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Medial prefrontal cortical extracellular dopamine responses after acutely experimenter-administered or orally self-administered ethanol

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Dedication

This dissertation is dedicated to my wonderful friends and family who have kept me sane over the many years required to achieve this degree.

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Medial prefrontal cortical extracellular dopamine responses after acutely experimenter-administered or orally self-administered ethanol

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Dopamine signaling in the prefrontal cortex is thought to play a role in ethanol abuse. However, little is known about how ethanol affects dopamine signaling in the region. There are a few rodent studies regarding the matter, but both the pharmacological effects of ethanol and the effects of self-administered ethanol on extracellular dopamine in the medial prefrontal cortex remain unclear. The goal of the studies conducted for this dissertation is to clarify these relationships. To accomplish this, we monitored both dialysate dopamine and ethanol concentrations in the medial prefrontal cortex of Long Evans rats while an experimenter administered or a rat operantly self-administered ethanol. In naïve rats, dopamine dose-dependently increased after the intravenous infusions of a 10% ethanol solution, while no changes were noted after saline infusions. In rats trained to orally self-administer drinking solutions, dopamine transiently increased at the initiation of consumption in both ethanol-plus-sucrose- and sucrose-solution-consuming rats. Dopamine concentrations remained significantly elevated for the entire 21-minute drinking period in the ethanol-plus-

sucrose-consuming group and for the first seven minutes of the drink period in the sucrose-consuming group. Additionally, in the ethanol-plus-sucrose-consuming group, dialysate ethanol concentrations were lowest at the initiation of drinking and then slowly increased, peaking 35 minutes after drinking commenced. Taken together, these data suggest that the mesocortical dopamine system is responsive to acute, intravenous and repeatedly, orally, self-administered ethanol. It appears that direct pharmacological effects of ethanol were responsible for the dopamine increase after acute, ethanol administration. Furthermore, while is it possible that the direct pharmacological effects of ethanol also bolstered the dopamine response seen after ethanol self-administration, we cannot firmly conclude by what mechanism ethanol elicited the differences. Overall, our clarifying and novel results support a role for the mesocortical dopamine system in ethanol abuse, which deserves continued investigation.

In addition to completing the two aforementioned data studies, we also published the methods we use to monitor dialysate ethanol concentrations, in a specific brain region, during ethanol self-administration in a video-methods journal. The methods are presented in both a detailed written protocol, as well as a video demonstrating how to perform the procedures.

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Chapter 1: General Introduction and specific aims

Overview

Alcoholism and alcohol (ethanol) abuse affects the individual and society as a whole. It is currently thought that around 18 million United States residents suffer from an ethanol-use disorder ("Alcohol Use Disorders", NIAAA, 2012). Worldwide, ethanol is the third highest risk factor for disease and disability, and is causally linked to 60 major types of illness and injury (World Health Organization, 2011). Therefore, this is an important research topic not only to better understand and provide treatments for alcoholics, but also to ameliorate the devastating effects the disorder has on society.

Ethanol use and abuse is associated with many cognitive deficits such as perseveration, poor decision-making and working memory, as well as difficulties maintaining goal-directed behaviors (Bechara and Van Der Linden, 2005; Fuster, 2008; Chanraud et al., 2007; Goldstein et al., 2004). These functions are inextricably linked with the prefrontal cortex (PFC), making the region an obvious, and yet understudied, target for ethanol research (Demakis, 2003; Takahashi et al., 2012; Slifstein et al., 2008; Mattay et al., 2003; Casey et al., 1997).

The PFC is part of the mesocortocolimbic dopamine system, which is believed to be important in ethanol abuse (Di Chiara, 1999; Gonzales et al., 2004; Volkow et al., 2009). Many subregions in the system such as the nucleus accumbens and ventral tegmental area are already well established as critically involved in the

reinforcing effects of ethanol, yet the medial prefrontal cortex (mPFC) has received little research effort by comparison (Czachowski et al., 2001, 2012; Doyon et al., 2003, 2005; Hodge et al., 1993, 1996; Howard et al., 2008, 2009; Samson and Chappell, 2001, 2003; reviewed in Gonzales et al., 2004; Hodge et al., 1993; Samson and Chappell, 2003). Our understanding of the effect of ethanol on mPFC extracellular dopamine falls short in two major areas: pharmacological and behavioral effects.

The pharmacological effect of non-contingent ethanol administration on extracellular dopamine activity in the mPFC is a debated subject. Some studies show that a hormone-ethanol interaction, intraperitoneal ethanol or ethanol microinjected into the posterior ventral tegmental area increase mPFC extracellular dopamine (Dazzi et al., 2002, 2007; Ding et al., 2011). However, other studies have found no extracellular dopamine response in the mPFC after acute intraperitoneal ethanol administration (Hegarty and Vogel, 1993; Bassareo et al., 1996; Engleman et al., 2006). The multiple experimental factors that could account for these disparate results will be addressed in Chapter 2, and our findings regarding the pharmacological effects of ethanol on extracellular dopamine in the mPFC will be presented.

Pharmacological manipulations of dopamine receptors in the mPFC modify ethanol self-administration behaviors; however, the exact effects of these manipulations on seeking and consumption behaviors are unclear (Hodge et al., 1996; Samson and Chappell, 2003). Furthermore, as pharmacological manipulations could cause compensatory changes in an intricately connected

system such as the mesocorticolimbic system, it is important to establish the inherent role of mPFC extracellular dopamine during ethanol self-administration. Therefore, we evaluated extracellular dopamine concentrations in the mPFC during ethanol self-administration. It should be noted that, to our knowledge, this is the first time any laboratory has monitored mPFC dopamine during ethanol self-administration. These finding are presented in Chapter 3.

The extremely concise manner in which methods sections are written in the life sciences field can sometimes unintentionally obscure the methods used in studies, making it difficult for other researchers to replicate the data or even just learn the techniques. Therefore, to allow other researchers access to learning a relatively complicated and unique set of techniques, and to increase the transparency of our experimental methods, we completed a video-methods-publication of most of the methods used in Chapter 3. The final written product is presented in Chapter 4 and the video may be viewed at http://www.jove.com/video/4142/microdialysis-ethanol-during-operant-ethanol-self-administration.

Specific aims

My dissertation centers on two specific questions, which lead to two specific experimental aims. Additionally, I chose to address issues regarding the explanation and transparency of experimental methods, leading to an additional non-data aim:

1) What are the pharmacological effects of ethanol on extracellular dopamine in the mPFC of the Long Evans rat?

Aim 1: Monitor extracellular dopamine concentrations in the mPFC during intravenous administration of saline and a 10% ethanol-saline solution.

2) What are the effects of ethanol self-administration, in a separate seeking-consummatory model, on extracellular dopamine in the mPFC of the Long Evans rat?

Aim 2: Monitor extracellular dopamine concentrations in the mPFC on the seventh day of operant self-administration of ethanol in the presence of sucrose.

3) Our laboratory performs extremely technical and complicated experiments, which are difficult to fully communicate in a traditional methods section. How can we best explain and make it clear to others how we perform our experiments?

Aim 3: Fully explain and demonstrate the methods necessary to monitor dialysate ethanol concentrations in a rat during an operant ethanol self-administration session in a manner publishable online.

Dopamine, prefrontal cortical executive functions and ethanol

The PFC is associated with many higher level "executive" functions, which are necessary for optimal interaction with and success in an animal's or human's environment. These functions include, but are not limited to, working memory, attention, set-shifting, ability to change strategy based on new information as opposed to perseverating and relying on previously rewarded strategies, behavioral inhibition, decision-making and goal-directed behavior (Demakis, 2003; Takahashi et al., 2012; Slifstein et al., 2008; Mattay et al., 2003; Casey et al., 1997). Deficits in these abilities, as well as decreased frontal cortical grey and white matter, are associated with ethanol abuse (Kubota et al., 2001; Pfefferbaum et al., 1997; Rando et al., 2011; Bechara and Van Der Linden, 2005; Fuster, 2008; Chanraud et al., 2007; Goldstein et al., 2004; Demakis, 2003; Sullivan et al., 1993; Bechara and Damasio, 2002; Bechara et al., 1996; Rossiter et al., 2012; Easdon et al., 2005). Literature regarding human and rodent studies will be briefly discussed.

<u>Human studies</u>

Work with PFC brain injury patients shows that an intact PFC is important for proper executive functioning. Behavioral tests, imaging and comparing drug addicts to brain lesion patients has also shown that the PFC and dopamine signaling in the PFC are important in drug abuse and specifically ethanol abuse. Basic findings regarding the topic are discussed below.

Working memory, attention and set-shifting

In humans, the Wisconsin card sorting test has been used to demonstrate that working memory, attention and set-shifting are associated with the PFC and PFC dopamine functioning. Patients with frontal lobe damage perform significantly worse on the Wisconsin card sorting test, achieving more perseveration errors, but not non-perseveration errors, than patients with brain damage in non-frontal regions (Demakis, 2003). Furthermore, within an intact prefrontal cortex, it appears that dopamine signaling optimizes executive functioning during tasks. Takahashi et al. (2012) found a "putative U-shaped" relationship between dopamine D_1 receptor availability in the prefrontal cortex and total errors made on the Wisconsin card sorting test. This is supported by work analyzing subjects with a valine/valine (val/val) or a methionine/methionine (met/met) catechol O-methyltransferase polymorphism. A val/val polymorphism leads to higher catechol O-methyltransferase enzyme activity, while met/met leads to lower enzyme activity. Because catechol O-methyltransferase is a significant factor in terminating PFC dopamine signaling, val/val carrying subjects are thought to have lower PFC dopamine, while met/met have higher PFC extracellular dopamine (Kaenmaki et al., 2010). The val/val subjects performed significantly worse on the Wisconsin card sorting test than the met/met subjects, but their performance substantially improved after amphetamine administration, while the drug decreased the met/met subjects' performance (Slifstein et al., 2008, Mattay et al., 2003). Taken together, these data suggest that the PFC and dopamine signaling in the PFC are associated with working memory, attention and set-shifting in humans.

It is probable that the prefrontal cortex is affected by and contributes to ethanol abuse, because deficits in executive functioning are common in chronic ethanol abusers (Sullivan et al., 1993; Chanraud et al., 2007). Sullivan et al. (1993) showed that chronic alcoholics perform worse on the Wisconsin card sorting task than controls. Chanraud et al. (2007) took this one step further and found that sober alcohol-dependent males made more perseveration errors during the Wisconsin card sorting test and showed up to a 20% reduction in dorsolateral frontal cortical volume compared to healthy counterparts. Finally, acute ethanol administration has also been found to diminish working memory (Ralevski et al., 2012). Overall, these data suggest that ethanol use affects executive functioning, and that this is likely attributable to prefrontal dysfunction.

Decision making

Patients with frontal lesions as well as "substance-dependent individuals" show deficits in decision making and have abnormal pre-decision physiological responses thought to guide behavior in humans (Bechara and Damasio, 2002, Bechara et al., 1996). The Iowa gambling task evaluates a subject's ability to successfully weigh risk and positive/negative outcomes to achieve optimal gains. Using this test, Bechara and colleagues (1996, 2002) found that patients with prefrontal cortical lesions, as well as drug abusers, have deficits in optimal decision making. Additionally, these groups did not show typical physiological indications of an affective response (skin conductance responses) prior to selecting a high risk option compared to healthy subjects. This suggests that persons with certain prefrontal dysfunctions do not display the same somatic responses that putatively guide "normal" humans in their decision making

("Somatic Marker Hypothesis"). Taken together, this suggests that while the prefrontal cortex does not act alone, it does play an integral part in appropriate long-term decision making, which may be guided by affective responses. Additionally, these data highlight the importance of researching how drugs of abuse, including ethanol, affect this region, and how deficits in the PFC might lead to uncontrolled drug-taking behavior.

Behavioral inhibition

The go/no-go task has been used to show that the PFC is associated with, and that ethanol use can attenuate, optimal behavioral inhibition. During a go/no-go task the dorsolateral and orbitofrontal cortices were activated in adults and children, suggesting that the PFC plays a role in behavioral inhibition (Casey et al., 1997). Acute ethanol administered to non-problem drinkers led to increased errors on this task (Easdon et al., 2005). Furthermore, Rossiter et al. (2012) found that heavy and moderate ethanol drinkers showed different levels of behavioral inhibition on a go/no-go task under a delayed-monetary reward or immediate punishment condition. Overall, these data suggest that behavioral disinhibition is associated with alcoholism, which supports the PFC as an important target in ethanol research.

Chronic, as well as acute, ethanol can diminish executive functions. This is not only an issue of behavioral control while intoxicated, but also leads to difficulties during recovery from ethanol addiction. Therefore, understanding how the PFC modulates executive functions, how ethanol affects the PFC, and how a compromised PFC affects ethanol addiction and recovery is imperative in

alcoholism research. There are significant limits to the types of experiments that researchers can use on humans, therefore, many aspects of ethanol abuse and executive functioning require animal models to fully examine the topic.

Animal models of executive function

There are many animal behavioral paradigms used to model executive function. Furthermore, more invasive procedures are possible and researchers can control lifetime drug exposure, environment and other extraneous factors in animal models that are often uncontrollable in human studies. Therefore, animal studies are invaluable for helping to elucidate the effects of ethanol on the PFC and executive functions. Findings pertinent to this dissertation topic are discussed below.

Working memory

Optimal prefrontal dopamine signaling is required for optimal working memory function and memory retrieval. Manipulation of dopamine D_1 receptors, but not typically dopamine D_2 receptors, leads to modifications of performance on working memory tasks (Floresco and Magyar, 2006). For example, during a short delay (10 – 30 minutes) between a training and recall radial maze trial, "supranormal" dopamine D_1 stimulation or attenuation degrades working memory performance (Zahrt et al., 1997; Seamans et al., 1998). However, Floresco and Phillips (2001) showed that the microinjection of a dopamine D_1 agonist in the mPFC diminished working memory performance when a short (30 minute) delay occurred between training and test trials, but improved performance after a 12-hour delay. This suggests that endogenous mPFC dopamine activity interacts

with the amount of time a representation needs to be held online to optimize task performance.

This theory is supported by a similar experiment in which Phillips et al. (2004) used an eight-arm radial maze test paired with microdialysis to directly show that working memory/memory retrieval is optimized via mPFC extracellular dopamine concentrations. Specifically, mPFC dialysate dopamine increased during training, as well as when animals entered the correct arms 30 minutes later during a recall session. Importantly, this dopamine increase was independent of whether rats received food in the correctly selected arm or not. Finally, when the delay period was unexpectedly extended (1 or 6 hours) recall test performance time-dependently decreased, and the recall-related dopamine increase was attenuated after 1 hour and abolished after 6 hours. Therefore, an increased delay period led to little or no mPFC dopamine increase and decreased working memory and retrieval performance.

Taken together, these data suggest that the effects of dopamine receptor signaling manipulation on working memory could be dependent on whether endogenous mPFC dopamine concentrations are currently optimal. As in humans, an inverted U-function is thought to best relate prefrontal dopamine concentration to optimal working memory (Floresco and Magyar, 2006). Unsurprisingly, ethanol dose-dependently decreases performance on a spatial working memory task (White et al., 1997), which suggests that this drug affects mesocortical dopamine functioning, and further supports the need to investigate the role of mPFC dopamine function in ethanol research.

Behavioral inhibition

Behavioral inhibition is associated with proper mPFC functioning and possibly mPFC dopamine signaling in rodent models. Rats trained on an alternating go/no-go task, which tests behavioral inhibition, showed reductions in correct responses after mPFC lesions, though it should be noted that the lesion was predominantly in the anterior cortex (Sakurai and Sugimoto, 1985). However, lesions in the infralimbic cortex led rats to increased premature responding, and decreased responding latency, suggesting infralimbic dysfunction can lead to increased impulsivity (Chudasama et al., 2003). Finally, a low dose of amphetamine enhanced inhibitory performance, while a high dose attenuated performance on a behavior inhibition task, suggesting that appropriate dopamine functioning optimizes behavioral inhibition (Gregoire et al., 2012). Overall, these data suggest that, similar to working memory, optimal inhibitory function requires an intact mPFC and optimal dopamine signaling.

Set-shifting and attention

The PFC and PFC dopamine signaling is associated with the ability to attend to appropriate stimuli and to modify behavior according to changing reward-predictive stimuli. Rats with lesions in or dopamine signaling manipulation of the infralimbic and prelimbic cortices showed deficits in extra-dimensional set-shifting, suggesting that the region and dopamine signaling in the region are important in higher-level behavior modification (Birrell and Brown, 2000; Floresco et al., 2006). Furthermore, rats with prelimbic cortex lesions showed increased perseverative responding during a five-choice serial reaction time task and

decreased ability to discriminate cues during an attention task, further implying that the mPFC plays a role in appropriate attention to cues and updating advantageous reward strategies (Chudasama and Muir, 2001). Finally, chronic intermittent ethanol exposure decreased attentional set-shifting performance in mice (Kroener et al., 2012), implying that ethanol affects the role of the mesocortical dopamine system in attentional and set-shifting behaviors.

Animal models of executive functions have furthered our understanding of how the PFC modulates these functions. Furthermore, these models allow for the elucidation of mechanisms of drug abuse that lead to regional dysfunction and behavioral deficits. Finally, these models will also allow for future testing of therapeutic drugs that could enhance PFC function in order to assist recovering addicts.

Anatomy of the prefrontal cortex and its connections

Dopamine, the dopamine receptor, mPFC anatomy and connections with other regions, as well as the putative actions of dopamine in the mPFC, are briefly discussed below.

Mesocortical dopamine system

The mesocortical dopamine system consists of dopaminergic neurons with cell bodies mostly in the ventral tegmental area (A10) that project to the PFC (Vertes, 2004; Sesack and Carr, 2002). Medial ventral tegmental area dopaminergic

afferents innervate PFC layers V and VI, while lateral ventral tegmental area innervate layers I – III afferents less densely (Gonzalez-Burgos et al., 2007). However, ventral tegmental area dopaminergic projections are not homogenous. Its dopamine afferents to the mPFC can be differently modulated and show unique firing patterns compared to those projecting to the nucleus accumbens (Margolis et al., 2006, 2008). Furthermore, the posterior ventral tegmental area has been shown to be more drug sensitive than the anterior ventral tegmental area (Rodd et al., 2007, 2008; Hauser et al., 2011). For example, Ding et al. (2011) showed that ethanol microinjected into the posterior, but not anterior, ventral tegmental area increased mPFC extracellular dopamine concentrations. Similarly, ethanol microinjection into the posterior, but not anterior, ventral tegmental area reinstated ethanol-seeking behaviors in alcohol-preferring rats (P rats); however, this effect was blocked by the co-infusion of a D2/3 agonist (Hauser et al., 2011). Finally, rats have been shown to intracranially self-stimulate when electrodes are placed in the mPFC (Corbett, 1992). Overall, the mesocortical dopamine system is well positioned to affect drug self-administration behaviors, and is currently implicated as a region of interest in research on drugs of abuse.

Infralimbic and prelimbic mPFC subregions

The infralimbic and prelimbic regions are subregions of the mPFC (Illustration 1.1). These are highly connected with limbic and striatal regions, as well as interconnected within the PFC (Vertes, 2004; Groenewegen and Uylings, 2000). The prelimbic subregion in the rodent is thought to be homologous to dorsolateral PFC in primates, which are more associated with cognitive processes. The

infralimbic subregion in the rodent is thought to be homologous to the orbitomedial PFC in primates, which are more associated with visceral and autonomic processing (Groenewegen and Uylings, 2000). Both subregions have been shown to be important in many drugs-of-abuse models, and yet the mPFC has received significantly less attention than the nucleus accumbens or ventral tegmental area in ethanol research (Gonzalez-Burgos et al., 2007; McFarland et al., 2003, 2004; Peters et al., 2008; Kroener et al., 2012; Groblewski et al., 2011; Dayas et al., 2007; Di Chiara, 1999; Gonzales et al., 2004).

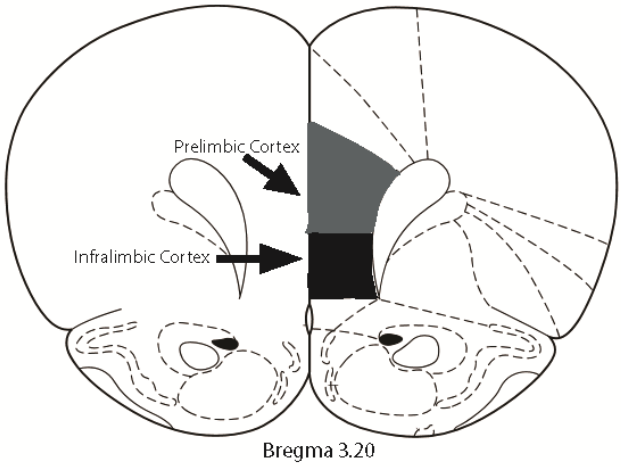

Illustration 1.1. mPFC subregions of interest: prelimbic cortex and infralimbic cortex. Coronal slice from a rat (bregma 3.20). Figure adapted from Paxinos and Watson (1998).

PFC interconnections

The PFC is highly interconnected with many brain regions, such as the ventral tegmental area and nucleus accumbens, which are both highly associated with drug abuse (Gonzalez-Burgos et al., 2007; Di Chiara, 1999; Gonzales et al., 2004). The PFC sends glutamatergic projections to the ventral tegmental area and the nucleus accumbens (Van Bockstaele and Pickel; 1995, Sesack and Pickel, 1992). The prelimbic cortex projects heavily to the core, but also to the shell of the nucleus accumbens, with the exception of the caudal shell. The infralimbic cortex predominantly targets the medial shell of the nucleus accumbens (Vertes, 2004; Sesack and Grace, 2010; Berendse et al., 1992). The prefrontal cortex-accumbens glutamate transmission itself is associated with drug-seeking behaviors (Kalivas and Volkow, 2005). Furthermore, output from the prefrontal cortex to the ventral tegmental area has been shown to modulate ventral tegmental area-derived extracellular dopamine concentrations in the nucleus accumbens (Karreman & Moghaddam, 1996; Jackson et al., 2001). Finally, the ventral tegmental area sends both dopaminergic and GABAergic projections to both the nucleus accumbens and medial prefrontal cortex (Vertes, 2004; Sesack and Carr, 2002; Carr and Sesack, 2000), and the nucleus accumbens sends GABAergic projections to the ventral tegmental area (Xia et al., 2011).

Dopamine and the mPFC

Dopamine is a catecholamine neurotransmitter that is derived from the amino acid tyrosine (Kandel et al., 2000), and binds to G protein coupled dopamine receptors (Cooper et al., 2003). There are two major classes of these receptors:

D_1-like (D_1 and D_5 dopamine receptors), and D_2-like (D_2, D_3, and D_4 dopamine receptors). D_1-like dopamine receptors increase, while D_2-like dopamine receptors inhibit adenylyl cyclase activity (Kebabian and Calne, 1979; Cooper et al., 2003). D_1-like and D_2-like dopamine receptors are present in the mPFC, located on both non-pyramidal and pyramidal neurons, and are more densely expressed in layers V and VI (Vincent et al., 1993; Seamans and Yang, 2004). However, D_1-like receptors substantially outnumber D_2-like receptors in the PFC (Gonzalez-Burgos et al., 2007). Finally, midbrain dopamine neurons are known to fire both, at a slow rate over a long period of time (tonic firing) and at a rapid rate for a short period of time (phasic firing) (Hyland et al., 2002). While tonic firing contributes to basal extracellular levels of dopamine in terminal regions, phasic firing can also increase extracellular dopamine concentrations (Suaud-Chagny et al., 1992).

