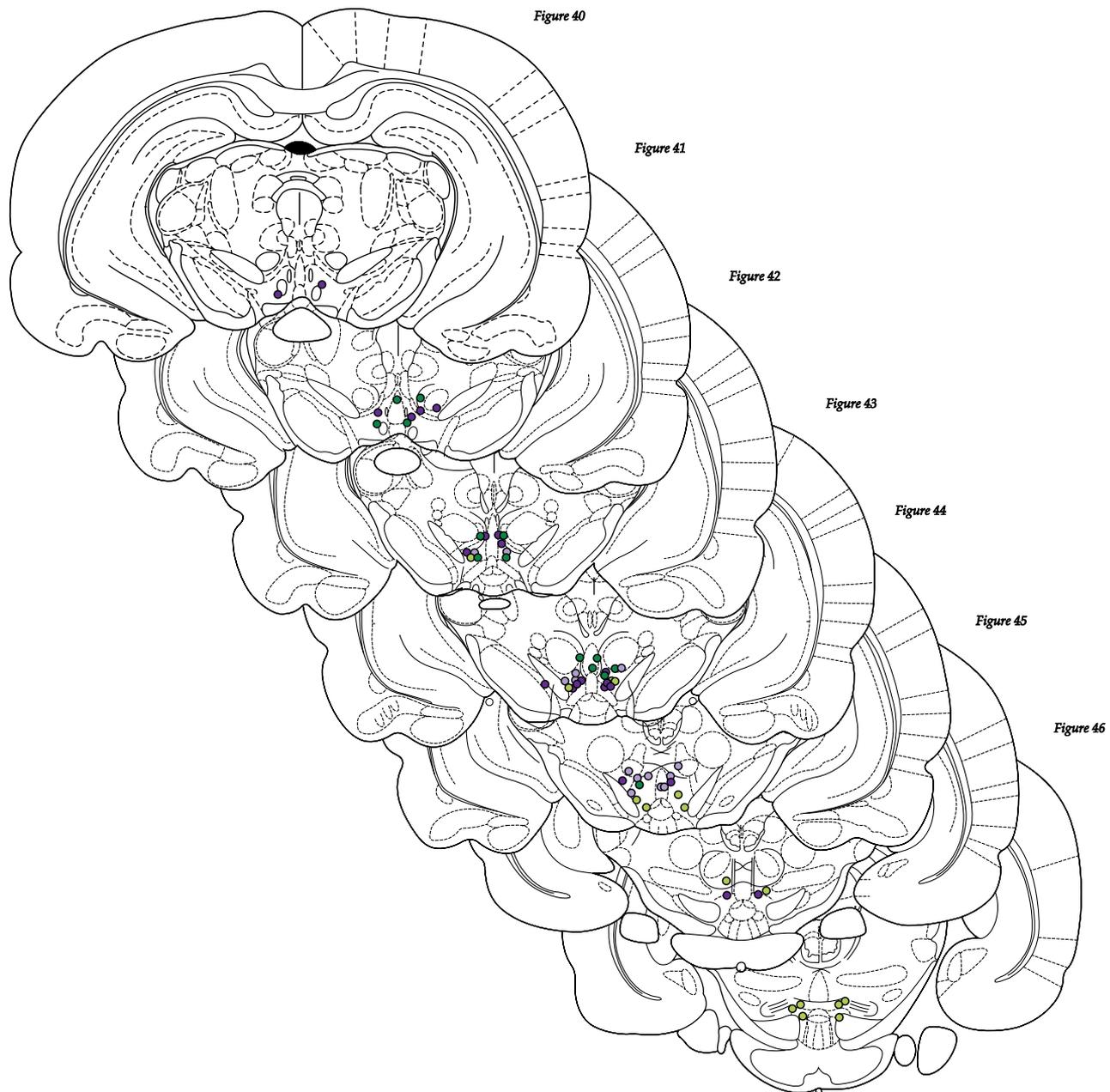


Fig. 2. Ventral tegmental area Histology demonstrating injection sites for place preference behavior. Purple = DPDPE and green = deltorphan. Lighter colors indicate ethanol (EtOH)-naïve animals and saturated colors indicate EtOH-consuming animals.

