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DOPAMINE CONCENTRATIONS IN THE NUCLEUS ACCUMBENS CORE-SHELL BORDER DURING THE EARLY STAGES OF OPERANT ETHANOL SELF- ADMINISTRATION

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by

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DEDICATION

I dedicate this work to my family, Ron Houseman, Violeta Alicia Soberanis, Jose Mario Carrillo, Jessica Carrillo, Autumn Sky Williams, Arrow Ray Williams, Damien Jay Williams, Estanislao Soberanis, and Joaquina Soberanis, for all the love they have given me.

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Mesolimbic dopamine plays an important role in ethanol reinforcement, and studies have shown that accumbal dopamine increases during operant ethanol self-administration. However, no one has ever studied this dopaminergic response during the acquisition of ethanol self-administration. Furthermore, some studies have shown that the dopamine signal does not correlate with the pharmacological effects of ethanol, but with the time during which the animal consumes the majority of the ethanol solution and when the sensory stimuli of ethanol are strongest. However, there is currently no direct evidence showing that the sensory stimuli of ethanol is indeed what causes the brief increase in accumbal dopamine during ethanol self-administration. The studies in this dissertation attempted to elucidate these issues.

We designed and tested a placebo spout, which was to be used to study the relationship between accumbal dopamine and the sensory stimuli of ethanol

during self-administration. Unfortunately, the placebo designs were either not feasible for performing microdialysis or did not show promising behavioral data. We also developed and tested a self-administration protocol in which the concentrations of ethanol (10%) were kept constant throughout the study. The new protocol was successful in initiating and maintaining ethanol self-administration, and the animals doubled their intake from day 1 to day 2 of ethanol consumption. Using this protocol, we trained male Long Evans rats to self-administer ethanol and measured accumbal dopamine during the first two days of ethanol self-administration through microdialysis. The behavioral and neurochemical data matched. A single exposure to ethanol was sufficient for the animals to double their ethanol consumption by day 2 and to cause an increase in accumbal dopamine during the first 5 minutes of ethanol self-administration. The dopamine response was observed during the time when the sensory stimuli of ethanol were strongest, but before ethanol reached peak concentrations in the brain. Overall, these results suggest that the dopamine response to ethanol self-administration may not be solely pharmacological and that a single exposure to ethanol is sufficient to learn the association between ethanol and its cues. These findings give us greater insight into mesolimbic dopamine's role in the early stages of ethanol reinforcement.

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Chapter One: General Introduction

Alcohol abuse and alcoholism are a major problem in our society, affecting the abuser, the family, and the community as a whole. In fact, approximately 1 in every 12 adults in the United States abuses alcohol or is alcohol dependent (NIAAA, 2007). People seek alcohol and become dependent for various reasons that are not well understood. However, behavioral changes and repeated drug seeking occur, and this suggests that the process of learning is implicated. Alcohol affects different neurotransmitters and systems, and the mesolimbic dopamine system is believed to be involved in the compulsive seeking and consumption of alcohol (Gonzales et al., 2004; Weiss and Porrino, 2002). It is therefore crucial to study dopamine and the role it plays in ethanol reinforcement, and to better understand the drug seeking and taking behavior, we should use a model that involves operant reinforcement.

The purpose of this dissertation was (1) to design a rat placebo spout to further examine the effect that the sensory stimuli of ethanol (taste and smell) have on dopamine in the nucleus accumbens during the self-administration of 10% ethanol + 10% sucrose (2) to develop and test a new operant self-administration protocol that would allow us to study the beginning stages of ethanol reinforcement, without the confounding factors introduced by varying concentrations of sucrose and ethanol (3) to use the new operant protocol to

investigate accumbal dopamine during the initiation of ethanol self-administration (the first two days of ethanol exposure).

Classical and Operant Conditioning

Behavior is greatly affected by drugs of abuse, including alcohol. Classical and operant conditioning paradigms have proven to be useful tools in our attempt to model human drug seeking and taking behaviors. Since much of this dissertation will focus on the operant self-administration of ethanol, it is important to give some background on this particular experimental method. The theories regarding classical and operant conditioning will be discussed in the following section, with emphasis on operant conditioning.

Classical Conditioning

Classical conditioning was first introduced by Ivan Pavlov, who paired a neutral stimulus (conditioned stimulus) with a stimulus that produced an autonomic response (unconditioned stimulus). Pavlov presented dogs with food and measured their salivary response, unconditioned response. He then began to ring a bell immediately before presenting the food. After repeated pairings, the dogs began to salivate when the sound of the bell was presented. The animals associated the sound of the bell with the presentation of the food and therefore

salivated as a response to the cue, a conditioned response (Pavlov, 1927, translation by Anrep). The food in this study would be the unconditioned stimuli because it provokes a response without training. The ring tone from the bell would be the conditioned stimuli, since the dogs respond to it after learning to associate it the unconditioned stimuli, and the act of salivating would be the conditioned response.

Operant Conditioning

Edward L. Thorndike was the first to extensively study operant conditioning (instrumental conditioning), which differs from classical conditioning in that it involves voluntary behavior. Instrumental conditioning forms an association between a behavior and a consequence. Thorndike observed the behavior of cats trying to escape from home-made puzzle boxes. He noticed that the cats took a long time to escape when they were first placed in the box, but with experience, the cats became faster at escaping from the box (Woodworth, 1952). Thus the cat's escape strategy was affected by whether or not it was able to escape from the box. Thorndike named this "The Law of Effect." Essentially, this law states that an effective outcome of the behavioral response tended to "stamp in" its connection with the situation at hand, whereas an ineffective outcome tended to "stamp out" the connection (Woodworth, 1952). Thus, some consequences strengthened behavior, while others weakened behavior.

B.F. Skinner advanced the concept of operant conditioning by stating that a reinforcer was any stimulus that increased the frequency of a specific behavior (an operant response) and by inventing the famous operant conditioning chambers (the Skinner box). Skinner formulated a more detailed description of instrumental conditioning. He proposed that as behaviors turn into goal directed responses, some stimuli will be able to predict when a reinforcer will occur, while others will not and will therefore be ignored (Labrador, 2004). Extinction happens when a behavior that had previously been reinforced loses its effectiveness and therefore ceases to exist. For example, if an animal is required to press a lever to receive a solution it finds rewarding, but if the solution is removed the animal will eventually stop pressing the lever.

