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Evidence that Vasopressin V_{1b} Receptors Mediate the Transition to Excessive Drinking in Ethanol-Dependent Rats

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Abstract

Alcoholism is a devastating condition that represents a progression from initial alcohol use to dependence. Although most individuals are capable of consuming alcohol in a limited fashion, the development of alcohol dependence in a subset of individuals is often associated with negative emotional states (including anxiety and depression). Since the alleviation of this negative motivational state via excessive alcohol consumption often becomes a central goal of alcoholics, the transition from initial use to dependence is postulated to be associated with a transition from positive to negative reinforcement mechanisms. Vasopressin is a neuropeptide known to potentiate the effects of CRF on the HPA axis, and emerging evidence also suggests a role for centrally located vasopressin acting on V_{1b} receptors in the regulation of stress- and anxiety-like behaviors in rodents. The present study determined state-dependent alterations in vasopressin/V_{1b}R signaling in an animal model of ethanol dependence. The V_{1b}R antagonist SSR149415 dose-dependently reduced excessive levels of ethanol self-administration observed in dependent animals without affecting the limited levels of ethanol drinking in non-dependent animals. Ethanol self-administration reduced V_{1b} receptor levels in the basolateral amygdala of non-dependent animals, a neuroadaptation that could theoretically facilitate the positive reinforcing effects of alcohol. In contrast, V_{1b}R levels were seemingly restored in ethanol-dependent rats, a switch that may in part underlie a transition from positive to negative reinforcement mechanisms with dependence. Together, our data suggest a key role for vasopressin/V_{1b}R signaling in the transition to ethanol dependence.

Keywords

Amygdala; Ethanol Dependence; Vasopressin

Introduction

In the United States, approximately 51% of people over the age of 12 consume alcohol (120 million), and of these current users, 7.7% (18 million) have met the criteria for Substance

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The authors declare no conflicts of interest.

Author Contributions

SE conducted all behavioral and biochemical experiments, analyzed the data, and wrote the manuscript. MG and OMG synthesized SSR149415 under the supervision of ER. GFK conceived and designed the study. All authors critically reviewed content and approved the final version for publication.

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Abuse or Dependence (Substance Abuse and Mental Health Services Administration, 2008). Thus, while most people consume alcohol recreationally for its positive rewarding effects, some individuals go on to develop problem drinking. The gradual development of excessive alcohol use is typically associated with the recruitment of a negative emotional state, including the emergence of anxiety and depression symptoms (Koob and Le Moal, 1997; Kreek and Koob, 1998; Edwards and Koob, 2010). To study this condition, preclinical models that are able to distinguish and describe neurobiological differences between dependent and non-dependent groups of animals are invaluable in understanding the important transition from initial to habitual drug use (Koob *et al*., 2004; Kalivas, 2005). In rodents, recreational alcohol drinking is often represented via limited access operant ethanol self-administration, where drinking tends to remain controlled over time, as is seen in non-dependent (recreational) human drinkers. Excessive, compulsive drinking can be produced in this model by the induction of dependence using experimental titration of blood alcohol levels (BALs) following chronic exposure to ethanol vapors. Use of this paradigm has characterized pharmacological agents that are capable of reducing drinking in both non-dependent and dependent groups (e.g., naltrexone; Walker and Koob, 2008), or selectively in dependent animals (e.g., corticotropin-releasing factor (CRF) receptor antagonism; Funk *et al*., 2007; Richardson *et al*., 2008). These studies have led to the specific hypothesis that a recruitment and potentiation of brain stress neurotransmission is responsible for driving a negative motivational state supporting ethanol dependence (Gilpin and Koob, 2008).

