CRF_1 Receptor Antagonist Injections Increase Medial Prefrontal Cortex Serotonin after Alcohol Self-Administration

Julia Carlson

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Julia Carlson, Isabel Quadros, Lara Hwa, Akiko Shimamoto, Joe DeBold, Klaus Miczek

Abstract

Aggressive behaviors comorbid with alcohol abuse are a public health crisis. These behaviors are historically correlated to an inverse concentration of serotonin in the central nervous system. Corticotropin-Releasing Factor (CRF) antagonists that mediate the physiological stress response have recently begun to be developed to treat alcohol abuse. As CRF modulates serotonergic release, it may affect aggressive behaviors. A previous behavioral study in our lab found that the CRF_1 antagonist CP-154,526 attenuated alcohol-heightened aggression in Carworth Farms Webster (CFW) mice. Based on this experiment, we decided to use in vivo microdialysis to evaluate the change in serotonin concentration in the medial prefrontal cortex following ethanol self-administration, CP-154,526 injection (17 mg/kg intraperitoneal and 0.6 µg microinjection into the dorsal raphe nucleus), and paired self-administration and acute drug injection. 1 g/kg ethanol self-administered was seen to significantly increase serotonin concentration. CP-154,526 i.p. administered had no overall effect, but trended towards increasing serotonin concentration. Acute injections following self-administration increased serotonin concentration, but the microinjection was only compared to its baseline as opposed to an inert vehicle injection. Overall, serotonin concentration increases following acute injection CP-154,526 and ethanol self-administration correlate to the anti-aggressive effects observed in the previous behavioral study. CP-154,526 may be a useful therapeutic in treating alcohol abuse comorbid with aggressive behaviors.

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Human Epidemiology of Alcohol and Violence

Violent and aggressive behaviors are a salient public health issue; according to the World Health Organization's (WHO) World Report on Violence and Health in 2000 approximately 1.6 million people worldwide died in a violence-related death, and is one of the leading causes of death in young people aged 15-29, particularly affecting young men. Aggression affects not only the victims, but the perpetrators and society at large, especially in the United States where 3.1% of the population or approximately 7.2 million offenders were under correctional supervision in 2009 (Glaze, 2010). Heuristically, criminals are defined as aggressive and violent individuals, and presently there are few viable treatment options besides incarceration (De Almeida, Ferrari, Parmigiani, & Miczek, 2005; Trainor, Sisk, & Nelson, 2009). Aggression is a broad term referring to a number of behaviors, and thus it is problematic to define by what the law denotes as aggressive.

Alcohol has been linked to escalated aggressive behaviors more than any other drug. Ethanol is considered one of two crime-inducing drugs, the other being phencyclidine (PCP or 'angel dust') (De Almeida et al., 2005; Virkkunen & Linnoila, 1993). According to the WHO's World Health Report on Substance Abuse (2002), approximately two billion people drink alcohol worldwide, and 76.3 million of them have diagnosable alcohol related disorders. They also report alcohol, like violence, as one of the leading factors relating to deaths of young people aged 15-29 worldwide, again affecting more men then women. In the United States, as reported in the Global Status Report on Alcohol (2004), approximately 7.7% of the population has

diagnosable alcohol addiction according to DSM-IV standards. Alcohol is also affecting the American criminal justice system, as 11% of all criminal incidents reported involved alcohol in the United States (Rand et al., 2010). As of 1996, nearly 36% of convicted offenders reported drinking alcohol during their crime (Greenfeld, 1998). Over time, there has been a decrease of incidents involving alcohol; as of 2008, only 19% of the victims of a violent crime perceived alcohol to be involved, reduced from a rate of 25% in 1997 (Rand et al., 2010). Alcohol is one of the most widespread drugs and has impact not only on health, but the criminal justice system as well.

Ethanol and Aggression

As a small and highly soluble molecule, ethanol once ingested affects every organ system, including the brain. Within the central nervous system, ethanol is well known to interact with a number of ligand-gated ion receptors (Table 1) (Starke, 1991; For a review, see Little, 1999). Once consumed, ethanol is broken down by liver enzymes in a highly conserved mechanism: first by alcohol dehydrogenase, and then usually by aldehyde dehydrogenase (Jacobson, 1952). After either oxidation step, acetaldehyde may enter the citric acid cycle (Figure 1) to be further metabolized (Westerfeld, 1961). Sensitivity, or the degree of physiological response, to the effects of alcohol may be linked to the activities of alcohol dehydrogenase or aldehyde dehydrogenase, as well as other genetic, sex, and environmental factors (Church, Fuller, & Dann, 1979; Sheppard, Albersehim, & McClearn, 1968). Ethanol presumably has many effects upon the body, some of which are well understood, and many of which have yet to be explored.

Ethanol has biphasic pharmacodynamics for many of its physiological and behavioral effects (De Almeida et al., 2005; Pohorecky, 1977). At low doses, it is excitatory and healthy, whereas at high doses it causes sedation and toxicity, and potentially death (Pohorecky, 1977). In humans, low doses of ethanol have behaviorally disinhibiting effects correlated by more frequent aggressive and impulsive behaviors (Pedersen, Aviles, Ito, Miller, & Pollock, 2002).

Aggression, a behavior frequently associated with alcohol ingestion at moderate doses, is defined as any behavior that intends to harm another individual emotionally or physically (Bailly & King, 2006; de Almeida, et al., 2005; Miczek, 2001; Niehoff, 1999). Specifically, pathological aggression as a maladaptive behavior is of interest to study in order to develop pharmaceuticals for better clinical treatment. Pathological aggression is also known as escalated aggression, or a rise in aggressive behaviors from an individual's baseline trait expression, and may be influenced by drugs, such as alcohol and other factors such as stress (de Almeida et al., 2005).

The prefrontal cortex (PFC) is one of the brain regions most associated with aggressive behaviors in both rodents and primates (Cambon et al., 2010). In humans, aggressive behaviors have been associated with deficits in the PFC, which is associated with controlling higher-order functions such as decision-making (Bassarath, 2001; Best, Williams & Coccaro, 2002; Golden, Jackson, Peterson-Rohne, & Gontkovsky, 1996; Siever, 2008; Veit et al., 2002). Lesioning this area during traumatic accidents is also often associated with personality changes that increase aggressive traits (Grafman et al., 1996). There may also be different roles of the primate prefrontal cortex depending on the subregion of the PFC involved, such as orbital, lateral, dorsal, or medial portions (Gansler et al., 2010; Lotze, Veit, Anders & Birmbaumer, 2007). Rodent studies support the role of the PFC, and particularly the infralimbic cortex, in influencing

aggressive behaviors (Halász et al., 2006; Haller et al., 2006). One of the most associated brain regions with aggressive behaviors is the PFC.

In humans, competitive games, administration of electric shocks against a fictitious opponent (Taylor paradigm), the point subtraction aggression paradigm, or functional MRI imaging paired with viewing of aggressive images or movies have been used to study alcohol-increased aggression (Bushman & Cooper, 1990; Cherek, 1981; Kareken et al., 2010; Pedersen et al., 2002; Taylor, 1967). In the Taylor paradigm, participants who consumed a moderate dose of ethanol were scored as more competitive and aggressive in the immediate period after consuming alcohol (the ascending limb) in contrast to either the descending limb of alcohol effects or sober control groups (Giancola & Zeichner, 1997). A small subpopulation of individuals who already scored a higher baseline level of aggression, did not show this same rise in aggression in a similar paradigm (Bailly & King, 2006), and the evidence for alcohol heightened aggression in both men and women in other studies has been mixed (Dougherty, Bjork, Bennet, & Moeller, 1999; Dougherty, Cherek, & Bennet, 1996; Moeller et al., 1998). Alcohol-induced aggression may also be influenced by several other factors in men including obesity and impulsivity (Cherek, Moeller, Doughter, & Rhoades, 1997; DeWall, Bushman, Giancola, & Webster, 2010). In humans most research has been limited by ethical restrictions; animal models allow for a better understanding of the molecular basis of alcohol-induced aggression.

Alcohol-induced or alcohol-heightened aggression is studied in a wide range of species, but most notably in mice, *Mus musculus*. Mice are often studied because they are easily induced to exhibit species-typical territorial aggressive behavior (Brain, 1975). This behavior is specific to males, is genetically selectable, and can be influenced by adolescent experiences in mice

(Miczek, Maxson, Fish, & Faccidomo, 2001; Quadros, Takahashi, & Miczek, 2010; Takahashi, Quadros, de Almeida, & Miczek, 2010; Van Oortmerssen & Bakker, 1981). From an ethological perspective, territorial aggression is a reactive aggressive behavior, which is adaptive for male mice as it benefits their reproductive opportunities both by protecting their female mates and by scattering other young male mice (De Almeida et al., 2005; Miczek & de Almeida, 2010; Miczek et al.,2001; Van Oortmerssen& Bakker, 1981). These male-male encounters also maintain the mouse social hierarchy, modeled in the laboratory as a resident-intruder paradigm (Miczek & O'Donnell, 1978). An intruder mouse is introduced into a resident's home cage, which quickly results in the resident male attacking the intruder (Miczek & O'Donnell, 1978). This paradigm can be easily adapted to study alcohol-heightened aggression.

Alcohol-heightened aggression has been observed in many species besides humans, including mice, rats, and monkeys (Miczek, Winslow, & DeBold, 1984). A specific subset of approximately 25-33% of individuals in a given population of subjects after consuming low to moderate doses of ethanol are classified as having alcohol-heightened aggression (Blanchard, Hori, Blanchard, & Hall, 1986; De Almeida et al., 2005; De Almeida, Saft, Rosa, & Miczek, 2010; Miczek, Barros, Sakoda, & Weerts, 1998; Miczek, Fish, & DeBold, 2003). Aggression is operationally defined in these papers, as in this one, as bite frequency, but some also considered other aggressive behaviors such as tail rattling, sideways threats, and chasing (Miczek & Barry, 1977). At a moderate dose range, there does not seem to be any significant changes to non-aggressive behaviors including grooming, motor function, and consciousness (De Almeida et al., 2010; Miczek et al., 1998). No simple relationship has been linked between the level of aggression in different individuals at the same dose of alcohol and alcohol metabolism using Blood Ethanol Content (BEC) (Benton & Smoothy, 1984; Miczek et al., 1998). As of yet, there

is a functional relationship between low to moderate doses of ethanol and heightened levels of aggression in a subset of animals but its mechanism has yet to elucidated.

Influence of Corticotropin-Releasing Factor on Alcohol Abuse and Aggression

Stress can be often associated with a relapse into alcohol abuse, and during withdrawal many alcoholics report feeling increased anxiety (Heilig & Koob, 2007; Valdez et al., 2002). Recently, neuropeptide drugs are beginning to be explored as possible therapies for alcohol abuse (Jupp & Lawrence, 2010). Corticotropin-releasing factor (CRF) is a 41-amino acid peptide neurotransmitter often associated with initiating the physiological stress response (Dunn & Swiergiel, 2008; Spiess, Rivier, Rivier, & Vale, 1981; Vale, Spiess, Rivier, & Rivier, 1981). The CRF receptor is a seven transmembrane-receptor with two subtypes (type 1 and type 2) coupled to a G-protein that activates adenylate cyclase (Chalmers, Lovenberg, & De Souza, 1995; Radulovic, Sydow, & Spiess, 1998; Spiess et al., 1998). CRF is secreted from the hypothalamus, and acts upon CRF_1 receptors to signal the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland. ACTH travels through the vascular system to reach the adrenal glands where it stimulates them to release glucocorticoids, cortisol in primates and corticosterone in many other species; this cascade is known as the hypothalamo-pituitary-adrenocortical (HPA) axis (Dunn & Swiergiel, 2008; Rivier, Brownstein, Spiess, Rivier, & Vale, 1982).

CRF_1 receptors have been found to specifically affect alcohol-drinking behaviors. In humans, CRF_1 receptor up-regulation is associated with binge drinking in both adolescents and adult alcoholics (Treutlein et al., 2006). Non-peptide therapeutic molecules that affect CRF_1 receptor action may be useful treatments for alcoholism (Jupp & Lawrence, 2010). Antalarmin,

a CRF_1 receptor antagonist, has already been approved by the Federal Drug Administration (FDA) for treatment of alcohol abuse (Funk, Zorilla, Lee, Rice, & Koob, 2007; Kehne & Cain, 2010). These compounds are thought to reduce physical anxiety during withdrawal and can limit drug seeking behavior that would lead to a relapse (Jupp & Lawrence, 2010).

In mice, there is also evidence to support CRF_1 receptor involvement with ethanol seeking and associated behaviors such as aggression. Like humans, rodents display foot-shock stress-induced relapse to alcohol seeking via self-administration if they are physically dependent (Le et al., 2000). CRF_1 receptor antagonists blocked the stress-induced relapse (Le et al., 2000). CRF_1 receptor antagonists are effective treatments in reducing the anxiety experienced during ethanol withdrawal in mice and rats (Funk et al., 2007; Huang et al., 2010; Jupp & Lawrence, 2010; Lowery et al., 2010; Overstreet, Knapp, & Breese, 2004; Rassnick, Heinrichs, Britton, & Koob, 1993; Valdez et al., 2002). In these studies, CRF_1 receptor antagonists did not have an effect upon anxiety during ethanol withdrawal in subjects that were not physically dependent upon alcohol (Heilig & Koob, 2007; Koob, 2010). Importantly, CRF_1 receptor knock-out mice, unlike wild-type controls, did not display increased ethanol self-administration during withdrawal after becoming physically dependent on ethanol (Chu et al., 2007).