The mPFC has lower extracellular dopamine concentrations and unique dopamine-signaling termination compared to other dopamine-rich regions. Engleman et al. (2006) determined that the mPFC extracellular dopamine concentration in a male Wistar rat is 4.8 ± 0.4 nM and in a P rat is 2.0 ± 0.4 nM. For comparison, Yim and Gonzales (2000) found that male Sprague-Dawley rats have around a 9 nM extracellular dopamine concentration in the nucleus accumbens, though studies using microelectrodes estimate accumbal dopamine extracellular concentrations to be closer to 18 or 20 nM (Shou et al., 2006; Owesson-White et al., 2012). However, dopamine re-uptake transporters are scarce in the prelimbic and infralimbic mPFC and are located farther from synapses compared to regions like the dorsal striatum (Sesack et al., 1998).

This combination leads to greater extracellular dopamine diffusion in the mPFC. This is unlike the nucleus accumbens where the dopamine transporter is responsible for a large portion of dopamine uptake and therefore significantly contributes to basal extracellular dopamine concentrations (Owesson-White et al., 2012).

The dopamine transporter is not the only mechanism which terminates dopamine signaling in the mPFC. Both the dopamine and norepinephrine transporters are able to take up dopamine (Carboni et al., 2006). In catechol-O-methyltransferase knock-out mice and wild-type littermates, inhibition of the norepinephrine transporter or monoamine oxidase leads to increases in dialysate dopamine concentrations. Yet, inhibition of the dopamine transporter in the same animals did not change dialysate dopamine concentrations (Kaenmaki et al., 2010). Furthermore, when no-net-flux is performed on catechol-O-methyltransferase knock-out mice, increases in basal extracellular dopamine are noted in the prefrontal cortex, but not the nucleus accumbens or the dorsal striatum. Additionally, inhibition of catechol-O-methyltransferase in wild-type littermates also leads to increases in dialysate dopamine, suggesting that catechol-O-methyltransferase is also a substantial mechanism for dopamine-signaling termination in the PFC (Kaenmaki et al., 2010). Taken together, this suggests that multiple mechanisms terminate dopamine signaling in the mFPC.

Dopamine in the PFC is thought to serve as a neuromodulator, affecting the likelihood and patterns of firing of PFC interneurons and pyramidal neurons (Gonzalez-Burgos et al., 2007). Moore et al. (2011) used an iontophoretic

current pulse application of dopamine to mimic phasic dopamine firing in brain slices and showed that, on average, lower dopamine concentrations increased PFC pyramidal neuron firing, but higher concentrations suppressed firing (10 mM dopamine in the pipette, 20-25 µm from soma, current range 20-600 nA, depolarized neurons). However, heterogeneous responses were noted within the population; yet, overall a D_1 agonist resulted in suppression of firing, while a D_2 agonist increased firing, suggesting an explanation for the differences. This agrees with data suggesting that D_2-like receptors have a higher affinity for dopamine than D_1-like receptors (Richfield et al., 1989). It also supports Moore and colleagues' hypothesis that the initial dopamine burst firing directly onto one set of mostly D_1-expressing pyramidal neurons temporarily suppresses firing, while the "spillover dopamine" later increases the excitability of D_2-expressing pyramidal neurons. Collectively, these data support that dopamine serves a modulatory role in the PFC.

Cues, drugs, learning and the PFC

Mesocorticolimbic dopamine signaling is thought to be important for cue learning, which facilitates an organism's survival and adaptation to ever changing surroundings. Drugs of abuse exploit similar, if not identical neural mechanisms. Therefore, to understand the reinforcing effects of ethanol, pertinent learning theory and drug-cue-brain relationships must be explored. Theories and findings relevant to these topics are discussed below.

Learning theories

Associative learning

Associative learning theory outlines the importance of dopamine in creating new associations between a new stimulus and response contingency. The PFC and dopamine signaling in the region are thought to be important for associative learning (Puig and Miller, 2012; Kehagia et al., 2010). For example, blockade of dopamine D_1 receptors in the lateral PFC of non-human primates led to deficits in learning new associations, but not in performing "familiar associations", suggesting that dopamine signaling in the PFC is important for associative learning but not necessarily for expression of previously learned associations. During learning, a population of PFC neurons showed an increase in firing after presentation of the conditioned cue associated with the reinforced response, as opposed to the un-reinforced response. A dopamine D_1 receptor antagonist appeared to disrupt this task-specific PFC neural-firing selectivity, which putatively guided the animal to respond correctly to the cue (Puig and Miller, 2012). Taken together, these data suggest that optimal PFC dopamine signaling facilitates associative learning and by extension behavioral flexibility.

One interesting aspect of this theory is that the mPFC extracellular dopamine response to a conditioned cue does not appear to decrease after learning. Bassareo et al. (2002) noted an mPFC extracellular dopamine increase after presentation of a conditioned cue, as well as after the consumption of the palatable food predicted by the cue. However, only three cue-food pairings

preceded this experiment, and therefore the pairing was not necessarily an extremely “familiar association”. Furthermore, mPFC dopamine increases are associated with many types of stimuli, both appetitive and aversive as well as conditioned and unconditioned. Therefore, it seems likely that dopamine signaling in this region serves a more general role in cue processing than solely dealing with initial cue-response associations (Abercrombie et al., 1989; Feenstra et al., 1998, 2000; Hegarty and Vogel, 1993; Segovia et al., 2008; Bassareo and Di Chiara, 1999; Bassareo et al., 2002, 2007).

Reward prediction error

The reward prediction error theory outlines the adaptive behavior of midbrain dopamine neurons (which project to striatal and prefrontal regions), and suggests how the activity of these neurons leads an organism to learn about and predict reinforcer availability in the environment (Vertes, 2004; Seasack and Carr, 2002; Schultz et al., 1993; Schultz, 1997; Contreras-Vidal and Schultz, 1999; Stuber et al., 2008). Schultz and colleagues used macaque monkeys to show that midbrain dopamine neurons fire in response to natural reinforcers. After repeated presentation of a stimulus prior to reinforcer delivery, neural firing began to coincide with the conditioned stimulus predicting the reinforcer instead of during reinforcer delivery. This remained true as long as the “reward prediction” remained constant. If the reinforcer was unexpectedly not delivered, a decrease in neural firing was noted. If an unexpected reinforcer was delivered, firing increased (Schultz et al., 1993; Schultz, 1997). This is thought to allow for behavioral adaptation as conditions change; essentially, dopamine is thought to serve as a teaching signal (Schultz, 2002). Though Schultz and colleagues did

not verify the terminal regions of the putative midbrain dopamine neurons tested, later work has shown that accumbal dopamine transients demonstrate a shift from activity during reinforcer receipt to cues predicting the reinforcer (Stuber et al., 2008). Additionally, neuronal activity in the PFC has also been shown to exhibit reward-prediction-error-like responses during conditioning (Wallis and Kennerley, 2011; Monosov and Hikosaka, 2012).

The size of the midbrain dopamine neuronal response appears to be linked to the relative value of the reinforcer (Roesch et al., 2007; Roesch and Bryden, 2011). Rats trained on a delay discounting task demonstrated that the level of cue-induced ventral tegmental area neuronal activity was dependent on the animal receiving the preferred-reinforcer cue for that specific type of trial. The reinforcer could have been exactly the same between two types of trials, but as long as it was the “better option” within that type of trial, it elicited a larger cue-induced response (Roesch et al., 2007). Therefore, both the availability and the relative value of a reinforcer can be encoded to allow for future reward prediction.

Both of these learning theories highlight the importance of dopamine in learning and behavioral adaptations. The maladaptive and difficult-to-change behaviors noted in drug abuse and the ability of drugs of abuse to elicit these behaviors could even be thought of as dysfunctional learning. Therefore, though there are most likely many factors at play in drug abuse disorders, understanding how drugs of abuse and their associated cues interact with cortical regions should be a paramount topic in ethanol and drug-abuse research.

Drug cues and the mesocortical system

It is well established that dopamine signaling in the prefrontal cortex is involved in cue, stress, and drug-primed reinstatement of drug-seeking behaviors in cocaine self-administration. Activation of the infralimbic prefrontal cortex suppressed cocaine-primed reinstatement (Peters et al., 2008), while antagonizing dopamine D_1/D_2 receptors in the dorsal prefrontal cortex, or blockade of the prelimbic cortex and anterior cingulate with $GABA_{a/b}$ agonists suppressed footshock-induced and cocaine-induced cocaine reinstatement, respectively. Furthermore, the prefrontal blockade also blocked the extracellular glutamate increase in the nucleus accumbens core, which was shown to accompany cocaine reinstatement (McFarland et al., 2003, 2004). Similarly, ventral tegmental area-prefrontal cortex dopamine transmission also appears to be essential for foot-shock- or drug-primed-cocaine reinstatement (Capriles et al., 2003; McFarland et al., 2004).

Drug-paired cues increase mPFC extracellular dopamine and can also enhance a drug-induced mPFC dopamine increase. Bassareo et al. (2007) found that a morphine- or nicotine-predictive cue increased mPFC dialysate dopamine during cue presentation and that drug-conditioned animals showed greater orientation, approach, and contact behaviors with the box compared to saline-conditioned or nicotine-unconditioned animals. Furthermore, the box presentation prior to nicotine administration led to an enhanced nicotine-induced mPFC dopamine increase compared to controls that receive the same nicotine dose (Bassareo et al., 2007). These findings support the assertion that mesocortical dopamine

signaling has a role in drug-cue association, and that drugs of abuse could affect the system.

Ethanol research regarding mesocortical dopamine functioning is not nearly as thorough as it is for other drugs of abuse. There is emerging evidence that the PFC is sensitive to ethanol-related cues and could play a role in ethanol abuse, as it does for other drugs of abuse. Ethanol-associated cues increased phosphorylation of cAMP response element-binding protein in the prelimbic and infralimbic subregions of the mPFC (Groblewski et al., 2011) and in a separate study, concomitantly induced reinstatement and increased c-fos expression in the prelimbic, infralimbic and anterior cortex subregions of the mPFC (Dayas et al., 2007). Chronic intermittent ethanol exposure resulted in the modification of mPFC NMDA-receptor dependent spike-timing-dependent plasticity and reduced attentional set-shifting performance (Kroener et al., 2012). Taken together, these findings suggest that the mPFC is responsive to ethanol-related cues, which could lead to drug-seeking behaviors as has been seen for other drugs of abuse.

The PFC and mesocortical dopamine system are associated with both learning and responding to drug-related cues. Yet, little work has been done evaluating the effects of ethanol on mesocortical dopamine system, or even just the PFC. One of the goals of this dissertation is to take the fledgling step and directly monitor the effects of self-administered ethanol on mPFC extracellular dopamine (Chapter 3).

Classical and Operant Conditioning

Behavioral models are used to study drugs of abuse because they are vital in gauging the reinforcing properties of a drug, as well as determining the external and internal factors that affect an organism's response to the drug. These models can also be used to test putative therapeutic drugs, or can be paired with other techniques to evaluate physical changes that accompany modifications in drug self-administration behavior. One aim of this dissertation uses an operant ethanol self-administration paradigm, and therefore, background information regarding the experimental model and general concepts is presented below.

Classical conditioning

Ivan Pavlov famously outlined the concept of classical conditioning in his 1927 publication "Conditioned Reflexes: An Investigation of the Physiological Activity of the Cerebral Cortex" (Pavlov, 1927, translation by G.V. Anrep). He noted that an animal's "inborn reflexes" (unconditioned reflexes) can be uncontrollably linked to a neutral stimulus when that stimulus is repeatedly presented with the inborn reflex. Once the neutral stimulus leads to the inborn reflex, Pavlov called it a "signal reflex" or rather a conditioned reflex. This type of learning inherently allows organisms to respond to and take advantage of their environment. For example, a young dog does not salivate when presented with meat if it has not previously consumed meat. However, once the sight and smell of the meat are associated with the chemical stimulus of the meat in the pup's mouth (which inherently leads to salivation and usually consumption), the sight and smell of the meat alone will cause the dog to salivate. Pavlov also initially demonstrated that

experimenters could manipulate these associations. When a dog was exposed to the sound of a metronome (conditioned stimulus) and then given food (unconditioned stimulus), eventually the sound of the metronome alone elicited increased salivation (conditioned response) (Pavlov, 1927, translation by G.V. Anrep).

Operant conditioning

While classical conditioning was an enormous advancement in understanding learning, it does not account for all animal (or human) learning and in particular, fails to account for the voluntary component of learning. The American psychologist Edward Lee Thorndike formulated his theory of "connectionism" after performing a set of experiments that laid the foundations for instrumental conditioning (operant conditioning). Thorndike put young cats inside of a closed wooden box that was equipped with a lever and an escape door that would only open when the cat repeatedly pressed the lever. The cats tried many instinctive actions while attempting to escape these "puzzle boxes" before they accidently hit the lever, causing the door to open. As training progressed, the cats took less and less time to press the lever and escape. Thorndike proposed that with increased experience being placed in the box (stimulus) and engaging in the "correct response" (conditioned response), as opposed to the earlier instinctive escape responses (incorrect response), that the connection between the stimulus and the incorrect response was "stamped out", and the connection between the stimulus and correct response was "stamped in". From this came Thorndike's most important law of learning, the law of effect, which essentially says that a stimulus-response relationship with a desired or positive response will be

strengthened, while one with an undesired or negative response will be weakened (Darity, 2008; Dewsbury, 1998).

Thorndike's experiments laid the ground work for later scientists who continued to develop the concept of instrumental conditioning. In 1938, Burrhus Frederic Skinner published "The Behavior of Organisms: An Experimental Analysis", which outlined his theory of operant conditioning. Like Thorndike, Skinner thought that there was more to learning than just involuntary conditioned responses:

> "*With the discovery of the stimulus and the collection of a large number of specific relationships of stimulus and response, it came to be assumed by many writers that all behavior would be accounted for in this way as soon as the appropriate stimuli could be identified…There is a large body of behavior that does not seem to be* ***elicited****, in the sense in which a cinder in the eye elicits closure of the lid, although it may eventually stand in a different kind of relation to external stimuli."* - B.F. Skinner, 1938.

Skinner conceptually and experimentally advanced our understanding of operant conditioning. Rightfully deemed the father of operant conditioning, Skinner outlined many concepts and associated definitions to describe and test this form of interaction with the environment. The most basic of these terms are as follows: A reinforcer is a stimulus that increases the likelihood of a particular behavior being performed. Positive reinforcement is when a stimulus (positive reinforcer) is presented after a particular behavior (operant response), which

increases the likelihood of the behavior being performed. Negative reinforcement is when a noxious stimulus (negative reinforcer) is presented until a particular action is performed (operant response) at which point the noxious stimuli is removed. This too will increase the frequency of the chosen operant response. In contrast, a punishment is a stimulus that decreases the likelihood of a particular behavior being performed. If a particular behavior is linked with a positive punishment, the occurrence of the behavior will decrease. A negative punishment is when a positive reinforcer is removed after a particular behavior is performed. Finally, extinction occurs when a previously reinforced behavior is no longer reinforced, leading to an eventual reduction or cessation of the behavior.

Skinner and colleagues formulated many schedules of reinforcement, and combinations of these schedules, to use during operant conditioning (Ferster and Skinner, 1957). The most basic and commonly used of these schedules are as follows: Non-intermittent schedules include continuous reinforcement, when each operant response is reinforced, and extinction, when no responses are reinforced. Intermittent schedules include fixed-ratio, variable-ratio, fixed-interval, and variable-interval. A fixed-ratio schedule requires a certain number of operant responses be performed before a reinforcer is provided, while a fixed-interval schedule requires a certain amount of time to pass between reinforcers before an operant response yields a reinforcer. A variable-ratio schedule requires a variable number of operant responses to be completed before the reinforcer is produced. The required number of responses is usually randomly selected from a range, but has a given mean. A variable-interval schedule requires that a variable amount of time passes between reinforced responses

before an operant response yields a reinforcer. The time interval is randomly selected from a range, but has a given mean. These schedules can be combined or linked in many ways to create more complex schedules (Ferster and Skinner, 1957).

In addition to outlining multiple paradigms under which the occurrence of a particular behavior could be increased or decreased, Skinner also designed an apparatus in which he could test these paradigms under tightly controlled conditions in a laboratory. The “Skinner box”, today referred to as an operant chamber, is a small chamber where an animal (typically a rat or pigeon) has access to a lever, target disk, or other object on which it can operate in response to reinforcement paradigms and schedules (Illustration 1.2).

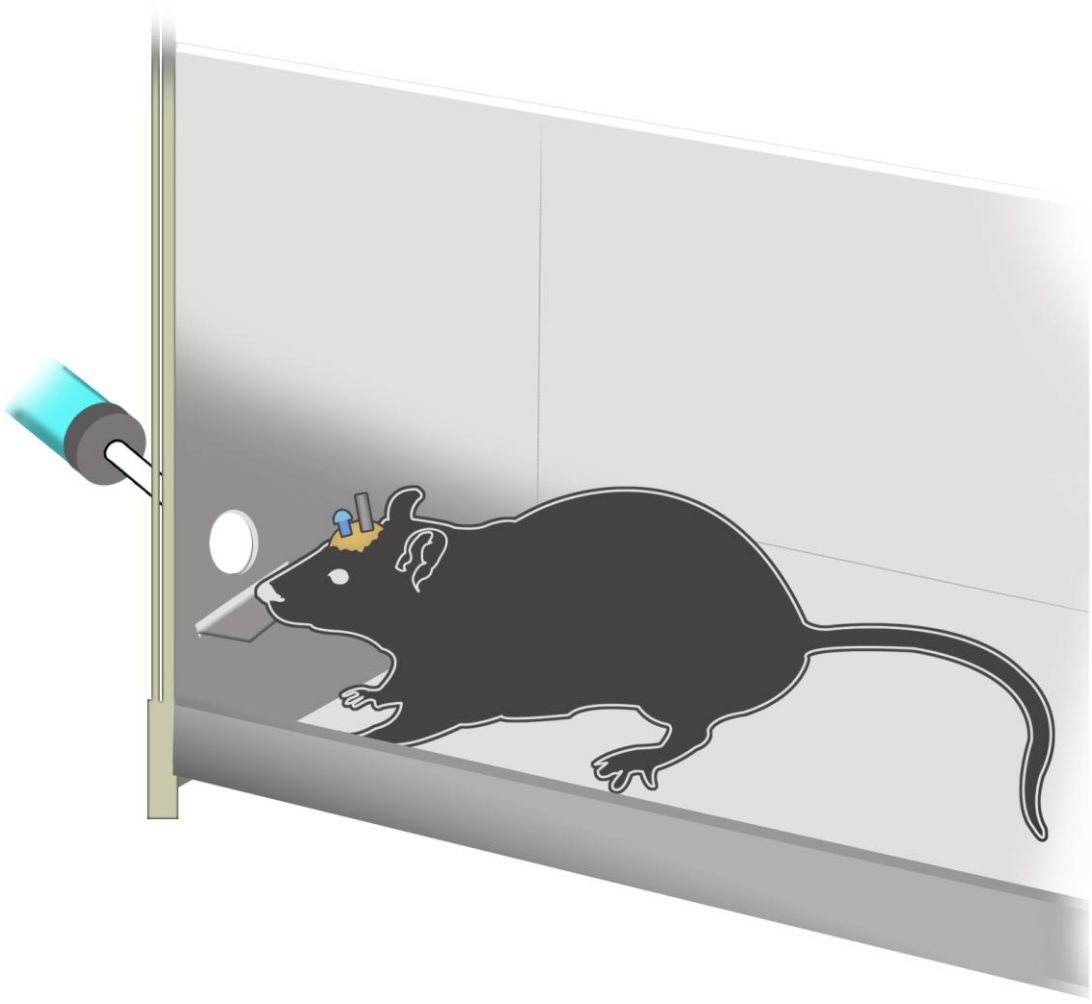

Illustration 1.2. Example of a Skinner box set up for liquid self-administration. Adapted from Schier et al., 2012b.

Skinner believed that only observable behavior should be evaluated; yet, he also acknowledged that internal states can affect behavioral output. He dealt with this phenomenon by adding a middle "state" step to his behavioral equation (Skinner, 1938). Basically, the internal state would determine the strength of the behavioral output. In this way, the state of the animal (e.g. the "drive" or

"emotion") can be inferred by the animal's operant response. For example, if a rat trained to lever press for a food reinforcer is presented with the lever, its decision to press the lever, or how many times it is willing to press the lever, could be dependent on whether or not the animal is hungry.

Classical and operant conditioning paradigms are both vital to drug abuse research. These principles allow for the creation of animal models that can be used to test numerous aspects of drug abuse. The uniting feature of these behavioral paradigms is that the subject's observable behavior is used to infer the effects of, or "state" produced by, the drug and experimental circumstances on the animal. Furthermore, the behavioral information provided by these conditioning models can also be paired with other techniques that evaluate physiological changes in a subject. Concomitant behavioral and physical changes observed in animal models can reveal the mechanisms leading to drug abuse.

In this dissertation (Chapter 3), we use an operant ethanol self-administration paradigm and microdialysis to evaluate the effects of self-administered ethanol on extracellular dopamine in the mPFC. In addition to analysis of dialysate dopamine and ethanol concentrations, multiple behavioral parameters are recorded. Our operant chambers are outfitted with a retractable lever the rat may push (operant response), a retractable bottle holder/sipper tube for drinking solution delivery (reinforcer) and a lickometer circuit (to monitor reinforcer consumption). This equipment is all under the control of a computer program that records the number and time of lever presses and individual licks on the

sipper tube. These techniques allow us to monitor extracellular dopamine and ethanol changes in the mPFC that are concomitant with pertinent operant behaviors and self-administration phases. Previous use of these techniques showed that the mesolimbic dopaminergic system responds to acute experimenter-administered and voluntary self-administered ethanol in a manner suggesting that the direct pharmacological effects of ethanol are not responsible for mesolimbic activation during repeated voluntary ethanol consumption (Doyon et al., 2005; Howard et al., 2008, 2009). In this dissertation, we attempt to determine if a similar divergence of pharmacological and self-administration effects of ethanol on dopamine occur in the mPFC, as has been shown in the nucleus accumbens (Chapter 3).

Ethanol, mesocortical dopamine system and rodent models

The mesocortical dopamine system has received little attention compared to the mesolimbic dopamine system in ethanol research. There are a handful of studies that evaluate the pharmacological and self-administration effects of ethanol on dopamine in the mPFC. However, the experimental designs of many of the studies could have led to the disparate results found. Specifics are discussed below.

Pharmacological studies

Previous microdialysis studies evaluating the pharmacological effects of ethanol on extracellular dopamine in the mPFC produced incongruent results. Some

studies show that acute ethanol increases mPFC extracellular dopamine, while others show that ethanol does not affect mPFC dopamine concentrations (Dazzi et al., 2002, 2007; Ding et al., 2011; Bassareo et al., 1996; Engleman et al., 2006; Hegarty and Vogel, 1993).

Ethanol stimulates mPFC dopamine increases

Dazzi et al. (2002) found that acute intraperitoneal ethanol elicited a dose-dependent biphasic effect on mPFC extracellular dopamine in male Sprague-Dawley rats, and that progesterone pretreatment led to a left shift of the dose-response curve (0.25, 0.5, 1.0 g/kg ethanol). Furthermore, acute ethanol administration increased dopamine in female Sprague-Dawley rats in estrus, but not in diestrus or proestrus (0.5 g/kg ethanol) (Dazzi et al., 2007). In a more recent study, Ding et al. (2011) microinjected 44 mM ethanol into the posterior ventral tegmental area of female Wistar rats which also led to a significant mPFC dialysate dopamine increase. These studies suggest that acute ethanol can stimulate mPFC dopamine increases and that a hormone-ethanol interaction affects mPFC dopamine.

