δ-Opioid Receptor Expression in the Ventral Tegmental Area Protects Against Elevated Alcohol Consumption

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Alcoholism is a complex and debilitating syndrome affecting ~140 million people worldwide. However, not everyone who consumes ethanol develops abuse, raising the possibility that some individuals have a protective mechanism that inhibits elevated alcohol consumption. We tested the hypothesis that the δ-opioid receptor (DOR) plays such a protective role. Here we show that DOR activity in the ventral tegmental area (VTA) robustly decreases ethanol consumption in rats and that these effects depend on baseline ethanol consumption. Intra-VTA microinjection of the DOR agonist DPDPE decreases drinking, particularly in low-drinking animals. Furthermore, VTA microinjection of the DOR selective antagonist TIPP-Ψ increases drinking in low, but not high, drinkers and this increase is blocked by comicroinjection of the GABA$_A$ antagonist bicuculline. Using electrophysiological techniques we found that in VTA brain slices from drinking rats DPDPE presynaptically inhibits GABA$_A$ receptor mediated IPSCs in low drinkers, but not in high drinkers or naive animals, most likely through activation of DORs on GABA terminals. This DOR-mediated inhibition of IPSCs also correlates inversely with behavioral correlates of anxiety measured in the elevated plus maze. In contrast, presynaptic inhibition of VTA GABA$_A$ IPSCs by the μ-opioid receptor agonist DAMGO is significantly reduced in both high- and low-drinking rats (<30%) compared with age-matched nondrinking controls (>70%). Together, our findings demonstrate the protective nature of VTA DORs and identify an important new target for therapeutic intervention for alcoholism.

Key words: ethanol; ventral tegmental area; δ-opioid; alcoholism; drinking; consumption

Introduction

The δ-opioid receptor (DOR) plays a protective role in cerebral ischemia, hypoxia, cardiac dysfunction, skeletal muscle damage, peripheral organ survival, and vulnerability to stress (Borlongan et al., 2004; Hebb et al., 2005; Hong et al., 2005; Saitoh et al., 2005; Chao et al., 2007; Förster et al., 2007). Interestingly, CNS expression of DORs is often dynamically induced following physiological challenge (Commons, 2003; Hack et al., 2005; Cahill et al., 2007) and trafficking to the plasma membrane is regulated through a variety of mechanisms, which are dependent on stimulus type and duration (Cahill et al., 2007). Although previous studies have identified a role of the DOR in opioid tolerance and dependence (Gomes et al., 2004; Zhang et al., 2006), and have suggested that DOR activity modulates both food consumption and drug reward (Duvauchelle et al., 1996; Kelley et al., 1996; Ragnauth et al., 1997; Suzuki et al., 1997; Lamonte et al., 2002;

Khaimova et al., 2004), to date no one has specifically investigated the possible protective effects of the DOR on drug or ethanol (EtOH) consumption.

The ventral tegmental area (VTA) has been implicated in the motivational actions of opioids (Bozarth and Wise, 1984) and EtOH (McBride et al., 1991): injection of a μ-opioid receptor (MOR) agonist into the VTA is reinforcing (Bals-Kubik et al., 1993; Olmstead and Franklin, 1997; Zangen et al., 2002; Terashvili et al., 2004) while MOR antagonists in the VTA block both opioid and EtOH place preference (Phillips and LePiane, 1980; Bechtholt and Cunningham, 2005). The major reinforcing process in the VTA is thought to be excitation of dopamine (DA) neurons (Schultz, 2007) and MOR agonist-mediated reinforcement in the VTA is thought to be due to inhibition of GABA release onto DA neurons (Johnson and North, 1992). Indeed, MOR agonists in the VTA increase DA levels in multiple VTA projection targets (Spanagel et al., 1992; Devine et al., 1993b; Yoshida et al., 1993) and it has been suggested that endogenous opioid release in the VTA in response to EtOH consumption is accessing the same mechanism to produce reinforcement (Nylander et al., 1994; Bechtholt and Cunningham, 2005).

Although many studies have reported a role for DORs in regulating EtOH intake, no consistent picture of its actions has emerged. DOR knock-out mice consume more EtOH than wild types (Roberts et al., 2001), yet pharmacologically blocking DOR signaling either attenuates EtOH consumption (Lê et al., 1993;
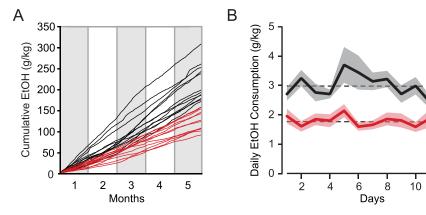
Figure 1. Drinking habits develop and stabilize over months. ***A***, Cumulative EtOH consumption for individual high (black; $n = 12$) and low (red; $n = 12$) drinking animals over the course of 5 months on a two-bottle free choice, continuous access paradigm. Drinking patterns are clearly established by the end of the fourth month. ***B***, Stable EtOH consumption plateau in high and low drinkers over the last 2 weeks before manipulation. Note that there is no crossover between high (black) and low (red) drinking populations. Broken horizontal lines indicate mean consumption over the 12 measurement points and shaded regions ±SEM for each group.