Relatively little work has been done to study the effect of CRF and CRF modulators upon aggressive or alcohol-heightened behaviors. CRF injected intracerebroventricularly (i.c.v.) into rainbow trout correlated with more victories during aggressive encounters against weight-matched conspecifics (Carpenter et al., 2009). Maternal aggression, but not intermale aggression, is impaired when CRF binding protein (a protein that acts extracellularly and affects the amount of CRF in the synaptic cleft) is knocked-out (Gammie, Seashotz, & Stevenson, 2008). CRF_2 receptor knock-out mice showed the same pattern of maternal and inter-male

aggression (Gammie et al., 2005). Interestingly, CRF_1 receptor knock-out mice showed no significant difference in their aggressive phenotypes in comparison to wild type mice, although they did express less anxiety-like behaviors (Gammie & Stevenson, 2006). The precise influence CRF may have on aggressive behaviors has yet to fully determined.

When studying CRF and ethanol influences upon behavior the mode of delivery is of the utmost importance. Any effects of stress due to delivery will directly interfere with the effects of ethanol and may obscure results of the CRF manipulation. Stress effects are most easily incurred by over-handling, which can increase heart rate and hypothermia (Peris & Cunningham, 1985). Self-administration, in contrast to gavage and intraperitoneal injection (i.p.) of ethanol, is less likely to cause stress during drug delivery (Spanagel, 2003). Although still associated with some stress, animal subjects will engage in voluntary consumption of ethanol when they are water deprived or bred for ethanol preference (McClearn & Rodgers, 1961; Spanagel, 2003).

The Role of Serotonin in Alcohol-Heightened Aggression

Serotonin or 5-hydroxytryptamine (5-HT), more than any other neurotransmitter has been linked to aggressive behaviors. 5-HT was first discovered in the 1940s as a powerful vasoconstrictor in the gut, and was first isolated from the bloodstream, providing the origin for its name (Rapport, Green, & Page, 1948). It was first posited to be a neurotransmitter in the 1950s, and was quickly implicated in a variety of mental disorders afterward, beginning the vast body of research about its purpose and activity in the central nervous system (Brodie & Shore, 1957; Woolley & Shaw, 1954). 5-HT is produced locally in the brain as a derivative of tryptophan with the rate-limiting step associated with tryptophan hydroxylase (Ichiyama, Nakamura, Nishizuka, & Hayaishi, 1970). Once released, 5-HT is removed from the synapse by

two mechanisms. The primary one is the 5-HT transporter (5-HTT), which re-uptakes 5-HT into the presynaptic terminal (Blakeley et al., 1991). The secondary mechanism is that monoamine oxidase type A (MAO-A) breaks down 5-HT in the synaptic cleft as well as presynaptically (Shimizu, Morikawa, & Okada, 1959). Both of these mechanisms can be selectively altered to affect serotonergic transmission and concentration in the central nervous system, most notably by selective serotonin reuptake inhibitors (SSRIs) and MAO inhibitors (MAOIs).

At the present time, fourteen different 5-HT receptors can be distinguished within eight different subtypes, although the precise number is still debated (Olivier, 2004). 5-HT receptors may act as autoreceptors or postsynaptically, and dependent on subtype, may be ligand-gated ion channels or g-protein coupled receptors triggering either the adenylate cyclase or phosphoinositol enzyme cascades (Olivier, 2004). The 5-HT_1 receptor family are all G-protein coupled receptors, and so both of these receptors are linked to an enzyme cascade that opens a potassium channel (Adell, Celada, Abellán, & Artigas, 2002; Olivier, 2004). All other serotonergic receptors are assumed to be acting postsynaptically at this time (Olivier, 2004). 5-HT_{2A}, 5-HT_{2C}, and 5-HT_3 receptors may also affect aggressive behaviors, but less is known about their role as there are still few pharmacological tools that are specific to these receptors at this time (Miczek & de Almeida, 2010; Quadros et al., 2010).

Although 5-HT is found throughout the brain, the source of the highest amount of 5-HT release from projections of the raphe nuclei (RN), and more specifically the median (MR) and dorsal (DR) subregions (Adell et al., 2002). The pathway most implicated in aggressive behaviors is from the DR to the medial prefrontal cortex (mPFC) (Olivier, 2004). When the DR is chemically lesioned, it can decrease 5-HT concentration measured by in vivo microdialysis and HPLC by 70-80% to this region, whereas a lesion to the MR only decreases the

concentration by 30% (Adell & Myers, 1995). This decrease has been seen to correlate with higher levels of aggression (Johansson, Bergvall, & Hansen, 1999).

Since the late 1960s, an inverse relationship between serotonin and aggression, known as the serotonin deficiency hypothesis, has had the most experimental support. This first notation was in isolated aggressive mice had lower levels of cortical serotonin (Giacalone, Tansella, Valzelli & Garattini, 1968). However, the study that increased awareness of this relationship studied marines with a history of aggressive behaviors and correlated this to 5-Hydroxy indole acetic acid (5-HIAA), a derivative of 5-HT, in their cerebrospinal fluid (CSF) obtained by lumbar puncture. In comparison to non-aggressive controls, these men had a significantly lower concentration of 5-HIAA in their CSF (Brown et al., 1979). Low levels of 5-HT, 5-HIAA, and prolactin (another metabolite of 5-HT) in CSF have also been correlated with impulsivity, personality disorder, and substance abuse, including alcoholism, which are often comorbid with aggressive behaviors (Coccaro, Lee, & Kavoussi, 2010; George et al., 2001; Kruesi et al., 1990; Linnoila et al., 1983; Miczek et al., 2001; Seo, Patrick, & Kennealy, 2008; Virkkunen & Linnoila, 1993). Due to this correlation, the few pharmacological treatments prescribed to the individuals with pathological aggression are often serotonergic drugs, such as SSRIs, but clinical evidence to their effectiveness is mixed and often obscured by these comorbidities (Jupp & Lawrence, 2010).

The serotonin deficiency hypothesis is also supported by evidence found from pharmacological and receptor knock-out studies using animal subjects (Table 2). Excitotoxic lesions to the basal forebrain that reduced cortical levels of 5-HT and 5-HIAA concentrations were correlated with high levels of aggression with and without ethanol present (Johansson, Bergvall, & Hansen, 1999). Subjects with alcohol-heightened aggression, in contrast to those that

fight at a species-typical level had a less dense population of 5-HT_1 and 5-HT_2 receptors (Chiavegatto et al., 2007; Miczek et al., 2007).

Of the serotonergic receptors, 5-HT_{1A} and 5-HT_{1B} receptors have both been extensively studied with regard to their role in aggressive behaviors because receptor agonists have been correlated with dose-dependent anti-aggressive effects (Chichinadze, Chichinadze, & Lazarashvili, 2011; Faccidomo, Bannai, & Miczek, 2008; Miczek, Fish, de Bold, de Almeida, 2002). The aggression-reducing doses of 5-HT_{1A} receptor agonists correlate to doses with sedative and other motor side effects (Bell & Hobson, 1993; Haug, Wallian, & Brain, 1990; Olivier, 2004; Takashi et al., 2010). In contrast, 5-HT_{1B} agonists have a more specific to reducing aggressive behaviors without having motor effects (De Boer & Koolhaas, 2005; Faccidomo et al., 2008; Olivier, 2004; Quadros et al., 2010; Takashi et al., & Miczek, 2010). 5-HT_{1A} receptors are localized presynaptically in the RN and postsynaptically on non-serotonergic neurons, and directly inhibit 5-HT release when activated (Olivier, 2004). 5-HT_{1B} receptors are also localized presynaptically as autoreceptors on the axons of serotonergic neurons and postsynaptically as heteroreceptors on non-serotonergic neurons where they directly inhibit 5-HT release by inhibiting cell firing (Boschert, Amara, Segu, & Hen, 1994; Olivier, 2004; Trainor et al., 2009).

In addition to the pharmacological evidence, 5-HT_{1B} receptor knock-out mice also displayed increased aggressive behavioral phenotypes when compared to their background strain (Brunner, Buhot, Hen, & Hofer, 1999; Brunner & Hen, 1997; Malleret et al., 1999; Saudou et al., 1994). Knock-out studies of the 5-HT_{1A} receptor have not yet been successful with regard to studying the effects of the receptor loss on aggressive behaviors (Takahashi et al., 2010).

There have also been in-vivo microdialysis studies that have shown support for the serotonin deficiency hypothesis. In-vivo microdialysis confers the added benefit of a real time neural chemical correlation, as opposed to post-mortem, when many early measurements that support the theory took place (Van Erp & Miczek, 2000). In 2000, Van Erp and Miczek demonstrated that 5-HT concentration decreased by 80% from baseline levels in the rat mPFC during and after a confrontation, but not before. In a follow-up study, also using rats, they found that a single aggressive encounter did not alter 5-HT concentrations in the mPFC; however, after ten days of regular aggressive encounters, on the eleventh day 5-HT concentration decreased in anticipation of the encounter (Ferrari, van Erp, Tornatzky, & Miczek, 2007). Changes in 5-HT concentration in the mPFC have been shown to correlate to aggressive encounters that support the inverse relationship of 5-HT and aggression.

Corticotropin-Releasing Factor Modulation of Serotonergic Pathways

CRF receptors are found throughout the central nervous system, including in the DR where CRF has extra-hypothalamic effects upon the serotonergic system (Day et al., 2004; Radulovic, Sydow, & Spiess, 1998). Linking the CRF and serotonergic systems appears intuitive, as physiological stress is related to many psychiatric disorders, including anxiety disorders, depression, and substance abuse (Lesch, 1991; Valentino, Lucki & Bockstaele, 2010). The source of CRF afferents to the DR is still unknown, although it may be from the bed nucleus stria terminalas, lateral hypothalamus, paraventricular hypothalamic nucleus, or some combination of the three (Lee, Kim, Valentino, & Waterhouse, 2003; Valentino et al., 2010). CRF has a biphasic effect upon 5-HT release, such that at low doses it increases 5-HT concentrations in various forebrain regions, and decreases release at higher doses in the same

areas (Kirby, Allen, & Lucki, 1995; Kirby, Rice, & Valentino, 2000; Valentino & Commons, 2005).

This biphasic effect may relate to the distribution of CRF receptors in the central nervous system (Valentino et al., 2010). CRF receptor subtypes have different levels of expression in the DR, with CRF_2 receptors highly expressed and CRF_1 receptors moderately detectable (Chalmers, Lovenberg, & De Souza, 1995; Valentino et al., 2010). This presumably leads to CRF receptors having opposing effects upon 5-HT release from the DR as measured in the nucleus accumbens by in vivo microdialysis (Lukkes, Forster, Renner, & Summers, 2008). Antagonism of CRF_1 receptors in the DR blocked a CRF-induced release of 5-HT in the nucleus accumbens, whereas CRF_2 receptor antagonism in the DR augmented the release (Lukkes, Forster, Renner, & Summers, 2008). Acute CRF injection into the DR has also been seen to specifically increase the concentration of 5-HT in the mPFC (Forster et al., 2006). This effect was blocked by CRF_2 receptor antagonism, but not by CRF_1 antagonism (Forster et al., 2008). CRF receptors appear to have opposing effects on 5-HT release from the DR to the nucleus accumbens and mPFC.

Meloni, Reedy, Cohen, and Carlezon (2008) provided strong evidence that CRF mediates the pathway from the DR to the mPFC associated with aggressive behaviors. Rats injected with CRF i.c.v. had increased c-Fos expression in the DR indicating increased activity in this location. They also used retrograde labeling coupled to the c-Fos staining and found that activated parts of the DR ipsilaterally deposited tracer into the infralimbic cortex (IL) within the mPFC, indicating this pathway as essential to CRF modulation of 5-HT. Based on previous evidence that 5-HT concentration and alcohol-heightened aggression have a correlational relationship, and that CRF can modulate the release of 5-HT, CRF may be able to influence aggressive behaviors through this pathway.

Objectives

In a previous experiment in our laboratory, CP-154,526 injected both systemically and intracerebrally using microinjection into the DR prevented escalated levels of aggression after a moderate (1 g/kg) dose of self-administered alcohol (Quadros et al., 2009). Systemically, the drug reduced baseline levels of aggressive behaviors; this effect was not present when locally injected to the DR (Quadros et al., 2009). These anti-aggressive effects were blocked when the 5-HT_{1A} receptor agonist 8-Hydroxy-*N*,*N*-dipropyl-2-aminotetralin (8-OH-DPAT) was infused into the DR. This drug is previously known to slow the release of 5-HT (Larson, Rényl, Ross, Svensson, & Ängeby-Möller, 1990). This result was confirmed by similar results with a second CRF_1 receptor antagonist, 3-(4-chloro-2-morpholin-4-yl-thiazol-5-yl)-8-(1-ethylpropyl)-2,6-dimethyl-imidazo[1,2-*b*]pyridazine (MTIP).