Several emerging lines of evidence have implicated the neuropeptide vasopressin in the pathophysiology of stress-related behaviors (Surget and Belzung, 2008; Chrousos, 2009). Vasopressin is a nine-amino acid peptide first known to be synthesized in the hypothalamus and transported to the posterior pituitary (Brownstein *et al*., 1980). Here, it is released into the bloodstream after appropriate stimulation (dehydration) to act on the kidneys for the purposes of water retention, acting on V_2 vasopressin receptors (Kaufmann *et al*., 2000). Vasopressin-synthesizing neurons are also centrally localized (Buijs, 1978; Buijs *et al*., 1983; De Vries and Buijs, 1983), originating within the paraventricular nucleus (PVN), bed nucleus of the stria terminalis (BNST), medial amygdala (MeA) and suprachiasmatic nucleus (SCN). Vasopressin cell bodies project extensively throughout the limbic system (Veinante and Freund-Mercier, 1997) and play a significant role in regulating various complex behaviors, including aggression, social affiliation, and sexual pair bonding. Its facilitatory role in limbic system plasticity is thought to underlie various aspects of affective processing (Caldwell *et al*., 2008), and early studies established a specific role for central vasopressin in aversive learning and memory mechanisms (de Wied and Versteeg, 1979; Koob and Bloom, 1982; Le Moal *et al*., 1984; Engelmann *et al*., 1996). More recently, the generation of small molecule, receptor subtype-selective antagonists has greatly advanced the characterization of the complex behavior regulated by the two major central vasopressin receptor subtypes: V_{1a} and V_{1b}. While V_{1a} receptors are believed to regulate social behavior and aggression, V_{1b} receptors are thought to play a role in generalized affective-like behaviors, as demonstrated by the anxiolytic- and antidepressant-like effects of the small molecule V_{1b} vasopressin receptor antagonist SSR149415 (nelivaptan, (2S,4R)-1-[(3R)-5-chloro-1-(2,4-dimethoxyphenyl)sulfonyl-3-(2-methoxyphenyl)-2-oxo-indolin-3-yl]-4-hydroxy-N,N-dimethyl-pyrrolidine-2-carboxamide; Griebel *et al*., 2002). Studies utilizing regional microinjections of SSR149415 have established a role for stress- and/or anxiety-like V_{1b}R signaling in the basolateral/central/medial amygdala (Salome *et al*., 2006), the lateral septum (Stemmelin *et al*., 2005), and the dorsal hippocampus (Engin and Treit, 2008).

A role for vasopressin/V_{1b}R signaling in drug dependence was recently hypothesized (Koob, 2008; Zhou *et al*., 2010). Vasopressin mRNA levels are increased selectively in the amygdala during early spontaneous withdrawal from chronic heroin exposure, and

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SSR149415 blocks footshock-induced reinstatement of heroin-seeking behavior, suggesting that vasopressin systems in the amygdala may represent a key component of the aversive emotional consequences of opioid withdrawal (Zhou *et al*., 2008). Given the literature suggesting that V_{1b}R antagonists have anxiolytic-like profiles and the fact that vasopressin and its receptors are highly expressed in the extended amygdala, we hypothesized that vasopressin systems in the extended amygdala or other limbic areas may have a role in the increased alcohol intake associated with dependence. In the present study, we show that a differential regulation of vasopressin/V_{1b} receptor signaling is involved in the transition from limited ethanol use to ethanol dependence, a transformation that is hypothesized to be regulated by activation of vasopressinergic neurons as part of the brain stress system.

Materials and Methods

Animals

Male Wistar rats (n=44, Charles River) initially weighting 250–275g were communally housed (2–3/cage) with food and water available *ad libitum*. The animals were housed in a temperature-controlled (21.5 C) vivarium and maintained on a 12 h light/dark cycle (lights on at 0800). Animals were handled daily for one week before the onset of operant conditioning. All experiments adhered to the guidelines provided in the NIH *Guide for the Care and Use of Laboratory Animals* and protocols were reviewed and approved by The Scripps Research Institute's Institutional Care and Use Committee.

Drug Administration

SSR 149415 was synthesized in the Department of Chemistry at The Scripps Research Institute. It was prepared as a fresh solution each test day in physiological saline (0.9%) containing 5% DMSO (Sigma, St. Louis, MO) and 5% Cremophor EL (Sigma) and injected intraperitonealy thirty minutes before testing.

Operant Chambers

The operant chambers (MedAssociates) used in this study had two retractable levers located 4 cm above a grid floor and 4.5 cm to each side of a two-well acrylic drinking cup that allowed for two solutions to be administered upon pressing of the appropriate lever. Recording of operant responses and subsequent solution delivery were controlled by custom software running on a PC computer. Each lever press resulted in activation of a 15 RPM Razel syringe pump (Sanford, CT) that delivered 0.1 ml of solution to the appropriate well over 0.5 s. Operant chambers were individually housed in ventilated, sound-attenuated cubicles to minimize environmental disturbances.

