Title
Changes in behavioral effects of delta opioid receptor agonists with ethanol consumption

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CHANGES IN BEHAVIORAL EFFECTS OF DELTA OPIOID RECEPTOR AGONISTS WITH ETHANOL CONSUMPTION

By

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DISSERTATION

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Changes in behavioral effects of delta opioid receptor agonists with ethanol consumption

Allison Coker

***Abstract*:**

Alcohol use disorders (AUDs) are a common problem with few effective treatment options. There are only three FDA-approved drugs for the treatment of AUDs, all of which show limited treatment efficacy and none alleviate comorbid anxiety and/or depression. Thus, there is a significant need to identify novel targets for the treatment of AUDs, and the delta opioid receptor (DOR) could represent such a target. DOR agonists have been demonstrated to reduce EtOH consumption in rodent models and have well-established roles in motivation and anxiety. Importantly, the expression and function of DORs are known to change dynamically with a variety of perturbations including EtOH exposure, stress, and pain. This manuscript examines some ways in which the DOR regulation of reward and anxiety behaviors changes with behavioral state. We examined the preference to the DOR-1 agonist DPDPE and the DOR-2 agonist deltorphin in the conditioned place preference paradigm in animals that were either naïve or ethanol consuming, as well as either stressed or un-stressed. While neither DPDPE nor deltorphin induced a place preference in naïve unstressed animals, deltorphin induced a significant place preference in unstressed ethanol consuming animals. In stressed animals, DPDPE does not induce a place preference whether or not the animals were consuming ethanol. In contrast, deltorphin induces a place preference in ethanol naïve stressed animals, however not in animals that were both stressed and ethanol consuming. We also examined the effects of the DOR-1 agonist TAN 67 and the DOR-2 agonist SNC 80 on anxiety-like behavior measured by the marble burying task in both naïve and ethanol consuming animals. We found that while

SNC 80 showed anxiolytic-like effects in naïve animals, TAN 67 did not. However, neither DOR agonist showed an anxiolytic-like effect in ethanol consuming animals. This is in contrast to previous findings with the light/dark transition test in which DOR agonists increased anxiolytic efficacy with ethanol consumption. Together these studies examine DOR effects on reward and anxiety behaviors and further our understanding of how the effects of DOR agonists are dynamically changed by physiological state to help to refine targets for therapeutic development.

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CHAPTER 1

Introduction

Alcoholism and treatment

Alcohol use disorders are very common, affecting over 17 million in the United States alone, and have a huge impact on our society (*1*). The economic burden of alcohol misuse is wide reaching, costing over $220 billion dollars annually (*2*). Alcohol misuse results in nearly 88,000 deaths annually, making it the 3rd leading causes of death in the United States (*3*).

Despite this devastating impact, the Food and Drug Administration has only approved three drugs to treat alcoholism: disulfiram, acamprosate, and naltrexone. Disulfiram, known by the trade name Antabuse, is an irreversible inhibitor of alcohol dehydrogenase. Resulting in a bevy of unpleasant physiological effects if combined with alcohol, disulfiram primarily works as a "punishment" if one consumes alcohol and is only effective in highly compliant patients. Acamprosate (Campral), which has a relatively unclear mechanism and Naltrexone (Revia), a non-specific opioid receptor antagonist, are both relatively effective in increasing abstinence and reducing alcohol consumption, however they are also prone to unpleasant side effects, keeping compliance, and therefore their overall treatment efficacy, low.

The pervasive societal health, safety and economic impacts of alcohol use disorders coupled with the inadequacy of the comparatively few currently available pharmacological

treatments necessitate the exploration of new or refined drugs to be investigated as potential new therapeutics.

Opioid receptors

The opioid receptors are a family of G-protein-coupled receptors widely distributed in the central nervous system. Though opioids have been used clinically as potent analgesics for centuries, our understanding of their extensive actions is still incomplete. There are four distinct classes of opioid receptors: the mu opioid receptor (MOR), the delta opioid receptor (DOR), the kappa opioid receptor (KOR) and the nociceptin receptor (ORL) (*4*). Opioids have a significant role in regulating ethanol (EtOH) consumption in both humans and rodent models and selectively targeting each of the opioid receptors can either promote or inhibit consumption. Agonists at the MOR can increase EtOH consumption (*5, 6*), while antagonists can decrease EtOH consumption (*7, 8*), and inhibit the expression of EtOH induced place preference (*9, 10*). Different agonists and antagonists at the DOR can both increase and decrease EtOH consumption in rodents (*11, 12*), and KOR antagonists also can increase or decrease ethanol consumption depending on the state of the animal (*13, 14*). MOR knock-out mice drink less EtOH than their wild type counterparts (*15*) and DOR knock-out mice drink more (*16*). Finally, in human subjects, release of endogenous opioids has been demonstrated following acute alcohol consumption (*17*).

Of the current pharmacological treatments, naltrexone is thought to be the most effective and is the most commonly prescribed. It has been repeatedly demonstrated to reduce alcohol consumption (*18*), craving (*19*), and relapse (*20*) in human alcoholics. This becomes

problematic since, not only does naltrexone also present a variety of aversive effects, but its treatment efficacy is strongly correlated with the degree of that aversion (*21*). While the naltrexone-induced aversion may be an important factor in its efficacy, it also has a large role in decreasing overall compliance and efficacy (*22*). Additionally, because naltrexone is a non-specific antagonist, it inhibits all of the opioid receptors, albeit to different degrees, each of which has differing roles in motivational processes and ethanol consumption. By parsing out which opioid actions are involved in the reduction of alcohol consumption and which are involved in the aversive effects, we can more specifically target a pharmacological substrate to improve treatment efficacy while reducing aversive effects.

The delta opioid receptor

Compared to its more familiar counterpart, the mu receptor, the enigmatic delta receptor has been relatively understudied. Following the development of selective DOR agonists we have been able to better investigate its functions *in vivo* and expand our knowledge of its varied roles. As novel functions emerge, the DOR appears to be an increasingly attractive therapeutic target for a breadth of neurological and psychiatric disorders.

The delta receptor has a diverse array of functions and pharmacological properties. DORs have been repeatedly demonstrated to play a protective role in a diverse variety of conditions including: cerebral ischemia, hypoxia, cardiac dysfunction, skeletal muscle damage, peripheral organ survival, and vulnerability to stress (*23-28*). Interestingly, while delta receptors have an assorted expression throughout the brain and body, many studies

report dynamic changes in DOR expression following with physiological perturbations or challenges such as stress, drug or alcohol exposure and pain (*11, 29-34*).

Although there is only one gene encoding the DOR (*35, 36*), DOR-specific ligands have been divided into two pharmacologically distinct classes: DOR-1 and DOR-2. These classes were initially defined by the absence of cross-tolerance to agonists of the other class (*37*), and an inability of antagonists of one class to block agonists from the other (*38, 39*). The metabolically stable enkephalin analog [d-Pen^2,d-Pen^5]-Enkephalin (DPDPE) is the prototypical selective DOR-1 agonist, while [D-Ala^2]-Deltorphin II (deltorphin) is the prototypical selective DOR-2 agonist (*37, 40, 41*), though other reports suggest they may interact more than initially thought (*42*). Though the underlying mechanism responsible for this two subtype classification is still poorly understood, DOR compounds continue to be classified and investigated as "DOR-1" and "DOR-2" due to the repeated demonstration of different and sometimes opposing pharmacological effects of the DOR ligands. DOR-1 classified ligands include DPDPE, TAN67, DALCE, and BNTX, while DOR-2 classified ligands include deltorphin, DSLET, naltriben, and 5'NTII (*43*).

The delta receptor and ethanol consumption

DOR agonists have been demonstrated to be effective at reducing ethanol consumption in both rats and mice (*11, 12*). Intra-VTA microinjection of the DOR-1 agonist DPDPE decreases EtOH consumption in a continuous access 2-bottle choice paradigm in chronically drinking rats, and this effect is the greatest in low-drinkers. The magnitude of the EtOH consumption is inversely correlated to the magnitude of DOR inhibition of GABA

release onto VTA neurons. Conversely, intra-VTA administration of the DOR antagonist TIPP-Ψ increases EtOH consumption, and this effect is blocked by local pretreatment with a GABA antagonist (*11*). In mice, systemic injections of the DOR-1 agonist TAN 67 decrease EtOH consumption in a limited access 2-bottle choice paradigm, and this effect is blocked by the DOR-1 antagonist BNTX, which is ineffective at changing consumption on its own. In the same paradigm, the DOR-2 agonist SNC 80 induces an increase EtOH consumption, while the DOR-2 antagonist naltriben induces a decrease. Importantly, co-administration of the DOR-1 agonist TAN 67 and the DOR-2 antagonist naltriben caused a greater decrease in drinking than either did alone, suggesting that TAN 67 and naltriben are acting on distinct targets to modulate EtOH consumption (*12*). These studies suggest that the DOR may be a potential treatment target for alcohol use disorders, and the VTA is one important region of action for DOR activity, however there are two important points to consider in targeting the DORs.

First, the different pharmacological classes of DOR agonists can have different, sometimes opposing, effects on the same behaviors. As described above, although some DOR agonists (DOR-1) curtail ethanol consumption, others (DOR-2) can actually increase it. Similarly, DOR-2, but not DOR-1, agonists have been demonstrated to be anxiolytic in naïve animals (*44*). These pharmacological distinctions are critical to the development of a treatment targeting the DOR receptor, but distinguishing them in cultured cells is not yet reliably achieved (*45*). Thus, it is vital to examine the differences in effects of DOR-1 versus DOR-2 agonists on behavior in whole animals. While decreasing ethanol consumption may be the primary measure, due to the shortcomings of other available treatments, it is also critical to

examine the effects of DOR-1 versus DOR-2 on the behavioral traits representative of conditions commonly comorbid with alcohol use disorders, such as anxiety and depression, as well examining aversive effects that may cause low compliance in human patients.

Second, the expression and function of delta opioid receptors varies dynamically with the animal's physiological state. Multiple lines of evidence have demonstrated that functional DORs in the CNS are up-regulated in response to exposure to perturbations such as morphine (*30*), cocaine (*46*), ethanol (*11*), and stress (*34*). This "moving target" presents both a challenge and a unique opportunity in pursuing the DOR as a treatment target, and makes it vital to consider the responses to DOR agonists across behavioral states of the animal. In this case, while ethanol naïve animals may have one response to a DOR agonist, after the dynamic redistribution of DORs following exposure to ethanol, this response may be altered. 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. Taken together, these points underscore that it is critical to evaluate changes in the effects of these DOR compounds across behavioral states that may shift over the course of treatment.

Dissertation outline

The preceding sections outlined the dearth of effective pharmacological treatments for alcohol use disorders and highlighted the potential of DOR agonists as therapeutics. As outlined below, the projects that I have worked on address these two important considerations for DOR treatment development. Briefly, since DOR agonists have been

demonstrated to reduce EtOH consumption and could potentially become a possible treatment target, I assess here several behaviors related to alcohol use disorders and how the DOR agonists might affect these behaviors if used as a therapeutic agent. Chapter 2 is focused on changes in the reward value of DOR-1 and DOR-2 agonists in naïve and EtOH consuming animals as measured by conditioned place preference. Chapter 3 examines changes in the reward value of DOR-1 and DOR-2 agonists in stressed animals and animals that are both stressed and consuming EtOH as measured by conditioned place preference. Chapter 4 outlines the anxiolytic-like effects of DOR-1 and DOR-2 agonists in naïve and EtOH consuming animals as measured by the marble burying assay. Chapter 5 summarizes the results from all of these studies and discusses the potential meaning of shifts in DOR function across behavioral states.

CHAPTER 2

Differential contributions to reward and ethanol consumption by delta opioid receptor subtypes*

Abstract

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 two 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 two 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.

Introduction

There are four distinct classes of opioid receptors: the mu opioid receptor (MOR), the delta opioid receptor (DOR), the kappa opioid receptor (KOR) and the nociceptin receptor (ORL). 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 knock-out mice drink less EtOH (*15*), while DOR knock-out mice drink more EtOH (*16*). In addition, KOR antagonists can either promote or reduce EtOH consumption, depending upon the behavioral state of the animal (*13, 14*).

The ventral tegmental area (VTA) is important for both opioid reward and regulation of EtOH consumption in rodents. Alcohol-preferring rats self-administer EtOH (*47*) and its primary metabolite acetaldehyde (*48*) directly into the VTA and VTA cFOS expression increases after exposure to an EtOH-associated context (*49*). Intra-VTA administration of either the non-selective opioid antagonist naltrexone (*21*) or the MOR selective antagonist CTOP reduces EtOH consumption in rats (*11*), while intra-VTA administration of the DOR antagonist TIPP-Ψ increases EtOH consumption (*11*). The non-selective opioid antagonist methylnaloxonium also attenuates EtOH place preference in mice (*9*).

Although there is only one gene encoding the DOR (*35, 36*), there are two pharmacologically distinct classes of DOR ligands: DOR-1 and DOR-2, both of which are blocked by the highly DOR-selective antagonist TIPP-Ψ (*50*). The metabolically stable enkephalin analog [d-Pen^2,d-Pen^5]-Enkephalin (DPDPE) is a highly selective DOR-1

agonist, while [D-Ala^2]-Deltorphin II (deltorphin) is a highly selectively DOR-2 agonist (*37, 40, 41, 51*). There is no cross tolerance between these two DOR agonists (*38, 39*), and DOR-1 and DOR-2 ligands have different, sometimes opposing, effects on a variety of behaviors (*12, 52-54*). We previously reported that DOR-1 activation in the VTA of the rat decreases EtOH consumption (*11*). However, in mice, systemic administration of the DOR-2 antagonist, naltriben, decreases EtOH consumption (*12*), suggesting opposing roles of DOR subtypes on drinking behavior. In order 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 have 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 (*11*). Furthermore, the DPDPE-induced presynaptic inhibition of GABA terminals is inversely correlated with EtOH consumption (*11*). 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 if 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 (Figure 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).

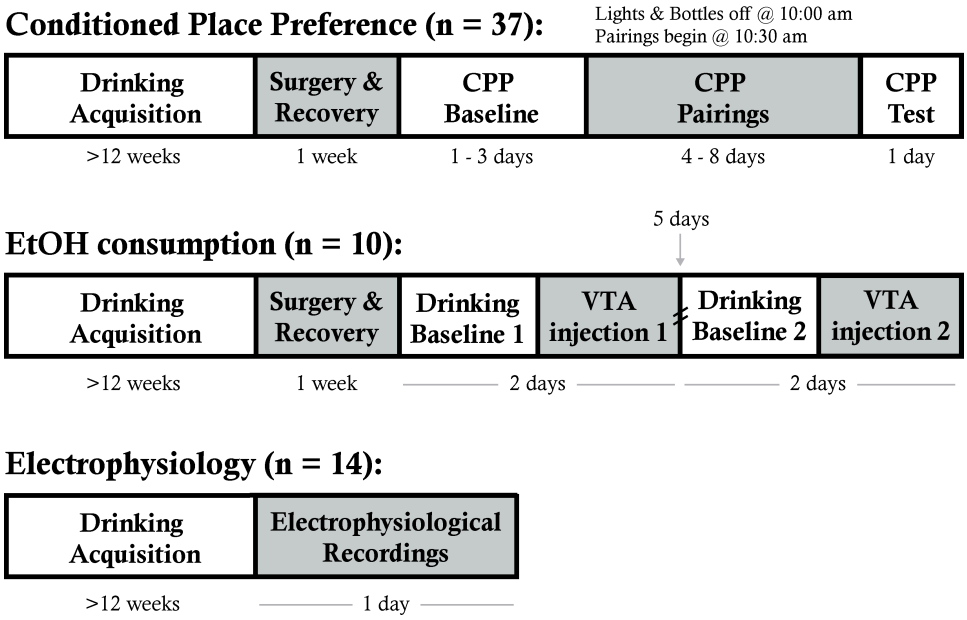

Figure 2-1: Experimental timeline for place preference behavior (n = 37), EtOH drinking experiments (n = 10), and electrophysiology (n = 14). Animals always had access to EtOH and H_2O bottles when not in the CPP apparatus.