CRF_1 receptor antagonists are associated with biphasic behavioral effects, and so doses used in our experiment were based on this previous study (Henrichs, Pich, Miczek, Britton, Koob, 1992; Lowery et al., 2010). CP-154,526, a specific CRF_1 receptor antagonist that easily crosses the blood-brain barrier, has been seen to be particularly effective in reducing alcohol-consumption, locomotor sensitization effects, and anxiety during withdrawal in mice, but has not been approved for clinical use by the FDA (Fee, Sparta, Picker, & Thiele, 2007; Heilig & Koob, 2007; Lowery et al., 2010; Schulz et al., 2006).

We postulate that this decrease in aggressive behavior may come from a change in 5-HT concentration in the mPFC, as it has been recently shown that CRF may exert some effect on the pathway between the DR and mPFC (Meloni et al., 2008) (Figure 2). As a follow-up experiment to this behavioral experiment, we decided to examine if there were neurochemical correlates to

this proposed pathway. We mimicked the previous experiment during the microdialysis experiment with the ethanol and drug manipulations present, but lacking the aggressive confrontation.

Methods

Subjects

Male CFW mice (Charles River Laboratories, Wilmington, MA), weighing 23-25g upon arrival were assigned to be either resident or intruder mice (n=68). Resident male mice were housed as breeding pairs with a female mouse in clear polycarbonate cages (28 × 17 × 14 cm) with pine shavings as bedding material. Intruder mice were housed with groups of seven to ten mice per large cage (48 × 26 × 14 cm) with corncob bedding. All mice were kept in a temperature and humidity controlled vivarium (21 ± 2°C, 35-40%) on a reversed twelve-hour light/dark cycle (lights off at 7:00 AM). For resident mice, food pellets (Purina, St. Louis, MO) were freely available; however, water was limited to three hours per day. Intruder mice had access to food and water ad libitum. The Institutional Animal Care and Use Committee of Tufts University approved all procedures. The animals were cared for according to the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011).

Drugs

Ethanol was prepared as a 6% w/v solution and administered 1g/kg for all experiments. CP-154,526 (N-butyl-N-ethyl-2,5-dimethyl-7-(2,4,6-trimethylphenyl)pyrrolo[3,2-e]pyrimidin-4-amine) (Tocris, Ellisville, MO) was administered 17 mg/kg and 0.6 ng for i.p and microinjection experiments, respectively. Both doses of drug were selected as most effective in previous

experimental data (Quadros et al., 2009). Drug vehicle for i.p. injection was 0.1% methyl-cellulose. Drug vehicle for microinjections was aCSF (aCSF: 147 mM NaCl, 1.2 mM $CaCl_2$, 0.85 mM $MgCl_2$, 2.7 mM KCl/ CMA Microdialysis, North Chelmsford, MA)

Ethanol Self-Administration

After a one-week habituation period, the mice were reinforced for responding at a nose poke device by delivery of 0.05 mL liquid. After 21 hours of water restriction, the female cage mate and any pups were removed from the home cage and placed in a holding cage for the duration of the experimental session. The male resident was then weighed while a custom designed aluminum panel (16.5 × 15.9 cm) was inserted into the resident's home cage (Miczek & de Almeida, 2001). The panel provided a central house light and a cue light on each side above a drinking trough (3 × 5 cm) inset past a beam-break sensor (Med Associates, Georgia VT).

The resident mice began with 1g/kg water as the liquid reinforcement after each fifth nose-poke (Fixed Ratio of five schedule, FR5). The goal for all mice was to receive this amount in less than five minutes. After the resident mice had successfully met this criterion, usually within five days, they were given 6% w/v ethanol as a liquid reward every other day.

Establishing Aggression

Once ethanol self-administration was successfully acquired, each resident male mouse confronted a male intruder mouse. The female cage mate and any pups were removed from the resident's home cage before the intruder was introduced. Time to the first attack bite by the resident (latency time) was recorded, and the fight was allowed to continue for five minutes

thereafter. Total number of attack bites was recorded for the five-minute duration. Resident-intruder confrontations took place no less than six times, until attack bite count stabilized. In the rare cases when the intruder attacked the resident, the encounter was immediately terminated. If this occurred, the resident was matched with a new intruder. If a switch was necessary, six screenings were performed until the number of attack bites stabilized. These initial confrontations were performed several hours after ethanol self-administration had occurred.

In the next phase, confrontations took place ten minutes following ethanol self-administration. If the resident drank in less than five minutes, the protocol continued after a ten-minute delay (Miczek et al., 1998). Each resident underwent three aggressive confrontations following each ethanol and water self-administration. Aggressive confrontations alternated post-ethanol and post-water self-administration. If the attack bites were not stable during three aggressive confrontations, a fourth one was performed and the three most consistent were used for analysis. Once all aggressive confrontations were completed for a resident mouse, his fighting averages were analyzed for characterization of alcohol-heightened aggression. This was defined as the bite attack average during ethanol confrontations being greater than two standard deviations above the water confrontation average. See Figure 3 for a schematic of the experiment.

Surgery and Cannulation

Following the establishment of a consistent history of aggression, residents were implanted with a CMA/7 guide cannula (CMA Microdialysis, Chelmsford, MA) aimed 2 mm above the infralimbic region within the medial prefrontal cortex (AP, ± 1.96 mm; ML, -0.3 mm; DV, -0.7 mm to bregma) according to a mouse brain atlas (Paxinos and Franklin, 2001) (Figure

4). Mice were anesthetized by i.p. injection of a mixture of 100 mg/kg ketamine HCL and 10 mg/kg xylazine, and placed in stereotaxis for implantation. Mice were housed individually for a minimum of three days following surgery to recover and were kept separate from their female.

For Experiment 4, in addition to the CMA/7 guide cannula, a second 26-gauge guide cannula (Plastics One Inc., Roanoke, VA) aimed 2 mm above the DRN (AP, -4.2 mm; ML, ±1.5 mm; DV, -1.9 mm to bregma; angled 26° to the vertical) was implanted for microinjection (Figure 5).

Microdialysis

Following recovery, the mice in Experiments 2, 3, and 4, had three days of self-administration practice. This practice used a panel that had larger openings (1.9 × 5 cm) to allow extra space for the head mount while nose poking. For the final practice, the dummy probe was removed from the microdialysis guide cannula and a modified version of the microdialysis probe was inserted and connected to a counter-weighted spring. This modified probe did not have an active membrane extending beyond the guide. Upon completion of the session, the modified probe was removed.

The evening prior to microdialysis, a CMA/7 probe (2 mm active membrane) was inserted into the mPFC under isoflurane inhalation anesthesia. The mice were kept in their home cage during the experiment without their female partner. Unless restricted for self-administration experiments, overnight the mice had access to a food pellet and water bottle. Overnight, the probe was infused with artificial cerebrospinal fluid (aCSF: 147 mM NaCl, 1.2 mM $CaCl_2$, 0.85 mM $MgCl_2$, 2.7 mM KCl/ CMA Microdialysis, North Chelmsford, MA) at a flow rate of 0.5 µl/min using an infusion pump (CMA Microdialysis, Chelmsford, MA). The flow rate was

increased the following morning, 15 hours post-insertion, to 1.5 µl/min, and a sample was collected every 20 min after one hour of stabilization. 7.5 µl of stabilizing agent that consisted of 20 mM phosphate buffer including 25 mM EDTA and 0.5 mM ascorbic acid was added to each sample vial to prevent monoamine degradation.

Following the end of the experimental session, the probe was removed from the guide cannula. The probe was then inserted into a 50 ng/mL standard solution of 5-HT, DA, and NE. One more sample was collected, and used to analyze probe recovery against a sample of the standard solution.

The changes in 5-HT concentrations were expressed as the percent change from the average baseline for each individual. Please see Figures 6 and 7 for a general timeline and visual representation of the microdialysis experiment set-up.

Experiment 1: Ethanol Self-Administration

A group of mice (n=11) self-administered ethanol (1g/kg) in order to examine changes in mPFC 5-HT concentration using in vivo microdialysis. Following collection of seven baseline samples, ten minutes into the seventh sample, the modified nose-poke panel was inserted into the home cage. The time delay was such that the dialysate collected from this time period would be collected as part of sample eight.

Mice followed the self-administration procedure outlined above (*Microdialysis*). If the subject did not finish drinking within ten minutes, the self-administration session was terminated. These mice were considered to be "low drinkers," as they drinked less than 1 g/kg of ethanol, but still completed the five experimental samples for a total of twelve samples.

Experiment 2: i.p. Injection of CP-154,526

A group of mice (n=6) were injected with vehicle and CP-154,526 i.p. following baseline sampling. Seven baseline samples were collected to measure changes in the mPFC in 5-HT concentration against post drug manipulation. Following baseline, 10 mg/kg of methyl-cellulose was injected intraperitoneally (i.p). The injection was performed five minutes prior to the end of sample seven in order to observe for potential injection effects. Following three samples post vehicle injection, a second injection of 17 mg/kg CP-154,526 was injected i.p. on the opposite side of the peritoneum. An additional five samples were taken to observe drug effects, for a total of fifteen samples.

Experiment 3: Ethanol Self-Administration and i.p. Injection of CP-154,526

A group of mice (n=22) were injected i.p. with either 0.01 mL/g 0.1% vehicle or 1.7 mg/mL CP-154,526 following 1 g/kg ethanol self-administration. Similar to the previous experiment, the modified panel was inserted into the home cage ten minutes prior to the start of sample eight. Upon completion of self-administration implementing the same criterion as above, the mouse was injected i.p. either with 17 mg/kg CP-154,526 or 10 mg/kg of methyl-cellulose. An additional five samples were collected following this injection to observe for drug effects for a total of twelve samples.

Experiment 4: Ethanol Self-Administration and Microinjection of CP-154,526

A group of mice (n=9) were microinjected with 0.6 ng CP-154,526 following 1 g/kg ethanol self-administration. Unlike the previous experiments, the nose-poke panel was not inserted halfway through the seventh sample. Following the seventh sample, the sample

collector was turned-off with an extra tube in place to collect any dialysate during this time. The mice then self-administered ethanol following the procedure previously described. Upon completion of ethanol self-administration, a microinjector was inserted into the second guide cannula aimed above the DRN and 0.2 μL of 0.3 ng/uL CP-154,526 was infused over two minutes at a rate of 0.1 μL/min. The sample collector was restarted once the microinjector had been removed. Five additional samples, for a total of twelve, were collected following this microinjection to observe for drug effects.

HPLC

After samples were taken, high performance liquid chromatography was used to measure the concentration of 5-HT, DA, and NE in the 37.5 μL dialysate samples collected from the mPFC. The system was equipped with an electrochemical detector (DECADE, Antec Leyden, Zoeterwoude, Netherlands) and a cation-exchange column (1.5 mm × 250 mm, 5 μm I.D., Shiseido, Tokyo, Japan) with the column temperature set at 30ºC. The limit of detection (LOD) for 5-HT with this system was 0.11 fmol and retention time between 17.5-21.5 min. Data collection and analysis of the sample was done using a software package designed for HPLC (ALEXYS, Antec Leyden, Zoeterwoude, Netherlands). The mobile phase, flowing at a rate of 0.2 ml/min, consisted of 150 mM ammonium acetate, 50 mM citric acid, 27 μM EDTA, 10% methanol, and 1% acetonitrile with pH adjusted to 4.6. 5-HT concentration was determined by comparison to standard curves created shortly before each microdialysis experiment by using a known amount of 5-HT ranging from 2.1-17 fmol. Under these conditions, the correlation coefficient for the standard solutions needed to exceed 0.99 to be used as a standard curve.

Histology

Following the termination of dialysate sample collection, mice were injected with a lethal dose of 100mg/kg ketamine HCL and 10 mg/kg xylazine mixture. Mice were then intracardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), and their brain was removed for analysis. After post-fixation in 4% PFA for at least 48 hours, brains could be sliced. Either a freezing microtome (American Optical Company, Buffalo) or cryostat (Leica Microsystems, Buffalo) were used to slice the brains into 60 µm sections, mounted on frosted slides (Fisherbrand) which were stained with cresyl violet to verify the placement of the microdialysis probe and/or guide cannula. The probe and injection sites for each animal are represented in Figures 10 and 11 for mPFC and DR, respectively.

Statistics

A baseline sample was calculated from the averaging all seven baseline samples, calculating the percent change from the average of those samples, and taking the three most similar and averaging those together. All comparisons of experimental manipulations were compared to this value as baseline.

In order to analyze Experiment 1 and 4, a repeated-measures one-way ANOVA was performed for either six data points (one baseline, five experimental samples following drug manipulation). For Experiment 2, a repeated-measures two-way ANOVA was performed on 9 data points (one baseline, three saline samples, and five samples after the i.p. injection of CP-154,526). Experiment 3 also used a repeated-measures two-way ANOVA on 5 data points, comparing drug and time effects.

Results

Acquisition of 6% Ethanol Self-Administration Behavior

Sixty-eight CFW mice were trained to self-administer alcohol using a nose-poke panel. Mice were initially trained using an frequency ratio of one (FR1) reward schedule with water for thirty minutes, and then moved to an FR2 schedule the next day again with water for thirty minutes. The mouse was then moved to an FR5 schedule and continued with water self-administration (1 g/kg) for a full week (Figure 8). All mice successfully attained this criteron, and were then given ethanol (1 g/kg) on alternating days. All mice stabilized to perform this behavior under the desired time of five minutes.