Ethanol does not affect mPFC dopamine

Engleman et al. (2006) did not find an mPFC extracellular dopmaine change after acute intraperitoneal administration of a 2.0 g/kg ethanol dose to male P rats or Wistar rats. Similarly, a 0.5 or 2.0 g/kg ethanol dose did not modify mPFC extracellular dopamine in male Sprague-Dawley rats (Bassareo et al., 1996, Hegarty and Vogel, 1993).

Taken together, these results suggest that the pharmacological effects of ethanol on mPFC dopamine are still unclear. Additionally, because the findings are so mixed, it is likely that factors other than the physiology of the mesocortical dopamine system are affecting these results.

Possible confounding factors

There are many experimental factors that could account for the disparate results regarding the pharmacological effects of ethanol on extracellular dopamine in the mPFC.

Hormone-ethanol interaction

A hormone-ethanol interaction can enhance the effects of ethanol on dopamine in the mPFC, which could enhance an otherwise undetectable mPFC dopamine increase (Dazzi et al., 2002, 2007). This could account for the ethanol-stimulated mPFC dopamine increase noted in some experiments in Dazzi et al. (2002, 2007). To this point, Ding et al. (2011) used female rats, yet did not track the rats' estrous cycles during experiments, and therefore their positive result could also have been contributed by an estrus-ethanol interaction. The three studies with negative results used male rats; therefore, an estrus-ethanol interaction was not possible. However, countering this argument, Dazzi et al. (2002) demonstrated an ethanol-stimulated mPFC dopamine increase in naïve, male rats.

Method of drug administrtion

Stress and physical handling can also cause an increase in mPFC extracellular dopamine (Abercrombie et al., 1989; Feenstra et al., 1998, 2000; Hegarty and Vogel, 1993; Segovia et al., 2008). While the aforementioned studies all used proper controls, it is still possible that the physical handling and stress involved in administering an intraperitoneal injection could have contributed to or induced an increase in mPFC dialysate dopamine concentrations. There are inherent differences between animal handlers and their ability to deliver an injection without perturbing or stressing an animal. It is also possible that the intraperitoneal injection of an ethanol solution is more irritating than the injection of saline. However, while we can speculate that these factors played a role in the inconsistent findings, it is impossible to determine if the manner in which the rats were injected, or the feeling of the injection, contributed to a dopamine increase in some studies and not others.

Temporal resolution

Temporal resolution of dopamine sampling could allow for the detection or masking of a transient mPFC extracellular dopamine change. All of the aforementioned studies, with the exception of Ding et al. (2011), used 20-minute dialysis samples. This is a long sampling period, which could have washed out a transient extracellular dopamine change. Therefore, it is possible that the studies which used male rats and found negative results did have a dopamine increase that was undetectable due to time resolution. The hormone-ethanol interaction, noted in many studies with postive results, might have bolstered the dopamine increase allowing for its detection using 20-minute sampling periods. Supporting

this possibility, Ding et al. (2011) used 10-minute dialysis samples and showed an ethanol-stimulated mPFC dopamine increase.

Overall, the pharmacological effects of ethanol on dopamine in the mPFC are still unclear. This may be partly due to differences in experimental designs or experiment execution. One of the main goals of the study outlined in Chapter 2 was to either experimentally account for or eliminate the possible confounding factors (stress, physical handling and estrous effects) that previous experimenters faced. To accomplish this, we employed a unique and technically challenging experimental design, which will be outlined in Chapter 2.

Ethanol and operant oral self-administration

Experimental design and factors affecting and associated with ethanol self-administration are first discussed. The relatively small literature about ethanol-mPFC self-administration follows.

Separation of seeking and consumption behaviors

When modeling ethanol self-administration, two types of behavior are often evaluated: seeking and consumption. A seeking behavior is any action or behavior that assists in attaining the desired object or action. Consummatory behavior is the actual consumption of the desired object or action (e.g. food, drug, sex) (Craig, 1917).

In the past and even now, many researchers in the field used a self-administration model in which the rat was required to lever press (seeking

behavior) to receive access to a sipper tube (or drinking cup) of sucrose or ethanol drinking solution (consummatory behavior) for a short period (Hodge et al., 1996; Samson and Chappell, 2003; Czachowski et al., 1999; Srinivasan et al., 2012; Echeverry-Alzate et al., 2012; Dayas et al., 2007). The rat can only drink a small amount of solution before the sipper tube retracts from the chamber. The rat then has to re-press the lever to once again receive access to the solution. This experimental design inextricably links the two behavioral phases, and therefore could complicate data interpretation in some studies.

Czachowski and colleagues (1999, 2001) created a self-administration model that separated the seeking and consummatory phases. Animals were first required to lever press (seeking phase), which then led to continuous access to the drinking solution (consummatory phase). Using this model, they found that dopamine D_2 receptor antagonism by raclopride in the nucleus accumbens more strongly affected seeking behaviors than consummatory behaviors. Seeking behaviors were delayed at all of the raclopride doses tested, while total ethanol consumption was only affected at the highest drug dose (Czachowski et al., 2001). It is plausible that the different behavioral phases of ethanol self-administration could elicit different dopamine responses in the mPFC. Therefore, in the operant self-administration paradigm described in Chapter 3, the seeking and consummatory phases are separated. Specifics about each phase and its relationship to the mPFC are detailed below.

Seeking behaviors and the mPFC

The mPFC and mPFC dopamine signaling have been associated with drug-seeking behaviors. Blocking mPFC activity or manipulating dopamine signaling in the region has been shown to affect cocaine reinstatement (Peters et al., 2008; McFarland et al., 2003, 2004; Capriles et al., 2003). Furthermore, ethanol-associated cues have been shown to induce reinstatement and increase c-fos expression in the prelimbic, infralimbic and anterior cortex subregions of the mPFC (Dayas et al., 2007). Finally, the presentation of a classically conditioned morphine- or nicotine-predictive cue concomitantly increased approach behaviors and mPFC dialysate dopamine concentrations in a rodent model (Bassareo et al., 2007). Altogether, these data suggest that the mesocortical dopamine system plays a role in drug-seeking behaviors.

Consummatory behaviors and the mPFC

Consumption of palatable food, as well as its related cues, increases medial prefrontal extracellular dopamine concentrations (Bassareo and Di Chiara, 1997; Bassareo et al., 2002). In classically conditioned rats, exposure to a food-paired cue led to significant mPFC dopamine increases, as did the voluntary consumption of the palatable food it predicted. Importantly, repeated feedings of the palatable food did not diminish the mPFC dopamine response (Bassareo and Di Chiara 1997). A similar initial peak and then decline in mPFC dopamine was found when experimenters intra-orally administered a 20% sucrose or chocolate solution into the rats' mouths. This suggests that the mPFC dopamine increase was not linked to the physical act of chewing food or choosing to consume the food, but rather to food related stimuli (taste and smell of the food).

Self-administration associated behaviors: Lick behavior and tail pressure-induced licking behavior

Oral self-administration of ethanol is an animal model with significant face value as it better replicates the conditions under which humans take this drug. However, there are many components involved in animal self-administration models that one must take into account in addition to the effects of the drug. Dopamine in the brain is thought to be connected to the action of licking. For example, systemically administered haloperidol (dopamine antagonist) decreases the rate, force and number of licks during water consumption (Ciucci et al., 2009). However, when the mPFC was specifically lesioned via aspiration, no differences were noted in tail pressure-elicited oral behaviors compared to the pre-lesion baseline (Shipley et al., 1980) or in post-surgical water consumption, as was seen after aspiration ablation of the ventrolateral prefrontal cortex (Nonneman and Kolb, 1979). Conversely, a unilateral 6-OHDA lesion in the striatum led to decreased tongue force while licking (Ciucci et al., 2011). Overall, while dopamine and some areas of the prefrontal cortex may be important for licking behaviors, the mPFC does not seem to be critical for licking behaviors.

Ethanol-mPFC self-administration microinjection studies

Manipulation of neurotransmitter signaling in the mPFC alters operant ethanol and sucrose self-administration behaviors. Microinjection of the D_2/D_3 agonist quinpirole into the mPFC led to a later onset and shorter bout of

responding/drinking, consequently decreasing ethanol intake. The microinjection of the D_2 antagonist raclopride into the mPFC led to a reduction in ethanol intake via a decrease in the rate of responding for ethanol, but failed to alter sucrose self-administration. Additionally, quinpirole and especially raclopride differentially affected responding for ethanol or sucrose, suggesting specificity for a mechanism of ethanol reinforcement (Hodge et al., 1996; Samson and Chappell 2003). Finally, microinjection of a $GABA_a$ agonist into the mPFC also decreased responding for ethanol by reducing the length of the initial drinking bout (Samson and Chappell, 2001). Overall, their data suggest that the mPFC is involved in ethanol self-administration. However, because rats were required to alternate between seeking (lever-pressing) and consummatory (drinking) behaviors to drink the desired amount of solution in these studies, it is difficult to ultimately determine the effects of mPFC receptor manipulations on the different behavioral phases (Hodge et al., 1996; Samson and Chappell, 2003). Furthermore, the pharmacological manipulation of receptors in one region in the mesocorticolimbic system could lead to compensatory changes in the system as a whole. Therefore, the observation of the role of dopamine in the mPFC during ethanol self-administration should be established prior to drug manipulation.

The main goal of Chapter 3 is to elucidate the relationship between mPFC extracellular dopamine and ethanol self-administration. The topic will be further discussed in the chapter.

Chapter summary

In summary, the mPFC is a highly connected, multi-functional brain region that has been implicated as an important target in ethanol research. There is currently limited research regarding the acute, pharmacological effects of ethanol, as well as the effects of repeatedly self-administered ethanol on dopamine signaling in the mPFC. The work presented in the following chapters will help clarify these topics, as well as assist other scientists with acquiring the techniques used to study these matters.

Chapter 2: Intravenous ethanol increases medial prefrontal cortical extracellular dopamine in the Long Evans rat

(This work was published in Alcoholism: Clinical and Experimental Research, In Press, by Christina. J. Schier, Geoffrey A. Dilly, and Rueben A. Gonzales)

Christina Schier contributed to the experimental design, data acquisition, analysis and interpretation, as well as drafting and revising the article. Dr. Rueben Gonzales contributed to the experimental design, article revision, and the analysis and interpretation of the data. Geoffrey Dilly substantially contributed to the data acquisition.

Abstract

Background: Ethanol affects prefrontal cortex functional roles such as decision making, working memory, and behavioral control. Yet, the pharmacological effect of ethanol on dopamine, a neuromodulator in the medial prefrontal cortex, is unclear. Past studies exploring this topic showed dopamine increases, decreases or no change after ethanol administration; however, a handful of factors (temporal resolution, method of drug administration, estrous cycle) could have contributed to these discrepancies. We sought to mitigate these factors in order to elucidate ethanol's pharmacological effects on medial prefrontal cortical dopamine in Long-Evans rats.

Methods: We used a jugular catheter to intravenously administer experimental solutions via a handling-free route, microdialysis to monitor dopamine in the medial prefrontal cortex, and male rats to avoid estrous cycle/ethanol interactions. Additionally, we doubled the temporal resolution, compared to most previous experiments, to capture possible transient dopamine changes. First, we

rapidly (~2.7 ml/min) or slowly (~0.6 ml/min) administered 1.0 g/kg ethanol and saline infusions, showing that the experimental methods did not contribute to dopamine changes. Then a cumulative dosing protocol was used to administer 0.25, 0.75, 1.50, and 2.25 g/kg intravenous ethanol doses to evaluate dose-response. Finally, we monitored dialysate ethanol levels during an oral ethanol self-administration session to compare the dialysate ethanol levels achieved during the pharmacological experiments to those seen during self-administration.

Results: Intravenous administration of a rapid or slow 1.0 g/kg ethanol infusion resulted in similar significant 55 ± 9% and 63 ± 15% peak dialysate dopamine increases, respectively. The 0.25, 0.75, 1.50, and 2.25 g/kg ethanol doses produced a non-significant 17 ± 5% and significant 36 ± 15%, 68 ± 19%, and 86 ± 20 % peak dopamine dialysate increases, respectively. Self-administration dialysate ethanol concentrations fell within the range of concentrations noted during the ethanol dose-response curve.

Conclusions: These experiments show that, using experimental methods which minimize possibly confounding factors, acute intravenous ethanol increases extracellular dopamine in the medial prefrontal cortex in a dose-dependent manner, thereby clarifying ethanol's pharmacological effects on the mesocortical-dopamine system.

Introduction

Human studies suggest that ethanol affects prefrontal cortex functions such as decision making, working memory, and behavioral control (reviewed in Abernathy et al., 2010). Chronic ethanol abusers often show decreased grey and white matter volume in the frontal cortex, which is thought to play a role in their loss of control of ethanol consumption (Chanraud et al., 2007; Rando et al., 2011). Furthermore, animal studies have shown that the mesocorticolimbic dopaminergic system plays a role in the reinforcing effects of ethanol (Czachowski et al., 2001; Hodge et al., 1993; Samson and Chappell, 2003), in particular the nucleus accumbens and ventral tegmental area (Czachowski et al., 2001, 2012; Doyon et al., 2003, 2005; Hodge et al., 1993; Howard et al., 2008, 2009; Samson and Chappell, 2003; reviewed in Gonzales et al., 2004). Conversely, the medial prefrontal cortex (mPFC) has received substantially less attention concerning these matters (Hodge et al., 1996; Samson and Chappell, 2003). Considering dopamine's pivotal role as a neuromodulator in the region, the current uncertainty about the pharmacological effect of ethanol on mPFC dopamine is a breach in ethanol abuse research (neuromodulation reviewed in Seamans and Yang, 2004).

Previous microdialysis experiments investigating the relationship between acute ethanol administration and extracellular dopamine concentration in the mPFC have shown mixed results. Dazzi and colleagues found that male Sprague-Dawley rats, ovariectomized female Sprague-Dawley rats pretreated with estrogen, as well as intact female Sprague-Dawley rats in estrous, showed a

significant increase in dialysate dopamine concentration after a 0.5 g/kg intraperitoneal (ip) ethanol injection (Dazzi et al., 2002, 2007). A recent study showed that the microinjection of 44 mM ethanol into the posterior VTA of female Wistar rats resulted in a significant medial prefrontal cortical increase in extracellular dopamine (Ding et al., 2011). Yet, other studies found no dopamine response in the mPFC after ethanol injections were delivered to male Sprague-Dawley rats (0.5 and 2.0 g/kg, ip), male P-rats (2.0 g/kg, ip) or male Wistar rats (2.0 g/kg, ip) (Bassareo et al., 1996; Engleman et al., 2006; Hegarty and Vogel, 1993). Furthermore, the 2002 Dazzi study noted a decrease in dopamine when male Sprague-Dawley rats received a 1.0 g/kg, ip ethanol injection (Dazzi et al., 2002). There are a variety of factors that could contribute to these disparate results. Most of these studies collected dialysate samples at 20-minute (min) intervals. This long sampling period could have masked a transient extracellular dopamine change, explaining the absence of a dopamine response in some studies. Ding et al. (2011) noted an increase in prefrontal cortical dialysate dopamine when using a 10-min sampling period, yet the female rats' estrous cycles were not monitored during experiments. Dazzi and colleagues' work suggests that changes in hormone levels during the estrous cycle affect the mPFC dopaminergic response to ethanol. Though Ding and colleagues attempted to randomize the estrus phase of rats during experiments, it is possible that an estrus phase and ethanol interaction could be responsible for the increases in dopamine noted in the study.

Stressors such as a tail shock or physical restraint, as well as physical handling increase dialysate dopamine concentrations in the mPFC of rats (Abercrombie et

al., 1989; Feenstra et al., 1998; 2000; Hegarty and Vogel, 1993; Segovia et al., 2008). Therefore, it is possible that physiological factors associated with handling required to administer the i.p. injection by Dazzi and calleagues in 2002 and 2007 could have contributed to the dopamine response observed in the prefrontal cortex, rather than solely the pharmacological effects of ethanol. However, the 2002 study did not see a significant dialysate dopamine increase after a vehicle or 0.25 g/kg ip ethanol injection, suggesting that ethanol, not stress or physical handling, contributed to the extracellular dopamine increase. A variety of hypotheses could be formulated to explain the conflicting results of these microdialysis studies, yet, the problem remains that there is not a clear understanding regarding the effects of ethanol on medial prefrontal cortical extracellular dopamine concentrations.

Our goal was to determine if pharmacological effects of ethanol cause changes in extracellular dopamine concentrations in the mPFC. To address past confounding factors, our experimental design incorporated the following: 1) higher dopamine time course resolution (10-min dialysis samples), 2) a handling-free route of drug administration, 3) use of male subjects to eliminate estrous cycle interactions, and 4) an additional experimental group to validate that our method of drug administration did not contribute to a dopamine response. We performed microdialysis in the mPFC of male Long-Evans rats during intravenous (iv) infusions of ethanol or saline. Both dialysate dopamine and ethanol concentrations were monitored to evaluate the effects of non-contingent, acute iv ethanol on the extracellular dopamine concentrations in the mPFC.

Material and Methods

Animals

Final analyses used a total of 41 male, Long-Evans rats (200 - 275 g upon arrival from Charles River Laboratories), which were housed under a 12 hour (hr) light/dark schedule, at 23 ± 2 ºC with *ad libitum* food and water. All procedures complied with the guidelines set by the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*, and were approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin.

Materials

The 10 % ethanol solution (weight/volume in saline) was prepared from 95 % ethanol (AAPER Alcohol and Chemical Co., Shelbyville, KY), NaCl (Fisher Scientific) and either distilled water or sterile saline, and then filtered with an Acrodisc 0.2 µm nylon membrane filter (Pall Corp., Ann Arbor, MI). Solutions had final NaCl concentrations of either 0.06 M or 0.15 M. The 10 % ethanol/10% sucrose solution was made from 95 % ethanol (AAPER Alcohol and Chemical Co., Shelbyville, KY), ultra-pure sucrose (MP Biomedicals, LLC, Solon, OH) and distilled water. Gentamicin (APP Pharmaceuticals, Schaumburg, IL), heparin (APP Pharmaceuticals, Schaumburg, IL), lidocaine (Hospira, Inc., Lake Forest, IL), carprofen (Pfizer, New York, NY), and Timentin (GlaxoSmithKline, Research Triangle Park, NC) were all required for surgical procedures.

Surgical procedures

Surgical procedures were performed as described in Howard et al., 2008 and Duvauchelle et al., 1998. Briefly, for experiments 1 – 3, after at least 3 days of handling and habituation to our facility, animals were surgically implanted with a jugular catheter and then placed in a stereotaxic frame and fitted with a 21 gauge guide cannula (Plastics One, Roanoke, VA) above the left mPFC (in mm relative to bregma and the skull surface): +3.0 antero-posterior, +0.6 lateral, -2.0 ventral (Paxinos and Watson, 1986). Probe active area began 0.25 mm below the guide cannula. Carprofen (5 mg/kg) was subcutaneously administered for post-surgical malaise. Catheters were flushed at least every 3 days with 0.1 ml of heparinized saline to maintain patency. Animals were allowed at least 5 days of recovery prior to experiments.

For experiment 4, animals were handled and habituated to the facility for at least a week prior to surgical cannulation above the left mPFC. These animals were allowed a 7-day recovery period prior to lever-press training.

Microdialysis

The afternoon before the experiment, we briefly sedated the rat with isoflurane and inserted a laboratory-constructed dialysis probe through the guide cannula into the rat's mPFC. For 2 animals in experiment 1 the active area was 2.75 mm long. We then adjusted the active area to 3.25 mm for the rest of the rats to increase dopamine dialysate recovery (18,000 molecular weight cut off

membrane). The probes were perfused with artificial cerebral spinal fluid (ACSF: 149 mM NaCl, 2.8 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.2 mM ascorbic acid, and 5.4 mM D-glucose) overnight at 0.2 µl/min, and increased to 1.0 µl/min in the morning at least 2 (hr) before dialysate sample collection began (Exmire Microsyringe, Ito Corp. Fuji, Japan; CMA 102 or CMA 100 microinjection pump, Acton, MA USA). Dialysis samples were collected every 10 min, immediately placed on dry ice, and later stored in a -80ºC freezer. Microdialysis set-ups and probe construction were modeled after Doyon et al., 2003, and Pettit and Justice, 1991. Ethanol infusions were given as a 10% weight/volume ethanol solution in saline. During the course of the experiments, a solution error was discovered. The iv ethanol solution administered was hypotonic (0.06 M NaCl), but the tonicity error was corrected (0.15 M NaCl) for subsequent experiments. The number of animals that received each solution is noted in the results section. The volume of ethanol infusions was determined by the animal's weight and the desired ethanol dose. Saline infusions were calculated in the same manner. Infusion rates and volumes are found in Table 1. At the conclusion of all experiments, we perfused calcium-free ACSF through the probe for 1-2 hr and collected additional samples. A minimum 40% dopamine concentration reduction in calcium-free ACSF samples was required for inclusion for analysis. Additionally, baseline samples were required to have a relative standard deviation < 0.25 for data inclusion. Animal behavior was visually observed and video recorded during experiments 1-3, and visually observed during experiment 4.

Experiment 1: Rapid 1.0 g/kg bolus ethanol infusion

Baseline samples were collected for 30 min before animals were intravenously infused with saline. Samples were taken for another 40 min and then animals were intravenously infused with 1.0 g/kg ethanol, and an additional 40 min of samples were collected. We used the 3 samples taken before the saline infusion, and the 2 samples taken before the ethanol infusion as the dopamine baseline to determine the dopamine response after the saline and ethanol infusions, respectively.

Experiment 2: Slow 1.0 g/kg ethanol infusion

Experiment 2 matched experiment 1 in every aspect except for the flow rate, manner, and habituation of the fluid infusion. Instead of rapid manual infusions (~2.7 ml/min), fluid was slowly (~0.6 ml/min) infused by a pump (CMA 400 or CMA 100 microinjection pump, Acton, MA USA). Additionally, at least 45 min prior to the beginning of the experiment, animals were infused with saline for 2 min to habituate them to the sound of the pump and the feeling of the slow fluid infusion.

Experiment 3: Cumulative ethanol or saline infusions

Baseline samples were collected for 30 min before animals began a series of either 10 % ethanol or saline infusions. In 20-min periods, we manually administered a 0.25, 0.5, 0.75, and then 0.75 g/kg infusion of ethanol or saline. This dosing scheme results in cumulative ethanol doses of 0.25, 0.75, 1.5 and 2.25 g/kg. On average, fluid was infused at a rate of 2.2 ml/min, and was completely administered within the first 1.5 min of the 10 min infusion sample.

Samples were taken for 30 min after the final infusion. We used the first 3 samples as the dopamine baseline to determine the dopamine response after all infusions.

Experiment 4: Ethanol self-administration

After recovery from surgery, animals were trained to lever press for a 10 % sucrose solution in a MedAssociates, Inc. (Vermont, USA) operant chamber outfitted with a retractable lever and bottle as described in Howard et al., 2009. After animals learned to lever press, they began an 8-session training protocol. During this protocol, ethanol was successively added to the rat's drinking solution, culminating in a 10 % sucrose/10 % ethanol solution. Microdialysis was performed during the 8th self-administration session. Seven-min samples were taken. The experimental time course is shown in Figure 2.5.