Acquisition of Operant Self-Administration

The timeline of experimental procedures is shown in Figure 1. Animals were first trained to self-administer ethanol by a sucrose-fading procedure (Samson, 1986), at the end of which animals were allowed to self-administer 10% (w/v) ethanol and water solutions until stable responding was maintained. Animals were then split into two groups that were matched for ethanol self-administration over their last 5 sessions, with one group designated as "ethanol-dependent" (vapor-exposed) and the other as "non-dependent" (air-exposed).

Ethanol Vapor Chambers

Ethanol vapor exposure has been demonstrated to be a safe, reliable method to titrate blood alcohol levels (BALs) for the induction of ethanol dependence (Gilpin *et al*., 2008). In this procedure, BALs are adjusted by the experimenter to maintain dependence-inducing BALs without jeopardizing animal health. Rats were made dependent by chronic, intermittent

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exposure to ethanol vapors (CIEV), and BALs were regulated between 38–49mM. Tail blood samples were taken and analyzed 1–2x week for BAL determination as previously described (Gilpin *et al.*, 2008). Animals underwent cycles of 14 hr on vapor and 10 hr off, with BALs being diminished by 6 hr withdrawal (Figure 1), when behavioral testing and tissue collection occurred. Importantly, at this time point animals exhibit a host of symptoms related to dependence, including both somatic withdrawal signs (Roberts *et al*., 2000; O'Dell *et al*., 2004) and negative motivational symptoms (Schulteis *et al*., 1995), and show excessive alcohol drinking compared to air-exposed animals.

Pharmacological Testing of Ethanol Self-Administration in Dependent and Non-Dependent Animals

Following acquisition of ethanol self-administration and induction of ethanol dependence by four-six weeks of CIEV exposure, dependent and non-dependent (air-exposed) animals were tested until post-vapor intake stabilization was reached (10 sessions). Post-vapor testing (both for behavior and biochemical analysis, see below) was conducted when dependent animals were in acute (6hr) withdrawal. Responses for both ethanol and water (a natural reinforcer) were recorded. After stable responding for ethanol, which included habituation to intraperitoneal injections of vehicle (5% DMSO/5% Cremophor EL in physiological saline), the effects of V_{1b}R antagonism on ethanol self-administration was tested in both ethanol-dependent and non-dependent (air-exposed) rats (n=8/group). Thirty minutes before each test session, animals were injected with the V_{1b}R antagonist SSR149415 (10, 20, 30 mg/kg, IP) or vehicle. This dose range was chosen based on its anxiolytic- and antidepressant-like effects in rodents (Griebel *et al*. 2002). Each dose (including vehicle alone) was tested in individual sessions conducted twice weekly (on non-cage changing days) according to a Latin square design. Between test sessions, animals were allowed to self-administer ethanol in non-test sessions to reveal any carry-over effects of previous V_{1b} antagonist dosing on subsequent ethanol drinking.

Western Blot Analysis

In separate groups of drug-naïve animals (n=7–8/group), we measured vasopressin V_{1b} receptor levels in five limbic brain regions previously implicated in the anxiolytic-and antidepressant-like effects of SSR 149415 of ethanol self-administering (dependent and non-dependent) and naïve animals. Naïve control animals were age/batch-matched and housed under identical conditions, were handled twice weekly, but did not undergo any operant training. One day following their last self-administration session (the tenth session post-dependence induction), animals were euthanized under light halothane anesthesia by decapitation. This time point was chosen since it corresponds to the period when animals were normally self-administering ethanol (i.e., six hours post-vapor for ethanol-dependent animals). Rat brains were rapidly dissected and snap frozen in isopentane. Regional tissue samples were obtained with 12–16 gauge punches from chilled coronal brain slices (0.5 mm thick) obtained by the use of a cryostat. Tissue processing for Western blot analysis was conducted as described previously (Edwards *et al*., 2009). Briefly, tissue samples were homogenized by sonication in lysis buffer (320 mM sucrose, 5 nM HEPES, 50 mM NaF, 1 mM EGTA, 1 mM EDTA, 1% SDS, with Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktails I and II diluted 1:100; Sigma, St. Louis, MO), boiled for 5 min, and stored at −80°C until determination of protein concentrations by the Lowry method. Samples of 20μg protein were subjected to SDS-polyacrylamide gel electrophoresis on 10% acrylamide gels using a Tris/Glycine/SDS buffer (Bio-Rad, Hercules, CA), followed by electrophoretic transfer to PVDF membranes (polyvinylidene; Amersham, Piscataway, NJ). Membranes were blocked overnight in 5% nonfat milk at 4° C, and incubated in primary antibody for the vasopressin V_{1b} receptor (1:1000; #AVP1B13-S, Alpha Diagnostic International, San Antonio, TX, USA) for 24 hrs at 4° C. This antibody has been extensively