Aggression History

58 CFW mice were screened for aggression in confrontations with a male intruder. One resident mouse did not fight, even after using social instigation and was excluded from the study (Fish, Faccidomo, & Miczek, 1999). As a general trend, confrontations after ethanol self-administration tended to have higher bite frequencies than confrontations after water self-administration, although this was not true for every individual subject (Figure 9). Of the mice that participated in the study, 17 subjects, or 29% expressed alcohol heightened aggression.

Microdialysis

The probe recovery for this experiment was 32.6% with a standard error of the mean of 6.34. The total attrition rate was 50%. There were a variety of reasons for exclusion, mostly for physical errors in the performance of the experiment (Table 3). If a subject was eliminated due to poor quality data, if there were less than three peaks within the baseline or more than two in

the experimental samples. Many subjects were also excluded after histological examination if the probe was not inserted into the infralimbic cortex or DR (Figures 10 and 11, respectively).

Experiment 1: Ethanol-Self Administration

Of the subjects (n=11) that self-administered ethanol, a subgroup (n=6) successfully completed the full procedure. Five subjects were excluded from final analysis; four for incorrect placement and one for lack of useable HPLC data. Following self-administration, 5-HT concentration in the mPFC increased for approximately 60 minutes (Figure 12) (One way repeated measures ANOVA; $F_{(1,70)}$=40.999, P < 0.001).

Experiment 2: i.p. Injection of CP-154,526

Of the subjects (n=6) that were injected with vehicle and CP-154,526 i.p a subgroup (n=4) successfully completed the full procedure. One subject was excluded from final analysis due to incorrect placement. There was no overall effect of vehicle and drug. When examined separately, there was an effect after the vehicle injection (Figure 13) (One way repeated measures ANOVA; $F_{(1,30)}$=29.719, p<0.001). There was no effect between baseline and drug.

Experiment 3: Ethanol Self-Administration and i.p. Injection of CP-154,526

Of the subjects (n=18) that were injected i.p. following ethanol self-administration, a subgroup (n=9) successfully completed the full procedure. Eleven subjects were excluded from final analysis; eight for incorrect placement, one for low drinking, two for probe malfunction, and one for lack of useable HPLC data. Following the self-administration and drug injection, there was a significant difference between vehicle and drug treatments (Figure 14) (Two way repeated measures ANOVA; $F_{(1, 47)}$= 7.014, p=0.011). There was no effect of time found. CP-154,526 following self-administration increased 5-HT concentration (One way repeated

measures ANOVA; $F_{(1, 68)}$= 47.573, P < 0.001). There was also an effect of vehicle injection following self-administration (One way repeated measures ANOVA; $F_{(1, 46)}$ = 8.962, p=0.004).

Experiment 4: Ethanol Self-Administration and Microinjection of CP-154,526

Of the subjects (n=9) that were microinjected into the DR following ethanol self-administration, a subgroup (n=5) successfully completed the full procedure. Four subjects were excluded from final analysis; three for placement and one for headmount malfunction. Following self-administration and microinjection, 5-HT increased in the mPFC for twenty minutes (M=117.264, SD= 33.937) and then returned to baseline levels(M=10, SD = 10.541) (Figure 15) (One way repeated measures ANOVA; $F_{(1,58)}$=38.431, p< 0.001. $t_{(18)}$=-9.545, p < 0.001).

Discussion

5-HT concentration in the prefrontal cortex was seen to increase after ethanol self-administration and CP-154,526 injections. Overall, as this increase in the mPFC correlates with the anti-aggressive effects of the compound according to the previous behavioral study (Quadros et al., 2009), these findings appear to be consistent with the serotonin-deficiency theory. However, when each experiment is considered separately, they do not necessarily completely support this hypothesis.

Experiment 1: Ethanol Self-Administration

We found that 1 g/kg ethanol operantly self-administered increased 5-HT concentration in the mPFC, correlating to the time that alcohol-heightened aggression occurs in mice (Figure 12). Our data match earlier studies, as ethanol i.p. has previously been found to increase the concentration of 5-HT in the mPFC and nucleus accumbens during in vivo microdialysis

dependent upon dose and rat strain (Montis et al., 2004; Langen, Dietze, & Fink, 2002; Selim & Bradberry, 1996; Yoshimoto, McBride, Lumeng, & Li, 1991). The data for changes of 5-HT concentration in the mPFC using in vivo microdialysis is mixed, and studies have found either no overall effect or an overall increase (Langen, Dietze, & Fink, 2002; Selim & Bradberry, 1996). Only one of these previous studies found a 5-HT concentration increase in the mPFC using ethanol self-administration; however, they did not use an operant procedure and they sweetened the ethanol with vanilla sugar (De Montis et al., 2004). No studies have been performed at this time using mice or an operant self-administration procedure during microdialysis. As the effect of ethanol by itself appears to depend on dose, genetics, and potentially method of administration, it is difficult to explain this finding of an increase especially as its apparent time course correlates with alcohol-heightening aggression effects. This correlation does not fit within the framework of the serotonin deficiency hypothesis.

More recently there has been evidence to dispel the dogma of the serotonin deficiency hypothesis as being more complicated than initially believed (De Boer & Koolhaas, 2005). In 2003, Van der Vegt et al. attempted to repeat the experiment of Brown et al. (1979) in rats by measuring their CSF metabolites and correlating it with aggressive traits. However, they found that rats that expressed aggressive traits had significantly increased CSF concentrations of 5-HT and 5-HIAA, opposite of what was found in humans. Another criticism is that the earliest pharmacological studies of 5-HT_{1A} and 5-HT_{1B} receptors utilized drugs that were neither receptor specific nor able to distinguish between auto- or post-synaptic receptors (de Boer & Koolhaas, 2005). Activating these 5-HT_{1A} and 5-HT_{1B} autoreceptors would be expected to decrease 5-HT concentration in projection sites. In contrast, activating them postsynaptically would be expected to increase 5-HT release. It is possible that ethanol may affect cells with

autoreceptors, by inhibiting their firing by acting upon ligand gated ion channels, such as $GABA_A$ receptors, that hyperpolarizes the membrane potential (Solder, Proctor, Dunwiddie, 1998). This could lead to an increase in release of 5-HT in the mPFC if the hyperpolarized cells normally exert inhibiting effects on serotonergic projects such as those from the DR. Further testing would have to be done in order to evaluate which receptors and pathways in order to determine if this is the mechanism by which we saw an increase in mPFC 5-HT concentration following ethanol self-administration.

Experiment 2: i.p. Injection of CP-154,526

Interestingly, i.p. CP-154,526 by itself had no overall effect upon 5-HT concentration. This corresponds to a previous study in rats that saw no change in 5-HT concentration over time (Isogawa et al., 2000), although our data appears to have a trend towards an increase (Figure 13). Both of our experiments show a high range of variability, potentially because of dose, administration, species, and brain location. There was a significant effect following the inert vehicle injection, which could be due to handling; however, previous studies have shown that stress effects due to handling usually correspond to increases in the mPFC, not a decrease as we observed (Fujino et al., 2002). No previous experiments have tested CP-154,526 affect on 5-HT concentration in the mPFC in mice. The lack of an effect in this experiment could also be due to low statistical power from a small number of subjects. In a previous study, it was found that CRF_1 antagonism did not block a release of 5-HT to the mPFC following acute injection of CRF into the DR (Forster et al., 2008). It is therefore possible that when administered by itself, CP-154,526 via CRF_1 receptor antagonism does not affect the release of 5-HT from the DR to the mPFC.

Experiment 3: Ethanol Self-Administration and i.p. Injection of CP-154,526

The increase in 5-HT concentration following ethanol self-administration and injection of CP-154,526 corresponded to the anti-aggressive effects seen in the previous study (Quadros et al., 2009). Although this increase had a high degree of variability as there appeared to be a secondary increase later than expected (Figure 14). This may be due to slower release of the drug from the injection site. From the inert vehicle i.p. control group there again seems to be either a handling effect or an effect due to alcohol. However, unlike Experiment 1, there was a significant decrease in 5-HT concentration, which is more similar to the results of the vehicle injection during Experiment 2. It appears that there is variability in the serotonergic response in the mPFC similar to the previous in-vivo microdialysis experiments with ethanol i.p. (Yoshimoto, McBride, Lumeng, & Li, 1991). This is not attributable to a group effect as all experiments included mice from different squads, but may instead relate to the strain being out-bred or at the dose of ethanol.

When comparing the vehicle i.p. to the CP-154,526 i.p. after ethanol self-administration, there is a significant difference between these groups (Figure 14), indicating an effect due to CP-154,526. As CP-154,526 may not exert its own effect, it may exert an augmented effect when administered after ethanol consumption. Presumably, this augmented effect relates to 5-HT_{1A} receptors as the anti-aggressive effects were blocked with a 5-HT_{1A}, and potentially to an inhibition of neurons by ethanol action described above, but the exact mechanism by which this could occur has yet to be elucidated.

Experiment 4: Ethanol Self-Administration and Microinjection of CP-154,526

There was a significant increase in 5-HT concentration in the mPFC twenty minutes following microinjection of 0.6 µg CP 154,526 into the DR post ethanol self-administration. The microinjection group shows the time course more clearly than the i.p. injection as a relatively transient effect (Figure 14). This may occur because systemic injections are less precise in their effects upon the brain than microinjections, which can provide evidence about a specific site's role in a behavior or pathway. As there is no inert vehicle control for the microinjection experiment, it was not possible to be certain that the observed effect was due to CP-154,526 and not just ethanol, or due to the DR; however, as an effect was observed due to CP-154,526 during Experiment 3, it is highly possible that a drug effect occurred during the microinjection experiment as well.

Methodological Limitations

Microdialysis Attrition

This experiment suffered from a high rate of attrition; half of the subjects had to be eliminated, most commonly for poor probe placements (Table 3). Using a larger species may partially alleviate this issue as desired brain sites should be correspondingly larger. Mice are not used as commonly as rats or other larger animal subjects for microdialysis experiments because relatively far less damage is done by the probe upon insertion and it is easier to hit a specific brain site in larger species (Boschi & Scherrmann, 2000). As well, due to the size of a mouse, it is too heavy to protect the influx and efflux tubing attached to the probe by a metal casing as is used with rats and other species, and so during the course of the experiment the subject may damage the tubing. Clots, lost headmounts, and post-surgery death are unavoidable portions of

any microdialysis experiment, but some of the attrition may be improved by using other species that can utilize different equipment.

Endogenous Concentration of Serotonin

5-HT, despite being projected to the IL from the DR, is still present at relatively low concentrations in the synaptic cleft at this brain site (Adell et al., 2002). Once released, 5-HT is also quickly acted upon by MAO-A and 5-HTT (Blakeley et al., 1991; Shimizu et al., 1959). This means that there is a very low amount of 5-HT to measure endogenously. This concentration happened to be near the limit of detection of our HPLC. In addition, measured 5-HT concentration was limited by how effective probes were at recovering extracellular neurotransmitters, and their recover ranged from approximately 15%-50%. Some previous microdialysis experiments have administered citalopram, a 5-HTT inhibitor, in order to try to increase the amount of 5-HT present to be taken-up by the probe (Adell et al., 2002). HPLC analysis was hampered by low endogenous 5-HT concentrations, limit of detection, and probe recovery.

Counter Balancing

This experiment was also not fully counterbalanced for all control groups. To better evaluate drug versus behavioral effects, ethanol drinking should be compared to water and the microinjection to a vehicle injection. Water drinking may not be necessary though for the comparison of CP-154,526 injections following ethanol drinking pending the results of an experiment using water drinking by itself. Eleven groups would have to be filled to fully counterbalance this experiment, requiring a very large number of subjects, not all of which may provide valuable insight. An inert vehicle microinjection following ethanol self-administration

for the microinjection experiment should be performed in order to complete this study and better evaluate the effect of the CP-154,526 microinjection.

Translation from Mouse to Human

Other limitations of studying alcohol-heightened aggression originate from the behavior methodologies. Most experiments examining aggression study a form of a species-typical behavior that may be easily induced and often relate to forming and maintaining a social hierarchy (Takahashi et al., 2010). These behaviors may be unique to this species, which then may be translatable to humans (Ramirez, 2000). The reverse is also true; humans have their own species-typical behaviors that are not translatable to an animal models, such as fantasizing about aggressive behaviors (Ramirez, 2000). Human males also not only attack other males, but they often attack females, a behavior that is relatively rare in mice (Nelson & Chiavegatto, 2001; Trainor Sisk, & Nelson, 2009). In addition to the aggressor expressing different behavior between species, it is not yet fully understood what the effect of an intruder mouse may exert upon the resident mouse (Trainor et al., 2009). Interestingly, there is evidence that sober, non-drugged, resident male mice will more frequently attack an ethanol-intoxicated intruder mouse than a sober intruder (Miczek et al.,1984)There are still limitations to the validity of this model in regard to its translation to human behavior, despite the two species having a genome similarity upwards of 90% (Ramirez, 2000; Trainor et al., 2009).