was defined as the 24 hours of drinking following drug or vehicle infusion into the VTA. CPP difference scores were calculated by subtracting the time spent in the vehicle-paired chamber from the time spent in the drug-paired chamber during a test session. A positive CPP difference score indicates place preference, while a negative score indicates place aversion. A paired *t*-test (difference scores at baseline vs. testing) was calculated for each group to determine significance of a preference effect. To compare between groups, a preference score was calculated by subtracting the difference scores between testing and baseline sessions:

$$(\text{Testing}_{\text{Paired}} - \text{Testing}_{\text{Unpaired}}) - (\text{Baseline}_{\text{Paired}} - \text{Baseline}_{\text{Unpaired}}).$$

For regression analysis, baseline drinking was defined as the 24 hours of drinking (10:00 AM to 10:00 AM) preceding the first day of CPP training.

For electrophysiology, the analyzed data were composed of the 4 minutes of baseline just preceding drug application and minutes 4 to 7 of drug application. Summary data are presented as mean \pm SEM. Paired *t*-tests, unpaired *t*-tests, and regression analyses were completed in Excel (version 11.4.1; Microsoft Corp., Redmond, WA). Alpha was set at 0.05 for all analyses.

RESULTS

To assess the rewarding effects of DOR agonists, we administered either DPDPE or deltorphin into the VTA in

both EtOH-naïve and EtOH-drinking animals. Intra-VTA DPDPE did not induce a significant place preference in either EtOH-naïve (Fig. 3A: $t = -1.29$, $p = 0.236$, $n = 8$) or EtOH-consuming Lewis rats (Fig. 3A: $t = -2.03$, $p = 0.069$, $n = 11$). Further, the preference in the drinking animals was not significantly greater compared with the EtOH-naïve animals (Fig. 3A: $t = -0.745$, $p = 0.466$, $n = 19$). In contrast, although intra-VTA deltorphin did not induce a place preference in EtOH-naïve rats (Fig. 3B: $t = 0.008$, $p = 0.994$, $n = 9$), it did induce a significant place preference in EtOH-consuming rats (Fig. 3B: $t = -18.96$, $p < 0.001$, $n = 9$). The preference seen in EtOH-consuming rats was also significantly greater than that seen in the naïve rats treated with deltorphin (Fig. 3B: $t = -5.77$, $p < 0.001$, $n = 18$).

Given that the rewarding effect of deltorphin emerged in drinking animals, we next assessed whether DOR place preference is related to magnitude of EtOH consumption. In fact, there was no correlation between CPP and baseline drinking (Fig. 4) for either the DOR-1 agonist DPDPE ($R = 0.185$, $p = 0.585$, $n = 11$) or the DOR-2 agonist deltorphin ($R = 0.074$, $p = 0.849$, $n = 9$), indicating that although EtOH consumption is necessary for the rewarding effects of DOR-2 agonists to emerge, DOR-2 place preference is not strictly regulated by level of EtOH consumption