The two main categories of operant conditioning are reinforcement and punishment, and both have a positive and a negative subcategory. Reinforcement, both positive and negative, strengthens a behavior. Positive reinforcement occurs when a behavior, such as pressing a lever, is followed by a stimulus, like a sucrose solution, causing the frequency of the behavior to increase. Negative reinforcement takes place when the behavior is followed by the removal of a stimulus, such as a foot shock, causing the frequency of the behavior to increase. Punishment, like reinforcement, is a stimulus paired with a specific behavior/response. However, both positive and negative punishment,

result in diminishing the frequency of the behavior. Positive punishment is when a behavior is followed by a negative stimulus, and negative punishment is when the behavior is followed by the removal of a positive stimulus. Thus both paradigms cause the frequency of the behavior to decrease.

Skinner introduced several schedules of reinforcement, and among the most popular are continuous, interval, and ratio. The interval and ratio schedules can be either fixed or variable. Continuous reinforcement is when the reinforcer is delivered every time a specific behavior is performed. Interval schedules require a minimum amount of time to pass before a response is reinforced. If the response occurs before the required amount of time has passed, it will not be reinforced. A fixed interval schedule has a specific amount of time required between reinforcers, such as exactly 30 seconds, while a variable interval schedule has a different amount of time between reinforcers, usually a range, but the time can vary from 15 seconds, 20 seconds, or 30 seconds, for example. Ratio schedules require a certain number of operant responses, such as 4 responses, before the next reinforcer is produced. A fixed ratio schedule has a set number of responses required from one reinforcer to the next, while with a variable ratio schedule, the number of responses needed from one reinforcer to the next may vary (Ferster, 2002; Skinner, 1958). However, it is important to remember that although Skinner developed and tested several schedules of reinforcement, new schedules continue to be created, tested, and used.

Overall, operant training is needed to create a successful behavioral model to study ethanol reinforcement and mesolimbic dopamine. In fact, several studies have shown that animals are willing to self-administer ethanol and that the consumption of ethanol causes an increase in dopamine in the nucleus accumbens (Doyon et al., 2003; Doyon et al., 2005; Gonzales and Weiss, 1998; Howard et al., 2009; Melendez et al., 2002; Weiss et al., 1996). This issue will be further discussed later in this dissertation, but first, we will review why mesolimbic dopamine is implicated in ethanol reinforcement.

Dopamine

Dopamine is a neurotransmitter that is important for many processes. For example, dopamine plays a role in control of movement, motivation, learning, and reward produced by natural stimuli and drugs of abuse, including alcohol (Gonzales et al., 2004). This neurotransmitter was first discovered in the brain in the 1950s by Arvid Carlsson (Andersen, 2009). Dopamine is synthesized by tyrosine hydroxylase from the amino acid tyrosine into DOPA (dihydroxyphenylalanine), which is then converted into dopamine via DOPA decarboxylase. Dopamine has five types of receptors: D_1 like (D_1 and D_5) and D_2 like (D_2, D_3, and D_4) (Verheij and Cools, 2008). The dopamine signal is transduced by G protein-coupled receptors. Activation of D_2 like receptors inhibits

adenylate cyclase, while D_1 like receptor activation is excitatory and stimulates adenylate cyclase. There are four major dopaminergic pathways: nigrostriatal, tuberoinfundibular, mesocortical, and mesolimbic. The nigrostriatal pathway connects the substantia nigra with the striatum, and it seems to be involved in motor processes. The tuberoinfundibular pathway connects the hypothalamus to the pituitary gland, and it is important in regulating the secretion of prolactin from the anterior pituitary gland (Swanson, 1982). The mesocortical and mesolimbic pathways are similar. Both systems originate in the ventral tegmental area (VTA), with the mesocortical pathway projecting to the frontal cortex (Swanson, 1982) and the mesolimbic system projecting to limbic regions like the nucleus accumbens, prefrontal cortex, amygdala, ventral pallidum, hippocampus, and olfactory tubercle (Pierce and Kumaresan, 2006; Swanson, 1982). Both systems appear to play a role in reward, memory, and emotional and motivational responses. However, since the role that dopamine in the nucleus accumbens plays in reinforcement is more established in the literature, the main focus of this introduction will be in the mesolimbic system, with emphasis on the nucleus accumbens.

Dopamine and the Nucleus Accumbens

The nucleus accumbens is part of the mesolimbic dopamine system. GABAergic medium spiny neurons are the main neuronal cells in the nucleus accumbens,

about 95% in rats and 70% in primates (Heimer et al., 1997; Zhang et al., 2006) and send inhibitory projections to areas such as the ventral pallidum, VTA, and substantia nigra (O'Donnell et al., 1999; Zhou et al., 2003). These spiny neurons are believed to receive convergent synaptic input from dopamine neurons in the VTA and glutamatergic neurons from areas like the hippocampus, medial prefrontal cortex, and amygdala (Brog et al., 1993; O'Donnell et al., 1999; Pierce and Kumaresan, 2006). The exocytotic release of dopamine from the VTA and its subsequent uptake by the dopamine transporters appears to maintain the extracellular concentration of dopamine at the terminal regions (Nirenberg et al., 1997; Sombers et al., 2009). *In vivo*, the firing rate of the dopamine cells in the VTA fluctuates between tonic single spike firing (about 4 Hz) and phasic burst firing, 15 to 20 Hz (Hyland et al., 2002). Phasic firing of the dopamine neurons can be caused by the presentation of a reward or a cue signaling the reward (Schultz, 2010).

Core, Shell, and Core-shell Border

The nucleus accumbens consists of subregions, which include the core, the shell, and the core-shell border area. Although in general the shell is slightly more medial and ventral than the core, part of the shell wraps around the core (figure 1), making these subregions anatomically distinct (Heimer et al., 1991; Ikemoto et al., 1995; Meredith et al., 1992; Usuda et al., 1998; Zahm and Brog,

1992; Zahm, 1999). In fact, the two can be differentiated using histochemical markers; the core stains much more darkly for calbindin than the shell subregion (Meredith et al., 1996; Brauer et al., 2000). The shell tends to innervate the extended amygdala and lateral hypothalamus, while the core connects with motor areas like the dorsal striatum (Zahm and Brog, 1992). In addition, the neurons in the core seem to have about 50% more surface area than the neurons in the shell, and the dendritic trees of cells in the shell are less spiny and have fewer branches than those in core (Meredith et al., 1992). The core-shell border, also known as the shore or the transition zone, is the region that separates the core and the shell of the nucleus accumbens, and very little is known about the anatomy and function of this subregion. However, as with the anatomy, the core and the shell appear to have different functions.