Current and Future Directions

The most immediate steps for this experiment are to continue to run subjects in order to bolster the statistical power of the observed effects, especially to the groups in Experiments 2

and 4. Adding subjects to Experiment 2 will provide better evidence about the effect of CP-154,526 by itself. As previously mentioned, it would be valuable to have a vehicle control for the microinjection to the DR in order to draw more powerful conclusions about the role of the site and in the effect of the CP-154,526.

As the observed effects appear to be transient, the best future experiment would be to attempt to replicate the findings related to CP-154,526 in a larger animal subject that is better suited to microdialysis, such as a rat, controlling for drug concentration and using self-administration. It would also be important to take the next step methodologically and try to completely replicate the behavioral component during microdialysis, similar to the Van Erp and Miczek (2000) paper using in vivo microdialysis during aggressive encounters. This experiment would have the mouse self-administer ethanol, injected with CP-154,526, and then encounter an intruder mouse to correlate the changes in 5-HT in the mPFC.

All subjects in this study, except for those used to study the effect of CP-154,526, had an aggression history. The current evidence about 5-HT in the mPFC in aggressive mice is mixed, some studies have found that there is a basal difference between aggressive and non-aggressive subjects and others have found no significant difference, although strain may have a role in this concentration (Caramaschi, de Boer, de Vries, Koolhaas, 2008; Carpenter et al., 2009; Giacalone, Tansella, Valzelli, & Garattini, 1968). As a single bout of aggression and social stress may have neurobiological effects (Koolhaas, DeBoer, De Ruiter, Meerlo, & Sgoifo, 1997), it would be valuable to see if naïve mice had a different observable change in 5-HT concentration in the mPFC.

Testing other doses of CP-154,526 in addition to non-aggressive animals may prove valuable. Another dose may reveal a different effect of the drug on 5-HT concentration, as CRF_1

receptor antagonists are known to have biphasic behavioral effects (Henrichs et al., 1992; Kirby et al., 1995; Valentino & Commons, 2005). If a higher dose potentially has a longer-acting effect, as long as it did not have undesireable side effects, could be translated to a therapeutic dose range. It would also be interesting to see what occurs with co-administration of CRF and CP-154,526 to the concentration of 5-HT in the mPFC, as our behavioral evidence shows that CP-154,526 successfully blocks the associated increase in aggression with CRF administration (Quadros et al., 2009), but the previous neurochemical evidence links CRF administration with increases in 5-HT forebrain concentrations (Forster et al., 2006; Kirby, Allen, & Lucki, 1995).

Using another drug entirely may also provide insight into the precise mechanism of the augmentation of 5-HT release with both CP-154,526 and ethanol as seen in Experiment 3. As these effects are linked to 5-HT_{1A} receptors, using S-15535, which is a 5-HT_{1A} autoreceptor agonist and 5-HT_{1A} postsynaptic receptor antagonist (de Boer & Koolhaas, 2005), could provide evidence as to whether or not the 5-HT increase is mediated by the auto or postsynaptic receptors.

Conclusion

According to the evidence from this study, CP-154,526, may not lend itself as a viable treatment for clinical aggression linked to alcohol abuse. At this dose, the drug may not last long enough to cause a valuable modulation of behavior. It is possible that the drug would have a different effect in humans, or it may be useful to treat other aspects of alcohol abuse such as anxiety and cravings.

Overall, both ethanol and CP-154,526 were seen to increase 5-HT concentration in the mPFC. This corresponds with a decrease in alcohol aggressive behaviors in mice administered

CP-154,526, lending support to the serotonin deficiency hypothesis. This study provides further evidence of CRF activity modulating the effects of serotonergic transmission from the DR upon the mPFC, specifically the IL. Further studies are needed to better understand the effects of CP-154,526 as a therapeutic agent; however, this study provides a promising start to those experiments.

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Figures

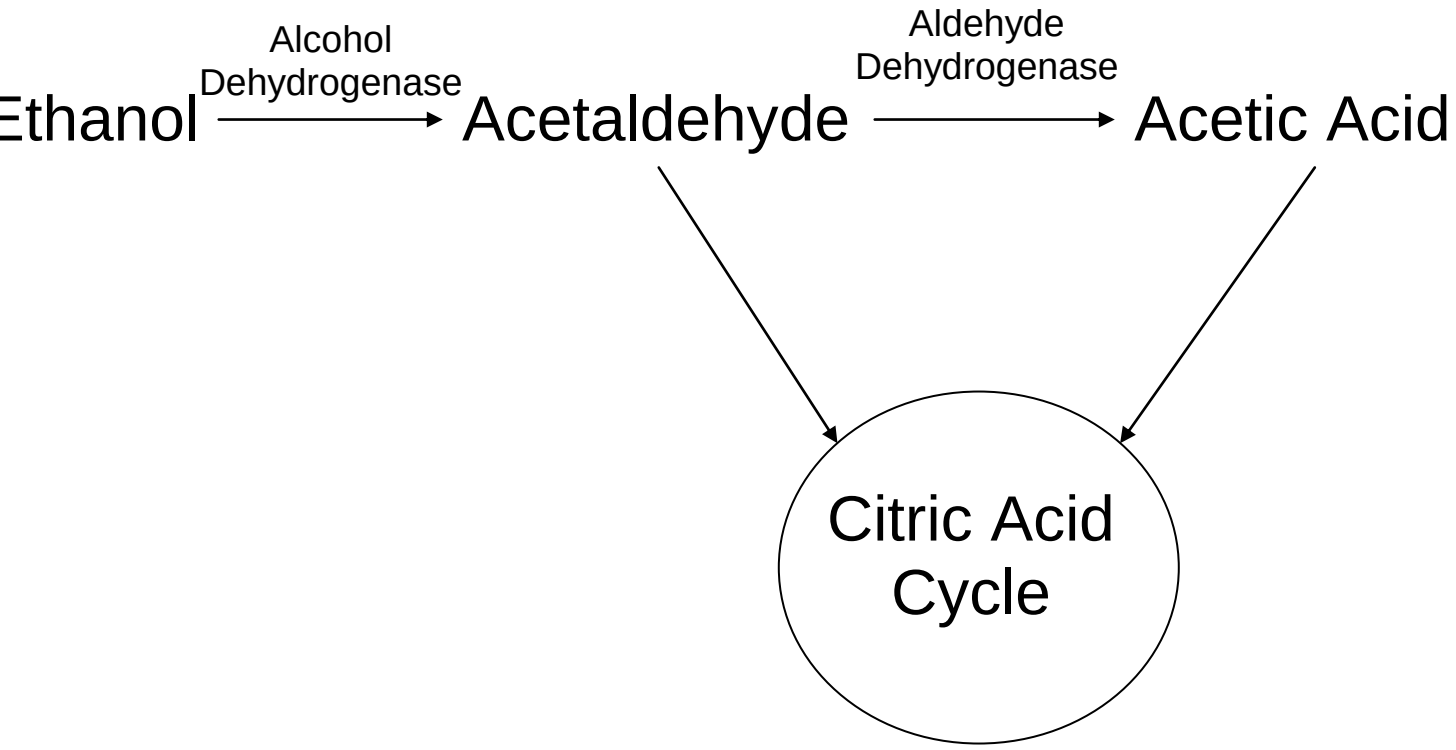


Figure 1. *General Scheme for Physiological Ethanol Metabolism*. Ethanol is first oxidized in the liver by alcohol dehydrogenase to acetaldehyde and then may enter the citric acid cycle for energy use or be further oxidized to acetic acid. Acetic acid also enters the citric acid cycle, and so ultimately ethanol will be utilized for its energy stores within its carbon-carbon bonds.

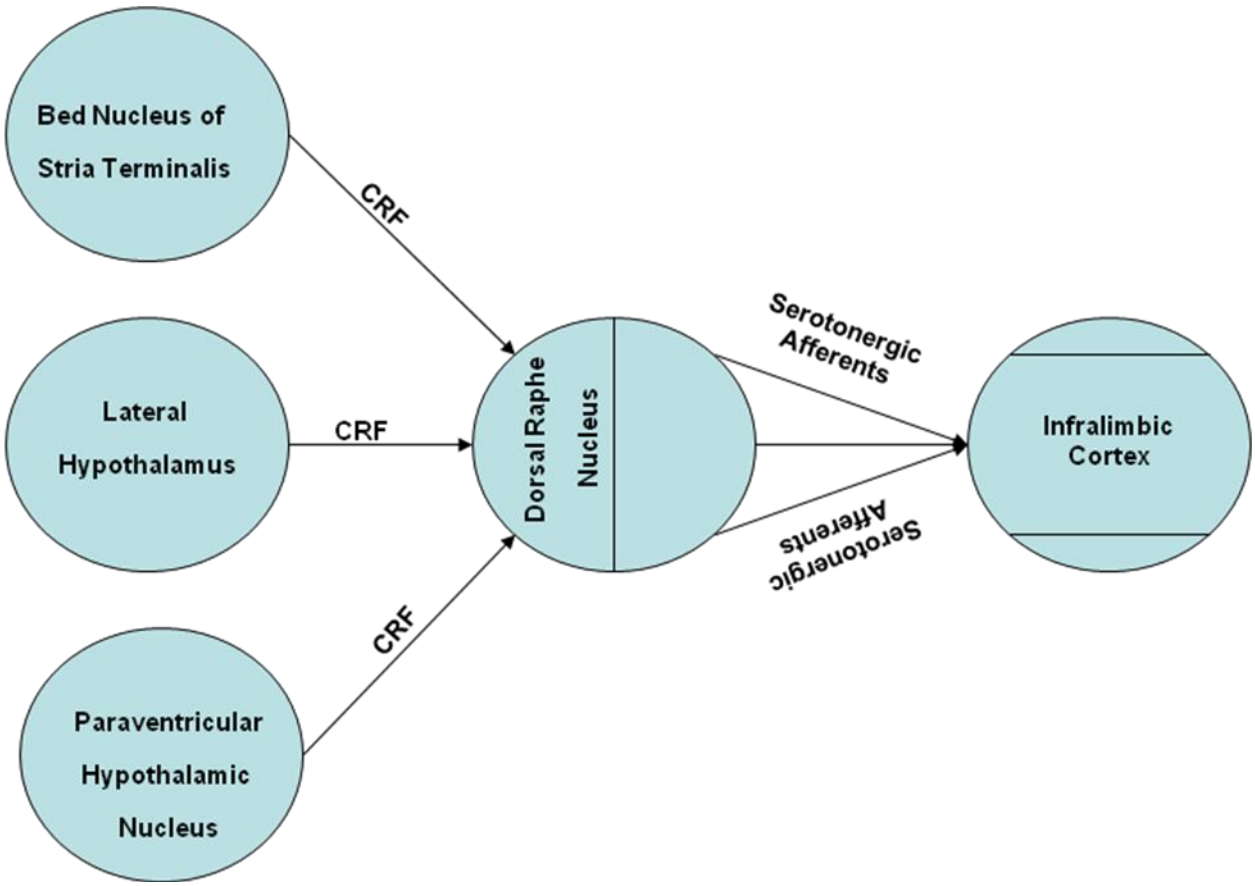


Figure 2. *CRF-influenced Serotonergic Pathway from Dorsal Raphe Nucleus projecting to the Infralimbic Cortex*. We postulate that aggressive behavior may be influenced by this proposed pathway from recent studies. The recent article by Meloni et al. (2008), showed that CRF influenced the release of 5-HT from the DR to the infralimbic cortex. As well, our recent behavioral experiment studied this same pathway, and found that aggressive behaviors were first decreased by CRF_1 antagonists and that this effect was blocked when $5\text{-}HT_{1A}$ receptors were acted upon by 8-OH-DPAT in the DR.

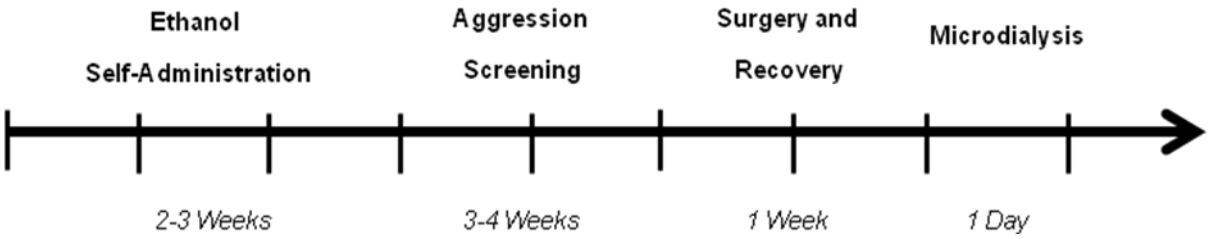


Figure 3. *Schematic and Approximate Timeline of Overall Methods***.** All subjects completed all stages of the experiment with the exception of those that were excluded after aggression screening or before microdialysis. Given times are approximate, and the shortest period of time the pieces could be accomplished.