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characterized in previous reports (Hurbin *et al*., 2002; Folny *et al*., 2003; Stemmelin *et al*., 2005). Membranes were washed and labeled with species-specific peroxidase-conjugated secondary (goat anti-rabbit, 1:10,000; BioRad) for 1 hr at 25° C. Following chemiluminescence detection (ECL plus; Amersham), blots were stripped and reprobed for beta-Tubulin (1:10,000 and 1:5000; Santa Cruz) as internal standards for protein levels. Immunoreactivity was quantified by densitometry (Scion Image) under conditions linear over at least a 3-fold concentration range.

Statistics

Blood alcohol levels between 0hr and 6hr withdrawal from ethanol vapors were analyzed by Student's t test. The relationship between preferred levels of ethanol self-administration before and after ethanol vapor exposure was determined by Pearson's linear correlation. The effects of vapor exposure and/or pharmacological challenges on ethanol self-administration were analyzed using a mixed two-way ANOVA with vapor treatment (ethanol or air) as the between-subjects factor and test day or $V_{1b}R$ antagonist dose as the repeated, within-subjects factor. In cases of a significant main effect, *post hoc* comparisons were performed with Bonferroni's *post hoc* tests. Western data were analyzed using a one-way ANOVA, and, in cases of a significant main effect, *post hoc* comparisons were performed with Newman-Keuls Multiple Comparison tests. All statistical analyses were performed with Prism 4.

Results

Figure 1A depicts the experimental timeline used in the present study. Animals (n=44) were initially trained to self-administer ethanol via a sucrose-fading procedure. Following stabilized acquisition of ethanol self-administration, half of the rats (ethanol-dependent group, n=22) were made dependent by exposure to intermittent ethanol vapor (14 hr/day for 4–6 weeks) and subsequently tested for ethanol self-administration (10% v/v, FR1, 30 minute sessions) six hours into withdrawal, a time point commensurate with diminished blood alcohol levels (Figure 1B) and heightened anxiety-like behavior (Roberts *et al*., 2000; O'Dell *et al*., 2004). The other half of the rats (non-dependent group, n=22) were exposed to air, and tested at the similar time of day as ethanol-dependent animals. All animals were tested on non-cage changing days to facilitate stable ethanol self-administration. Ethanol self-administration behavior five days before and after dependence induction is presented in Figure 2A. Ethanol vapor exposure significantly elevated alcohol drinking in ethanol-dependent animals (group × day interaction, $F_{9,378} = 25.15$). Additionally, there was a positive correlation between individual preferred levels of drinking before and after chronic intermittent vapor exposure ($r = 0.644$, $p = 0.001$, Figure 2A inset), suggesting a preservation of individual differences in dependent animals following vapor exposure, albeit at magnified levels.

After stable responding for ethanol, which included habituation to intraperitoneal injections of vehicle (5% DMSO/5% Cremophor EL in physiological saline), the effects of $V_{1b}R$ antagonism on ethanol self-administration was tested in both ethanol-dependent and non-dependent (air-exposed) rats (n=8/group). Thirty minutes before each test session, animals were injected with the $V_{1b}R$ antagonist SSR149415 (10, 20, 30 mg/kg, IP) or vehicle. This dose range was chosen based on its anxiolytic- and antidepressant-like effects in rodents (Griebel *et al*. 2002). Each dose (including vehicle alone) was tested in individual sessions conducted twice weekly (on non-cage changing days) according to a Latin square design. Between test sessions, animals were allowed to self-administer ethanol in non-test sessions to reveal any carry-over effects of previous $V_{1b}R$ antagonist dosing on subsequent ethanol drinking, and such effects were not observed. SSR149415 dose-dependently reduced responding for ethanol selectively in dependent rats (Figure 2B, group × dose interaction,