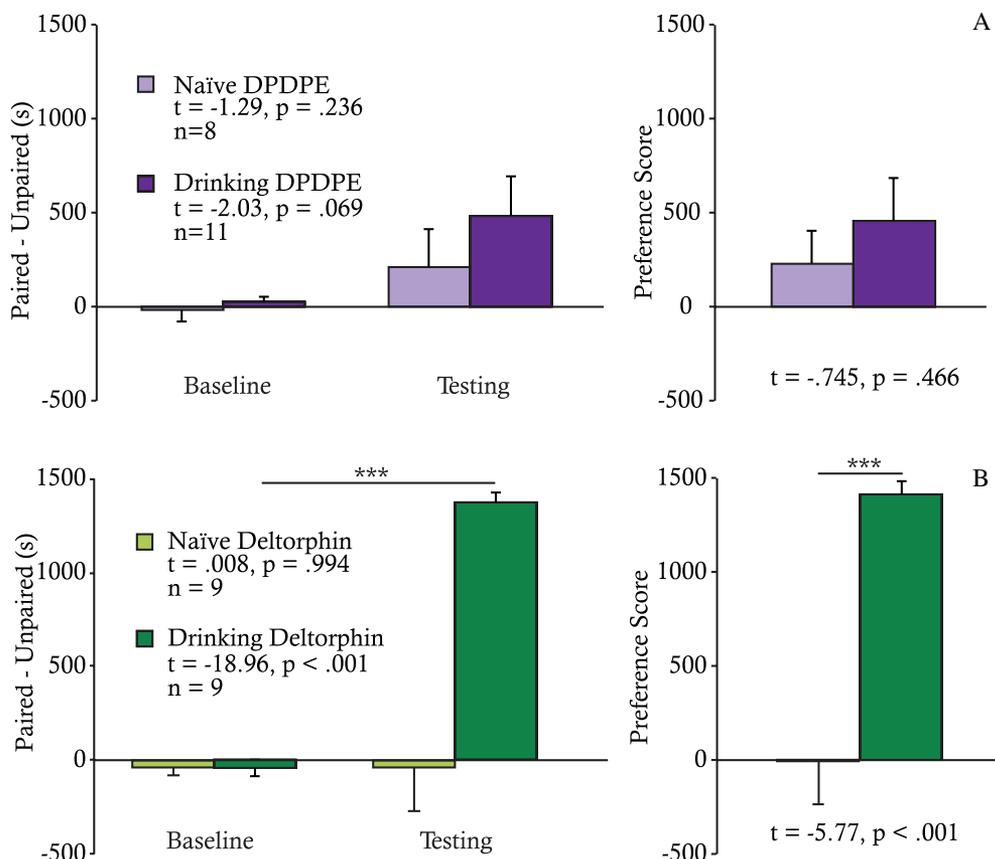


Fig. 3. Conditioned place preference following intraventricular tegmental area administration of the delta opioid receptor (DOR)-1 agonist DPDPE (10 mM; $n = 8$ naïve animals and $n = 11$ ethanol (EtOH)-drinking animals; **A**) and DOR-2 agonist deltorphin (2.5 mM; $n = 9$ naïve and $n = 9$ EtOH-drinking animals; **B**). Data are expressed as both paired—unpaired data within groups and as preference score data between groups.

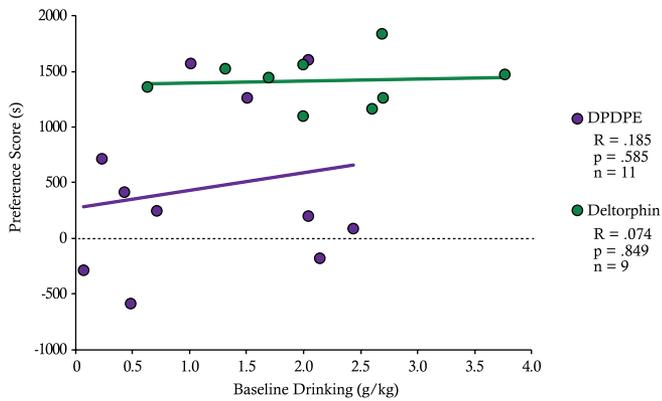


Fig. 4. Comparison of baseline drinking to conditioned place preference (CPP) preference score for both DPDPE (10 mM; $n = 11$; purple) and deltorphin (2.5 mM; $n = 9$; green) in drinking animals. There is no significant correlation between either DPDPE or deltorphin CPP and baseline ethanol consumption.

at these behaviorally and electrophysiologically relevant doses.

We previously demonstrated that intra-VTA administration of the DOR-1 agonist DPDPE decreases EtOH consumption. In contrast, here, we found that intra-VTA infusion of the DOR-2 agonist deltorphin had no significant effect on EtOH consumption (Fig. 5), confirming that subtype selectivity is a critical factor in DOR modulation of EtOH consumption.

We also previously demonstrated that DPDPE's ability to decrease EtOH consumption depends upon its inhibition of GABA release in the VTA and that the magnitude of the inhibition of GABA release is inversely correlated with EtOH consumption (Margolis et al., 2008). However, as deltorphin microinjections into the VTA did not affect EtOH consumption, we hypothesized that we would not observe an effect of deltorphin on VTA GABA release. We measured electrically evoked and spontaneous GABA_AR-mediated IPSCs in VTA neurons from EtOH-naïve and EtOH-drinking animals. On average, deltorphin (1 μ M) caused a very small inhibition of both evoked IPSC amplitude and spontaneous IPSC (sIPSC) frequency (Fig. 6). However, the effect