EtOH self-administration

EtOH was self-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. Animals were weighed daily and the amount of EtOH and water consumed was measured at the same time daily (10:00 AM). Bottles were identical and their positions were counterbalanced and rotated daily. Animals were maintained on the two-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 H_2O 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 (*55*). 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 (IM, 1 mg/kg) and topical antibiotics. A stainless steel dummy cannula (Plastics One, Roanoke, VA) was inserted into each guide cannula and remained in place when the guide cannulas were not in use. All animals were allowed a minimum one-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 restabilized (at least one week).

Conditioned place preference (CPP)

Place preference training began after bottles were measured (10:30 AM). Animals were trained in three chamber place conditioning boxes (Med Associates, St. Albans, VT) in which two chambers (28 × 21 × 21 cm) that differed in color (one black, one 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 three chambers for a period of 30 min. Beam breaks, entries, and time spent in each chamber were automatically recorded using infrared beams. Animals were given a maximum of three 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 s. During each conditioning session, animals were injected with either drug or saline then immediately confined to one of the two larger end-chambers for 30 min. Animals received two conditioning sessions (separated by five hours) per day for four days. Due to timing constraints, one 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 vs. white box. Animals were tested for expression of CPP one day after the final conditioning session.

VTA microinjections

Each injection was made using a 1 μL syringe (Hamilton, Reno, NV) attached to 20 cm of PE 50 tubing connected to a 33-gauge injection cannula (Plastics One, Roanoke, VA). Microinjections of 0.5 μL volumes were given at a rate of 0.25 μL/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 min following microinjections to minimize the backflow of drug solution. For CPP experiments, microinjections were made directly prior to placement of animals into one of the two 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 μm thick) containing the VTA were prepared using a vibratome (Leica Microsystems). Slices were submerged in artificial cerebrospinal fluid solution containing (in mM): 126 NaCl, 2.5 KCl, 1.2 MgCl, 1.4 NaH_2PO_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 hr. 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 2.5-5MΩ 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_3GTP (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 MOhms, or 15% of baseline, were excluded from analysis. $GABA_AR$ mediated IPSCs were pharmacologically isolated with 6,7-dinitroquinoxaline-2,3 (1*H*,4*H*)-dione (DNQX: 10 μM), strychnine (1 μM), and sulpiride (10 μM). We previously confirmed that this approach isolates $GABA_AR$ signaling with both picrotoxin (*11, 34*) and gabazine (*34*). To measure drug effects on evoked IPSCs, paired electrical pulses (50 ms interval) were delivered once every 10 s through stimulating electrodes placed 80-250 μm 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 (*11*).

Drugs and doses

EtOH (100%; Gold Shield, Hayward, CA) 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. Deltorphin II (2.5 mM, Sigma Aldrich) was dissolved in physiological saline or ddH_2O. Deltorphin 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 non-paired chamber. For electrophysiology, deltorphin II (1 μM final concentration; Sigma, St. Louis, MO) was applied by bath perfusion (stock solution in H_2O). 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 μm, 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 (Figure 2).

Data analysis

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

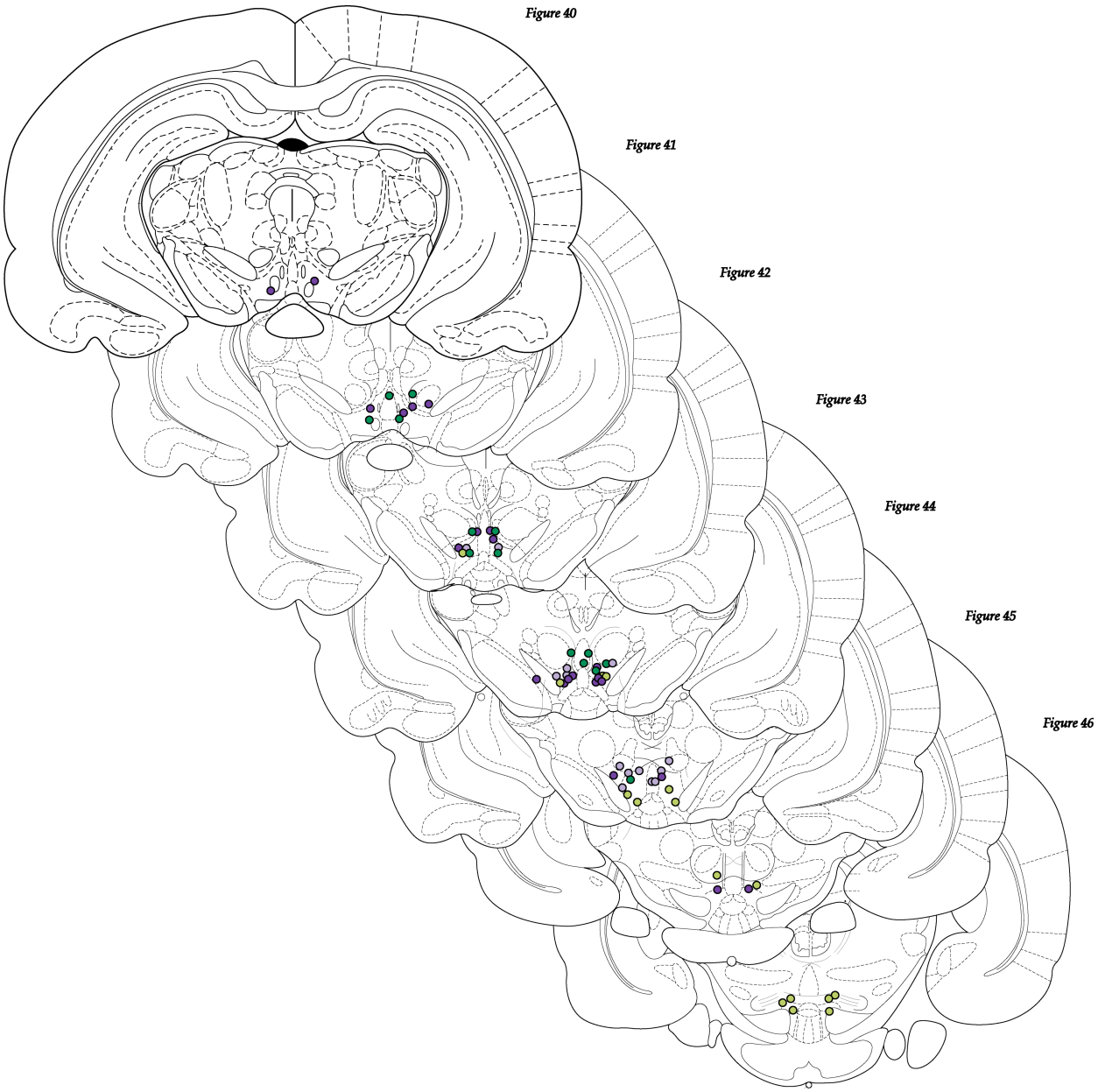

Figure 2-2: VTA Histology demonstrating injection sites for place preference behavior. Purple = DPDPE and green = deltorphin. Lighter colors indicate EtOH naïve animals and saturated colors indicate EtOH consuming animals.

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 am to 10 am) preceding the first day of CPP training.

For electrophysiology, the analyzed data was composed of the 4 min of baseline just preceding drug application and minutes 4-7 of drug application. Summary data are presented as mean ± SEM. Paired t-tests, unpaired t-tests, and regression analyses were completed in Excel (v.11.4.1). Alpha was set at .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 (Figure 2-3A: $t = -1.29$, $p = .236$, $n = 8$), or EtOH consuming Lewis rats (Figure 2-3A: $t = -2.03$, $p = .069$, $n = 11$). Further, the preference in the drinking animals was not significantly greater compared to the EtOH naïve animals (Figure 2-3A: $t = .745$, $p = .466$, $n = 19$). In contrast, although intra-VTA deltorphin did not induce a place preference in EtOH naïve rats (Figure 2-3B: $t = .008$, $p = .994$, $n = 9$) it did induce a significant place preference in EtOH consuming rats (Figure 2-3B: $t = -18.96$, $p < .001$, $n = 9$). The preference seen in EtOH consuming rats was also significantly greater than that seen in the naïve rats treated with deltorphin (Figure 2-3B: $t = -5.51$, $p < .001$, $n = 18$).

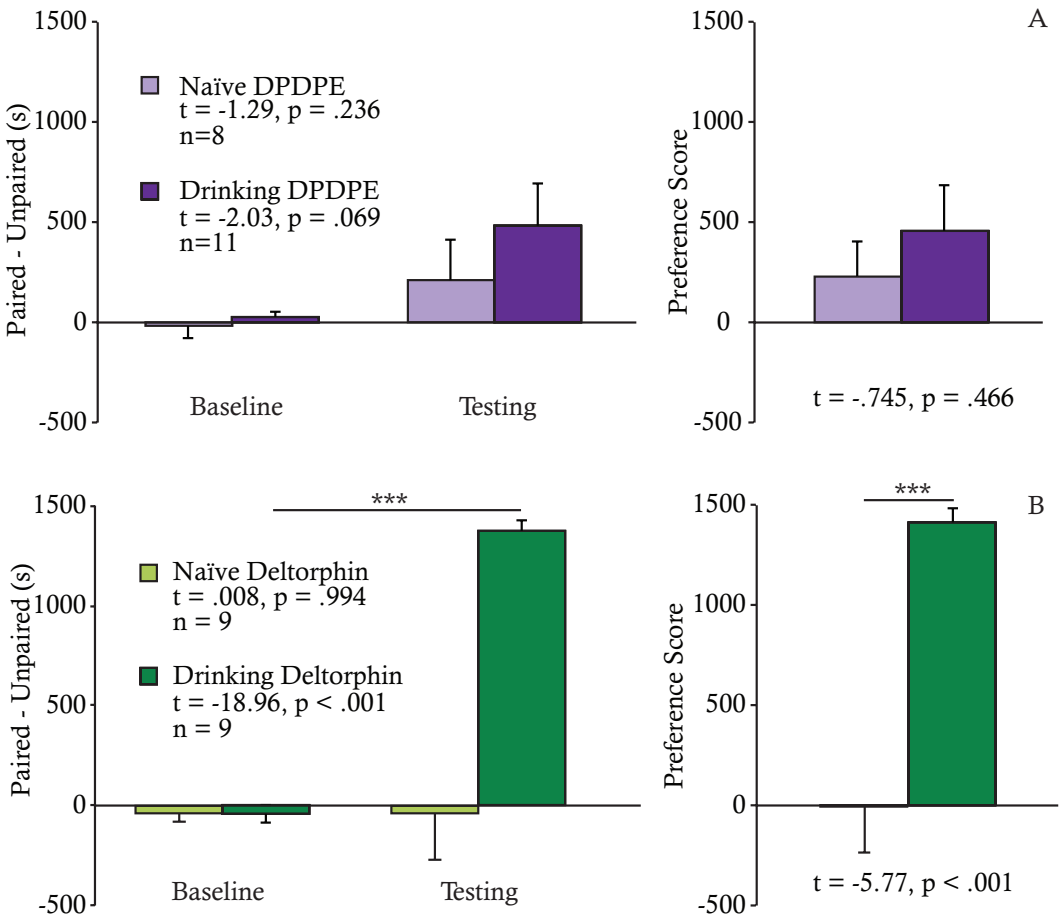

Figure 2-3: Conditioned place preference following intra-VTA administration of the DOR-1 agonist DPDPE (10 mM; n = 8 naïve animals & n = 11 EtOH drinking animals; A) and DOR-2 agonist deltorphin (2.5 mM; n = 9 naïve & n = 9 EtOH drinking animals; B). Data are expressed as both paired – unpaired data within groups and as preference score data between groups.

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 (Figure 2-4) for either the DOR-1 agonist DPDPE (R = .185, p = .585, n = 11) or the DOR-2 agonist deltorphin (R = .074, p = .849, n = 9), indicating that EtOH consumption is necessary for the rewarding effects of DOR-2 agonists to emerge.

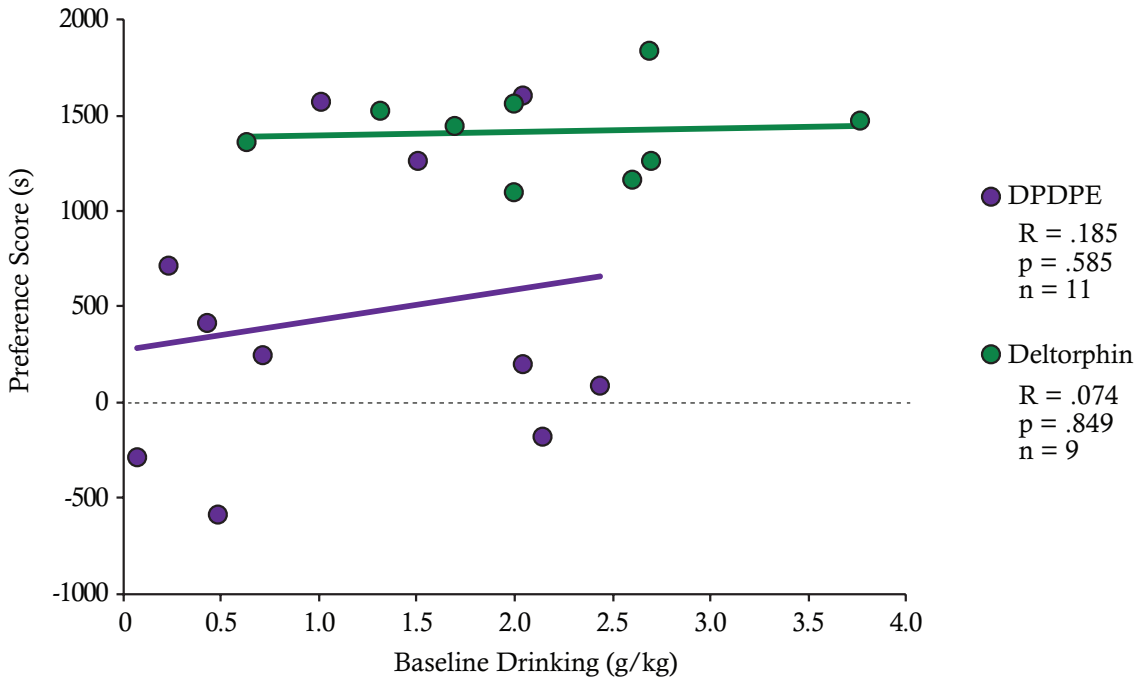

Figure 2-4: Comparison of baseline drinking to CPP preference score for both DPDPE (10 mM; n = 11; purple) and deltorphin (2.5 mM; n = 9; green) in drinking animals. There is no significant correlation between either DPDPE or deltorphin CPP and baseline EtOH consumption.

