The Role of Corticotrophin-Releasing Factor Receptor-1 and Ethanol in Aggressive Motivation in Male Mice

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Abstract

In the United States, there are over 56,000 deaths each year resulting from violence--from the Center of Disease and Prevention. More than half these violent acts are associated with the consumption of alcohol. Understanding the neurobiology underlying the manifestation of specific types of aggression is essential for improving treatment options for violent offenders. Although there has been much research on the neurobiology of reactive aggressive outbursts, neural processes that control rewarding aggression remain unclear.

Here, aggression and the motivation to commence aggressive acts were quantitatively examined in mice under the following conditions. First, male mice were trained to respond on a schedule of reward, for the opportunity to fight another male mouse. The demands established by the reward schedule allow for observations of the motivation to obtain unconditioned reinforcement. Second, the role of corticotrophin releasing hormone (CRH) receptors was examined on aggressive arousal and performance of aggressive acts. Third, mice were trained to self-administer alcohol and then gain access to fighting by their successful completion of behavioral demands.

Increasing doses of the selective CRF receptor subtype 1 antagonist CP 376395 completely abolished motivational aspects of aggressive behavior without influencing the escalation of attack behaviors during confrontations with an intruder. As expected, self-administered alcohol dose-dependently modulates both the motivation to engage in aggression and fighting performance. The results provide valuable insight towards the neural mechanisms involved in seeking aggressive opportunities, and suggest a dissociation from those required to facilitate aggressive acts.

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Key words of Title: aggression, CRF, alcohol, FI-motivation

Introduction

Violence in the United States is a significant public health burden and there are over 56,000 deaths each year resulting from violence (CDCP, 2016). Alcohol is linked to half of all violent assaults as shown consistently over the course of the past 60 years in epidemiological studies (Rossow & Bye, 2013; Miczek et al. 2015). The relationship and underlying mechanisms of the motivation for aggressive behaviors induced by alcohol is still poorly understood. While much aggression is fear-driven, aggressive behaviors can be rewarding—leading many individuals to commit violent acts (Scott, 1950; Scott, 1958). This rewarding effect of aggression can be linked to the rewarding effects of corticosterone, a stress steroid (Deroche et al. 1993; Piazza et al. 1993). Corticotropin-releasing factor (CRF), a neuropeptide, is the initial link in the activation of the hypothalamic–pituitary–adrenal axis (HPA axis) and causes the downstream release of corticosterone by the adrenal cortex into the bloodstream. This present study is concerned with the extra-hypothalamic CRF found in the amygdala, bed nucleus of the stria terminalis, dorsal raphe nucleus, locus coeruleus, and the ventral tegmental area. The aim of the study was to investigate the motivation to perform an aggressive act in male resident mice, the novel role of CRF in the ventral tegmental area (VTA) in its potential reward-enhancing effects, and the mediation of alcohol in escalating aggressive motivation and performance.

Animal Models

Preclinical models of aggressive behavior are understudied because of their complexity (Miczek et al., 2013). In aggression research, it is essential to differentiate

adaptive and maladaptive forms of aggression. Ethological aggression research create experimental conditions that display adaptive, species-specific aggressive behaviors—like inter-male and maternal aggression (Barnett, 1967; Drickamer, Vandenbergh, & Colby, 1973; Levin, Vandenbergh, & Cole, 1974). Engaging in these aggressive behaviors can be advantageous—to obtain specific goals and defend against threats (Miczek, Fish, De Bold, & De Almeida, 2002). In addition, the research investigates experimental models of maladaptive aggression. These escalated and abnormal forms of aggression can be characterized by prolonged and frequent attack bites and aggressive behaviors with brief latencies (Miczek et al., 2002; Takahashi & Miczek, 2014). But a challenge of animal models of maladaptive aggression is the differentiation and characterization of distinctive neural mechanisms between those governing species-typical aggressive behavior (Miczek, de Boer, & Haller, 2013).

Preclinical models of aggression must be strategic and the translation from preclinical data to clinical populations is relevant when models focus on specific key features of a psychiatric disorder (Miczek & de Wit, 2008). The most valuable models achieve homology between the experimental preparation and cardinal symptoms of the clinical condition in terms of construct validity, face validity, and predictive validity (Kornetsky 1989; McKinney 1989). Escalated aggression models can achieve construct validity by the fact that the pathological aggressive phenotype is obtained in a minor fraction of individuals upon positively reinforcing social experiences (Miczek et al., 2013). Face validity in these models has been achieved by the reported findings of brain serotonergic dysfunction and violent behaviors. In understanding the neurological underpinnings of pathological aggression and developing clinical treatments increases the

predictive validity. The ultimate goal of aggression research is to understand the biological, neural, and behavioral components of pathological, escalated aggression to improve therapeutic interventions in human and non-human patients.

The preclinical model of aggression of this study focuses on male mice because of the established resident-intruder paradigm in this species, where a territorial male resident mouse confronts a male intruder (Crawley, 1977; Miczek and O'Donnell, 1978). This animal model is relevant because adult male mice will establish their territory and defend it—an adaptive behavior. Maladaptive behaviors can also be modeled when male residents continually win confrontations against an intruder ("winner" effect) (Ginsburg & Allee, 1942). This escalated form of aggression, an exaggeration of species-typical behavior, may translate into violent, premeditated aggression in humans (Takahashi & Miczek, 2014).

Aggression as a Reinforcer

The present study investigates aggressive behaviors that are intrinsically reinforced by the opportunity to fight another male mouse (Fish, DeBold, & Miczek, 2002). Although aggression and its rewarding effects have been examined in the past, the underlying neural mechanisms are still poorly investigated (Looney & Cohen, 1982). In mouse models, reinforcing properties of various forms of aggression have been demonstrated (Lagerspetz, 1964; Tellegen et al., 1969; Legrand, 1970; Tellegen & Horn, 1972; Connor, 1974; Fish et al., 2002). Victorious rats and mice in aggressive confrontations were more likely to behave aggressively in the future, demonstrating that aggression can be a positive reinforcer (Ulrich 1938; Ginsburg and Allee 1942). The reinforcing effects of aggression may be adaptive because the increase in probability of

future aggression may facilitate the individual in asserting territory or a position in dominance hierarchy (Fish et al., 2002). In human studies, aggression as a reinforcer has clinical relevance because of the reports of aggressive pleasure (Baron, 1979; (Berkowitz, 2012). The more intense the level of pain that male participants supposedly inflicted on their provocateur, the greater pleasure participants reported (Berkowitz, 1993). The rewarding effects of aggression in humans are of interest in investigation of possible therapeutic interventions for violent populations. Serotonin and dopamine are two neurotransmitters of interest because of different receptor subtypes have been found to mediate aggressive behaviors.

Aggression and Serotonin

Serotonin, or 5-hydroxytryptamine (5-HT), is a monoamine neurotransmitter that is biochemically derived from tryptophan and has been extensively studied in its link to aggression. The first evidence for the role of serotonin in aggression, where serotonin antagonists and pharmacological 5-HT depletion reduced isolation-induced aggression came in the 1960s (Malick & Barnett, 1976). By the 1990s, molecular studies started to delineate cellular activity in corticolimbic neural structures (Lesch & Merschdorf, 2000; Tecott & Barondes, 1996). The use of pharmacology and selective drugs have been a vital tool in differentiating receptors. The 5-HT1A receptor has been investigated for its role in aggression through pharmacological studies. The treatment of 5-HT1A agonists, such as 8-hydroxy- 2-(N,N-di-n-propylamino)tetralin (8-OH-DPAT), effectively and potently attenuate aggressive behaviors in various animal species from rodents to non-human primates {Miczek, 1998; Tompkins et al. 1980; Dompert et al. 1985; Lindgren and Kantak 1987; Blanchard et al. 1988; McMillen et al. 1988; Haug et al. 1990;

Nikulina et al. 1992; Sanchez et al. 1993; Bell and Hobson 1994; Sanchez and Hyttel 1994; Muehlenkamp et al. 1995; de Almeida and Lucion 1997; Joppa et al. 1997; de Boer et al. 1999, 2000; Van Der Vegt et al. 2001}. In addition, reversal of these effects by antagonists point to its role in modulating aggression (de Boer, Lesourd, Mocaer, & Koolhaas, 2000; Mendoza, Bravo, & Swanson, 1999; Miczek, Hussain, & Faccidomo, 1998). 5-HT1B receptors are expressed in serotoninergic neurons in the raphe that project to the striatum and hippocampus. These 5-HT1B receptors are localized primarily at axonal terminals and may regulate neurotransmitter release (Sari, 2004). In mouse studies, 5-HT1B agonists have been shown to mediate anti-aggressive effects (Fish, Faccidomo, & Miczek, 1999; Fish, McKenzie-Quirk, Bannai, & Miczek, 2008)(de Almeida et al. 2001b; Miczek and de Almeida 2001). Because the action can be limited to the central nervous system, 5-HT1B receptors offer a promising target for reducing impulsive aggressive behavior (Miczek et al., 2002).

Another serotonin receptor of interest is 5-HT2A in promoting impulsive and aggressive behaviors in animal studies. Pharmacological 5-HT2A antagonism attenuates aggressive behaviors (Higgins, Enderlin, Haman, & Fletcher, 2003; Winstanley, Theobald, Dalley, Glennon, & Robbins, 2004) and this significant antagonist action holds promise in the management of psychotic aggressive patients (Fava, 1997). Compared to the other 5-HT receptors, 5-HT3 receptor is the only 5-HT receptor that functions as a ligand-gated ion channel and is found both pre- and post-synaptically. The 5-HT receptor is particularly relevant because of its regulation of mesolimibic dopamine release, which plays a key role in motivation and reward (De Deurwaerdere, Moison, Navailles, Porras,

& Spampinato, 2005). The antagonism of 5-HT3 receptors leads to an attenuation of aggressive behaviors (Cervantes & Delville, 2009).

Aggression and Dopamine

The preclinical literature for the role of dopamine systems in modulating aggressive behaviors is less conclusive and at times contradictory. In pharmacological studies where the roles of D1, D2, and D3 have been investigated, both agonists and antagonists decrease aggressive behaviors. At the D1 subtype, D1 agonists--dihydrexidine and SKF 383393--and D1 antagonist—SCH 23390—reduced aggressive behaviors (Rodriguez-Arias, Minarro, Aguilar, Pinazo, & Simon, 1998; Tidey & Miczek, 1992b). In the D2 receptor subtype, D2 agonist quinpirole and D2 antagonist raclopride both decrease mouse aggressive behavior (Aguilar, Minarro, Perez-Iranzo, & Simon, 1994; Couppis & Kennedy, 2008; Rodriguez-Arias, Pinazo, Minarro, & Stinus, 1999; Tidey & Miczek, 1992a). This evidence suggests that dopamine is not necessary for the initiation or execution of aggressive behavior (Miczek et al., 2007).

But mesocorticolimbic dopaminergic circuits are activated in the initiation and response to aggression. The major evidence of dopamine's permissive functions were the changes in mesocorticolimbic dopamine in anticipation, during execution, and in recovery from aggressive encounters in rodents (Miczek, DeBold, & van Erp, 1994). Following 20 minutes after the aggressive encounter, there is an increase in dopamine that may be involved in the motivational aspects of aggression (van Erp & Miczek, 2000). This increased dopamine activity in the nucleus accumbens is also reflected in other motivated social and nonsocial behaviors, like feeding and copulation (Hernandez & Hoebel, 1988; Pfaus et al., 1990; Westerink, Teisman, & de Vries, 1994; Yoshida et

al., 1992). These findings suggest that the motivational component of aggressive behaviors is reliant on the mesocorticolimbic dopaminergic circuits.

Alcohol

Alcohol, specifically ethanol is the oldest and most consumed psychoactive drug across human cultures for all of recorded history (McGovern 2009; Tramacere et al. 2012b, c). The consumption of alcohol is featured in salient and celebratory events, but the violent and destructive nature of alcohol's effects is of concern to the criminal justice system and a major public health issue (Holder, 2008; Shield, Parry, & Rehm, 2013; Rossow & Bye, 2013). Although the motivational mechanisms of initial alcohol consumption and possible motivation of addictive alcohol consumption have been well studied, the motivational component of alcohol on aggressive behaviors are understudied (Tabakoff & Hoffman, 2013).