Dopamine analysis

We analyzed dialysate dopamine concentrations via reversed-phase high performance liquid chromatography with electrochemical detection. Over the course of the experiments, 3 different systems were used. System 1 used a Polaris 50 x 2 mm column (C18, 3-µm particle size; Varian, Lake Forest, CA), an Alexys autosampler (Antec Leyden, Netherlands) and a 2mm glassy carbon working electrode electrochemical detector (SenCell, Antec Leyden, Netherlands) at potential + 345 mV. The autosampler injected 7 µl of dialysate along with ascorbate oxidase into the system. Mobile phase flow rate was 0.3 ml/min. Systems 2 and 3 used a Luna 50 x 1.0 mm column (C18, 3-µm particle size; Phenomenex, Torrance CA), an 8125 manual injector (Rheodyne, Cotati,

CA) or an automated Valco Valve (Valco Instruments Co. Inc., Houston, TX), and a 2 mm glassy carbon working electrode (SenCell or VT03 with ISAAC reference electrode, Antec Leyden, Netherlands) at potential + 345 or + 395 mV. Samples were thawed and then 7 µl of sample were manually mixed with 1.5 µl of ascorbate oxidase, and then 5 – 7 µl were injected into the system. Mobile phase flow rate was 0.1 ml/min. All systems used the following mobile phase: 1 liter of approximately 2.1 mM octanesulfonic acid, 0.05-1.5 mM decanesulfonic acid (adjusted as needed throughout the experiments), 0.34 mM ethylenedianimetetraacetic acid, 71 mM sodium phosphate monobasic dehydrate, and 60 mM potassium chloride aqueous solution that was then adjusted to 5.60 pH with 1 M sodium hydroxide, after which 150 mL methanol was added. All systems used EZChrom Elite software to record and analyze chromatograms. External standards (0.0625 or 0.125 – 0.5 or 1.0 nM**)** were used to quantify the dopamine concentrations (linear standard curve, typically R^2 > 0.99). All samples were required to have a signal to noise ratio above 3. Dopamine dialysate data are represented as a percent of their respective baseline samples (mean ± standard error of the mean (S.E.M.)).

Ethanol analysis

For experiments 1-3, two samples before the ethanol infusion and all samples after had 2 µL of dialysate removed for ethanol analysis. For experiment 4, two samples before the rat began drinking the 10% ethanol/ 10% sucrose solution and all samples after had 1 µL of dialysate removed for ethanol analysis. The aliquot of dialysate was placed into a 2 mL glass chromatography vial that was then sealed with an air-tight septum and analyzed for ethanol concentrations via

gas chromatography with flame ionization detection. The system consisted of a Varian CP 3800 gas chromatograph with flame ionization detector, a Varian 8200 or 8400 headspace autosampler (65 or 50 ºC), and an HP Innowax capillary column (30 m x 0.53 mm x 1.0 µm film thick), with helium mobile phase. Varian Star Chromatography Workstation software was used to record and analyze chromatograms. External standards (0.3 – 20 mM for experiments 1, 2 and 4, and 0.3 - 40 mM for experiment 3) were used to quantify the ethanol concentrations (linear standard curve, typically $R^2 > 0.99$). Ethanol dialysate data are represented as the concentration of ethanol in the dialysate (mean ± S.E.M.).

Histology

Animals were overdosed with sodium pentobarbital (150 mg/kg, ip) and then intracardially perfused with saline followed by 10% formalin in saline. The brain was removed, coronally sectioned (100 µm thick) using a vibratome (Leica, Nussloch, Germany), and stained with cresyl violet. Correct placement of the probe active dialysis area was confirmed and subregional placement was noted (Paxinos et al., 1999; Paxinos and Watson, 1986).

Statistical Analysis

Data were analyzed with repeated measures analysis of variance (ANOVA), and ANOVA. Bonferroni corrections were used for all post hoc analyses. Statistical analyses required $p < 0.05$ to assign significance. For experiments 2 and 3, due to a lack of homogeneity of variance in the dopamine data, all statistics were performed on transformed dopamine data. In experiment 3, natural log

transformation of the data corrected the homogeneity of variance violations. In order to correct the homogeneity of variance violations in experiment 2, the pre-saline and saline infusion dopamine data was squared, while the pre-ethanol and ethanol dopamine data were natural log transformed. Experiment 2 saline and ethanol data were then analyzed separately. In experiment 3, Spearman's rho was used to test for correlations between ethanol dose or dialysate concentration, and dialysate dopamine concentration.

Results

Experiment 1: Rapid 1.0 g/kg bolus ethanol infusion

A rapid iv infusion of ethanol (1.0 g/kg) resulted in concurrent increases of mPFC dialysate ethanol and dopamine concentrations (Figure 2.1). The dopamine response peaked at 55 ± 9 % above baseline, and remained significantly elevated for 20 min after the infusion ($F_{5,68}$=12, individual 10 and 20 min time points $F_{2,68}$=27, $F_{2,68}$=4.9, respectively, $p<0.05$). A rapid infusion of saline had no significant effect ($F_{6,68}$ = 0.5, ns) (Figure 1A). Five of the 8 animals showed pronounced loss of motor control after ethanol infusion. All animals received hypotonic ethanol solution.

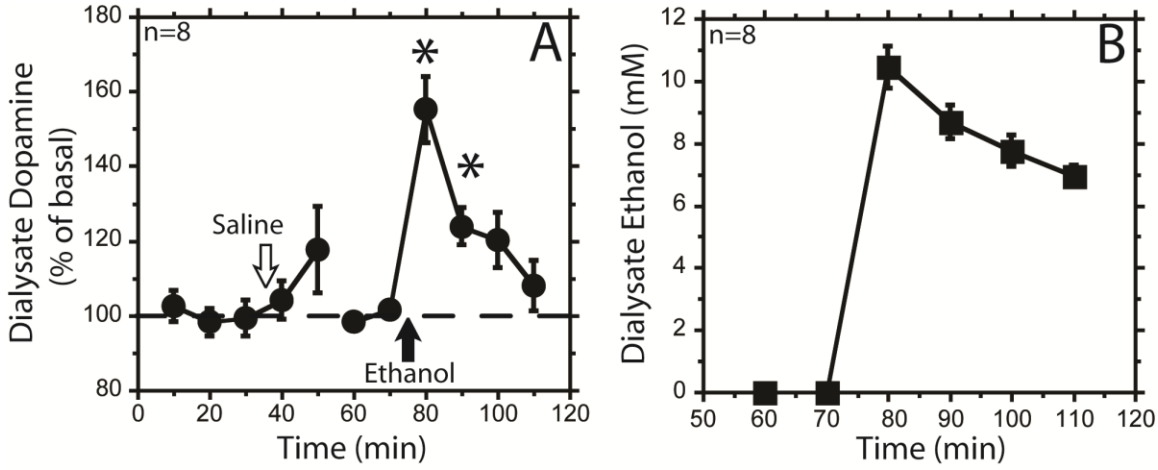

Figure 2.1: Intravenous bolus infusion of ethanol (1 g/kg) increases dialysate dopamine and ethanol concentrations. Panel A shows the percent baseline dialysate dopamine (circles) over the course of an intravenous (iv) saline and then iv 1.0 g/kg 10% ethanol infusion. **Open arrow** represents iv saline infusion. **Closed arrow** represents iv ethanol infusion. In **panel B** the ethanol dialysate data (squares) are represented as the concentration of ethanol in the dialysate. Data are represented as mean ± standard error of the mean. * signifies $p<0.05$ compared with baseline.

Experiment 2: Slow 1.0 g/kg ethanol infusion

To control for possible effects of the rapid bolus infusion of relatively large volumes of fluid, we repeated the experiment with a slower infusion rate. In addition, we also included a separate saline infusion before the experiment to habituate the rat to the infusion procedure. The isotonic and hypotonic ethanol solution groups' dialysate dopamine and ethanol time courses were not significantly different; therefore, the groups were collapsed for further analysis (saline treatment dopamine time course $F_{1,9} = 0.11$, ethanol treatment dopamine time course $F_{1,9} = 1.5$, ethanol dialysate time course $F_{1,9} = 0.06$, ns). A slow pump-driven infusion of ethanol (1.0 g/kg) resulted in concurrent increases of mPFC dialysate ethanol and dopamine concentrations (Figure 2.2). Dopamine

peaked at 63 ± 15% above baseline, and remained significantly elevated for 30 min after the infusion ($F_{5,44}$ = 15, individual 10, 20, 30 min time points $F_{2,44}$ = 26, $F_{2,44}$ = 22, $F_{2,44}$ = 8.8, respectively, $p<0.05$). The magnitude and pattern of the extracellular dopamine increase after the slow ethanol infusion was similar to that observed previously with the rapid bolus infusion method, though the infusion parameters were substantially different (Table 2.1). A slow pump-driven infusion of saline had no significant effect ($F_{6,51}$ = 0.5, ns) (Figure 2.2A). Six of the 11 animals showed pronounced loss of motor control after ethanol infusion.

Experiment	Infusion Volume Range (ml)	Infusion Time Range (seconds)	Average Infusion Rate (ml/minute)
Experiment 1: Rapid Infusion	3.0 – 4.6	58 – 102	2.7
Experiment 2: Slow Infusion	3.3 – 4.8	332 - 473	0.6

Table 2.1: Experiment 1 and 2 Infusion Rates. Ethanol (1.0 g/kg) was infused at different rates in experiments 1 and 2. Similar volumes of fluid were used in both experiments, but experiment 1 used a rapid manual infusion, while experiment 2 used a slow pump-delivered infusion.

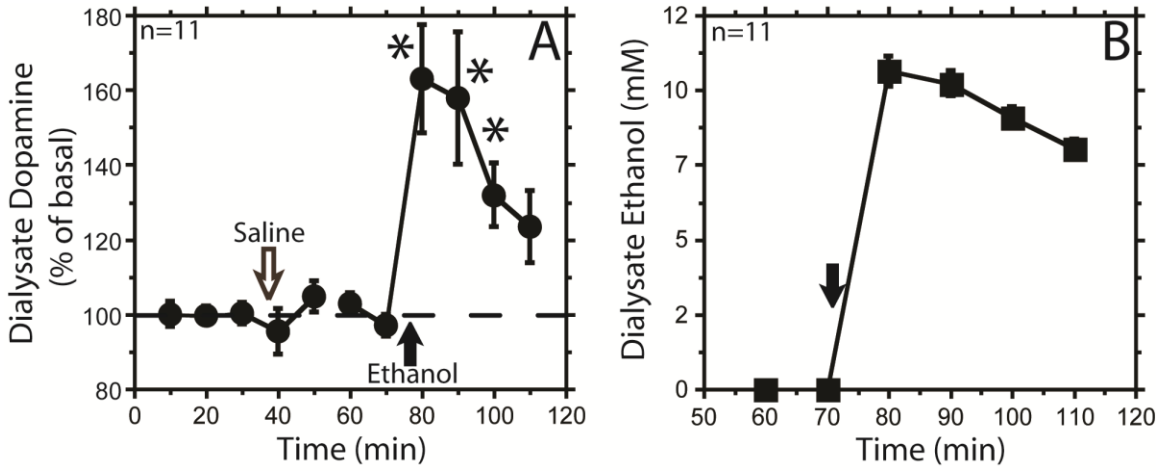

Figure 2.2: Intravenous slow infusion of ethanol (1 g/kg) increases dialysate dopamine and ethanol concentrations. Panel A shows the percent baseline dialysate dopamine (circles) over the course of a slow pump-driven intravenous (iv) saline and then iv ethanol infusion. **Open arrow** represents iv saline infusion. **Closed arrow** represents iv ethanol infusion. In **panel B** the ethanol dialysate data (squares) are represented as the concentration of ethanol in the dialysate. Data are represented as mean ± standard error of the mean. * signifies $p<0.05$ compared with baseline.

Experiment 3: Cumulative ethanol or saline infusions

In order to determine the dose-dependent effects of iv ethanol infusion, we completed a cumulative dosing experiment. Administration of 0.25, 0.75, 1.50, and 2.25 g/kg cumulative doses of ethanol resulted in concurrent increases of mPFC dialysate ethanol and dopamine concentrations (Figure 2.3). The isotonic and hypotonic ethanol solution groups' dialysate dopamine and ethanol time courses were not significantly different; therefore, the groups were collapsed for further analysis ($F_{1,9}$=0.0001, $F_{1,9}$=1.3, respectively, ns). The 0.75, 1.50 and 2.25 g/kg doses of ethanol resulted in significant dopamine increases above baseline ($F_{4,162}$=9.2, $F_{4,162}$=34, $F_{5,162}$=46, respectively, $p<0.05$), while infusions of saline

and the 0.25 g/kg dose of ethanol did not ($F_{11,162}$=0.6, $F_{4,162}$=2.8, respectively, ns) (Figures 2.3A and 2.4).

Significantly different peak dialysate ethanol concentrations were demonstrated for each ethanol dose (Figure 2.3B), confirming that the cumulative dosing protocol produced 4 different doses of ethanol ($F_{3,43}$=153, $p<0.05$). There was a main effect of ethanol dose on dialysate dopamine concentration, as well as a significant correlation between the dialysate ethanol and dopamine concentrations, and between ethanol dose and dopamine concentrations ($F_{4,109}$ = 12, $p<0.05$; $r = 0.63$, $r = 0.61$, $p<0.01$, respectively). The dialysate ethanol concentrations reported are not corrected with an extraction fraction, and therefore do not represent ethanol tissue concentrations. Two of the 11 animals that received ethanol began showing pronounced loss of motor control after the first infusion (0.25 g/kg dose), 1 of the 11 after the second infusion (0.75 g/kg dose), 5 of the 11 after the third infusion (1.5 g/kg dose), and 3 of the 11 after the fourth infusion (2.25 g/kg dose). Saline infusions did not cause obvious changes in behavior.

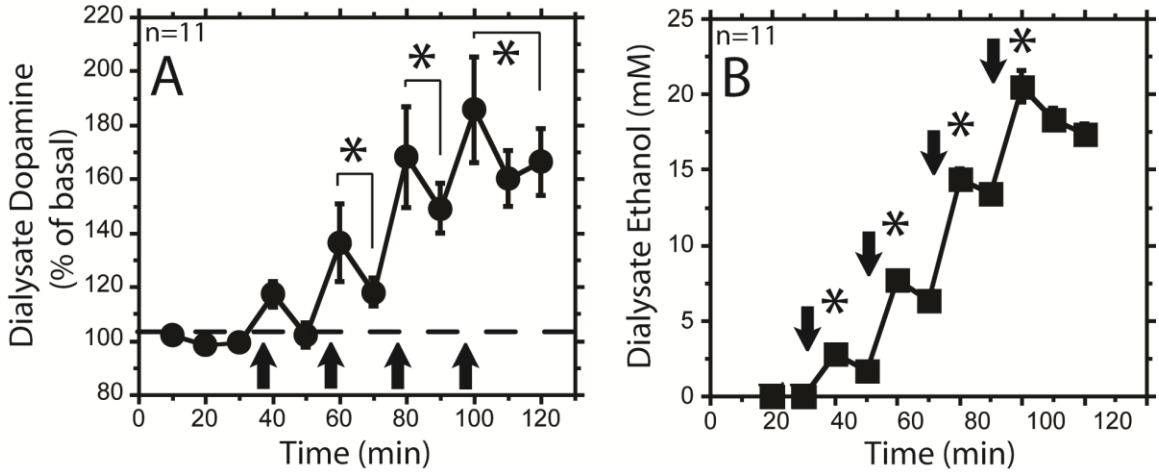

Figure 2.3: Dose-dependent stimulation of dialysate dopamine and ethanol concentrations using a cumulative dosing procedure. Panel A shows the percent baseline dialysate dopamine (circles) over the course the cumulative ethanol infusions with cumulative doses of 0.25, 0.75, 1.5 and 2.25 g/kg. **Closed arrows** represents intravenous (iv) ethanol infusions. * signifies $p<0.05$ compared with baseline. In **panel B** the ethanol dialysate data (squares) are represented as the concentration of ethanol in the dialysate. * signifies $p<0.05$ compared with each other dose. All data are represented as mean ± standard error of the mean.

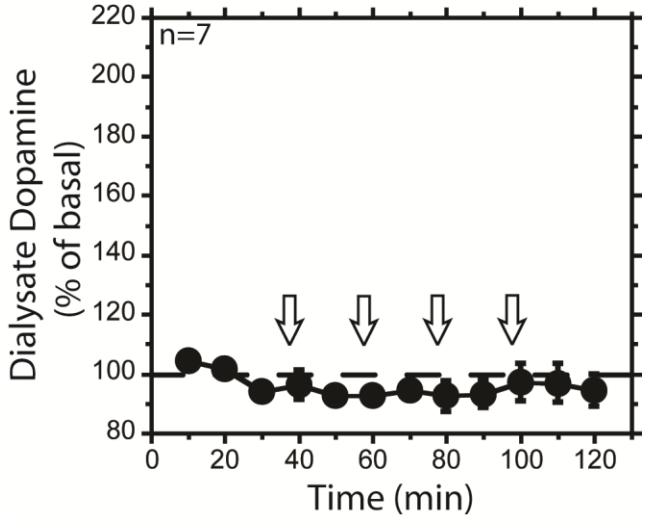

Figure 2.4: Experiment 3 saline percent basal dopamine time course. Percent baseline dialysate dopamine over the course the cumulative saline infusions. **Open arrows** represent intravenous saline infusions. Data are represented as mean ± standard error of the mean.

Experiment 4: Ethanol self-administration

In order to relate the brain ethanol concentrations from acute experiments 1 - 3 to self-administration experiments, we completed an ethanol self-administration study using similar microdialysis and ethanol analysis conditions as experiments 1-3. Rats orally self-administered an average 1.2 ± 0.08 g/kg ethanol dose on the day of dialysis. Dialysate ethanol concentrations were lowest in the first drink sample and gradually increased over the drink and post-drink session, peaking at 4.14 ± 0.6 mM dialysate ethanol concentration during the post-drink period (Figure 2.6). The dialysate ethanol concentrations reported are not corrected with an extraction fraction, and therefore do not represent ethanol tissue concentrations.

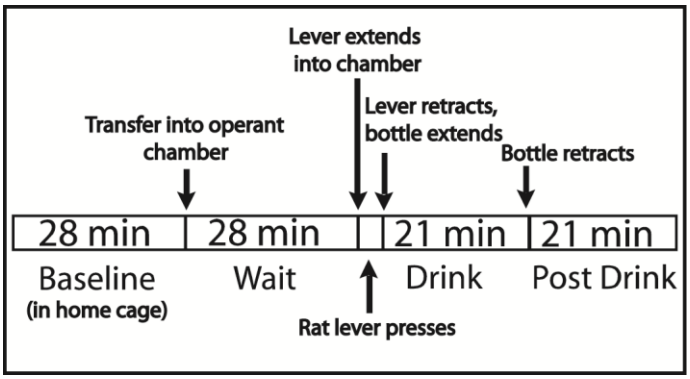

Figure 2.5: Experimental time course for ethanol self-administration. On the 7th day of ethanol self-administration dialysis was performed. Seven-minute samples were taken during these behavioral phases.

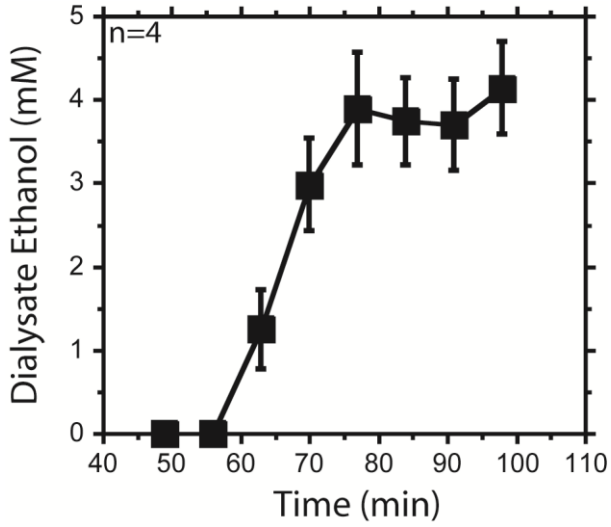

Figure 2.6: Ethanol time course for ethanol self-administration. Ethanol dialysate data show the concentration of ethanol in the dialysate. Data are represented as mean ± standard error of the mean. Animals began drinking around 56 minutes (min). The bottle retracted from the chamber around 77 min. Seven-min samples were taken.

Experiment	Saline (nM)	Ethanol (nM)
Experiment 1: Rapid Infusion	0.16 ± 0.02	0.17 ± 0.02
Experiment 2: Slow Infusion	0.12 ± 0.01	0.12 ± 0.01
Experiment 3: Cumulative Ethanol or Saline Infusion	0.14 ± 0.01	0.19 ± 0.01

Table 2.2: Dopamine Baseline Concentrations by Experiment. Experiments 1 and 2 show the pre-saline and pre-ethanol dopamine baseline averages seen within the groups. Experiment 3 shows the dopamine baseline averages for the two separate saline and ethanol groups.

Histological analysis and dopamine basal averages

Figure 2.7 represents the placements of the microdialysis probe active areas. At least 50% of the active dialysis area was in the infralimbic and prelimbic subregions of the mPFC for all animals. Most probes also sampled from the

cingulate cortex, and minimally from the dorsal peduncular cortex and motor cortex. There were no significant differences in average baseline dopamine concentrations between the isotonic and hypotonic slow infusion groups, or the isotonic ethanol, hypotonic ethanol and saline cumulative dosing groups (Table 2.2) ($F_{1,10} = 0.2$, $F_{2,17} = 2.7$, respectively, ns).

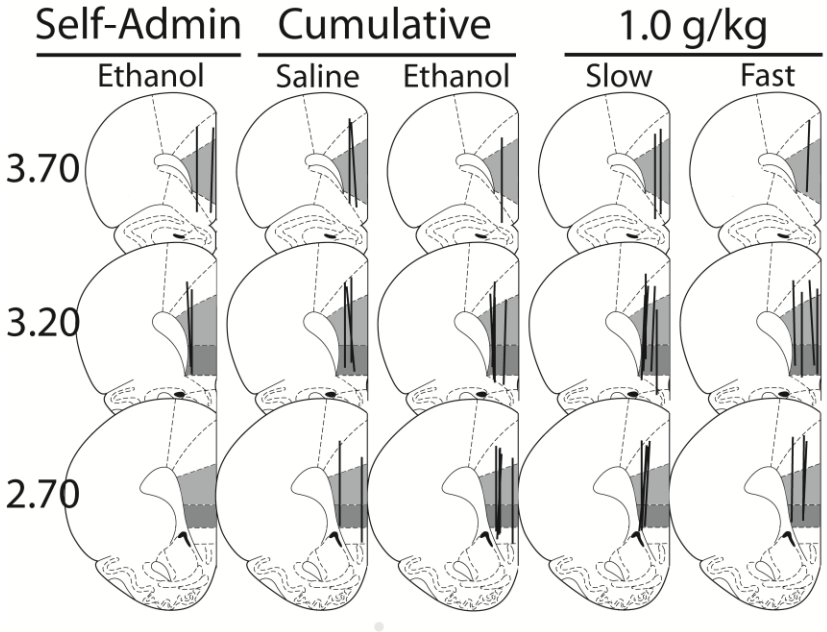

Figure 2.7: Microdialysis probe placements. Coronal slices representing microdialysis probe placements for all experimental groups. Line shows active dialysis area. Light gray shows prelimbic subregion. Dark gray shows infralimbic subregion. Numbers to the left indicate distance relative to bregma (mm). Figure adapted from Paxinos & Watson (1998).

Discussion

Overall, our results suggest that acute non-contingent iv ethanol administration increases extracellular dopamine in the mPFC of the male Long-Evans rat. Previous studies arrived at incongruent results, but factors such as long sampling times, estrous cycle interactions, handling of the animals during experimentation, and drug administration by ip injection could explain, at least in part, the disparities. Our experimental design diminished these factors, and produced findings that clarify how acute ethanol affects the mesocorticolimbic dopaminergic system.