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 (Figure 2-5), 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 (*11*). However, since deltorphin microinjections into the VTA did not affect EtOH consumption, we

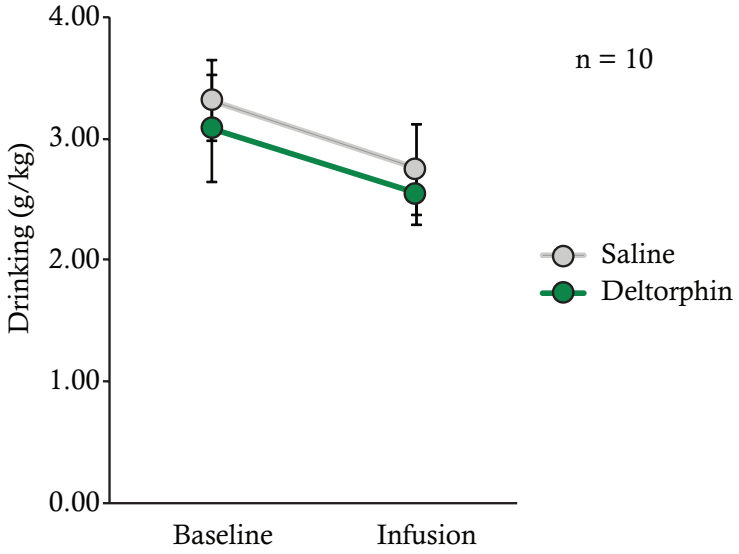

Figure 2-5: Deltorphin (2.5mM, n = 10) has no significant effect on EtOH consumption across animals following microinjection into the VTA.

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 drinking animals. On average, deltorphin (1 μM) caused a very small inhibition of both evoked IPSC amplitude and sIPSC frequency (Figure 2-6). However, the effect 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 (Figure 2-6C, 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 = .65) or drinking (27.6 ± 3.0 pA baseline; 24.2 ±2.6 pA deltorphin; n = 11; p = .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

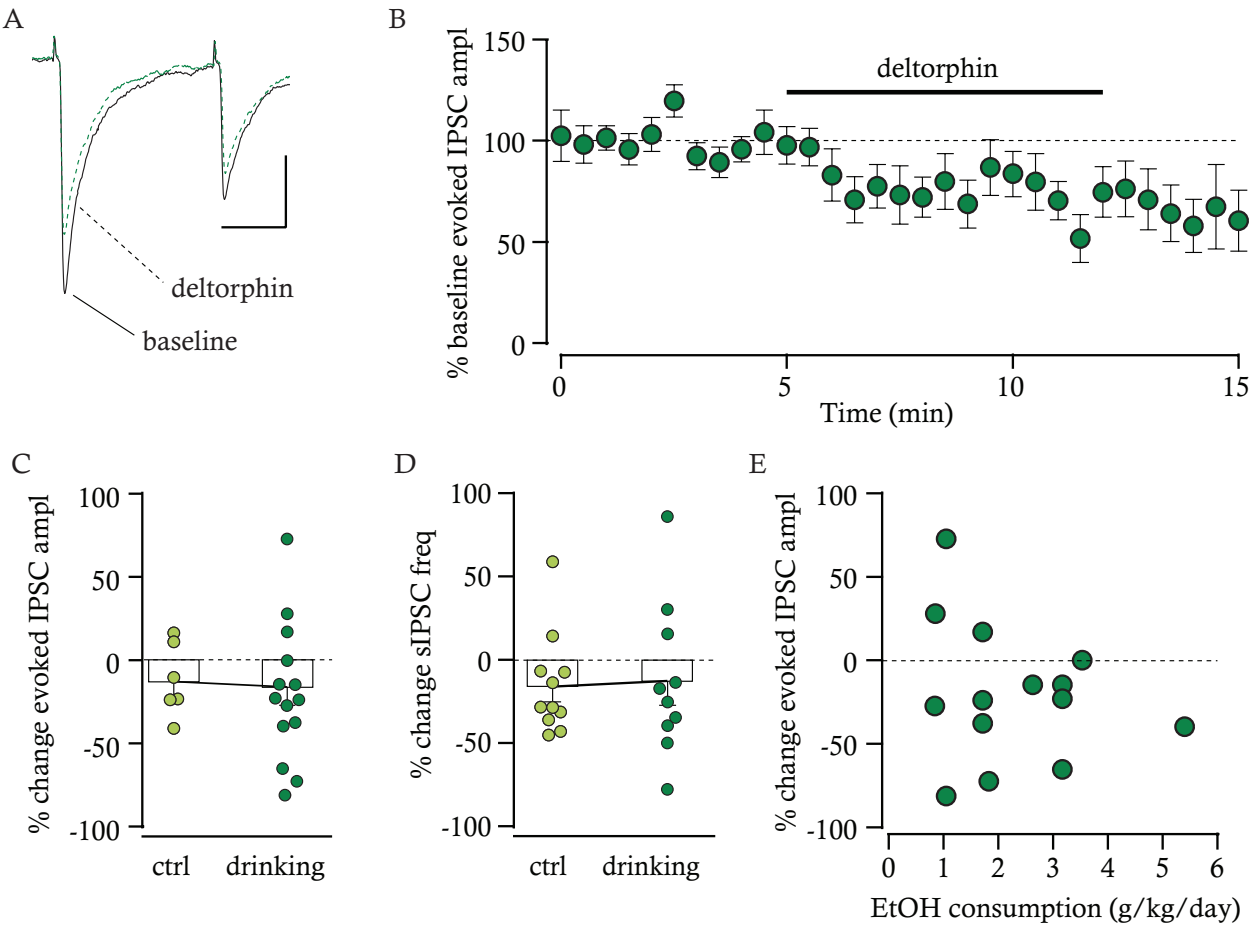

Figure 2-6: Deltorphin modulates $GABA_AR$ synaptic transmission in VTA neurons of naïve and EtOH consuming animals. Example evoked IPSC traces from a VTA neuron from a drinking animal in which bath application of deltorphin (1 μM; A) caused a small inhibition. Average time course of deltorphin effects 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 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 hr period prior to the electrophysiology experiments (n = 14 neurons from 9 drinking animals; E).

sIPSC frequency (p = .004). However, there was also a significant correlation between the change in evoked IPSC amplitude and sIPSC amplitude (p = .03) and no change in the paired pulse ratio in neurons from drinking animals (.93 ± 0.11 baseline; .99 ± 0.11 deltorphin; n = 14; paired t-test p = .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 (Figure 2-6E). 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 to a non-selective ligand, a DOR-1 agonist could retain therapeutic efficacy but with reduced addiction potential.

Our data are in keeping with recent findings in non-human primates, indicating that the DOR-2 agonist SNC-80 significantly enhances the discrimination of low to intermediate doses of EtOH (*56*). Taken together with data showing that the DOR selective antagonist

naltrindole attenuates both operant (*57*) and home cage (*58*) 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 previous findings in mice, which show that neither a DOR-1 agonist (TAN-67) nor a DOR-2 agonist (SNC-80) are rewarding following EtOH consumption (*59*), 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 to 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 post-presynaptic 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 (*34*). Further, in contrast to previous findings with DPDPE (*11*), 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 (*11*), 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 (*60-62*). In addition, anxiety levels are potentiated in DOR knock-out mice (*63*). We recently reported that following stress, activation of the DOR augments, rather than inhibits, $GABA_AR$ IPSCs in VTA neurons (*34*). 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 if 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 HPA response to stress compared to other rat strains (*64*), 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 co-morbid anxiety disorders (*65, 66*).

The two-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 (*11, 33*) 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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CHAPTER 3

Stress and ethanol consumption alter delta opioid reward*

Abstract

Delta opioid receptor (DOR) agonists modulate both ethanol (EtOH) consumption and stress and anxiety, conditions that are frequently co-morbid in humans. We previously found that ventral tegmental area (VTA) microinjection of the DOR-2 selective agonist deltorphin produces a strong conditioned place preference (CPP) in EtOH-consuming rats, while the DOR-1 selective agonist DPDPE does not. Here we examine the interaction of VTA-microinjected DOR-1 and DOR-2 selective ligands and footshock stress in both EtOH naïve and EtOH consuming Lewis rats, as reflected in place preference and EtOH consumption. We find that the DOR-1 agonist DPDPE does not induce CPP in stressed animals, whether or not they are consuming EtOH. In contrast, the DOR-2 agonist deltorphin induces CPP in EtOH naïve stressed rats but not in ETOH consuming stressed rats. These data affirm the critical importance of subtype selectivity of DOR agonists in developing pharmacotherapies for both comorbid and independent anxiety and alcohol abuse. While DOR-1 agonists are particularly useful for reducing EtOH consumption independent of stress, DOR-2 agonist efficacy for anxiety is variable and may be reduced by EtOH consumption.

Introduction

Both stress and anxiety affect EtOH consumption. In rats, alcohol drinking increases after uncontrollable life events (*67, 68*), while in non-human primates, stressful experiences early in life lead to heightened alcohol intake in adulthood (*69*). Stress and anxiety also contribute

to human alcohol consumption: Alcohol consumption typically increases following periods of stress or anxiety (*70*) and many human alcoholics report that they drink to alleviate both anxiety and stress (*71, 72*). Unfortunately, abstinence from alcohol often potentiates these symptoms and abstinent alcoholics frequently relapse to drinking following stressful life events (*73, 74*). Another complication is that chronic alcohol consumption itself can produce elevated stress and anxiety (*75*). Therefore, evaluating interactions between stress, anxiety, and alcohol consumption is an important consideration when assessing the potential clinical utility of pharmacological targets for alcohol abuse and dependence.

The delta opioid receptor (DOR) is one of four known members of the opioid receptor family. Selectively targeting different opioid receptors can either promote or inhibit EtOH consumption. For example, mu opioid receptor (MOR) knock-out mice drink less EtOH (*15*), while DOR knock-out mice drink more EtOH (*16*). The midbrain ventral tegmental area (VTA) is critically involved in opioid regulation of EtOH consumption in rodents. Intra-VTA administration of the non-selective opioid antagonist naltrexone (*21*), the DOR-1 selective agonist DPDPE, or the MOR selective antagonist CTOP reduces EtOH consumption in rats (*11*), while intra-VTA administration of the DOR antagonist TIPP-Ψ increases EtOH consumption (*11*). Additionally, intra-VTA injection of the non-selective opioid antagonist methylnaloxonium attenuates EtOH place preference in mice (*9*).

Although there is only one known gene encoding the DOR (*35, 36*), there are two pharmacologically distinct classes of DOR ligands: DOR-1 and DOR-2, both of which are blocked by the DOR selective antagonist TIPP-Ψ (*50*). The metabolically stable enkephalin analog [d-Pen^2,d-Pen^5]-Enkephalin (DPDPE) is a highly selective DOR-1 agonist, while [D-

Ala[2]]-Deltorphin II (deltorphin) is a highly selectively DOR-2 agonist (*37, 40, 41, 51*). There is no cross-tolerance between these two DOR agonists (*38, 39*), which have different, sometimes opposing, effects on a variety of behaviors (*12, 52-54*).

Both DOR-1 and DOR-2 agonists are anxiolytic, while the DOR selective antagonist naltrindole is anxiogenic (*60-62*). We recently reported that stress and ethanol have distinct mechanisms of action on VTA DOR receptors (*34*). Therefore, it is critical to determine if 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.

While DOR agonists hold promise as potential therapeutics for alcohol abuse (*76*), it is essential to examine the abuse liability of these opioid agonists. Here we used the conditioned place preference paradigm (CPP) to assess the reward potential of both DOR-1 and DOR-2 agonists in footshock-stressed animals with and without access to EtOH. By evaluating the effects of DOR drugs in both stressed EtOH naïve and stressed EtOH drinking animals, we can model the behavioral states of target treatment populations in an effort to determine which DOR subtype is most likely a viable therapeutic with reduced abuse potential.

Materials and methods

Animals

Fourty-four male Lewis rats (Harlan Laboratories, Hayward, CA) weighing between 275-300 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). All experiments were performed during the dark portion of the cycle. Rat chow and water were available *ad libitum*. 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).

EtOH self-administration

EtOH was self-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. Animals were weighed daily and the amount of EtOH and water consumed was measured at the same time daily (10:00 AM). Bottles were identical and their positions were counterbalanced and rotated daily. Animals were maintained on the two-bottle choice paradigm for at least 12 weeks until consumption had stabilized (less than 15% change in drinking over 3-day bins) before surgery and experimentation commenced. For CPP experiments, animals always had access to EtOH and H_2O 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) directed at the VTA (AP, −5.8; ML, ±0.5 to 0.75; DV, −7.0) based on the atlas of Paxinos and Watson (1997). Cannulae were implanted 2 mm above the VTA to prevent any trauma to the region during the surgical procedures. Cannulae were secured to the skull with stainless steel screws and dental cement. At the end of the surgical procedure, animals were treated with penicillin (1 mg/kg), acetaminophen (4mg solution/1ml H_2O), and topical antibiotics. A stainless steel dummy cannula (Plastics One, Roanoke, VA) was inserted into each guide cannula and remained in place when the guide cannulas were not in use. Animals were allowed a one-week recovery period before baseline testing. EtOH bottles were removed from cages 12 hours prior to surgical procedures to minimize interactions with anesthesia. Bottles were replaced the morning following surgery. Drinking and CPP experiments did not commence until drinking had restabilized (at least one week).

Footshock stress

Footshock stress (0.8 mA for 0.5 s every 40 s for 15 min) was administered once daily for seven days (9:00 AM) in operant chambers fitted with electrically scrambled stainless steel rod floors (Med Associates, St. Albans, VT). Shock sessions began the day after the final baseline CPP session, which was 3 days prior to the first CPP conditioning session. Shock sessions took place for 7 consecutive days, throughout CPP conditioning. On CPP conditioning days, footshock sessions occurred immediately prior to the first drug infusion. No footshock took place on the test day (Figure 3-1).

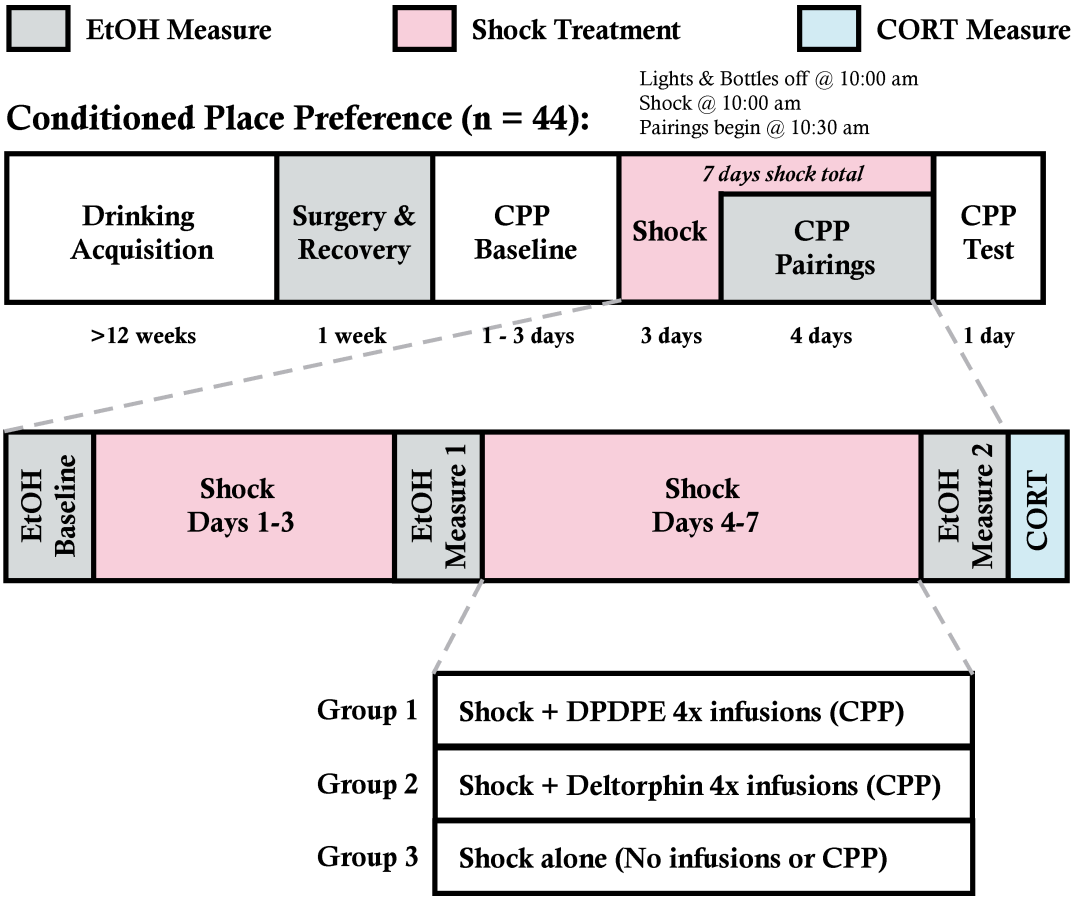

Figure 3-1: Experimental timeline for place preference and stress experiments. Place preference was assessed following intra-VTA administration of either a DOR-1 agonist (DPDPE) and a DOR-2 agonist (deltorphin) in both "stressed" and "stressed and drinking" groups of animals.