Before the conceptualization of alcohol and its neurobiology, temperance leaders advocated that alcohol itself was the root of alcoholism and supported prohibition of the product (Tabakoff & Hoffman, 2013; Huss, 1849). Drunkenness by alcohol was described as a disease in losing control over drinking behavior, and total abstinence was the only effective cure (Rush, 1808). Advocates for prohibition resulted in the ratification of the Eighteenth Amendment to the U.S. Constitution in 1920 and the period of prohibition. This period lasted until 1932 and reduced scientific investigation into the effects of alcohol (Tabakoff & Hoffman, 2013). By the 1960s, the concept of alcoholism as a disease related to the loss of control over alcohol consumption became codified (Jellinek, 1960). In addition, the focus on disordering effects were shifted from the lipid

component of cell membranes to the action of ethanol on protein components in neuronal membranes by the 1980s (Meyer, 1899; Overton, 1901; Tabakoff & Hoffman, 2013).

Acute exposure to alcohol results in a biphasic dose and time-dependent relationship between alcohol and most of its behavioral, physiological, and neurochemical effects (Pohorecky, 1977). Low doses of alcohol on the ascending limb of the dose-effect curve can result in high energy and disinhibition (Pohorecky, 1982). But with increasing doses, these effects decrease and there are sedative effects in humans, non-human primates, and rodents (Hendler, Ramchandani, Gilman, & Hommer, 2013). The transition from alcohol drinking to alcohol addiction involves learning and memory, which involves dopaminergic systems—suggesting to the rewarding effects of alcohol consumption (Salemink & Wiers, 2014).

Alcohol as Reward- Dopamine and Serotonin

Alcohol consumption has its own reinforcing effects via dopaminergic neurons whose cell bodies reside in the ventral tegmental area (VTA) (Arias-Carrion, Stamelou, Murillo-Rodriguez, Menendez-Gonzalez, & Poppel, 2010). These dopamine populations are modulated by GABAergic and glutamatergic afferents, which project to the core and shell of the nucleus accumbens and medial prefrontal cortex (mPFC) (Spanagel & Weiss, 1999). Ethanol activates these neurons by disinhibiting dopamine neurons from the effects of GABA neurons (Beckstead & Phillips, 2009; Ron & Messing, 2013). The mapping of the dopaminergic neurons of the brain from the VTA to the nucleus accumbens (NAc) began with the development of histochemical methods by (Carlsson, Falck, Hillarp, & Torp, 1962). This mesolimbic pathway has been well established as a central component of reward in response to an action by an individual (Berridge, 2007).

This brain reward system plays a role in drug reinforcement and the development of addiction (Soderpalm & Ericson, 2013).

Using dialysis techniques to measure dopamine release in the NAc, there was a statistically significant increase in dopamine release even at the lowest concentration of ethanol as injected by the experimenter (Wozniak, Pert, Mele, & Linnoila, 1991). In rat studies, systemic administration of ethanol produced an increase in firing of dopaminergic neurons in the VTA and dopamine release in the NAc (Di Chiara & Imperato, 1988; Gessa, Muntoni, Collu, Vargiu, & Mereu, 1985). Neural activity in the VTA and increased dopamine in the NAc suggests the rewarding effects of ethanol (Brodie, Pesold, & Appel, 1999; Brodie, Shefner, & Dunwiddie, 1990; Di Chiara & Imperato, 1988). Ethanol-induced dopamine stimulation and dopamine release in the NAc can be mediating the learning of behaviors and rewarding specific behaviors (Morikawa & Morrisett, 2010).

Serotonin may also have a role in modulating the reinforcing effects of ethanol in the NAc (Yoshimoto & McBride, 1992). In rat models, ethanol was found to increase the release of serotonin in the nucleus accumbens (Weiss et al., 1996; Yoshimoto, McBride, Lumeng, & Li, 1992a, 1992b). Investigation into serotonergic systems may provide valuable insight into alcohol consumption and explain the difference in alcohol intake and abuse (Lovinger, 1991; Lovinger & Zhou, 1994; Tikkanen et al., 2009).

Alcohol and CRF

Extensive evidence supports alcohol's actions on the corticotrophin-releasing factor (CRF) systems—modulating both hypothalamic-pituitary-adrenal (HPA) axis function and extra-hypothalamic CRF in different brain regions. Corticotrophin-releasing

factor, CRF, is a 41-amino acid neuropeptide first identified by Vale and colleagues (1981). CRF is crucial for neural, endocrine, and behavioral responses to stress and interacts with its two G-protein coupled receptors—CRF-Receptor 1 and CRF-Receptor 2 and binding proteins (Bale & Vale, 2004). Acute alcohol exposure has been found to promote activation of the HPA axis by dose-dependent increases on plasma ACTH and corticosterone (Rivier, Bruhn, & Vale, 1984; Rivier & Lee, 1996; Zhou et al., 2000). In extra-hypothalamic sites, like the central amygdala, there is also an increased release of CRF peptide resulting from acute alcohol administration (Lam & Gianoulakis, 2011).

With repeated alcohol exposure, there is development of tolerance in the HPA axis response, which is mostly mediated by CRFR1 (Logrip et al., 2013; Lowery-Gionta et al., 2012; Ogilvie, Lee, & Rivier, 1997; Zhou et al., 2000). After repeated exposure, rats present hyperactive extra-hypothalamic CRF activity—indicated by an increase in CRF immunoreactivity and mRNA for CRF in the amygdala nuclei and the BNST (Merlo Pich et al., 1995; Olive, Koenig, Nannini, & Hodge, 2002; Rivier, 1996). This is consistent with the upregulation of CRFR1 subtype after alcohol exposure (Dallman, 2005; Lee & Rivier, 1997a, 1997b). In human studies, the activational effects of alcohol on the HPA axis has been demonstrated by the increases in plasma cortisol after acute administration (Jenkins & Connolly, 1968). Similar to other drugs of abuse, studies further suggest a direct modulation of brain reward function by CRF signaling in the ventral tegmental area, nucleus accumbens, and the prefrontal cortex (Phillips, Reed, & Pastor, 2015). The rewarding effects of alcohol exposure mediated by CRF may play a role in the dopaminergic systems induced by alcohol. Alcohol activation of CRF suggest

its importance in conditions of alcohol taking, seeking, and development of alcohol abuse (Quadros, Macedo, Domingues, & Favoretto, 2016).

Connection of Aggression and Alcohol

The connection between the consumption of alcohol and aggression is found in a small subgroup of individuals. There is great variation in those that become more aggressive under alcohol's effects, while the same amount of alcohol has no effect or leads to pro-social overtures (Pernanen, 1976; Cherek, Steinberg, & Manno, 1985; Miczek, Faccidomo, et al., 2004; Rosell & Siever, 2015). This is a major public health burden because alcohol has been linked to at least half of all violent assaults, cases of child abuse, and other incidents of violence (Rossow & Bye, 2013). The Task Force on College Drinking of the National Institute of Alcohol Abuse and Alcoholism (NIAAA) estimates that each year 600,000 U.S. college students are assaulted by another student related to alcohol consumption (Hingson, Heeren, Zakocs, Kopstein, & Wechsler, 2002). Although this alcohol-escalated aggression is only found in a subgroup of the population that consumes alcohol, the neurobiological processes underlying this link are poorly understood, and there is no effective clinical intervention.

Preclinical studies have been able to identify subgroups of individuals where a moderate dose of alcohol escalates aggressive behaviors—reflecting the subpopulations of human individuals (Miczek, Fish, De Almeida, Faccidomo, & Debold, 2004). The critical determinant of alcohol's effects is the phase of action because alcohol has biphasic effects on endocrine, neural, and behavioral parameters (Pohorccky, 1977). But it is important to note that animal species differ considerably in the fate of alcohol and the functional characteristics of neural sites of action. In translating preclinical studies to

humans, it is necessary to consider doses and time of peak effect across species. In mice, alcohol (1.0 g/kg) heightens aggressive behaviors by at least two standard deviations of the individual's vehicle control in 27% of the mice (Miczek, Barros, Sakoda, & Weerts, 1998). This subgroup of the population tested is referred to as alcohol-heightened aggressors (AHA). In other individuals, the same dose of alcohol has no effect or decreases aggressive behaviors. In rats and squirrel monkeys, alcohol produces large aggression-stimulating effects in a subgroup of animals (Weerts & Miczek, 1996; Weerts, Tornatzky, & Miczek, 1993; Winslow & Miczek, 1985). These preclinical models of alcohol-escalated aggression are valuable in systemic and controlled drug manipulations in understanding alcohol's relationship to aggression.

Key features for modeling pathological aggression in experimental animals are the lack of threats preceding injurious attacks and the indiscriminate targets for inflicting bodily harm (Haller & Kruk, 2006; Miczek et al., 2013; Newman et al., 2015). In characterizing alcohol-escalated aggression in mice, a key feature is the persistent, large increase in injurious attack behaviors after every exposure to a moderate 1.0 g/kg dose of alcohol (Miczek & de Almeida, 2001). The aggressive behaviors are also characterized differently—where alcohol self-administering resident mice or rats aim their attack bites at vulnerable parts of the intruder's body, such as the ventrum and face (Newman et al, 2013). This alcohol-heightened aggression may involve serotonin transmission from the dorsal raphe nucleus (DRN) and involve different serotonin receptors (Chiavegatto, Quadros, Ambar, & Miczek, 2010; Faccidomo, Bannai, & Miczek, 2008; Faccidomo, Quadros, Takahashi, Fish, & Miczek, 2012). In mice, 5-HT1A agonist dose-dependently decreases alcohol-heightened aggression (de Boer & Koolhaas, 2005; Stein, Miczek,

Lucion, & de Almeida, 2013). Anti-aggressive effects are engendered by 5-HT1B agonist, CP-94,253, in the presence of alcohol (Fish et al., 1999).

Repeated exposure to alcohol promotes neuroadaptation in brain reward pathway, similar to other drugs of abuse (Robinson & Berridge, 1993, 2001). The changes render the brain more vulnerable and sensitive to drug-induced reward and stimulation. This behavioral sensitization is defined as progressively augmented motor stimulation responses in the presence of similar drug doses (Abrahao & Souza-Formigoni, 2012; Masur & Boerngen, 1980; Quadros, Nobrega, Hipolide, de Lucca, & Souza-Formigoni, 2002). Alcohol sensitization is associated with alterations in mesocorticolimibic dopamine and glutamate activity (Broadbent & Weitemier, 1999; Souza-Formigoni et al., 1999). The investigation of alcohol sensitization is critical to understanding how behaviors are changed and how repeated exposure can lead to alcohol dependence in humans (Newlin & Thomson, 1991, 1999).

In mice, repeated alcohol exposure sensitizes its effects, including motor stimulation, drinking, and heightened aggression (Lessov & Phillips, 1998). Although alcohol-induced locomotor sensitization in rats is not easily demonstrated (Correa, Arizzi, Betz, Mingote, & Salamone, 2003; Hoshaw & Lewis, 2001), locomotor sensitization is well described in mouse strains (Camarini & Hodge, 2004; Masur, Oliveira de Souza, & Zwicker, 1986; Phillips, Dickinson, & Burkhart-Kasch, 1994). After alcohol-induced sensitization, twice as many mice were characterized as alcohol-heightened aggressors (Fish et al., 2002). Behavioral sensitization by repeated alcohol exposure may require CRF (Pastor et al., 2008; Pastor et al., 2012). In CRFR1 knockout mice, alcohol sensitization failed to develop and show a reduction in the acute response to alcohol

(Olive et al., 2003). Corticotrophin-releasing factor may mediate the effects of alcohol and may be required in the rewarding effects of aggression (Caramaschi, de Boer, de Vries, & Koolhaas, 2008; Hsu, Earley, & Wolf, 2006; Quadros et al., 2014).

Corticotrophin-releasing Factor (CRF)

Corticotrophin-releasing factor, or CRF, is the molecule responsible for the activation of the neuroendocrine stress cascade of the hypothalamic-pituitary-adrenal (HPA) axis (Rivier & Vale, 1983a, 1983b). A stressor triggers the activation of the medial dorsal parvocellular region of the periventricular nucleus (PVN) of the hypothalamus to release CRF (Armario, 2006, 2010). This induces the release of adrenocorticotropic hormone (ACTH) by corticotrope cells of the anterior pituitary. ACTH travels in the blood stream and activates the secretion of glucocorticoid from the zona fasciculate of the adrenal cortex (Herman et al., 2003)—leading to physiological responses in the individual.