Because past investigations have reported an increase, decrease, or no change in extracellular dopamine concentrations in the mPFC after acute ethanol administration, it is important that we address how previous studies' experimental designs and results compare to ours (Bassareo et al., 1996; Dazzi et al., 2002, 2007; Ding et al., 2011; Engleman et al., 2006; Hegarty and Vogel, 1993). Most of the previous studies took 20-min microdialysis samples. This long sampling time could have masked a transient change in extracellular dopamine, which could explain why some investigators did not see an ethanol-induced dopamine change. To address this we doubled the time resolution. To assure that the decreased sample volume did not compromise the quality of the dopamine chromatography, we required a signal-to-noise ratio > 3. Ding et al. (2011) also used these criteria, and they too reported an ethanol-stimulated dopamine increase, strengthening the argument that increased time resolution unveils a previously un-measureable extracellular dopamine change.

The interaction of ovarian steroid hormone levels and mPFC dopaminergic response to ethanol might also have contributed to varied experimental outcomes. The effects of estrus, or treatments that mimic it, could have enhanced an ethanol-induced dopamine increase so that it was visible using 20-min sampling periods (Dazzi et al., 2002, 2007). Furthermore, one cannot rule out a possible contribution of estrus effects to the dopamine increase seen in Ding et al., 2011, as the authors did not account for this factor. Using male rats, we show that an ethanol-induced mPFC dialysate dopamine increase can occur in the absence of estrous or estrogen pretreatment.

Intraperitoneal injection is a non-optimal method of drug administration when monitoring extracellular dopamine in the mPFC, because physical handling of rats, as well as stressors such as physical restraint or tail shock, can increase extracellular dopamine in the mPFC (Abercrombie et al., 1989; Feenstra et al., 1998; 2000; Hegarty and Vogel, 1993; Segovia et al., 2008). Unlike previous experiments, we avoided this potential confounding factor by using a jugular catheter to administer saline and ethanol infusions. The catheter inlet tubing was suspended above the rat during the experiment; therefore, the experimenter never touched the animal during dialysis, ruling out that physical handling, or injection stress contributed to the observed extracellular dopamine increase.

While iv drug administration allowed us to avoid many previously noted confounding factors, iv delivery of substantial volumes of fluid could have contributed to our observed dopamine increase. After a rapid 1.0 g/kg bolus

infusion of ethanol, dopamine significantly increased for 20 min, while infusion of saline did not significantly alter dialysate dopamine concentrations (experiment 1). This suggests that the dopamine increase seen after the ethanol infusion was specific to the effects of ethanol, not the infusion of fluid. Nevertheless, 3 to 4.6 ml of 10% ethanol infused at a rate of 2.7 ml/min could have engendered physiological effects that may have augmented the extracellular dopamine increase (Abercrombie et al., 1989; Hegarty and Vogel, 1993; Segovia et al., 2008). Therefore, to clarify whether or not the iv infusion protocol interacted with the pharmacological effects of ethanol to increase dopamine, we slowed the 1.0 g/kg ethanol infusion from an average 2.7 ml/min to an average 0.6 ml/min (experiment 2). This reduction in infusion flow rate should lessen any physiological changes that may have been produced by the infusion. Yet, the increases in dialysate dopamine concentrations after the slow and rapid infusions of ethanol were similar. These results not only imply that the pharmacological effects of ethanol, rather than the method of administration, led to the dopamine increase in the mPFC, they also substantiate the use of iv catheter drug administration while monitoring extracellular dopamine changes in the mPFC.

Finally, the strongest evidence that the pharmacological effects of ethanol, rather than physiological factors, are predominantly responsible for the dopamine increases is the dose-dependence of the response (experiment 3). This, along with the significant correlation of dialysate dopamine and ethanol concentrations, suggests that ethanol concentrations in the brain play a significant role in the systemic ethanol-stimulated dopamine response in the mPFC.

It is probable that excitation of VTA dopaminergic neurons projecting to the mPFC are responsible for ethanol-induced mPFC dopamine increases. Ding et al. (2011) found that a microinjection of ethanol in the posterior VTA led to an increase in extracellular mPFC dopamine. These authors attempt to reconcile their findings with previous studies, in which systemic ethanol administration did not cause an mPFC dopamine increase, by purporting that systemic ethanol administration may allow compensatory responses to suppress a mPFC dopamine increase. In light of our findings, it seems more likely that either local posterior VTA or systemic ethanol administration leads to increased mPFC dialysate dopamine concentrations in ethanol-naïve rats, and that a lack of time resolution accounts for previous negative results.

An important question raised by our findings of a dose-dependent stimulation of mPFC dopamine after non-contingent ethanol is whether a similar effect occurs with the ethanol concentrations achieved during self-administration. The ethanol self-administering rats from experiment 4 drank an average 1.2 g/kg ethanol, which is essentially in the middle of the cumulative ethanol dose range (0.25 to 2.25 g/kg). While the pharmacokinetics of the two routes of administration are quite different, with iv ethanol delivery leading to higher peak brain ethanol concentrations than that seen after the oral consumption of the same dose of ethanol, the dialysate ethanol concentrations after the 0.25 and 0.75 g/kg doses of iv ethanol in experiment 3 encompass the range of dialysate ethanol concentrations in experiment 4. Therefore, the current pharmacological data will be a useful reference to compare with future studies evaluating extracellular dopamine changes in the mPFC during ethanol self-administration.

Our findings provide a knowledge base regarding how ethanol acutely affects extracellular dopamine concentrations in the mPFC. It remains to be seen how repeated, voluntary ethanol consumption and mPFC dopamine relate. However, when this area is studied, these acute data will allow for comparison of the pharmacological effects of ethanol and the effects of ethanol self-administration associated cues, ultimately clarifying the role of the mesocorticolimbic dopamine system during ethanol reinforcement.

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Chapter 3: Medial prefrontal cortical dopamine reponses during the oral self-administration of ethanol

(This work has been submitted for publication to The Journal of Neurochemistry, by Christina. J. Schier, Geoffrey A. Dilly, and Rueben A. Gonzales)

Christina Schier contributed to the experimental design, data acquisition, analysis and interpretation, as well as drafting and revising the article. Dr. Rueben Gonzales contributed to the experimental design, article revision, and the analysis and interpretation of the data. Geoffrey Dilly substantially contributed to the data acquisition.

Abstract

The mesocortical dopamine system has received little attention in ethanol research, even though prefrontal dopamine signaling is associated with executive functioning and affects drinking behavior. Specifically, medial prefrontal cortical extracellular dopamine has never been directly observed during ethanol self-administration. To address this, we used microdialysis to monitor extracellular dopamine in the medial prefrontal cortex during the self-administration of an ethanol-plus-sucrose or sucrose solution by male Long Evans rats. At the initiation of drinking, ethanol-plus-sucrose- and sucrose-consuming rats showed transient peak 34 ± 6% and 23 ± 5% dialysate dopamine increases, respectively. Dopamine was significantly elevated for 21 minutes in the ethanol-plus-sucrose group, but only for seven minutes in the sucrose group, suggesting that the addition of ethanol to the sucrose solution differentially affected the extracellular dopamine response in the medial prefrontal cortex. Dopamine was also significantly elevated in drinking rats, as well as handling controls, during transfer from the home cage into the operant chamber. Overall, these results directly

show that the mesocortical dopamine system differentially responds to the operant self-administration of an ethanol- and non-ethanol-containing solution, suggesting that this system plays a role in ethanol self-administration.

Introduction

Prefrontal cortex (PFC) dysfunction, which is frequently noted in heavy alcohol consumers, is associated with increased impulsivity and perseveration, as well as deficits in executive functions (e.g. working memory, decision-making, attention, goal-directed behavior) (Bechara and Van Der Linden, 2005; Fuster, 2008; Chanraud et al., 2007; Goldstein et al., 2004; Demakis, 2003; Sullivan et al., 1993; Bechara and Damasio, 2002; Bechara et al., 1996). Similarly, acute ethanol can also disrupt working memory (Ralevski et al., 2012). Chronic alcohol abusers often have decreased frontal cortical grey and white matter, the former correlating with earlier time to relapse in alcoholics (Chanraud et al., 2007; Kubota et al., 2001; Pfefferbaum et al., 1997; Rando et al., 2011). Furthermore, drug addicts have been shown to display reduced activity in many PFC regions (Volkow et al., 2009); yet, when exposed to drug-related cues, detoxified alcoholics showed significantly greater medial prefrontal cortex (mPFC) activation compared to controls (Heinz et al., 2004). These findings suggest that ethanol use may be associated with enhanced sensitivity to drug-related cues and decreased behavioral control due to compromised prefrontal cortical functions.

Altered dopaminergic activity in the PFC has been shown to modify executive functions, alter reinstatement of drug-seeking behaviors, and affect cue-oriented learning (Seamans et al., 1998; Granon et al., 2000; McFarland et al., 2004; Capriles et al., 2003; Puig and Miller, 2012; Kehagia et al., 2010). However, the effects of ethanol on mPFC dopamine and the effects of mPFC dopamine on ethanol self-administration are still unclear. The pharmacological effects of ethanol on mPFC extracellular dopamine have been previously debated (Dazzi et al., 2002, 2007; Hegarty and Vogel, 1993; Bassareo et al., 1996; Engleman et al., 2006), although more recent studies suggest that acute ethanol administration increases extracellular dopamine in the mPFC of naïve rats (Schier et al., 2012a; Ding et al., 2011). Modulation of dopamine receptor signaling in the mPFC has been shown to change ethanol self-administration behaviors, but due to the experimental designs used it is unclear if mPFC dopamine was important for drug-seeking or consummatory behaviors (Hodge et al., 1996; Samson and Chappell, 2003). Additionally, as the mesocorticolimbic dopaminergic system is an intricate interconnection of many structures, the pharmacological alteration of one region could cause compensatory changes in the system that make it difficult to determine the role of the mPFC in self-administration behavior. Therefore, the evaluation of the effects of operant ethanol self-administration on mPFC extracellular dopamine, during which seeking and consummatory behavioral phases are separated and no additional drugs are administered, could reveal the inherent role of mPFC extracellular dopamine during ethanol self-administration.

Based on our laboratory's previous work that suggests ethanol-associated stimuli can increase extracellular dopamine in the nucleus accumbens during operant ethanol self-administration (Doyon et al., 2005; Howard et al., 2009; Carrillo and Gonzales, 2011), we hypothesized that extracellular dopamine would also increase in the mPFC when an experienced, non-dependent, ethanol self-administering rat is exposed to drinking-associated stimuli. To test this, we used microdialysis to monitor extracellular dopamine during an operant self-administration session, in which rats drank a 10% ethanol plus 10% sucrose solution (10S10E), a 10% sucrose solution (10S), or no solution (handling group). Additionally, our operant self-administration sessions separated seeking and consummatory behavioral phases. Therefore, we were able to monitor the effects of both contextual cues during the transfer of the rat into the drinking environment (operant chamber) and stimuli experienced during the drink period (presumably the taste and smell of the solution).

Materials and Methods

Materials

Drinking solutions (10% sucrose (w/v) or 10% sucrose + 2, 5, or 10% ethanol (v/v)) were made from 95% ethanol (AAPER Alcohol and Chemical Co., Shelbyville, KY), ultra-pure sucrose (MP Biomedicals, LLC, Solon, OH) and distilled water. Carprofen (Pfizer, New York, NY) and gentamicin (APP Pharmaceuticals, Schaumburg, IL) were used during surgery.

Animals

Final statistical analyses used 27 male, Long Evans rats from Charles River Laboratories (Portage, MI or Raleigh, NC, USA; 200-225 g upon arrival). Animals were maintained on a 12-hour light/dark schedule, at 23 ± 2 ºC, with ad libitum food and water (except where noted). All animal procedures complied with and were approved by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee of the University of Texas at Austin, respectively. ARRIVE guidelines were also consulted.

Surgery

After a week of habituation to the facility and animal handlers, we anesthetized the animals with isoflurane (5% induction, 2.5% maintenance) and, using a stereotaxic apparatus, surgically placed a 21-gauge guide cannula (Plastics One, Roanoke, VA) above the left medial prefrontal cortex (in mm relative to bregma and the skull surface: +3.0 antero-posterior, +0.6 lateral, -2.0 ventral (Paxinos and Watson, 1986)). Three skull screws and dental cement were used to secure the guide cannula and a tether bolt to the animal's skull. We administered carprofen (5 mg/kg, subcutaneously) to minimize post-surgical malaise, placed a dummy cannula into the guide cannula to prevent blockage, and monitored the animal's weight and health over a seven-day recovery period prior to beginning operant training.

Self-Administration training and protocols

Groups

Handling animals were exposed to the same physical handling, water deprivation during the lever-press training period, time in the operant chamber, tethering, and dialysis as their respective experimental groups; but, they were not exposed to drinking solutions or operant training. The other groups were the ethanol-plus-sucrose-consuming group (10S10E group) and sucrose-consuming group (10S group). These animals received all of the aforementioned treatments, but were also trained to lever press for access to a 10% sucrose solution, and then continued to receive drinking solutions (ethanol + sucrose or sucrose) throughout their operant sessions.

Lever-press training and operant protocol

A week after surgery, animals were habituated to Med Associates, Inc. (Vermont, USA) operant chambers and then trained to lever press for a 10% sucrose solution (10S10E and 10S groups). Water deprivation (maximum 22 hours/day) was used to expedite lever-press training. Animals typically learned to lever press within three training sessions (one session/day), after which they regained ad libitum access to water for the remainder of the experiment. Chambers were as previously described by Howard et al. (2009). Briefly, chambers were outfitted with a retractable lever and sipper tube bottle, house light, and lickometer circuit. Chambers were contained in sound-attenuating boxes with the front doors removed for microdialysis purposes. Operant programs were run and data were collected using Med Associates, Inc. software.

Once trained to lever press, animals began an eight-session training schedule during which a pre-lever-press wait period was successively extended, and the response requirement was increased from two to four. For the 10S10E group, ethanol was introduced at a 2% (v/v) concentration in the 10% (w/v) sucrose solution, and gradually increased to a final 10% ethanol in 10% sucrose solution (10S10E) (Table 1). This procedure is a modification of the Samson, 1986 sucrose fading procedure; however, we did not fade sucrose out of the solution. Sessions were run once a day, four to six days per week to facilitate staggering of dialysis experiment days. Animals received a total of three to four, but never more than two sequential, days off from training once the eight-session protocol began. The training parameters for each session are noted in Table 1. The handling animals completed the same programs with the exception that the drinking solution was not available and, while the animals had the opportunity to, they were not required to lever press for inclusion in analysis. Solution consumption was determined by measuring the volume of solution before and after the drinking session (to the nearest 0.25 ml), while pattern of consumption was monitored using the lickometer. Spillage was accounted for by placing a dish under the sipper tube to catch spilled solution to add back to the bottle before post-session volume measurement. Rats were weighed each day and grams of ethanol was calculated from the volume consumed to determine g/kg ethanol consumed.

Table 3.1: Eight-session protocol parameters

Training Day	Wait Period (min)	Lever Presses Required	Ethanol in 10% Sucrose Drinking Solution (Ethanol group only)*
1	2	2	0%
2	5	2	2%
3	8	2	2%
4	13	2	5%
5	18	4	5%
6	23	4	10%
7 (Tethered)	28	4	10%
8 (Dialysis)	28	4	10%

* Sucrose animals only received 10% sucrose drinking solution, and handling animals did not receive drinking solution and are not required to lever press.

Microdialysis

Approximately 48 hours before dialysis, a spring was attached to the tether bolt on the rat's head and connected to a swivel suspended above the rat by a counter-balance lever arm (procedure details are described in Schier et al., 2012b). For the remainder of the experiment, rats were tethered in their home cages (placed next to their operant chamber in the operant room) and during their operant sessions to facilitate habituation to the apparatus and environment. After their seventh operant session rats were briefly anesthetized for implantation of a lab-constructed microdialysis probe (3.25 mm active area, 13,000 MW cutoff, constructed as described in Pettit and Justice, 1991). Probes were perfused with artificial cerebral spinal fluid (ACSF: 149 mM NaCl, 2.8 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.2 mM ascorbic acid, and 5.4 mM D-glucose), set to a 0.2 µl/min flow rate overnight and to a 1.0 µl/min flow rate the following day at least two hours prior to the dialysis experiment. For the 10S10E group, two samples before the lever extended into the chamber and all samples after were evaluated

for ethanol concentration by pipetting 1 µl of dialysate into a 2-ml glass vial that was then sealed with an air-tight septum. The remainder of these samples, and all other samples, were immediately frozen on dry ice and later stored in a freezer at -80 ºC until they were analyzed for dopamine concentration.

Experimental Timeline

Microdialysis samples were manually taken every seven minutes before and during the eighth operant session as depicted in Figure 1. Four baseline samples were taken while the rat was still in its home cage. During the last minute of the fourth baseline sample, the rat was transferred to the operant chamber. The operant program was started, turning on the house light and sound-attenuating fan, as the sample was changed to the first wait-period sample. Four wait-period samples were taken. The time it took the rat to meet the response requirement of four lever presses was collapsed into the last wait sample. The wait/lever-press sample was changed to the first drink sample as the drinking bottle entered the chamber. Three samples were taken during the drink period, after which the bottle retracted and the house light turned off. Then three samples were taken during the post-drink period. The rat was then returned to its home cage, and the ACSF was changed to calcium-free ACSF. Approximately 1.5 hours later, two additional samples were taken.

Dopamine analysis

We evaluated the dopamine concentration in all samples using reverse-phase high performance liquid chromatography with electrochemical detection. All sample sets were run with accompanying external standards (0.03125 or 0.0625

nM to 0.5 or 1.0 nM dopamine). Samples and standards were run using an 8125 manual injector (Rheodyne, Cotati, CA), a Luna 50 x 1.0 mm column (C18, 3-µm particle size; Phenomenex, Torrance CA) or a Haisil 100 50 x 0.5 mm column (C18, 3-µm particle size; Higgins Analytical Inc., Mountain View, CA), a 2 mm glassy carbon working electrode (SenCell or VT03 with ISAAC reference electrode, Antec Leyden, Netherlands) at potential + 345 or + 395 mV. Mobile phase was comprised of a one-liter aqueous solution of approximately 2.1 mM octanesulfonic acid, 0.04 - 0.3 mM decanesulfonic acid (adjusted as needed to optimize chromatography), 0.34 mM ethylenedianimetetraacetic acid, 71 mM sodium phosphate monobasic dihydrate, and 60 mM potassium chloride that was then adjusted to 5.60 pH with 1 M sodium hydroxide. Prior to use, 150 mL of methanol was added, and the solution was sparged with helium. Mobile phase flow rates ranged from 0.1 to 0.12 ml/min for 50 x 1.0 mm columns, and 0.025 – 0.032 ml/min for 50 x 0.5 mm columns. Four to 5.5 µl of dialysate was mixed with 1 to 3 µl of ascorbate oxidase (EC 1.10.3.3; 102.3 U/mg) prior to injecting 5 µl of the mixture (via partial or full 5 µl loop) into the system. EZChrome Elite software was used for chromatogram acquisition and peak integration. Dopamine peaks were required to have a signal-to-noise ratio > 3.

Ethanol analysis

Appropriate samples were analyzed for ethanol concentration as described in Schier et al., 2012b. Briefly, 1 µl aliquots of dialysate or external standards (0.3125 to 20 mM ethanol) were sealed in 2 ml glass vials, heated in an autosampler tray (65 or 50 ºC), and analyzed for ethanol content by a gas chromatograph with flame ionization detection.

Histological analysis

Within three days of dialysis, animals were overdosed with an intraperitoneal injection of sodium pentobarbital (150 mg/kg) and perfused through the heart with saline and then 10% formalin in saline prior to brain removal. The brains were post-fixed with 10% formalin in saline, coronally sliced (100 µm thick), and later stained with cresyl violet to visualize the probe track for correct probe placement confirmation (Paxinos et al., 1999).

Exclusion criteria

For animals to be included in the final data analysis, dopamine concentrations in the samples taken prior to initiation of the operant program (home-cage baseline) were required to have a relative standard deviation < 0.25. We also required a 40% decrease in dopamine dialysate concentration in calcium-free ACSF samples compared to standard ACSF samples, verifying that dopamine release was exocytotic. Animals were required to acquire the lever-press behavior within six training sessions, and ethanol animals were required to consume at least 0.8 g/kg on the day of dialysis. Animals that failed to lever press on the day of dialysis, or failed to complete all operant sessions, were also excluded. Failure of dialysis equipment such as flow-rate irregularity was also grounds for exclusion. Finally, missing dopamine values (generally due to contamination) at samples before and after the transfer into the operant chamber, or in the solution-consuming rats, the samples before and after the initiation of drinking were also grounds for exclusion.

Statistical analysis

Dopamine concentrations (nM) were natural log transformed to achieve homogeneity of variance between groups and then analyzed using repeated measures analysis of variance. For clarity, data are presented in the figures as percent of baseline ± standard error of the mean. Samples taken in the home cage before transfer to the operant chamber were used as the baseline to compare dopamine changes during the wait period. The final two wait-period samples were used as the baseline to compare dialysate dopamine changes in the drink and post-drink periods. If a time x group interaction was detected in a behavioral phase, then simple effects of time, group, and time x group interactions were performed, as well as appropriate post hoc analyses. If no group effect or time x group interaction was detected but an effect of time was detected, then we collapsed the groups for post hoc analysis of individual time points compared to baseline. Bonferroni corrections were used for all post hoc analyses. Due to sample contamination, five of the 313 dialysate dopamine points were dropped from analysis. We suspected but could not confirm contamination in one of the 313 dialysate dopamine points and therefore confirmed and dropped the point as an outlier using the Q-test. The values used for these points were estimated by averaging the points before and after it. The degrees of freedom were corrected appropriately in each analysis to account for this loss of data. Data were analyzed using SPSS® software. Significance was assigned if $p < 0.05$.

Results

Lever-press behavior and dopamine time course prior to consumption

We began sampling while the rats were in their home cages, next to their operant chamber, where they had spent the previous night. Average home-cage baseline dialysate dopamine concentrations were stable across time and similar between groups (10S10E = 0.15 ± 0.02 nM, n=9; 10S = 0.24 ± 0.03 nM, n=9; handling = 0.22 ± 0.03 nM, n=9) (time: $F_{3,167}$=1; group: $F_{2,26}$=0.018; ns). After collecting the baseline samples, the rats were placed in their operant chamber and the operant program was initiated. During the home-cage baseline, transfer into the operant chamber, and pre-lever press wait period in the operant chamber, dopamine concentrations did not significantly differ by group (group: $F_{2,24}$=2.7; group x time: $F_{14,167}$=1.3, ns), though dopamine concentrations significantly changed over time (time: $F_{7,167}$=15, $p<0.05$). The data from the three groups were collapsed and post hoc analyses showed that extracellular dopamine significantly increased above the home-cage baseline during the wait period in the operant chamber (Figure 2) (wait period points 1 – 4: $F_{4,167}$=21; $F_{4,167}$=8.7; $F_{4,167}$=5.0; $F_{4,167}$=4.0, $p<0.05$). At the end of the wait period, the lever was presented to all groups. Ethanol-plus-sucrose- (10S10E) and sucrose- (10S) consuming rats were required to lever press four times for access to the drinking solution. 10S10E rats began lever pressing significantly sooner than 10S rats after lever presentation, yet both groups showed similar lever-press rates (Table 2) ($F_{1,17}$=5.2, $p<0.05$; $F_{1,17}$=1.1, ns). While the lever was presented to handling rats, the animals generally did not press the lever and therefore were dropped from lever-press analysis. The time taken to complete the lever presses

was accounted for in the final wait-period sample, making that sampling period variable (7 minutes and 3 seconds to 10 minutes and 4 seconds).