Conditioned place preference (CPP)

Place preference conditioning began after bottles were measured (10:30 AM). Animals were conditioned in three chamber place conditioning boxes (Med Associates, St. Albans, VT) in which two chambers (28 × 21 × 21 cm) that differed in color (one black, one white), pattern, light level, and floor texture were separated by a neutral gray chamber (12 × 21 × 21 cm). Importantly, these chambers were visually and tactilely distinct from the chambers in which the animals received footshock. During the initial baseline period, animals were placed in the neutral chamber and were allowed to freely explore all three chambers for a period of

30 min. Beam breaks, entries, and time spent in each chamber were automatically recorded using infrared beams. Animals were given a maximum of three baseline sessions on sequential days to demonstrate that no chamber bias was present, and were excluded from the study if bias exceeded 250 s. During each conditioning session, animals were injected with either drug or saline then immediately confined to one of the two larger end-chambers for 30 min. Animals received two conditioning sessions, separated by five hours, per day for four days. Animals were counterbalanced such that an equal number of animals received drug injections in the black vs. white box and the preferred vs. non-preferred chamber. Animals were tested for expression of CPP one day after the final conditioning session (Figure 3-1).

VTA microinjections

Each injection was made using a 1 μL syringe (Hamilton, Reno, NV) attached to 20 cm of PE 50 tubing connected to a 33-gauge injection cannula (Plastics One, Roanoke, VA). Microinjections of 0.5 μL volumes were given at a rate of 0.25 μL/min using a syringe pump (kd Scientific, Holliston, MA) into both sides of the VTA. Injection cannulae extended 2 mm beyond guide cannula to reach a depth of 9.0 mm and were left in place for 1 min following microinjections to minimize the backflow of drug solution. Microinjections were made directly prior to placement of animals into one of the two pairing chambers. The first microinjection began approximately 10 minutes after the termination of footshock exposure.

Drugs and doses

EtOH (100%; Gold Shield, Hayward, CA) was diluted to 10% (v/v) for self-administration. DPDPE (10 mM, Sigma Aldrich, St. Louis, MO or American Peptide Company, Sunnyvale, CA) and Deltorphin II (2.5 mM, Sigma Aldrich) were dissolved in physiological saline or double deionized water (ddH_2O). DPDPE and Deltorphin II doses were chosen based on pharmacological effects on drinking demonstrated in previous studies (*11, 77*). DMSO (2.64%) was added when necessary to dissolve DPDPE. Physiological saline was always injected for conditioning sessions in the non-paired chamber.

Perfusions and histology

At the conclusion of the experiment, and immediately following the final CPP session, 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 μm, mounted and stained with cresyl violet or neutral red. Only animals with confirmed injection sites within the VTA were included in the analysis (Figure 3-2).

Corticosterone (CORT)

To analyze CORT levels, trunk blood was collected at the time of sacrifice (1.5 mL), spun for 10 minutes (7000 RPM), and plasma was removed and frozen until the end of the study. Samples were then thawed, diluted 1:40, and plasma was run through a commercial assay (Corticosterone EIA kit, Assay Designs, Ann Arbor, MI) and read on a Spectra Max 190

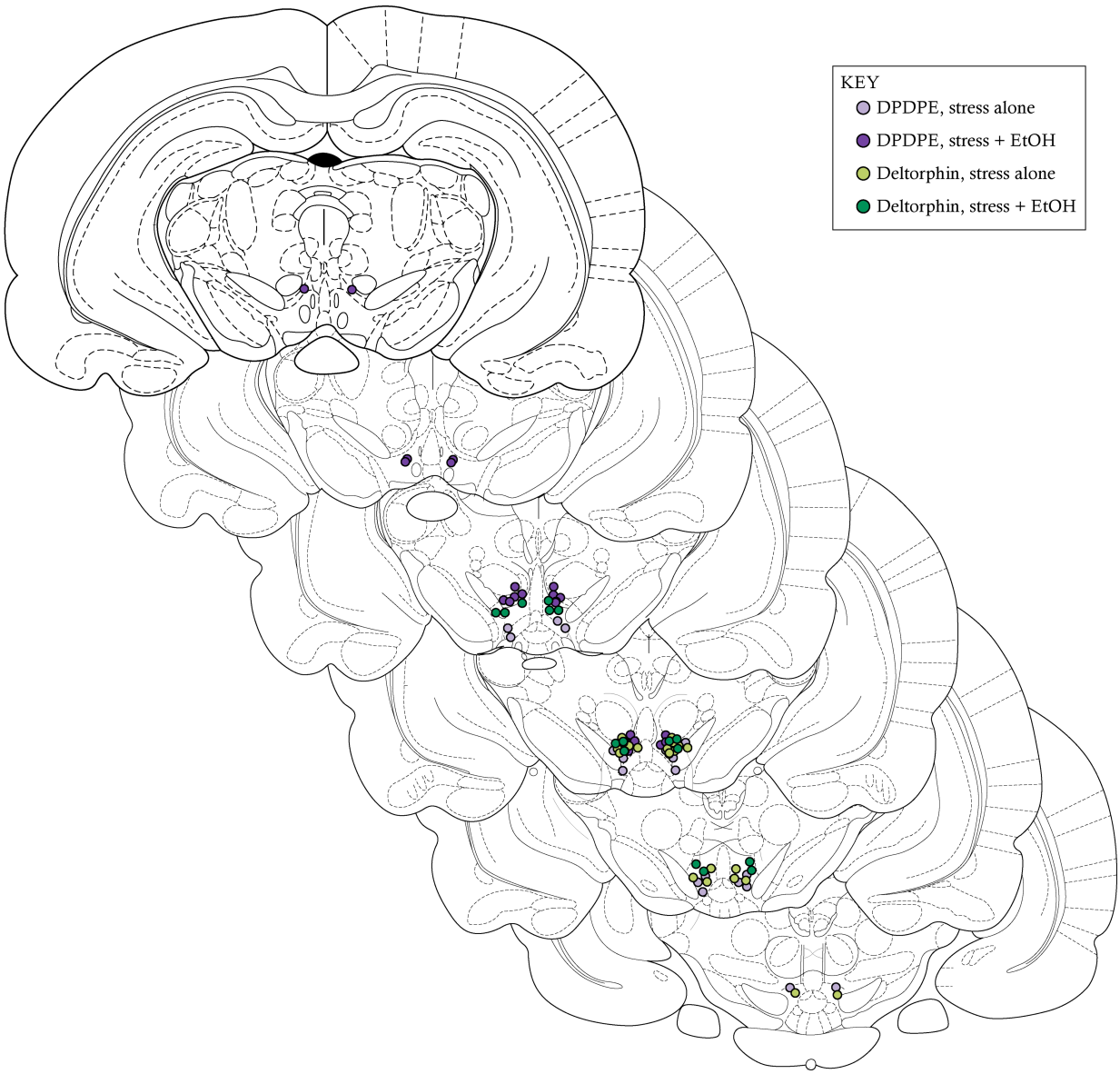

Figure 3-2: Ventral tegmental area histology. VTA injections sites are indicated for "stressed" (light purple) and "stressed and drinking" (dark purple) animals receiving the DOR-1 agonist DPDPE as well as for "stressed" (light green) and "stressed and drinking" (dark green) animals receiving the DOR-2 agonist deltorphin.

plate reader (Molecular Devices, Sunnyvale, CA) at 405 nm. Due to time constraints, CORT data was not collected from 4 animals in the *DPDPE stress alone* group.

Data analysis

For consumption data, drinking was analyzed using 24-hour time points (10 a.m. to 10 a.m.). CPP difference scores were calculated by subtracting the time spent in the saline paired chamber from the time spent in the drug paired chamber during a test session. A positive score therefore indicates place preference while a negative score indicates place aversion. A paired t-test (difference scores in baseline vs. testing) was calculated for each group. To compare between groups, a "preference score" was calculated by subtracting the difference scores between testing and baseline sessions:

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

Summary data are presented as mean ± SEM. Comparisons and regression analyses were completed in Excel (v.11.4.1).

Results

Histology

Histology (n = 44) is presented in Figure 2. No differences were observed in effects of injections into the anterior versus posterior VTA.

DOR, place preference, and stress

We previously found that microinjection of the DOR-1 agonist DPDPE into the VTA did not produce CPP in either naïve or EtOH-consuming rats (Chapter 2 and (*77*). Similarly, here we found no place preference in response to intra-VTA administration of the DOR-1

agonist DPDPE in either EtOH naïve stressed animals (n = 12, t = -0.22, p = 0.83) or in EtOH drinking stressed animals (n = 13, t = -1.48, p = 0.16), nor was there a significant difference in DPDPE place preference between these two groups (t = -1.03, p = .31; Figure 3-3A). In examining potential individual differences, we also found no significant correlation between baseline drinking and CPP for stressed EtOH-consuming animals that receive intra-VTA DPDPE (n = 13, R = .036, F = .014, p = .91; Figure 3-4A).

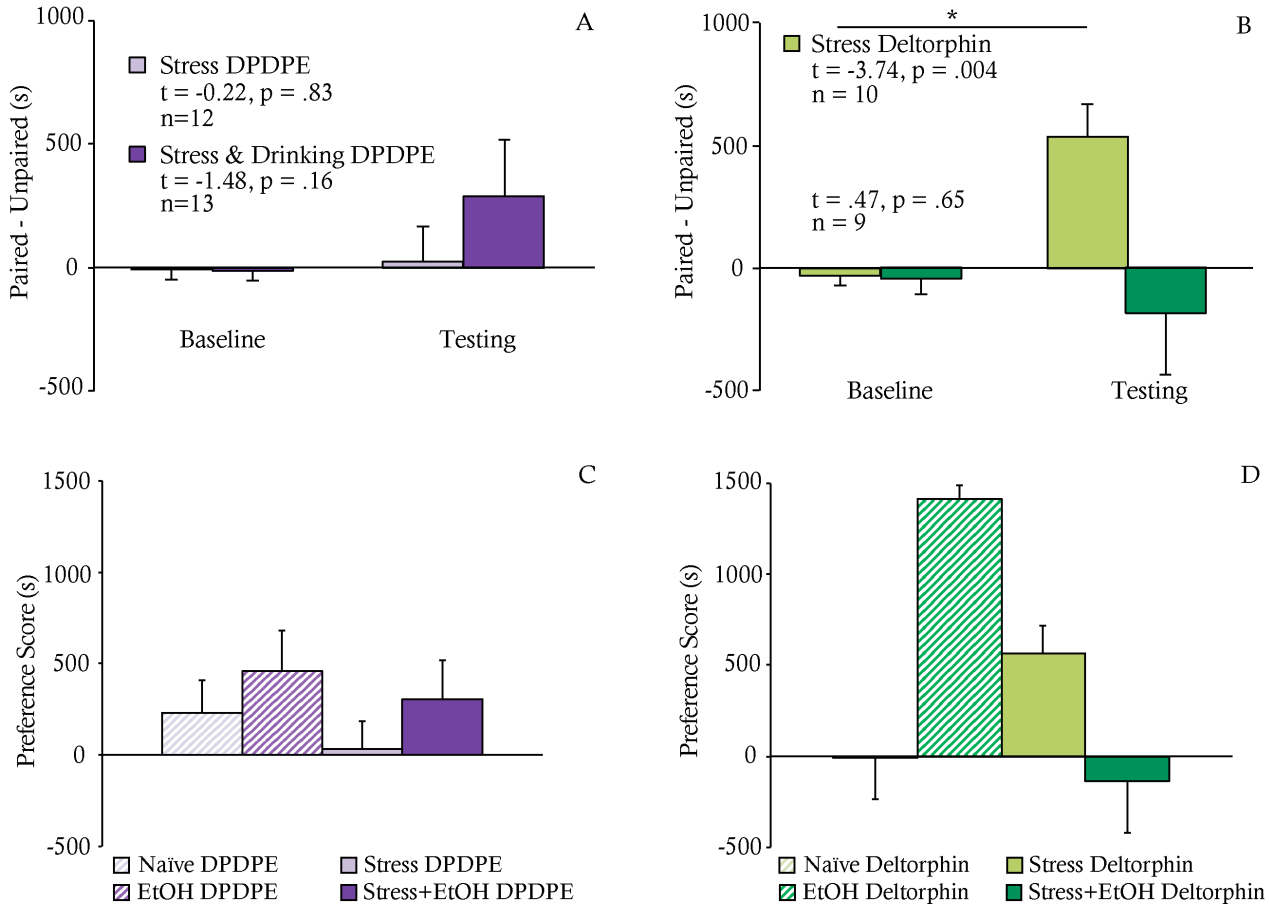

Figure 3-3: DOR place preference in stressed versus stressed EtOH consuming animals. The DOR-1 agonist DPDPE does not induce a place preference in either "stressed" or "stressed and drinking" animals (A), while the DOR-2 agonist deltorphin induces CPP in "stressed" but not in "stressed and drinking" animals (B). When compared with previously published data from "unstressed" drinking animals (*77*), stress does not significantly alter DPDPE-induced CPP in either "stressed" or "stressed and drinking" animals (C), while deltorphin-induced CPP is affected by both stress and EtOH consumption.

In contrast to the effects of a DOR-1 agonist, we previously reported that the DOR-2 agonist deltorphin produces CPP in EtOH-consuming, but not in EtOH naïve, rats (Chapter 2 and (*77*). Here we found a significant CPP in response to intra-VTA administration of the DOR-2 agonist deltorphin in EtOH naïve stressed rats ($n = 10$, $t = -3.74$, $p = 0.004$). Surprisingly, however, deltorphin CPP was not observed in EtOH drinking stressed rats ($n = 9$, $t = 0.47$, $p = 0.65$; Figure 3-3B). However, although there was no net deltorphin CPP in EtOH drinking stressed animals overall, there was a robust inverse correlation between intra-VTA deltorphin CPP score and EtOH consumption, such that high drinkers found deltorphin aversive ($n = 9$, $R = .915$, $F = 36.166$, $p = .0005$; Figure 3-4B).

To further examine the effect of stress on DOR CPP, we measured blood CORT levels at the time of sacrifice. There was no significant correlation between blood CORT levels and DPDPE CPP in either stressed animals ($n = 8$, $R = 0.537$, $p = .170$), or stressed EtOH drinking animals ($n = 13$, $R = 0.076$, $p = .806$), suggesting that CORT levels (and, by inference, degree of response to a stressor) are not related to the rewarding effects of DPDPE under stressful conditions, irrespective of EtOH consumption. Similarly, CORT levels (collected at time of sacrifice) did not predict deltorphin CPP in either stressed animals ($n = 10$, $R = 0.392$, $p = .262$), or stressed EtOH drinking animals ($n = 9$, $R = 0.074$, $p = .850$).

