The role of CRF type-1 receptors in the VTA and DRN in excessive alcohol drinking in rats and mice.

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Abstract

The dysregulation of brain stress systems, specifically extrahypothalamic action of corticotropin-releasing factor (CRF) via the type-1 receptor (CRF-R1), is thought to mediate escalated, alcohol drinking. The current study modeled excessive drinking in rodents by offering adult male Long-Evans rats and C57BL/6J mice a choice of 20% ethanol or water for 24 hours intermittently throughout the week. After escalated baseline drinking was established, animals received several doses of a CRF-R1 antagonist into either the ventral tegmental area (VTA) or the dorsal raphé nucleus (DRN) aiming to decrease alcohol drinking. CP-154,526 selectively reduced ethanol drinking in the VTA in all animals whereas only the high alcohol preferring animals, the mice and high-preferring rats, showed this decrease in the DRN. Since CP-154,526 reduced water intake in the DRN, we demonstrated a differentiation between brain sites in how selective CRF-R1 antagonism can act to suppress elevated ethanol drinking in some individuals but not others.

Key words: ethanol, intermittent access, CRF-R1, VTA, DRN

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VI. Figures and Table

The role of CRF-R1 in the VTA and DRN in excessive alcohol drinking in rats and mice.

Alcohol dependence is an addictive disorder that includes neuropsychological compulsivity absent in social alcohol use. The Diagnostic and Statistical Manual for Mental Disorders (DSM-IVR) defines alcohol dependence as alcohol abuse – the repeated use despite recurrent adverse consequences – combined with tolerance, withdrawal and an uncontrollable drive to drink (VandenBos, 2006). The World Health Organization has a similar definition, though uses the term alcohol dependence syndrome rather than alcoholism (WHO, 2011). The harmful use of alcohol has dire costs both to the individual consumer and to society. Alcohol abuse results in 2.5 million deaths worldwide per year (WHO, 2011). Key questions regarding alcoholism are why do some susceptible individuals undergo a transition from casual alcohol drinking to compulsive patterns of alcohol abuse, and why do alcoholics find it so difficult to stop using alcohol (Edwards, 1981)?

Current theories driving alcohol dependence

To address these questions requires consideration of how use of abusive substances is influenced by neural systems. Much research on the transition to dependence has targeted brain systems that mediate the rewarding effects of potentially addictive drugs and how these brain systems are changed by drug use. A leading proposal posits that addictive drugs usurp neural circuitry normally controlling pleasure, incentive motivation, and learning (Wise, 1989; Robbins and Everitt, 1996; Berridge and Robinson, 1998; Di Chiara, 1999; Kelley, 1999; Hyman and Malenka, 2001; Kelley and Berridge, 2002). These neural circuits, often referred to as brain reward circuitry, include dopamine (DA) projections from the ventral tegmental area (VTA) and substantia nigra to the ventral nucleus accumbens (NAcc) and striatum, as well as glutamate

inputs from the prefrontal cortex (PFC), amygdala, olfactory tubercle, septal area and hippocampus (Wise, 1989; Koob, 1992; Ikemoto, 2007).

This brain circuitry may have evolved to regulate behavior that is beneficial for survival – i.e. consumption of nutrients and water, reproduction, and safety – with psychologically rewarding properties. These circuits can also be stimulated by unnatural rewards like drugs of abuse (Kelley and Berridge, 2002). However, substances like alcohol not only engage these brain reward systems, often more potently than natural rewards, but they can also change them (Robinson and Berridge, 2003). Persistent neuroadaptations in NAcc-related circuitry after repeated drug taking have been found at molecular, cellular, and neural system levels (Nestler et al., 1993; Robinson and Berridge, 1993, 2000; Vanderschuren and Kalivas, 2000; Hyman and Malenka, 2001; Everitt and Wolf, 2002; De Vries and Shippenberg, 2002). One working hypothesis is that these alcohol-induced neuroadaptations are critical in the transition to dependence (Koob, 2003; Krystal et al., 2003; Siggins et al., 2005). However, it is not well understood which psychological functions are changed as a consequence of these alcohol-induced neuroadaptations, or how those changes lead to alcoholism.

The traditional view is that drugs are initially taken because they are pleasant, but with repeated drug use, homeostatic neuroadaptations lead to tolerance and dependence, such that unpleasant withdrawal symptoms ensue upon the cessation of use (Koob and Le Moal, 1997). Drug taking is then maintained, according to this framework, to avoid aversive withdrawal symptoms. The opponent processes model (Solomon and Corbit, 1973; Solomon, 1980) characterizes the drug addiction cycle as consisting of three components: preoccupation-anticipation, binge-intoxication, and withdrawal-negative affect (Koob and Le Moal, 1997). In some cases, a resulting descent into psychological distress occurs, where an initial failure of self-

regulation leads to emotional distress, setting the foundation for additional cycles of repeated self-regulatory failures each with augmenting negative affect (Baumeister et al., 1994). Several hypotheses involving the opponent processes posit the same logic that addictive drugs are taken initially simply to achieve enjoyable drug "highs," and throughout the transition to addiction, to escape withdrawal "lows" (Wikler, 1948; Solomon, 1980; Koob et al., 1997; Koob and Le Moal, 1997, 2001).

Solomon's opponent-process theory (1980) also identifies several key factors that can strengthen or weaken the opponent "b" / negative reinforcement process. Using distress behavior in ducklings, he deduced the following ways that it can be strengthened: increasing the intensity of the initial stimulus exposure, increasing the duration of the stimulus, or shortening the interstimulus interval. In the case of drug taking, if reinforcing drugs are presented at interstimulus intervals greater than the 'critical decay duration,' then the opponent process will fail to grow (Solomon, 1980). Thus, apt scheduling of intermittent access to alcohol in translational models may wield a large influence on their imposed transition to dependent-like drinking.

Animal models of alcohol abuse

Early stages of excessive alcohol use are characterized by impulsive drinking to intoxication in binge-like episodes (Hasin et al., 2001; Bonomo et al., 2004). Escalated alcohol drinking tends to be highly episodic in both humans and animals (Breese et al., 2005a). Intermittent access to alcohol represents a repeated cycling of high ethanol consumption followed by deprivation, which usually engenders more ethanol intake over time (Sinclair and Senter, 1968). In other words, there are "on" drug-taking periods with subsequent "off" drug abstinence periods.

One classic theory for how alcohol dependence develops is the kindling hypothesis (Ballenger and Post, 1978). The term kindling was modeled after the increasingly heightened responses (i.e. sensitization) to repeated electrical brain stimulation (Goddard et al., 1969). In the context of alcohol dependence, each withdrawal-induced episode of CNS hyperexcitability may serve as a stimulus, in a kindling-like process (Ballenger and Post, 1978). A vicious cycle of escalated alcohol drinking and alcohol abstinence/withdrawal may develop over time, where individuals drink increasing amounts to manage escalating bodily stress. An increase in drinking can be due to tolerance and/or physical dependence (Ritzmann and Tabakoff, 1976; Kurtz et al., 1996). Although this theory is generally accepted, we cannot ignore observations in animals that increase their drinking behavior to a maximum (Hwa et al., 2011). This asymptote in drinking behavior suggests that kindled increases in the progression to dependence may be exclusive to symptoms of withdrawal without corresponding increases in alcohol drinking.

A notable portion of the human population drinks alcohol in a binge pattern (Wechsler and Issac, 1992; Wechsler et al., 2000). According to the kindling hypothesis, binge drinking may not initially result in serious, or even noticeable, withdrawal symptoms. However, it is the repeated episodes of this pattern of alcohol intoxication followed by alcohol abstinence or deprivation and withdrawal that may lead to a worsening of future withdrawal-related symptoms (Becker, 1998). Consequently, a kindling process may underlie the commonly observed progression of withdrawal symptoms from relatively mild responses (i.e. irritability and tremors) characteristic of initial withdrawal episodes to more severe symptoms (i.e. seizures) associated with subsequent withdrawal episodes (Ballenger and Post, 1978). According to this model, it is the repeated experience of alcohol withdrawal and “off” drug periods, rather than repeated alcohol exposures, which underlie the progressive intensification of negative symptoms. The

deprivation can increase the likelihood of withdrawal-induced relapse and also increase the amount of alcohol consumed during the relapse episode (Sinclair and Senter, 1968).

This kindling-driven schedule of access to alcohol may generate dependence over repeated on-and-off cycles (Ballenger and Post, 1978), and importantly, the present studies aim to model the phenomenon in rodents. It remains a challenge to induce standard laboratory animals to drink aversive-tasting ethanol solutions without the potentially confounding uses of sucrose fading (Samson, 1986), or food and water deprivation (Meisch and Thompson, 1972). It is also difficult to produce substantially high alcohol drinking when a second fluid choice is given – termed 2-bottle choice. Historically, one-bottle paradigms, or ethanol liquid diet, have been used to create elevated alcohol drinking where an animal would not have a choice of a non-alcoholic drinking fluid (Lieber et al., 1963; Lieber and DeCarli, 1982). To date, ethanol exposure via forced vapor inhalation has been the current prototypical method for rendering rodents dependent on alcohol (Rogers, Wiener & Bloom, 1979; O'Dell et al., 2004; Becker and Lopez, 2004; Roberts et al., 2000; Schulteis et al., 1995). The problem with the aforementioned procedures is the need to incorporate free-choice preferential alcohol drinking.

Therefore, intermittent access to alcohol provides the greatest external validity for comparing excessive alcohol drinking in rodents to the human condition. Intermittent access appears to be a cardinal feature of many conditions that promote drinking large amounts of alcohol (Le Magnen, 1960; Rodd-Henricks et al., 2000; Sinclair and Senter, 1968). As previously described, these episodic presentations of alcohol represent the repeated cycling of ethanol access and deprivation that seem necessary to create dependence. Since the 1970s, studies in rats have demonstrated that intermittent access to ethanol generates higher voluntary ethanol

consumption than continuous access to ethanol (Pinel and Huang, 1976; Wayner and Greenberg, 1972; Wise, 1973; Breese et al., 2005b; Tomie et al. 2006).

More recently, Simms and colleagues (2008) demonstrated the strength of intermittency by giving Long-Evans rats intermittent access to 2-bottle choice 20% ethanol and water, which led to escalated voluntary ethanol intake. In fact, the scheduled Monday, Wednesday, Friday alcohol access over several weeks led to an average alcohol consumption of 6 grams/kilogram (g/kg) in 24 hours (Simms et al., 2008). Though 6 g/kg/24h may not be enough alcohol to induce dependence, this amount has been amongst the highest reported voluntary drinking in non-preferring outbred rats. Since rats were never water restricted and the ethanol solutions were never sweetened, a possible explanation for this relatively high drinking can be the intermittent schedule of access. In the same study, rats genetically bred for high alcohol preference (P rats) consumed around 8 g/kg/24h in the intermittent access procedure. This confirms the principle of intermittency as a likely generator of escalated drinking behavior, even in animals not bred for alcohol preference.

An advantage of using Long-Evans rats is that they represent a common laboratory animal that offers a diverse genetic pool. Herein, we may be able to study individual differences in drinking behavior, which could be practical for approximating the human condition. However, as mentioned earlier, the Long-Evans strain does not prefer the taste of alcohol nor drink large quantities voluntarily. This is a key disadvantage in using this outbred strain as an animal model for the transition to alcohol dependence. Therefore, the present studies include animals that naturally show escalated alcohol drinking, as a serendipitous consequence of their inbreeding is that they do not find the taste of alcohol aversive. This C57BL/6 strain has been used in alcohol

research for more than five decades for their high alcohol preference (Belknap et al., 1993; McClearn and Rodgers, 1959).

Using similar methods that conserved the principle of intermittent access, our laboratory recently translated the procedure from outbred rats to C57BL/6J (B6) mice (Hwa et al., 2011). Therefore, the pairing of the escalating intermittent access schedule with high-preferring mice was shown to generate high alcohol drinking to more than 20 g/kg in 24 hours (Hwa et al., 2011). Since the human equivalent of this amount of consumption would be around 80 standard beers for the average weighing man, we aim to better approximate the transition to dependence with the extraordinary alcohol consumption of these animals in this paradigm. Based on the external validity of this 2-bottle choice paradigm, and the respective advantages for both different strains/species, intermittent access to alcohol in Long-Evans rats and B6 mice will be the method to generate high alcohol drinking for the proposed study.

As earlier discussed, it is highly instructive to develop translational models of excessive alcohol drinking to ultimately understand human alcohol use. With preclinical research, we can more easily identify the underlying neurobiological differences between those who consume alcohol in excess and those who do not (McBride and Li, 1998).