In the mammalian brain, CRF is also found outside of the hypothalamus in the amygdala, bed nucleus of the stria terminalis (BNST), hippocampus, thalamus, locus coeruleus, and the raphé nucleus (Bale & Vale, 2004; Merchenthaler, Vigh, Petrusz, & Schally, 1982; Merchenthaler, Vigh, Schally, Stumpf, & Arimura, 1984; Morin, Ling, Liu, Kahl, & Gehlert, 1999; Shepard, Schulkin, & Myers, 2006; Swanson, Sawchenko, Rivier, & Vale, 1983; Tran & Greenwood-Van Meerveld, 2012). This extra-hypothalamic CRF contributes to the integration of endocrine, sympathetic, behavioral, and cognitive responses to stress (Gilpin, 2012; Hauger, Risbrough, Brauns, & Dautzenberg, 2006; Muller et al., 2003; Walker, Miles, & Davis, 2009). The present study is interested in extra-hypothalamic CRF because of its role in aggression and alcohol brain circuitry.

In the ventral striatum (i.e. nucleus accumbens), CRF is found on GABAergic neurons and in the midbrain (i.e. ventral tegmental area), CRF is found on dopaminergic neurons (Bonfiglio et al., 2011; Lemos et al., 2012; Refojo et al., 2011). The CRF system modulates dopaminergic neurons by activating both CRF-R1 and CRF-R2 (Tagliaferro & Morales, 2008; Van Pett et al., 2000; Wang & Morales, 2008). The VTA receives CRF projections mostly from the limbic forebrain and PVN of the hypothalamus (Rodaros, Caruana, Amir, & Stewart, 2007) and CRF increases the firing rate of VTA dopaminergic neurons (Korotkova, Brown, Sergeeva, Ponomarenko, & Haas, 2006; Wanat, Hopf, Stuber, Phillips, & Bonci, 2008). In addition, the VTA sends projections to the NAc—involved in the acute pleasure effects of drug taking behaviors (Koob & Nestler, 1997). This circuit modulated by CRF may play an important role in reinforcing behaviors that include drug taking and aggression (Holly & Miczek, 2016).

The activation of CRF systems may be necessary in the motivation of reinforced behaviors—including addiction-like behaviors (Zorrilla, Logrip, & Koob, 2014). Stressors, like reward seeking behaviors, can affect dopamine-dependent circuits modulated by CRF (Wanat, Bonci, & Phillips, 2013). This motivated behavior is facilitated by activation of the mesolimbic dopamine projections from the VTA to the NAc (Salamone, Correa, Farrar, Nunes, & Pardo, 2009). CRF may be needed in initiating other reinforcing behaviors, like food reward (Liu, 2015; Teegarden, Scott, & Bale, 2009). The involvement of CRF in the motivational component of salient reinforcing behaviors may include aggression as a reinforcer—as the present study investigated.

Fixed-Interval Schedule of Reinforcement

The present study used the fixed-interval schedule of reinforcement (Skinner & Fester, 1957), where the opportunity for an aggressive confrontation with an intruder male mouse is reinforced (Cherek & Thompson, 1973). This behavioral paradigm captures the motivational component of a behavior because fighting is contingent upon completing a nose-poking task. Individuals are reinforced for the first response that occurs after a fixed time interval has elapsed (Fry, Kelleher, & Cook, 1960). Previous responses during the interval are recorded, but have no specified consequence. The fixed-interval schedule produces a characteristic response patterns—low response rates in the beginning of the interval and then gradually accelerating rates as the interval progresses (Skinner 1938; Dews 1970). The scallop effect of the fixed-interval schedule of reinforcement quantifies the motivational component of the rewarding behavior (Dews, 1970). The advantages of this behavioral paradigm is its robust nature, its ability to be easily manipulated by pharmacological studies, and its potential in novel neuroscience methodologies.

Previous studies have utilized the fixed-interval schedule of reinforcement in primates and pigeons—but rarely in mice (Bowen & Balster, 1998; Cherek & Thompson, 1973; Ripley, Horwood, & Stephens, 2001; Burke et al. 1994). The time interval was arbitrarily chosen, but is based on previous studies that utilized the protocol in mice (Fish et al., 2002). Relative to other behavioral paradigms, the conditioning process of the fixed-interval schedule can be extensive and time-consuming. In the present study, cohorts of mice were gradually conditioned up to the FI10 schedule for at least five weeks before experiments began. The observation of the change in response rate over the

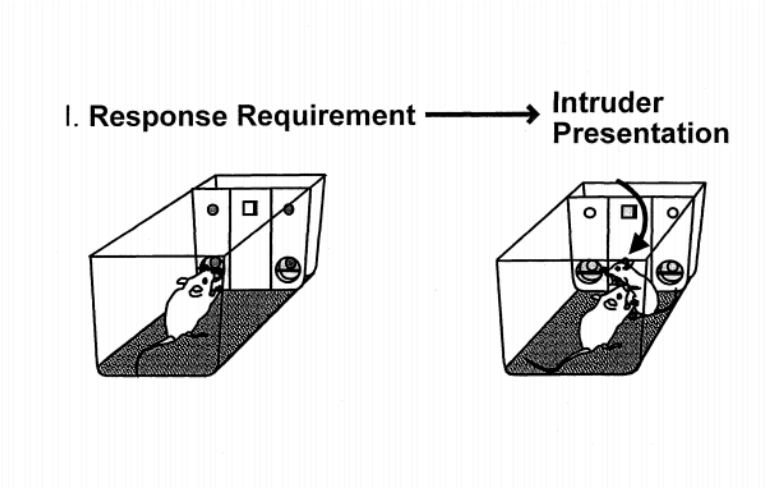
course of the interval suggests anticipation and motivation for the reinforced behavior—crucial for understanding reward-seeking behaviors and neural mechanisms activated.

Methods

Subjects

Male C57BL/6J mice (Charles River Breeding Labs Wilmington, MA, USA) weighing 23-25 grams upon arrival were housed in polycarbonate cages (28 × 17 × 14 cm) lined with pine bedding (Shepherd's Specialty Blend Alpha-dri). Food (Lab Diet 5001 Rodent Diet, PMI Nutrition International, Brentwood, MO, USA or Purina Rodent Chow) and water were available at all times. For study of aggression, male mice were pair-housed with females of the same strain to facilitate aggressive behaviors and avoid social isolation (Miczek and O'Donnell 1978). Pups born were weaned at 3 weeks of age. Once stable levels of aggression were reached (see below), female mice were removed and male residents were singly housed. Additional male C57BL/6J mice were group-housed as "intruders" in large polycarbonate cages (46 × 24 × 15 cm). The vivarium was maintained at 21 ± 1°C, 30–40% humidity, and 12-h reverse light/dark cycles (lights on at 1730 hours). Experimental procedures were approved by the Tufts Institutional Animal Care and Use Committee following the Guide for the Care and Use of Laboratory Animals (National Research Council 2011).

Materials



Aluminum operant conditioning panels were inserted into the male resident's home cage, as described by (Miczek and de Almeida 2001). The panel contained a house light (ENV-315W; Med Associates, St. Albans, VT, USA) located in the center of the panel. Cue lights were located on both sides of the house light and nose poke response units (ENV-313W; Med Associates) were located underneath. Each panel was connected to a computer running software (MED-PC for Windows, version 4.1; Med Associates) that controlled lights and recorded nose poke responses.

Drugs

CP 376,395 was obtained from Tocris Bioscience (Avonmouth, Bristol, United Kingdom). It was first dissolved with 10% dimethyl sulfoxide (Sigma-Aldrich Corporation, Natick, MA) and diluted with dH2O. CP 376,395 was injected intraperitoneally (i.p.) in a volume of 10 ml/kg of body weight. For ethanol procedures, ninety-five percent ethyl alcohol was purchased from Pharmco-AAPER Products, Inc (Brookfield, Connecticut, USA) and diluted with tap water to obtain 10%, 15%, and 18% ethanol concentration (w/v). It was administered via gavage in a volume of 10 ml/kg of body weight.

Blood Collection

Upon completion of experiments, blood was collected 10-minutes post ethanol 1.0 g/kg administration via gavage. Using a heparinized capillary tube, 0.1ml of blood was collected by submandibular bleed. Blood samples were centrifuged at 4°C for 10 min at 3000 r.p.m. Alcohol concentrations in plasma were measured by ultraviolet spectroscopy using an alcohol dehydrogenase enzyme assay (Analox Instruments Inc., Lunenburg, MA).

Fixed-Interval Responding Reinforced by Aggression

After resident male mice were housed in pairs with females for a minimum of three weeks, their aggressive behavior against an intruder was assessed (Miczek and O'Donnell, 1978). The female cage-mate and pups were removed and the male intruder introduced. The latency to attack and number of attack bites were recorded. The confrontation lasted 5 minutes after the first bite or 5 minutes if no bite occurred. These confrontations were scheduled daily for 10 days. The resident encountered different intruders in each confrontation to avoid habituation (Winslow and Miczek, 1983). To maintain consistent fighting levels and reduce potential harm to intruders, intruders were socially defeated 5-8 times before confrontation with male residents.

When male residents engaged in reliable levels of aggression, they were trained on a fixed-interval schedule of reinforcement. Specifically, each resident mouse performed nose-poke responses for the opportunity to fight an intruder (Fish, 2002). The female cage-mate and pups were removed and the operant panel was inserted into the male resident's home cage. A cover was positioned on top of the cage and a shade separated the operant chamber to reduce external stimulus. After 10-minutes into the

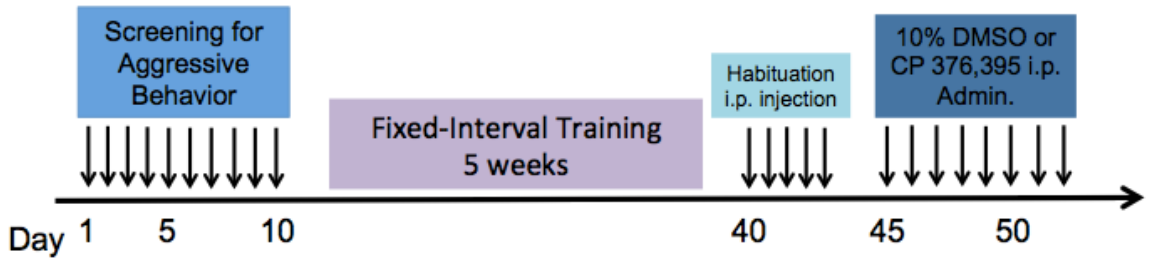
interval, the next response on the active nose poke caused the house light to be turned on and the experimenter introduced an intruder. On training days, the confrontation only lasted 5 attack bites by the male resident to reduce excessive stress and harm to the intruders and avoid exhaustion and habitation of the male resident. On experimental days, the confrontation lasted for 60-seconds. Latency to first bite and time it took resident to make 5 attack bites were recorded.

Video Analysis

Agonistic mouse behavior was recorded using a digital webcam (Logitech® HD Pro Webcam C920, Newark, CA). A trained observer (intra-observer reliability: $r > 0.95$) analyzed video recordings during the fixed-interval and the aggressive encounter of the male residents using *The Observer XT* software (Noldus, v. 8.0.330 or v. 9.0.436; Wageningen, The Netherlands). The first 60-seconds of the fixed-interval, the last 60-seconds of the fixed-interval, and the 60-seconds of the aggressive confrontation were analyzed. Key presses on a custom-made keyboard coded the frequency, duration, and latency of each operationally defined behavior (see Table). Aggressive behaviors included attack bites and sideways threat. Non-aggressive behaviors included anogential and nasal contact, pursuit, self-grooming, rearing, and walking (Grant and Mackintosh 1963; Miczek and O'Donnell 1978). Arousal behaviors included tail rattle, digging, and jumping (Kršiak & Steinberg, 1969). Each video recording was analyzed twice to ensure reliability of the measurements.