Froehlich, 1995; Krishnan-Sarin et al., 1995a,b; June et al., 1999; Hyytiä and Kiianmaa, 2001; Ciccocioppo et al., 2002) or has no effect (Hyytiä, 1993; Ingman et al., 2003). The reasons for this variability are not known; however, one possibility is that DOR efficacy changes dynamically during EtOH exposure. To determine whether dynamic regulation of the DOR affects EtOH intake in chronic drinkers, we studied the effect of DOR ligands on EtOH consumption in high- and low-drinking rats following long-term voluntary EtOH consumption. We performed this investigation in the VTA because opioid signaling in this brain region is sufficient to modulate EtOH reinforcement (Bechtholt and Cunningham, 2005).

Materials and Methods

Animals. Fifty-three male Lewis rats (Harlan Laboratories) weighing between 275 and 300 g on arrival were housed individually in a temperature controlled colony room (21°C) on a 12 h reversed light/dark cycle (lights off at 10 A.M.). All experiments were performed during the dark portion of the cycle. Rat chow, water, and 10% EtOH (Gold Shield) were available *ad libitum*. Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and were approved by the Ernest Gallo Clinic and Research Center Committee on Animal Research. Animals were never food or water deprived.

EtOH self-administration. EtOH was administered via a two-bottle continuous access, free-choice paradigm in which one bottle contained 10% EtOH (v/v) and the other bottle contained water. Sucrose was never added to the EtOH solution. The amount of EtOH and water consumed was measured at the same time daily (10 A.M.). Stable self-administration was achieved in 12–14 weeks (Fig. 1). Experiments began after a stable level of EtOH self-administration was achieved for all animals. Animals were weighed daily. No significant differences or changes in weight were observed between high and low drinkers or between any treatment groups. Bottles were identical and their positions were counterbalanced and rotated daily.

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 and were implanted with bilateral 26-gauge stainless steel chronic guide cannulas (Plastics One) into the VTA (AP, −5.8; ML, ±0.5; DV, −7.0) based on the atlas of Paxinos and Watson (1997). Cannulas were secured to the skull with dental cement. At the end of the surgical procedure, animals were treated with penicillin and topical antibiotics. A stainless steel dummy cannula (Plastics One) was inserted into each guide cannula and remained in place when the

guide cannulas were not in use. Animals were allowed a 1 week recovery period before behavioral testing.

VTA microinjections. Each injection was made using a 1 μl syringe (Hamilton) attached to 20 cm of PE 50 tubing connected to a 33-gauge injection cannula (Plastics One). Microinjections of 0.5 μl volumes were given at a rate of 0.5 μl/min using a syringe pump (kd Scientific) into each side of the VTA, except for bicuculline injections, where 0.25 μl was injected per side. Injection cannulas extended 2 mm beyond guide cannulas and were left in place for 1 min following microinjections to minimize the backflow of drug solution. In addition to drug microinjections, physiological saline microinjections were made in every rat to measure the effect of the injection manipulation alone on drinking. Drug injections were randomized and counterbalanced. Change in drinking due to drug microinjections was calculated by comparing to both the previous day's consumption ("baseline") and to saline injection. At the conclusion of the experiment, animals were anesthetized with pentobarbital and perfused intracardially through the ascending aorta with 0.1 M phosphate buffered saline followed by 10% formalin. Brains were sectioned coronally at 50 μm, mounted and stained with cresyl violet. Only animals with confirmed injection sites within the VTA were included (see supplemental figure, available at www.jneurosci.org, for placements). No behavioral differences were observed between animals with anterior compared with posterior cannula placements.

Elevated plus-maze. Before electrophysiological slice recordings, animals were run in an Elevated Plus-Maze (Med Associates) for 5 min. The main platform was elevated 75 cm from the ground and consisted of four arms (50 × 10 cm each) joined by a central platform (10 × 10 cm) with floor lines delineating the entrance to each arm. Enclosed arm walls were 40 cm high. Amount of time in open and closed arms, and number of rearings in open and closed arms were recorded for analysis. Animals began testing when placed in the central hub with their head facing toward a closed arm.

Slice preparation and electrophysiology. Lewis rats were maintained on 2 bottle choice, as described for the behavioral experiments, until their drinking stabilized. Electrophysiological experiments were completed blind to EtOH consumption levels. Recordings were made throughout the VTA. To commence electrophysiological experiments, rats were anesthetized with isoflurane and their brains were removed. Horizontal brain slices (200 μm thick) containing the VTA were prepared using a vibratome (Leica Microsystems). Slices were submerged in artificial CSF solution containing (in mM): 126 NaCl, 2.5 KCl, 1.2 MgCl, 1.4 NaH$_2$PO$_4$, 2.5 CaCl$_2$, 25 NaHCO$_3$, and 11 glucose saturated with 95% O$_2$– 5% CO$_2$ and allowed to recover at 32°C for at least 1 h. Individual slices were visualized using a Zeiss Axioskop microscope with differential interference contrast optics and infrared illumination. Whole-cell patch-clamp recordings were made at 32°C using 2.5–5 MΩ pipettes containing (in mM) 128 KCl, 20 NaCl, 1 MgCl$_2$, 1 EGTA, 0.3 CaCl$_2$, 10 HEPES, 2 MgATP, and 0.3 Na$_3$GTP (pH 7.2, osmolarity adjusted to 275), plus 0.1% biocytin or lucifer yellow to label the recorded neuron. Signals were amplified using a Multiclamp 700B amplifier (Axon Instruments), filtered at 2 kHz, and collected at 5 kHz using IGOR Pro (Wavemetric). I_h was measured by voltage clamping cells and stepping from −60 to −40, −50, −70, −80, −90, −100, and −120 mV. 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. GABA$_A$ IPSCs were pharmacologically isolated with 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX: 10 μM), strychnine (1 μM), and sulpiride (10 μM). Picrotoxin (100 μM) was added at the end of some recordings to confirm the remaining signal was due to GABA$_A$ receptor activation. Stimulating electrodes were placed 80–250 μm away