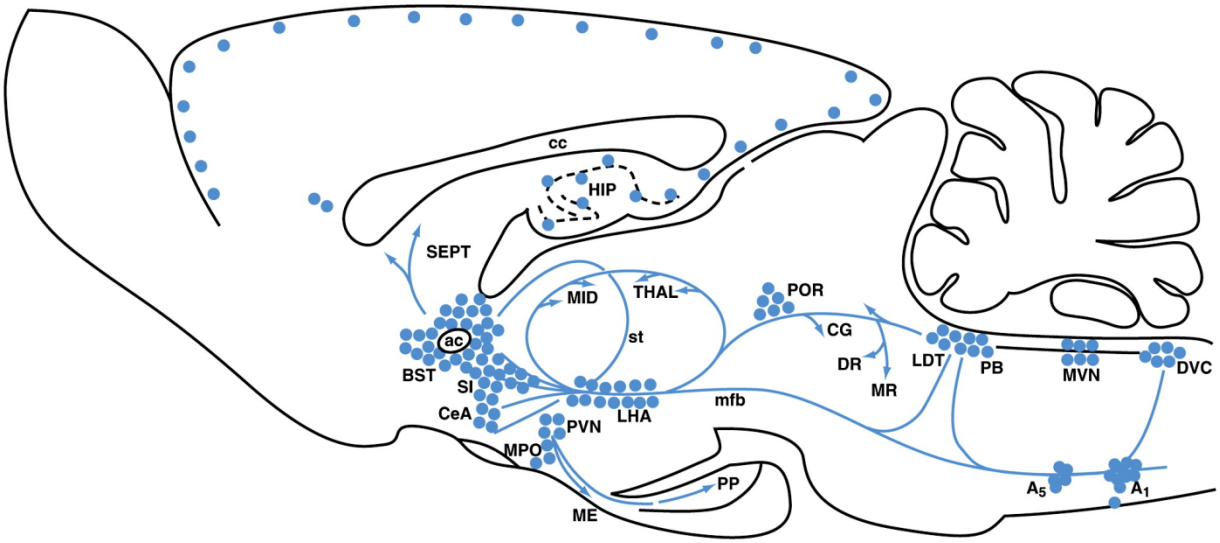
Proposed neural mechanisms for excessive drinking

To study the transition from casual alcohol use to excessive alcohol drinking, we look to behavioral theories rooted in neural mechanisms. Neural substrates of the hedonic opponent process theory have been offered, most prominently by Koob and colleagues (Koob et al., 1997; Koob and Le Moal, 1997, 2001). For example, Koob and Le Moal (1997) suggested the positive reinforcement process is caused by activation of mesolimbic DA projections to the NAcc and amygdala that mediate the acute reinforcing effects of drugs. However, the intensity and duration

of the ‘high’ and inter-drug intervals are additional variables that mediate the neuroadaptative process to dependence. As previously mentioned, Solomon’s theory (1977) describes that each opponent process has an inherent decay behavior, and the opponent ‘b’ process may be hindered depending on the half-life of the neurotransmitters, hormones, or receptor behavior involved. Thus, there may be other contributors in the transition to dependence that should be more promoted in addition to the dominating tenets of the opponent process theory.

During the transition to dependence, repeated drug use induces tolerance or downregulation in the mesolimbic DA system, decreasing the drug positive state (Koob and Le Moal, 1997). Cessation of drug use would cause DA and serotonin (5-HT) neurotransmission to further drop, resulting in a dysphoric negative state of withdrawal. Finally, Koob and colleagues put forward that repeated drug use also activates an additional negative process via the hypothalamic-pituitary axis stress system, causing release of corticotropin releasing factor (CRF) in the amygdala, as well as other stress responses (Koob et al., 1997; Koob and Le Moal, 1997).

CRF orchestrates the stress response in the endocrine, autonomic, immune, and behavioral systems through the activation of the hypothalamic-pituitary-adrenal (HPA) axis and extrahypothalamic pathways (Bourke and Owens, 2010). The peptide is highly conserved across species, and its evolutionary role is to recruit appropriate energies and behavior(s) in response to a stressor. Vale and colleagues first isolated and characterized CRF in 1981. CRF neurons are highly distributed but selectively clustered in the CNS (Schematic below adapted from Koob, 2008).



The major CRF-stained cell groups (dots) and fiber systems in the rat brain. Most of the immunoreactive cells and fibers appear to be associated with systems that regulate the output of the pituitary and the autonomic nervous system and with cortical interneurons. Most of the longer central fibers course either ventrally through the medial forebrain bundle and its caudal extension in the reticular formation, or dorsally through a periventricular system in the thalamus and brainstem central gray. The direction of fibers in these systems is unclear because they appear to interconnect regions that contain CRF-stained cell bodies. Three adjacent CRF-stained cell groups—laterodorsal tegmental nucleus, locus coeruleus, parabrachial nucleus—lie in the dorsal pons. Uncertain is which of these cell groups contributes to each of the pathways shown, and which of them receives inputs from the same pathways. ac, anterior commissure; BST, bed nucleus of the stria terminalis; cc, corpus callosum; CeA, central nucleus of the amygdala; CG, central gray; DR, dorsal raphe; DVC, dorsal vagal complex; HIP, hippocampus; LDT, laterodorsal tegmental nucleus; LHA, lateral hypothalamic area; ME, median eminence; mfb, medial forebrain bundle; MID THAL, midline thalamic nuclei; MPO, medial preoptic area; MR, median raphe; MVN, medial vestibular nucleus; PB, parabrachial nucleus; POR, perioculomotor nucleus; PP, peripeduncular nucleus; PVN, paraventricular nucleus; SEPT, septal region; SI, substantia innominata; st, stria terminalis. Figure and caption from Koob, 2008.

For example, the densest region of CRF is the parvocellular region of the paraventricular nucleus of the hypothalamus that project to the median eminence. There, CRF is released into the portal vessel system supplying the anterior pituitary to initiate the HPA axis endocrine response to stress. Also, large numbers of CRF cell bodies are localized in the central nucleus of the amygdala, the bed nucleus of the stria terminalis (BNST), and the locus coeruleus where CRF influences behavioral and autonomic responses (Owens and Nemeroff, 1991).

Bioavailability of CRF may be largely modulated by CRF-binding protein (Kemp et al., 1998), but it binds exclusively to CRF receptor type-1 (CRF-R1) and CRF receptor type-2 (CRF-R2) to produce its intracellular effects (De Souza, 1995). In situ hybridization in mouse and rat tissue showed CRF-R1 mRNA expression predominately in the cerebral cortex, sensory relay nuclei, and in the cerebellum and its major afferents (Van Pett et al., 2000). CRF-R2 expression was more restricted than that of CRF-R1 including aspects of the olfactory bulb, lateral septum, BNST, ventromedial hypothalamic nucleus, medial and anterior cortical nuclei of the amygdala, ventral hippocampus, mesencephalic raphe nuclei, and localizations in the nucleus of the solitary tract and area postrema (Van Pett et al., 2000).

In clinical studies, circulating CRF levels are often dysregulated in patients suffering from a variety of psychiatric illness including post-traumatic stress disorder (PTSD), early life trauma, and major depressive disorder (MDD) (Nemeroff et al., 1984). Along these lines, there is a large and growing body of clinical and preclinical evidence suggesting an important, complex relationship between stress, anxiety, and alcohol-use disorders (AUDs) (Kushner et al., 2000; Piazza and Le Moal, 1998; Roberts et al., 2000; Weiss et al., 2001). CRF is hypothesized to play a key role in the anxiety-like effects of acute withdrawal, anxiety-like effects of protracted abstinence, and relapse to drug taking during protracted abstinence induced by stressors.

CRF might have important reward-related actions in the VTA because the mesocorticolimbic DA system is implicated in both responsiveness to stress (Deutch et al., 1985, 1991; Thierry et al., 1976) and in the pleasurable effects of drugs of abuse, including ethanol (Di Chiara and Imperato, 1988; Gonzales et al., 2004; Koob, 1992; Koob et al., 1998; McBride et al., 1999; Wise, 1996). Many have studied the important interaction between CRF and DA neurons in the VTA. This interaction was first suggested by the histological studies of Swanson et al.

(1983) and the intracranial injection studies of Kalivas et al. (1987), and has been more recently confirmed by the electrophysiological studies of Ungless et al. (2003) and current microdialysis and anatomical studies (Wise and Morales, 2010). Researchers have identified CRF-DA synapses (Tagliaferro and Morales, 2008) and detected and quantified stress-induced CRF release (Wang et al., 2005, 2007) in the VTA. Specifically, CRF-containing axons and varicosities have been identified in the VTA (Swanson et al., 1983), and CRF showed behavioral actions when administered in the VTA (Kalivas et al., 1987). More recently, the Bonci group showed that the interaction of CRF and DA neurons in the VTA included glutamate-induced excitatory responses on DA neurons (Saal et al., 2003; Ungless et al., 2001; 2003). However important DA may be in the rewarding actions of ethanol, no neurotransmitter acts in isolation. For example, dopamine release can be influenced by and interact with serotonin transmission.

Another important monoamine in the field of alcohol research is the dorsal raphé serotonin (5-HT) system. Since the dorsal raphé nucleus (DRN) contains the largest collection of forebrain-projecting 5-HT neurons (Molliver, 1987; Jacobs and Azmitia, 1992), it is a likely site of 5-HT dysfunction (Heils et al., 1997; Arango et al., 2002; Zalsman et al., 2006). The DRN 5-HT system is a compelling target of CRF given the established role of this system in stress-related psychiatric disorders (Cowen, 1993; Heils et al., 1997; Lesch, 1991; Mann, 1999; Nordstrom and Asberg, 1992; van Praag, 1984). Given the overlap of 5-HT and mood dysregulation, it is valid that 5-HT has been implicated in the regulation of alcohol preference and intake in animals (for review see Higley and Bennett, 1999; McBride and Li, 1998; Naranjo et al., 1986; Sellers et al., 1992) and humans (Gorelick and Paredes, 1992; Underwood et al., 2004; Wong et al., 2003).

Given this significant interaction with 5-HT, CRF has been well-studied in the DRN. CRF-immuno-reactive fibers densely innervate the DRN in a topographically organized manner (Kirby et al., 2000; Sakanaka et al., 1987; Swanson et al., 1983). Whereas CRF1 receptors predominate in most brain regions, the DRN is distinct in its relatively high expression of CRF2 receptors (Chalmers et al., 1995). Because of the neurochemical heterogeneity of the DRN and the presence of multiple CRF receptor subtypes on both 5-HT and non-5HT neurons, the regulation of the DRN-5-HT system by CRF has been difficult to unravel (Valentino, Lucki, & Van Bockstaele, 2010).

The action of how stress influences the DRN-5-HT system and subsequent substance abuse can be through several suggested mechanisms. One means by which this can occur is through CRF1 mediated inhibition of the DRN-5-HT that occurs during acute stress (Price et al., 1998; Price and Lucki, 2001). The hyposerotonergic state that results from CRF1 mediated inhibition could contribute to the initiation of substance abuse, perhaps by promoting impulsive behavior. Price and colleagues (1998; Price and Lucki, 2001; Lukkes et al., 2008) have shown that low doses of CRF, which is more selective for CRF-R1, led to decreased extracellular 5-HT in the forebrain. In contrast, high doses of CRF, which begin to activate CRF-R2, or selective CRF-R2 agonists led to higher concentrations of serotonin (Amat et al., 2004). In line with this hypothesis, serotonergic deficits produced by acute stress and CRF1 activation promote active behavior, and this hyposerotonergic state has been linked to the increased impulsivity that is a component of drug seeking behavior (Virkkunen and Linnoila, 1990). Studies in animals (Branchey et al., 1981; Murphy et al., 1982; Weiss et al., 1996) and humans (Ballenger et al., 1979; Tabakoff, Balat, & Anderson, 1975) have implicated a suppressed serotonergic state with an alcoholic phenotype.