Coded Behavior	Definition
Attack Bites	Any bite made by resident on the intruder
Sideways Threat	Sideways movement—with possible threat function
Anogenital and Nasal Contact	Nasal-anogenital contact or nasal-nasal contact by resident with intruder
Pursuit	Resident following behind intruder
Self-Grooming	Resident grooming behaviors- facial, body, and tail grooming
Rearing	Two front paws of resident off the ground
Walking	Any forward movement of resident in home cage
Tail Rattle	Rattling of resident's tail
Digging	Resident pushing bedding forward or behind itself
Jumping	Four paws of resident off the ground

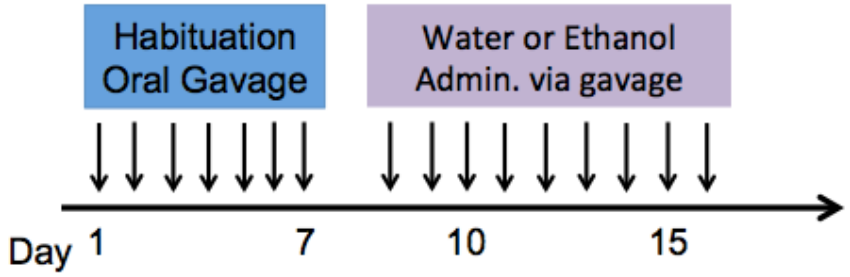
Experiment I: Modulation of CRF R1 by CP 376,395



Before the first administration of CP 376,395, male residents were habituated to intraperitoneal injections of 0.9% saline 1-hour prior to the fixed-interval. On test days, male residents were given either vehicle (10% DMSO) or various doses of CP 376,395 (0, 3, 10, 17 mg/kg i.p.) 50-mintues prior to the fixed-interval in an unsystematic sequence. Each resident was tested at least three times in the vehicle condition (control) and once at every drug dose. Forty-eight hours separated drug doses to prevent residual

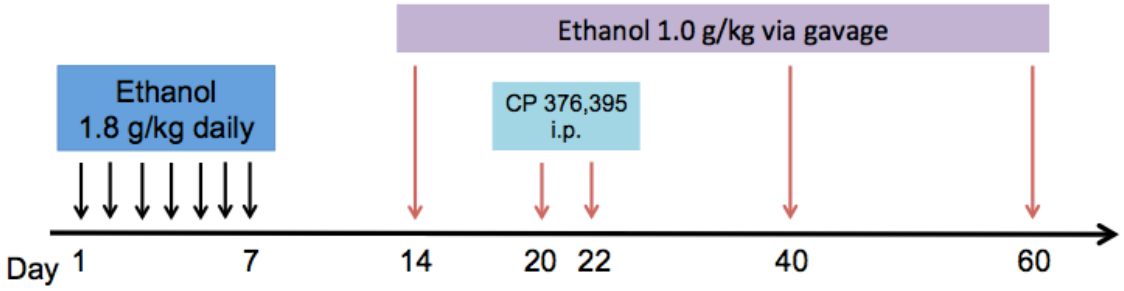
effects on behavior. Male residents continue to be tested on fixed-interval schedule in-between drug doses to maintain stable responding behavior.

Experiment II: Ethanol Dose Response on Aggressive Arousal



Following Experiment I and a one-week wash-out period, male residents were habituated to oral administration of tap water via a gavage 30-minutes prior to performing the nose poke response under the control of the fixed-interval schedule. On test days, male residents were given either tap water or various doses of ethanol (0.5, 1.0, 1.8 g/kg) 10-minutes prior to the fixed interval in an unsystematic sequence. The oral administration took ca. 10-seconds and the rate of nose poke behavior stabilized before ethanol was administered. Each resident was tested at least three times in the vehicle condition and once at every ethanol dose. Forty-eight hours separated drug doses to prevent residual effects on behavior.

Experiment III: Ethanol Sensitization and Heightened Aggressive Arousal



Following ethanol dose response determinations, male residents were given seven consecutive days of ethanol (1.8 g/kg/day gavage) 10-minutes prior to the session assessing response rates under the control of a fixed interval schedule. This dose was chosen because it initially attenuated both nose poke responding schedule and aggressive behavior.

Through the repeated administration of ethanol over seven days, there was an emergence of ethanol sensitization for the rate of nose poke responding. To observe the lasting effects of ethanol sensitization, male residents were administered ethanol (1.0 g/kg gavage) fourteen, forty, and sixty days after the last dose of ethanol (1.8 g/kg). Ethanol (1.0 g/kg) dose was chosen as it was the intermediate dose between the three doses previously administered. Three days prior to each ethanol challenge, residents were re-trained on the fixed interval and habituated to the tap water via oral gavage.

Experiment IV: Ethanol Sensitization Modulated by CP-376,395

Following ethanol sensitization, the novel role of CP-376,395 on responding that was maintained by the fixed interval schedule was investigated. Male residents were given either vehicle (10% DMSO) or various doses of CP 376,395 (0, 3, 10, 17 mg/kg i.p.) 50-mintues prior to the session assessing response rates under the control of the fixed

interval schedule in an unsystematic sequence. Male residents were habituated to intraperitoneal injections before CP-376,395 was given. In addition, residents were administered ethanol (1.0 g/kg) 10-minutes prior to the fixed interval schedule.

Results

Data Analysis

The mean number of cumulative responses and the rate of responding (responses/min) on the active nose poke hole were measures of the performance on the FI10-min schedule of reinforcement. Duration (seconds) and frequency were calculated for both aggressive and non-aggressive behaviors. Measures of fixed interval schedule responding, aggressive behaviors, and non-aggressive behaviors were analyzed using one-way repeated measures analysis of variance (ANOVA). Post-hoc Holm-Šídák multiple comparison tests were used for ANOVA effects with $p<0.05$ in comparing drug treatments to vehicle baseline.

Experiment I. Results

The cohort of mice trained on the fixed interval of reinforcement actively responded and initiated aggressive behaviors upon intruders after the completion of the interval. Before the administration of CP 376,395, mice were habituated to i.p. injections and exposed to 1-minute aggressive confrontations.

The CRF R1 antagonist, CP 376,395 dose-dependently decreased the rate of responding in the active nose poke hole ($F(3,14) = 8.824$, $p=.001$; Figure 1). Post-hoc analysis revealed that the mice responded significantly lower in the active hole after lowest dose, 3.0 mg/kg. The highest dose, 17.0 mg/kg, showed a significant decrease relative to the vehicle condition, but rate of response was similar to 10.0 mg/kg. The scalloping effect of the fixed interval of responding was maintained with increasing doses.

Behavioral analysis of the aggressive confrontation after the fixed interval schedule revealed no significant difference in the frequency of sideways threat and attack bites ($F(3,14) = 1.865$, $p=.15$; Figure 2) with increasing doses of CP 376,395. There was no significant difference in behaviors analyzed in the first minute and the last minute of the fixed interval due to the effect of the CP 376,395 administration (Table 1).

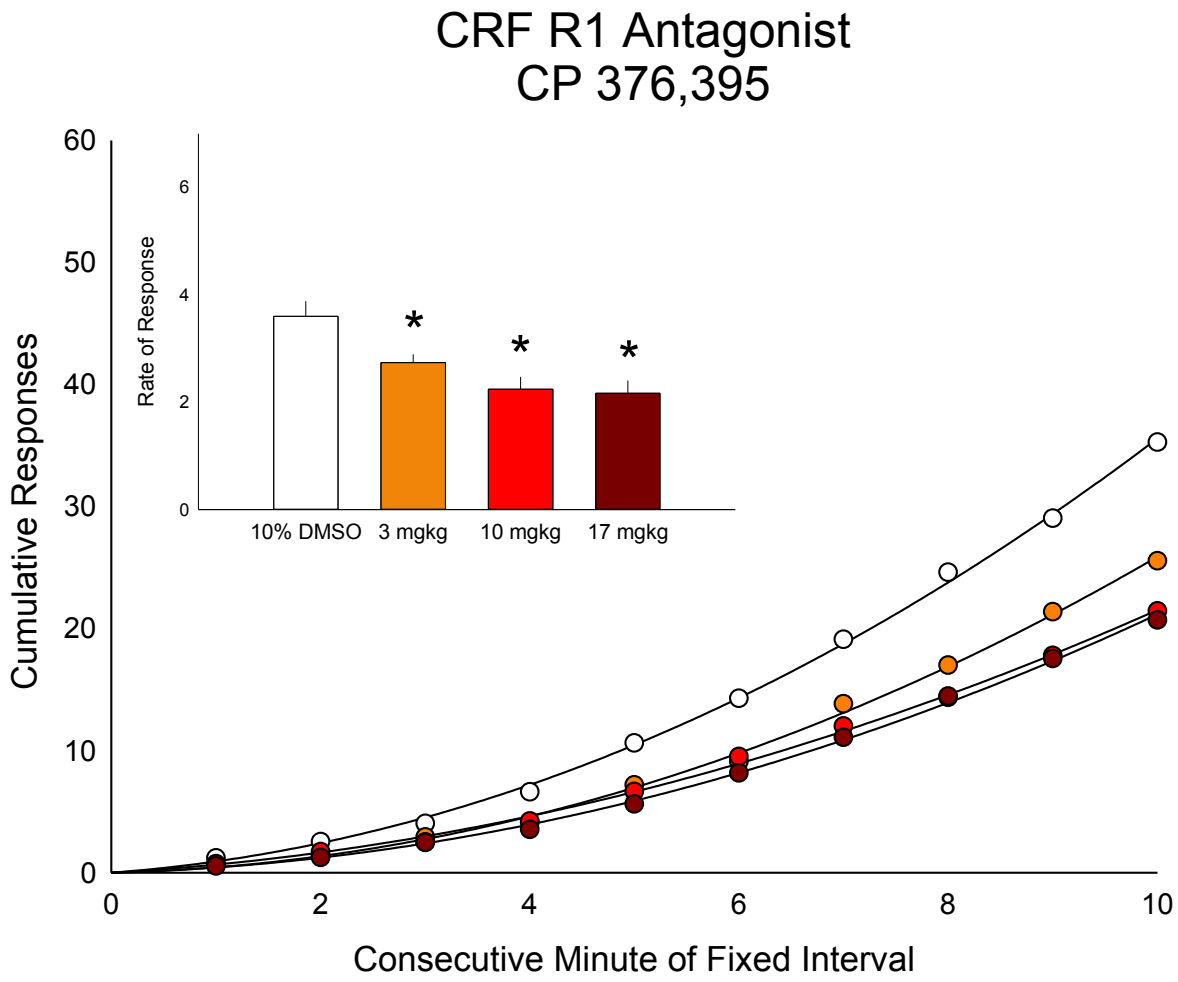


Figure 1. shows the effect of CP 376,395 on measures of responding during the FI10-min schedule reinforced by presentation of an intruder. The scatter plot represents the average cumulative responses through the duration of the FI10-min schedule with administration of 10% DMSO or CP 376,395 i.p. A second-order regression curve was superimposed. The bar graph represents the mean rate of response during the FI10-min ± SEM (vertical lines). Asterisks denotes values that are significantly different from vehicle, $p<0.05$.

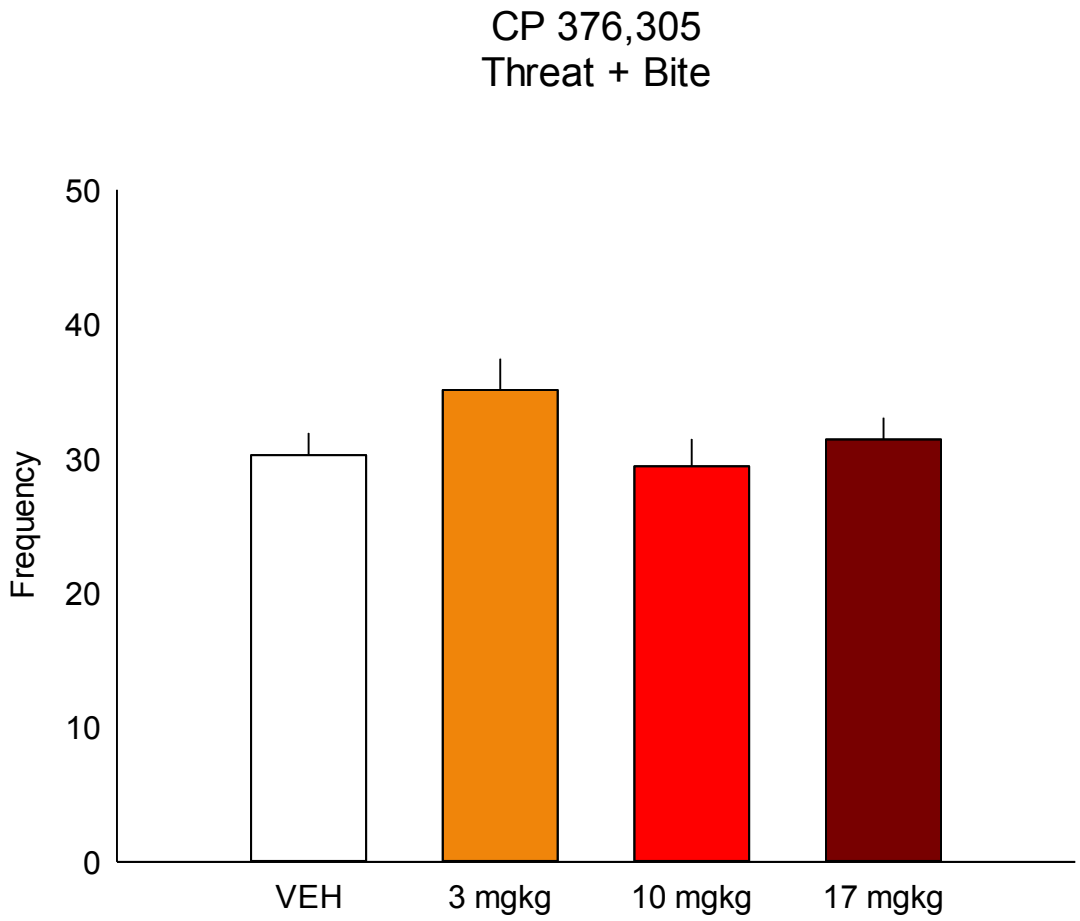


Figure 2. shows the effect of CP 376,395 on the frequency of sideways threat and attack bites during the aggressive confrontation with an intruder upon completion of the FI10-min. The aggressive confrontation lasted for 60-seconds. SEM is represented by the vertical lines. Asterisks denotes values that are significantly different from vehicle, $p<0.05$.