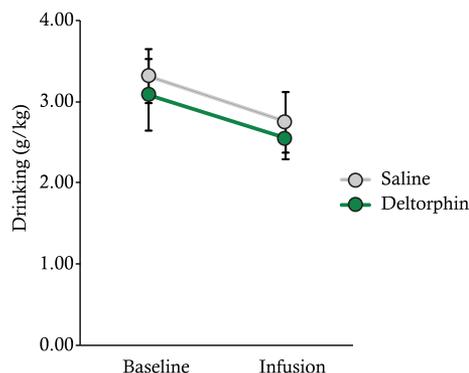


Fig. 5. Deltorphin (2.5 mM, $n = 10$) has no significant effect on ethanol consumption across animals following microinjection into the ventral tegmental area.

magnitudes varied greatly from cell to cell, and in a subset of neurons, deltorphin even caused an increase in evoked IPSC amplitude and sIPSC frequency (Fig. 6C,D). There was no significant effect of deltorphin on the amplitude of sIPSC events in control (29.7 ± 4.2 pA baseline; 31.1 ± 5.9 pA deltorphin; $n = 10$; $p = 0.65$) or drinking (27.6 ± 3.0 pA baseline; 24.2 ± 2.6 pA deltorphin; $n = 11$; $p = 0.23$) animals, consistent with the dominant effect being presynaptic. Also consistent with a presynaptic site of action, there was a significant correlation between the inhibition of evoked IPSC amplitude and inhibition of sIPSC frequency ($p = 0.004$). However, there was also a significant correlation between the change in evoked IPSC amplitude and sIPSC amplitude ($p = 0.03$) and no change in the paired pulse ratio in neurons from drinking animals (0.93 ± 0.11 baseline; 0.99 ± 0.11 deltorphin; $n = 14$; paired t -test $p = 0.35$), making it unclear whether the effects of deltorphin are purely presynaptic under these conditions. Finally, we tested whether there was a relationship between the effect of deltorphin on GABA_AR signaling in VTA neurons and how much animals were drinking just prior to the electrophysiology experiments. Unlike DPDPE, there was no relationship between modulation of GABA_AR signaling by deltorphin and EtOH consumption (Fig. 6E). These differences between the effects of DPDPE and deltorphin on GABA_AR signaling in VTA neurons from drinking animals are consistent with the different behavioral effects of these drugs described above and suggest different mechanisms of action for DOR-1 and DOR-2 in the VTA.

DISCUSSION

The data presented here show that at a behaviorally effective concentration, the DOR-1 agonist DPDPE does not induce CPP in either EtOH-consuming animals or EtOH-naïve animals, suggesting that DPDPE is not rewarding at this therapeutic dose. In contrast, the DOR-2 agonist deltorphin induces a robust CPP in drinking animals, but not in EtOH-naïve animals, while having no effect on EtOH consumption. Together, these data suggest that while DOR-1 agonists are potentially beneficial as treatments for alcohol-related disorders and may not induce additional addiction liabilities, DOR-2 agonists appear to have a reward potential in actively drinking animals. This suggests that, compared with a nonselective ligand, a DOR-1 agonist could retain therapeutic efficacy but with reduced addiction potential.

Our data are in keeping with recent findings in nonhuman primates, indicating that the DOR-2 agonist SNC-80 significantly enhances the discrimination of low to intermediate doses of EtOH (Platt and Bano, 2011). Taken together with data showing that the DOR-selective antagonist naltrindole attenuates both operant (Hyttiä and Kiianmaa, 2001) and home cage (Krishnan-Sarin et al., 1995) responding for EtOH in high drinking rats, these data suggest a possible potentiation of EtOH reward by DOR-2 agonists. Additionally, while our data may at first appear to be in conflict with