Given the established role of the VTA and DRN pathways in escalated alcohol consumption, neuropeptides that regulate both neural systems are compelling targets for stress-related modulations of alcohol drinking. Furthermore, both pathways are seemingly connected; the DRN 5-HT system regulates impulse flow in DA neurons and also DA discharge in terminal regions (Bubar and Cunningham, 2008; Di Matteo et al., 2008; Weiss et al., 2001). The multiplicity of 5-HT receptors and their presence on different neurons within reward circuitry suggests a complex regulation of the DA release that ultimately drives alcohol-seeking behavior (Valentino, Lucki, & Van Bockstaele, 2010). Most 5-HT receptor subtypes including 5-HT1A, 5-HT1B, 5-HT2A, 5-HT3 and 5-HT4 have excitatory effects on DA release in the NAcc and/or discharge activity of DA neurons in the VTA that project to the NAcc (Di Matteo et al., 2008). In contrast, 5-HT2C receptors exert tonic inhibitory control over DA release in the NAcc (Bubar and Cunningham, 2008; Leggio et al., 2009). Other drugs of abuse, like cocaine, increase 5-HT and DA in the NAcc (Andrews and Lucki, 2001; Parsons et al., 1995).

Other than expression in the VTA and DRN, CRF is highly concentrated in the hypothalamus, brainstem, and amygdala (Swanson et al., 1983). Both acute and chronic ethanol exposures notably activate central CRF pathways (Koob et al., 1993; Rasmussen et al., 2000; Rivier et al., 1984). Increased levels of CRF are observed in the amygdala during ethanol withdrawal (Merlo-Pich et al., 1995), and the anxiogenic effects of ethanol withdrawal in dependent rats can potently be reversed by CRF receptor antagonists (Breese et al., 2004; Knapp et al., 2004; Overstreet et al., 2004; Rassnick et al., 1993).

CRF-R1 antagonists and alcohol drinking

Recent pharmacological and genetic evidence support the hypothesis that CRF exerts its effects on ethanol consumption through activation of the CRF-R1, rather than through action of

the CRF-R2 or CRF binding protein. In addition to lessening the stress-related symptoms of acute ethanol withdrawal (Baldwin et al., 1991; Valdez et al., 2003), CRF-R1 antagonists have shown to reduce voluntary alcohol drinking long after forced alcohol exposure (Rimondini et al., 2002; Valdez et al., 2002; Gehlert et al., 2007). A prominent feature of all CRF-R1 antagonists tested to date (Heilig and Koob, 2007) is their ability to selectively diminish excessive rates of conditioned response behavior for alcohol reinforcement, or to block excessive two-bottle free-choice alcohol consumption in post-dependent animals (Funk et al., 2007, Gehlert et al., 2007).

For example, systemic injections of selective non-peptide CRF-R1 antagonist, MTIP, decreased lever presses for ethanol reinforcement in ethanol-dependent rats to non-dependent levels of consumption, while rats without a history of dependence were importantly not affected by any drug dose (Gehlert et al., 2007). Recently, Sparta and colleagues (2009) also dose-dependently reduced operant responding for ethanol with systemic injections of the prototypic CRF-R1 antagonist CP-154,526 (Schultz et al., 1996) in C57BL/6J mice that had undergone 4 days of alcohol deprivation. Given the wealth of preclinical evidence, several research groups continue to encourage CRF-R1 antagonists as pharmacological treatments for alcoholism (for recent reviews, see Heilig and Koob, 2007; Lowery and Thiele, 2010).

Hypotheses

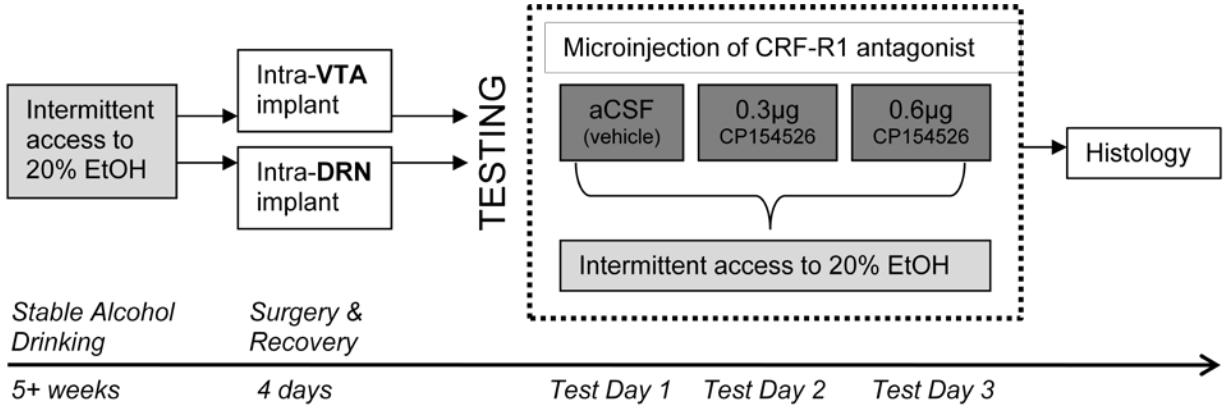
Altogether, observations like these suggest that central CRF receptor signaling modulates increased ethanol drinking in dependent-like animals, and thus makes CRF a promising target in the modulation of escalated, intermittent alcohol drinking. The current study aimed to distinguish high alcohol-consuming individuals from low alcohol-consuming individuals through neurobiological manipulations in C57BL/6J mice and Long-Evans rats given intermittent access to ethanol. By using two species that display different preferences for ethanol, we were able to

provide some insight as to how CRF acts in non-dependent versus dependent-like excessive drinking. Since both dopamine and serotonin have been suggested to affect alcohol drinking, we investigated action of CRF in the two different cell populations. We hypothesized that intermittent alcohol drinking will be differentially affected by CRF-R1 antagonism of the dopaminergic neurons in the VTA compared to CRF-R1 antagonism of serotonergic action in the DRN. Additionally, we were able to explore differences in how prominent the role of CRF-R1 antagonism is in the intermittent access procedure across species, given the natural differences in ethanol preference in outbred Long-Evans rats and inbred C57BL/6J mice.

Methods

Study Design

Stable alcohol drinking was established in adult male Long-Evans rats and C57BL/6J mice given intermittent access to 20% ethanol without sweeteners after at least 4 weeks. Subjects were randomly divided into groups for stereotaxic cannula implants targeting either the VTA or DRN. In a within-subjects design, individuals underwent 3 microinjections total (aCSF, 0.3 µg, and 0.6 µg CP-154,526) on 3 consecutive ethanol drinking test days with at least 48 hours apart according to Simms and colleagues (2008) intermittent access protocol for rats and Hwa and colleagues (2011) protocol for mice. All animals received microinfusions of aCSF on the first test day to control for vehicle effects. On the later test days, drug doses were counterbalanced. Ethanol intake (g/kg), volume of ethanol and water consumption (ml) and ethanol preference (%) were measured 2 hours and 24 hours after microinfusion. A summary of overall study design is illustrated below.



Animals and Housing

Adult, male Long-Evans rats (Charles River, Wilmington, MA) weighing 225-250 g upon arrival were preliminarily group-housed in large plastic cages (8 x 10 x 18.5 in.) with wire mesh lids and pine shavings as bedding. Ethanol-naive animals were given 1 week to habituate to the vivarium conditions on a 12-hour reversed light/dark cycle (lights off at 8am) and maintained temperature (70±5ºF) and humidity (25%). Adult, male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME), aged 8 weeks old upon arrival, were also initially group-housed in a similar manner. Mouse vivarium environmental conditions were identical, except the 12-hour reversed light/dark cycle had lights off at 7am.

After initial acclimatization, animals were individually housed for the duration of the study. The rat homecages were custom-made, cube-like, Plexiglas chambers (25 x 25 x 30 cm) lined with Cellu-Dri^{TM} pellet bedding (Shepherd Specialty Papers, Kalamazoo, MI). Homecage chambers had interchangeable side panels, so the surrounding sides consisted of a rear panel with ventilating wire mesh, 2 non-ventilated side panels to minimize contact with neighboring cages, and a front panel that included 2 angled holes for drinking spouts. Mouse homecages were polycarbonate cages (28 x 17 x 12 cm) with stainless steel wire mesh lids and pine shavings covering the floor. Importantly, rodent chow (LabDiet 5001 Rodent diet) and water were

available *ad libitum*. All proposed procedures were approved by the Tufts University Institutional Animal Care and Use Committee and were in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*.

Ethanol Intake Procedures

Ethanol solutions (w/v) were prepared in tap water from 95% ethyl alcohol (Pharmaco-AAPER, Brookfield, CT). Fluids were presented to rats in 100-ml graduated plastic cylinders with no. 6 rubber stoppers containing stainless steel ball-bearing sippers. Graduated cylinders were inserted through the 2 drinking spouts in the homecage and secured with stainless steel springs 3 hours into the dark cycle. For mice, fluids were presented in 50-ml plastic centrifuge tubes (Nalgene) with no. 5 rubber stoppers (Fisher Scientific, Agawam, MA). Centrifuge tubes were securely held through the wire mesh cage lid and presented to mice 3 hours into the dark cycle. Peak fluid consumption in outbred mice (Goldstein and Kakihana, 1977) and one-bottle 20% ethanol consumption in C57BL/6J mice has been shown to be at this time of day compared to earlier or later into the dark cycle (Rhodes et al., 2005).

To assess fluid consumption, we measured differences in bottle weights before and after 2-bottle choice to ethanol and water. Bottles were weighed to the nearest hundredth of a gram 24 hours after the fluids were given. To control for extraneous spillage due to experimenter handling or evaporation, weekly “drip” averages (loss of fluid in cage with no animal present) were subtracted from individual fluid intakes.

On test days, 2-hour fluid intake was measured in addition to 24-hour intake. Although animals were given 24-hour total access, it was informative to monitor the initial binge-like drinking during the first hours of alcohol access after the 24-hour deprivation. Also, acute 2-hour measurements allowed us to make drug comparisons influenced by shorter-acting

microinjections that were not able to be observed after the total 24 hours. Differences in volume for rats were visually assessed on the graded graduated cylinders to the nearest 0.5 of a milliliter. 2-hour bottle weights were assessed for mice. Rats and mice were weighed before every ethanol drinking session to calculate the grams of ethanol intake per kilogram of body weight. To determine the ethanol preference (%), ethanol intake (ml) was divided by total fluid intake (ml).

Intermittent-Access 20% Ethanol 2-Bottle Choice Drinking Paradigm

Rats were given intermittent access to 20% ethanol without sweeteners in a recently revived drinking paradigm (Simms et al., 2008) established by Wise (1973). Mice were given intermittent access to 20% ethanol in a similar procedure adapted by our laboratory (Hwa et al., 2011). Every Monday, Wednesday, and Friday, animals were presented with one bottle of 20% ethanol and one bottle of water for 24 hours, 3 hours into the dark cycle (10am). At the same hour the following day, bottles were removed, weighed, and the ethanol solution in one bottle was thoroughly washed out and replaced by water. The two water bottles remained in place until the next alcohol drinking session. Ethanol bottle placement – left side vs. right side – was alternated each ethanol drinking session to avoid side preferences.

Stereotaxic Surgery

After at least 4 weeks of stable alcohol drinking (less than 15% variability between three alcohol sessions per individual), animals underwent stereotaxic surgery for permanently indwelling cannulae in either the VTA or DRN. Rats weighed approximately 400-500 grams at the time of surgery while mice weighed 25-30 grams. A combination of ketamine (100 mg/ml) and xylazine (6 mg/ml) combination and carprofen (5 mg/kg) were administered to rats as anesthetic (1 ml/kg, i.p.) and analgesic (1 ml/kg, s.c.), respectively, preceding surgical procedures on stereotaxic frame (Kopf Instruments, Tujunga, CA). A less concentrated ketamine (10 mg/ml) and xylazine

(1 mg/ml) combination and carprofen (1 mg/ml) were administered to mice as anesthetic (10 ml/kg, i.p.) and analgesic (10 ml/kg, s.c.), respectively, preceding surgery.

Rats will be randomly divided into two groups and will be either bilaterally implanted with 11 mm, 26 ga. guide cannulae (Plastics One, Roanoke, VA) aimed at the VTA (AP-5.2, ML+1.8, DV-7.5mm from bregma) at a 10º angle, or unilaterally aimed at the DRN (AP-7.5, ML+3.2, DV-5.8mm from bregma) at a 28º angle (Franklin & Paxinos, 1986). Dental cement (Jet Acrylic, Lang Dental, Wheeling, IL) and two 0.5 mm tapered screws (Small Parts, Lexington, KY) anchored cannulae permanently to the skull surface. Obdurators were fitted at a depth of 0.5 mm beyond the guide cannulae immediately after surgery and will remain in place except during testing.