from the soma. To measure drug effects on evoked IPSCs, paired pulses (50-ms interval) were delivered once every 10 s. The IPSC amplitude was calculated by comparing a 2 ms period around the peak to a 2 ms interval just before stimulation. The paired-pulse ratio (PPR) was calculated by dividing the amplitude of the second IPSC by that of the first, after averaging together 8 consecutive trials. 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. Dose–response experiments were completed in the presence of a MOR antagonist (1 μM CTAP) to insure the observed effect remained DOR selective at the 10 μM dose of DPDPE.

All neurons were identified as $I_h(+)$ or $I_h(-)$. Wherever possible, $I_h(+)$ neurons were immunocytochemically processed for tyrosine hydroxylase (TH) content as a marker for DA neurons (see below). However, since no results differed across any of these cell groups, the data were grouped together for analysis.

Immunocytochemistry. Immediately after electrophysiological recording, slices were fixed in 4% formaldehyde for 2 h and then stored at 4°C in PBS. Slices were preblocked for 2 h in PBS plus 0.3% (v/v) Tween, 0.2% BSA and 5% normal goat serum and then incubated at 4°C with a rabbit anti-tyrosine hydroxylase polyclonal antibody (1:100). The slices were then washed thoroughly in PBS with 0.3% Tween and 0.2% (w/v) BSA before being agitated overnight at 4°C with Cy5 or FITC anti-rabbit secondary antibody (1:100). For cells filled with biocytin, fluorescein (DTAF)-conjugated streptavidin (3.25 μl/ml) was also added during this step. Sections were rinsed and mounted on slides using Bio-Rad Fluoro-guard Antifade Reagent mounting media and visualized under a Zeiss LSM 510 META microscope. Primary antibodies were obtained from Chemicon International, secondary antibodies from Jackson ImmunoResearch Laboratories, and all other reagents from Sigma Chemical.

Drugs and doses. For behavioral experiments, DPDPE (10 mM; Sigma), TIPP-Ψ (5 μM; NIDA), Bicuculline (1 mM; Sigma), DAMGO (0.2 mM; Sigma), and CTOP (10 mM; Tocris) were prepared in physiological saline for microinjection into the VTA. For electrophysiology, all drugs were applied by bath perfusion. Stock solutions were made and diluted in artificial CSF immediately before application. All chemicals were obtained from Sigma or Tocris except TIPP-Ψ, which was acquired from NIDA.

Data analysis. Results are presented as mean ± SEM where appropriate. For behavioral data, drinking was analyzed using 24 h time points. Raw drinking data were used for paired Student's t test comparisons probing drug effects on drinking compared with baseline or saline injections, a more conservative comparison than analyzing normalized data. Drinking comparisons and regression analyses were completed in Excel (v.11.4.1; Microsoft). Because TIPP-Ψ induced a long-lasting, robust increase in EtOH consumption in low-drinking animals (see below), cross-over saline data could not be obtained for three animals that received the TIPP-Ψ injection before saline. Baseline data were substituted for these three animals for benefit of statistical comparison. For electrophysiology, the analyzed data were composed of the 4 min of baseline just preceding drug application and minutes 4–7 of drug application. Comparisons across electrophysiology groups were made with one-way ANOVA followed by the Student–Newman–Keuls (SNK) method for multiple comparisons where appropriate using SigmaStat software (SPSS). $p < 0.05$ was required for significance in all experiments.

Results

DOR activation in the VTA attenuates EtOH consumption

We initially investigated the effects of DOR activation on EtOH consumption by microinjecting DOR selective compounds into the VTA of chronically drinking Lewis rats. The DOR selective agonist DPDPE (10 mM) decreased drinking across all animals compared with drinking the day before treatment ($n = 15$, $t = 2.14$, $p = 0.008$) (Fig. 2A). This effect was also evident compared with control saline microinjections in the same rats, and was particularly prominent in low drinkers ($n = 7$, $t = 2.45$, $p = 0.02$) (Fig. 2B). This DPDPE effect on drinking was only at trend level in high drinkers ($n = 8$, $t = 2.36$, $p = 0.059$) (Fig. 2B). Addition-
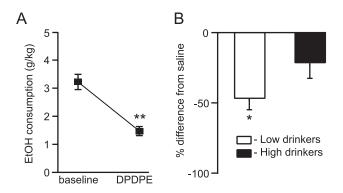
Figure 2. Chronically drinking rats decreased their EtOH consumption following DOR activation in the VTA. *A*, EtOH drinking decreased across the whole population of rats when the DOR agonist DPDPE (10 mM) was microinjected bilaterally into their VTA compared with the preceding 24 h. *B*, This decrease in drinking was also evident when compared with control saline injection effects and was particularly pronounced in low-drinking animals. *p < 0.05; **p < 0.01.
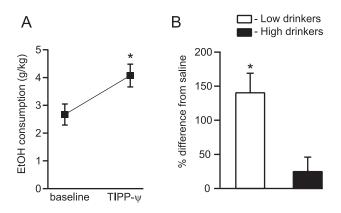
Figure 3. Chronically drinking rats increased their EtOH consumption when DOR signaling was blocked in the VTA. *A*, Microinjecting the DOR selective antagonist TIPP-Ψ (5 μM) into the VTA increased drinking compared with the preceding 24 h of consumption. *B*, This increase in drinking was also apparent compared with saline injections and was driven by low-drinking animals. *p < 0.05.