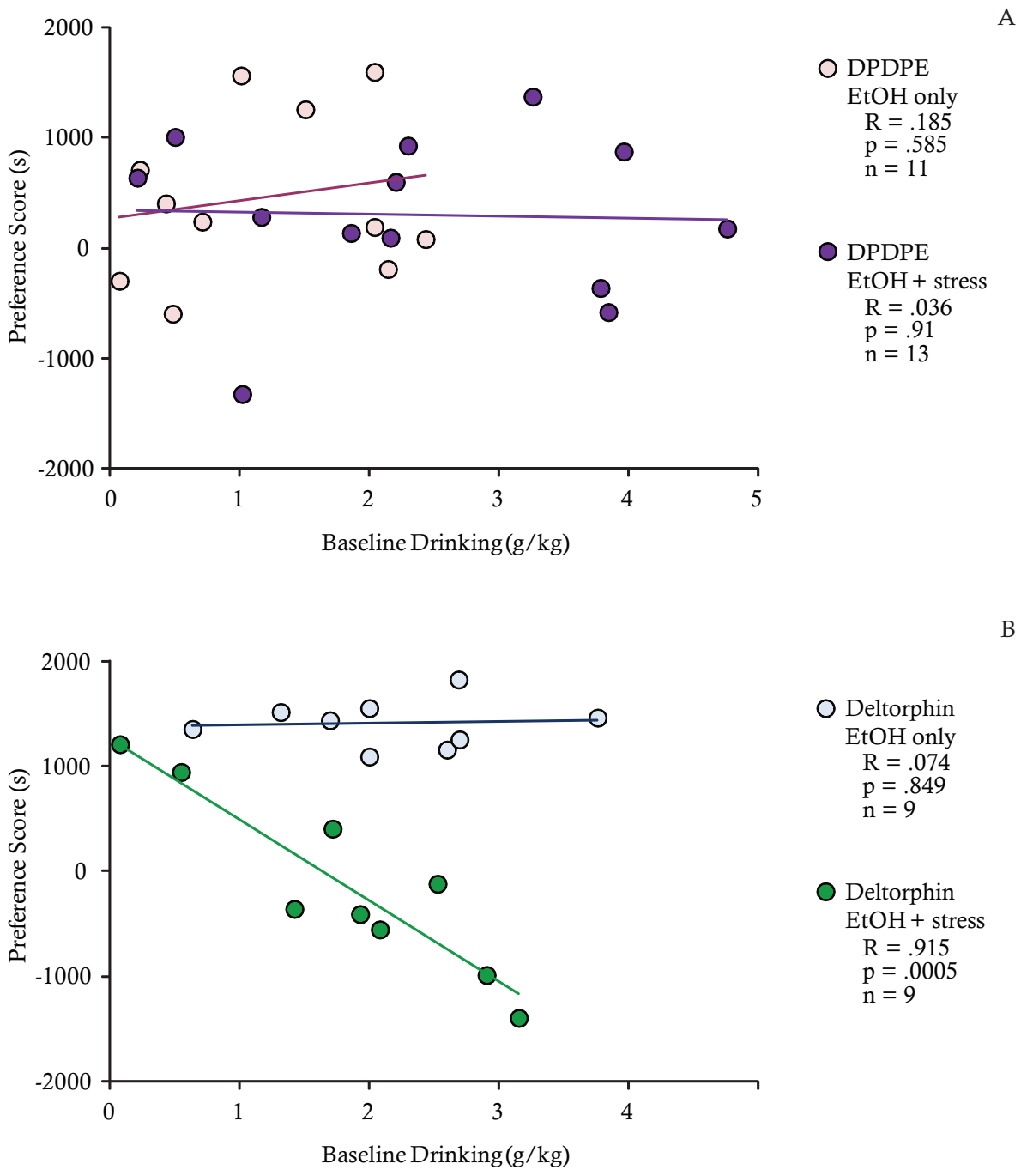

Figure 3-4: DOR place preference and EtOH consumption. There is no relationship between DOR-1 agonist induced place preference and baseline drinking in either "stressed" or "unstressed" animals (A). In contrast, following stress, there is a significant correlation between DOR-2 agonist place preference and baseline drinking (B). Previously published data from "unstressed" drinking animals is reproduced here for comparison (Chapter 2, (*77*).

DOR, EtOH consumption, and stress

Although EtOH consumption was unaffected by either 3 or 7 days of footshock stress in non-DOR treated animals (Figure 3-5A,B), repeated intra-VTA administration of either the DOR-1 agonist DPDPE or the DOR-2 agonist deltorphin had a robust effect on EtOH consumption. Specifically, when animals were stressed, low drinkers drank more and high drinkers drank less following either intra-VTA DPDPE ($n = 13$, $R = .670$, $p = .012$; Figure 5C) or intra-VTA deltorphin ($n = 9$, $R = .798$, $p = .010$; Figure 3-5D), suggesting that administration of DOR agonists triggers a change in drinking behavior in stressed EtOH consuming rats.

DOR, footshock, and CORT

Baseline EtOH consumption in non-DOR treated animals did not predict CORT levels measured at sacrifice ($n = 8$, $R = .519$, $p = .188$). Additionally, in non-DOR treated animals, change in EtOH consumption induced by footshock did not predict CORT levels at sacrifice ($n = 8$, $R = .070$, $p = .870$; Figure 3-6A). However, if footshock was followed by administration of DPDPE, a significant positive correlation emerged between footshock-induced change in EtOH consumption and CORT levels measured at sacrifice ($n = 13$, $R = .721$, $p = .005$; Figure 3-6B). No such relationship developed with deltorphin ($n = 9$, $R = .108$, $p = .783$; Figure 3-6C). More simply, animals that increased their drinking the most following footshock also showed the highest CORT levels at sacrifice if they have been treated with DPDPE but not if they have been treated with deltorphin, suggesting that DOR-1 and DOR-2 differentially affect the interaction between EtOH consumption and stress.

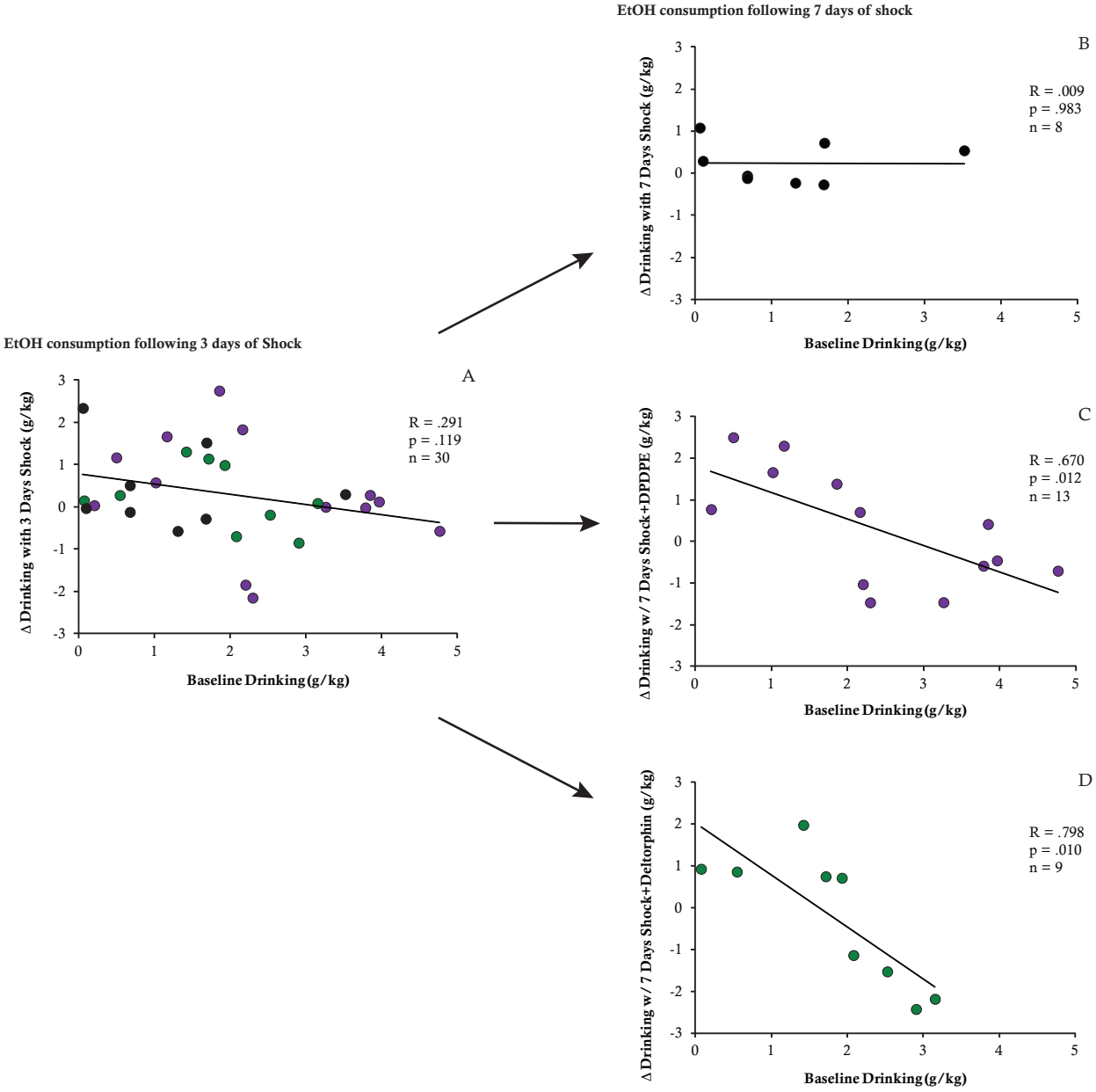

Figure 3-5: Change in EtOH consumption with shock. While 3 or 7 days of shock (A and B) are ineffective at altering EtOH consumption in control animals, administration of either the DOR-1 agonist DPDPE (C) or the DOR-2 agonist deltorphin (D) to shocked animals results in a negative correlation between baseline drinking and change in drinking following shock, such that shocked high drinking animals drink less following DOR agonist administration.

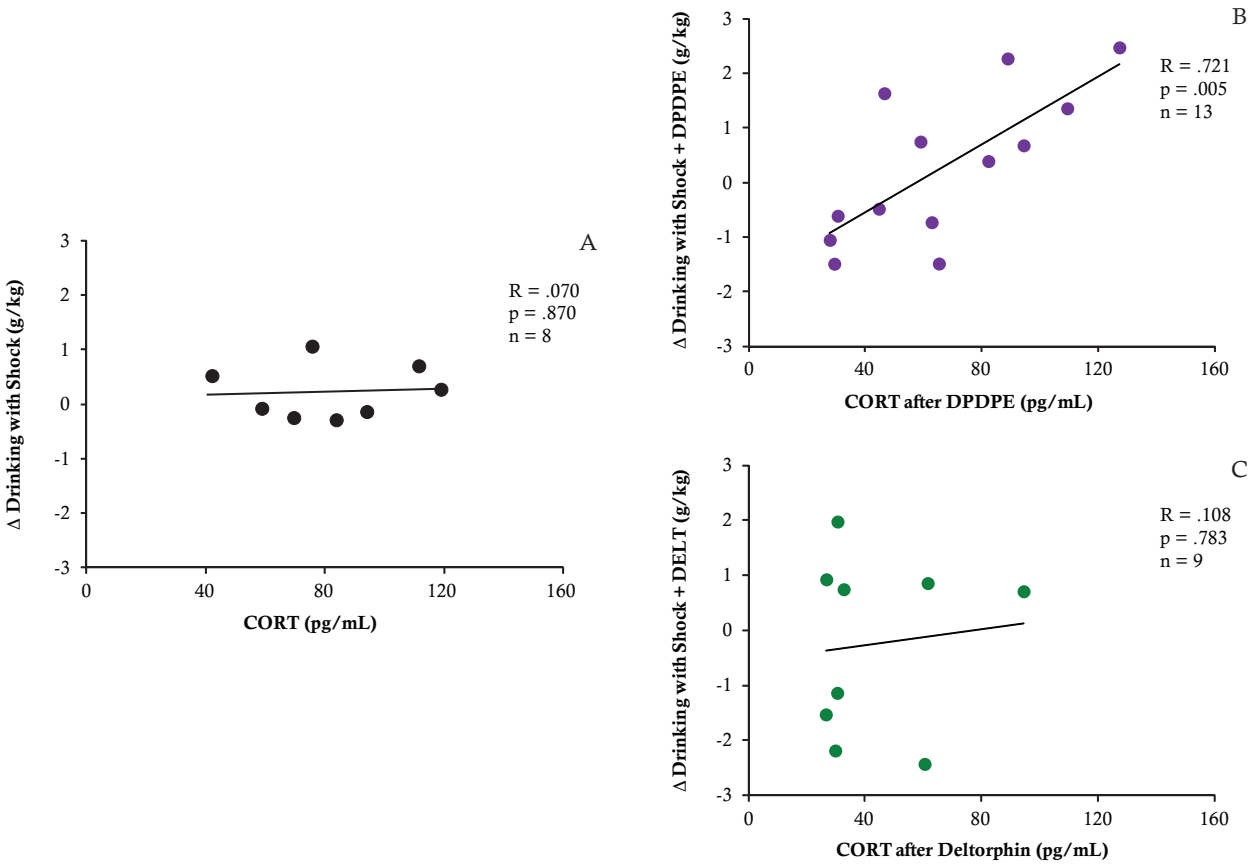

Figure 3-6: Shock and CORT levels. Change in drinking following shock does not correlate with CORT measured at sacrifice in control animals (A) or in animals treated with the DOR-2 agonist deltorphin (C). In contrast, a positive correlation emerges between change in drinking and CORT following treatment with the DOR-1 agonist DPDPE (B).

Discussion

We have previously demonstrated that deltorphin induces a robust CPP in EtOH-drinking animals, and that this DOR-2 CPP is independent of the level of EtOH consumption (Chapter 2 and (*77*). The current data extend these findings in several important ways. First, we now show that deltorphin CPP also emerges following footshock stress. Second, and perhaps counter-intuitively, we show that when animals are exposed to *both* EtOH and footshock stress, CPP to deltorphin is no longer observed. Lastly, we show that these effects are specific to a DOR-2 agonist, as DOR-1 agonist place preference is not modulated by stress, by EtOH consumption, or by the combination of stress and EtOH consumption.

Together with our previous results, the present data show that intra-VTA injection of the DOR-1 agonist DPDPE has neither rewarding nor aversive properties, irrespective of whether an animal is stressed, EtOH-consuming, or both. In contrast, deltorphin is rewarding in a CPP paradigm across a range of EtOH drinkers, but following stress deltorphin becomes aversive to high drinking animals. Therefore, the therapeutic use of deltorphin, or similar DOR-2 agonists, for anxiety could be hindered by their interaction with EtOH consumption, whereas DOR-1 agonists such as DPDPE may be effective therapeutics for EtOH abuse in part because their effects do not appear to be attenuated by stress. Indeed, although our previous research indicated that a DOR-1 agonist was most effective at curtailing EtOH consumption in unstressed low drinkers (*11*), the present findings show that, when animals are stressed, a DOR-1 agonist is most effective at curtailing EtOH consumption in high drinking rats (Figure 3-5).

In *stressed* animals, baseline EtOH consumption predicts deltorphin CPP; high drinkers show aversion and low drinkers show preference. This correlation between deltorphin CPP and baseline EtOH consumption was not seen in *unstressed* animals. Furthermore, we did not observe a correlation between CPP and EtOH consumption following DPDPE administration in *either* stressed or unstressed animals. These data indicate that, when stressed, high drinkers shift from preferring deltorphin to avoiding it, while low drinkers appear to find deltorphin positively reinforcing, whether or not they are stressed.

Interestingly, CORT levels taken at the time of sacrifice correlated with shock-induced change in drinking following DPDPE administration. However, no such pattern emerged following deltorphin administration. Since CORT could only be measured once, via blood at the time of sacrifice, it is possible that we are unaware of a stress bias in the DPDPE group. In other words, it is possible that the animals that were the most stressed by footshock had the most robust increases in CORT and that this preceded DPDPE administration entirely. However, it is statistically unlikely that this would result in a significant correlation, leading us to instead speculate that DPDPE is somehow modulating the relationship between drinking and stress and inducing a positive correlation between stress-induced change in drinking and CORT.