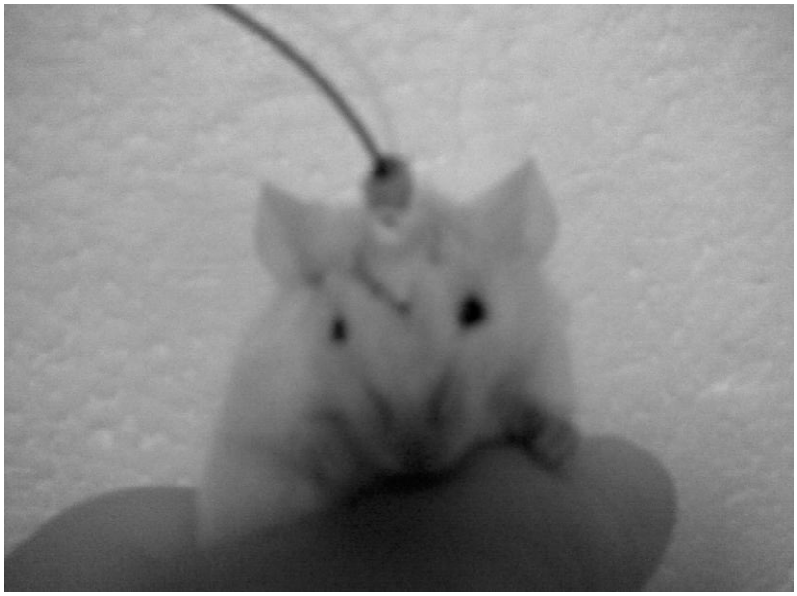


Figure 4. *Single Cannulation to the Medial Prefrontal Cortex of CFW Mouse*. The cannula was aimed for the infralimbic cortex, and stabilized the probe once it was inserted. The blue tube carried the influx of aCSF and the clear tube carried the efflux to the automated sample collector.

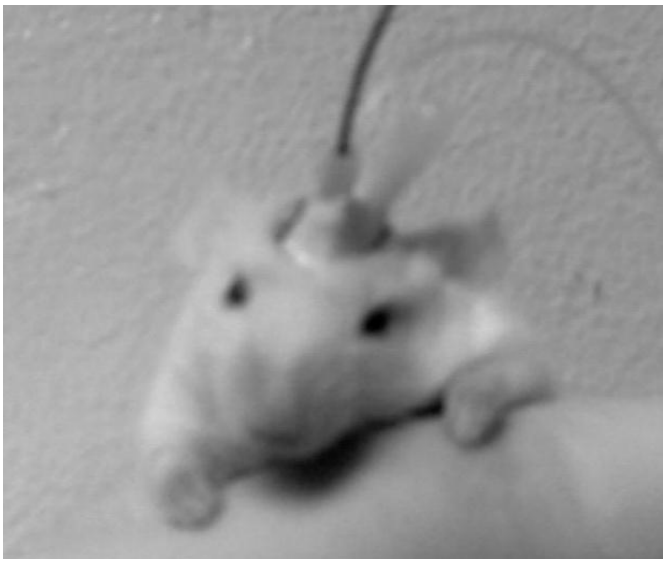


Figure 5. *Double Cannulation to the Medial Prefrontal Cortex and Dorsal Raphe Nucleus of a CFW Mouse.* On the anterior part of the brain, there is a probe inserted into the mPFC as in the single cannulation. The posterior cannula is angled to reach the DR for the microinjection.

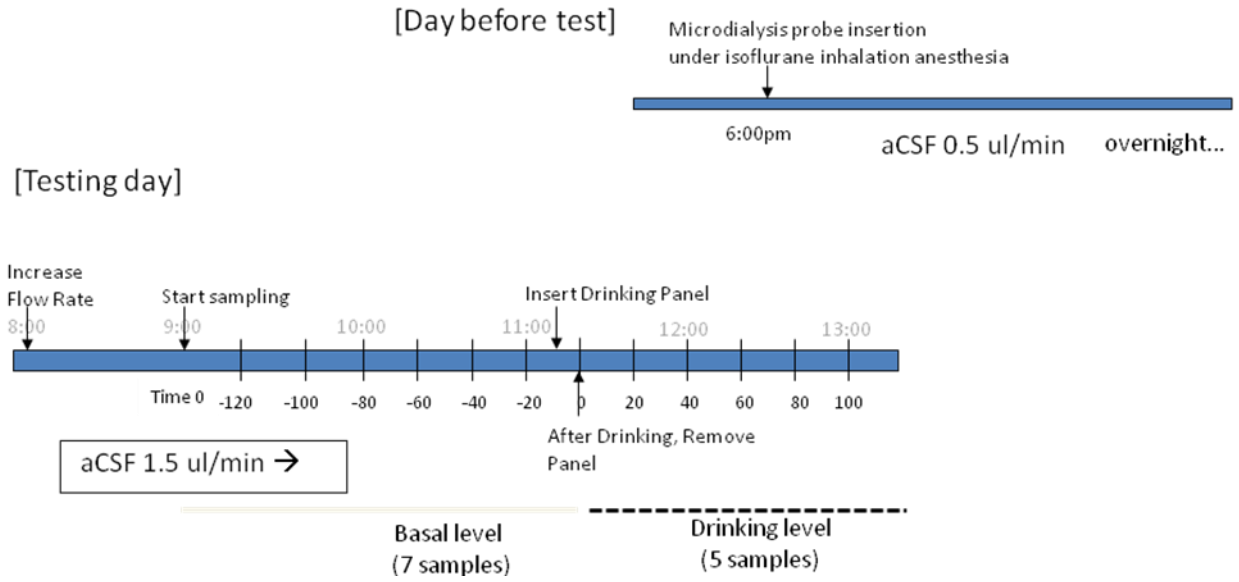
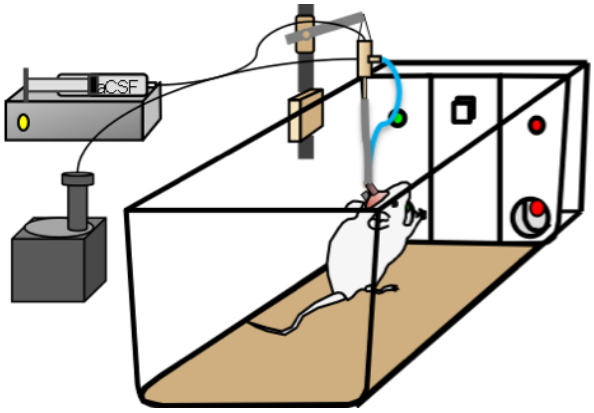


Figure 6. *Microdialysis Testing Day Schedule.* Microdialysis experiments were set-up the evening prior to self-administration and/or drug injection in order to allow for probe equilibration. The flow rate was increased the following morning to the experimental rate (1.5 μL/min) and allowed to equilibrate for an additional hour before experimental sampling began.



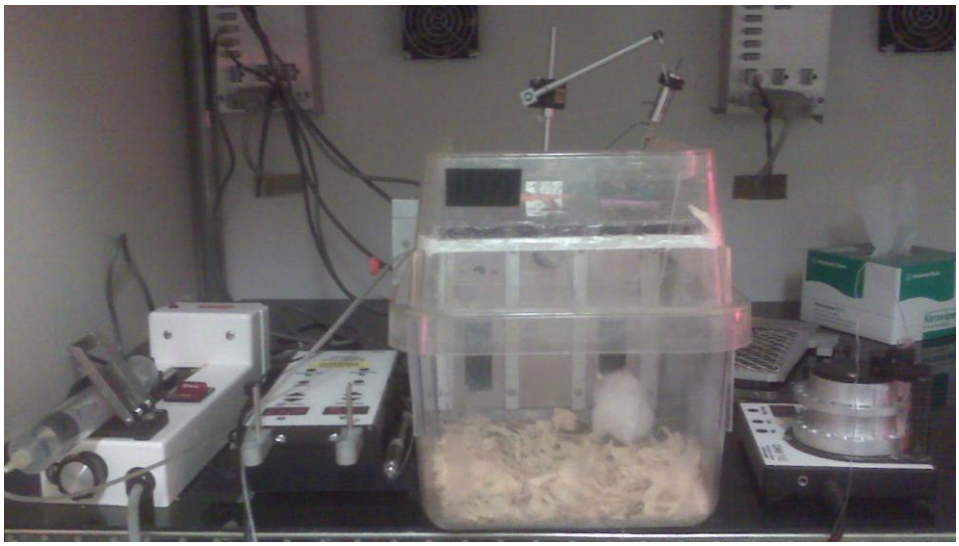


Figure 7. *Microdialysis Set-Up.* Please note that the far left pump and syringe in the photograph is a part of the self-administration procedure, and the slightly smaller black pump and syringe immediately to its right are flowing aCSF through the probe. Also note that autosampler is usually kept in a freezer box with ice packs, not next to the cage, but was taken out for visualization purposes.

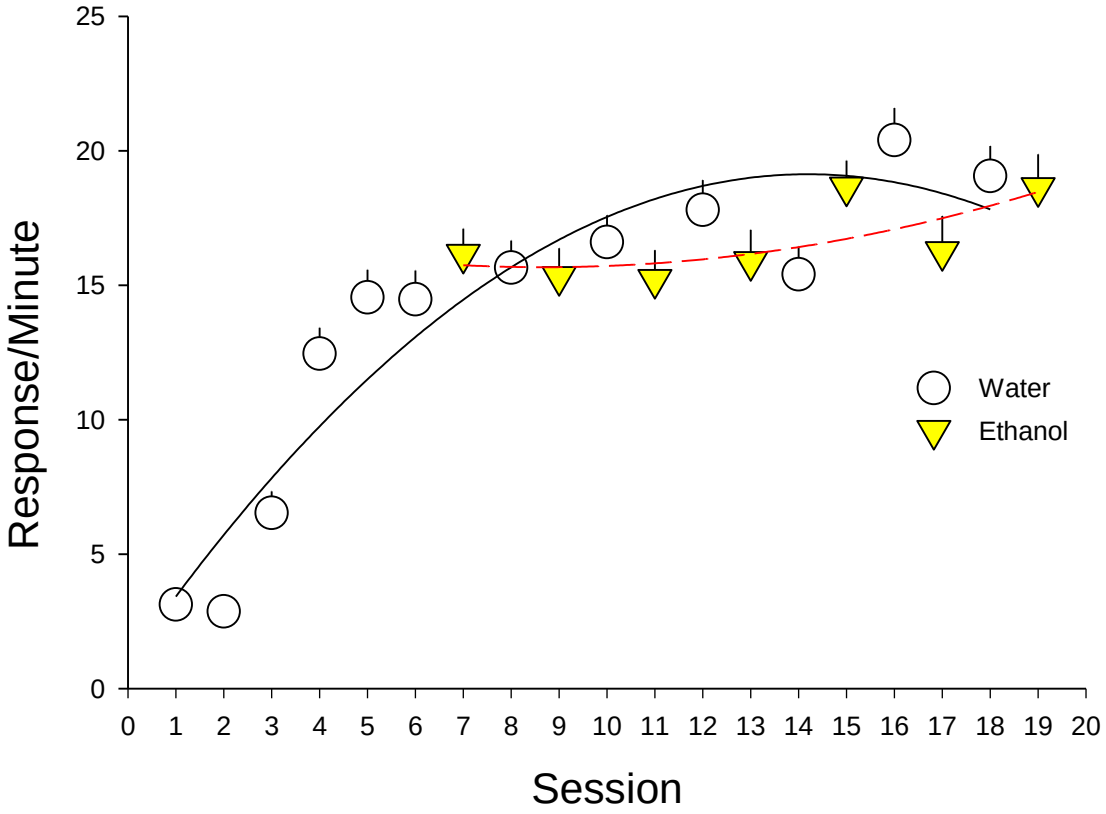


Figure 8. *Self-Administration Behavioral Acquisition.* Over twenty sessions, mice acquired and stabilized for ethanol self-administration behavior. Session 1 was on a FR1 reinforcement schedule, and subjects poked thirty minutes for water. Session 2 was on a FR2 reinforcement schedule, and again subjects were allowed to poke for thirty minutes for water. From Session 3 onward, mice were on an FR5 schedule and were removed once they had completed administration of 1 g/kg of either water or ethanol. The ethanol and water administration behavior stabilized over time.