ally, there was an inverse correlation between baseline EtOH consumption and % baseline drinking following DPDPE administration ($n = 15$, $F = 6.35$, $r = 0.57$, $p = 0.02$), which demonstrated that the lowest drinking animals were the most affected by DPDPE. Consistent with this result, the DOR selective antagonist TIPP-Ψ (5 μM) microinjected into the VTA increased drinking across all animals compared with EtOH consumption the day preceding treatment ($n = 14$, $t = 2.16$, $p = 0.006$) (Fig. 3A). This was also the case compared with saline injections, and a median split of the data revealed that this effect was again driven by the low drinkers ($n = 7$, $t = 2.45$, $p = 0.0002$ for low drinkers vs $n = 7$, $t = 2.45$, $p = 0.32$ for high drinkers) (Fig. 3B). These data suggest that DOR activation by endogenous opioids released in the VTA normally suppresses EtOH intake. Moreover, some animals appear to lack this protective mechanism, resulting in increased EtOH intake.

DOR activation decreases GABA release in the VTA in chronically drinking animals

Our behavioral observations indicate that DOR activation in the VTA is interacting with EtOH reinforcement. One possibility is that in low drinkers EtOH is reinforcing at a lower concentration. Since attenuation of GABA$_A$ signaling in the VTA is reinforcing (Ikemoto et al., 1997) and presynaptic opioid receptor activation
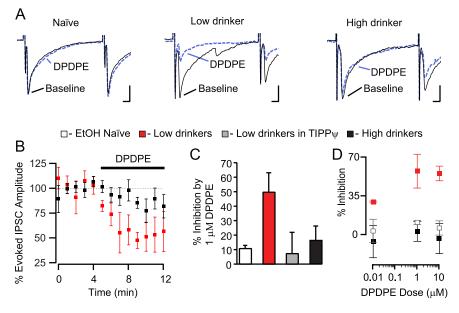
Figure 4. DOR agonists inhibit evoked GABA$_A$ signaling in the VTA of drinking rats. ***A***, Example traces of evoked GABA$_A$ IPSCs from VTA neurons from EtOH naive, low and high drinkers. Only in the cell from a low drinker did the selective DOR agonist DPDPE (1 μM) decrease the amplitude of the evoked IPSC. Calibration: 50 pA, 5 ms. ***B***, Time course of the DPDPE effects in VTA neurons from drinking animals. ***C***, The effect of DPDPE in low drinkers ($n = 4$) was significantly larger than that in naive ($n = 3$) or high drinkers ($n = 6$). The effect in low drinkers was blocked by the DOR selective antagonist TIPP-Ψ ($n = 3$). ***D***, Dose–response data were collected from each group, 2–4 neurons per data point.

can inhibit neurotransmitter release (Williams et al., 2001), we examined whether DOR activation decreases GABA release onto VTA neurons in drinking animals. We measured pharmacologically isolated evoked and spontaneous GABA$_A$ IPSCs in brain slices from animals with drinking histories similar to those used in the behavioral experiments. We confirmed that the evoked and spontaneous IPSCs were due to GABA$_A$ receptor activation, as the GABA$_A$ receptor selective antagonist picrotoxin (100 μM) completely blocked both evoked and spontaneous IPSCs ($n = 4$, data not shown).

DPDPE (1 μM) significantly inhibited both evoked (Fig. 4) and spontaneous (Fig. 5) IPSCs in VTA neurons from drinking animals. In contrast, in age-matched, ethanol-naive, control animals there was no effect of DPDPE on GABA$_A$ IPSCs (Figs. 4*A,C,D*, 5*B,C*). The DPDPE effect in drinking animals was blocked by the DOR selective antagonist TIPP-Ψ (1 μM), indicating that the agonist was acting through the DOR (Fig. 4*C*). Application of the antagonist alone had no effect on either evoked IPSC amplitude (6.8 $\pm$ 9.0% decrease from baseline, $n = 3$) or spontaneous IPSCs (8.6 $\pm$ 2.4% decrease in frequency and 2.6 $\pm$ 1.6% decrease in amplitude, $n = 2$), suggesting that there is no tonic activation of DOR in this slice preparation. Importantly, for both evoked IPSCs and spontaneous IPSCs there was an inverse correlation between DPDPE induced inhibition and amount of EtOH consumed, and this relationship was particularly strong for spontaneous IPSC frequency (Fig. 6). We also collected dose–response data to probe whether elevated EtOH consumption shifted the IC$_{50}$ of this DOR-mediated effect or whether the maximal effect was smaller in high-drinking and EtOH naive animals. We found that in all animal groups 1 μM DPDPE was a saturating dose with no apparent shift in the IC$_{50}$ between groups (Fig. 4*D*).