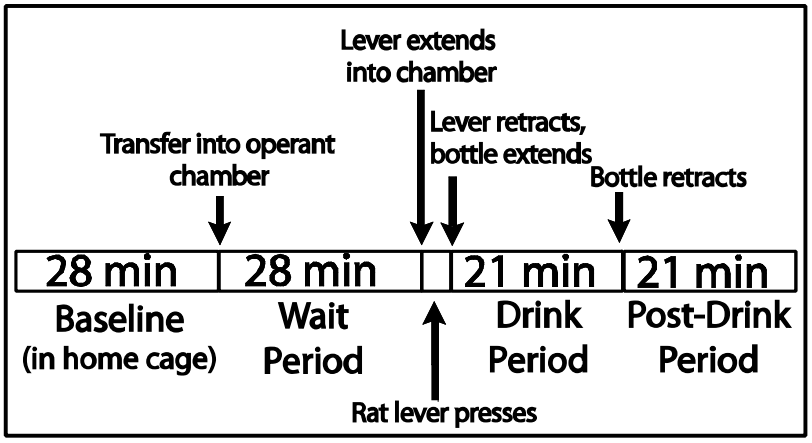

Figure 3.1: Experimental time course for operant self-administration. On the eighth day of operant sucrose or ethanol plus sucrose self-administration dialysis was performed. Seven-min samples were taken during these behavioral phases. This figure was adapted from Schier et al., 2012a.

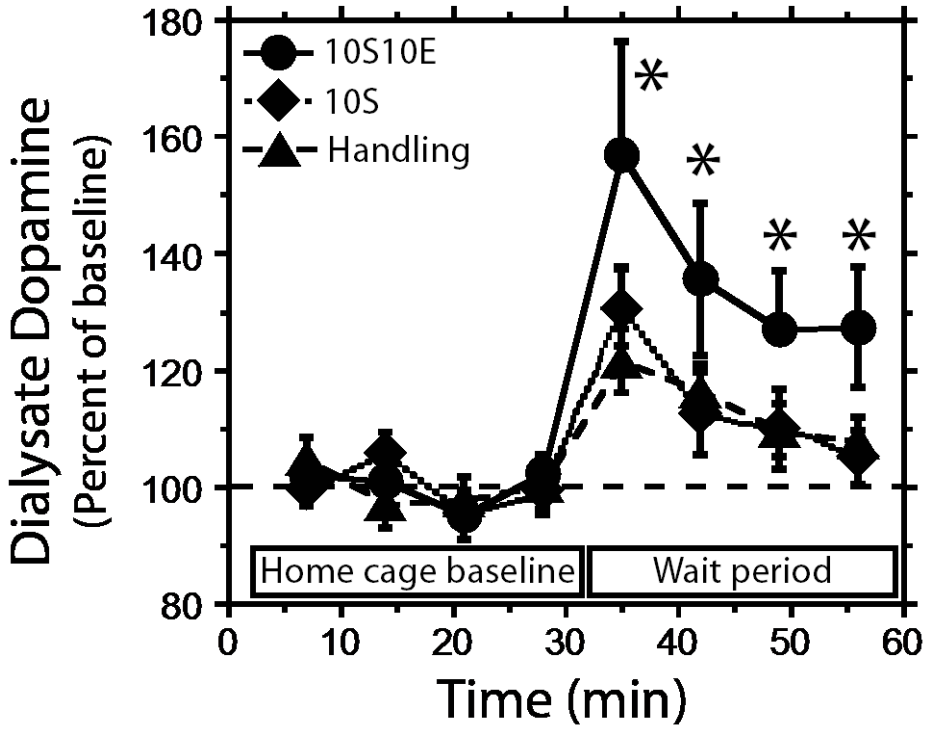

Figure 3.2: Dialysate dopamine home-cage baseline and wait period time course shown as percent of the home-cage baseline. Ethanol group **(circles, n = 9)**, sucrose group **(diamonds, n = 9)**, and handling group **(triangles, n = 9)** were not significantly different, and therefore the data were collapsed for post hoc analyses. **Asterisks (*)** represent significant increase in dopamine above home-cage baseline in collapsed data. Data are represented as mean ± SEM.

Dopamine time course and behavioral activity during consumption

Once the response requirement was completed, the lever retracted and the bottle entered the chamber, at which time the dialysis sample was changed and the drink period began. The 10S10E and 10S rats showed similar latency to drink, rate of licking during the first drinking bout, and total number of drinking bouts (bout defined as at least 25 licks with less than a two-minute break while drinking) ($F_{1,17}$=2.2; $F_{1,17}$=2.6; $F_{1,17}$=0.46, ns). The 10S10E animals drank significantly less solution, had significantly fewer licks overall and during the first

bout, and a significantly shorter first bout than 10S animals (Table 2) ($F_{1,17}$=22; $F_{1,17}$=18; $F_{1,17}$=15; $F_{1,17}$=9.5, $p<0.05$). When licks were binned to align with their respective seven-minute dialysis sample, 10S10E animals had significantly fewer licks during the first two seven-minute bins than the 10S animals, but the groups had similar licks during the final sample (Figure 5) (seven-minute bins 1-3: $F_{1,16}$=27, $F_{1,16}$=5.3, $p<0.05$; $F_{1,16}$=0.4, ns). On dialysis day, 10S10E animals consumed an average 1.65 ± 0.14 g/kg ethanol.

The last two wait-period samples were used as the new dialysate dopamine baseline for all consumption and post-consumption analyses. The data from the handling group was analyzed in the same fashion, though the animals did not consume a drinking solution. The new baseline was stable across time and the baseline averages were similar between groups (10S10E = 0.19 ± 0.01 nM, 10S = 0.25 ± 0.03 nM, handling = 0.24 ± 0.03 nM; time: $F_{1,92}$=0.5, group: $F_{2,25}$=1.6, ns). During the drink period, each group had a unique dialysate dopamine time course. Dialysate dopamine significantly increased 34 ± 6% above baseline in the first drink sample and remained significantly elevated for the remainder of the drink period in the 10S10E group (Figure 3) (drink points 1-3: $F_{2,92}$=31; $F_{2,92}$=8.4; $F_{2,92}$=5.8, $p<0.017$). In the 10S group, dialysate dopamine was only significantly elevated above baseline in the first drink sample (23 ± 5%) and then declined toward baseline (drink point 1: $F_{2,92}$=19, $p<0.017$). The handling group did not show a dialysate dopamine increase at the beginning of the “drink session” when the lever extended into the chamber, but the last drink-period sample did significantly decrease 11 ± 4% below baseline ($F_{2,92}$=5.4, $p<0.017$).

After the 21-minute drink period, the bottle retracted from the chamber and the house light extinguished, but the rat remained in the operant chamber for a 21-minute post-drink period. During the post-drink period dialysate dopamine concentrations did not significantly differ between groups, but did show an effect of time (group: $F_{2,24}$=1.1, group x time: $F_{8,95}$=1.8, ns; time: $F_{4,95}$=3.4, $p<0.05$). The data from the three groups were collapsed and post hoc analyses showed that extracellular dopamine significantly decreased below baseline in the last post-drink sample (Figure 3) ($F_{2,95}$=5.5, $p<0.017$).

Table 3.2: Behavioral parameters

Parameter	Ethanol Group	Sucrose Group
Time to complete presses (sec)	24 ± 16	25 ± 3.2
Latency to press (sec)	7.0 ± 3.8*	18.3 ± 3.2*
Latency to drink (sec)	6.1 ± 2.2	2.8 ± 0.2
Length of first bout (min)	4.9 ± 0.3*	8.4 ± 1.1*
Licks in first bout	1239 ± 80*	2478 ± 310*
First bout lick rate (licks/min)	259 ± 21	305 ± 20
Solution consumed (ml)	8.8 ± 0.7*	13.8 ± 0.9*
Total licks	1503 ± 100*	2704 ± 265*

Asterisks (*) represent significant difference in behavioral parameter between 10S10E and 10S groups. Data are represented as mean ± SEM.

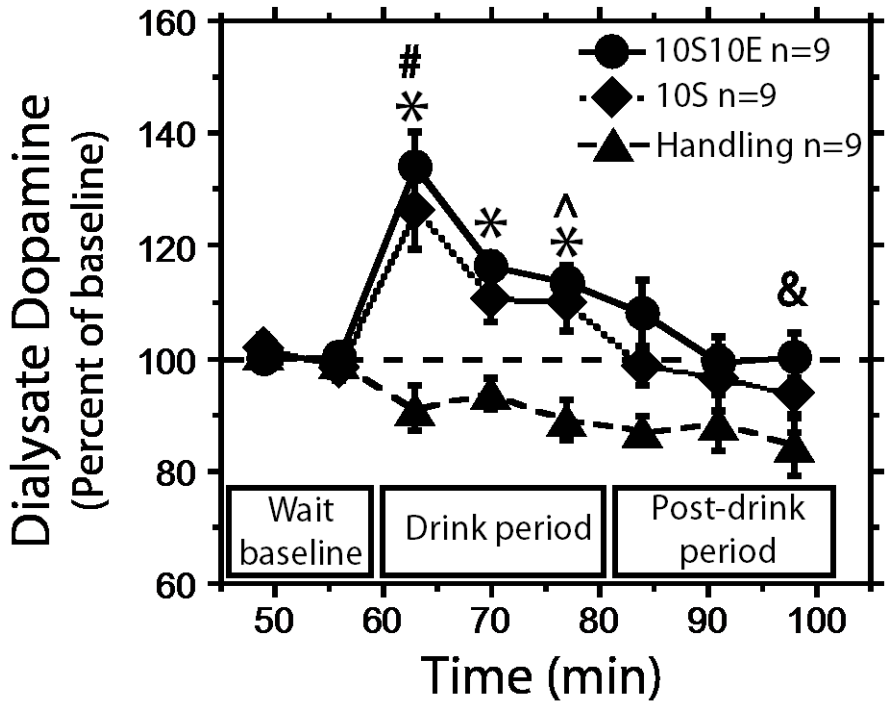

Figure 3.3: Dialysate dopamine wait-period baseline, drink period and post-drink period time course shown as percent of wait-period baseline. 10S10E group **(circles, n = 9)**, 10S group **(diamonds, n = 9)**, and handling group **(triangles, n = 9)** showed a significant interaction during the drink period, and therefore post hoc analyses were performed on each group. Specific symbols signify a significant difference from wait-period baseline with-in each group **(* for 10S10E, # for 10S, ^ for handling)**. The post-drink period was not significantly different between groups and therefore the data were collapsed. **Ampersand &** represents significant change in dialysate dopamine from home-cage baseline in collapsed data. Data are represented as mean ± SEM.

Dialysate ethanol concentrations during and after consumption

In the 10S10E group, we saw a mismatch between the dialysate dopamine and ethanol time courses. At the initiation of drinking, dopamine peaked and then declined throughout the drink and post-drink periods, while ethanol was lowest at the initiation of drinking and then increased throughout the drink and post-drink period, peaking at 5.2 ± 0.8 mM dialysate ethanol during the post-drink period

(Figure 4). Dialysate ethanol concentrations are not corrected with an in vivo extraction fraction, and therefore are lower than the true tissue ethanol concentration.

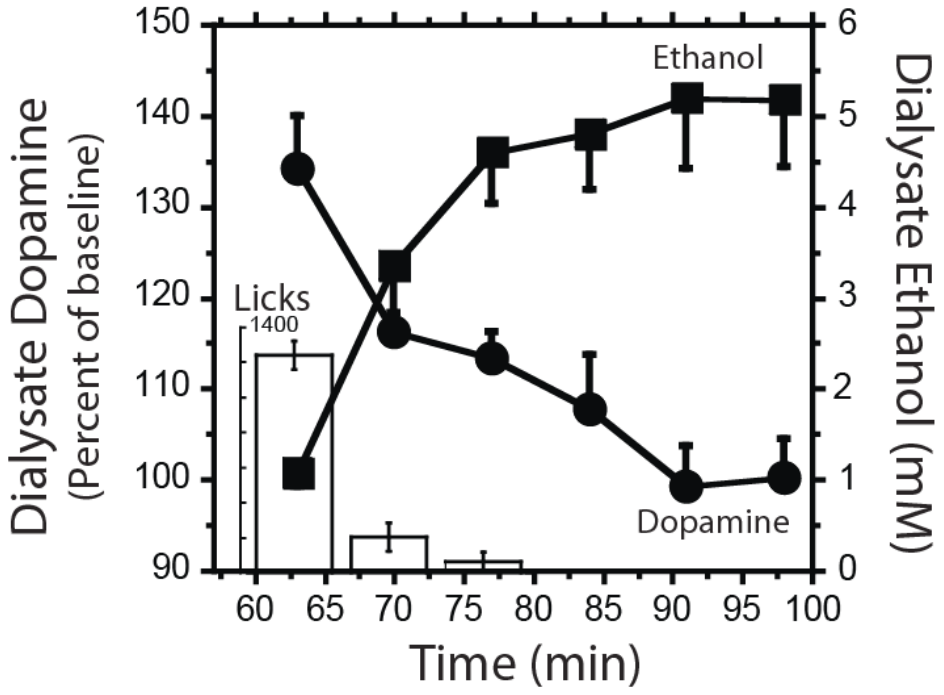

Figure 3.4: Dialysate dopamine and ethanol time courses during the drink and post-drink periods of 10S10E self-administration. Left y-axis shows the percent change in dopamine concentration **(circles)** during the drink and post-drink periods compared to the preceding wait period. **Right y-axis** shows the dialysate ethanol concentration **(squares)**. Bottle filled with 10S10E solution entered the chamber ~56 min. Bottle retracted ~77 min. Inset histogram shows average licks per 7 min sample during the drink period. Data are presented as mean ± SEM. (n=9)

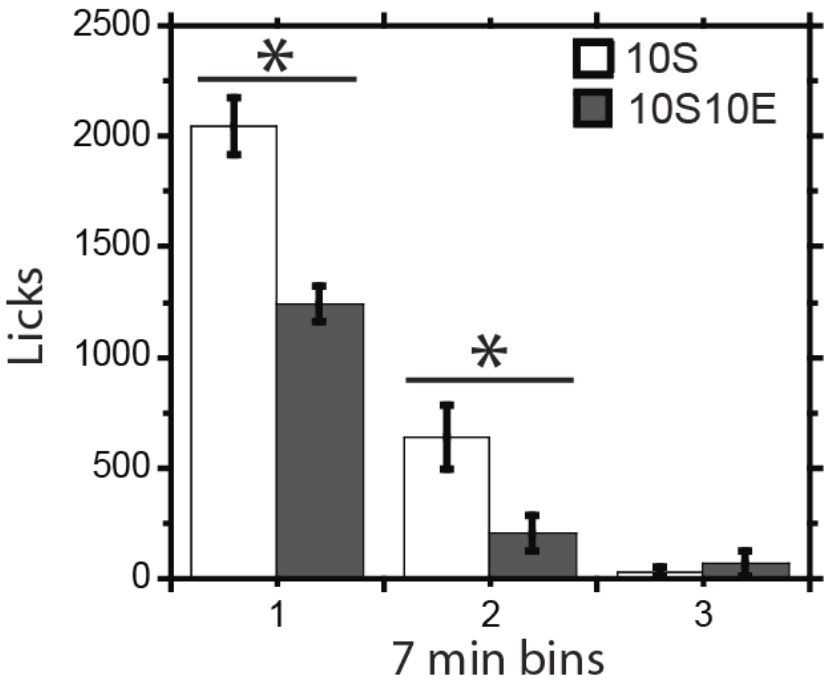

Figure 3.5: Licks shown in seven-minute bins that align with dialysis samples. Asterisks (*) represent significant difference in licks between 10S10E (n = 9) and 10S (n = 9) groups. Data are represented as mean ± SEM.

Histologies, body weights, and calcium-dependent dopamine decrease

Neither total percent of probe active area within the target infralimbic and prelimbic regions, nor animal body weight at dialysis, were significantly different between groups ($F_{2,26}$=2.1; $F_{2,26}$=2.7, ns, respectively). Probe active area was required to be at least 50% in the infralimbic and prelimbic regions of the mPFC. 10S10E, 10S, and handling groups were 76 ± 3%, 66 ± 5%, and 65 ± 5% within these regions, respectively (Figure 6). Animal weights on the day of dialysis in the 10S10E, 10S, and handling groups were 418 ± 45 grams, 388 ± 36 grams, 382 ± 17 grams, respectively. Calcium-dependent dopamine release was confirmed by a minimum 40% dialysate dopamine concentration decrease when

calcium-free ACSF was perfused through the probe. 10S10E, 10S, and handling groups each showed an average 67 ± 3%, 57 ± 4%, and 61 ± 5% dopamine decrease in calcium-free ACSF samples compared to dopamine concentrations at the conclusion of the operant session.

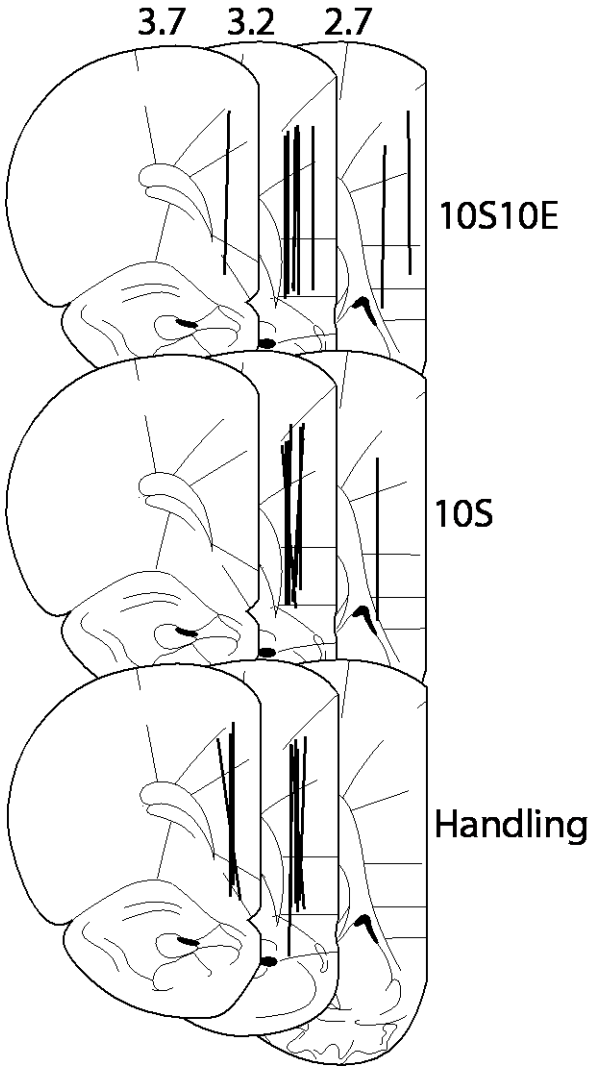

Figure 3.6: Microdialysis probe placements. Coronal slices 2.7, 3.2, and 3.7 mm from bregma showing microdialysis probe placements for all experimental groups. Lines represent 3.25 mm active dialysis area. Figure adapted from Paxinos & Watson (1998).

Discussion

Although previous studies suggested that mPFC dopamine signaling was increased after ethanol administration, we now provide for the first time evidence that a robust dopamine response occurs during operant self-administration of either a sucrose (10S) or sucrose-plus-ethanol solution (10S10E). While both solution-consuming (10S10E or 10S) and non-drinking control rats (handling group) showed dialysate dopamine increases when transferred into the operant chamber, only solution-consuming rats showed dialysate dopamine increases during the drink period. Furthermore, both 10S10E- and 10S-consuming rats had a significant increase in extracellular dopamine within the first seven minutes of solution consumption. However, dialysate dopamine concentrations remained significantly elevated for the entire drink period (21 minutes) in the 10S10E group, but only significantly increased during the first seven minutes of the drink period in the 10S group. Moreover, dialysate dopamine concentration in the 10S10E group peaked at the initiation of consumption and then declined, while dialysate ethanol concentration was lowest in the first drink sample and then continued to rise throughout the remainder of the operant session. Overall, our data suggest that in an operant paradigm, consumption-associated stimuli (presumably the taste and smell of the drinking solution) transiently increase extracellular dopamine in the mPFC of the Long Evans rat.

Consumption-associated stimuli contributed to the drink-period dialysate dopamine increase

The peak in dialysate dopamine at the initiation of consumption could be attributed to the presence of sucrose in both solutions. Previous work has demonstrated that consumption of palatable food and its associated cues increase extracellular dopamine in the mPFC. Bassareo et al. (2002) found that when an experimenter administered an initial unexpected intra-oral 20% sucrose or chocolate solution, mPFC dialysate dopamine increased within ten minutes of ingestion. Additionally, a conditioned stimulus (empty plastic box) preceding the feeding of palatable food (Fonzies©) increased mPFC dopamine, as did the following feeding of Fonzies© (Bassareo and Di Chiara, 1997). We now show that mPFC extracellular dopamine also increases during the consumption of 10S and 10S10E in an operant paradigm. Taken together, these results suggest that, in both solution-consuming groups, the drink-period dopamine increase is at least partly related to sucrose consumption and consumption-associated stimuli (i.e. the taste and smell of the drinking solutions).

Possible pharmacological contributions to the drink-period dialysate dopamine increase

The pharmacological effects of ethanol could have contributed to the extension of the drink-associated dopamine increase in the 10S10E group. Previous pharmacological data from our laboratory show that, in ethanol-naïve rats, ethanol dose-dependently increases extracellular dopamine concentrations in the mPFC, and that dialysate ethanol and dopamine concentrations largely correlate

(Schier et al., 2012a). The 10S10E animals achieved over 3 mM dialysate ethanol concentration in the second drink-period sample (7-14 minutes after consumption began) and peaked around 5 mM during the post-drink period. These concentrations fall between the dialysate ethanol concentrations measured after administration of a 0.25 g/kg and 0.75 g/kg intravenous ethanol dose in our earlier microdialysis study. The 0.75 g/kg dose led to a significant dialysate dopamine increase, while the 0.25 g/kg dose did not (Schier et al., 2012a). Thus, it is possible that the pharmacological effects of ethanol contributed to dialysate dopamine remaining significantly elevated during the last 14 minutes of the drink period. However, the pharmacokinetics of intravenous and oral ethanol administration are different, and therefore, it is not certain that the mesocortical dopamine system would respond identically to these routes of administration, even when the same dialysate ethanol concentration is achieved. Furthermore, multiple experimenter-administered microinjections of ethanol into the dopamine cell body region in the ventral tegmental area were reported to sensitize the dopamine response in the nucleus accumbens (Ding et al., 2009). Therefore, a lower ethanol concentration could have caused a larger dopamine increase in the mPFC in the ethanol-experienced rats in the present study compared to naïve rats. Overall, if there was a pharmacological contribution during the second two drink points it was transient, as the dopamine increase ultimately returned to baseline by the time dialysate ethanol concentrations peaked during the post-drink period. Further work will be needed to distinguish between the contributions of possible direct pharmacological effects of ethanol from the possible adaptations due to previous ethanol experience.

mPFC dopamine response to contextual stimuli is not drinking-solution dependent

Both drinking rats and handling controls showed a dialysate dopamine increase when transferred into the operant chamber compared with the previous home-cage baseline concentrations. We attribute this dopamine increase to the physical handling of the rat and environmental change, as opposed to contextual-drinking stimuli, since the response occurred in all groups. Physical handling increases extracellular dopamine in the mPFC, as does transfer into a novel environment (Feenstra et al., 1998, 2000). While the operant chamber was not a novel environment, it was an environmental change. Therefore, it is reasonable to surmise that this could lead to an extracellular dopamine increase in the mPFC.