CRF R1 Antagonist, CP 376,395

	First Minute of FI-10				Last Minute of FI-10				Minute of Intruder Presence			
	10% DMSO	3.0 mg/kg	10.0 mg/kg	17.0 mg/kg	10% DMSO	3.0 mg/kg	10.0 mg/kg	17.0 mg/kg	10% DMSO	3.0 mg/kg	10.0 mg/kg	17.0 mg/kg
Latency to 1st Bite Duration (s)	-	-	-	-	-	-	-	-	2.1 ± .11	3.2 ± .76	2.5 ± .33	2.5 ± .37
Pursuit Frequency	-	-	-	-	-	-	-	-	3.5 ± .68	3.3 ± .64	3.8 ± .99	3.4 ± .72
Tail Rattle Frequency	-	-	-	-	-	-	-	-	5.6 ± .74	7.0 ± .68	6.6 ± .69	6.9 ± .86
Contact Frequency	-	-	-	-	-	-	-	-	0.067 ± .07	0	0.167 ± .14	0
Walking Duration (s)	20.1 ± 1.9	21.2 ± .84	20.0 ± .82	19.7 ± .77	14.4 ± .71	13.9 ± .6	12.8 ± .99	14.3 ± .89	16.8 ± .81	18.3 ± .78	18.1 ± .72	16.5 ± .72
Rearing Duration (s)	12.3 ± .86	13.3 ± .57	12.2 ± .62	10.7 ± .84	11.0 ± .95	13.1 ± .98	10.1 ± .79	13.3 ± 1.5	2.1 ± .69	1.8 ± .53	2.8 ± .73	3.1 ± .52
Self-Groom Duration (s)	2.48 ± .18	2.3 ± .49	2.8 ± .59	3.0 ± .56	1.7 ± .54	1.3 ± .55	3.2 ± 1.09	1.7 ± .77	0.96 ± .85	0.24 ± .14	0.04 ± .03	0.07 ± .05
Digging Duration (s)	1.54 ± .5	0.69 ± .22	1.9 ± .76	2.4 ± .8	6.7 ± 1.35	6.3 ± 1.6	8.8 ± 1.9	5.4 ± 1.9	0	0.1 ± .1	0.03 ± .03	0
Jumping Frequency	5.8 ± 1.96	2.7 ± .62	2.6 ± .68	3.5 ± .88	4.9 ± .63	6.4 ± 1.7	2.1 ± .52	4.2 ± .84	0	0.72 ± .52	0.2 ± .17	5.9 ± 3.4

Table 1. shows the effect of CP 376,395 on the first minute and last minute of the FI10-min schedule and the minute of the intruder present on behaviors analyzed. Duration (seconds) or frequency of specific behaviors are shown with ±SEM. Bolded values denote significant difference form vehicle, $p<0.05$.

Experiment II. Results

After Experiment I, there was a 1-week washout period, where mice were habituated to oral gavage before the administration of ethanol. The administration of ethanol via gavage dose-dependently decreased the rate of responding in the active nose poke hole ($F(3,13) = 14.238$, $p=.001$; Figure 3). Post-hoc analysis revealed no significant difference compared to water in response rate at the lowest dose, 0.5 g/kg. With increasing doses of ethanol, 1.0 g/kg and 1.8 g/kg, there was a significant decrease in rate of response. With administration of 1.8 g/kg of ethanol, the scalloping effect of the FI10-min schedule is not present and the second-order regression plot appears as a linear line.

Behavioral analysis of the aggressive confrontation after the fixed interval schedule revealed no significant difference in the frequency of sideways threat and attack bites ($F(3,13) = 7.375$, $p=.001$; Figure 4) with increasing doses of ethanol. But at the lowest dose, 0.5 g/kg, there is an increase in frequency of sideways threat and attack bite compared to the water condition. With administration of the highest dose of ethanol, 1.8 g/kg, there was a significant increase in latency to first bite ($F(3,13) = 4.225$, $p=.011$; Table 2) and frequency of contact ($F(3,13) = 7.113$, $p=.001$; Table 2) and a significant decrease in frequency of tail rattle ($F(3,13) = 9.006$, $p=.001$; Table 2).

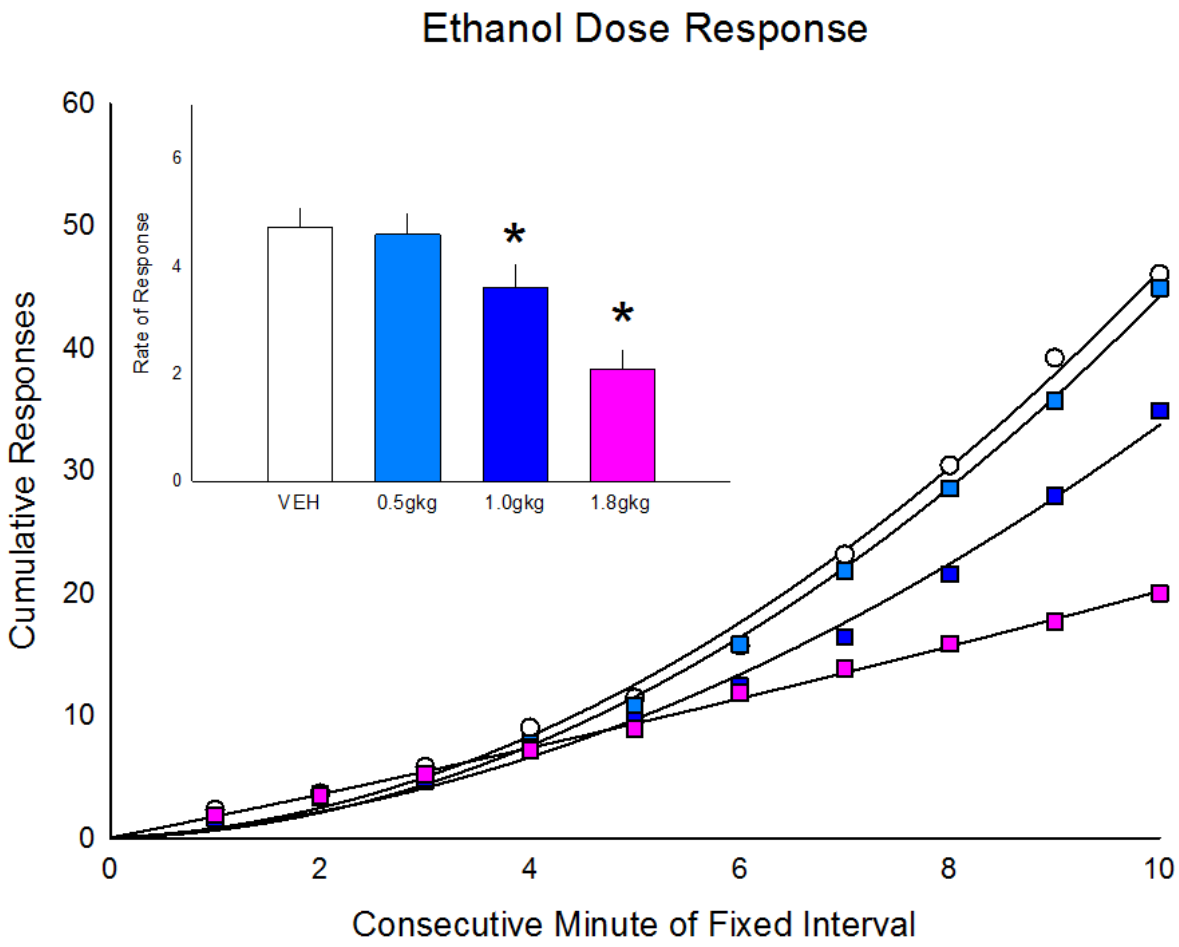


Figure 3. shows the effect of ethanol on measures of responding during the FI10-min schedule reinforced by presentation of an intruder. The scatter plot represents the average cumulative responses through the duration of the FI10-min schedule with administration of water or ethanol via oral gavage. A second-order regression curve was superimposed. The bar graph represents the mean rate of response during the FI10-min ± SEM (vertical lines). Asterisks denotes values that are significantly different from vehicle, $p<0.05$.

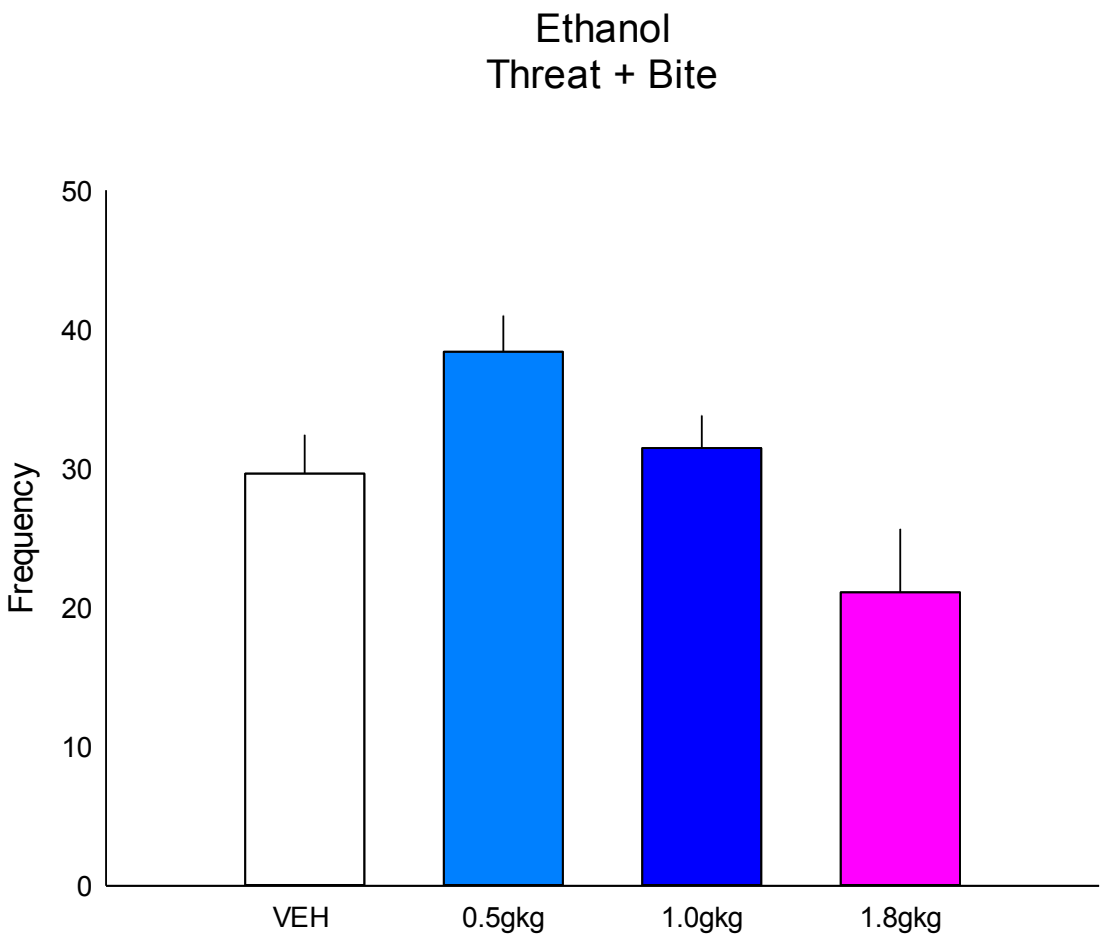


Figure 4. shows the effect of ethanol on the frequency of sideways threat and attack bites during the aggressive confrontation with an intruder upon completion of the FI10-min. The aggressive confrontation lasted for 60-seconds. SEM is represented by the vertical lines. Asterisks denotes values that are significantly different from vehicle, $p<0.05$.