To confirm that these DOR-mediated effects were mediated by presynaptic receptors located on GABAergic terminals synapsing onto recorded neurons, we examined additional properties of the DPDPE effect in EtOH consuming animals. An in-

crease in PPR of evoked postsynaptic currents suggests a decrease in the probability of release of neurotransmitter (Manabe et al., 1993). There was a significant increase in the PPR of evoked IPSCs following DPDPE bath application (baseline: 0.85 $\pm$ 0.08; DPDPE: 1.18 $\pm$ 0.1; $p = 0.03$). DPDPE also inhibited spontaneous IPSC frequency but not amplitude, across neurons (Fig. 5*B,C*). Moreover, the change in spontaneous IPSC frequency correlated with the inhibition of evoked IPSCs (slope = 0.75, intercept = 3.0; $F = 7.17$, $r = 0.74$, $p = 0.04$). We also measured miniature IPSCs (mIPSCs) recorded in the presence of tetrodotoxin (TTX; 500 nM) to block possible action potential driven events. DPDPE decreased the frequency of mIPSCs and sIPSCs similarly in neurons from low-drinking animals (Fig. 5*B*). Also, as with the spontaneous events, across cells there was no change in the amplitude of mIPSCs with DPDPE (Fig. 5*C*). We therefore conclude that these DPDPE effects on IPSCs are due to inhibition of GABA release by presynaptic DORs on GABA terminals.

While there was no significant change in the amplitude of spontaneous IPSCs following DPDPE application, a subset of neurons showed a decrease in spontaneous IPSC amplitude. This change was driven by a loss of the largest events (see example in Fig. 5*A*), which could result from events onto or very close to the soma or from simultaneous release of multiple vesicles. Since these large events persist in the presence of TTX (Fig. 5*D$_i$*), they are not action potential dependent. At some synapses the amplitude distributions of IPSC events are sensitive to changes in the probability of release (Behrends and ten Bruggencate, 1998). Another alternative is that DORs may be expressed on a subset of presynaptic terminals. Consistent with this possibility are the example distributions of mIPSC event amplitudes showing that DPDPE selectively inhibits the frequency of large events in one cell (Fig. 5*D$_i$*) and small events in a different cell (Fig. 5*D$_{ii}$*).

DOR mediates EtOH consumption through GABA$_A$ signaling
If presynaptic inhibition of GABA release plays a role in our observed intra-VTA DOR modulation of EtOH consumption, then the TIPP-Ψ induced increase in drinking should be blocked by coinjection of a GABA$_A$ receptor antagonist. In fact, when the GABA$_A$ receptor antagonist bicuculline (1 mM) was coinjected with TIPP-Ψ (5 μM) into the VTA, it completely blocked the TIPP-Ψ induced increase in EtOH consumption in low-drinking animals (Fig. 7). Bicuculline also produced a small overall decrease in drinking across all animals following coadministration ($n = 15$, $p = 0.035$). However, a median split revealed that this effect was carried by high-drinking animals ($n = 7$, $t = 2.45$, $p = 0.017$) (Fig. 7) in whom TIPP-Ψ alone had no significant effect. Furthermore, TIPP-Ψ and bicuculline cotreatment had no effect on EtOH consumption in low-drinking animals ($n = 8$, $t = 2.36$, $p = 0.79$) (Fig. 7). Therefore, VTA GABA neurotransmission plays a critical role in DOR mediated control of EtOH consumption in low-drinking animals.

DOR modulation of GABA in the VTA correlates with anxiety-like behaviors

Because anxiety has been linked to EtOH consumption (Boyd et al., 1989) we also measured behavioral correlates of anxiety using an elevated plus maze prior to electrophysiological experiments. More time spent in the open arms and more open arm rearings suggest an animal is less anxious. We found that the animals that spent more time in the open arms also expressed greater DPDPE-induced inhibition of GABA$_A$ sIPSC frequency (Fig. 8A). There was also a significant correlation between the number of rearings in the open arms and DPDPE inhibition of sIPSC frequency (Fig. 8B). These correlations suggest that animals that are less anxious have more functional DORs on GABA terminals in the VTA during chronic drinking, and raise the possibility that activation of these DORs is anxiolytic.

MOR antagonists in the VTA cause a decrease in EtOH consumption

Naltrexone's therapeutic effects on drinking have been proposed to be mediated through the MOR (Ghozland et al., 2005; Weerts et al., 2008), and blocking opioid signaling in the VTA diminishes EtOH reinforcement (Bechtholt and Cunningham, 2005). To evaluate the role of VTA MORs in drinking and to compare with our DOR findings, we completed similar behavioral and electrophysiological experiments with MOR ligands. In contrast to DOR, microinjection of the MOR agonist DAMGO (0.2 mM) into the VTA did not affect drinking (Fig. 9A). However, the MOR selective antagonist CTOP (10 mM) significantly decreased drinking (Fig. 9B). In contrast to the DOR antagonist mediated enhancement of ethanol consumption in low drinkers, this MOR antagonist mediated decrease was similar in high- and low-drinking animals.