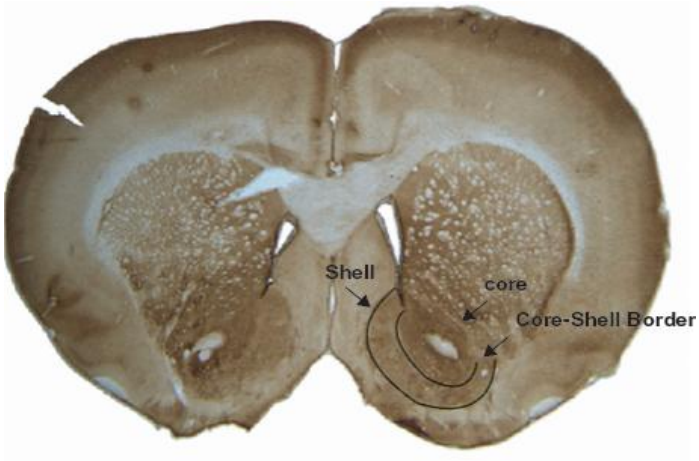

Figure 1. Calbindin staining of a coronal slice of a rat brain (Bregma 1.9). The nucleus accumbens core subregion is wrapped around the anterior commissure, while the shell wraps around the core. The core is also more darkly stained than the shell.

Studies have shown that the core plays an important role in motor processes, such as associative conditioning and expression of instrumental behaviors (Bassareo & Di Chiara, 1999; Day et al., 2007; Hernandez et al., 2002; Kelley et al., 1997). For example, Bassareo and Di Chiara showed that when rats had been previously exposed to food, the food stimuli alone stimulated the transmission of dopamine in the core (1999). In addition, protein synthesis is important for a memory to form, and Hernandez and colleagues micro-infused anisomycin, a protein synthesis inhibitor, in the core, immediately after each training session for 5 days (2002). The micro-infusions disrupted the consolidation of lever-pressing for food. The rats were unable to associate lever pressing behavior with receiving sugar pellets (Hernandez et al., 2002). Also, intracerebral microinfusions of AP-5, an antagonist of NMDA receptors, into the

core of the nucleus accumbens impaired response-reinforcement learning or lever pressing for food in rats; a much smaller impairment was seen in the shell (Kelley et al., 1997).

On the other hand, the shell subregion has been reported to play a critical role in novelty (Bassareo and Di Chiara, 1999; Rebec, 1997, 1998). For example, when rats were presented with a novel food, unpredicted consumption of the food stimulated dopamine transmission in the shell (Bassareo and Di Chiara, 1999). Furthermore, Rebec demonstrated that unlike in the core subregion, entry into a novel compartment caused an increase in dopamine in the shell (2002).

It should be noted, though, that some studies do not report a difference between the core and shell. A study showed that there was a similar increase in dopamine in the core and shell subregions during the presentation of a conditioned stimulus (white noise) followed by an unconditioned stimuli (food pellets) and again when later presented with the white noise only (Cheng et al., 2003). However, the rodents in this study were food deprived and this could have caused extra stress to the animals and therefore an aberrant response. Furthermore, some of the probe placements in the study seemed to be in the core-shell border and not in the shell or core subregions. It is possible that the location of the probes affected the results. In addition, a study by Bassareo and colleagues reported a similar increase in dopamine in the core and in the shell of the nucleus accumbens in

rodents when exposed for the first time to a sucrose plus chocolate solution (2002). However, some of the probe placements designated to the shell appear to be crossing the core-shell border as well, and the microdialysis samples were 10 minutes long. Perhaps the dopaminergic response observed in the shell was affected by the location of the probes not being restricted to the shell subregion. Additionally, a shorter sampling time may have possibly revealed a greater initial dopaminergic response in the shell versus the core.

Unlike the core and shell of the nucleus accumbens, only a handful of studies have targeted the core-shell border as the region of interest, but it may represent a functionally independent accumbal subregion. In fact, dopamine in this subregion appears to respond differently to cocaine, opioids, and more recently, to alcohol than dopamine in the core and shell subregions (Fabbricatore et al., 2009; Hipolito et al., 2008; Howard et al., 2009; Rebec et al., 1997). Rebec et al. showed that when rodents were first introduced to a novel environment, there was an increase in dopamine in the shell and core-shell border, but not the core (1997). Furthermore, the increase in dopamine seen in the shell was higher and more rapid than the response seen in the core-shell border (Rebec et al., 1997). A more recent study reported that there was an increase in extracellular dopamine during operant sucrose plus ethanol self-administration in the core-shell border, but not in the shell or core subregions (Howard et al., 2009).

Theories of Mesolimbic Dopamine and Reinforcement

There is abundant evidence indicating that dopamine plays a role in drug reinforcement (Leshner and Koob, 1999; Pierce and Kumaresan, 2006). However, the precise function of mesolimbic dopamine is still not fully understood. Over the years, several theories have shed light on dopamine's role in the brain. One of the earliest and most influential theories was proposed by Roy Wise in the late 1970s and early 1980s. This theory suggested that dopamine mediates hedonic processes (Wise, 1978). The anhedonia theory was attractive because the mesolimbic dopamine system is activated by many rewards, like food and drugs (Everitt and Robbin, 2005; Roitman et al. 2004), but the premise has lost support over the years.

The anhedonia (also hedonia) hypothesis was based on the belief that dopamine mediates pleasure derived from a reinforcer or associated stimuli (Berridge and Robinson, 1998). For example, Franklin and McCoy showed that rats that were treated with pimozide (a neuroleptic with strong dopamine receptor antagonist properties) stopped responding for intracranial electrical stimulation (1979). Xenakis and Sclafani also found that pimozide decreased lick rate and consumption of a saccharin-glucose solution, which the animals once found rewarding (1981). On the other hand, there are studies that have shown that dopamine is not essential for a pleasurable experience. For instance, a study

demonstrated that mice that cannot synthesize dopamine prefer sucrose to water in a preference test (Cannon and Palmiter, 2003), suggesting that dopamine is not necessary for the animals to perceive sucrose as more rewarding than water. Most recently, a study reported that patients with Parkinson's disease and the control counterparts did not differ in subjective pleasure ratings for sweet food, like chocolate and sucrose (Sienkiewicz-Jarosz et al., 2005). These studies show that dopamine is not necessary for experiencing reward and that hedonic processing is not directly mediated by dopamine. As evidence against the anhedonia theory grew, the theory lost support and three modern theories about accumbal dopamine's role gained recognition. The focus of each theory is different, but it is important to note that they are not mutually exclusive.