The present data clearly demonstrate that the effects of DOR drugs are dependent on the behavioral state of the animal, however it is still necessary to consider how and why these changes are occurring. It has previously been suggested that high EtOH drinkers have lower basal levels of enkephalin, an endogenous opioid peptide with high affinity for the DOR and MOR, and may therefore drink to stimulate enkephalin release (*78-80*). This potentiation in enkephalin release could induce DOR internalization selectively in high EtOH drinkers (*33*), rendering them less protected against further EtOH consumption. Previous studies have also shown heterogeneity in DOR internalization and trafficking (*81, 82*) and have demonstrated that some internalized DORs require MOR activity to resurface (*83-86*). Additionally, stress induces release of another endogenous opioid peptide that activates MOR and DOR, β-endorphin (*87-89*), but not enkephalin (*90, 91*), in a number of brain regions. Taken together, these findings raise the possibility that stress induced β-endorphin release could

preferentially activate the MOR and enable DOR re-expression in high EtOH drinkers. In contrast, in low EtOH drinkers, it is possible that the MOR is already fully stimulated by enkephalin so that as stress induces β-endorphin release, further activating the MOR, the complex is internalized and DOR signaling is diminished. Though a variety of factors undoubtedly influence this interaction, differences in basal endorphin levels provide one possible model to explain the variability in DOR effects in response to stress and EtOH exposure.

While the National Comorbidity Survey has shown a strong familial aggregation of general anxiety disorder and substance abuse (*92*) and many alcoholics suffer from co-morbid anxiety (*75, 93*), very few pharmacotherapies have been explored for this population. DOR compounds have the advantage of being potentially useful therapeutics for both anxiety and alcoholism. Additionally, the ongoing pharmaceutical interest in DOR agonists as pain therapeutics suggests that pharmaceutical companies may have candidate compounds available that could accelerate development of DOR ligands for new indications. Additional research is imperative to determine whether existing compounds could be beneficial for treating alcohol abuse, stress, and anxiety.

In conclusion, we find that DOR-1 agonists such as DPDPE reduce EtOH consumption in stressed high drinking animals. Importantly, there is no correlation between drinking and DPDPE CPP in either stressed or unstressed animals, suggesting that the reward value of DPDPE is independent of level of ongoing EtOH consumption and raising the possibility that a DOR-1 agonist could be a safe and effective therapeutic for EtOH abuse and

dependence, particularly in the presence of co-morbid anxiety. In contrast, while DOR-2 agonists such as deltorphin can also curtail EtOH consumption in stressed high drinking animals, the effects of DOR-2 compounds are less predictable and more dependent on behavioral state. These data support the hypothesis that DOR-1 agonists will be effective therapeutics for alcohol abuse disorders and may be particularly beneficial to anxious alcoholics.

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CHAPTER 4

Ethanol consumption can alter the effects of delta opioid agonists and antidepressants on marble burying*

Abstract

Animal models of anxiety and depression have been critical to our development of effective pharmaceutical treatments for use in humans. The marble burying test taps into rodents natural defensive burying behavior and has been studied as a measure of anxiety behavior. Here, we examine the effects of chronic ethanol exposure on behavior in the marble burying task and whether chronic ethanol exposure alters the efficacy of classic antidepressants and the DOR agonists TAN-67 and SNC-80. We demonstrate that the marble burying test can detect ethanol induced changes in baseline behavior as well as changes in drug efficacy. In naïve animals, desipramine, escitalopram, and SNC 80 all reduced marble burying as compared to vehicle controls, suggesting an anxiolytic-like effect. No effect of bupropion or TAN 67 on marble burying was observed. Ethanol consumption induced a reduction in the number of marbles buried, indicating an anxiolytic-like effect. In drinking animals, only desipramine displayed an anxiolytic-like effect—the effects seen with escitalopram and SNC 80 in naïve animals were no longer present in drinking animals. This is in contrast to previous findings with the light/dark transition test in which DOR agonists increased anxiolytic efficacy with ethanol consumption. Importantly, we show here that ethanol alters both baseline anxiety-like behavior and drug efficacy in different ways depending on the assay utilized. Taken together, these results underscore the importance of determining which animal models will be most predictive for efficacy in treatment seeking alcoholics.

Introduction

Alcohol use disorders are very common, affecting over 17 million in the United States alone, and have a huge impact on our society (*1*). The economic costs of excessive drinking are estimated to be over 200 billion dollars per year (*94*). Furthermore, excessive drinking was responsible for one of every ten deaths among working age adults from 2006-2010 (*95*). Despite these alarming statistics, there are only three FDA-approved drugs for the treatment of alcohol use disorders and all show limited efficacy and are plagued by side effects that limit compliance. Furthermore, none of these medications alleviate the anxiety and depression that are comorbid in the majority of treatment seeking alcoholics (*96, 97*).

The comorbidity of depression and anxiety disorders with alcohol use disorders is prevalent (*97*). There are a plethora of FDA-approved medications for the treatment of anxiety and depression, many of which are used in the management of recovery in treatment seeking alcoholics. While both selective serotonin reuptake inhibitors (SSRIs) and benzodiazepines are used to treat anxiety disorders in alcoholics, there is mounting preclinical and clinical evidence that their efficacy is reduced in alcoholic subjects. Furthermore, many anxiolytics are themselves habit-forming and have been shown to increase both the palatability of alcohol and alcohol consumption (*98*). Thus, there is a significant need to identify novel targets for the treatment of both alcohol use disorders and the anxiety and depression that are so often co-morbid with alcoholism.

The delta opioid receptor (DOR) could represent such a target, as its activity regulates both ethanol (EtOH) consumption and anxiety. Specifically, DOR agonists have been shown to

reduce EtOH consumption in both rats (*11*) and mice (*12*). Furthermore, DOR agonists have well-established anxiolytic properties (*44, 60, 99*). Importantly, the expression and function of the DOR is known to change dynamically with a variety of physiological perturbations, including EtOH exposure (*11, 12, 33, 44*), stress (*34*), and pain (*32*). These studies demonstrate that the effects of DOR ligands can vary widely depending on the history and physiological state of the subject.

Animal models of anxiety and depression have been critical to our development of effective pharmaceutical treatments. Preclinical testing for antidepressant effects typically fall to the classic Porsolt or "forced swim test" and the tail suspension test. Drug effects in these tests have demonstrated predictive validity for antidepressant drugs in humans. Both tests measure the amount of time the animal struggles versus remaining immobile during the test. Drugs that are effective antidepressants in humans increase the amount of time the animal spends struggling (and decrease immobility time) in these tests. The most commonly used anxiety tests for rodents are the elevated plus maze, open field test, and light/dark transition test. These tests focus on changes in exploratory behavior as a measure of anxiety; animals that spend more time in the open arms, center of the field, or in the light side of the box are considered "less anxious" and drugs that induce these behaviors are "anxiolytic." Depression and anxiety are known to be closely related and have very high comorbidity rates; and importantly many antidepressants have also been demonstrated to have anxiolytic effects in both animal models (*100*) and human subjects (*101*).

Defensive burying is a natural rodent behavior in which rats and mice use their forepaws to

tread bedding material directed toward noxious, or threatening objects (*102*). The burying of novel, seemingly harmless objects, such as glass marbles, taps into this natural behavior and has been studied as an additional measure of anxiety. Unlike other anxiety measures, marble burying does not rely on exploratory behavior or locomotion to assess anxiety. Critically, the marble burying test has been established to be sensitive the effects of classic anxiolytics, such as benzodiazepines, showing decreases in the number of marbles buried, as well as demonstrating a similar anxiolytic effect of antidepressants, such as SSRIs (*103, 104*). Further, both chronic and acute stress have been demonstrated to increase marble burying behavior (*105*). However, no studies have examined the effects of chronic EtOH consumption on marble burying behavior nor has this test been utilized to examine the effects of DOR ligands.

Here, we examined the effects of chronic EtOH exposure on behavior in the marble burying task. We also examined whether chronic EtOH exposure altered the efficacy of classical antidepressants and the DOR agonists TAN-67 and SNC-80.

Materials and methods

Animals

Male C57BL/6 mice (Taconic Laboratories) between 7 to 10 weeks old upon arrival were housed individually in a temperature controlled colony room (21° C) on a 12-hour reversed light/dark cycle (lights off at 10 a.m.). All experiments were performed during the dark (active) cycle. Food and water were available *ad libitum*. Animals were given 1 week to acclimate before the start of any experiments. During EtOH self-administration periods (see

below) 10% EtOH (v/v Gold Shield, Hayward, CA) was also available *ad libitum* to some of the animals. Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH) and were approved by the UCSF Institutional Animal Care and Use Committee. Animals were never food or water deprived.

EtOH self-administration

The limited-access two-bottle choice paradigm was performed as described previously (van Rijn and Whistler, 2009). EtOH was administered during the dark cycle via a two-bottle, limited-access (4 h/day, 5 days/week) drinking paradigm in which one bottle contained 10% EtOH (v/v) and the other bottle contained water. Sucrose was never added to the EtOH solution. Bottles were identical and their positions were weighed and rotated daily. Animals were not used in experiments until they had been drinking EtOH for a minimum of 4 weeks (25 drinking sessions minimum). All behavioral testing was conducted 24 hours following the animal's last limited-access EtOH session. Consumption was measured after the 4-hour limited access period by bottle weight and recorded as g/kg (w/w).

Behavioral testing

All tests were performed during the animals' dark cycle. Animals were allowed to habituate to the room and lights for 2 hours before the start of the experiment.

Defensive marble burying

20 minutes following drug or vehicle injection, animals were placed in a new plexiglass home cage with 5 cm high bedding (Aspen shavings, Kaytee Products, Chilton, WI) with 15

(3 x 5) evenly spaced blue marbles. Animals remained in the cage with the marbles for 20 minutes before being removed. At the conclusion of the 20-minute session, the number of marbles exposed was counted and the number of marbles buried (defined as more then 2/3 covered with shavings) was determined. Bedding was replaced and marbles were cleaned with 70% EtOH between animals.

Swimming

At the conclusion of the marble burring task, mice were placed in a swimming task. Cylinders measured 30 cm in diameter and 36 cm high, water depth ranged from 22 -24 cm and temperature was 36.0 ± 0.2 degrees Celsius. Mice were placed into the center of the bucket and allowed to swim freely for 6 minutes before they were removed and towel dried.

Locomotor exploration

After towel drying, mice were placed in a locomotor exploration task. Clear plexiglass cubes measuring 19.5 cm in each direction were inserted in the corner of standard 43 cm locomotor chambers (Med Associates, St Albans, VT). Mice were placed into the cube and locomotor activity was digitally counted for 60 minutes.

Drugs and doses

EtOH solution was prepared in tap water using 95% (v/v) EtOH (Gold Shield Chemical Company, Hayward, CA). TAN 67 and SNC 80 were selected as "DOR-1" and "DOR-2" agonists respectively, in keeping with our previous work (*12, 43, 44*). Our previous findings suggest that TAN 67 effects are dependent on both MOR and DOR activation, while SNC

80 effects are only dependent on DOR activation (*12*). TAN 67 (25 mg/kg) was purchased from Tocris Biosciences (Ellisville, MO). SNC 80 (20 mg/kg), desipramine (20 mg/kg), bupropion (4 mg/kg), and escitalopram (3 mg/kg) were purchased from Sigma-Aldrich (St. Louis, MO). All compounds were dissolved in saline and administered i.p. 20 minutes before the beginning of each experiment.

Data analysis

Results are presented as mean ± SEM where appropriate. ANOVA and multiple comparisons tests were performed in Prism. T-test and regression analysis were performed in Excel.

Though data is divided below for the sake of clarity, statistical analyses were performed as a two-way ANOVA on both naïve and EtOH consuming animals combined. Individual groups were compared to saline treated animals and corrected for multiple comparisons. The ANOVAs revealed an overall effect of drug injection ($p < 0.0001$) with a significant interaction of drug injection and EtOH exposure ($p = 0.0279$) on marble burying and an overall effect of drug injection ($p < 0.0001$) on locomotion. Individual comparisons are reported in the results sections of Figures 4-1 and 4-3.

Results

Consistent with previous reports, we found that classic antidepressants could produce anxiolytic-like effects in the marble burying task. There was a significant decrease in the number of marbles buried following injections of either desipramine or escitalopram (Figure

4-1a). However, this anxiolytic-like effect was not produced by all the antidepressants tested. Specifically, there was no change in the number of marbles buried following bupropion injection (Figure 4-1a). This variability in efficacy is in contrast to previous studies in which desipramine and bupropion showed similar efficacy in behavioral tests thought to measure depression, rather than anxiety, such as the forced swim test and tail suspension test (*106*).

The DOR agonists also demonstrated variability in their anxiolytic-like effects on the marble burying task. Specifically, there was a significant decrease in the number of marbles buried following injections of the DOR agonist SNC 80, but not the DOR agonist TAN 67 (Figure 4-1a). This is consistent with previously reported anxiolytic-like effects of SNC 80 but not TAN 67 in the light/dark transition test in naïve mice (*44*).

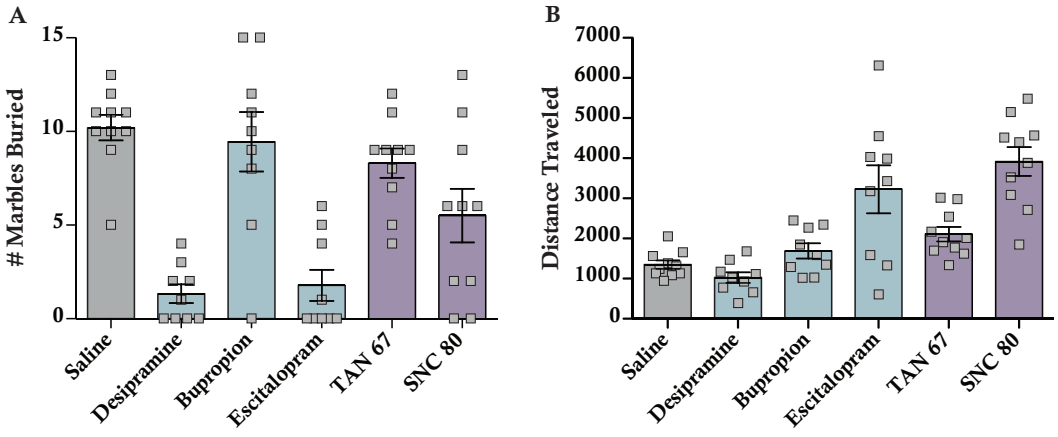

Figure 4-1: Drug effects on marble burying in naïve animals. A) Desipramine, escitalopram, and SNC 80 significantly decreased marble burying, as compared to saline, in naïve animals. B) Escitalopram and SNC 80 significantly increased locomotor activity as compared to saline, in naïve animals.

These effects on marble burying are unlikely driven by drug-induced changes in locomotor activity. While there was a significant increase in distance traveled following injections of escitalopram and SNC 80 (Figure 4-1b), there was no relationship between the number of marbles buried and the distance traveled in any of the groups (data not shown). Similarly,

while desipramine had the most robust effect on marble burying, it had no significant effect on locomotion, while bupropion, which did not have a significant effect on marble burying, induced locomotor activity to a degree between that seen with desipramine and escitalopram.