MOR agonist effects on GABA release are diminished in drinking animals

Electrophysiologically we found that, compared with EtOH naive animals, DAMGO (1 μM) had a relatively small effect on evoked and spontaneous GABA$_A$ signaling in drinking animals (27 ± 10% in drinkers versus 74 ± 5% decrease in evoked GABA$_A$ amplitude in naive animals) (Fig. 10A). However, there was great variability among these effects (Fig. 10B) and there was no apparent relationship between DAMGO effect and either EtOH consumption or cell type. The DAMGO effects on evoked IPSC amplitude and spontaneous IPSC frequency were correlated within each cell (Fig. 10B), suggesting a presynaptic site of MOR agonist action on GABA$_A$ signaling.
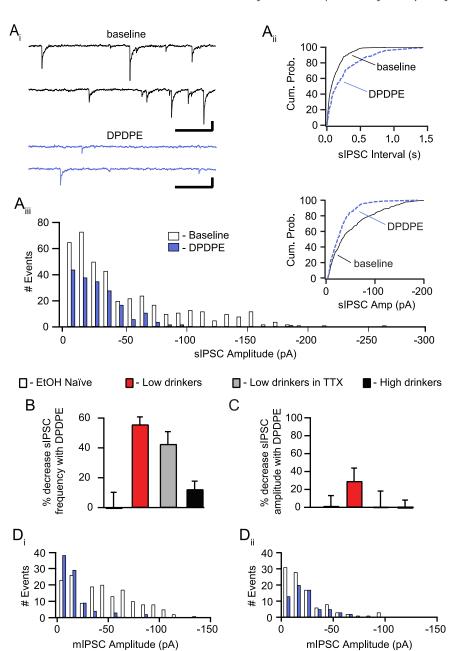

Figure 5. DOR agonists inhibit spontaneous GABA$_A$ signaling in the VTA of drinking rats. **A$_i$,** Example traces from the baseline period and DPDPE (1 μM) application from a cell from a low-drinking animal showing spontaneous IPSCs. Calibration: 50 pA, 200 ms. **A$_{ii}$,** The cumulative probability plot of event interval from the same neuron shows a rightward shift in interval length in the presence of DPDPE in this case. **A$_{iii}$,** A histogram of the event amplitudes shows a decrease in the frequency of the events of all amplitudes with DPDPE. Inset, The cumulative probability plot of the event amplitudes shows a shift to the left with DPDPE indicating a decrease in event amplitudes in this cell. **B,** There was an overall greater DPDPE-induced inhibition of sIPSC frequency in neurons from low-drinking animals (n = 5) than in naive (n = 4) or high drinkers (n = 7), and this effect was also present in neurons from low drinkers when the experiment was conducted in the presence of TTX (500 nM; n = 5). **C,** There was a nonsignificant trend for DPDPE to decrease the sIPSC amplitude in low-drinking animals. **D,** Example event amplitude histograms from mIPSC experiments collected in the presence of TTX (500 nM). Very large events persist in the presence of TTX (**D$_i$**), and DPDPE can either decrease (**D$_i$**) or increase (**D$_{ii}$**) the mean amplitude of mIPSCs within a particular neuron.

Baseline GABA$_A$ signaling following chronic EtOH

Melis et al. (2002) reported an augmentation of probability of release of GABA onto VTA $I_h(+)$ neurons 24 h after EtOH injection in mice. We examined our data to see whether such changes persist following chronic EtOH consumption. We found no difference in baseline spontaneous IPSC frequency in $I_h(+)$ neurons between drinking rats (3.0 ± 0.6; n = 23 neurons) compared with EtOH naive rats (2.3 ± 0.4; n = 11
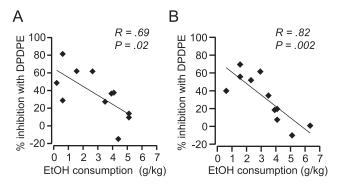
Figure 6. DOR-mediated inhibition of GABA$_A$ signaling correlates with EtOH consumption. **A**, The magnitude of the DPDPE inhibition of evoked IPSCs was inversely correlated with EtOH consumption. **B**, There was also an inverse correlation between the extent of the DPDPE inhibition of sIPSC frequency and the amount of EtOH the animal consumed.
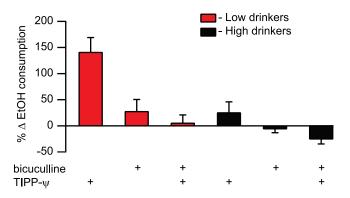

Figure 7. DOR mediates drinking in the VTA through a GABA$_A$ mechanism. Microinjection of the GABA$_A$ antagonist bicuculline (1 mM) into the VTA does not alter drinking when compared with baseline consumption. Coinjection of bicuculline with TIPP-Ψ (5 μM) blocks the TIPP-Ψ induced increase in drinking.
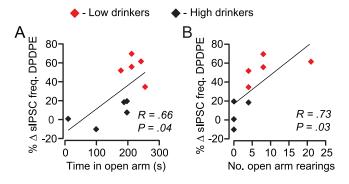

Figure 8. DOR agonist effects on spontaneous IPSC frequency correlate with anxiety measures in drinking animals. Before recordings, animals were tested in the elevated plus maze. The behavioral measures of time in the open arm (**A**) and number of rearings (**B**) correlated with the DOR mediated inhibition of spontaneous GABA$_A$ IPSC frequency.