Incentive Salience

In 1993 Robinson and Berridge presented the theory of incentive sensitization. The theory focuses on neuronal adaptations that occur after repeated drug exposure, making environmental stimuli more desirable. The "liking" and "wanting" of a drug are separated. "Liking" is the pleasure derived from exposure to the drug, while "wanting" of incentive salience is craving of the drug. As the drug administration continues, the incentive salience attributed to the drug becomes stronger and sensitization may occur (Robinson and Berridge, 1993). A study by Taylor and Horger found that repeated cocaine use may induce long-

term neuronal adaptations that result in increased sensitivity to the behavioral or motivational effects of stimulant drugs (1999). Another study using hyperdopaminergic mice showed that the animals had a higher "wanting" but not "liking" of sweet rewards. The mutant mice ate and drank more in their home cages, appeared to be more focused on obtaining the sweet reward, and learned to complete a task for a sweet reward in fewer trials than the wild type mice. However, the mutant mice failed to elicit higher orofacial “liking” reactions (Pecina et al., 2003). In addition, amphetamine microinjections in the nucleus accumbens increased the sucrose-related incentive salience attributed to a sucrose cue, causing it to trigger increased “wanting” but not “liking” for sucrose (Wyvell and Berridge, 2000).

An advantage of this theory is that it separates the “liking” from “wanting.” The incentive salience theory helps explain the long lasting changes seen in addicts, causing them to excessively “want” drugs. Thus, even when the rewarding effects of the drug have diminished, the drug seeking behavior persists. However, a disadvantage of this theory is that the basic mechanisms of “wanting” and “liking” are not well understood. Also, the majority of the studies supporting this theory are performed with psychostimulants. Since, other drugs, like alcohol, do not affect dopamine signaling as strongly as psychostimulants, it is not clear if the mechanisms proposed by the incentive sensitization theory can be applied to all drugs of abuse.

Associative Learning

Another theory about the role of dopamine in drug abuse is associative learning, which is based on making associations between a new response and a particular stimulus. For example, the first time the pharmacological effects of a drug are experienced, the individual begins to form associations between these effects and taking the drug. The euphoric experience the drug produces causes it to become a powerful reinforcer, which in turn increases the probability of future drug taking. According to this theory, the stage of acquisition of drug reinforcement is the most important (Di Chiara, 2002). In fact, dopamine signaling in the nucleus accumbens shell increases during initial administration of a drug (Pontieri et al., 1995; Pontieri et al., 1996). Studies have also shown that cue-evoked dopamine release in the nucleus accumbens core developed more rapidly when the cue is paired with cocaine, than when it is paired with a natural reward like sucrose (Day et al., 2007; Stuber et al., 2008). Unlike with non-drug rewards, dopamine increases induced by drug administration do not undergo habituation upon repeated drug exposure (Di Chiara, 2002); instead, the association between the drug taking and the drug experience is strengthened.

One of the strengths of this theory is that the lack of habituation of the drug induced dopamine response helps explain the difference in dopaminergic

increases resulting from drugs of abuse versus those caused by natural rewards (Bassareo and Di Chiara, 1999; Bassareo et al., 2002). In addition, the associative learning theory also helps elucidate the long lasting incentive value given to the environmental stimuli associated with drug administration. However, a weakness of the theory of associative learning is that an intact dopamine system does not seem to be necessary for associative learning to occur. A saccharin and polycose solution, which is well liked by rodents, was paired a lithium chloride injection, which makes the animals sick. The experimental group consisted of rats that had been depleted of 98 – 99% of their dopamine. The dopamine deficient rats and their control counterparts were able to make the association between the sweetened solution and the lithium chloride injections after three pairings (Berridge and Robinson, 1998).

Reward Prediction Error

The third theory about the role of dopamine in drug abuse is called reward prediction error, and it focuses on the predictability of a reinforcer in the environment. The mesolimbic dopaminergic system plays an important role in the process of learning about the availability of rewards in the environment, which is done by monitoring errors in the prediction of rewards. The evidence suggests that the value of the reward is influenced by its magnitude, probability, and timing (Abler et al., 2006). Schultz and colleagues recorded from midbrain dopamine

neurons during the presentation of natural rewards and showed that neuronal firing activity coincided with the presentation of a reward or the cues that predicted reward (1997). For example, VTA neurons show an increase in firing rate when unpredicted appetitive stimuli are presented (Mirenowicz & Schultz, 1994; Hollerman & Schultz, 1998). Dopamine neurons fire to signal an error in prediction because the animal did not expect the reward. Furthermore, after repeated pairings between a reward and a cue, the dopamine neuronal activity shifts from the time that the reward is given to the time that the cue occurs (Day et al., 2007; Schultz et al., 1997; Schultz, 1998; Schultz, 2007). Thus, after the association between cue and reward is made, the reward is no longer unexpected and there is no change in neuronal firing. However, the cue is now the unpredicted event and this causes the dopamine neurons to fire. In addition, if a reward is omitted after repeated pairing, an error in prediction is signaled by a decrease in dopaminergic activity (Abler et al., 2005; Abler et al., 2006; Fiorillo et al., 2003; Mirenowicz and Schultz, 1996; Schultz and Dickinson, 2000).

The reward prediction error theory can be directly applied to the learning theory, as dopamine is believed to act as a teaching signal (Schultz, 2002). However, a disadvantage is that this theory works under the assumption that the drug taking experience is rewarding, but fails to explain how reward occurs. Furthermore, many of the early studies were performed on water or food deprived animals (Mirenowicz and Schultz, 1994; Hollerman and Schultz, 1998; Hollerman et al.,

1998), and it is not clear what impact this type of physiological stress and drug-seeking behavior has on motivation. Fortunately, this is not the case in many of the later studies (Abler et al., 2006; Day et al., 2007).

Ethanol and Mesolimbic Dopamine

Mesolimbic dopamine plays a role in drug reinforcement (Pierce and Kumaresan, 2006). More specifically, several studies have shown that dopamine in the nucleus accumbens is enhanced by ethanol administration (Doyon et al., 2003, 2005). This dissertation will focus on the elucidating the role that dopamine plays in ethanol reinforcement.

Dopamine and Ethanol Administration

Evidence suggests that ethanol activates dopamine neuron firing in the VTA and that this increases dopamine release in the nucleus accumbens (Brodie et al., 1990; Yim et al., 1998). For example, intravenous administration of ethanol, in paralyzed rats, increases the firing rate of VTA dopamine neurons, A9 and A10 (Gessa et al., 1985). Extracellular single unit studies in dissociated neuron cultures have also shown that ethanol directly excites dopamine neurons (Brodie et al., 1999). More recently, a study using non-dependent P rats showed a significant increase in the number of spontaneously active dopamine neurons in

the posterior VTA, 24 hrs after the last session of voluntary ethanol consumption (Morzorati et al., 2010). Due to the connections between the nucleus accumbens and the VTA, it is possible that accumbal dopamine release due to ethanol is mediated by the VTA. Ding et al recently reported that ethanol administration in the posterior VTA increased dopamine levels in the nucleus accumbens shell (2009).