Mice were also implanted with unilateral 6 mm, 26 ga. guide cannulae (Plastics One, Roanoke, VA) aimed at the DRN (AP-4.6, ML+0.5, DV-1.9mm from bregma) at a 26° angle. Injectors protruded 0.5 mm beyond the guide cannula, and fitted obdurators prevented debris from clogging the cannula for the duration of experiments other than test time. A dual cannulae system was used to bilaterally target the mouse VTA (Plastics One, Roanoke, VA) with no angle from bregma (AP-3.2, ML±0.5, DV-4.5mm). VTA dual injectors protruded 0.1mm past the cannula, and dummy injectors and dust caps remained in place other than testing.

Animals were allowed 3-8 days of recovery before resuming the intermittent access to 20% ethanol drinking protocol. In these drinking sessions before testing, obdurators were handled before fluid presentation to habituate animals to testing conditions.

Intracerebral CRF-R1 manipulation of intermittent ethanol drinking

On test days, 0.3 µg or 0.6 µg of CRF-R1 antagonist CP-154,536 (Tocris, Ellisville, MO) was freshly suspended in 0.5 ml artificial cerebral spinal fluid (aCSF) vehicle. For all animals,

injector(s) were connected via flared polyethylene tubing (size P50, CMA Microdialysis, North Chelmsford, MA) to a glass syringe controlled by an automatic CMA/100 microinjection pump (CMA Microdialysis, Sweden). For rats, a 33 ga., 11 mm injector(s) microinfused a 0.5 µl volume of aCSF, 0.3 µg, or 0.6 µg CP-154,526 at a flow rate of 0.1667 µl/min into target brain sites. For mice, a 33 ga. injector microinfused 0.2 µl volume of 0.3 µg, or 0.6 µg CP-154,526 at a flow rate of 0.1 µl/min into the DRN. Since the mouse VTA sites were bilateral and a small population of neurons, we microinfused 0.1 µl volume on each side for a total of 0.2 µl total at a flow rate of 0.075 µl/min. Animals were able to move about freely during the infusion process. After, injectors remained in place for 1 min to allow for diffusion and to minimize vertical action up the cannula. 9 min later (total of 10 min after drug administration), the alcohol and water bottles were given to animals to assess CRF-R1 manipulation of alcohol drinking in the VTA and DRN.

Drugs

CP-154,526 [butyl-(2,5-dimethyl-7-[2,4,6-trimethylphenyl]-7H-pyrrolo[2,3-d]pyrimidin-4-yl)-ethylamine] (Tocris, Ellisville, MO) was freshly suspended in a vehicle of artificial cerebral spinal fluid (aCSF). CP-154,526 displays high affinity for the CRF-1 receptor (K_i < 10 nM) and blocks CRF-stimulated adenylate cyclase activity in rodent pituitary and cortical membranes (Lundkvist et al., 1996; Schulz et al., 1996). Peripheral administration of CP-154,526 crosses the blood–brain barrier and reaches peak brain concentrations 20 minutes after administration with significant levels of the drug observed in the cortex, striatum, cerebellum, and hippocampus (Keller et al., 2002). Importantly, previous research found that systemic administration of a 10 mg/kg dose of CP-154,526 effectively reduced anxiety-like behavior in mice (Griebel et al., 1998).

Histology

After testing, rats were deeply anesthetized with an injection (1 ml/kg, i.p.) of sodium pentobarbital (100 mg/ml). Mice received a lethal dose (0.5 ml, i.p.) of the ketamine/xylazine combination (10 mg/ml ketamine, 1 mg/ml xylazine). Animals were intracardiacally perfused with 0.9% saline and 4% paraformaldehyde, so brains could be removed. Tissue was allowed to further solidify by being submerged in 4% paraformaldehyde for at least 2 days. Brains were later frozen and sliced in 60 µm coronal sections using a Cryostat (Leica CM1900, Bannockburn, IL). Afterward, slices were mounted on gelatin-coated slides, dried, and finally Nissl stained to check placement of guide cannulae. Animals with incorrect placements into target sites (VTA required two correct placements; DRN required one correct placement) were excluded from analysis.

Statistical Analyses

All statistical analyses were performed using SigmaStat version 3.1 (Systat Software, San Jose, CA). Ethanol consumption (g/kg), volume of ethanol intake (ml) and water intake (ml) after microinjection of CP-154,526 doses were analyzed using one-way analysis of variance (ANOVA) with repeated measures, followed by Bonferroni post hoc analyses if significant effects were found ($p < .05$). Since the rats showed large differences in fluid preference, we chose to analyze ethanol drinking separate from water drinking in all tests. Due to the inherently large differences between the Long-Evans rats and B6 mice baseline drinking, data for the two species were analyzed separately. Brain sites were also analyzed separately for all statistical tests. Fluid consumption, both (g/kg) and (ml) were assessed 2 hours after microinjection and 24 hours after microinjection.

Due to the large individual differences in baseline ethanol drinking in the outbred rats, 2-hour fluid intake (ml) and 24-hour fluid intake was converted to ethanol preference (%), defined as the volume of ethanol consumed (ml) divided by total volume consumed (ml) multiplied by 100. A change in ethanol preference due to the highest dose of CP-154,526 was calculated based on percent change from ethanol preference after vehicle microinfusion. This allowed for greater comparisons with dissimilar baseline values. As well, two-way ANOVA with repeated measures, and additional Bonferroni post hoc tests if necessary, were as used to assess differences in percent change from baseline consumption between the target brain sites and between high ethanol-preferring rats and low ethanol-preferring rats.

Results

Acquisition of 20% ethanol drinking under intermittent access conditions

Long-Evans rats

A total of 24 Long-Evans rats were given a 24-hour, 2-bottle choice of 20% ethanol or water on a fixed Monday, Wednesday, Friday schedule for at least 4 weeks. Ethanol intakes (g/kg) steadily increased over time, and after approximately 20 ethanol drinking sessions, mean ethanol consumption averaged 5.2 ± 1.2 g/kg/24h [Figure 1]. Of the 24 total rats, 10 received VTA implants, and 14 received DRN implants. One rat in the VTA group and 5 rats in the DRN group were eliminated due to missed cannula placements [Figure 6a]. Correct placement in targeted brain sites have been depicted in Figure 6b.

After recovery from stereotaxic surgery, animals maintained stable drinking levels. Long-Evans rats showed large individual differences for ethanol preference. Although the average 24-hour ethanol preference after vehicle infusion was 25% ± 4%, individuals ranged from exhibiting

high preference (64%) to low preference (7%) [Figure 2]. Similarly, for the initial 2-hour 2-bottle choice access, rats also displayed differences between high (36%) and low (4%) preferences during this time period. A small proportion of individual rats were characterized as high-preferring animals, since 2-hour ethanol preference positively correlated with 24-hour ethanol preference [$r^2 = 0.56$; Figure 2]. The high-preferring group (VTA n=3, DRN n=2) showed at least 25% preference during both the initial 2-hour access period and the 24-hour access period. Together, this high-preferring subgroup (n=5) demonstrated 45% ± 5% ethanol preference compared to lower-preferring rats (n=13) which exhibited 18% ± 3% preference over 24-hours. This high-preferring minority also showed increased ethanol drinking behavior (31% ± 2% preference) during the initial 2-hour access versus the lower-preferring majority (17% ± 2% preference).

C57BL/6J Mice

A total of 24 male C57BL/6J mice were given 24-hour 2-bottle choice to ethanol and water in the same weekly intermittent access schedule as the rats. These mice acquired high ethanol drinking levels rapidly over the first week and maintained escalated ethanol consumption for the remainder of experimentation at 24.3 ± 0.7 g/kg/24h [Figure 1]. Individual mice from this inbred strain showed little variation in drinking behavior. Mice also maintained a high ethanol preference at 65% ± 2% [not shown]. Of the 24 total mice, 12 received VTA implants, and 12 received DRN implants. 2 VTA mice did not survive the cannulation surgery. 2 DRN mice died before implantation, and another 2 DRN mice had missed placements [Figure 7a]. Photomicrographs of correct placements are shown in Figure 7b.

Intra-VTA modulation of ethanol drinking

Long-Evans rats

A group of rats (n=9) were microinjected into the VTA with the prototypic CRF-R1 antagonist, and fluid intake was measured at 2 time points. A one-way ANOVA with repeated measures indicated that there was a dose-dependent attenuation of ethanol drinking (g/kg) in the first 2 hours after drug infusion [$F(2,8) = 22.37$, $p < .001$, Figure 4a]. Post-hoc Bonferroni tests showed that both doses of CP-154,526 intra-VTA significantly reduced ethanol intake (g/kg) in the 2-hour time frame [aCSF vs. 0.3ug: $t = 4.41$, $p < .001$; aCSF vs. 0.6ug: $t = 6.56$, $p < .001$]. Importantly, microinjections of CP-154,526 did not alter total ethanol consumption (g/kg) overnight, 24 hours after drug infusion [$p = .26$; Table 1].

One-way ANOVA with repeated measures also demonstrated a dose-dependent decrease in the volume of ethanol drinking (ml) [$F(2,8) = 21.94$, $p < .001$; Figure 5a] for rats in the VTA, compared to no significant change in water drinking (ml) [$F(2,8) = 0.67$, $p = .53$; Figure 5a] in the first 2 hours of access to 2-bottle choice. By the 24-hour time point, intra-VTA infusions of CP-154,526 did not alter total ethanol volume consumed (ml) across drug doses [$F(2,9) = 1.69$, $p = .22$, Table 1], though total water consumed (ml) was affected differentially by the different CP-154,526 doses [$F(2,8) = 4.65$, $p < .05$; Table 1]. Further Bonferroni tests revealed that the significant difference in 24-hour water drinking (ml) in rats occurred between the 0.3ug dose and the 0.6ug dose of CP-154,526 [$t = 3.05$, $p < .05$], but these were not different from aCSF vehicle.

Rats with VTA microinjections were previously characterized as high ethanol-preferring individuals or low ethanol-preferring individuals according to baseline ethanol preference ratios after 2-hour access and 24-hour access. A two-way ANOVA with repeated measures (Preference characterization × Drug dose) showed that the highest dose of CP-154,526 significantly reduced ethanol preference in the initial 2-hour access [$F(1,7) = 26.04$, $p < .01$; Figure 3a] by an 85% average reduction. There were no significant differences between the high-preferring group and

the low-preferring group (p = .85) for 2-hour intra-VTA infusions. Though percent change from baseline preference showed a similar reduction overnight, this trend was not statistically significant [Figure 3c]. Still, high-preferring rats receiving CP-154,526 intra-VTA changed an average of 39% ± 26% from baseline high preference over 24 hours whereas low-preferring rats showed an average suppression of 19% ± 13% from baseline low preference over 24 hours [Figure 3c].

C57BL/6J Mice

The group of mice that received intra-VTA microinfusions (n=10) also showed a similar acute dose-dependent suppression of ethanol drinking (g/kg) behavior [$F(2,9) = 17.33$, $p < .001$; Figure 4c] in the initial 2 hours. Both intra-VTA doses significantly decreased ethanol drinking (g/kg) in the mice compared to vehicle [aCSF vs. 0.3ug: $t = 4.60$, $p < .001$; aCSF vs. 0.6ug: $t = 5.48$, $p < .001$]. Different from the rats, intra-VTA CP-154,526 continued to reduce ethanol intake (g/kg/24h) overnight [$F(2,9) = 10.08$, $p < .01$; Table 1], specifically at the highest drug dose [$t = 4.04$, $p < .05$].

CRF-R1 antagonist microinjections into the mouse VTA reduced volume of ethanol consumed (ml) in a similar fashion [$F(2,9) = 17.40$, $p < .001$; Figure 5c]. Post-hoc t-tests confirmed that ethanol intake (ml) was lower after both CP-154,526 doses compared to ethanol intake (ml) after aCSF infusion [aCSF vs. 0.3ug: $t = 4.57$, $p < .001$; aCSF vs. 0.6ug: $t = 5.51$, $p < .001$]. Acute intra-VTA CP-154,526 also reduced water drinking (ml) [$F(2,9) = 6.83$, $p < .01$; Figure 5c], where the moderate drug dose reduced water intake most effectively [$t = 3.66$, $p < .01$]. 24 hours after microinfusion, mice continued to show a reduction in total ethanol consumption (ml) [$F(2,9) = 10.09$, $p < .01$; Table 1]. Ethanol intake after the highest intra-VTA CP-154,526 dose was significantly different from both aCSF [$t = 3.63$, $p < .01$] and the moderate

0.3ug dose [$t = 4.11$, $p < .01$]. Overnight water drinking (ml) in mice was not affected by intra-VTA microinjections of the CRF-R1 antagonist [$p = .34$; Table 1].