Overall conclusions

The present data suggests that dopamine signaling in the mPFC is activated differentially during various stages of operant self-administration of 10S and 10S10E solutions. The dopamine response during the early (seeking) phase of the operant session was similar in consuming and non-consuming rats, suggesting that a general activation of the mesocortical dopamine system that was not specific to drinking context occurred. In contrast, there was a differential response of the mesocortical dopamine system to consumption of drinking solutions compared to non-consuming rats, indicating that stimuli associated with drinking contributed to the response. Our data clearly showed that the mPFC dopamine response occurred in both 10S- and 10S10E-drinking rats. This is in marked contrast to the dopamine response in the accumbens, which occurs with

ethanol consumption, but not sucrose consumption (Doyon et al., 2005; Howard et al., 2009; Carrillo and Gonzales, 2011). Therefore, the present data indicate that the mesocortical and mesoaccumbal pathways perform different functions during operant self-administration of sucrose. In addition, we also uncovered an extension of the dopamine response during consumption due to the addition of ethanol to the sucrose solution. It is plausible that the pharmacological effects of ethanol bolstered the dopamine increase in the 10S10E group, although our experiments do not allow us to firmly conclude the exact mechanism by which ethanol produced this effect. Overall, we present novel data using a behaviorally relevant time course to show that mPFC dopamine signaling is differentially enhanced during operant self-administration of 10S and 10S10E solutions. These data provide an initial step toward elucidating the role of the mesocortical dopamine system in the reinforcing effects of ethanol.

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substantially contributed to the data acquisition. The authors have no conflicts of interest.

Chapter 4: A protocol publication: Microdialysis of ethanol during operant ethanol self-administration and ethanol determination by gas chromatography.

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Christina Schier contributed to the manuscript and movie script composition, movie production, animal preparation for the movie production, and project organization. Regina Mangieri contributed to the manuscript and movie script composition, and movie production. Geoffrey Dilly contributed to the movie production, and animal preparation for the movie production. Rueben Gonzales contributed to the manuscript composition.

To view the video, please visit http://www.jove.com/video/4142/microdialysis-ethanol-during-operant-ethanol-self-administration

Abstract

Short Abstract

A method to determine the time course of ethanol concentration in the brains of rats during operant ethanol self-administration is described. Gas chromatography with flame ionization detection is used to quantify ethanol in the dialysate samples, because it has the sensitivity required for the small volumes that are generated.

Long Abstract

Operant self-administration methods are commonly used to study the behavioral and pharmacological effects of many drugs of abuse, including ethanol. However, ethanol is typically self-administered orally, rather than intravenously

as are many other drugs of abuse. The pharmacokinetics of orally administered drugs are more complex than intravenously administered drugs. Because understanding the relationship between the pharmacological and behavioral effects of ethanol requires knowledge of the time course of ethanol reaching the brain during and after drinking, we use in vivo microdialysis and gas chromatography with flame ionization detection to monitor brain dialysate ethanol concentrations over time.

Combined microdialysis-behavioral experiments involve the use of several techniques. In this article, stereotaxic surgery, behavioral training and microdialysis, which can be adapted to test a multitude of self-administration and neurochemical-centered hypotheses, are included only to illustrate how they relate to the subsequent phases of sample collection and dialysate ethanol analysis. Dialysate ethanol concentration analysis via gas chromatography with flame-ionization detection, which is specific to ethanol studies, is described in detail. Data produced by these methods reveal the pattern of ethanol reaching the brain during the self-administration procedure, and when paired with neurochemical analysis of the same dialysate samples, allows conclusions to be made regarding the pharmacological and behavioral effects of ethanol.

Protocol Text

1) Stereotaxic Surgery

1.1) All procedures follow the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

1.2) Using a stereotaxic apparatus, well-handled Long-Evans rats, anesthetized with isoflurane, are implanted with a 21-gauge cannula (for later microdialysis probe insertion) (Plastics One, Roanoke, VA) above the brain region of interest, and a tether bolt is built into the head stage (used to support microdialysis equipment).

1.3) Monitor rats carefully during and after surgery. Make sure that all animals receive at least one week of post-surgical care and recovery and are healthy before beginning the following procedures. A JoVE video of the stereotaxic surgery method is available (Geiger et al., 2008).

2) Operant Training

2.1) After a week of post-surgical recovery, animals are trained to lever press for 10% sucrose solution in a Med Associates operant chamber (MedAssociates, Inc., Vermont, USA) outfitted with a lickometer, retractable lever, and sipper tube (as previously described in Howard et al., 2009). Software from MedAssociates is used to design all operant programs (MedAssociates, Inc., Vermont, USA).

2.2) Once trained to respond for sucrose, begin rats on an appropriate training schedule during which ethanol is gradually added into the solution over multiple drinking sessions.

2.3) For example, our lab currently uses an eight-day training schedule where ethanol is faded into the solution culminating in a 10% ethanol/10% sucrose drinking solution. Control animals only receive 10% sucrose or no drinking solution. The specific type of operant schedule to be used can vary widely, but the general procedures described below for microdialysis sampling can still be carried out (Doyon et al., 2003).

3) Pre-microdialysis Procedures: Tethering

3.1) The night before the second-to-last training session (in our example this is the seventh training session), animals are habituated to the tether spring, which attaches to the tether bolt on their head stage. The spring attaches to the microdialysis swivel and counter balance lever arm, which is suspended next to the cage with a ring stand and clamps so that the animal can move freely within its cage. Tethered animals spend the night in the operant room, in their home cage with ad libitum food and water, to habituate them to the tethering set-up.

3.2) The following day, the rat completes its operant program with the tether in place, to habituate the animal to the feeling of the tether while performing its behavioral tasks. Mount the tethering apparatus to the wall of the operant chamber near the top to allow suspension of the tether and swivel above the center of the roof of the operant chamber. All of this is placed within the sound attenuating chamber. After the session, return the rat to its home cage in the operant room to await microdiaysis probe insertion.

4) Pre-microdialysis Procedures: The microdialysis probe is inserted the day before the microdialysis experiment, after the rat has completed behavioral training for the day

4.1) The day before the microdialysis experiment, after the rat has completed an operant session while tethered, anesthetize the rat with isoflurane and remove the obturator from the guide cannula. Slowly (over ~ five minutes) insert a microdialysis probe, perfused with artificial cerebral spinal fluid (ACSF), through the cranial guide cannula into the brain region of interest. We use laboratory-constructed probes and microdialysis procedures modeled after Doyon et al., 2003, and Pettit and Justice, 1991.

4.2) Use the previously discussed tethering apparatus to suspend the inlet and outlet lines of the probe above the animal.

4.3) Turn the probe flow rate down to 0.2 µl/min. Once again, the rat spends the night in the operant room.

5) Microdialysis Procedures: Collection of samples during self-administration session with appetitive and consummatory phases separated

5.1) At least two hours before the experiment begins, turn up the probe to its working flow rate. We use either 1.0 or 2.0 µl/min depending on the brain region. Check that the probe flow rate is consistent, and at least 90% of the set flow rate by using a Hamilton syringe to measure volume over time.

5.2) Dialysis samples (five or seven minute) are taken before, during, and after the operant session. The sampling interval depends on the brain region, neurotransmitter being analyzed, dialysate concentration of the analyte, and sensitivity of the analytical chemistry equipment to be used for the analysis. The behavioral phases are as follows:

5.2.1) Baseline: At beginning of experiment, collect dialysis samples in the animal's home cage (four samples).

5.2.2) Transfer: After collection of home cage baseline samples, transfer the rat to the operant chamber. Transfer of the tethered rat requires extreme care to make sure that the microdialysis fluid transfer line does not become tangled, and that the rat remains calm. Immediately after the transfer, activate the operant program as you change from the baseline/transfer sample to the first wait sample.

5.2.3) Wait: Continue to collect samples as the rat waits for the lever to extend into the chamber (three to four samples depending on the brain region).

5.2.4 Drink: After the lever is presented and pressed, a bottle of drinking solution (10% sucrose/10% ethanol or 10% sucrose) is made available to the rat for around 20 minutes (three to four samples).

5.2.5) Post-drink: After drink period, the bottle retracts, but the rat remains in the operant chamber for around 20 minutes (three to four samples).

6) Microdialysis Procedures: Preparation of microdialysis sample for ethanol analysis

6.1) Two samples before animals self-administer the ethanol solution, and all samples after, are evaluated for ethanol concentration. Pipette either a 1 or 2 µl aliquot from the sample of interest into a 2 ml glass vial. Then seal the vial with an air tight septum (9 mm Red Poly Screw Cap, PTFE/Sil Septa, Agilent Technologies). The volume of the ethanol analysis aliquot (1 or 2 µl) depends on the total sample volume, and the sample volume required for any additional analyses.

6.2) If the samples will be used for later neurochemical analysis, store the sample appropriately after pipetting the aliquot for ethanol quantification.

6.3) For example, our lab analyzes the samples for dopamine. To accomplish this, place the samples on dry ice during the experiment and then, store the samples at -80ºC after the experiment. We use 2 µl aliquots for ethanol analysis of a five minute sample collected using a flow rate of 2.0 µl/min for probes placed in the nucleus accumbens. This allows at least 7 µl remaining for later analysis of dopamine by high performance liquid chromatography with electrochemical detection.

6.4) For samples taken from the prefrontal cortex, which has much lower extracellular dopamine concentrations, we use a 1 µl aliquot for ethanol analysis taken from a seven-minute sample using a flow rate of 1.0 µl/min.

7) Post-microdialysis procedures

7.1) After the conclusion of the microdialysis experiment, anesthetize the rat and remove the probe. Replace the obturator if the animal is not immediately euthanized. Collect the brain within three days of the experiment. Otherwise, visualization of the probe tract may not be possible.

7.2) The brain should be removed in accordance with approved animal use protocols. We use sodium pentobarbital (150 mg/kg, ip) overdose, followed by cardiac perfusion with saline and then formalin in saline. Submerge brain in a formalin-saline solution for at least 12 hours before sectioning.

7.3) Coronally section the brain into 100 µm slices. Stain slices with cresyl violet, and examine for correct probe placement (Paxinos et al., 1986, 1998).

8) Analysis of samples for ethanol concentration

8.1) The collected samples as well as external standards (0.3125 – 20 mM ethanol) are run on our gas chromatograph with flame ionization detection system. This system is comprised of a Varian CP 3800 gas chromatograph with a flame ionization detector, a Bruker (Varian) 8400 headspace autosampler heated to 50ºC, and an HP Innowax capillary column (30 m x 0.53 mm x 1.0 µm film thick), with helium as mobile phase.

8.2) Chromatograms are recorded and analyzed with chromatography software such as the Varian Star Chromatography Workstation software that will be specifically discussed here.

8.3) To prepare the system for ethanol analysis, heat the autosampler plate using a recirculating water bath, and open the two additional gas tanks (air and hydrogen; helium is always left running to preserve the wax column).

8.4) Record the gas tank pressures, as well as the number of samples you intend to run so that you can keep count of the number of times the fiber and septum have been used so that they can be changed when appropriate (fiber-500 injections; septum-100 punctures).

8.5) Initiate the start-up method, which prepares the system to run samples (program parameters in Table 2). Wait for the system to report that it is “Ready”.

8.6) Initiate a 20-minute “burn,” which prepares the fiber for sample analysis by subjecting it to a high temperature to desorb any contaminates it has absorbed while at rest.

8.7) Standards are made by diluting 238 µl of 95% ethanol with water to a final volume of 100 mL using a volumetric flask in a chemical fume hood. This creates a 40 mM ethanol solution. We use a 1:1 serial dilution to create 20, 10, 5, 2.5, 1.25, 0.625, 0.3125 mM standards. For each concentration of standard, use the

same volume aliquot that will be taken from the dialysis samples. Pipette the aliquot into a 2 ml glass vial, and then seal the vial with an air tight septum.

8.8) All ethanol samples are heated in the autosampler until the entire liquid aliquot has been vaporized. We heat our samples for about 30 minute.

8.9) This section describes the Star Workstation software that we use with our gas chromatograph. Other software may be used, but the description below may not be applicable.

8.10) To run samples, create a sample list noting which sample is in which autosampler slot, and how many times the sample should be punctured. Be sure to route the data files to your selected folder. Then, select the method of choice for your samples, and begin the run. We use the running parameters noted in Table 2.

8.11) Our dialysate ethanol program absorbs the sample for three minutes and desorbs into the helium stream, which feeds into the wax column, for one minute. However, programs can be written to accommodate your specific needs.

8.12) The sample components separate in the wax column, and then go through the flame ionization detector where the carbon containing-compounds, such as ethanol, combust and release ions. This results in increased electron flow from the detector's anode to the cathode creating a current, which is converted to voltage and recorded resulting in chromatograms like the one shown below

(Figure 1). The change in voltage is proportional to the amount of carbon passing through the detector across time (Skoog et al., 1998).

8.13) After the system is finished running the samples you will need to check the integration of each peak. For Star Workstation software, click on the blue peak icon in the tool bar. Click on each peak's color and drag the arrows to adjust the baseline. Re-integrate each peak before moving on to the next group of peaks. The peak analysis software can be any general chromatography software.

8.14) For Star Workstation software, click on the batch report icon in your tool bar. Drag each sample from your specified folder to the batch report to print the sample reports. Reporting software can be customized with most common chromatography software systems.

8.15) The reports can be programed to show the information of your choice. We currently use peak height, but the reports can also be programed to use peak area.

8.16) To shut down the system, initiate the shut-down method (parameters noted in Table 2), turn off the water bath, and close the hydrogen and air tanks when the column oven temperature reaches 30ºC.

9) Ethanol Data

9.1) Plot the peak height as a function of each known external standard ethanol concentration (Figure 2A). Use the linear function given by these points to calculate the ethanol concentration from the peak height given by each dialysate sample.

9.2) By plotting the concentration of ethanol in the dialysate over time, we can see the pattern of ethanol levels in the brain region of interest during our behavioral session. Example data, shown below (Figure 2B), are represented as the concentration of ethanol in the dialysate across time during the self-administration session.

10) General Maintenance: The fiber should be changed every 500 punctures, and the septum every 100 punctures

10.1) To change the fiber:

10.1.1) On the gas chromatograph key pad press Menu, select 8400, press enter; select change syringe, press enter. The autosampler will position itself to allow the fiber (SPME Fiber Assembly, 75 µm Carboxen™-PDMS, Supelco Analytical, Bellefonte, PA) to be removed.

10.1.2) Open the door covering the fiber. Unscrew the lock nut, and move the latch to allow the fiber assembly to be removed. Take the fiber assembly out. Unscrew the nut that holds the fiber in the assembly, and then unscrew the fiber.

10.1.3) Replace the old fiber with a new one, and reassemble the fiber assembly. Replace the assembly in the autosampler, re-latching and screwing the assembly into place. When you are finished, press “change done” on the key pad, and the autosampler will ready itself for use.

10.2) To change the septum

10.2.1) First, unscrew the cap covering the septum (Hi-Temp .450 Dia. Generic Conditioned Septa, Varian) and then remove the old septum. Seat the new septum down into the fitting. Re-screw the cap and tighten it with the wrench. Then, use an injection needle to puncture the septum so that the fiber will not break the first time the septum is punctured.

Representative Results

Figure 1 shows example chromatograms for three concentrations of ethanol standards and for a rat dialysate sample collected at the end of the ethanol self-administration session. Ethanol peaks should be relatively symmetric, have consistent retention times, and a signal-to-noise ratio > 10. Failure to meet these criteria means that your system requires maintenance. Quality chromatography and correctly prepared standards produce a linear standard curve ($R^2 \geq 0.99$; Figure 2A) that is used to calculate the ethanol concentration of dialysate samples collected from ethanol self-administering animals over the course of their self-administration session (Figure 2B).

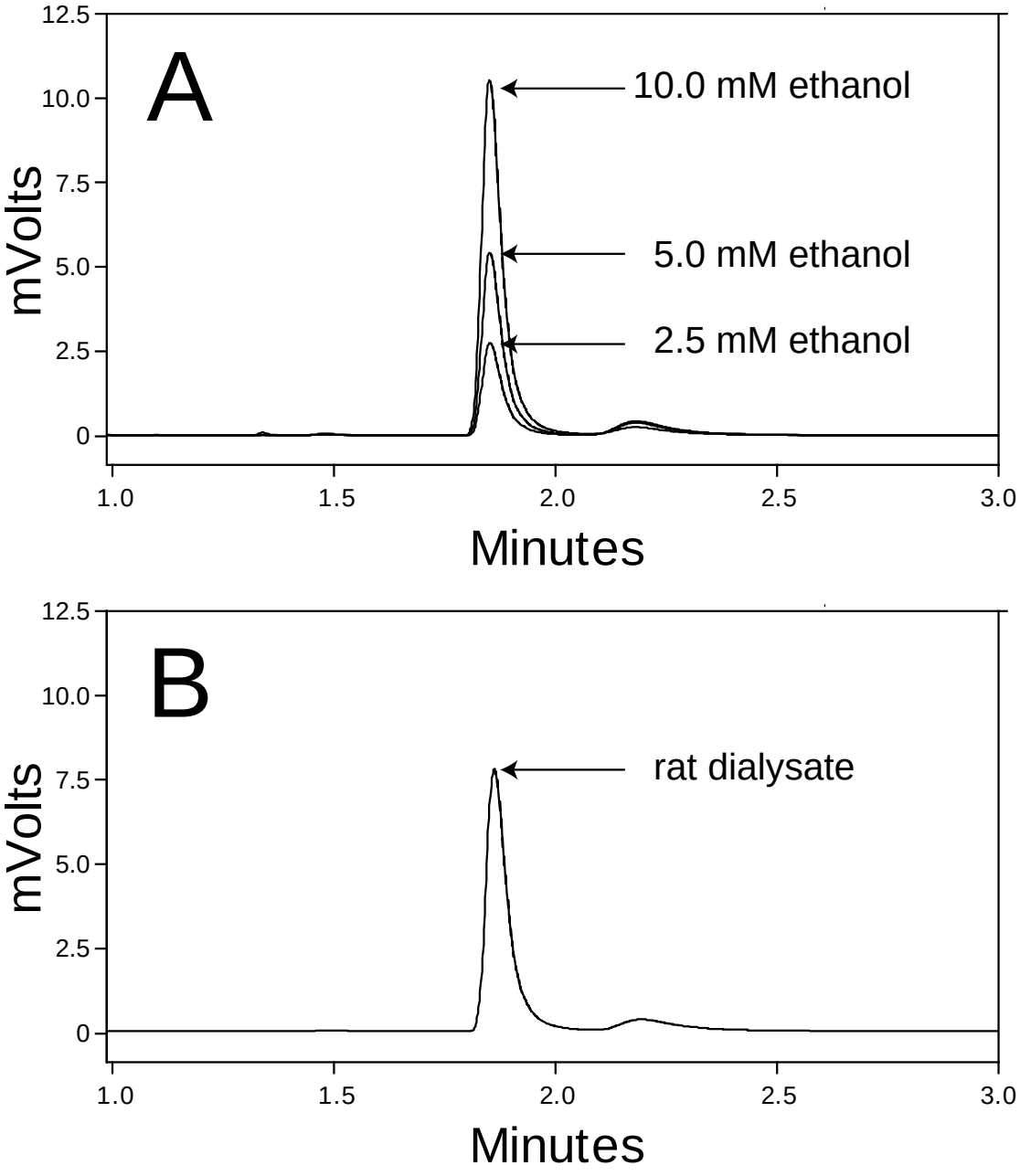

Figure 4.1: Example gas chromatography with flame ionization detection chromatograms. One microliter of ethanol standard or dialysate sample was loaded into a gas chromatograph vial and analyzed as described in the text. A) Overlay of peaks generated from 2.5, 5.0 and 10 mM ethanol standards. B) Peak generated from a dialysate sample from a rat that has self-administered ethanol.

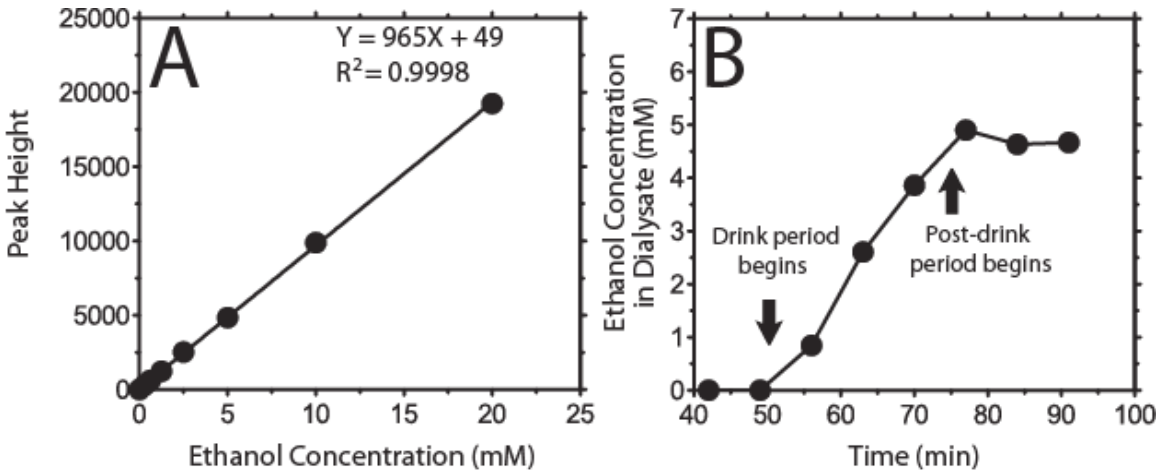

Figure 4.2: Graphical results from example experiment shown in Figure 4.1. A) Ethanol standard curve. B) Time course of dialysate ethanol concentration across an ethanol self-administration session.

Table 4.1: Specific reagents

Name of the reagent	Company	Catalogue number
95% Ethanol	AAPER Alcohol and Chemical Co., Shelbyville, KY	E190, 111000190
Ultra-Pure Sucrose	MP Biomedicals, LLC, Solon, OH	821721
Ultra High Purity Helium	Air Gas, Austin, TX	HE UHP300
Air	Air Gas, Austin, TX	AIZ300
Hydrogen	Air Gas, Austin, TX	AIZ300
GC 2 ml vials	Agilent	#8010-0015
GC vial caps with PTFE/silicone septa	Agilent	#8010-0084

Table 4.2: System parameters*

Hardware	Parameter	Start-up setting	Running setting	Resting setting
8400 Bruker (Varian) Autosampler				
	Injection mode	Spme	Spme	n/a
	Solvent penetration depth	0%	20%	n/a
	Sample penetration depth	20%	20%	n/a
	Adsorption time	0.01 min	3 min	n/a
	Desorption time	19 min	2 min	n/a
	Clean mode solvent source	I	I	n/a
	Clean mode adsorption and desorption time	0.01 min	0.01 min	n/a
	Water bath (heats autosampler)	50ºC	50ºC	off
CP-3800 Varian Gas Chromatograph				
Oven	Oven power	On	On	On
	Coolant	Off	Off	Off
	Enable coolant at	250ºC	250ºC	250ºC
	Coolant time out	20 min	20 min	20 min
	Oven temperature	250ºC	220ºC	30ºC
Column Oven	Coolant	Off	Off	Off
	Enable coolant at	50 C	50 C	50 C
	Coolant timeout	20 min	20 min	20 min
	Stabilization time	0.10 min	0.10 min	0.10 min
	Temperature	65ºC	65ºC	30ºC
Column	Mobile phase flow rate	8.5 ml/min	8.5 ml/min	n/a
	Column	~ 6 psi	~ 6 psi	≥ 0.1 psi
FID detector	Over power	On	On	On
	Temperature	220ºC	220ºC	120ºC
	Electronics	On	On	Off
	Time constant	Fast	Fast	Fast
	Range	11	11	11
	Autozero	Yes	Yes	Yes

*Table 4.2 shows Gas chromatograph with flame ionization detection system parameters. This table shows the parameters for the three programs used to prepare (start-up settings), run (running settings) and maintain the system while not in use (resting settings).