Systemic ethanol administration causes an increase in dopamine in the nucleus accumbens (Imperato & Di Chiara, 1986; Yoshimoto et al., 1992; Heidbreder & De Witte, 1993; Samson et al., 1997; Yim et al., 1998; Yim et al., 2000). For example, Heidbreder and De Witte showed that an intraperitoneal injection of 1 g/kg ethanol significantly increased extracellular levels of dopamine in the nucleus accumbens in male Wistar rats (1993). Yim and colleagues also found that an intraperitoneal injection of 1 g/kg ethanol (1998, 2000) increased dopamine relese in the nucleus accumbens, but local perfusion of ethanol in the nucleus accumbens (1998) using biologically relevant doses did not.

It is difficult to directly relate neurochemical findings to behavior with systemic administration of ethanol. Intraperitoneal injections can cause added stress to the animals that can inflate the dopaminergic response seen (Samson et al., 1997). In addition, drug seeking behavior cannot be assessed with forced administration

of ethanol. Operant ethanol self-administration is needed to investigate dopamine's function in the nucleus accumbens in a behavioral context.

Operant Ethanol Self-Administration and Dopamine

The relationship between mesolimbic dopamine transmission and ethanol self administration as assessed via the use of dopamine receptor agonists and antagonists is contradictory. Some studies suggest that dopamine receptor antagonists decrease ethanol mediated behaviors (Hodge et al., 1997; Liu and Weiss, 2002) and that dopamine receptor agonists facilitate ethanol mediated behaviors (Hodge et al., 1992; D'Souza et al., 2003). In contrast, another study showed that ethanol self-administration was reduced with quinpirole, a D2 agonist, and reinstated with sulpiride, a D2 like antagonist (Rodd et al., 2004). In addition, the use of the D2 antagonist, pimozide, failed to alter ethanol consumption in Maudsley Reactive and Wistar rats (Goodwin et al., 1996). Dopamine D1 receptor agonists have also been shown to decrease lever pressing responses needed to self-administer ethanol (Cohen et al., 1999).

These studies may have appeared inconsistent due to the methodological differences, such as differences in the antagonists and agonists used, the drug doses, the rat strain, the route of administration, or the brain region studied. For example, the studies by Rodd et al. (2004) and Hodge et al. (1992, 1997)

reported contradicting findings even though both studies used quinpirole. Rodd et al. (2004), however, used male Wistar rats that intracranially administered ethanol and quinpirole into the posterior VTA, while Hodge and colleagues (1992, 1997) trained male Long Evans rats to self-administer ethanol and then microinjected quinpirole into the nucleus accumbens of the rodents brains. These differences between the studies could account for the opposing results.

Studies using neurotoxin-induced lesions to reduce dopamine in the mesolimbic system have also been inconsistent. Induced lesions with 6-hydroxydopamine in the nucleus accumbens have increased ethanol self-administration (Quarfordt et al., 1991), decreased ethanol self-administration (Ikemoto et al., 1997), or failed to disrupt ethanol self-administration (Rassnick et al., 1993). The different results are likely due to the massive depletion of dopamine. Diminished amounts of dopamine in an animal can affect other neurotransmitters, which can compensate for the lack of dopamine. Confounding factors in this type of study make results difficult to interpret. Overall, it is evident that dopamine plays a role in the self-administration of ethanol, but that the exact role of dopamine remains complex.

On the other hand, microdialysis studies have consistently shown that dopamine in the nucleus accumbens increases during operant ethanol self-administration (Weiss et al., 1993; Weiss et al., 1996). However, these studies did not separate

the appetitive and consummatory phases of the self-administration sessions. The appetitive phase of the experiment represents the drug seeking behavior, and it is monitored in multiple ways, such as counting the number of lever presses in the session or by calculating the time between the presentation of a cue that predicts the availability of the lever. The consummatory phase is the actual consumption of the drinking solution, and it is calculated by measuring the volume of the drinking solution consumed or by counting the number of ethanol licks within the session. In studies like the ones above by Weiss and colleagues that did not separate between these two phases, the animals were required to meet a specific number of responses (i.e., 1) before gaining access to a small amount of the drinking solution (i.e., 0.1 ml), and the animals repeated this procedure multiple times throughout the session. Therefore, the appetitive and consummatory phases were measured simultaneously in each dialysate sample. Without separating these two phases, it is difficult to determine if the changes in dopamine activity were caused by the drug seeking behavior or the actual consumption of the solution (pharmacological effects of ethanol).

Over the years, the operant ethanol self-administration paradigm was modified to separate the appetitive and consummatory phases of the experiment. Samson proposed that the drug seeking behavior can be separated from the actual consumption of ethanol (Samson et al., 1998a). The animals were allowed to complete the required amount of lever presses for the delivery of the ethanol

solution before gaining uninterrupted, continuous access to the solution. Thus, some samples measured dopamine during the appetitive phase, while other samples measured dopamine strictly during the consummatory phase of the self-administration session (Samson et al., 1998a; Czachowski & Samson, 1999; Czachowski et al., 2002). Using this model of self-administration, some studies have shown that cues associated with self administration may be responsible for the increase in dopamine levels. There is an increase in accumbal dopamine during operant ethanol self-administration that is seen after the ethanol solution has been introduced and consumption begins, but this increase does not appear to be caused by the pharmacology of ethanol (Doyon et al., 2003; Doyon et al., 2005).

Previous data from our laboratory suggests that the increase in dopamine levels in the nucleus accumbens during the self-administration of 10% ethanol + 10% sucrose is not due to the pharmacological effects of ethanol, but to its sensory stimuli (Doyon et al., 2005). There is a transient increase in dopamine in the nucleus accumbens during ethanol self-administration with dopamine levels peaking within five minutes of drinking the solution. However, ethanol levels in the nucleus accumbens peaked forty minutes after the drinking period commenced. While the dopamine levels in the brain are decreasing, the ethanol levels are increasing, which is not consistent with a pharmacological effect. Interestingly, the number of licks correlated with the increase in dopamine levels

(Doyon et al., 2005). It was hypothesized that dopamine levels increased due to the sensory stimuli of the ethanol solution as the rat began to drink. The rats in this study had only been trained for about six days, making ethanol tolerance very unlikely. The control group from this study indicated that dopamine concentrations did not increase during the self-administration of 10% sucrose, ruling out that sucrose contributed to the differences between the dopamine and ethanol time courses (Doyon et al., 2005). Furthermore, lever pressing prior to drinking does not appear to elicit a dopamine response in the nucleus accumbens during ethanol self-administration (Doyon et. al., 2003). These results suggest the intriguing possibility that adaptations in dopamine signaling occur during the development of ethanol reinforcement, perhaps during the early stages of acquisition when ethanol's pharmacological effects are learned.