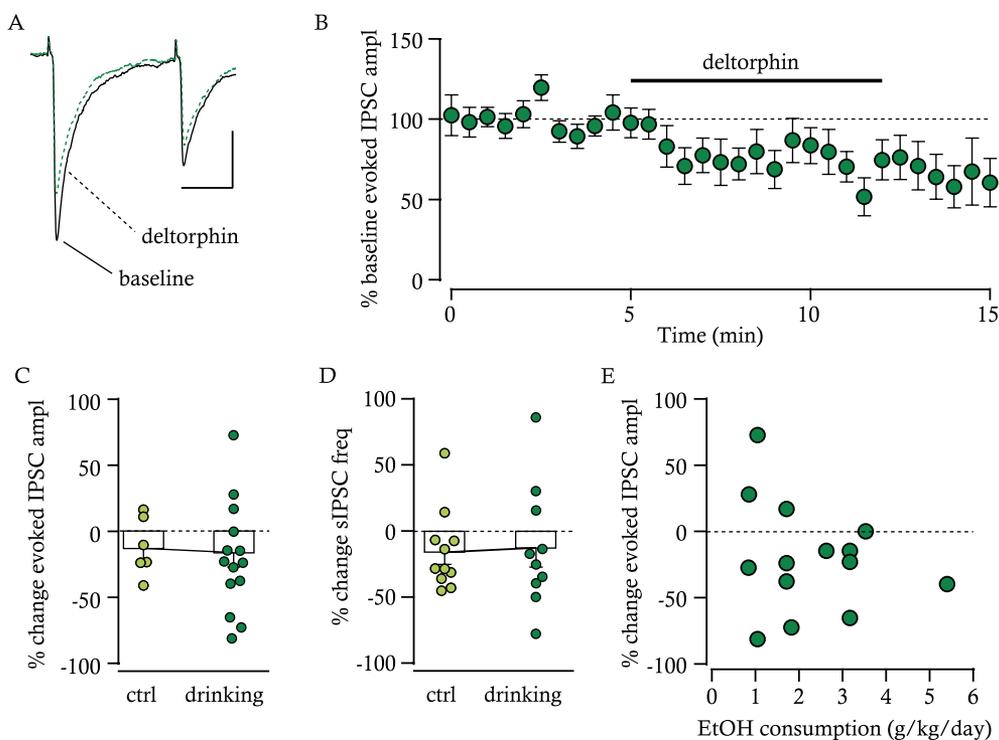


Fig. 6. Deltorphin modulates GABA_AR synaptic transmission in ventral tegmental area (VTA) neurons of naïve and ethanol (EtOH)-consuming animals. Example evoked inhibitory postsynaptic current (IPSC) traces from a VTA neuron from a drinking animal in which bath application of deltorphin (1 μ M; **A**) caused a small inhibition. Average time course of deltorphin effects across VTA neurons from drinking animals ($n = 14$ neurons from 9 EtOH-drinking animals; **B**). While on average deltorphin caused a slight inhibition of evoked IPSC amplitude ($n = 6$ neurons from 5 control animals and 14 neurons from 9 EtOH-drinking animals; **C**) and spontaneous IPSC (sIPSC) frequency ($n = 11$ neurons from 5 control animals and $n = 10$ neurons from 9 EtOH-drinking animals; **D**), there was a wide distribution of effects across neurons. There was no relationship between the effect of deltorphin on the evoked IPSC amplitude and EtOH consumption during the 24-hour period prior to the electrophysiology experiments ($n = 14$ neurons from 9 EtOH-drinking animals; **E**).

previous findings in mice, which show that neither a DOR-1 agonist (TAN-67) nor a DOR-2 agonist (SNC-80) is rewarding following EtOH consumption (van Rijn et al., 2012), a more detailed look at the published mouse data suggests an important similarity: while there was no potentiation of EtOH CPP in the mouse following SNC-80 versus saline administration, SNC-80 did induce CPP when compared with the effects of the DOR-1 agonist TAN-67. These data stress important differences in the rewarding effects of DOR-1 versus DOR-2 agonists in models of alcohol consumption and favor development of DOR-1-selective agonists for therapeutic use.

In contrast to the presynaptic effect previously found for the DOR-1 agonist DPDPE, our current data suggest that the effects of the DOR-2 agonist deltorphin are most likely a mix of pre- and postpresynaptic mechanisms. Change in sIPSC frequency significantly correlates with change in evoked IPSC amplitude, suggesting a presynaptic mechanism. We also observed some augmentations of GABA_AR signaling and have previously reported a postsynaptic mechanism for such effects in VTA neurons (Margolis et al., 2011). Further, in contrast to previous findings with DPDPE (Margolis et al., 2008), we saw no relationship between EtOH consumption and change in GABA_AR signaling with

deltorphin, even though deltorphin, like DPDPE, induced a wide distribution of effects on GABA_AR-mediated IPSCs. These data reinforce the differences between DOR-1 and DOR-2 on EtOH consumption and again suggest that an important relationship between drinking and change in IPSCs contributes to the effects of DOR-1, but not DOR-2, agonists in the VTA.