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$F_{3,42} = 5.601$), reaching significance at the 30 mg/kg dose ($q = 6.365$, $p<0.01$ vs. vehicle in ethanol-dependent group, Newman-Keuls Multiple Comparison Test). Although SSR149415 has been demonstrated to exhibit a somewhat limited brain penetrability (Schonberger *et al.*, 2010), antidepressant-like effects of the drug were still observed at 30 mg/kg in hypophysectomized rats (Griebel *et al.*, 2002), indicating a probable action at extrahypothalamic $V_{1b}R$ brain sites at this dose. In contrast, there was no effect of the antagonist on non-dependent animals, suggesting a relative potentiation or sensitization of $V_{1b}R$ signaling in dependent animals. Responses for water were not changed in either group by $V_{1b}R$ antagonism (Table 1). It is worth noting again that the effects of vasopressin (also known as anti-diuretic hormone) on fluid dynamics in the kidney are mediated by vasopressin V_2 receptors (Kaufmann *et al*., 2000).

To determine possible neuroadaptations associated with the differential responsiveness to $V_{1b}R$ antagonism between dependent and non-dependent groups, we measured $V_{1b}R$ protein levels in five brain regions previously implicated in SSR149415-mediated reductions in antidepressant-and/or anxiolytic-like behavior in separate groups of ethanol-dependent and non-dependent animals and naïve controls (n=7–8/group). Ethanol self-administration significantly reduced $V_{1b}R$ levels in the basolateral amygdala by 43% without effecting receptor levels in the other four brain regions studied (Figure 3A, $F_{2, 21} = 6.421$; $q = 4.201$, $p < 0.01$ non-dependent group vs. naïve controls, Newman-Keuls Multiple Comparison Test). In the ethanol-dependent group, this neuroadaptation was completely reversed, as $V_{1b}R$ levels in dependent animals were up-regulated near levels observed in naïve animals ($q = 4.597$, $p < 0.05$ non-dependent vs. dependent group, Newman-Keuls Multiple Comparison Test).

Discussion

The transition to ethanol dependence can be thought of as advancement along a timeline, where initial alcohol use is associated with and maintained by positive reinforcement mechanisms. Some individuals go on to escalate their drinking behavior, and at such a point consumption may be driven more so by negative reinforcement, whereby individuals attempt to ameliorate negative withdrawal symptoms via excessive alcohol use (Gilpin *et al*., 2008). Such a conceptualization posits that distinct stages/levels of drinking may be accompanied by incremental neuroadaptations that underlie these states (Heilig and Koob, 2007; Edwards and Koob, 2010).

In the present study, we used an animal model of ethanol dependence that recapitulates many of the symptoms of alcoholism (Gilpin *et al*., 2008). In this paradigm, dependent animals display excessive ethanol self-administration at a time point (6–8 hrs WD) commensurate with increased somatic withdrawal signs and anxiety-like behavior (Roberts *et al*., 2000; O'Dell *et al*., 2004), decreased brain reward thresholds (Schulteis *et al.*, 1995), and diminished BALs (Figure 1). In comparison, non-dependent animals self-administer limited amounts of alcohol, and model the vast majority of human alcohol consumers. We show here that vasopressin $V_{1b}R$ antagonism reduces ethanol self-administration in dependent animals close to levels observed in non-dependent animals (Figure 2). In comparison, ethanol drinking in non-dependent animals was not altered, suggesting a relative potentiation of vasopressin/$V_{1b}R$ signaling after the induction of ethanol dependence. Supporting this hypothesis, although moderate levels of ethanol self-administration in non-dependent animals were associated with reduced $V_{1b}R$ protein levels in the basolateral amygdala compared to ethanol-naïve animals, $V_{1b}R$ levels were seemingly restored in ethanol-dependent animals (Figure 3). Given the proposed role of $V_{1b}R$ signaling in the BLA in mediating both depression- and anxiety-like behaviors (Salome *et al.*, 2006), a reduction in $V_{1b}R$s may contribute to the positive rewarding properties (anxiolytic- or

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antidepressant-like effects) of ethanol self-administration. In ethanol-dependent animals, BLA $V_{1b}R$ levels were up-regulated compared to non-dependent animals, a neuroadaptation that may in fact reflect a switch in motivation for alcohol from positive to negative reinforcement. The precise mechanism for differential $V_{1b}R$ regulation between ethanol-dependent and non-dependent animals is unknown, although there are several candidate possibilities.