Ethanol Dose Response

	First Minute of FI-10				Last Minute of FI-10				Minute of Intruder Presence			
	Water	0.5 g/kg	1.0 g/kg	1.8 g/kg	Water	0.5 g/kg	1.0 g/kg	1.8 g/kg	Water	0.5 g/kg	1.0 g/kg	1.8 g/kg
Latency to 1st Bite Duration (s)	-	-	-	-	-	-	-	-	3.1 ± .44	3.3 ± .7	7.8 ± 2.0	**11.8 ± 3.5**
Pursuit Frequency	-	-	-	-	-	-	-	-	2.7 ± .56	2.0 ± .42	2.79 ± .53	1.4 ± .54
Tail Rattle Frequency	-	-	-	-	-	-	-	-	6.9 ± .97	7.6 ± .63	6.3 ± .87	**3.1 ± .59**
Contact Frequency	-	-	-	-	-	-	-	-	0.32 ± .18	0.18 ± .12	0.8 ± .34	**2.5 ± .86**
Walking Duration (s)	20.0 ± .96	20.5 ± .98	20.3 ± 1.3	19.6 ± 1.4	13.3 ± 1.0	13.2 ± .63	12.8 ± .95	10.6 ± 1.2	22.1 ± .69	20.7 ± .64	19.8 ± .72	18.2 ± 1.7
Rearing Duration (s)	9.2 ± .95	12.1 ± .84	8.9 ± .89	6.9 ± .91	12.3 ± 1.7	13.0 ± 1.1	9.5 ± 1.0	5.1 ± .82	2.5 ± .59	2.1 ± .5	2.0 ± .63	1.5 ± .68
Self-Groom Duration (s)	3.1 ± .63	2.8 ± .64	5.0 ± .87	2.8 ± .68	1.7 ± .86	2.2 ± .82	1.8 ± .81	1.6 ± .89	0.09 ± .06	0.04 ± .04	0.02 ± .02	0
Digging Duration (s)	0.92 ± .29	1.68 ± .53	0.35 ± .17	0.46 ± .16	2.5 ± .61	2.3 ± .89	2.1 ± .41	2.0 ± .56	0.05 ± .05	0.08 ± .08	0.06 ± .04	0
Jumping Frequency	1.0 ± .63	2.9 ± .70	0.57 ± .39	0.29 ± .19	2.2 ± .59	5.5 ± .83	4.0 ± .98	2.8 ± 1.0	0.01 ± .01	0.07 ± 0.07	0.04 ± .04	0

Table 1. shows the effect of ethanol on the first minute and last minute of the FI10-min schedule and the minute of the intruder present on behaviors analyzed. Duration (seconds) or frequency of specific behaviors are shown with ± SEM. Bolded values denote significant difference form vehicle, $p<0.05$.

Experiment III. Results

After Experiment II, the cohort of mice was administered ethanol 1.8 g/kg daily for six days 10-minutes before the start of the FI10-min schedule. The initial dose of ethanol 1.8 g/kg in Experiment II significantly decreased the cumulative response rate during the FI10 schedule reinforced by presentation of an intruder (Figure 3). But repeated doses of ethanol 1.8 g/kg increased the response rate and there was a significant increase after Day 3 ($F(7,17) = 12.392$, $p=.001$; Figure 5). This significant increase rate of response was present after expression challenges of ethanol 1.0 g/kg, fourteen and sixty days after the repeated ethanol 1.8 g/kg ($F(3,13) = 4.55$, $p=.009$; Figure 5).

Behavioral analysis of the aggressive confrontation after the fixed interval schedule revealed a significant decrease in the frequency of sideways threat and attack bites ($F(3,13) = 12.701$, $p=.001$; Figure 6) with repeated daily doses of ethanol 1.8 g/kg. On Days 1, 3, and 5 of repeated doses, there is a significant increase in latency to first bite ($F(3,13) = 15.599$, $p=.001$; Table 3) and frequency of contact with the intruder ($F(3,13) = 6.156$, $p=.002$; Table 3), while the frequency of tail rattle significantly decreases ($F(3,13) = 9.907$, $p=.001$; Table 3).

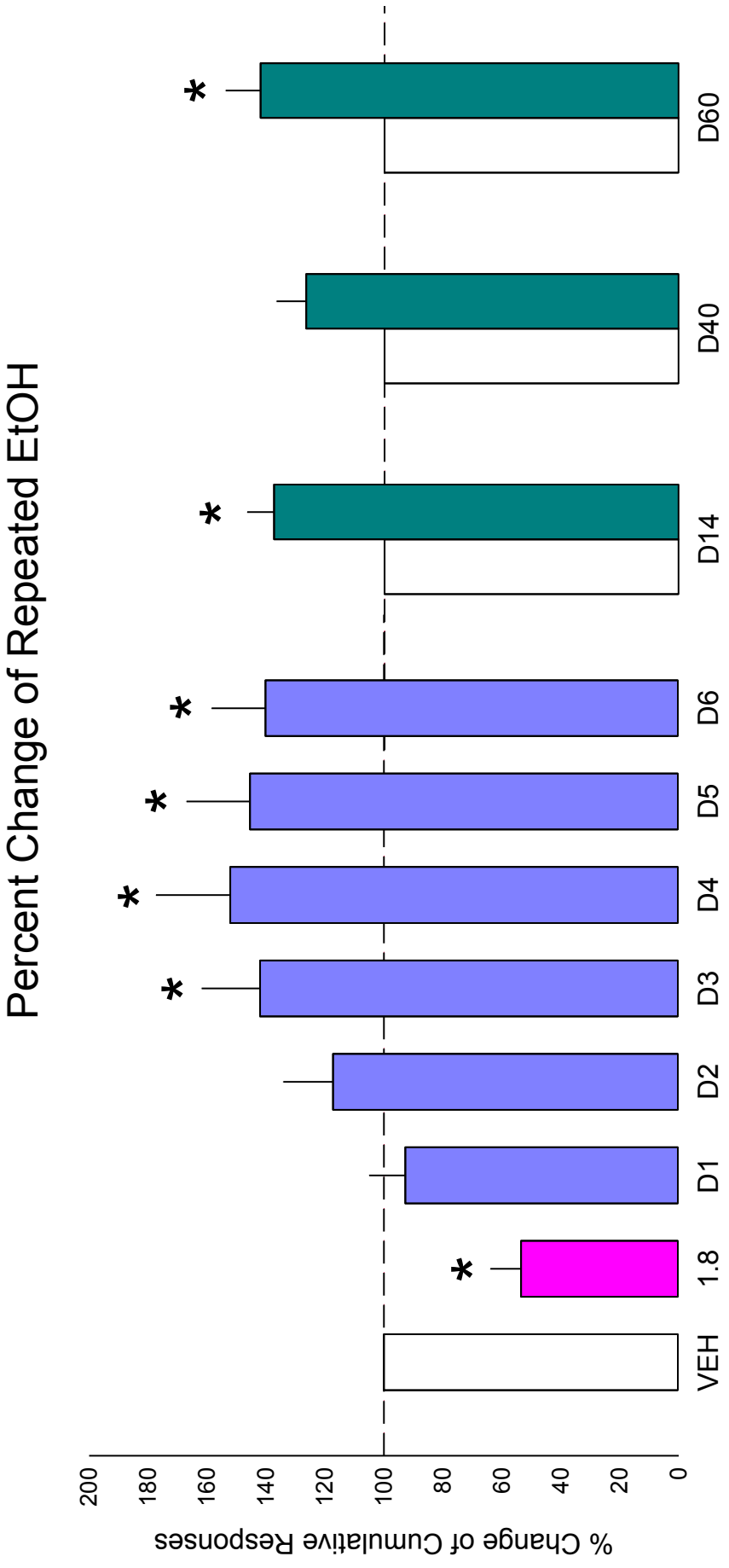

Percent Change of Repeated EtOH
% Change of Cumulative Responses
200
180
160
140
120
100
80
60
40
20
0
VEH
1.8
D1
D2
D3
D4
D5
D6
D14
D40
D60
*
*
*
*
*
*
*

Figure 5. shows the effect of repeated doses of ethanol 1.8 g/kg and expression challenges of ethanol 1.0 g/kg on the responding rate during the FI10-min schedule reinforced by presentation of an intruder. All of the data are expressed as mean percent change from baseline (i.e. averaged vehicle values for each individual mouse) ± SEM (vertical lines). Asterisks denotes values that are significantly different from vehicle, $p<0.05$.

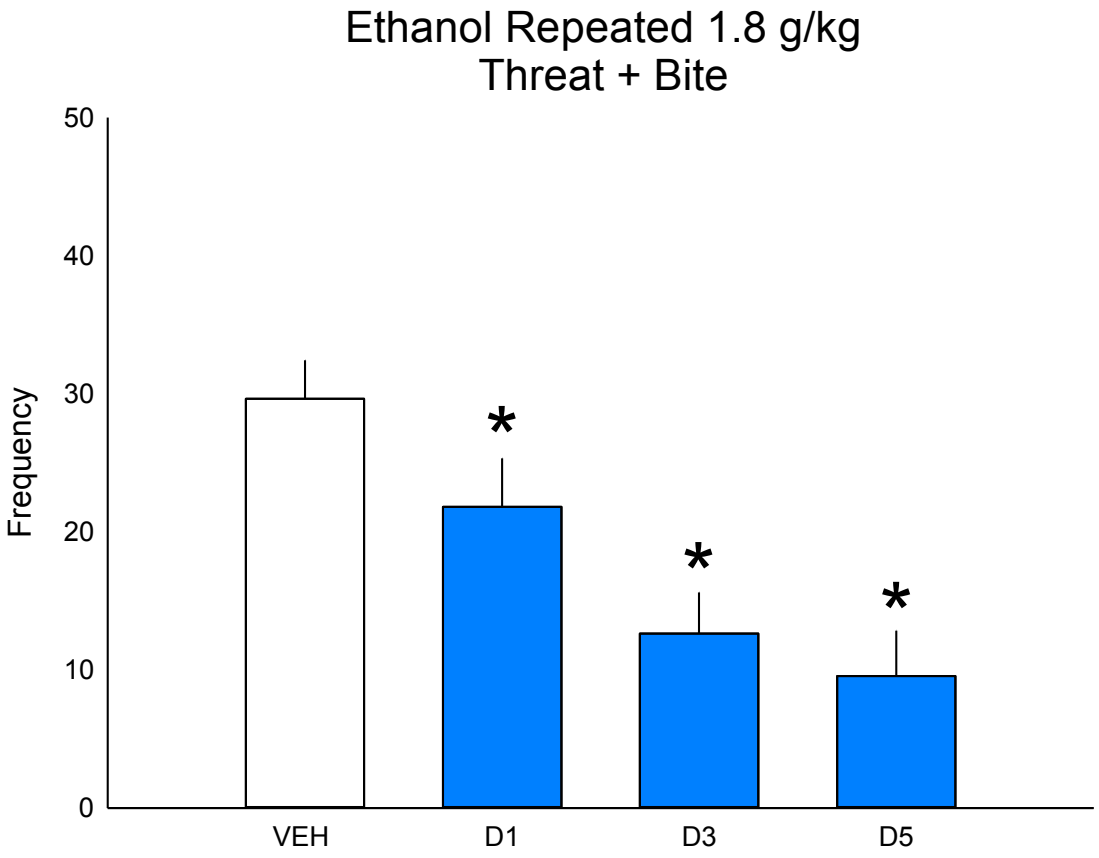


Figure 6. shows the effect of repeated ethanol 1.8 g/kg/daily on the frequency of sideways threat and attack bites during the aggressive confrontation with an intruder upon completion of the FI10-min. The aggressive confrontation lasted for 60-seconds. SEM is represented by the vertical lines. Asterisks denotes values that are significantly different from vehicle, $p<0.05$.