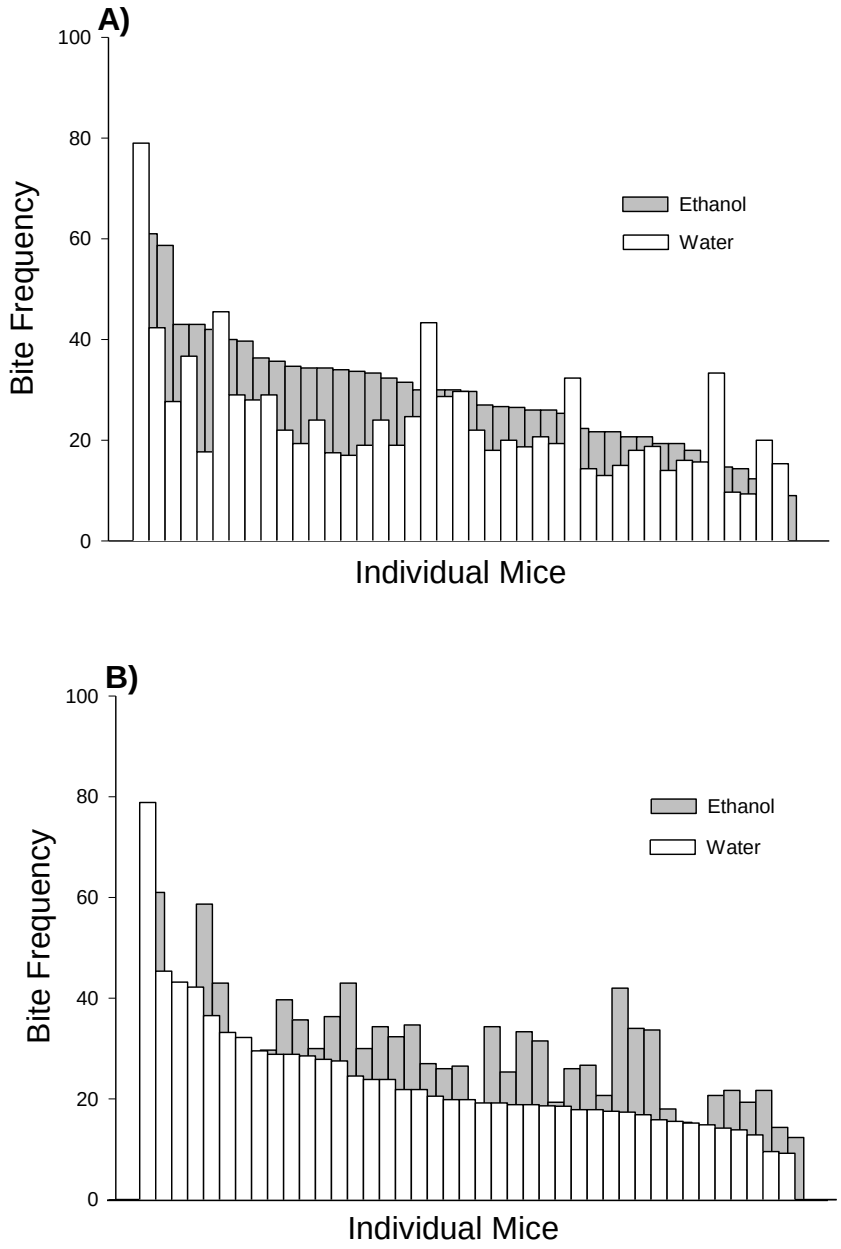


Figure 9. *Average Bite Frequency during Aggression Encounters.* All subjects that went through aggression screening had average bite counts under each self-administration condition. A) Ranks the subjects from highest to lowest according to average ethanol bite frequency. B) Ranks the subjects from highest to lowest bite according to average water bite frequency. Note that as a general trend, subjects were more aggressive under the influence of ethanol than after water self-administration.

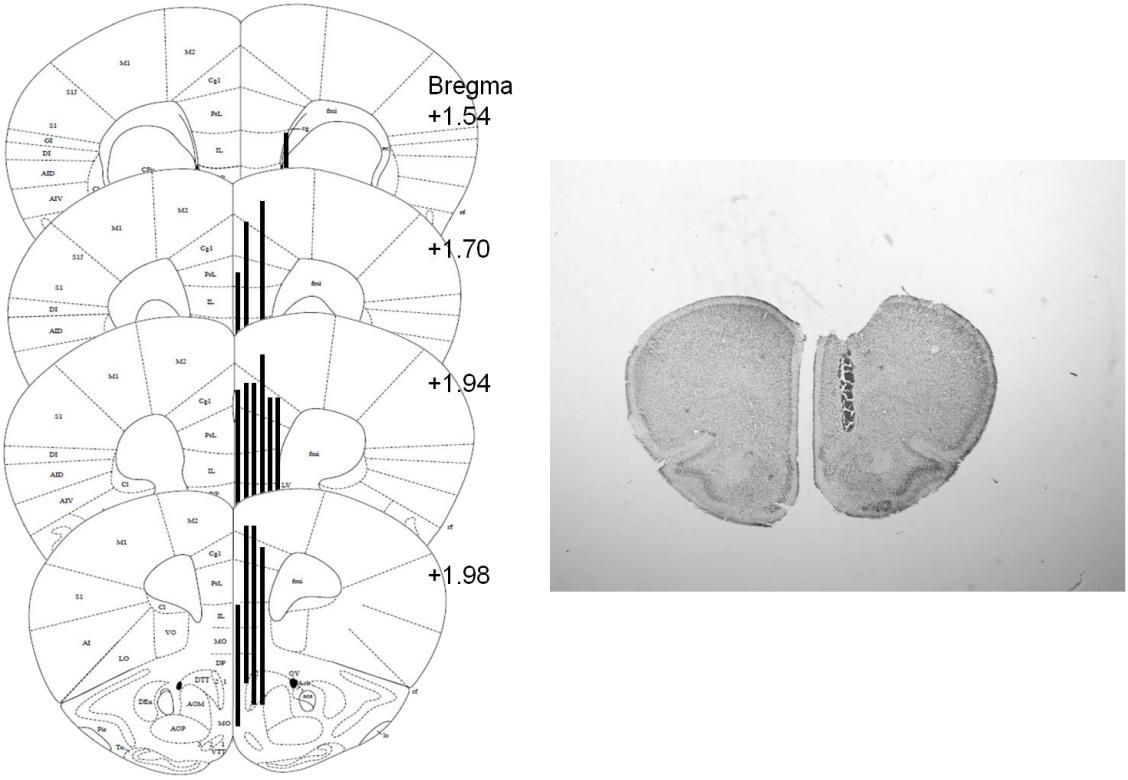


Figure 10. *Medial Prefrontal Cortex Placement Histology Summary*. Probes are denoted by black bars, and length and width are approximately accurate to scale. Subjects that had a correct probe placement that passed through the infralimbic cortex were included for statistical analysis. Missed placements are not included in this summary.

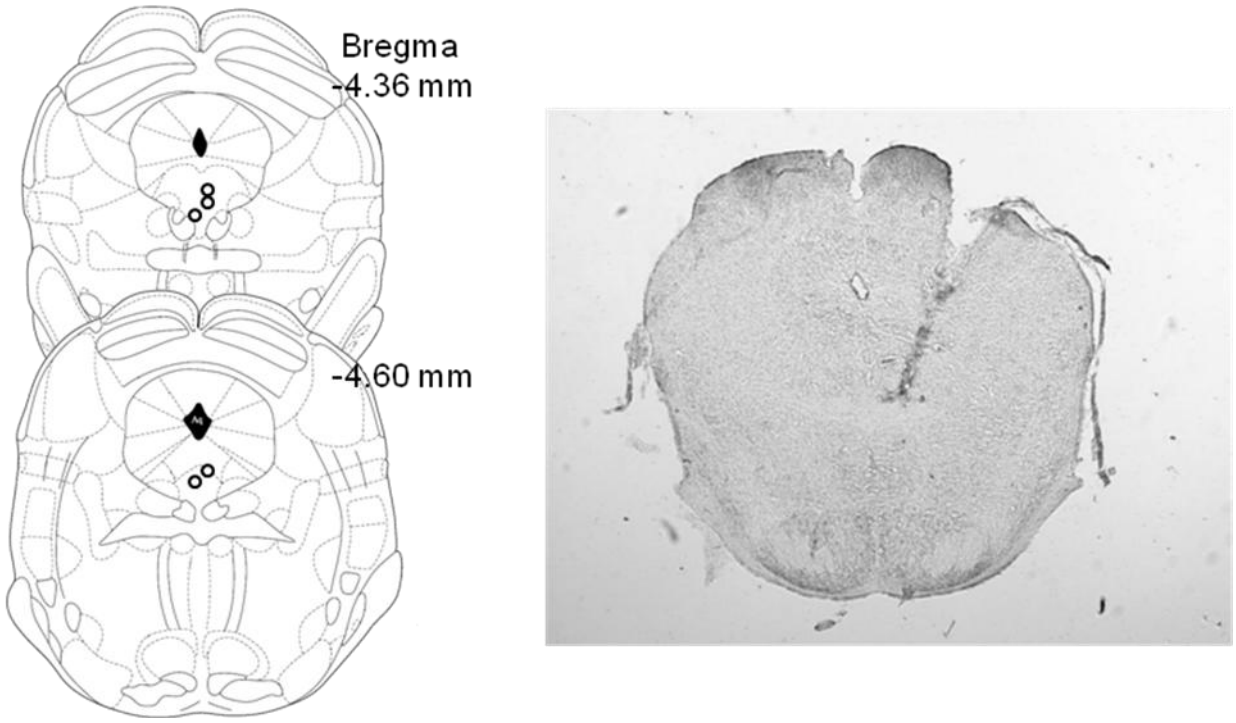


Figure 11. *Dorsal Raphe Nucleus Placement Histology Summary*. Injector placements are represented by the circles. Subjects that had a correct injector placement that infused drug into the DR were included for statistical analysis. Missed placements are not included in this summary.

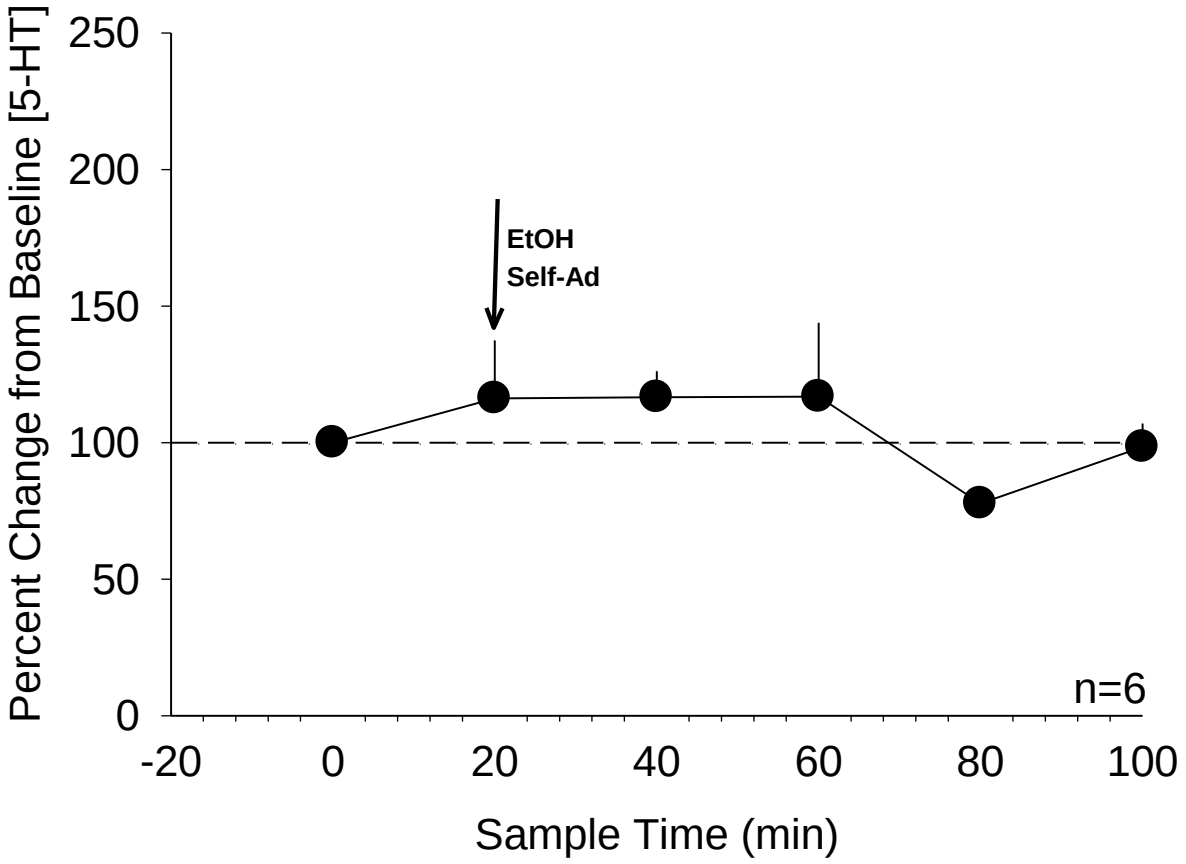


Figure 12. *Percent Change from Baseline Concentration of 5-HT in the mPFC Following Ethanol Self-Administration.* Following seven baseline samples, mice were allowed to self-administer 1 g/kg ethanol. Ethanol slightly increased 5-HT concentration for an hour following self-administration ($F_{(1,70)}$=40.999, P < 0.001).