neurons; $p = 0.45$). Furthermore, the baseline PPR was similar in $I_h(+)$ neurons from drinking (0.91 ± 0.10; $n = 21$) compared with EtOH naive (0.97 ± 0.09; $n = 12$; $p = 0.70$) rats. We also observed no difference in the mean amplitude of spontaneous IPSCs between drinking (32 ± 4 pA; $n = 23$ neurons) and EtOH naive rats (25 ± 3 pA; $n = 11$ neurons; $p = 0.23$). Therefore, the changes in GABA signaling in the VTA reported in response to acute EtOH administration appear to not persist following prolonged EtOH consumption.
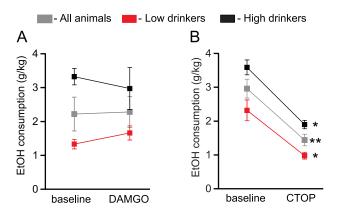

Figure 9. MOR antagonists in the VTA decrease drinking. **A**, The MOR agonist DAMGO (0.2 mM) microinjected into the VTA does not change EtOH consumption. **B**, The MOR antagonist CTOP (10 mM) microinjected into the VTA decreases EtOH consumption. *$p < 0.05$; **$p < 0.01$.
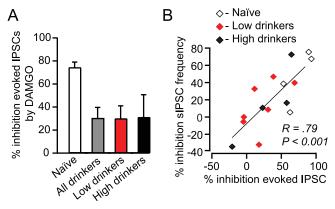

Figure 10. Chronic EtOH consumption decreases presynaptic MOR inhibition at VTA GABA terminals. **A**, Mean DAMGO (500 nM) effects on evoked IPSC amplitude by treatment group. EtOH naive animals ($n = 5$) showed a greater inhibition of evoked IPSC amplitude with DAMGO than either high ($n = 4$) or low ($n = 8$) drinking animals. **B**, There is a correlation between the effects of DAMGO on evoked IPSC amplitude and spontaneous IPSC frequency but not drinking behavior.

Discussion

Our results demonstrate that DOR activation in the VTA inhibits EtOH consumption in actively drinking rats. In low-drinking rats, VTA microinjection of the DOR agonist DPDPE significantly decreases EtOH consumption, while the DOR selective antagonist TIPP-Ψ significantly increases drinking. These effects were not seen in high-drinking rats. Our data implicate presynaptic modulation of GABA release by DOR in these behavioral effects since DPDPE inhibits evoked, spontaneous, and miniature GABA$_A$ IPSCs in low-drinking animals, but does not change evoked or spontaneous GABA$_A$ IPSCs in high-drinking or naive animals, and also because the TIPP-Ψ induced increase in EtOH consumption in low drinkers is blocked by comicroinjection of the GABA$_A$ antagonist bicuculline.

The emergence of functional DORs following exposure to drugs of abuse and other physiological challenges is a prevalent theme in DOR research. Ultrastructural studies (Cahill et al., 2007) show that in many brain regions DORs have a primarily cytoplasmic location. These cytoplasmic DORs only become available to bind most ligands when they translocate to the cell surface. Such translocation may underlie the emergence of functional DOR on GABA terminals in the periaqueductal gray following 48 h of exposure to morphine (Hack et al., 2005). Translocation of DORs to the cell membrane also occurs in the nucleus

accumbens following exposure to psychostimulants (Ambrose-Lanci et al., 2008). With respect to EtOH, we show here an increase in DOR function on GABA terminals in the VTA following chronic drinking, and this increase in DOR appears to protect against elevated EtOH consumption. This is consistent with the protective effects of DORs in other systems.

Previous research in EtOH preferring and/or high-drinking rats indicates that a DOR selective antagonist can either have no effect on EtOH consumption (Stromberg et al., 1998; Ingman et al., 2003) or can decrease EtOH consumption (Krishnan-Sarin et al., 1995a,b; June et al., 1999). The present data are consistent with the former findings. However, our current data demonstrate that, in contrast, there is significant DOR action in low-drinking animals. These results highlight the importance of considering individual differences in behavior relative to specific brain functions and offer a possible explanation for the previously reported heterogeneous effects of DOR manipulation on EtOH consumption. Furthermore, since stress and pain also cause increases in functional DORs (Besse et al., 1992; Hao et al., 1998; Ploj et al., 2003), previous experiences may influence DOR ligand modulation of drinking.

Although the anxiolytic properties of DOR selective agonists have been reported previously (Saitoh et al., 2004; Perrine et al., 2006; Jutkiewicz, 2007), the potential electrophysiological mechanisms by which these effects occur have yet to be investigated. Behaviorally, DOR knock-out mice display greater measures of anxiety than matched controls (Filliol et al., 2000), and selective δ antagonists can attenuate the anxiolytic actions of benzodiazepines (Primeaux et al., 2006). However, to our knowledge, ours is the first examination of an electrophysiological process linking anxiety-like behaviors to specific synaptic actions of DOR activation. We demonstrate that EtOH consuming animals with lower levels of anxiety have greater DOR function on GABA terminals in the VTA, raising the possibility that DOR activation in the VTA is anxiolytic. Furthermore, since less DOR inhibition of GABA release in the VTA correlates with both heightened drinking and higher behavioral anxiety scores, DOR expression in the VTA may be a potential physiological link between anxiety behaviors and EtOH consumption. These data are also intriguing in light of recent results showing an interaction between corticotrophin releasing factor (CRF), a physiological indicator of stress, and EtOH consumption, such that blocking CRF receptor activation decreases EtOH consumption (Heilig and Koob, 2007; Marinelli et al., 2007; Lowery et al., 2008). Additional studies are necessary to investigate the relationship between different types of stress, anxiety, EtOH consumption, and DOR function and to determine the time course of these effects.