Repeated EtOH 1.8g/kg

	First Minute of FI-10				Last Minute of FI-10				Minute of Intruder Presence			
	Water	D1	D3	D5	Water	D1	D3	D5	Water	D1	D3	D5
Latency to 1st Bite Duration (s)	-	-	-	-	-	-	-	-	3.1 ± .44	**24.7 ± 5.6**	**28.4 ± 6.7**	**41.9 ± 6.7**
Pursuit Frequency	-	-	-	-	-	-	-	-	2.7 ± .56	3.3 ± .90	1.5 ± .56	1.79 ± .92
Tail Rattle Frequency	-	-	-	-	-	-	-	-	6.9 ± .97	**4.3 ± 1.0**	**2.4 ± .55**	**1.9 ± .63**
Contact Frequency	-	-	-	-	-	-	-	-	0.32 ± .18	**2.4 ± .87**	**2.0 ± .6**	**2.9 ± .66**
Walking Duration (s)	20.0 ± .96	19.9 ± 1.1	21.3 ± .88	22.3 ± .87	13.3 ± 1.0	13.3 ± 1.1	14.4 ± 1.0	22.3 ± 1.3	22.1 ± .69	21.6 ± 1.1	22.2 ± .51	34.1 ± 2.78
Rearing Duration (s)	9.2 ± .95	9.8 ± 1.1	9.8 ± .66	9.6 ± .69	12.3 ± 1.7	6.3 ± .81	6.6 ± .54	6.2 ± .87	2.5 ± .59	3.9 ± .75	8.3 ± .87	8.6 ± 1.2
Self-Groom Duration (s)	3.1 ± .63	4.5 ± .77	3.5 ± .75	3.6 ± .29	1.7 ± .86	3.8 ± 1.2	0.91 ± .52	2.2 ± 1.1	0.09 ± .06	0	0.14 ± .14	0.02 ± .02
Digging Duration (s)	0.92 ± .29	0.45 ± .18	0.90 ± .25	1.0 ± .2	2.5 ± .61	1.97 ± .51	2.6 ± .52	3.1 ± .66	0.05 ± .05	0	0.07 ± .06	0
Jumping Frequency	1.0 ± .63	0.54 ± .32	1.4 ± .40	3.7 ± .93	2.2 ± .59	2.8 ± .75	7.8 ± 1.7	6.9 ± 1.4	0.01 ± .01	0.39 ± .22	0.57 ± .16	1.2 ± .37

Table 3. shows the effect of repeated ethanol 1.8 g/kg/daily on the first minute and last minute of the FI10-min schedule and the minute of the intruder present on behaviors analyzed. Duration (seconds) or frequency of specific behaviors are shown with ± SEM. Bolded values denote significant difference form vehicle, $p<0.05$.

Experiment IV. Results

The cohort of mice was habituated again to i.p. injections and oral gavage. Twenty days after repeated ethanol 1.8 g/kg, administration of CP 376,395 i.p. and ethanol 1.0 g/kg were given to assess the role of the CRF R1 antagonist on the increased rate of response induced by ethanol (Figure 7). With the administration of ethanol alone, there was a statistically significant increase in the cumulative responses compared to control. When CP 376,395 i.p. was given in conjunction with ethanol, there was a decrease in cumulative responses compared to ethanol alone. At the lowest dose of CP 376,395 (3 mg/kg), there was still a significant increase in cumulative responses compared to vehicle condition ($F(2,13) = 6.856$, $p = .004$). At the highest dose of CP 376,395 (10 mg/kg), the cumulative responses were lower than the vehicle condition, but were not significant.

Behavioral analysis of the aggressive confrontation after the fixed interval schedule showed no significant difference in the frequency of sideways threat and attack bites when ethanol was given in conjunction with CP 376,395 (Figure 8). When ethanol and CP 376,395 were administered, there was a significant increase in the frequency of tail rattles ($F(2,13) = 10.103$, $p = .001$; Table 4). There was a significant increase in the duration of contact when CP 376,395 (3 mg/kg) was given with ethanol ($F(2,13) = 3.459$, $p = .048$).

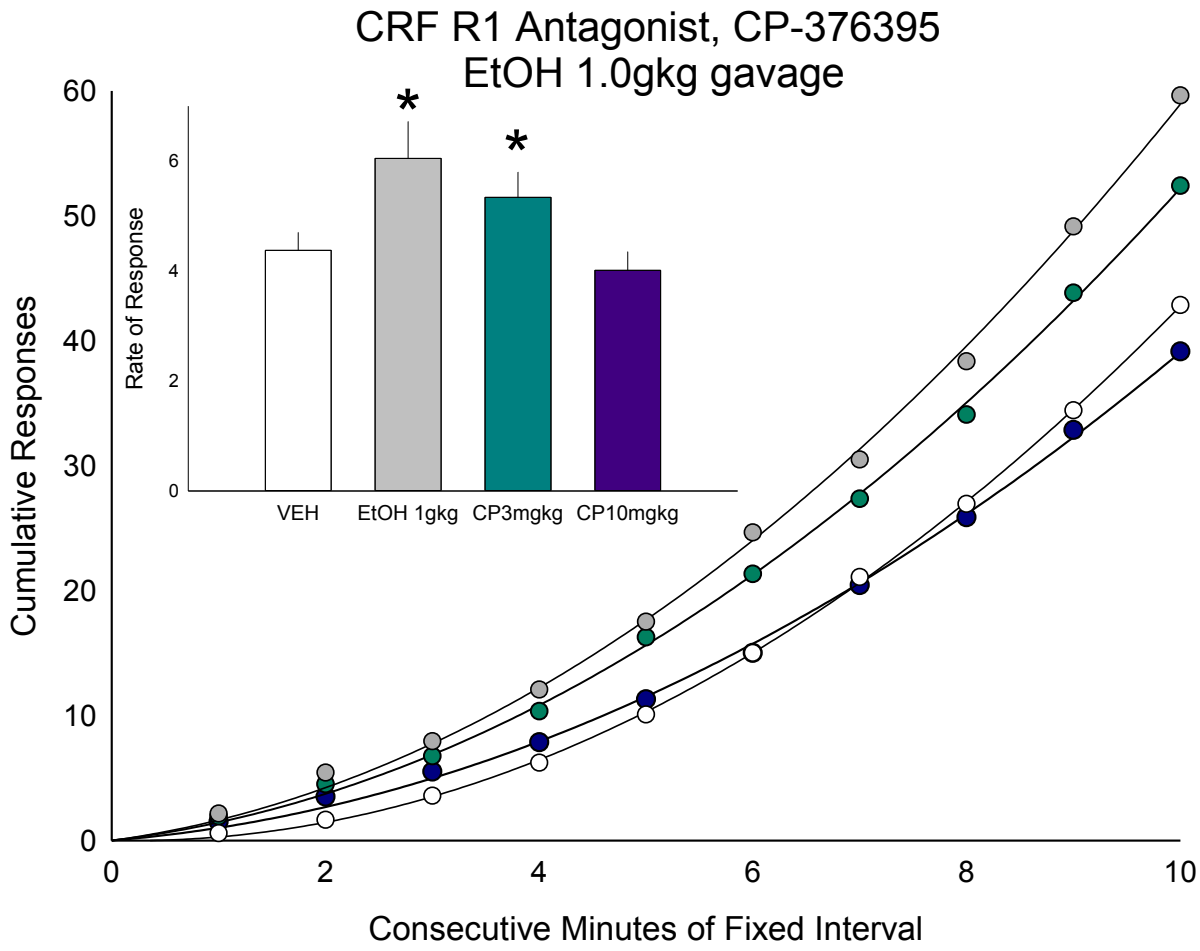


Figure 7. shows the effect of CP 376,395 in conjunction with ethanol (1 g/kg) on measures of responding during the FI10-min schedule reinforced by presentation of an intruder. The scatter plot represents the average cumulative responses through the duration of the FI10-min schedule with administration of 10% DMSO i.p. and water via gavage or CP 376,395 i.p. and ethanol via gavage. A second-order regression curve was superimposed. The bar graph represents the mean rate of response during the FI10-min ± SEM (vertical lines). Asterisks denotes values that are significantly different from vehicle, $p<0.05$.

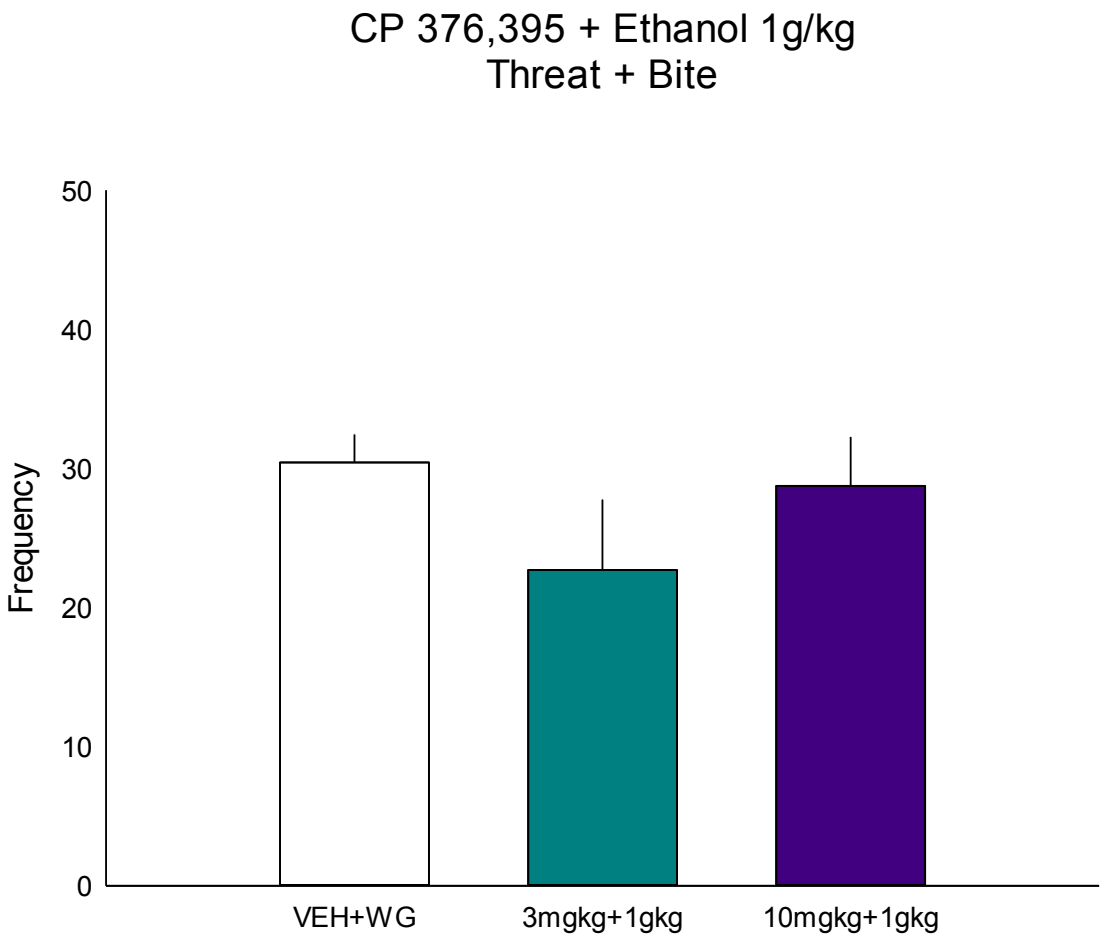


Figure 8. shows the effect of CP 376,395 in conjunction with ethanol (1 g/kg) on the frequency of sideways threat and attack bites during the aggressive confrontation with an intruder upon completion of the FI10-min. The aggressive confrontation lasted for 60-seconds. SEM is represented by the vertical lines. Asterisks denotes values that are significantly different from vehicle, $p<0.05$.