Intra-DRN modulation of ethanol drinking

Long-Evans rats

Another group of rats received cannula implants targeting the DRN (n=9). They also were microinjected with the CRF-R1 antagonist to reduce ethanol drinking in the intermittent access schedule. Fluid intake after CP-154,526 microinfusions was assessed after 2 hours and 24 hours of 2-bottle choice access. A one-way ANOVA with repeated measures indicated no significant attenuation of 2-hour ethanol drinking (g/kg) due to microinjections of CRF-R1 antagonist in the DRN [$F(2,8) = 0.20$, $p = .82$; Figure 4b]. Similarly, CP-154,526 did not change ethanol intake (g/kg) overnight during the 24-hour access period either [$F(2,8) = 0.67$, $p = .52$; Table 1].

Comparing the two fluid choices (ml), one-way ANOVA with repeated measures showed no significant drug effect on volume of ethanol intake (ml) in the first 2-hours of fluid access [$F(2,8) = 0.30$, $p > .05$; Figure 5b]. However, CP-154,526 administered in the rat DRN significantly decreased water drinking (ml) during this time period [$F(2,8) = 4.94$, $p < .05$; Figure 5b], specifically at the highest dose compared to vehicle [$t = 3.12$, $p < .05$]. Intra-DRN infusions of CP-154,526 did not alter total 24-hour fluid consumption (ml) [ethanol: $F(2,8) = 0.89$, $p = .43$; water: $F(2,8) = 0.31$, $p = .74$; Table 1.] at any dose in rats.

Rats with DRN microinjections were also previously characterized as high or low ethanol-preferring individuals according to baseline preference ratios after 2-hour access and 24-hour access. An additional two-way ANOVA with repeated measures failed to reveal significant interactions between ethanol preference and percent change in preference due to microinjection

[p = .30; Figure 3b]. There were no significant differences between the high-preferring group and the low-preferring group (p = .33) for 2-hour intra-DRN infusions, though the average change from baseline preference for low-preferring rats was a 15% ± 21% increase. In contrast, the average change from baseline preference for high-preferring rats was a 33% ± 40 % decrease due to intra-DRN drug [Figure 3b]. Percent change from baseline preference showed a similar trend overnight, where response to drug may vary according to baseline ethanol preference, but this trend was not statistically significant [p = .22; Figure 3d].

C57BL/6J mice

A group of mice (n=8) also received intra-DRN microinjections of CP-154,526 in a repeated measures design. In contrast to the lack of change in rats, mice displayed a significant reduction of ethanol drinking (g/kg) behavior in the initial 2 hours [$F(2,7) = 7.04$, $p < .01$; Figure 4d]. Post hoc tests showed a difference in ethanol drinking (g/kg) at the highest dose compared to vehicle [$t = 3.69$, $p < .01$]. However, this reduction of ethanol consumption (g/kg) was not apparent by the 24-hour time point [$F(2,7) = 0.88$, $p = .44$; Table 1].

As shown in the reduction of ethanol drinking behavior, intra-DRN CP-154,526 similarly reduced volume of ethanol consumed (ml) [$F(2,7) = 7.01$, $p < .01$; Figure 5d]. This effect was most apparent at the highest dose compared to vehicle [$t = 3.67$, $p < .01$]. Water drinking (ml) seemed to be attenuated by acute CP-154,526 in the mouse DRN, but this effect was not statistically significant [$F(2,7) = 2.90$, $p = .09$; Figure 5d]. After 24-hour 2-bottle choice, mice showed no significant changes in fluid consumption (ml) due to drug microinjections in the DRN [ethanol: $F(2,7) = 1.01$, $p = 0.39$; water: $F(2,7) = 0.37$, $p = .70$; Table 1].

Discussion

Key findings

In a cross-species comparison, this set of experiments manifested excessive alcohol drinking in animals. Outbred Long-Evans rats given intermittent access to 20% ethanol voluntarily consumed increasing amounts of alcohol over time, though they showed a spectrum of individual differences for ethanol preference. Most rats displayed low to moderate ethanol preference, while a minority consistently displayed high ethanol preference, both in the initial access (‘binge’) to alcohol and in overnight access, mimicking the high ethanol preference in C57BL/6J mice. Overall, all animals were potently affected by CP-154,526 in the VTA whereas only the high alcohol preferring animals, the mice and high-preferring rats, were affected by CRF-R1 antagonism in the DRN. Thus, the data suggest a differentiation between brain sites in how selective CRF-R1 antagonism can act to produce a decrease in elevated ethanol drinking in some individuals but not others.

Utility of cross-species comparisons

Comparative genomics is essential for increasing the external validity of therapeutic drugs. The rat has been the long-standing model organism, primarily because of its larger size and translatable physiological responses to natural stimuli (Boguski, 2002). Likewise, mice are typically used in mammalian genetics and are often the first-pass screen for pharmaceutical development (Dews, 1972). The Human Genome Project named the C57BL/6J mouse and the standard rat as two model organisms to have their genomes initially sequenced, and discovered that 99% of the 30,000 or fewer human genes have a sequence match to these rodents (Boguski, 2002). Still, they are not without significant differences in brain architecture – the ratio of total

cortex to subcortex in the rat is 0.82 while the ratio in mice is only 0.66 (Rosenzweig and Bennett, 1969).

Nevertheless, a proposed ‘triangulation’ strategy (Jacob and Kwitek, 2002) powerfully leverages the advantages of all three organisms – mice, rats, and humans – for studies of human disease. In the case of modeling alcohol dependence, a comparative study showed that man, rat, and mouse all exhibit tremors, tonic-clonic seizures, fatal convulsions, hyperactivity and startle to noise (Mello, 1973). Thus, it is valuable to study excessive drinking in two distinct species, which ultimately brings us closer to understanding the neural mechanisms underlying the alcoholic human condition.

The current intermittent access paradigm aims to translate voluntary, preferential excessive drinking in animals. Under unrestricted access conditions, C57BL/6J (B6) mice have been historically known to voluntarily consume more than 10 g/kg per day of ethanol (Fuller, 1964; McClearn and Rodgers, 1959). In the contemporary intermittent access procedure, B6 mice exhibit consistent escalated drinking at greater than 20 g/kg per day (Hwa et al., 2011). Though significantly less, the current paradigm generated increased daily drinking in outbred rats, approximately 6 g/kg/24h, confirming previous studies (Simms et al., 2008; Wise, 1973).

Unlike the consistently high alcohol-preferring mice, the Long-Evans strain of rat provides us with the opportunity to also study individual differences in intermittent access drinking. Not only did we distinguish certain individuals – via genetic strain or via individual preference – as high ethanol-preferrers or low ethanol-preferrers, but also we observed that CRF-R1 antagonism produced distinct effects on drinking depending on the animal’s ethanol preference predisposition. Thus, the cross-species comparison was most advantageous to confirm that blockade of CRF receptors was able to reduce escalated ethanol drinking in dependent

rodents, but had no effect on moderate levels of ethanol consumption in non-dependent rodents (Chu et al., 2007; Finn et al., 2007; Funk and Koob, 2007; Funk et al., 2007; Funk et al., 2006; Gehlert et al., 2007; Valdez et al., 2002). We hoped to model this dependent-like state with the intermittent access paradigm in the high ethanol-drinking individuals and confirm that CRF-R1 antagonists exert their effects most potently on escalated, dependent-like drinking rather than low-rate, non-dependent drinking.

Selective suppression of ethanol drinking with VTA manipulation

The present investigation found that microinfusion of CRF-R1 antagonist into the VTA selectively decreased ethanol drinking without altering water drinking. A considerable effort has been focused on understanding the impact of drugs of abuse on the mesolimbic dopamine system, in particular the dopamine neurons of the VTA (Borgland et al., 2006), as this site is thought to serve as a common intersection for all drug-seeking behaviors. Ethanol has been shown to excite rat dopaminergic VTA neurons in vivo (Gessa et al., 1985), in brain slices (Brodie, Shefner, & Dunwiddie, 1990) and in an acutely dissociated neuron preparation (Brodie, Pesold, & Appel, 1999). In behavioral experiments, DA antagonists reduce ethanol self-administration (Pfeffer and Samson, 1986), but DA agonists will also reduce self-administration and suppress ethanol-induced hyperlocomotion (Rassnick, Pulvirenti, & Koob, 1993; Carlsson et al., 1974). Additionally, the observation that rats will self-administer ethanol directly into the VTA (Gatto et al., 1994; Rodd et al., 2004) further substantiates that the VTA is a critical site of the action of ethanol on reward circuitry of the brain.

Electrophysiological work to date also suggests an essential role for synaptic plasticity in the VTA in the early behavioral responses following initial drug exposures, as well as in triggering long-term adaptations in regions innervated by DA neurons of the VTA (Kauer, 2004;

Kauer and Malenka, 2007). Abundant evidence (Wolf, 1998; Everitt and Wolf, 2002; Kalivas, 2009) supports the notion that excitatory synaptic function within mesolimbic DA circuits is crucial for the behavioral responses to drugs of abuse, particularly involving the excitatory neurotransmitter glutamate. In a kindling-like process, enhanced excitatory neurotransmission, through influential glutamate-DA interactions in the VTA, may contribute to the exacerbated withdrawal responses resulting from repeated withdrawal experience (Becker, 1998). This, together with the finding that NMDAR blockade specifically within the VTA (but not the NAcc) effectively prevents both behavioral sensitization and conditioned place preference, further supports the idea that NMDAR-dependent processes in the VTA might have a pivotal role in the development of addiction (Aston-Jones and Harris, 2004; Kalivas and Alesdatter, 1993; Harris et al., 2004).

An increased ability of the glutamate system in the VTA to induce bursting activity after repeated drug exposure may contribute to drug seeking and enhance the vulnerability to relapse during ethanol withdrawal (Chergui et al., 1994; Floresco, Todd, & Grace, 2001; Grillner and Mercuri, 2002; Grace, 2000; Schmidt and Pierce, 2006; Tupala and Tiihonen, 2004). In addition to altered glutamate neurotransmission, withdrawal from chronic ethanol leads to substantial decrements in VTA DA neuron activity (Diana, Gessa, & Rossetti, 1992; Shen and Chiodo, 1993) and extracellular NAcc DA levels (Rossetti et al., 1992; Weiss et al., 1996) that can last up to 6 days after ethanol withdrawal (Bailey et al., 2001). This research suggests that chronic ethanol exposure causes mesolimbic DA hypofunction, a condition hypothesized to be vital for maintenance of addiction: less extrasynaptic DA generates increased ethanol intake in order to compensate for its decreased efficacy on DA release, and by motivating relapse during withdrawal to reverse DA deficits (Weiss and Porrino, 2002).

Although the brain circuitry underlying addiction is complex, it is undisputed that the mesolimbic dopamine system, as well as associated limbic structures, is a critical substrate for the neuronal adaptations that underlie addictions. We have known for several decades that alcohol-induced DA release within the NAcc is critically involved in the initiation of alcohol reinforcement processes (DiChiara and Imperato, 1988; Spanagel and Weiss, 1999). The present study in rats and mice fully supports the crucial role of the VTA in voluntary ethanol drinking. Also, this set of experiments adds to previous findings that CRF potently influences DA neurons in the VTA, which increases rats' likelihood for drug relapse (Wang et al., 2005). However, the finding that 6-OHDA-induced lesion of the mesolimbic tract failed to alter voluntary self-administration in rats suggests a less central role of DA in maintaining alcohol consumption (Rassnick, Stinus, & Koob, 1993). Therefore, other neurotransmitter networks, such as 5-HT, may also contribute to the maintenance of intermittent ethanol drinking.

Reduction of fluid consumption with DRN manipulation

We found that CP-154,526 infusion in the DRN of the rat produced no significant change in ethanol drinking and instead, an immediate decrease of water drinking. Because of the neurochemical distinctiveness of the numerous DRN nuclei and both CRF-R1 and CRF-R2 on 5-HT and non-5-HT neurons (O'Hearn and Molliver, 1984; Waterhouse et al., 1986; Jacobs, 1987; Molliver, 1987; Simpson, Waterhouse, & Lin, 2003), the regulation of the DRN-5-HT system by CRF has been difficult to disentangle (recently reviewed by Valentino et al., 2010), as evidenced in the current study. A significant element to consider is that the CRF-R2 subtypes are more abundant than CRF-R1 in the DRN, adding an additional layer of complexity.