Behaviorally, we found that MOR and DOR selective antagonists have opposing effects on EtOH self-administration: intra-VTA microinjection of the MOR selective antagonist CTOP decreases consumption while the highly selective DOR antagonist TIPP-Ψ increases consumption. One possible explanation for this difference is that MORs and DORs regulate EtOH consumption through actions at distinct cellular sites within the VTA. Unlike DORs that emerge with EtOH exposure, EtOH downregulates MORs in the VTA (Mendez et al., 2001), in particular diminishing the ability of MOR agonists to decrease $GABA_A$ signaling. Therefore the dominant effect of MOR activation in drinking animals may be shifted to postsynaptic cell bodies and/or presynaptic glutamate terminals. Microdialysis studies in naive rats show that microinjecting either the MOR agonist DAMGO or the MOR antagonist CTOP into the VTA increases DA levels in the nucleus accumbens (Devine et al., 1993a), sug-

gesting a highly complex function of VTA MORs. In naive animals, MORs also modulate glutamate release and postsynaptically inhibit subsets of both DA and non-DA VTA neurons (Cameron et al., 1997; Bonci and Malenka, 1999; Manzoni and Williams, 1999; Margolis et al., 2003, 2005). Thus, MOR modulation of EtOH consumption may be due to a combination of these different synaptic effects in the VTA.

$GABA_A$ signaling in the VTA participates in a circuit involved in motivation and reinforcement (Ikemoto et al., 1997). Although VTA $GABA_A$ signaling is required for the DOR antagonist mediated increase in EtOH consumption, the downstream consequences of DOR activation are unclear since DOR-mediated inhibition of GABA release is similar onto DA and non-DA neurons. Therefore, *in vivo* activation of DORs on GABA terminals could disinhibit of all classes of VTA neurons, including glutamate, GABA, and DA neurons (Dahlstroem and Fuxe, 1964; Van Bockstaele and Pickel, 1995; Yamaguchi et al., 2007). Since there are DA-independent outputs of the VTA that can contribute to behavioral reinforcement (Nader and van der Kooy, 1997; Laviolette et al., 2004), it is possible that projecting glutamate or GABA VTA neurons are responsible for the modulation of drinking reported here. DA may also be involved in this modulation, since DA agonists and antagonists alter drinking (Pfeffer and Samson, 1988; Weiss et al., 1990; Rassnick et al., 1992, 1993; Cohen et al., 1998, 1999). If DA does play a role in DOR-mediated EtOH consumption, our data are consistent with an inverted u-shaped relationship between DA level and reward value. According to this model, animals self administer drugs or consume EtOH to achieve an optimal level of DA release. Therefore, animals with low basal DA or systems that are more resistant to processes that increase DA release tend to self administer larger quantities of drugs or EtOH, while animals with higher basal DA or more responsive DA systems tend to self administer less (Weissenborn et al., 1996; Volkow et al., 1999; Caine et al., 2000; Mattay et al., 2003; Meyer-Lindenberg et al., 2005; Cohen et al., 2007). Our data are consistent with this model in that endogenous opioids released when the animal is drinking should disinhibit DA neurons by activating DORs on GABA terminals, thereby increasing DA release and attenuating EtOH consumption in low-drinking animals. Blocking the DOR with TIPP-Ψ increases GABA release, thereby decreasing DA neural activity in low-drinking animals, which drives these animals to consume more EtOH to achieve optimal DA release. High-drinking animals, in which these DOR effects are minimal, may drink more because their basal DA levels are lower (Murphy et al., 1983, 1987; Bustamante et al., 2008) or because higher EtOH concentrations are required to increase DA release.

The nonspecific opioid antagonist naltrexone (Revia) is the most widely prescribed FDA approved treatment for alcohol abuse. However, its actions at multiple receptor types are likely a drawback to its use as a therapeutic for alcoholism. Understanding the contribution of each opioid receptor to EtOH consumption could lead to the development of a more effective therapeutic by permitting more selective targeting of the appropriate receptor(s). For example, MOR selective antagonists reduce EtOH intake (Krishnan-Sarin et al., 1998) while KOR selective antagonists can increase EtOH intake (Mitchell et al., 2005; but see Walker and Koob, 2008) in rats. Therefore, antagonizing all opioid receptors simultaneously is not an optimal treatment strategy. We show here that activating, not antagonizing, DORs may be most appropriate for decreasing EtOH consumption. Therefore an improved therapeutic efficacy would likely result from a compound that acts as both a DOR agonist and MOR antagonist.

Together, our findings demonstrate a protective action of the VTA DOR on alcohol consumption and identify an important new target for therapeutic intervention.

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