CP 376,395 + EtOH 1g/kg

	First Minute of FI-10			Last Minute of FI-10			Minute of Intruder Presence		
	10%DMSO + EtOH 1g/kg	CP 3mg/kg + EtOH 1g/kg	CP 3mg/kg + EtOH 1g/kg	10%DMSO + EtOH 1g/kg	CP 3mg/kg + EtOH 1g/kg	CP 3mg/kg + EtOH 1g/kg	10%DMSO + EtOH 1g/kg	CP 3mg/kg + EtOH 1g/kg	CP 3mg/kg + EtOH 1g/kg
Latency to 1st Bite Duration (s)	-	-	-	-	-	-	3.6 ± .52	18.6 ± 7.3	12.2 ± 5.3
Pursuit Frequency	-	-	-	-	-	-	2.0 ± .66	0.08 ± .08	1.2 ± .44
Tail Rattle Frequency	-	-	-	-	-	-	7.9 ± .84	**3.4 ± .83**	**4.7 ± .74**
Contact Frequency	-	-	-	-	-	-	0.32 ± .12	**3.0 ± 1.1**	1.5 ± .92
Walking Duration (s)	16.8 ± .7	19.8 ± .73	20.5 ± .8	11.8 ± .99	13.3 ± 1.2	13.8 ± 1.3	20.2 ± .77	20.0 ± 1.1	21.2 ± .9
Rearing Duration (s)	12.6 ± .88	10.8 ± .62	10.6 ± .88	13.8 ± 1.2	11.2 ± 1.2	9.2 ± .93	6.0 ± .64	6.8 ± 1.1	7.3 ± 1.3
Self-Groom Duration (s)	3.2 ± .65	4.0 ± .31	2.5 ± .60	1.8 ± .89	0.47 ± .47	3.6 ± 1.5	0.35 ± .35	0.02 ± .02	0.02 ± .02
Digging Duration (s)	1.9 ± .46	1.7 ± .56	0.93 ± .23	2.8 ± .9	5.6 ± 1.5	2.1 ± .6	0.14 ± .08	0.02 ± .02	0
Jumping Frequency	3.1 ± .66	2.8 ± .63	1.9 ± .46	3.8 ± .65	6.3 ± 1.3	4.9 ± .9	0.29 ± .13	0.42 ± .2	0.36 ± .19

Table 4. shows the effect of CP 376,395 in conjunction with ethanol (1 g/kg) on the first minute and last minute of the FI10-min schedule and the minute of the intruder present on behaviors analyzed. Duration (seconds) or frequency of specific behaviors are shown with ± SEM. Bolded values denote significant difference form vehicle, $p<0.05$.

Blood Ethanol Concentration Analysis

Blood Ethanol Concentration (mg/dl)	
Mouse ID	BEC (mg/dl)
39	76.7
40	97.1
41	80.9
42	108.0
43	70.1
46	98.6
49	124
50	104.5
51	105.6
52	126.2
53	125.5

Table 5. shows the blood ethanol concentration within blood samples of each individual 10-minutes post ethanol 1.0 g/kg. Blood ethanol concentration is measured in mg/dl.

Discussion

Male mice will work for the opportunity to engage in an aggressive confrontation under a fixed-interval schedule of reinforcement. This operant conditioning methodology generates behaviors that lead up to the aggressive confrontation, which reliably quantifies measures aggressive motivation. Aggressive arousal and the anticipation of the aggressive encounter can be observed in the low response rates in the beginning of the interval and an acceleration of the rate of response as the interval progresses (Skinner 1938; Dews 1970). The rate of response under the fixed-interval schedule of reinforcement may be a quantitative measure of the motivational component of an aggressive confrontation. The present study supports previous evidence that aggression may be reinforcing in animals and humans (Ginsburg and Allee 1942; Thompson 1969; Connor 1974; Potegal 1979; Martinez et al 1995; Fish et al. 2002). The present results highlight the dual role of CRF R1 and alcohol in modulating the motivation for an aggressive encounter and the aggressive performance.

Corticotrophin-releasing factor may be upregulated during operant responding on the fixed-interval schedule because of the slight elevated levels of corticosterone under this schedule of reinforcement where aggression is the reinforcer (Fish, DeBold, & Miczek, 2005). Stressors, like reward seeking behaviors, can affect dopamine-dependent circuits modulated by CRF (Wanat et al., 2013). This motivated behavior is facilitated by activation of the mesolimbic dopamine projections from the VTA to the NAc (Salamone et al., 2009). Previous studies demonstrated that corticosterone is involved in the maintenance of operant responding and has its own reinforcing effects (Deroche et al. 1993; Piazza et al. 1993). In Experiment I, the results show that CRF R1 antagonism can

decrease the rate of response under the fixed-interval schedule of reinforcement. The lowest dose of CP 376,395, 3.0 mg/kg, significantly reduced the rate of responding under the FI10 schedule. With increasing doses, there is also a significant reduction in rate of response. Although there is a reduction in the motivational component captured by the FI10 schedule, there is no significant difference in aggressive and nonaggressive behaviors due to the administration of CP 376,395. Overall, the results of Experiment I are consistent with previous studies that demonstrate corticosterone is necessary for operant responding under the fixed-interval schedule. The results also suggest the role of CRF in modulating the motivation for an aggressive confrontation.

The consumption of alcohol has been strongly linked to the escalation of aggression in a subgroup of humans—half of all violent incidents, including assaults, child abuse, and domestic abuse (Rossow & Bye, 2013). The motivation to seek out violence can be linked to the rewarding effects of aggression and alcohol—where alcohol modulates dopaminergic neuron whose cell bodies reside in the ventral tegmental area (VTA) (Arias-Carrion et al., 2010). Preclinical studies reflect this subgroup of human individuals, where a moderate dose of alcohol escalates aggressive behaviors (Miczek, Fish, et al., 2004). In CFW strain mice, alcohol (1.0 g/kg) heightens aggressive behaviors by at least two standard deviations of the individual's vehicle control in 27% of the mice (Miczek, Barros, et al., 1998). This subgroup of the population tested is referred to alcohol-heightened aggressors (AHA). In Experiment II, varying doses of alcohol were administered to investigate the effects of alcohol on performance of the fixed-interval schedule of reinforcement and performance of the aggressive behaviors. The results show that increasing alcohol doses attenuate both aggressive motivation on the FI10 schedule

and aggressive behaviors. This can be due to the biphasic effects of alcohol, where low doses can facilitate behaviors while higher doses can have sedative effects (Pohorccky, 1977). In the lowest dose administered (0.5 g/kg), it had no significant effect on rate of response during the FI10 schedule and increased the frequency of sideways threat and attack bites compared to water control. This increase in threats and bites were not significant, but it suggests that 0.5 g/kg is the peak alcohol dose that heightens aggressive behaviors in the C57BL/6J mouse strain.

Repeated alcohol exposure promotes neuroadaptation in brain reward pathways (Robinson & Berridge, 1993, 2001). Repeated alcohol dosing alters mesocorticolimibic dopamine and glutamate activity (Broadbent & Weitemier, 1999; Souza-Formigoni et al., 1999). The investigation of repeated exposure to alcohol is critical to understanding how behaviors are changed over time. In Experiment III, daily administration of the moderate dose (1.8 g/kg) of alcohol significantly increased the rate of response on the FI10 schedule of reinforcement. This significant increase in rate of response is long lasting and persists for 60 days post-repeated alcohol administration. The present study shows that repeated alcohol (1.8 g/kg) administration increases the motivation for the opportunity to fight another male mouse, but it significantly decreases the frequency of sideways threat and attack bites. The attenuation of aggressive behaviors may be due to exhaustion because the aggressive confrontations lasted for 60-seconds for six consecutive days—which has not been previously investigated. Leshner and Nock (1976) observed that mice prevented from fighting to "satiety" were more aggressive on subsequent confrontations. This could be a possible reason why the present study observed a decrease in the aggressive performance.

The neuroadaptative changes due to repeated alcohol exposure may require CRF (Pastor et al., 2008; Pastor et al., 2012). Corticotrophin-releasing factor may mediate the effects of alcohol and may be required in its rewarding effects of aggression (Caramaschi et al., 2008; Hsu et al., 2006; Quadros et al., 2014). This is consistent with the upregulation of CRFR1 subtype after alcohol exposure (Dallman, 2005; Lee & Rivier, 1997a, 1997b). In Experiment IV, the results show that the increased rate of response due to repeated alcohol administration can be modulated by the CRF R1 antagonist, CP 376,395. The administration of CP 376,395 (3 mg/kg) and alcohol (1.0 g/kg) is significantly increased compared to vehicle control. Compared with the CP 376,395 (10.0 mg/kg) and alcohol (1.0 g/kg) condition, there is a decrease in the rate of response due to a higher dose of CP 376,395. But there is no significant effect on the performance of the aggressive behavior. The results suggest that the increased rate of response due to administration of alcohol depends on the activation of CRF R1. Previous studies suggest that CRF directly modulates brain reward circuitry in the ventral tegmental area, nucleus accumbens, and the prefrontal cortex (Phillips et al., 2015). The rewarding effects of alcohol exposure mediated by CRF may play a role in the dopaminergic systems induced by alcohol. Aggressive behaviors, acute alcohol administration, and CRF may be necessary in the motivation of rewarding behaviors by modulating similar brain circuitry—captured by the FI10 schedule of reinforcement.

The challenge is to now to identify specific CRF-containing circuits that mediate the effects found in the present study. The systemic administration of CP 376,395 was useful in investigating the role of CRF R1 in aggressive motivation and performance, but it is crucial to further delineate the neural mechanisms involved. Recent technological

advances in viral-mediated gene transfer and optogenetics have made it possible to investigate the role of specific neuron populations in vivo (Williams & Deisseroth, 2013; Witten et al., 2011). Extra-hypothalamic CRFergic neurons are found in the central nucleus of the amygdala (CeA), bed nucleus of the stria terminialis (BNST), locus coerruleus, ventral tegnmental area (VTA), and nucleus accumbens (NAc), which may be important brain regions that mediate the motivation for and the initiation of aggression (Merchenthaler et al. 1982, 1984; Morin et al. 1999; Steckler & Holsboer 1999; Swanson et al. 1983). The dopaminergic pathway from the VTA to the NAc and the mesolimbic pathway are implemented in reward and reinforcement of behaviors (Ungless et al., 2003; Wang et al., 2007a; Hahn et al., 2009; Berridge, 2007). CRF may be colocalized in these dopaminergic pathways that are modulating the motivation and rewarding effects of aggression. Optogenetic protocols using a light-activated cation channel, channelrhodopsin (ChR2), can be used to differentiate the aggression-specific DA cells from those that mediate other motivated behaviors (Russo & Nestler, 2013).

The present study utilizes an operant conditioning protocol, the fixed-interval schedule of reinforcement, which quantitatively captures the motivation for an aggressive confrontation with a male intruder mouse (Skinner 1938; Dews 1970). The fixed-interval schedule of reinforcement is a robust behavioral paradigm, but there are limiting previous studies. The present study trained and maintained the FI10 schedule within a cohort of mice over a span of three months, where animals were tested daily. Because of the understudied nature of the fixed-interval schedule of reinforcement, it is unknown whether repeated testing affects the motivation for a rewarding behavior. In previous studies that utilized the fixed-interval schedule for the opportunity to run on a running

wheel, there is a quantitative difference in how much animals nose-poked compared to the results of the present study (Fish et al., 2008). In Fish et al (2002), mice were continuously allowed to perform on the operant conditioning panel for the opportunity to run on a wheel. But in the present study, mice were only tested once daily. The repeated testing over the span of three months may have consequences on the present study and what was quantified. The investigation of other salient rewarding behaviors and its motivational component can utilize the fixed-interval schedule of reinforcement to delineate the specific brain circuits necessary in motivating different rewarding behaviors—like the opportunity to copulate and to exercise on a running wheel (Everitt & Stacey, 1987). Current experiments are investigating other reinforced behaviors, including sexual behavior. In this experiment, male mice are trained on the fixed-interval schedule for the opportunity to mate—where a cycling female mouse is introduced into the cage. The fixed-interval schedule can also be a novel behavioral paradigm in understanding drug abuse and the motivation for drug taking (Robbins et al., 1989; Rezayof, Zarrindast, Sahraei, & Haeri-Rohani, 2002).

In conclusion, CP 376,395 produced a behaviorally specific reduction in the rate of response under the fixed-interval schedule of reinforcement, where the opportunity for an aggressive confrontation with an intruder mouse was reinforced. Although the motivational component for aggression was reduced, the performance of the aggressive behaviors was not affected. Repeated administration of alcohol can augment the rate of response of the fixed-interval schedule and this effect is long-lasting—suggesting that alcohol may be sensitizing the motivation for aggression. Moreover, CP 376,395 can attenuate this sensitized effect induced by alcohol. The present study supports the role of

CRF R1 in modulating the motivational component of aggression, which makes this type of compound particularly intriguing as a potential pharmacotherapeutic option. Using new methodologies in neuroscience, it may be possible to differentiate between brain circuitry and specific cell type neurons involved in the motivation for an aggressive confrontation (Dobrzanski & Kossut, 2017; Russo & Nestler, 2013).

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