The role of mesolimbic dopamine transmission during the acquisition of ethanol reinforcement remains largely unstudied. Most self-administration protocols gradually increase the concentration of ethanol in the drinking solution and fade out the sweetener over several days. Varying the ethanol concentrations throughout the multiple phases of the sucrose-fading protocol makes it difficult to interpret the changes in dopamine activity during this period. To our knowledge, there is only one study that has investigated the actual initiation of operant ethanol self-administration, and it is described in Chapter 4. Determining the

changes in dopamine transmission that occur during this period might be helpful in understanding the plasticity involved in motivated ethanol drinking.

Ethanol and Other Neurotransmitters

It is important to note that there are other neurotransmitters aside from mesolimbic dopamine that play a direct role or interact with dopamine in ethanol reinforcement. However, the focus of this dissertation is on the mesolimbic dopamine system. Thus, only a couple of other neurotransmitters and their implications in ethanol reinforcement will be very briefly discussed.

Glutamate and GABA are the major excitatory and inhibitory neurotransmitters, respectively, in the central nervous system and these two neurotransmitters seem to be involved in ethanol reinforcement. For example, MPEP, a mGluR5 antagonist, has been shown to reduce ethanol self-administration in male C57BL/6J mice (Hodge et al., 2006). In addition, male Long Evans rats have been shown to reduce ethanol seeking when injected with MPEP (Backstrom et al., 2004). These studies suggest that glutamate may play a role in the motivation of ethanol self-administration. Furthermore, Liang et al showed that an allosteric modulator, CGP7930, of GABA reduced ethanol self-administration (2006). Additionally, using whole-cell voltage clamp, Theile et al reported that ethanol increases GABAergic transmission in the VTA (2008). Thus, although ethanol is

a drug that is regulated by several neurotransmitters, the studies presented in this dissertation will be investigating the role dopamine plays in ethanol reinforcement.

Specific Aims

Ethanol self-administration has been shown to increase dopamine activity in the mesolimbic pathway, in particular the nucleus accumbens, and previous studies in our laboratory have suggested that the increase in dopamine levels in the nucleus accumbens is related to the process of consuming ethanol and not due to the ethanol concentration reaching the brain following consumption. However, there are still many questions that remain unanswered. Thus, the goal of this dissertation was to further investigate the relationship between dopamine and ethanol reinforcement. The specific aims are as follows:

1. Determine if the transient increase in dopamine in the nucleus accumbens during operant ethanol self-administration is due to the taste and smell of ethanol. We will attempt to design and test a placebo spout, which we plan to use to determine if there is a dopamine response in the nucleus accumbens due to the sensory stimuli of ethanol during operant self-administration.

2. Create and test a self-administration protocol that can be used to study the actual initiation of ethanol self-administration. Male Long-Evans rats will be trained to lever-press with a 10% sucrose solution for 20 minutes of access to the drinking solution. After four sessions with 10% sucrose as the drinking solution (one session per day), the rats will be exposed to a three-day period of 10% ethanol + 10% sucrose operant self-administration. The sucrose will be gradually faded out of the drinking solution, and the animals' final drinking solution will be a 10% ethanol drinking solution. The establishment of ethanol self-administration will be determined by ethanol intake, 2-bottle choice preference tests (before and after exposure to the self-administration protocol), and extinction trials.

3. Determine the effect of ethanol on dopamine in the nucleus accumbens core-shell border during the initiation of operant ethanol self-administration and help elucidate if the early stages of ethanol self-administration are involved in reinforcement. The experiment will be separated into an appetitive and a consummatory phase to distinguish between the drug seeking and drug taking behaviors, respectively. Male Long-Evans rats will be trained to lever-press for 20 minutes of access to either 10% sucrose or 10% ethanol + 10% sucrose using the novel self-administration protocol mentioned in aim 2. There will be four groups: day 1 10% ethanol + 10% sucrose, day 1 10% sucrose, day 2 10% ethanol + 10% sucrose, and day 2 10% sucrose. Five minute samples will be collected from the core-shell border during the microdialysis session, which will

consist of five phases: baseline, transfer, wait, drink, and post-drink. The licks will be counted using a lickometer and dopamine and ethanol concentrations will be measured using High Pressure Liquid Chromatography and Gas Chromatography, respectively.

Chapter Two: Placebo Spout

This chapter is a summary of work done towards developing a placebo spout in which the rodent can initially smell and taste alcohol but is actually drinking extremely low concentrations of ethanol, resulting in a negligible pharmacological dose. We developed this placebo spout to test the hypothesis that the taste and smell of ethanol are responsible for the release of dopamine in the nucleus accumbens during operant ethanol self-administration. Previous data collected in our laboratory suggests that the increase in dopamine levels in the nucleus accumbens during the self-administration of 10% ethanol + 10% sucrose is not due to the pharmacological effects of ethanol. Dopamine levels peak within five minutes of drinking the solution, while ethanol levels peak forty minutes after the drink period begins (Doyon et al., 2005). However, this increase in accumbal dopamine matches the time period during which the animals consume the majority of the drinking solution and appears to be specific to ethanol consumption. Rats self-administering 10% sucrose (Doyon et al., 2005) or water (Doyon et al., 2003) did not have a significant rise in accumbal dopamine during consumption. It is therefore possible that the sensory stimuli of ethanol (taste and smell) are serving as cues for the pharmacological effect of ethanol and that the dopamine response is elicited by this cues, which are specific to ethanol.

We created and tested two different placebo spouts. Unfortunately, the first design was not effective for microdialysis, and the second placebo design did not produce promising behavioral data. The following will be a description of the work that was done in our attempt to create a successful placebo spout.

Place Spout One

The placebo spout was a regular spout with two alterations. First, a band of filter paper was saturated with 10% ethanol + 10% sucrose solution to provide the smell of ethanol and was placed around the spout. The bottle was completely filled with 10% sucrose, and the spout in a rubber stopper was inserted into the bottle. Second, immediately after the rat met the required response of 20 presses, the 10% sucrose solution in the spout was removed with a syringe and the spout was filled with a with 3.1 mls of 15% ethanol + 10% sucrose, using a similar syringe. The bottle was placed in the chamber at approximately a 45° angle to deliver the drinking solution to the rat (Figures. 2 and 3).

Figure 2. Placebo spout in chamber. Drinking bottle filled with 10% sucrose and placebo spout placed in chamber. The tip of the spout was inside the chamber, so that the animals could have access to the drinking solution. After the required response was met, the bottle with the drinking solution was delivered to the animals for the complete drink period. The filter paper was strategically placed so that it was close enough for the rats to smell it, but far enough for them not to reach it.