Possible mechanisms of $V_{1b}R$ regulation

Post-transcriptional regulation of $V_{1b}R$ expression represents one mechanism by which receptor levels are regulated, and it is estimated that $V_{1b}R$ content is primarily regulated at the translational level (Volpi *et al.*, 2006). The $V_{1b}R$ gene contains several glucocorticoid response elements, and receptor levels in the pituitary are bi-directionally regulated by circulating glucocorticoids (Aguilera and Rabadan-Diehl, 2000). Restraint stress increases $V_{1b}R$ promoter activation and $V_{1b}R$ expression in the pituitary (Volpi *et al.*, 2002). It is also known that post-transcriptional mechanisms play a major role in regulating $V_{1b}R$ levels, including small upstream open reading frames in $V_{1b}R$ mRNA that code for active peptides capable of inhibiting $V_{1b}R$ translation (Rabadan-Diehl *et al.*, 2007). Overactivation of the extracellular signal-regulated kinase (ERK) cascade also drives $V_{1b}R$ gene transcription (Volpi *et al.*, 2006), and this pathway is activated in the amygdala of ethanol-dependent (but not non-dependent) animals during acute withdrawal (Sanna *et al.*, 2002).

Interactions of Vasopressin Levels and Ethanol

In accordance with the receptor-level changes revealed in the present study, there exists evidence for bidirectional changes in vasopressin peptide levels between initial and chronic ethanol exposure. Although acute ethanol is known to reduce systemic vasopressin release, plasma AVP levels are increased in alcoholics (Beard and Sargent, 1979). Hoffmann *et al.* (1990) have further speculated that elevated vasopressin levels could result from the stress of ethanol withdrawal, since in one study plasma AVP was increased only in alcoholics showing symptoms of withdrawal (Eisenhofer *et al*., 1985). Another study revealed that sons of ethanol-dependent fathers are more sensitive to ethanol's ability to blunt the AVP/ACTH psychosocial stress response, possibly heightening the risk for alcohol use disorders in these individuals (Zimmermann *et al*., 2004). Treatment of animals with vasopressin both during and after chronic ethanol administration also leads to a long-term maintenance of tolerance to the hypothermic and sedative effects of ethanol (Hoffman *et al*., 1978; Le *et al*., 1982; Hoffman *et al*., 1990), and this effect was linked to a central V1-subtype of vasopressin receptor (Szabo *et al*., 1988). In contrast, vasopressin-deficient Brattleboro rats fail to develop ethanol tolerance (Pittman *et al*., 1982). Therefore, it is possible that elevated vasopressin release in dependent individuals during withdrawal could foster a negative emotional state that in turn facilitates an escalation in levels of drinking that lead to alleviation of that state.

Recruitment of amygdala stress-related peptide signaling in ethanol dependence

Our results further demonstrate a role for stress-related peptide signaling in the amygdala in ethanol dependence. The effects of $V_{1b}R$ blockade on excessive ethanol self-administration closely resemble inhibition of the CRF_1 receptor system on this behavior (Funk *et al*., 2007). From a clinical perspective, excessive AVP and CRF signaling through V_{1b} and CRF_1 receptors (respectively) may contribute to anxiety and depression, as systemic administration of small-molecule antagonists for these receptors is effective in animal models of these conditions (Hodgson *et al*., 2007). In addition to CRF, AVP also stimulates the hypothalamic-pituitary-adrenal (HPA) axis by its actions on $V_{1b}Rs$ located on anterior pituitary corticotropes (Lolait *et al*., 2007). While CRF is the predominant HPA axis regulator, AVP synergizes with CRF to release adrenocorticotropin hormone (Antoni, 1993),