A prominent theory put forth by Valentino and colleagues (2005) has suggested a scheme whereby CRF-R1 and CRF-R2 regulate the DRN-5-HT system in opposing manners. During

acute stress, CRF acts on CRF-R1, causing neuronal inhibition and decreased 5-HT release. With increasing CRF concentrations and repeated stress, the inhibitory effect on both neuronal activity and 5-HT release in the forebrain is lost, and neuronal inhibition is replaced by excitation (Kirby et al., 2000; Lukkes et al., 2008; Price and Lucki, 2001). This has been attributed to the engagement of CRF-R2. CRF-R2 activation has also been associated with decreases in feeding and decreased stress responsiveness (Spina et al., 1996; Pelleymounter et al., 2000; Ho et al., 2001; Takahashi et al., 2001; Fekete and Zorrilla, 2007). This change in CRF receptor signaling may occur during the intermittent access procedure, since we hypothesize that the increased drinking is generated as a result of repeated, stress-inducing cycles of binging and withdrawal.

A separate finding was that we observed an additional trend that individual rats were differentially affected by CRF-R1 antagonist infusion depending on their baseline ethanol preference. Although there was an overall insignificant drug effect for CP-154,526 intra-DRN on ethanol drinking in rats, when we separately analyzed a subgroup of higher-preferring individuals, we saw divergent effects on ethanol preference. High-preferring rats, in a similar fashion as high-preferring B6 mice, showed moderate decreases in ethanol drinking. In contrast, CP-154,526 infused intra-DRN of low-preferring rats seemed to have an increasing effect on ethanol preference (Figure 3b & 3d). This finding further substantiates the literature confirming CRF-R1 suppression in individuals that drink excessively without affecting low consumption levels. Importantly, this interaction was only apparent in the DRN, as identical doses in the VTA significantly suppressed ethanol intake in all individuals, regardless of baseline ethanol preference.

Role of CRF in intermittent access to alcohol

Collectively, many have included CRF-R1 signaling as an element of the neuroadaptational mechanisms involved with the progression to ethanol dependence, theoretically stemming from repeated ethanol exposure and withdrawal. All drugs of abuse perturb the HPA axis during acquisition of drug taking and again during acute withdrawal through activation of CRF in the paraventricular nucleus of the hypothalamus. As the episodic cycles of drug taking and withdrawal continue, the HPA axis response becomes blunted, but the repeated exposure of the brain to high levels of glucocorticoids can have detrimental, toxic effects on the extrahypothalamic brain stress systems (Sapolsky, Krey, & McEwen, 1984; Koob et al., 2004). Strong evidence suggests that glucocorticoids "sensitize" the CRF system particularly in the amygdala (Imaki et al., 1991; Makino, Gold, & Schulkin, 1994). During ethanol withdrawal, extrahypothalamic CRF systems become hyperactive, shown by a potent accumulation of extracellular CRF within the central nucleus of the amygdala and BNST in dependent rats (Funk et al., 2006; Merlo-Pich et al., 1995; Olive et al., 2002). This dysregulation of brain CRF systems is hypothesized to fuel both exacerbated anxiety-like behavior and the consequent enhancement of ethanol self-administration during ethanol withdrawal (Koob, 2010).

The current intermittent access drinking paradigm intends to model the cyclic nature of ethanol drinking and deprivation, necessitating a role of the hypothalamic and extrahypothalamic CRF systems. Sensitization of CRF systems in the extended amygdala occurs in which they contribute to a stress component of the shift from homeostasis to pathophysiology associated with drug addiction (Koob, 2010). This stress component may reflect a feature of the opponent process response to excessive activation of reward systems, termed anti-reward (Koob, 2008). Viewed this way, repeated activation of the CRF system during binge drinking episodes leads to

a progressive and chronic upregulation of CRF-R1 signaling which culminates in alcohol dependence. Our study further confirmed the important role of CRF-R1 in excessive drinking. Blocking CRF-R1 in extrahypothalamic brain sites in rats and mice given intermittent access to ethanol leads to acute decreases in drinking and preference.

Future of CRF-R1 antagonists

The present experiments confirm that treating binge drinking with CRF-R1 antagonists may be an effective strategy for slowing the progression to ethanol dependence. Yet, however strong the preclinical findings advocate for the potential of CRF-R1 antagonists in the treatment of alcohol abuse disorders, it is important to consider why they have not been readily available as a pharmacotreatment (recently reviewed by Lowery and Thiele, 2010; Zorrilla and Koob, 2010). First, CRF has been implicated in the modulation of numerous neurobiological systems, including those that regulate feeding, anxiety and depression, HPA axis signaling and ethanol consumption (Hauger et al., 2006; Heilig and Koob, 2007; Arborelius et al., 1999; Heinrichs and Richard, 1999; Smith and Vale, 2006; Menzaghi et al., 1994; Kehne, 2007; Kuperman and Chen, 2008; Ryabinin and Weitemier, 2006). Chronic dampening of the CRF system may also have unwanted side effects on other vital bodily systems like the changes in gastromotility and decreased immune response (Heinrichs and Richard, 1999; Jain et al., 1991).

Similarly, the etiology of alcoholism is complex and multifaceted. The effectiveness of CRF-R1 antagonists may be limited to specific sub-populations of clinically diagnosed alcoholics or limited to specific aspects of relapsed drinking. For example, CRF-R1 antagonists protected against stress-induced reinstatement in rats, but were ineffective in blocking reinstatement induced by stimuli associated with ethanol (Liu and Weiss, 2002). Thus, these compounds may not defend against all components of the complex psychopathological disorder.

Most notably, clinical development of nonpeptide CRF-R1 antagonists has been hindered because most leads were undesirably lipophilic, with poor water solubility and pharmacokinetic properties (Zorrilla and Koob, 2004, 2010; Chen, 2006). Companies such as Sanofi-Aventis (Griebel et al., 2002), Neurocrine Biosciences (Guo et al., 2005), GlaxoSmithKline (Di Fabio et al. 2008), Pfizer (Chen et al., 2008a; Chen et al., 2008b) and Eli Lilly, in collaboration with NIAAA (Gehlert et al., 2007), have made compounds that exhibited more favorable overall pharmacokinetics. As a recent example, escalating doses of R121919 in an open-label Phase IIa trial exhibited a good overall safety profile, normalized sleep EEGs and reduced depressive and anxious symptoms in depressed patients (Zorrilla and Koob, 2004). As a result, clinical anticipation of CRF-R1 antagonists has been high. However, R121919 development was discontinued because of isolated instances of elevated liver enzymes in a parallel trial (Zorrilla and Koob, 2010).

Despite major efforts, no subsequent CRF-R1 antagonist has successfully completed a definitive Phase III trial. Several studies have tested the therapeutic potential of CRF-R1 antagonists for anxiety, depression and irritable bowel disorder, which may eventually pave the way for clinical evaluation in addictive disorders. It would be also helpful to determine how CRF-R1 antagonists fare as treatments for addiction to other drugs of abuse, like cocaine or heroin, as well as other disorders of excess, such as binge-eating disorder or obesity.

In conclusion, the present findings suggest that intermittent access to ethanol recruits CRF within distinct neural circuits. Specifically, the VTA and DRN pathways, and their transmission to projection sites like the NAcc, BNST, and PFC, are important internal mechanisms that may be heavily regulated by external schedules of drug access as in the current study. The function of CRF in these networks may play a part in determining the neurobiological

differences between individuals who consume alcohol in excess and those who do not. Both dopamine and serotonin may be involved with neural processes governing intermittent access to alcohol, though we suggest the CRF-dopaminergic connections may be most relevant to this specific alcohol drinking paradigm in Long-Evans rats and B6 mice. Not only did we help support the model that CRF-R1 antagonists could be useful in the development of treatments for alcohol-use disorders, but we also gained insight into the specific neural pathways are involved in escalated, episodic alcohol drinking.

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Figure 1.

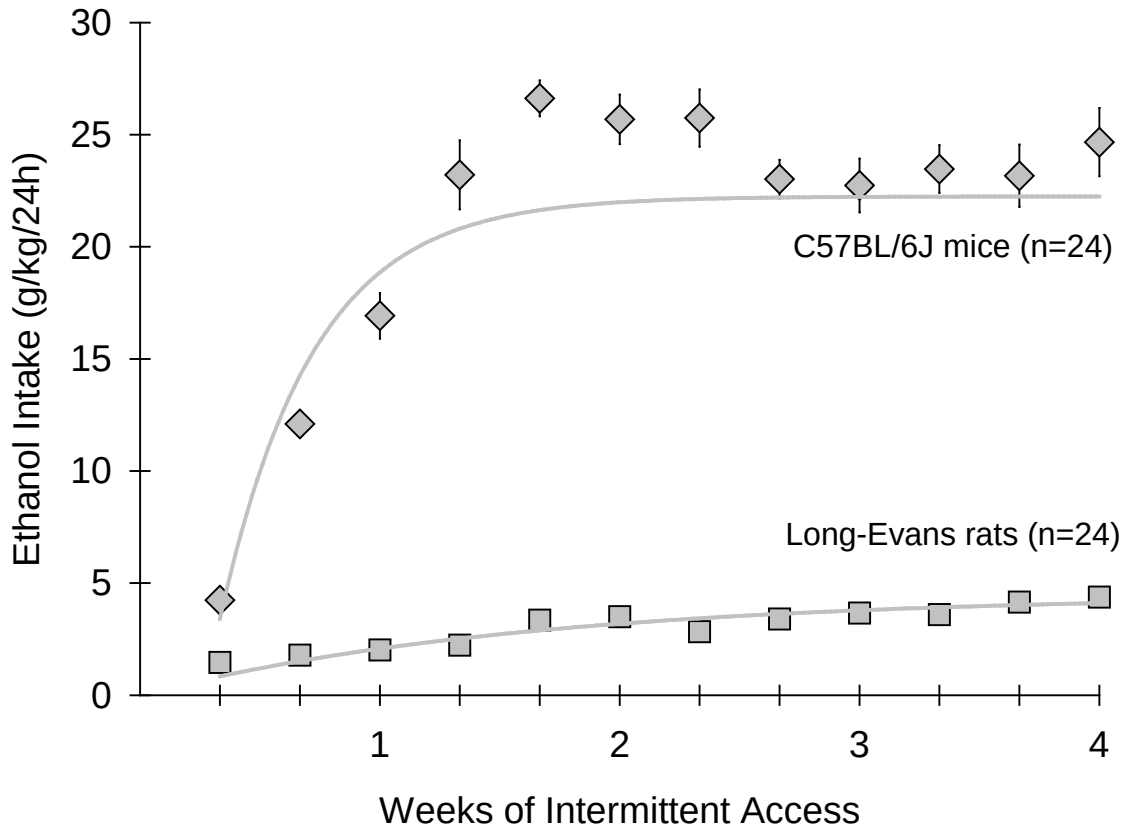

Figure 1. Acquisition of escalated ethanol drinking in the intermittent access to ethanol procedure in adult male Long-Evans rats (n=24) and C57BL/6J mice (n=24). Animals were given 2-bottle choice to 20% ethanol (w/v) and water on Mondays, Wednesdays, and Fridays for 24 hours for at least 4 weeks before cannulation surgery. Mean ethanol intake is measured in grams of ethanol per kilogram of body weight in the 24-hour access period (g/kg/24h) ± SEM.

Figure 2.