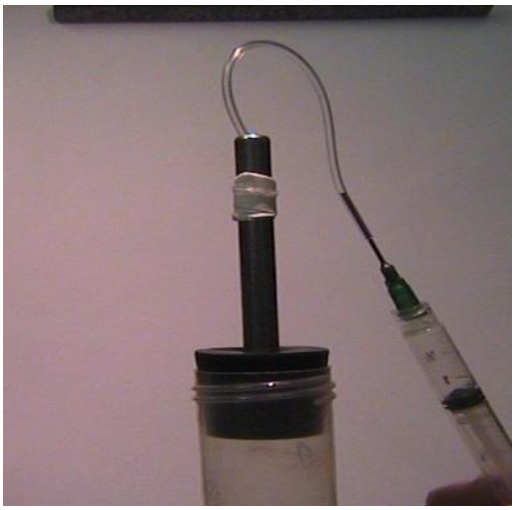

Figure 3. Placebo spout with syringe. Drinking bottle filled with 10% sucrose with rubber stopper and placebo spout. The tygon tubing was connected to the syringe to enable us to reach inside the spout completely. The syringe was used to extract the 10% sucrose solution in the spout. A similar syringe was used to add the 3.1 mls of 15% ethanol + 10% sucrose. The filter paper was saturated with 10% sucrose + 10% ethanol.

The concentration of ethanol in the spout decreased as the 15% ethanol + 10% sucrose solution began to diffuse into the 10% sucrose solution in the bottle. Therefore, we measured the concentration of ethanol in the spout at different time points. Originally, we tried adding a 10% ethanol + 10% sucrose solution into the spout so that the solution used could match the one the rats were trained to self-administer, but the final ethanol concentration was too low. Using the gas chromatograph with flame ionization detection, we determined that after 30 seconds, the ethanol concentration in the spout had dropped to about 1.5% (Figure 4). Therefore, we tried a higher concentration of ethanol, 15%. Three trials were run to determine the ethanol concentration in the placebo spout at intervals of 30 seconds after placing the bottle in the chamber. After 30 seconds, the ethanol concentration in the spout had dropped to 3.8%, and after 1 minute, it was about 0.8% (Figure 5). These results showed us that when using the placebo spout during the self-administration sessions, the rats would be exposed to the taste of ethanol, but would not ingest a pharmacologically relevant dose.

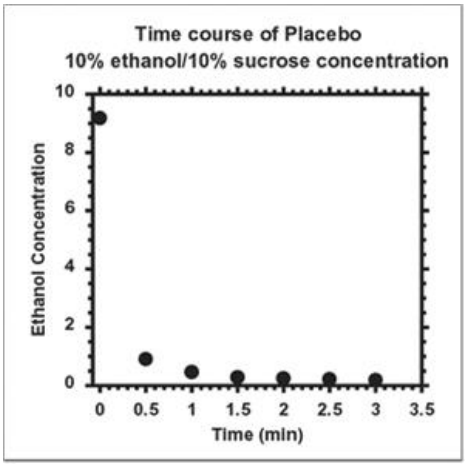

Figure 4. Ethanol concentration of a 10% ethanol + 10% sucrose solution, when added to the spout of a 10% sucrose bottle, was determined using the gas chromatograph. After 30 seconds, the concentration of ethanol had dropped to about 1.5% and continued to drop rapidly.

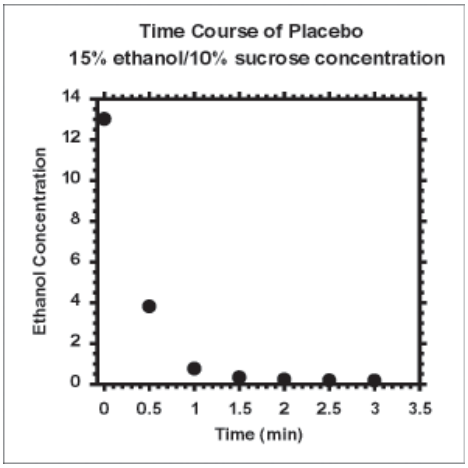

Figure 5. Ethanol concentration of a 15% ethanol + 10% sucrose solution, when added to the spout of a 10% sucrose bottle, was determined using the gas chromatograph. After 30 seconds, the concentration of ethanol had dropped to about 3.8% and continued to drop rapidly.

Behavioral Data

The protocol used to train the animals to self-administer a 10% ethanol + 10% sucrose (10% w/v) solution was a modified version of a sucrose-fading procedure demonstrated by Carrillo et al. (2008). Fourteen male Long-Evans rats were trained to lever-press with a 10% sucrose drinking solution. There were 6 rats in the control group and 8 rats in the placebo group. The rats were water deprived for 16-22 hours before each lever press training session to increase motivation. Once the animals were trained to lever press, the rats had *ad libitum* access to water. A 15-minute wait period was established over a four day period and followed the progression of 2, 6, 10, and 15-minutes. The cues utilized included a chamber light and a fan (both turning on) to begin the session. The required response was increased to 20 lever-presses over seven days with a single session per day, and the progression utilized was 2, 4, 4, 8, 12, 16, 20 presses. Upon completion of the required response, the lever retracted and a drink period began. During this period, animals had access to the drinking solution for 5 continuous minutes. This was followed by a 35-minute post-drink period, during which time neither the cues nor the spout were available. After 4 days of 10% sucrose self-administration, the rats self-administered 10% ethanol + 10% sucrose for the next 5 operant sessions. On the fifth day of ethanol exposure, the animals in the placebo group received a small amount of ethanol in their spout and a 10% sucrose solution in the drinking bottle instead of a 10% ethanol + 10%

sucrose solution. The rats in the ethanol group continued to self-administer 10% ethanol + 10% sucrose. Licks, milliliters of fluid consumed, and body weights were recorded daily.

Before performing a microdialysis experiment, we wanted to collect some behavioral data to determine if there was a difference in the drinking behavior between the animals using the placebo spout and those using the regular spout. The results from the behavioral data showed that there was no difference in the drinking behavior of the control and experimental groups (table 1). The ethanol intake, as measured by number of licks, was almost identical between the two groups ($p > 0.05$ by t-test). The drinking rate was also found to be similar between the two groups ($p > 0.05$ by t-test). We concluded that the placebo spout design would be effective for testing whether the stimuli of ethanol are strong enough to elicit a transient increase in dopamine without the pharmacological effects of ethanol. Therefore, we proceeded to the next step in this experimental design: microdialysis.

Table 1. Lickometer parameters on experiment day:

Parameter	Placebo spout (n=8)	Regular spout (n=6)
Total licks	1404 ± 93	1406 ± 222
Lick rate (15 sec) [a]	399 ± 18	381 ± 26
Lick rate (30 sec) [a]	398 ± 11	374 ± 16
Lick rate (1 min) [a]	373 ± 12	356 ± 15

[a] Rate is determined by the number of licks divided by time to complete licks.