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although whether this synergy exists centrally (i.e., at extrahypothalamic sites) is unknown. Previous studies have demonstrated a recruitment of CRF in the amygdala as a critical element driving the excessive ethanol intake observed during both acute and protracted withdrawal times (Heilig and Koob, 2007). These studies implicated enhanced CRF signaling in discrete amygdala nuclei, including the central (Lack *et al*., 2005; Funk *et al*., 2007) and basolateral (Sommer *et al*., 2008) nuclei as neuroanatomical substrates driving ethanol dependence. The amygdala is regulated by extrinsic (cortical) and intrinsic (BLA) excitatory projections, and it was recently demonstrated that these connections are modulated in opposite manners by oxytocin and vasopressin (Huber *et al*., 2005), with vasopressin acting to facilitate BLA-CEA communication. Accordingly, either in addition to or in cooperation with CRF, vasopressin activity may represent another mechanism whereby negative reinforcement mechanisms regulate ethanol dependence. Finally, one study found that SSR149415 exhibited significant antagonism at human recombinant oxytocin receptors (OTRs, Griffante *et al*., 2005). How oxytocin regulates ethanol drinking is unknown, while the effects of this neuropeptide on affective-like behaviors are equivocal. For example, although OTR stimulation inhibits the HPA axis (Neumann *et al*., 2000), intra-amygdala microinjection of an OTR antagonist produces antidepressant-like effects (Ebner *et al*., 2005). Thus, future studies to determine the specific roles of these closely related peptides are needed.

Conclusion

In the present study, we demonstrated that limited ethanol self-administration causes a reduction in V_{1b} receptors, a neuroadaptation that was seemingly reversed in ethanol-dependent animals. Further, V_{1b}R antagonism specifically blocked excessive drinking levels in ethanol-dependent animals. Presumably, an ideal therapeutic for alcoholism would not affect either recreational (i.e. non-dependent) drinking or motivation for natural rewards. Given the role of the AVP/V_{1b}R system in anxiety- and stress-related behaviors, reductions in V_{1b} receptors in the basolateral amygdala may be involved in the natural, positive reinforcing effects of alcohol. The restoration of V_{1b} receptor levels in ethanol-dependent animals may in part represent a transitional shift in the motivational state (positive to negative) underlying ethanol self-administration. Importantly, this study extends previous work reporting altered vasopressin signaling in the amygdala of opiate-dependent animals (Zhou *et al.*, 2008), and further supports V_{1b}R antagonism as a valid therapeutic strategy for drug dependence.

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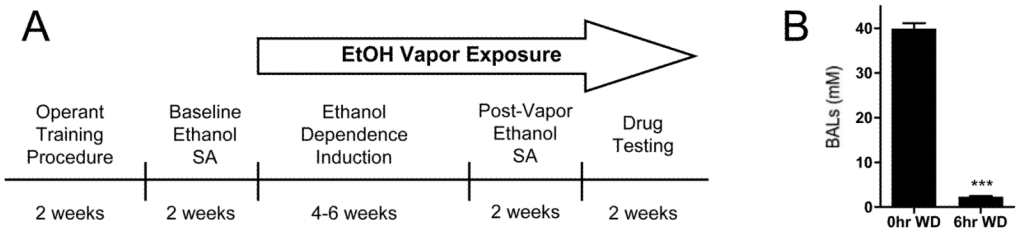

Figure 1.

Experimental timeline and blood alcohol level cycling. (A) Pharmacological testing and time points for tissue collection for Western analysis occurred after approximately 8–10 weeks of chronic intermittent ethanol vapor exposure. (B) All analyses were conducted when ethanol-dependent animals were in acute (6hr) withdrawal, a time point coinciding with diminished blood alcohol levels (BALs) and increased ethanol self-administration behavior. Asterisks indicate ***$p<0.001$ significant effect of withdrawal time point.

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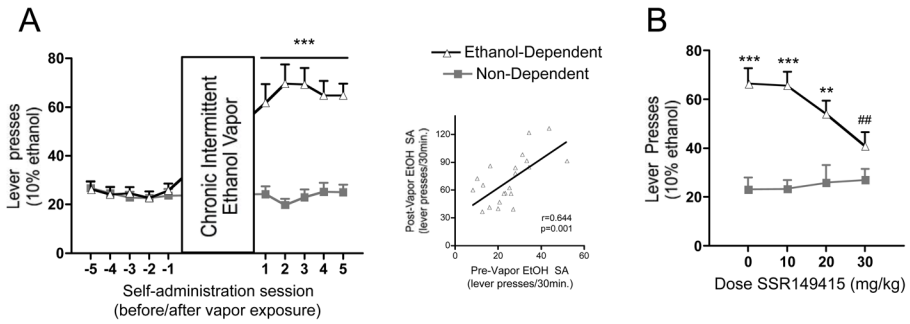

Figure 2.