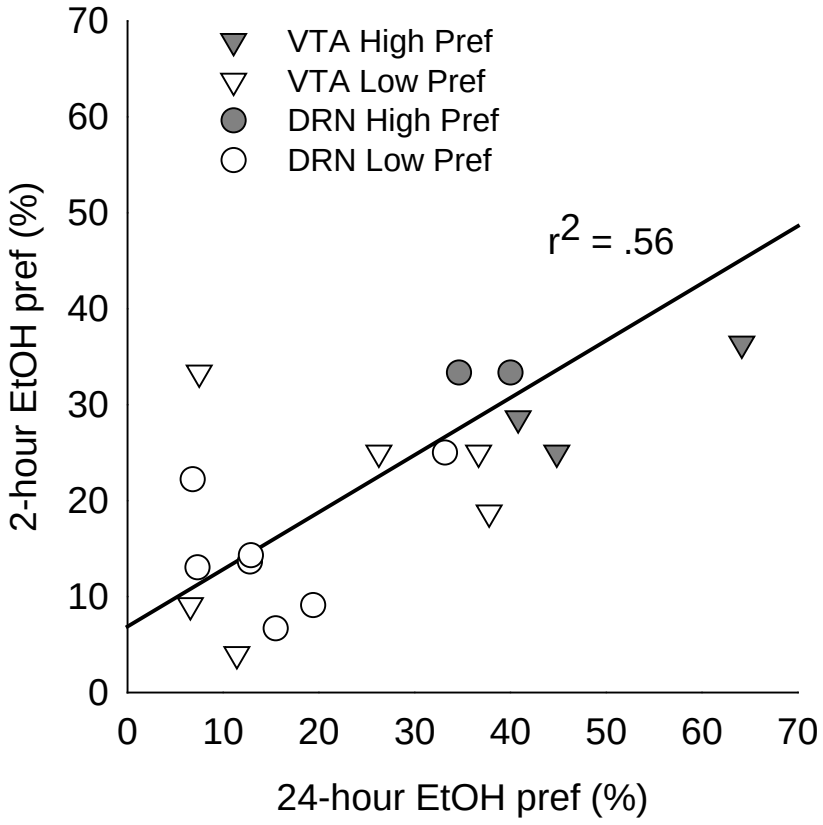

Figure 2. Adult male Long-Evans rats (n=18) were implanted with cannulae targeting either the VTA (triangles) of DRN (circles) and given intermittent access to 20% ethanol and water. Ethanol preference (EtOH pref), defined as ethanol consumed in milliliters divided by total fluid consumed in milliliters multiplied by 100 (%), in the initial 2 hours of 2-bottle choice access was conserved over the 24-hour access period. Some individuals demonstrated high ethanol preference (grey) while others displayed low ethanol preference (white).

Figure 3.

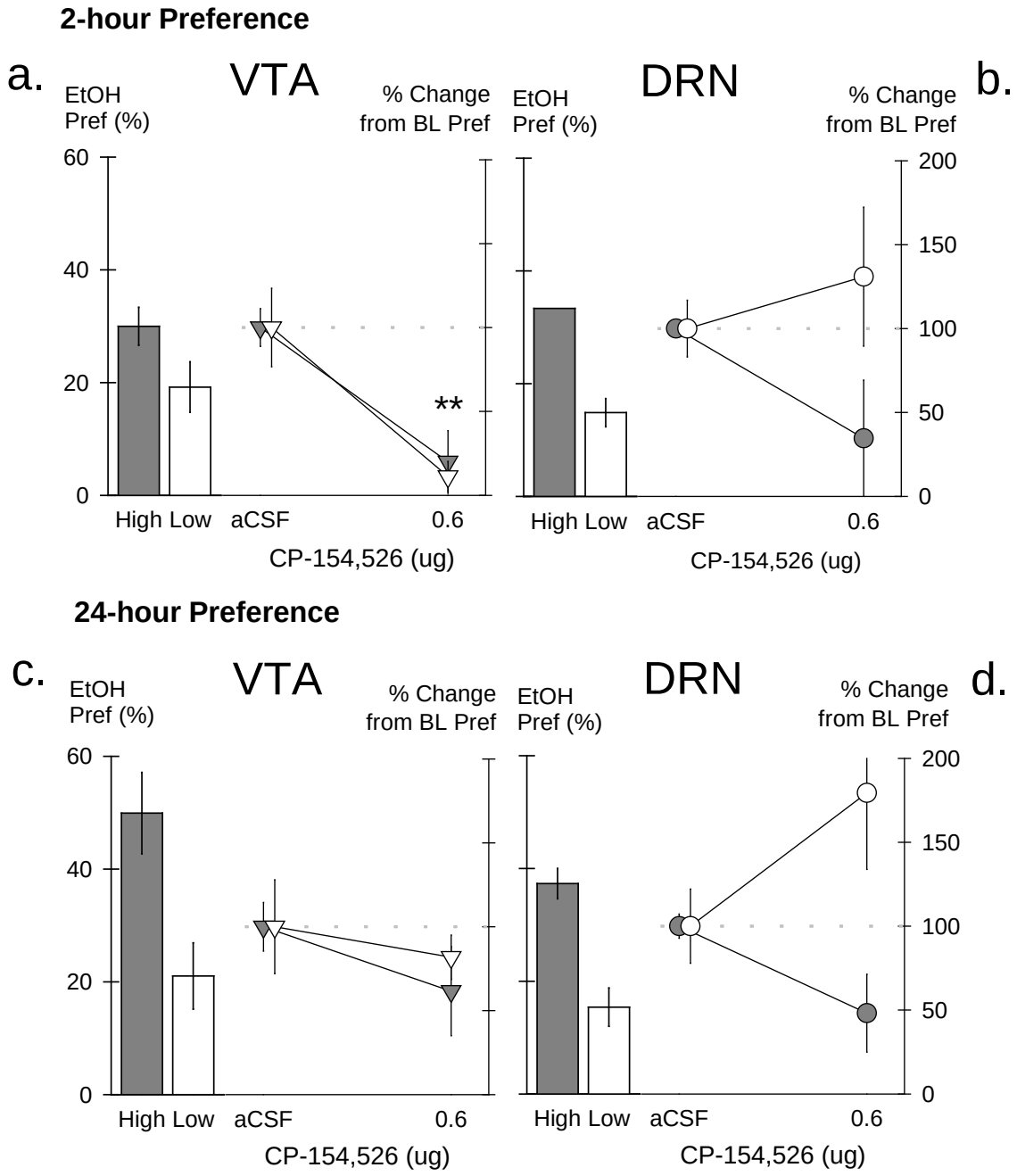

Figure 3. Long-Evans rats were implanted with cannulae targeting the VTA (n=9) or the DRN (n=9). Of the VTA rats (**Panel A & C**), individuals were characterized as high ethanol-preferring rats (grey; n=3) versus low ethanol-preferring rats (white; n=6). DRN rats (**Panel B & D**) were also characterized as high (n=2) or low (n=7) ethanol-preferring. After microinjection of 0.6 ug CP-154,526, ethanol preference changed from baseline preference (% Change from BL Pref) over the initial 2 hours access to fluid (**Panel A & B**) and over the total 24 hours access to fluids (**Panel C & D**). Data are mean values ± SEM. ** $p < .001$.

Figure 4.

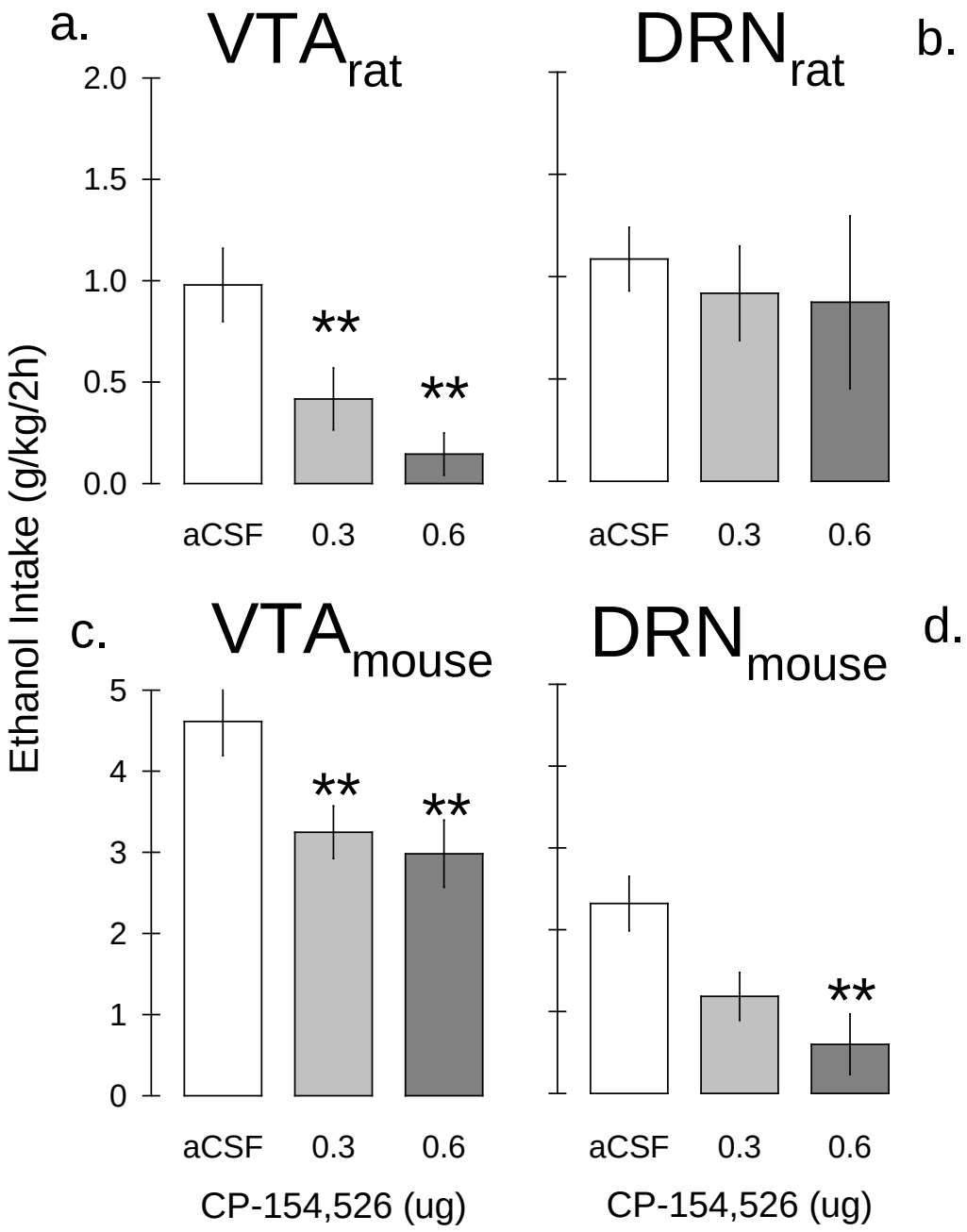

Figure 4. Doses of CRF-R1 antagonist CP-154,526 were microinjected into the VTA (**Panel A & C**) of Long-Evans rats (n=9) and C57BL/6J mice (n=10) or the DRN (**Panel B & D**) of rats (n=9) and mice (n=8). Mean ethanol intake in grams per kilogram of bodyweight was measured 2 hours post-infusion (g/kg/2h). Bars are mean intake ± SEM. ** $p < .001$.

Figure 5.