There was no relationship between baseline EtOH consumption and CPP score for either the DOR-1 or DOR-2 agonist, even though the DOR-2 agonist produced a robust CPP in EtOH-experienced rats. These data show that DPDPE, at a dose that is effective at curtailing EtOH intake (Margolis et al., 2008), is not rewarding across a group of variable drinkers. In contrast, while deltorphin induced a robust CPP across animals, it had no effect on EtOH consumption at this same dose. These data lend further support to the importance of DOR-1 agonist selectivity in the therapeutic use of DOR compounds for alcohol abuse and to the possibility that DOR-1 agonists could be developed as therapeutics without incurring additional addiction liabilities.

Previous data illustrate an important relationship between anxiety and stress and the DOR. Both DOR-1 and DOR-2 agonists are anxiolytic, while the DOR-selective antagonist naltrindole is anxiogenic (Perrine et al., 2006;

Randall-Thompson et al., 2010; Saitoh et al., 2004). In addition, anxiety levels are potentiated in DOR knockout mice (Filliol et al., 2000). We recently reported that following stress, activation of the DOR augments, rather than inhibits, GABA_AR IPSCs in VTA neurons (Margolis et al., 2011). These augmentations appear to be due to insertion of additional GABA_ARs into the plasma membrane. Together, these data suggest that alcohol and stress affect DOR signaling in different ways. Therefore, it is critical to next determine whether and how DOR-1 and DOR-2 compounds affect EtOH consumption in stressed animals and whether stress changes the effects of DOR agonists on drinking and preference. Additionally, as Lewis rats express greater basal anxiety and a blunted hypothalamic–pituitary–adrenal response to stress compared with other rat strains (Cohen et al., 2006), it will be necessary to compare the effects of stress on drinking across strains and in additional animal models. Therapeutic value would be enhanced by identification of a DOR agonist that is able to attenuate both anxiety and EtOH consumption, as many individuals with alcoholism also suffer from comorbid anxiety disorders (Grant et al., 2004; Schneier et al., 2010).

The 2-bottle choice continuous access methodology presented here generates lower levels of rodent EtOH consumption than EtOH studies that utilize intermittent or limited EtOH access, food or water restrict animals, sweeten or flavor the EtOH solution, or use alcohol-preferring animal lines. Furthermore, we do not know the precise blood alcohol levels of our animals at the various stages of testing. Additional experiments would be necessary to determine differences in DOR effects in higher drinking animals or in animals with high target blood alcohol levels. However, the objective of the current model is to allow for individual differences in drinking that may be obscured by methods that induce or impel EtOH consumption and thereby minimize variability in drinking. Previous studies (Margolis et al., 2008; Mitchell et al., 2012) indicate that the present methodology can render robust electrophysiological and behavioral effects that are significantly correlated during moderate levels of EtOH consumption.

In summary, the data presented here indicate that while neither the DOR-1 agonist DPDPE nor the DOR-2 agonist deltorphin induces a significant place preference in EtOH-naïve Lewis rats, EtOH consumption significantly potentiates deltorphin, but not DPDPE, place preference. Although the DOR-1 agonist DPDPE decreases EtOH consumption and this decrease inversely correlates with a DPDPE-induced inhibition of GABA release, we found no net effect of deltorphin on drinking and no relationship between drinking and the effect of deltorphin on GABA_AR signaling. In conclusion, these findings further support the hypothesis that DOR-1 agonists have potential as therapeutics for alcohol abuse but that DOR-2 agonists themselves may have abuse potential in alcoholics. Thus, development of DOR-based therapeutics for alcohol abuse should focus on DOR-1 agonist subtype selectivity.

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DISCLOSURES

The authors JMM, EBM, HLF hold a pending patent that relates to the identification of DOR-1- and DOR-2-selective compounds.

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