As previously discussed, EtOH is known to affect anxiety-like behaviors, so we next determined whether the marble burying test could detect a change in anxiety-like behavior as a consequence of EtOH exposure. Here, we found that there was a significant decrease in the number of marbles buried in the EtOH consuming animals, as compared to the naïve animals (both groups received vehicle injections, Figure 4-2a, $t = 2.347$, $p = 0.035$, $n = 10$), indicative of reduced anxiety-like behavior. These findings are in contrast to previously published studies, which reported increases in anxiety-like behavior after chronic EtOH consumption (*107-109*). There was no significant difference observed in the distance traveled for the naïve and EtOH consuming animals, suggesting that the decrease in marble burying in the EtOH consuming animals is not due EtOH induced changes in locomotor activity (Figure 4-2b).

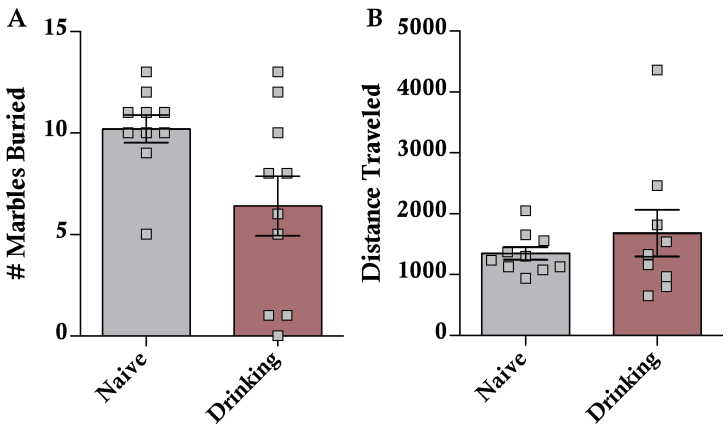

Figure 4-2: Effect of ethanol consumption on marble burying. A) The number of marbles buried was significantly decreased following ethanol consumption compared to naïve animals. B) There was no significant change in locomotor activity following ethanol consumption compared to naïve animals.

Because EtOH exposure has been demonstrated to influence behavioral effects of both classic antidepressants and DOR agonists on other measures of anxiety and motivation (*33, 44*), we next examined the effects of these compounds on marble burying following EtOH consumption. In EtOH consuming animals, while desipramine still induced a significant decrease in the number of marbles buried, neither escitalopram nor SNC 80 induced this effect following EtOH consumption (Figure 4-3a). In contrast to previous reported findings as measured by the light/dark transition test (*44*), here we found that TAN 67 did not become more effective at reducing anxiety-like behavior following EtOH exposure. Once again, these changes in drug induced anxiolytic-like effects are unlikely due to changes in locomotor activity. Specifically, only SNC 80 induced a significant increase in distance traveled in EtOH consuming animals, despite the fact that its anxiolytic-like effect on marble burying was absent (Figure 4-3b). The locomotor effect of escitalopram observed in naïve animals was no longer observed with EtOH exposure, nor did desipramine, which maintained its anxiolytic effect, demonstrate any significant locomotor alterations (Figure 4-3b).

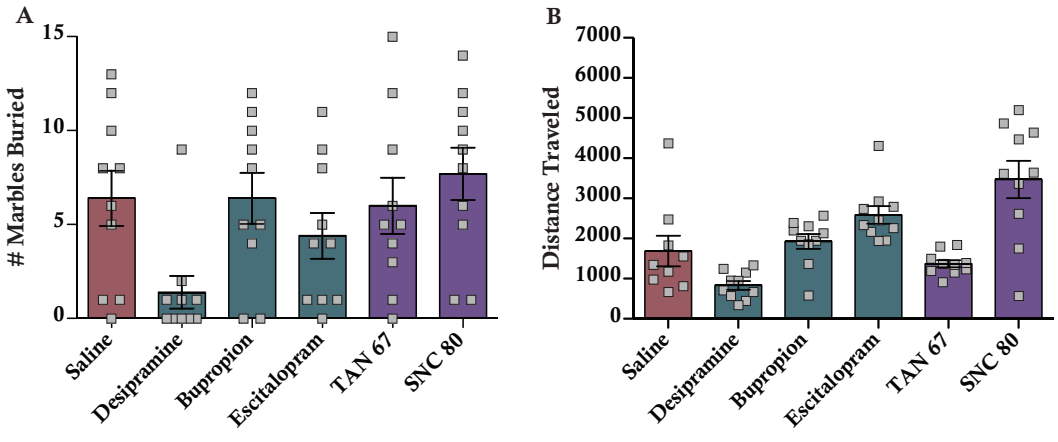

Figure 4-3: Drug effects on marble burying in ethanol consuming animals. A) Only desipramine significantly decreased marble burying, as compared to saline, in ethanol consuming animals. B) Only SNC 80 significantly increased locomotor activity as compared to saline, in ethanol consuming animals.

There was no significant effect of the drugs tested or ethanol exposure observed on immobility time in the swim test (data not shown).

Discussion

Our results here confirm previous reports that the marble burying assay is a sensitive measure for detecting anxiolytic-like effects of some commonly used antidepressants in naïve animals. The fact that some but not all classic antidepressants demonstrate effectiveness in the marble burying task here may reflect the differences in the neurotransmitter systems affected by these drugs. Indeed, the drugs escitalopram, desipramine, and bupropion were selected specifically because of their different pharmacological targets. Escitalopram is a second generation SSRI that acts though enhancing serotonin transmission and is commonly prescribed as a first line of treatment. Desipramine is a tricyclic antidepressant that acts through predominately enhancing norepinephrine transmission; while tricyclics are sometimes prescribed for depression, they have been largely replaced as a first-line treatment by SSRIs. Finally, bupropion had demonstrated efficacy for a number of conditions including depression, bipolar disorder, seasonal affective disorder, ADHD, as well as smoking cessation and obesity, however its mechanism of action is not entirely clear. It is thought to enhance both dopamine and norepinephrine transmission, as well as inhibiting acetylcholine receptors, but the full extent of its activity and which pharmacodynamics are responsible for which effects remains unclear. Based on our results here and those of previous studies using other behavioral outcomes, it seems likely that some but not all of these drugs would demonstrate dual efficacy for the amelioration of anxiety and depression. For example, SSRIs have been

repeatedly demonstrated to be effective at reducing depression and anxiety in both humans and animal models (*100, 101*), and have been previously reported to demonstrate efficacy in the marble burying test (*103, 104*). Since measures of anxiolytic-like effects may vary with the type of behavior tested, and it is still unclear which assay will have best predictive value for use in an alcoholic population, it's important to assess the behavioral effects of pharmacological treatments in different paradigms.

Similarly, here we demonstrate that DOR agonists also have different effects on the marble burying task in naïve animals. Consistent with our previous findings in the light/dark transition test (*44*), here we showed an anxiolytic-like effect of SNC 80 but not TAN 67 on marble burying in naïve animals. Interestingly, in our previous report, neither SNC 80 nor TAN67 demonstrated anxiolytic activity in the elevated plus maze test in naïve animals (*44*), underscoring the variable expression of anxiety-like behaviors and effects across paradigms. Though SNC 80 is known to increase locomotor activity, as we demonstrate again here, it is unlikely to be responsible for the effect on marble burying. Unlike the light/dark transition test and elevated plus maze, marble burying is much less dependent on exploratory and locomotor behavior to reveal an effect on anxiety. Though SNC 80 did increase locomotor activity in the animals tested, there was no relationship between the anxiolytic-like and locomotor effects. This, however, underscores the value of assessing anxiolytic-like efficacy in tasks like the marble burying test, which are less locomotor dependent than other tests designed to measure anxiety in rodent models.

We demonstrated for the first time here that the marble burying test is sensitive enough to detect an effect of EtOH consumption on anxiety-like behavior. We showed that EtOH drinking animals showed decreased anxiety-like behavior in the marble burying task compared to the EtOH naïve cohort. This is in contrast to our previous findings, where using the elevated plus maze we demonstrated that EtOH drinking animals showed increased anxiety-like behavior (*44*). It is also in contrast to other reports demonstrating EtOH withdrawal induced anxiety-like behavior (*107-109*). These differences could be due to differences in the withdrawal state in the current study compared to previous studies. Specifically, here the animals were not in a withdrawn state, as they were not without EtOH for any loner than their regular weekday schedule. However, it is also possible that this difference is simply due to differences in the behavioral model used to assess anxiety. Furthermore, it is also possible that the EtOH exposure shifts the dose response curves of these drugs such that an effective dose in naïve animals is no longer effective in drinking animals, but other doses may remain effective. Further studies are needed to determine if anxiolytic-like effects are available at difference dose ranges in ethanol consuming animals.

EtOH is known to have effects on locomotor behavior and coordinated motor impairment (*110*) and it is possible that locomotor impairment is presented differently in the measures designed to test exploratory versus defensive anxiety-like behaviors. However, EtOH consumption in the drinking in the dark model used here has been shown to induce tolerance to EtOH-induced ataxia and locomotor effects (*111*), indicating that motor impairment is likely minimal.

One of the most intriguing findings here is that EtOH exposure has variable effects on the efficacy of the anxiolytics and antidepressants tested here. Specifically, we demonstrate that, while escitalopram has an anxiolytic-like effect in naïve animals in the marble burying task, this effect was lost in EtOH drinking animals. In contrast, desipramine displayed an anxiolytic effect in both naïve and drinking animals. Taken together, these results suggest that a tricyclic antidepressant might show better efficacy for treating anxiety and/or depression in treatment seeking alcoholics than would an SSRI.

For the DOR agonists, addition of the marble burying test has broadened our understanding of how changes in DOR function during EtOH consumption alters behavioral responses to these drugs. We previously reported that, in naïve animals, SNC 80, but not TAN 67, was anxiolytic in the light/dark transition test. Furthermore, following EtOH consumption, both SNC 80 and TAN 67 became more effective anxiolytics (*44*). In the marble burying task here, we found that only SNC80 was effective in naïve animals, and that this effect was actually lost after drinking. This difference is significant because it illustrates that not only does the efficacy of these drugs vary with the physiological state of the animal (naïve versus drinking) but also that the anxiolytic-like effects of these same compounds vary across the behavioral measures utilized to detect anxiety-like behavior. Taken together, these results suggest that the efficacy of these drugs may vary based on the specific context of the stimuli. They also suggest these distinct anxiety-like behaviors may be modulated by different circuits, all expressing the DOR, since in one case (the light/dark transition test) EtOH exposure increased the efficacy of DOR agonists while in the other (the marble burying test)

EtOH decreased the efficacy of the DOR agonists. It is still unclear which of these assays would be most predictive for efficacy in treatment seeking alcoholics.

Furthermore, it is important to note that none of the anxiolytic-like effects corresponded to a decrease in locomotion. Though escitalopram increased locomotion in the naïve animals, where it also decreased marble burying, desipramine decreased marble burying in both groups with no significant change in locomotion. SNC 80, previously reported to be a locomotor stimulant (*112*), increased locomotor activity in both the naïve and drinking animals even though it did not affect anxiety-like behavior in the drinking animals.

In conclusion, we demonstrate here that the marble burying test, which captures defensive rather than exploratory anxiety, can detect EtOH-induced changes in baseline behavior as well as changes in drug efficacy. Importantly, we show that EtOH alters both baseline anxiety-like behavior and drug efficacy in different ways depending on the assay utilized. Taken together, these results underscore the importance of determining which animal models will be most predictive for efficacy in treatment seeking alcoholics.

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****Publication status***

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> **Coker, AR** & Whistler, JL (*Submitted*). Ethanol consumption alters the effects of delta opioid agonists and antidepressants on marble burying.

CHAPTER 5

Summary, discussion, and future directions

This manuscript has investigated the delta opioid receptor as a potential therapeutic target for alcohol use disorders, in particular those that may be co-morbid with anxiety or depression. In short, DOR agonists have been demonstrated to reduce EtOH consumption in both rats (*11*) and mice (*12*). Further, the DORs have well-established anxiolytic properties and like the other opioid receptors have a variable but significant role in reward and reinforcement. Perhaps most significantly, the expression and function of the DOR is known to change dynamically with a variety of physiological perturbations which share an important connection to alcohol use disorders, including EtOH exposure (*11, 12, 33, 44*), stress (*34*), and pain (*32*). This manuscript has examined some of the ways in which the DOR-mediated regulation of reward and anxiety behaviors may change with variations in the history and physiological state of the subjects. Below is a brief summary of the results described in each chapter.

Chapter 2: Differential contributions to reward and ethanol consumption by delta opioid receptor subtypes

This chapter looked at the relative preferences to the DOR-1 agonist DPDPE and the DOR-2 agonist deltorphin in the conditioned place preference paradigm in both naïve and ethanol consuming animals. Here we found that while neither DPDPE nor deltorphin induced a place preference in naïve animals, deltorphin induced a significant place preference in ethanol consuming animals. Further, there was no correlation between the amount of ethanol consumption and the magnitude of the preference for either DPDPE or deltorphin.

These data stress the important differences in the modulation of reward by DOR-1 versus DOR-2 agonists, which could help to better refine targets for therapeutic development in models of alcohol consumption.

Chapter 3. Stress and ethanol consumption alter delta opioid reward

This chapter looked at the relative preferences to the DOR-1 agonist DPDPE and the DOR-2 agonist deltorphin in the conditioned place preference paradigm in stressed animals either with or without ethanol consumption. Here we found that in animals that had been exposed to footshock stress, DPDPE does not induce a place preference, whether or not the animals were consuming ethanol. In contrast, deltorphin induces a place preference in ethanol naïve stressed animals, but not in ethanol consuming stressed animals. In fact, although there was no net deltorphin induced CPP, there was a robust inverse correlation between deltorphin preference score and EtOH consumption, such that high drinkers that were stressed found deltorphin aversive. While deltorphin induces a preference across EtOH consuming animals that were unstressed (Chapter 2 and (*77*), as well as a preference in the ethanol naïve stressed animals, intriguingly, when these factors come together, not only is the overall place preference now absent, but high drinking animals, that found deltorphin rewarding without stress, demonstrate an aversion to it following stress exposure. These findings demonstrate that the effects of DOR agonists can be highly dependent on behavioral state, and emphasize the importance of assessing the changes in these effects to avoid any unintended interactions when considering these targets for therapeutic potential.

Chapter 4. Ethanol consumption can alter the effects of delta opioid agonists and antidepressants on marble burying

This chapter looked at the effects of the DOR-1 agonist TAN 67 and the DOR-2 agonist SNC 80, as well as a selection of "classic" antidepressants, on the anxiety-like behavior measured by the marble burying task in both naïve and ethanol consuming animals. Here we found a variety of effects: while SNC 80 showed anxiolytic-like effects in naïve animals, TAN 67 did not. However, neither DOR agonist showed an anxiolytic-like effect in the drinking animals. This is an notable finding, as in previous work (*44*), we have demonstrated that these DOR-agonists can become more effective anxiolytics in drinking animals. Also of note, the classic antidepressants showed similar variability in effects: desipramine showed an anxiolytic-like effect in both the naïve and drinking animals, while bupropion did in neither. Escitalopram, however, showed an anxiolytic-like effect in naïve animals, which was absent in the drinking animals. Taken together, these findings underscore the importance of understanding and evaluating the distinctions in our "predictive" preclinical measures of affective behaviors and how these differences determine and affect the responses observed in order to assess which animal models will be most predictive of efficacy in our target populations.

Discussion

These data begin to dissect some of the differences in how DOR agonist effects are modulated by the behavioral state and history of the animal and how these changes may differ between DOR-1 and DOR-2 agonists. While the underlying patterns are still only beginning to emerge, a few overall insights can be gleaned from the accumulation of these

results. First, and most apparently, the effects of DOR agonists on behavior are dynamic and change significantly with the state and history of the animal. Second, the behavioral effects of DOR-1 agonists seem to be less dependent on changes in behavioral state than those of DOR-2 agonists. And finally, the shits in behavioral effects of the DOR agonists observed not only depend on the state of the animals but also the nature of the behavioral measure and the potentially differential role of DORs in the circuits that control these behaviors.