Discussion

Applications and limitations

Drug self-administration is used in rodents to model drug addiction. Many drugs of abuse that are modeled in this fashion can be administered intravenously, in which the drug is delivered directly to the central compartment. This allows for close monitoring of dose over a self-administration session. Since ethanol is usually orally self-administered, it is much harder to monitor drug levels due to individual differences in absorption and metabolism. By using microdialysis to sample from the brain region of interest, we are not only able to monitor the pattern of ethanol reaching the region, but we are also able to simultaneously monitor neurotransmitter changes in the same region over time during each self-administration phase.

Neurochemical alterations and drug-induced responses in the brain are associated with drug abuse and dependence; thus, the ability to concurrently measure neurochemical and drug concentrations during specific self-administration phases provides a very powerful and unique tool. One issue to keep in mind for correlating dialysate concentrations of analytes with behavior is the physical characteristics of the microdialysis plumbing. Specifically, the time it takes for fluid to be transferred from the lumen of the microdialysis probe to the collection tube is critical, and shorter times are better. In our lab, we construct the probes so that the time is about 90 seconds. Using these conditions, we have found that there is an accumbal dopamine increase at the initiation of ethanol consumption, which decreases over the course of the drink and post-

drink periods as the dialysate ethanol concentration increases (Howard et al., 2009; Doyon et al., 2003, 2005; Carrillo and Gonzales, 2011). These experiments, when combined with data from pharmacological studies, have allowed us to parse out the strictly pharmacological effects of ethanol and self-administration associated environmental cues on changes in neurotransmitter concentrations.

It should be noted that this particular application of combined behavioral-microdialysis techniques is suited to the current research interests of our laboratory. It is designed to evaluate the temporal pattern of ethanol reaching the brain in comparison to the pattern of neurotransmitter changes in the same region, so that we can relate these measures to synchronous self-administration behaviors. The derived dialysate ethanol concentrations are not corrected for in vivo probe recovery, and are only a fraction of the brain ethanol tissue concentration. If quantitative microdialysis of ethanol is required, the extraction fraction of ethanol that diffuses from the extracellular space into the probe should be experimentally determined. See previous publications from our laboratory for methods and further discussion (Howard et al., 2008; Robinson et al., 2000; Gonzales et al., 1998).

Although this protocol illustrates the use of gas chromatography along with solid phase microextraction of ethanol from the headspace of microdialysis samples, other methods for analysis of the ethanol content of the microdialysis sample could be used. However, alternative methods may suffer from some disadvantages. For example, less sensitive analytical methods may require a

larger sample volume which necessitates sampling times greater than the five-to-seven-minute sampling times illustrated here. The type of system discussed here uses a solid phase microextraction that concentrates the ethanol in the vapor phase in the sealed sample vial by allowing absorption to the fiber placed in the vial headspace. This improves the detection limit compared with direct headspace sampling which typically allows 50-100 microliters of the vapor to be injected. Another major advantage of the headspace method is that the sample injected for analysis is extremely clean and free of salts. Direct injection of the liquid microdialysis sample may also be used with higher sensitivity, but this will require more instrument down time due to regular maintenance required for cleaning out the injected salts.

Troubleshooting and other notes

1. Before your experiment begins, give yourself plenty of time to check that your microdialysis set-ups are functioning properly and to troubleshoot any issues. We suggest that you have an extra set-up, perfused with ACSF, ready to switch out with a malfunctioning set-up to save yourself time, as most operant sessions tend to occur at a specific time every day.

2. Be sure that the microdialysis probes are inserted over a period of about five minute to minimize the tissue damage produced by shearing of the tissue as the probe penetrates the brain.

3. When transferring the rat into the operant chamber it is helpful to have a second person assist so that the transfer line (part of the microdialysis set-up)

does not becoming tangled, and so that the operant program can be initiated on time.

4. Make sure that you give the autosampler water bath plenty of time to reach the appropriate temperature. Our system typically requires two hours to reach 50ºC. Also, assure that your samples are fully vaporized before analyzing the samples. We typically visually confirm that this has occurred before initiating sample analysis.

5. Standard good analytical chemistry practices should be followed. These include, but are not limited to, validation of individuals, equipment, and procedures. In brief, we employ the following guidelines: Individual users should demonstrate the ability to generate reproducible peak height values for standard concentrations and linear standard curves ($R^2 \geq 0.99$) across multiple days. To verify that the GC-FID system is working correctly, standards should always be analyzed before any dialysate samples are injected. The standard curve generated should have an $R^2 \geq 0.99$.

6. Most of the equipment we use was purchased through Varian, Inc., which was acquired by Agilent Technologies in 2010. At this time, Bruker Scientific Instruments obtained Varian's laboratory gas chromatography instruments business. For future purchases, consult either company.

7. The present protocol shows operant behavior with rats, but the viewer should be aware that more disruption of behavior is likely to occur with the smaller

mouse model. Another issue to be aware of is that placement of a microdialysis probe into brain regions other than the medial prefrontal cortex or nucleus accumbens may disrupt operant behavior to a greater degree than shown here. It is important to closely examine numerous behavioral parameters to determine if the damage caused by probe placement produces severe changes in behavior.

Disclosures

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Chapter 5: General summary and discussion

This dissertation work produced multiple important outcomes. First, we showed that acute, intravenous ethanol dose-dependently increases extracellular dopamine in the mPFC. Second, to our knowledge, we are the first laboratory to monitor dopamine in the mPFC during ethanol self-administration. Third, we found that the mesocortical dopamine system differentially responds to the operant self-administration of a sucrose or an ethanol-plus-sucrose solution. Fourth, we published our methods for monitoring dialysate ethanol concentrations during ethanol self-administration in an online-methods journal in written and video formats.

Technical challenges

During these studies, the largest challenge was monitoring extracellular dopamine in the mPFC. The low concentration of dopamine in this region makes successful dopamine analysis using biologically or behaviorally relevant sampling periods difficult (Engleman et al., 2006). It is possible that previous pharmacological studies failed to detect ethanol-stimulated dopamine increases in the mPFC because of long sampling periods. In our pharmacological study (Chapter 2), I presented data that used 10-minute microdialysis sampling periods, which, compared with many studies, is double the time resolution. I believe that this is part of the reason we were able to detect a dopamine change in the mPFC, showing that ethanol stimulates the mesocortical dopamine system. In our self-administration study (Chapter 3), I presented data that used seven-minute microdialysis sampling periods. This three-minute reduction in

sampling time allowed us to use the most behaviorally relevant time course possible and to take more than two samples per operant-behavior phase. The main source of attrition in these studies was due to contamination or loss of dialysate dopamine samples.

During these studies, the second greatest challenge was to successfully combine microdialysis, two analytical chemistry techniques, and, in one study, animal behavior. The success of all three or four components is required in each subject for the subject to be included for data analysis. Therefore, experiments of this nature generally have a high attrition rate; yet, due to the increased difficulty analyzing low dopamine concentrations, the studies presented here had around a 50% attrition rate. However, because of the novelty of the subject matter, and need to investigate the role of the mesocortical dopamine system in alcohol-consumption models, we felt that the completion of the study was worthwhile.

Due to the complicated and technically challenging nature of our methods, it is often difficult to fully explain how we perform our experiments to other scientists outside of our field. Therefore, when our laboratory was approached by The Journal of Visualized Experiments regarding publishing our operant ethanol self-administration/dialysate ethanol concentration analysis protocol, I accepted heading the project. I felt that publishing the protocol would be advantageous for other researchers interested in learning these techniques. In addition to recording a synopsis video of our experiment, we were also asked to provide a step-by-step protocol explaining how to perform the experiment (Chapter 4).

Furthermore, this protocol was paired with a traditional abstract, as well as less traditional discussion and troubleshooting sections that directly address typical experimental pitfalls and possible applications of the techniques. Once the written manuscript and video of the experiment were completed, it became obvious that this novel publication method greatly enhanced the transparency of our laboratory's experiments and should allow others to more easily and quickly learn and apply our techniques than is traditionally typical during technique acquisition.

Pharmacological effects of ethanol on the mesocorticolimbic dopamine system

In our first experiment (Chapter 2), we elucidated the previously unclear pharmacological effects of ethanol on extracellular dopamine in the mPFC by accounting for or addressing experimental factors that could have contributed to previous incongruent results. Additionally, due to the novelty of intravenous ethanol administration, we vetted our experimental methods to ensure that they did not confound the results. Due to this experimental design and rigorous testing, I believe that we can firmly state that intravenous ethanol dose-dependently increases extracellular dopamine in the mPFC of naïve Long Evans rats. For further discussion and support of these statements please refer to Chapter 2.

Our pharmacological study (Chapter 2), along with previous work in our lab, suggest that the mesocortical and mesoaccumbal dopamine systems are sensitive to the pharmacological effects of intravenous ethanol in naïve rats. However, there were a handful of methodological differences between the

current and previous intravenous-ethanol-administration study that could have affected the results in the previous work (Howard et al., 2008). Howard et al. (2008) used approximately a 4 ml/min infusion rate and administered a hypotonic-ethanol-experimental solution, but an isotonic-saline-control solution. The experiment presented in Chapter 2 tested both hypotonic- and isotonic-ethanol solutions along with an isotonic-saline-control solution. Additionally, the experiment also used a slightly slower manual infusion rate (average 2.5 ml/min), as well as an extremely slow pump-driven infusion rate (0.6 ml/min).

The methodological modifications and experimental outcomes in the current pharmacological study (Chapter 2), suggest that previous experimental shortcomings did not affect the experimental outcomes (Howard et al., 2008). Data presented in Chapter 2 show that similar dopamine responses occurred after the administration of either a hypotonic- or isotonic-ethanol solution, suggesting that, in the mPFC, intravenous solution tonicity did not affect the extracellular dopamine increase. Furthermore, in order to avoid agitating the rats during fluid infusions, we decreased the manual infusion rate to an average 2.5 ml/min. Additionally, we also included a control experiment in which we drastically reduced the infusion rate and used pumps to infuse the solution, showing that neither the infusion rate nor an inconsistent infusion (due to human error) contributed to the ethanol-induced mPFC dialysate dopamine increase. The mPFC is sensitive to stress, and therefore between the mPFC and the nucleus accumbens, if intravenous-infusion rate or solution tonicity were confounding experimental factors, it should be evident in this region (Feenstra et al., 1998, 2000). Therefore, the results of the current pharmacological study not

only show that ethanol dose-dependently increases extracellular dopamine in the mPFC, they also lead me to believe that, in spite of experimental shortcomings in our previous work, intravenous ethanol dose-dependently increases extracellular dopamine in the nucleus accumbens.

Effects of operant self-administered ethanol on the mesocorticolimbic dopamine system

Our current self-administration data (Chapter 3), along with previous laboratory data, suggest that the mesocortical and mesoaccumbal dopamine systems respond differently to operant self-administration (Doyon et al., 2003, 2005; Howard et al., 2009; Carrillo and Gonzales, 2011). Briefly, the data are as follows: The study that most closely followed the experimental design used in Chapter 3, Howard et al. (2009), showed that rats trained to consume 10S10E had significant dialysate dopamine increases in the nucleus accumbens core and shell when transferred into a drinking environment compared to rats trained to consume 10S. Furthermore, the nucleus accumbens core-shell border showed a solution-specific dopamine response at the initiation of consumption: Dopamine peaked within the first five minutes of 10S10E consumption, while there was a delayed dopamine increase (10-15 minutes after consumption began) in 10S-consuming rats. The core and shell did not show a significant dopamine response during consumption of either solution. This stands in contrast to the results noted in Chapter 3, where solution-consuming rats (both 10S and 10S10E) and non-consuming rats (handling control) showed similar mPFC dialysate dopamine increases when transferred into the drinking environment. Furthermore, rats trained to consume 10S and 10S10E solutions showed a

dopamine peak within the first seven minutes of consumption (though the response differed for the 14 minutes after the peak). Therefore, both systems respond during self-administration, but each show different durations of response.

The most noticeable difference between the dopaminergic responses of the mesocortical and mesoaccumbal systems was during the operant self-administration of sucrose. Extracellular dopamine in the mPFC immediately increased at the initiation of sucrose consumption, while the dopamine in the nucleus accumbens showed a smaller, delayed increase during sucrose consumption. Work by Bassareo et al. (2002) showed that the initial consumption, or intraoral administration, of palatable food or chocolate-drinking solution led to a transient dopamine peak and decline in both the mPFC and nucleus accumbens. The second time rats consumed these palatable items, the dopaminergic response in the mPFC and nucleus accumbens core was similar to that noted during the first exposure; however, the nucleus accumens shell no longer showed a significant dopamine increase (Bassareo et al., 2002). According to the histology figure in Bassareo et al. (2002), some of the shell probes also crossed the core-shell border, though the core probes did not. Therefore, it is not particularly surprising that we noted an initial dopamine peak during 10S consumption in the mPFC but not the accumbens core-shell border, as dialysis was performed (at least) the tenth time rats consumed the sucrose solution. Taken together, this implies that the core-shell border of the nucleus accumbens could habituate to natural reinforcers during operant self-administration, while the mPFC does not appear to do so.

Our data provide novel information regarding the roles of the mesocortical and mesoaccumbal dopamine systems during operant self-administration. Previously, Bassareo et al. (2002) used non-contingent intraoral administration, or free feeding of the palatable items, while monitoring extracellular dopamine in the mPFC and nucleus accumbens. The use of intraoral administration does not allow for choice, and neither intraoral administration nor free feeding incorporate anticipation or cue association in the models. Therefore, this study solely tested the effects of gustatory stimulation and caloric intake on these systems. Bassareo and Di Chiara (1997) use a classical conditioning model to show that food-associated cues, as well as palatable food can also increase mPFC extracellular dopamine; however, this model does not require the rat to expend any effort to receive the food. Conversely, by using an operant self-administration paradigm, our animals could anticipate the availability of the reinforcer, and then choose to work to receive access to and then consume the reinforcer. Because of this, our design allows the animals to make cue-associations with the reinforcer, self-regulate intake of the reinforcer, and allows the experimenter to manipulate the work required for the animal to receive the reinforcer. This makes our operant self-administration paradigm a more complete model with which to test drug or food reinforcement. Therefore, our data extend the current knowledge base by providing evidence that the mesocortical and mesoaccumbal dopamine systems play different roles during the operant self-administration of a natural reinforcer. This information, along with the ability to manipulate aspects of the operant model, create a superb starting point for future food and drug reinforcement research, and suggest that

these systems should be individually investigated, because it is likely that they serve different functions during operant self-administration.

The current self-administration data (Chapter 3) suggests that the mPFC dopamine response during transfer of the rat into the operant chamber is a solution- and training-independent effect. Similar results were originally noted in the nucleus accumbens (Doyon et al., 2003, 2005); however, later studies, with subregion specificity, larger group numbers and shorter probe active area, have shown drinking-solution-dependent differences during this time period in the nucleus accumbens (Howard et al., 2009). Therefore, while we currently attribute dopamine increases in the mPFC during this period to physical handling and environmental change, we cannot rule out that future studies could detect subtle differences that this study was unable to detect.

Ethanol self-administration mechanistic speculations

Although the results from the self-administration study (Chapter 3) provided important, novel information, we were not able to determine the exact mechanism by which ethanol led to dialysate dopamine remaining significantly elevated (compared to baseline) 14 minutes longer in the 10S10E group than in the 10S group during consumption. As previously stated, it is possible that a transient pharmacological effect of ethanol bolstered the dopamine increase seen in the 10S10E group (for further discussion please refer to Chapter 3); however, dialysate dopamine and ethanol concentrations do show a mismatched time course in this study. Dialysate ethanol concentrations are lowest at the initiation of drinking and then increase, peaking 35 minutes after drinking

commenced at which point dialysate dopamine concentrations had returned to baseline. Because the data do not show a clear-cut pharmacological response like the dose-dependent response seen in Chapter 2, it is tempting to speculate about other mechanisms (besides the direct pharmacological effects of ethanol), which could have led to this differential response.

I speculate that repeated ethanol consumption, after the presentation of drinking-associated stimuli, could have led to the enhanced dopamine response in the 10S10E group compared to the 10S group. This speculation is partly supported by Bassareo et al. (2007), who used a classical conditioning paradigm to show that drug-predictive cues can elicit a mPFC dialysate dopamine increase. This experiment's design greatly differs from that of our operant study, since the study used nicotine and morphine, had an experimenter administer the drugs, used classical conditioning, and separated cue presentation from drug administration. Therefore, we cannot draw strong parallels between their results and ours; yet, the study does suggest that mPFC dopamine signaling is sensitive to drug cues. Therefore, it is not unreasonable to speculate that stimuli predicting the later pharmacological effects of ethanol contributed to increased mPFC extracellular dopamine above that noted for the sucrose-consuming group. However, further investigation is required to tease apart the exact contributions of ethanol and sucrose to the stimuli-induced mPFC extracellular dopamine change.

The concept of cue-driven dopamine responses is not new territory in natural- or drug-reinforcer research. Schultz has previously shown that midbrain dopaminergic projections can shift from firing after a reinforcer is consumed, to

firing after reinforcer-predictive stimuli are presented before the reinforcer is consumed (Schultz, 1997). In our self-administration study, I suspect that we could be seeing a similar effect, though the current data set does not directly support this assertion. Shultz used a natural reinforcer (juice) that was reinforcing during the initial exposure. Though we currently cannot show that an initial, voluntary, oral consumption of ethanol increases mPFC dopamine, our pharmacological data (Chapter 2) show that intravenous ethanol stimulates the mesocortical dopamine system in naïve rats. While we cannot necessarily regard this initial intravenous exposure to ethanol as reinforcing (though rats have been shown to intravenously self-administer ethanol (Gass and Olive, 2007)), this, nonetheless, is an initial exposure to a substance that increases mesocortical dopamine signaling concurrent with administration. Therefore, it is likely that the initial consumption of ethanol would produce a similar pharmacological response in the mPFC. After conditioning, Schultz showed that midbrain dopmaine neurons fired after presentation of reinforcer-predictive stimuli. By the seventh day of ethanol self-administration, concomitant changes in mPFC dialysate dopamine and ethanol concentrations were no longer evident, as they were during the initial ethanol exposure in the pharmacological study (Chapter 2). Instead, a dialysate dopamine peak was seen at the initiation of 10S10E consumption, when less than 1 mM dialysate ethanol was present, suggesting that something other than the pharmacological effects of ethanol led to this initial dopamine increase. It is plausible that ethanol-related stimuli contributed to this initial dopamine increase, and also bolstered the increase during the drink period, as was previously proposed. Therefore, although there are significant differences in experimental design between our studies and those

of Schultz, and our data does not provide direct support for the idea, it is still tempting to speculate that a shift from ethanol to ethanol-associated stimuli stimulating mPFC dopamine signaling occurs in the mesocortical dopamine system during operant ethanol self-administration.

Finally, the 10S10E group showed a trend toward having a lower basal dopamine concentration during their home-cage baseline ($p=0.064$). Since optimal PFC dopamine concentrations are thought to optimize executive functioning, I believe that this "happenstance" is worth future investigation (Floresco and Magyar, 2006; Zahrt et al., 1997; Seamans et al., 1998; Phillips et al., 2004; Gregoire et al., 2012; Slifstein et al., 2008; Mattay et al., 2003; Takahashi et al., 2012). The current self-administration study exposed rats to low or moderate doses of ethanol seven times and only monitored dopamine change in response to drug and behavior challenges, not the true extracellular dopamine concentrations. If we were to extend training, allowing rats to chronically self-administer ethanol, and then run a no-net-flux experiment, we should be able to clearly see if basal mPFC dopamine levels are decreased in operant-ethanol-consuming rats, compared to non-ethanol-consuming rats. If basal dopamine concentrations were to be significantly decreased in the ethanol-consuming rats, then we could use the behavioral paradigm to test microinjected dopaminergic drugs to possibly identify a specific mPFC dopaminergic target that modifies drinking behaviors. Ultimately, this could lead to the identification of one of ethanol's mechanisms of action and possibly suggest appropriate drug therapies for recovering alcoholics.

One hypothesis regarding the PFC is that optimal function requires optimal dopamine concentrations. Level of proper functioning is thought to follow an inverted-U shape in relation to PFC dopamine concentration (Takahashi et al., 2012; Floresco and Magyar, 2006) (Figure 5.1). Too little or too much dopamine leads to disruptions in functioning.

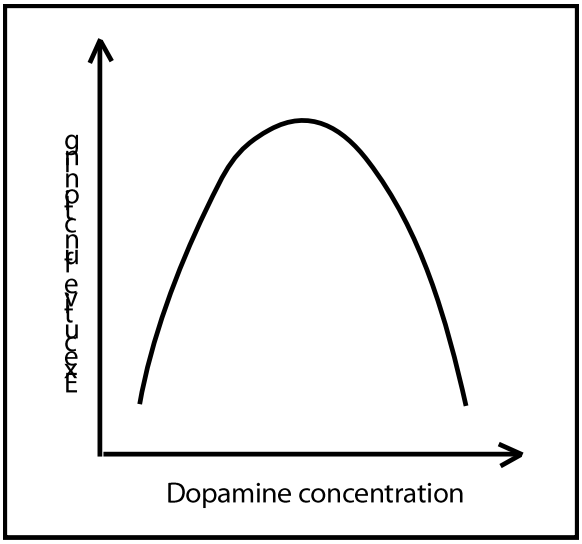

Figure 5.1: Theoretical inverted-U executive functioning in relation to PFC dopamine concentration.

When considering my data in light of this hypothesis, I would speculate that animals in my pharmacological study (Chapter 2) began with optimal mPFC dopamine concentrations. However, when ethanol was administered, dopamine sharply rose in the region. This suggests that acute ethanol could contribute to executive function dysfunction by increasing mPFC extracellular dopamine above an optimal level. Or it is also possible that this increase was within an optimal range of dopamine concentrations that biased appropriate responding to the situation. Yet, these are naïve animals, so how the dopamine response seen in these animals after multiple intravenous administrations of ethanol could be

different. It is possible that this dopamine response could be similar to the response noted in my self-administration study (Chapter 3) where dopamine increases coincided with the initiation of drinking. As previously discussed, one could speculate that in this study mPFC baseline dopamine concentrations were lower in the ethanol-consuming group. If the dopamine increase at the initiation was due to drinking-associated stimuli, the initially low concentration and sharp rise in dopamine, a neurochemical overreaction, after the stimuli exposure could lead to a behavioral overreaction to the stimuli compared to what would occur in a healthy individual. This could suggest that hypodopaminergic functioning in the PFC could contribute to the executive dysfunctions chronic alcohol abusers face even while sober. However, as there is no way to determine that the currently non-significant differences in the baseline-sample dopamine concentrations reflect truly different baseline dopamine concentrations, we must also consider the alternative. Assuming that our self-administration animals had optimal dopamine concentrations, then the spike in dopamine at the initiation of drinking could increase dopamine concentrations above optimal levels. Or it could also serve to modify signaling to other regions to allow the initiation of drinking, or be related to feed back during the initiation of drinking, as it could fall within a range of optimal dopamine concentrations that bias behavioral output in an appropriate manner. However, until we determine if ethanol self-administration modifies the baseline dopamine concentration in the mPFC, it is rather difficult to speculate where on the inverted-U curve this drinking-related shift in dopamine lies.

Conclusions

In conclusion, the data presented in this dissertation suggest that the mesocortical dopamine system plays a role in the reinforcing effects of ethanol. While the pharmacological effects of acute ethanol clearly stimulate the system, the mechanism leading to the differential effects of ethanol on the mesocortical dopamine system during operant self-administration is less clear. However, these results do suggest that the mesocortical and mesoaccumbal dopamine systems play unique roles in operant reinforcer self-administration. This supports the need to specifically investigate the role of the mesocortical dopamine system in drug and natural reinforcement, because it could lead to novel mechanisms for the treatment of not only ethanol addiction, but also addiction to other reinforcers.

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