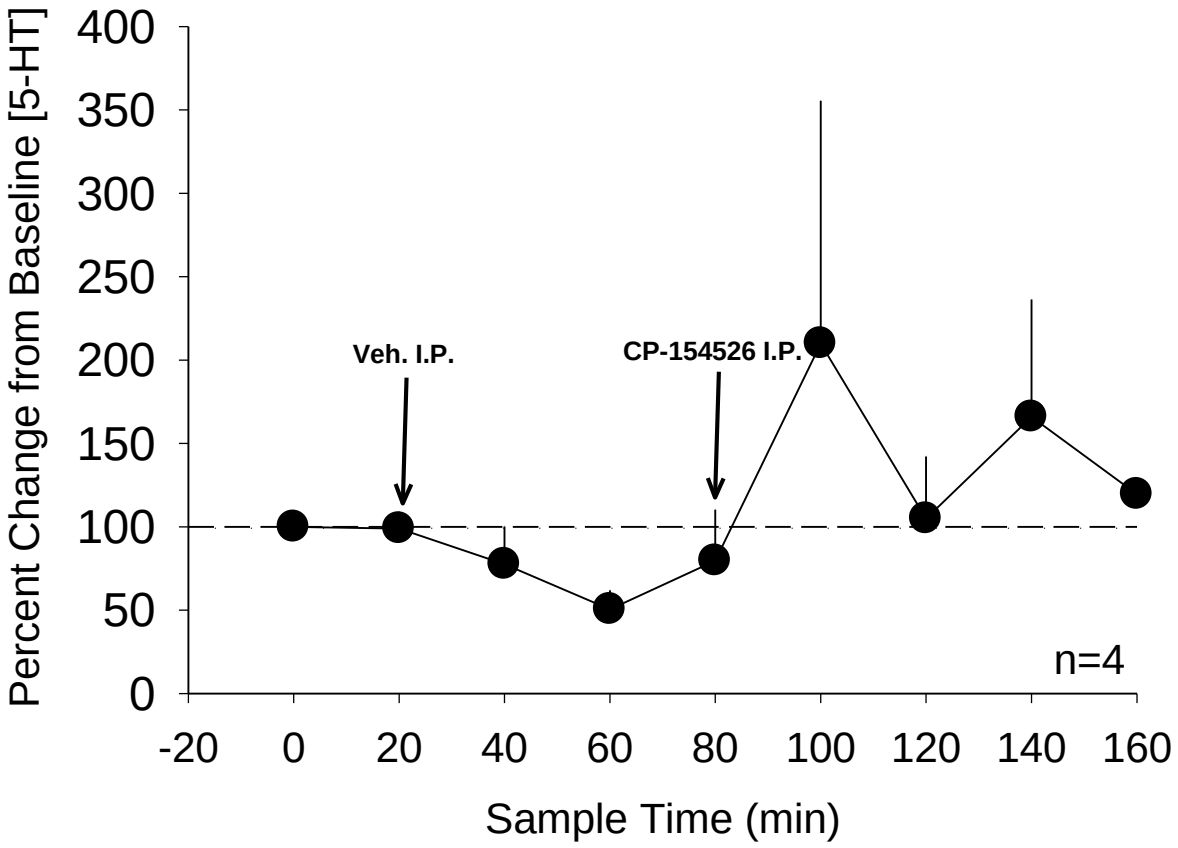


Figure 13. *Percent Change from Baseline Concentration of 5-HT in the mPFC Following Vehicle and CP-154,526 i.p. Injection.* Following seven baseline samples, mice were injected with vehicle, and after three samples were collected, an injection of 17 mg/g CP-154,526, and another five samples were collected. Overall, there was no significant change in 5-HT concentration in the mPFC. When evaluated separately, 5-HT concentration changed following vehicle injection (One way repeated measures ANOVA; $F_{(1,30)}$=29.719, p<0.001), but not after CP-154,526 injection.

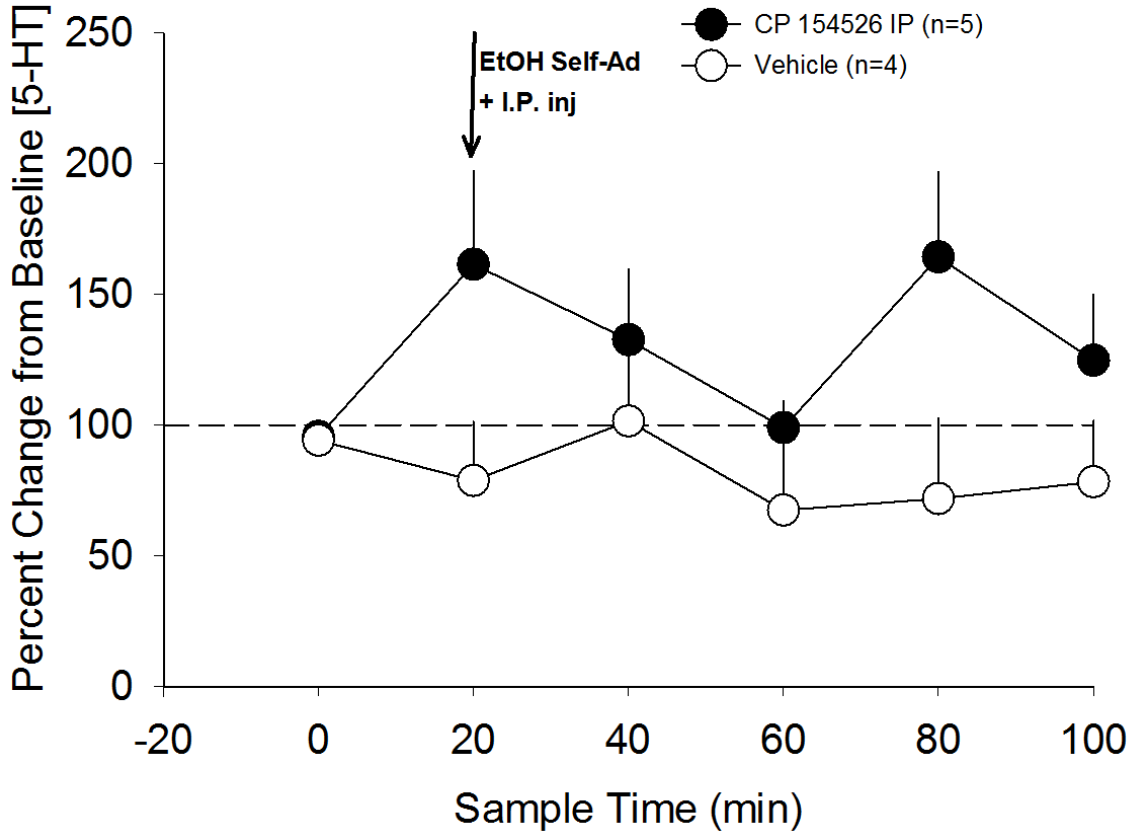


Figure 14. *Percent Change from Baseline Concentration of 5-HT in the mPFC Following Self-Administration and Vehicle or CP-154,526 i.p. Injection.* Following seven baseline samples, mice were injected with 0.01 mL/g of either 0.01% vehicle or 1.7 mg/mL CP-154,526 and another five samples were collected. Both vehicle and CP-154,526 affected 5-HT concentration following self-administration and injection ($F_{(1, 68)}$= 47.573, P < 0.001; $F_{(1, 46)}$ = 8.962, p=0.004). There was also a significant difference between drug treatments ($F_{(1, 47)}$= 7.014, p=0.011).

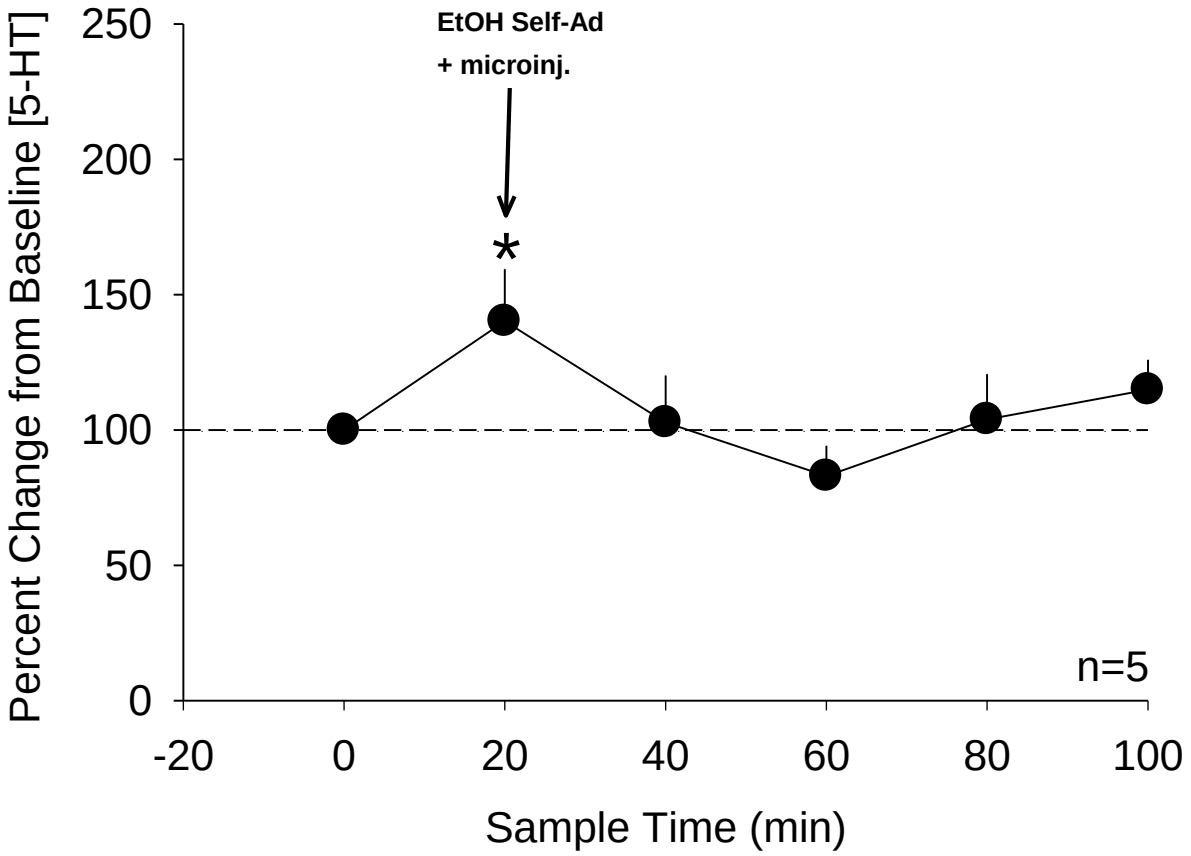


Figure 15. *Percent Change from Baseline Concentration of 5-HT in the mPFC Following Self-Administration and CP-154,526 DR microinjection.* Following seven baseline samples, mice were microinjected with 0.6 ng CP-154,526 and another five samples were collected. 5-HT concentration in mPFC increased following self-administration and microinjection of CP-154,526 ($F_{(1,58)}$=38.431, p< 0.001). Star indicates a value significantly higher than baseline concentration of 5-HT.

Tables

Table 1.

Ligand Gated Ion Channels Implicated in the Pharmacodynamics of Ethanol.

Neurotransmitter System	Receptor Subtype	References
γ-Aminobutyric Acid (GABA)	$GABA_A$	Nestoros, 1980; Takada et al., 1989; Procter et al., 1992
Acetylcholine (Ach)	Nicotinic	Mancillas et al. 1986; Yu et al. 1996; Covernton and Connelly 1997
Glutamate (Glu)	NMDA	Lovinger et al., 1989; Swartzwelder et al., 1995; Wong et al., 1997
Glycine (Gly)	Gly-R	Williams et al., 1995; Ye et al., 1998; Perkins et al., 2010
Serotonin (5-HT)	$5\text{-}HT_3$	Lovinger, 1991; Lovinger & White, 1991; Lovinger & Zhou, 1994; Zhou et al., 1998

Table 2.

General Effects of Serotonergic Drugs o n Aggression.

Drug Type	Examples	Effect on Aggression	References
5-HT_{1A} R agonist	Buspirone 8-OH-DPAT Flesinoxan	⇓	Haug et al., 1990; Bell & Hobson, 1994; de Almeida & Lucion, 1997; Ferris et al., 1999; Olivier, 2004; De Boer & Koolhaas, 2005; Clotfelter et al., 2007; Centenaro et al., 2008; Veiga et al., 2010
5-HT_{1A} R antagonist	WAY 100,635	No Effect	De Boer & Koolhaas, 2005
5-HT_{1A} autoR agonist, 5-HT_{1A} postsynaptic R antagonist	S-15535	⇓	De Boer & Koolhaas, 2005
5-HT_{1A}/5-HT_{1B} R agonist	Eltopranazine	⇓	Mos et al., 1993
5-HT_{1B} R agonist	Anpirtoline CGS12066 CP-93,129 CP-94,253 Eltoprazine TFMPP Zolmitriptan	⇓	Cologer-Clifford et al., 1997; Miczek & de Almeida, 2001; Miczek et al., 2002; Bannai et al., 2007; Faccidomo et al., 2008
5-HT_{1B} antagonist	GR-127935	No Effect	De Boer & Koolhaas, 2005
MAOA-Inhibitor	Phenelzine Isocarboxazid Tranylcypromine	⇓	DaVanzo et al., 1966; Valzelli et al., 1967; Welch & Welch, 1968; Sofia, 1969
5-HTT antagonist (SSRI)	Fluoxetine Fluvoxamine Sertraline Citalopram	⇓	Olivier et al., 1989; Delville et al., 1996; Pinna et al., 2003; Caldwell & Miczek, 2008; Carillo et al., 2009

Table 3.

Attrition Table

Exclusion Criterion	Number of Subjects
Placement	13
Headmount	9
Failed to Finish Drinking	4
Died	5
Bit Through Tubing	2
Refused to Fight	1
Total	34