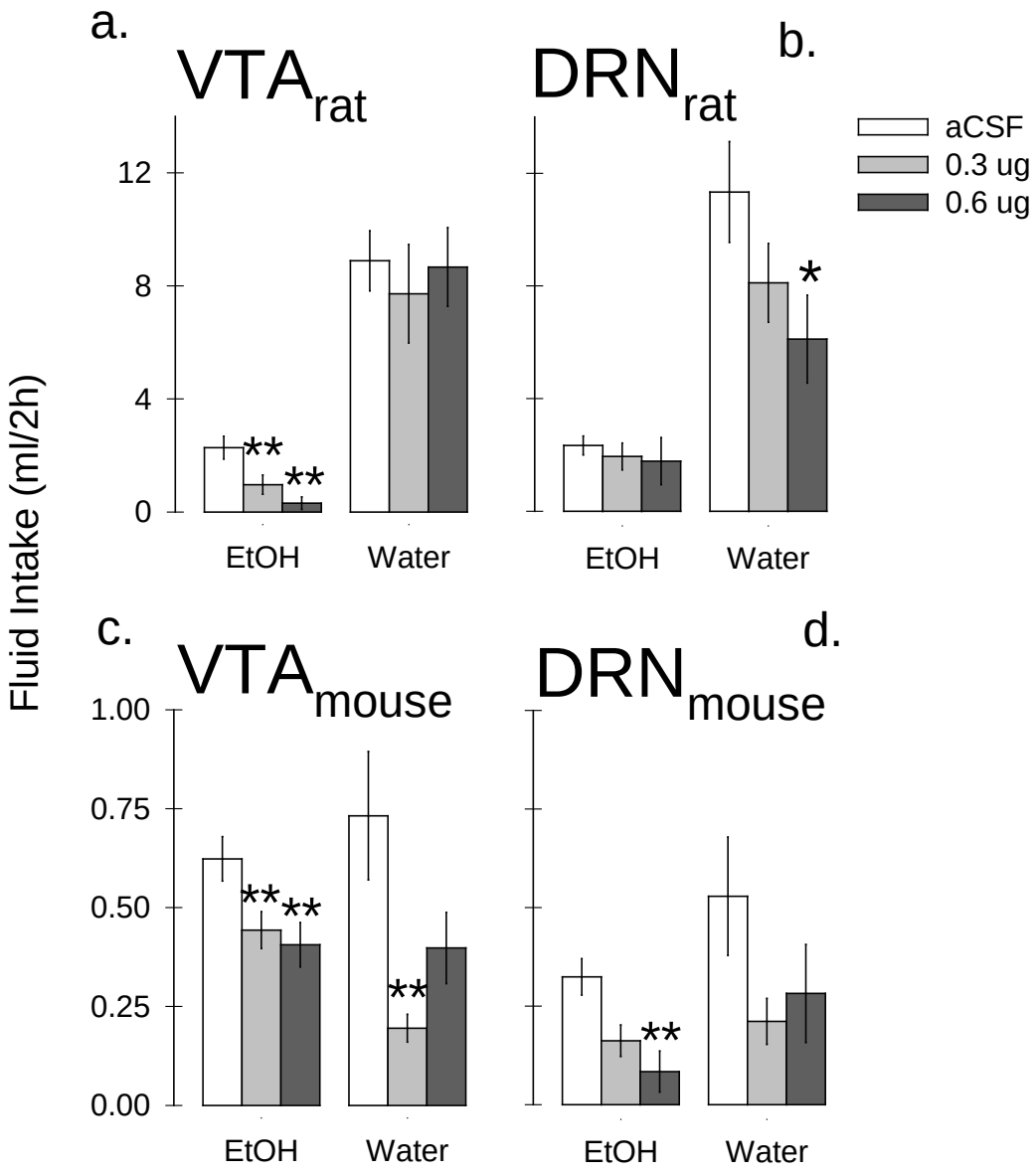

Figure 5. Doses of CRF-R1 antagonist CP-154,526 were microinjected into the VTA (**Panel A & C**) of Long-Evans rats (n=9) and C57BL/6J mice (n=10) or the DRN (**Panel B & D**) of rats (n=9) and mice (n=8). Mean fluid consumption over the initial 2-hour access period (2h/ml) is displayed ± SEM. Both ethanol (EtOH) and water intake were measured in the 2-bottle choice procedure. * $p < .01$, ** $p < .001$.

Figure 6.

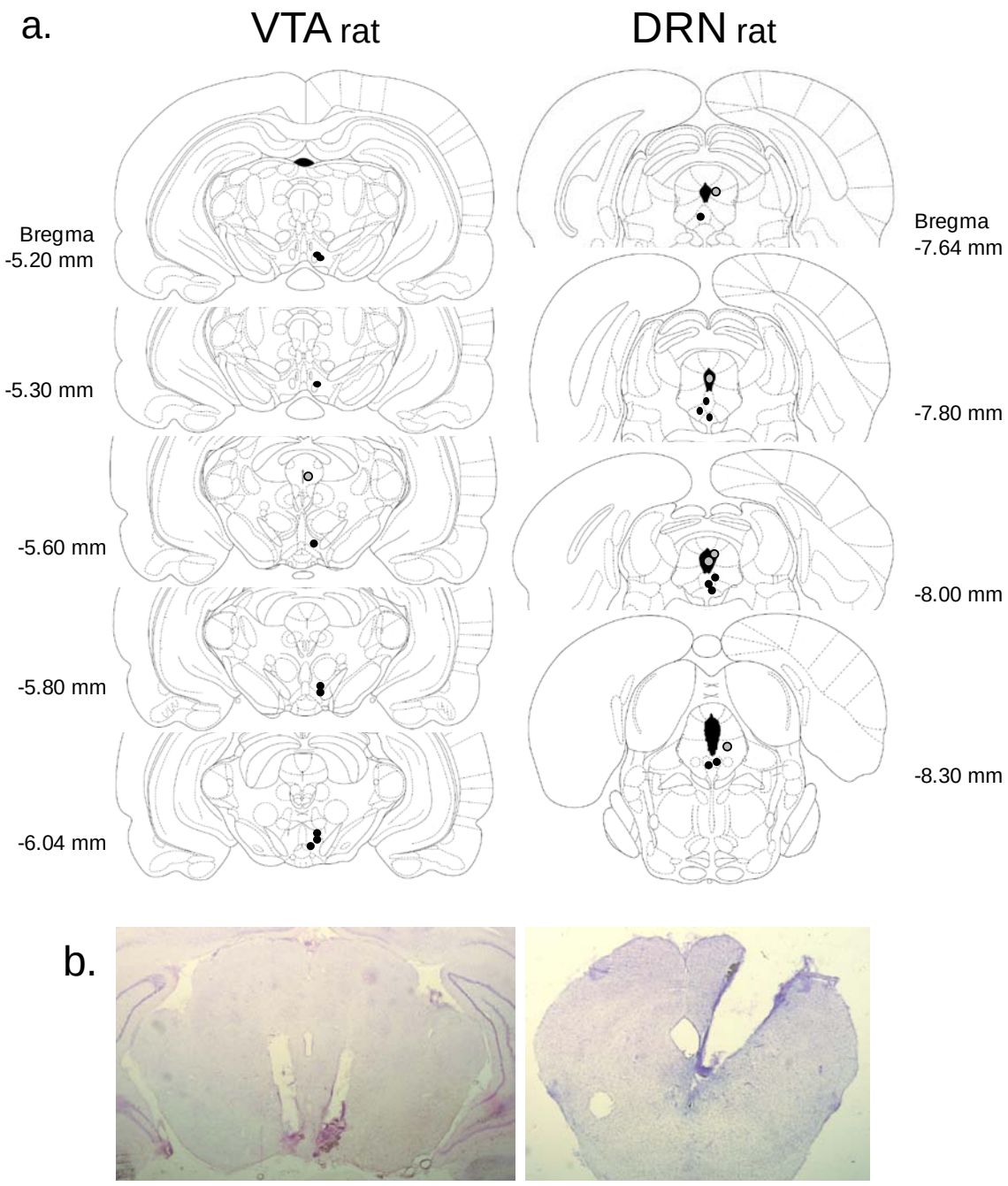

Figure 6. Correct (black circles) and incorrect (grey circles) cannula placements are depicted in representative coronal sections surrounding the VTA or DRN for Long-Evans rats (**Panel A**). VTA placements are bilateral though only one of two sides is shown. DRN placements are unilateral. Photomicrographs of correct placements after Nissl staining these brain regions are in **Panel B**.

Figure 7.

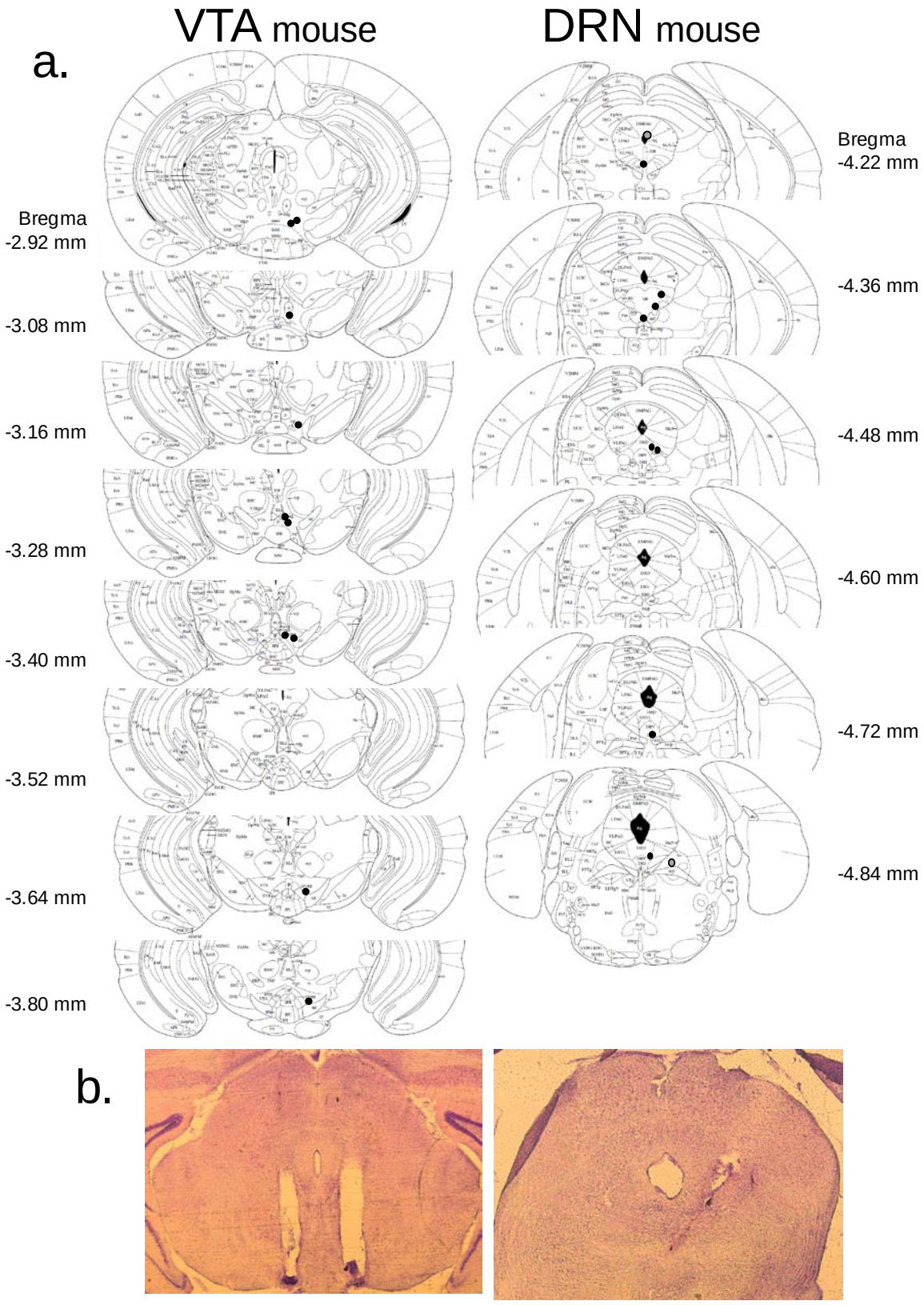
VTA mouse
DRN mouse
a.
Bregma
-2.92 mm
-3.08 mm
-3.16 mm
-3.28 mm
-3.40 mm
-3.52 mm
-3.64 mm
-3.80 mm
Bregma
-4.22 mm
-4.36 mm
-4.48 mm
-4.60 mm
-4.72 mm
-4.84 mm
b.

Figure 7. Correct (black circles) and incorrect (grey circles) cannula placements are depicted in representative coronal sections surrounding the VTA or DRN for C57BL/6J mice (**Panel A**). VTA placements are bilateral though only one of two sides is shown. DRN placements are unilateral. Photomicrographs of correct placements after Nissl staining these brain regions are in **Panel B**.

Table 1.

		CP-154,526 dose	EtOH (g/kg)	20% EtOH (ml)	H2O (ml)
Long-Evans Rats	**VTA** (n=9)	aCSF	4.42 ± 0.98	10.11 ± 2.14	29.17 ± 5.25
		0.3	5.00 ± 1.00	11.54 ± 2.20	25.59 ± 5.05
		0.6	4.07 ± 1.11	9.16 ± 2.39	32.73 ± 5.08
	DRN (n=9)	aCSF	3.00 ± 0.62	6.11 ± 1.36	27.90 ± 4.47
		0.3	3.41 ± 0.43	6.96 ± 0.92	32.05 ± 2.80
		0.6	3.59 ± 0.78	7.65 ± 1.61	27.65 ± 4.35
C57BL/6J Mice	**VTA** (n=10)	aCSF	23.42 ± 1.92	3.17 ± 0.26	2.17 ± 0.46
		0.3	23.80 ± 1.58	3.25 ± 0.22	1.94 ± 0.41
		0.6	18.81 ± 1.69**	2.57 ± 0.24**	1.74 ± 0.48
	DRN (n=8)	aCSF	21.27 ± 3.05	2.97 ± 0.42	2.17 ± 0.36
		0.3	18.57 ± 2.29	2.54 ± 0.30	1.75 ± 0.48
		0.6	23.61 ± 2.48	3.30 ± 0.36	2.10 ± 0.83

Table 1. 24-hour fluid consumption of rodents in the intermittent access, 2-bottle choice procedure after microinjection of CRF-R1 antagonist CP-154,526 into 2 distinct brain regions, the ventral tegmental area (VTA) and dorsal raphé nucleus (DRN). Ethanol intake (EtOH) in grams per kilogram of body weight (g/kg), volume of 20% ethanol in milliliters (ml), and volume of water (H2O) in milliliters (ml) were measured 24 hours after infusion. Values are mean ± SEM. ** $p < .001$.