Dynamic changes in DOR receptors

The dynamic nature of the DOR is an underlying thread has been repeatedly demonstrated by multiple lines of evidence: behavioral studies of DOR agonist effects discussed here, cellular studies of DOR expression, as well as electrophysiological studies of DOR function. While the studies herein focus primarily on changes in behavioral effects, to understand why these changes occur, we must examine other lines of evidence. Examining the cellular underpinnings of these dynamic changers can help us to better understand both when and how DOR function is altered following changes to the animal's behavioral state that may then alter DOR agonist effects on behavior.

As previously discussed, while the DORs are expressed throughout brain, the VTA is an ideal locus to examine the dynamic changes in DOR function with ethanol and stress exposure as previous studies have demonstrated. Studies assessing these changes on a cellular and circuit level can help provide context and suggest explanations for the behavioral effects described here. To summarize: In naïve animals there is little to no effect

of DOR agonists on GABA signaling onto VTA neurons. In chronically drinking animals, however, DOR effects of both DPDPE and deltorphin on GABA signaling emerge, wherein these DOR agonists inhibit GABA signaling onto VTA neurons. The degree of this DOR-mediated inhibition is highly variable, however the inhibitions by DPDPE are significantly inversely correlated to the amount of ethanol consumed (*11*), while there is no correlation between deltorphin inhibitions and ethanol consumption (Chapter 2 and (*77*).

The shifts in DOR agonist effects across behavioral states combined with cellular data together indicate that the "experience"– EtOH, stress, pain, etc, alters where DORs are expressed in the circuits and/or how DORs that may already be present can signal. In the absence of these stimuli, as in naïve animals, the DORs are distributed and signal in their own distinct patterns. That distribution and/or signaling is altered following EtOH exposure, resulting in changes in behaviors and DOR effects that are modulated by these circuits.

For example, in naïve animals some effects of DOR agonists have been observed in VTA neurons, while other effects of the agonists are absent. Specifically, while the effects of either DOR agonist on GABA transmission onto VTA neurons are negligible in naïve animals, there are direct post-synaptic effects of DOR-1 and DOR-2 agonists on a subset of VTA neurons. However, following chronic EtOH consumption, a DOR-mediated effect on GABA signaling of these same agonists emerges (*11*), now allowing the DOR agonists to modulate GABA transmission onto VTA neurons, where they could not in the naïve animals. This indicates that DOR agonists that are administered *in vivo* after chronic EtOH

exposure will similarly have different, previously unavailable, receptor targets become available to act on and modulate the animals' behavior, which result in the "shifts" in effects that we observe on behavior.

Further, understanding the time course of when these DOR-mediated effect on GABA signaling develops following EtOH exposure and how these effects relate to the changes on behavior might provide us with further insight into the nature of these dynamic changes. We have recently demonstrated that the DPDPE-mediated inhibition of GABA signaling in the VTA actually occurs rapidly after initial ETOH exposure—with as little as 3 days of EtOH exposure (*33*). This increase in DPDPE-mediated inhibition of GABA occurs in animals that were both self-administering EtOH as well as animals that were administered EtOH via oral gavage by the experimenter, with the degree of inhibition increasing over 3, 7, or 14 days of exposure. Critically, the magnitude of these DPDPE-mediated inhibitions, even at these early time points, were significantly correlated to relevant behavioral measures of anxiety and intoxication, but only if EtOH was self-administered (*33*). Over the first 14 days of EtOH self-administration, there is little variability in the amount of EtOH consumed between animals. This suggests that similar levels of EtOH consumption may induce differential changes in DOR-function in animals with different baseline phenotypes, such as high or low anxiety (*33*). If the differential DOR changes induced alter subsequent EtOH consumption and opioid actions, this could lead to further changes in DOR receptor expression and/or function that may contribute to escalating EtOH consumption. Examining these relationships can help us better understand the dynamic changes in DOR function as an animal transitions from EtOH naïve to escalating chronic EtOH

consumption, and may help us to better predict how DOR agonist effects will "shift" across behavioral states.

Distinctions and constancies between data sets

The data presented here have many divergent variables that may speak to some of the differences observed between experiments. One set of experiments uses the peptide compounds DPDPE and deltorphin to assess DOR-1 and DOR-2 differences; these studies were all completed in rats, with intra-VTA drug infusions, and 24-hour free EtOH access. The other set of experiments uses the non-peptidergic, small molecule compounds TAN 67 and SNC 80 to assess DOR-1 and DOR-2 differences; these studies were all completed in mice with systemic injections and limited 4-hour daily EtOH access. These distinctions may account for some of the differential effects observed both between the experiments discussed here and with those of previously published works.

Importantly, despite these numerous and varied differences, many of the fundamental findings are consistent between these data sets. First, the initial findings on changes in EtOH consumption previously reported are consistent between the different data sets: both DPDPE and TAN 67 (classified as "DOR-1 agonists") induced a reduction in ethanol consumption (*11, 12*). Similarly, SNC 80, a "DOR-2 agonist" induced an increase in ethanol consumption, and while the DOR-2 agonist deltorphin had no overall effect on ethanol consumption, it did increase consumption in low drinking animals (*11, 12, 77*). Additionally, the place preference experiments reported here and in previously published work show similar findings as well: DPDPE and TAN 67 both demonstrated no overall preference or

aversion in naïve or EtOH consuming animals (*59, 77*). Similarly, SNC 80 and deltorphin both resulted in no overall preference or aversion in naïve animals. While deltorphin, but not SNC 80, induced a highly significant place preference in EtOH consuming animals, both drugs showed a significantly stronger preference than did their DOR-1 counterparts in the drinking animals (*59, 77*). These parallel findings are observed despite the number of experimental distinctions between the experiments in species, drug type, injection location, and ethanol exposure paradigms. Therefore, although DOR action in the VTA is sufficient to alter preference and EtOH consumption, as demonstrated by the direct injections, the differential results with the systemic injections in the mouse model may also be due to additional DOR actions in other regions that may influence these same behaviors.

Overall, these results consistently demonstrate that though behavioral history and physiological state modulate DOR response, the behavioral effects of DOR-1 agonists are less dependent on changes in behavioral state than those of DOR-2 agonists. The DOR-1 agonist DPDPE showed similar levels of place preference, all non-significant, across behavioral states: naïve, drinking, stressed, and animals that were stressed while drinking (Chapter 2 and 3, and see (*77*). However the DOR-2 agonist deltorphin showed no preference in naïve animals, a highly significant preference in drinking animals, a moderate preference in stressed animals, and a slight though non-significant aversion in animals that were stressed while drinking (Chapter 2 and 3, and see (*77*). Similarly across the behavioral measures to assess "anxiety-like" behaviors tested here and in previous findings (such as in the elevated plus maze and the marble burying test), the DOR-2 agonist SNC 80 is more likely to demonstrate differential effects when tested in naïve versus drinking animals, while

the DOR-1 agonist TAN 67 showed consistent effects between the naïve and drinking animals in these same tests. These differential effects are illustrated in Table 5-1 below, which includes both the tasks described here (red) and some from previous works (black). Though many of these behaviors have been evaluated across naïve and EtOH consuming conditions in this panel of drugs, there are still many effects that remain untested.

			DELTA DRUGS				ANTIDEPRESSANTS		
			DOR 1		DOR 2		Tricyclic		SSRI
			DPDPE	TAN 67	deltorphin*	SNC 80	desipramine	bupropion	escitalopram
	ETOH consumption		↓*	↓	✖*	↑			
"Anxiety"	Plus Maze	N		✖		↓ ✖	↓ ✖	✖	↓
		D		✖		↓			
	Light/Dark Box	N		✖		↓			↓
		D		↓		↓			
	Marble Burying	N		✖		↓	↓	✖	↓
		D		✖		✖	↓	✖	✖
"Depression"	Forced Swim	N	↓	✖	↓	↓ ✖	↓ ✖	↓ ✖	↓ ✖
		D		✖		✖	✖	✖ ✖	✖
	Tail Suspension	N					↓	↓	
		D					✖		
	Place Preference	N	✖*	✖	✖*	✖			
		D	✖*	✖	↑*	✖			

N = naïve * = intra-VTA ✖ = no effect observed
D = drinkg ↑ / ↓ = increased / decreased
Data included in thesis manuscript **Previous published data**

Table 5-1: Summary of drug effects on preclinical measures of "depression" and "anxiety" in naïve and ethanol consuming animals. Arrows indicate the treatment effect (down arrow = decrease in depression/anxiety like behavior). Data contained in this manuscript indicated in red, previous publications are indicated in black.

These varied states of ethanol consumption, stress, and anxiety are important factors in treating human alcoholic populations, and the differences in the changes of DOR effects with these behavioral states are important considerations in evaluating the delta receptor as a treatment target. The proposal that the DOR-1 agonists would demonstrate greater potential as a treatment target than DOR-2 agonists is reinforced by these data, which

illustrate that the DOR-1 effects on reward, anxiety, and stress are less influenced by certain behavioral and state changes relevant to human alcoholic populations.

Predicting efficacy in alcoholics

The behavioral tests commonly used to assess "depression" and "anxiety" in animal models utilize different contexts and parameters to model the etiology and expression of observed behavioral changes. For example in measuring anxiety-like behavior in animal models, there are several commonly used tests of anxiety that each evaluates these behaviors in different ways. The elevated plus maze, open field test and light/dark transition test focus on changes in exploratory behavior as a measure of anxiety; they measure the animals movement, exploration, and where they spend their time (open/bright vs closed/dark areas of the apparatus). The marble burying test focuses on defensive behavior as a measure of anxiety; the burying behavior is taken as a measure of a defensive anxiety response to novel objects. Though both tests are thought to measure "anxiety-like" behavior in rodents through these different behavioral changes, they have limited construct validity to model the human condition. Instead they are chosen primarily for their predictive validity (i.e. their ability to identify drugs that will be effective in humans) and are generally successful in this endeavor. Drugs that are anxiolytic in humans (such as benzodiazepines and SSRIs) reliably decrease anxiety-like behavior in these animal models.

Though these preclinical behavioral tests demonstrate excellent predictive validity of drug efficacy for depression and anxiety in "normal" humans, not all of these predictions extend to people with alcohol use disorders. Despite the fact that antidepressants and anxiolytics

show high efficacy in subjects with a single diagnosis, the treatment of these same disorders in subjects with comorbid alcohol use disorders remains a challenge. While not often explicitly tested in human subjects, anecdotal evidence suggests that drugs that are effective for depression and anxiety in "normal" humans show limited or reduced efficacy in subjects with comorbid alcohol use disorders. Indeed, a meta-analysis of antidepressant treatments in subjects with comorbid alcohol and substance use disorders revealed that the efficacy of these commonly prescribed antidepressants is decreased in this comorbid population (*113*). Though SSRIs are now generally considered the first line of treatment for patients with both depressive disorders and even some chronic anxiety disorders, they can be less effective in subjects with alcohol use disorders. This analysis revealed that an older line of antidepressants, the tricyclics (such as desipramine), which is now rarely prescribed as a first-line treatment, may demonstrate greater efficacy for depressive symptoms in subjects with alcohol use disorders, and may in fact even begin to help reduce alcohol consumption as the depression symptoms are diminished (*113*).

To better treat this population, we must develop more targeted predictive criteria for efficacy. The variety of preclinical screening tests could represent specific differences in the behavioral constructs that may correspond to fundamental differences in the human conditions we are attempting to model. Or more simply, some tests could be more or less effective at predicting human behavior and drug efficacy. To better target or develop new treatments for alcohol abusing subjects, it is essential to identify which of these tests, or which combination of these tests, are most effective at predicting efficacy in this population,

with the understanding that these predictions may not necessarily match in subjects without alcohol use disorders.

By comparing the efficacy of known compounds (such as classic antidepressants) in people with alcohol use disorders to efficacy in "predictive" preclinical studies using ethanol consuming animals we can begin to determine which behavioral assays will be most predictive for efficacy in treatment seeking alcoholics. For example, in the experiments described here, we examined several of these typical antidepressants in both naïve and EtOH consuming mice on one behavioral measure of anxiety-like behavior, the marble burying test. Though both desipramine and escitalopram (an SSRI) reduced anxiety-like behavior in naïve mice, only desipramine retained its efficacy in the drinking animals. This coherence with the clinical data is significant, as previous studies with other behavioral measures such as the tail suspension test (summarized in Table 5-1 above) have reported that desipramine lost its efficacy as an antidepressant in EtOH consuming mice (*106*). This suggests that though the tail suspension test has excellent predictive validity for depressed patients without comorbid alcohol use disorders, the marble burying test is a better predictor of clinical efficacy in those patients that have comorbid alcohol use disorder and depression. The alignment of the preclinical and clinical findings demonstrating that the tricyclic antidepressant retains its efficacy over the SSRIs in both the human population and the preclinical marble burying test underscores the utility of this model in drinking subjects. Similar comparisons with other preclinical tests can help us to develop a more targeted preclinical battery for developing compounds for treatment of subjects with alcohol use disorders.

Exploration of these various behavioral measures has broadened our understanding of how EtOH consumption alters behavioral responses to DOR agonist drugs as well. Previous studies demonstrated that in exploratory tests of anxiety-like behavior, the DOR agonists SNC 80 and TAN 67 became more effective anxiolytics (*44*). However, in the data described here, in the marble burying task examining defensive anxiety-like behavior, only SNC80 was effective in naïve animals, and this effect was actually lost after drinking. This difference is significant because it illustrates that not only does the efficacy of these drugs vary with the physiological state of the animal (naïve versus drinking) but also that the behavioral effects of these same compounds varies across behavioral measures, which can be significant for developing targeted treatments. These differences in behavioral effects are summarized in Table 5-1 above. As the findings with the classic antidepressants demonstrated that the marble burying test is more predictive of clinical efficacy for subjects with alcohol use disorders, this behavioral measure is more indicative of the potential changes in DOR mediated effects in human alcoholics. However, a more complete set of predictive behaviors needs to be developed to further treatment development for human subjects. Because of the importance of these differences in specific behavioral measures in affecting the direction of these changes in DOR effects, further testing would be necessary to determine any changes in these effects in these other behaviors that have not yet been evaluated.

Future directions

Though we have begun to gain some insight into the variations and possible site at which DOR-mediated behavioral effects are changed with an animal's physiological state and history, the underlying patterns are still only beginning to emerge. Because these changes do not always follow a predictable pattern, it will remain important to continue to examine relevant behavioral states that may affect potential treatment possibilities. A significant one of these states is relapse.

The potential for relapse is one of the biggest challenges in treating alcohol use disorders. A variety of cues and stressors can trigger relapse in recovering alcoholics. Not only is it critical to identify treatments that will specifically curtail relapse, it is also important to assess the potential for treatments in active drinkers to affect their potential for relapse. If a pharmacological treatment is effective in curtailing drinking, it is important that its efficacy is not diminished with reduction in consumption or relapse.

Environmental cues and stressors can modulate or induce drug-seeking (or relapse) behavior, and the DORs are involved in both of these (*34, 114*). Importantly, following up to 14 days of abstinence, the EtOH induced alterations in DOR function in the VTA persist, suggesting the potential that the DOR would remain a viable treatment target (*33*). However, as the work described herein illustrates, though DORs are dynamically responsive to changes in behavioral state, the direction and outcome of which is not always predictable when different states are in concert, it is critical that the effects of potentially therapeutic DOR agonists on relapse behavior be examined.

Though this "moving target" phenomenon of DOR dynamic expression can appear to make the DOR an untenable treatment target, it could also become an asset in developing a pharmacological substrate. If we can better determine and understand the underpinnings of how DORs change with ethanol exposure and stress, we could potentially use its dynamic nature as an advantage in treatment development to improve efficacy while reducing aversive effects.

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