Ethanol self-administration in ethanol-dependent and non-dependent animals. (A) Induction of ethanol dependence and correlation of limited ethanol self-administration before and excessive drinking after dependence induction following chronic intermittent ethanol vapor exposure. (B) Effects of vasopressin V_{1b}R antagonism on ethanol self-administration, showing a dose-dependent reduction in ethanol drinking in dependent, but not non-dependent, animals. Responses are graphed as mean ± SEM, with correlative data points representing individual averages of the five sessions before vs. five sessions after dependence induction. Asterisks indicate ***$p<0.001$ significant effect of group × test session (A) or ***$p<0.001$, **$p<0.01$ significant effect of group (B). Pound signs indicate ## $p<0.01$ significant effect of dose in ethanol-dependent group.

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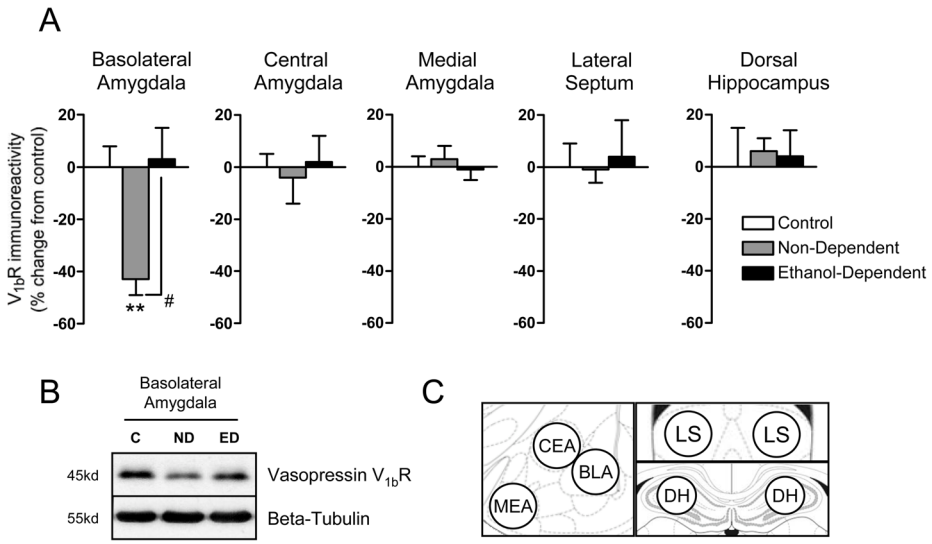

Figure 3.

Regulation of vasopressin $V_{1b}R$ levels by ethanol self-administration. (A) In the basolateral amygdala, $V_{1b}R$ immunoreactivity is significantly reduced in the non-dependent ethanol self-administering group (versus naïve control animals) and seemingly restored in ethanol-dependent animals. (B) Representative immunoblot showing changes in $V_{1b}R$ immunoreactivity with no change in beta-tubulin levels. (C) Diagram of regions collected by the slice/punch technique and analyzed by Western blot. MEA (medial nucleus of the amygdala), CEA (central nucleus of the amygdala), BLA (basolateral nucleus of the amygdala), LS (lateral septum), DH (dorsal hippocampus). Immunoreactivity (% change from naïve control group) is graphed as mean ± SEM. Asterisks indicate **$p<0.01$ significant decrease in $V_{1b}R$ immunoreactivity in non-dependent group vs. naïve controls. Pound sign indicates $p<0.05$ significant increase in $V_{1b}R$ immunoreactivity in ethanol-dependent vs. non-dependent group.

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Table 1

Water Responses

Group	SSR 149415 (mg/kg)			
	Vehicle	10	20	30
Ethanol-Dependent	13.8 ± 4.4	11.3 ± 3.0	12.5 ± 4.0	13.1 ± 3.7
Non-Dependent	11.5 ± 5.3	13.1 ± 5.1	15.5 ± 7.7	12.1 ± 6.1