Microdialysis

The microdialysis section of this study consisted of 5 different phases: baseline, transfer/wait, lever press, drink, and post-drink. Six 5-minute samples were collected during the baseline period, while the rats were still in their home cage. Four more samples were collected before the animal gained access to the drinking solution: Three 5 minute samples were collected during the transfer/wait period. One of the samples was taken while the animal was transferred into the operant chamber and the other two while the animal waited for the lever to come out. The fourth sample was collected during the lever press period. The sample was longer than 5 minutes and the time varied per animal (6-11 minutes) because it included 5 minutes of wait time in the chamber before the rats had access to the lever and the time it takes the rat to meet the required response. After the required response was met, the drinking solution was delivered for 5 minutes, and 1 dialysis sample was collected. The bottle then retracted, and a

35-minute post-drink period, in which seven 5 minute samples were collected, began (Figure 6).

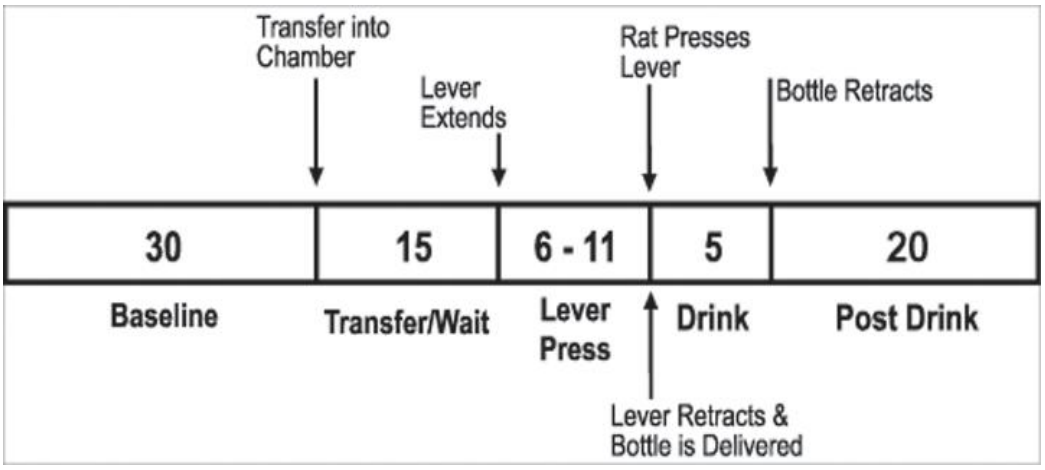

Figure 6. Timeline for 1st placebo spout microdialysis session. Phases include: Baseline, Transfer/Wait, Lever Press, Drink, Post Drink. Numbers indicate the time in minutes.

Multiple steps had to be performed simultaneously in order to use the placebo spout during microdialysis. First, to minimize diffusion of the ethanol solution into the bottle containing the sucrose solution, the 15% ethanol + 10% sucrose solution had to be added to the spout of the drinking bottle immediately after the rat met the required response to release the lever. Then, the drinking bottle had to be placed in the chamber so that the drinking solution became available to the animals. Lastly, a dialysate dopamine sample had to be collected. The tube holding the dopamine sample had to be switched extremely fast to assure that each dopamine sample was representative of each individual time period, especially because in our experiments, we separate the drug seeking and taking

behaviors. Unfortunately, because of the multiple steps that had to be performed, while switching the last sample in the wait period (drug seeking), we were unable to switch the sample tube on time and the sample contained dopamine from both, the wait and drink periods. We also tried having two people help with the procedure, but most animals became visibly stressed or distracted. The rats stopped drinking and either curiously looked at the experimenters or froze. Thus, our data could not be trusted.

The limitations of our design were not foreseen during the development of the placebo spout and proved to be major flaws. However, we decided to use the data collected from the first placebo spout design to improve the design of our study. We changed the self-administration protocol and created a placebo spout that was appropriate for performing microdialysis.

Placebo Spout Two

The second placebo spout design also consisted of a band of filter paper that was saturated with 10% ethanol + 10% sucrose to provide the smell of ethanol. However, the taste of ethanol was introduced differently in this design. A 30 ml syringe filled with 25 ml of 10% sucrose was connected to the drinking spout of the bottle by 27 cm of tygon tubing. We placed 2 cm of the tygon tubing inside the rubber stopper in the drinking spout and glued the tygon tubing to the outside

of the rubber stopper. During the self-administration session, the tygon tubing was connected to the 30 ml syringe containing 25 ml of 10% sucrose and to another syringe with 2.3 ml of 10% ethanol + 10% sucrose by a Discofix 3-way Stopcock valve (Figure 7). First, the 10% ethanol + 10% sucrose solution was pushed into the tygon tubing and drinking spout. Then, the syringe was removed and replaced with one containing air so that 3 cm of air, which took about 1 second to pass through, were injected into the tygon tubing. Once the session started, the syringe with 25 ml of 10% sucrose was pushed by a Model R-E syringe infusion pump at approximately 2.6 ml per minute (Figure 8). The 2.3 ml of 10% ethanol + 10% sucrose solution was pushed through first, followed by the air, and then the 10% sucrose solution. It is important to note that this procedure was followed throughout the entire training protocol. However, the 30 ml syringe was filled with 25 ml of 10% ethanol + 10% sucrose and the solution was only replaced with the 10% sucrose solution on the 7^{th} session, which was when the placebo spout was used. Also, although the animals received 2.3 ml of the 10% ethanol + 10% sucrose solution in the spout, they were not able to consume all of the solution because the pump was constantly flowing. Thus, the rats were able to taste the solution, but not consume pharmacologically relevant amounts of ethanol. The pump was also used throughout the protocol to habituate the animals to the sound. In addition, the wall of the operant chamber from which the drinking spout was delivered, was modified. We shorten the length of the wall so that we could add a tray underneath the wall to collect the fluid that was flowing

through the drinking spout but that was not being consumed by the animal. The tray was not within reach of the rodent, since it was underneath the grid floor (Figure 8).

Figure 7. Placebo spout. The 30 ml syringe was filled with 25 ml of 10% sucrose and placed on a pump. A three way valve connected this syringe to another syringe containing 2.3 ml of 10% ethanol + 10% sucrose and to the tygon tubing in the drinking spout. The 10% ethanol + 10% sucrose solution was pushed into the tygon tubing, followed by air, and the 10% sucrose solution during the drinking session.

Figure 8. Placebo spout with tray underneath wall of chamber. The fluid collection tray was placed underneath the wall of the chamber to collect the drinking solution flowing out of the drinking spout and not consumed by the animal.

Behavioral Data

The protocol used to train the animals to self-administer a 10% ethanol + 10% sucrose (10% w/v) solution was a modified version of the standard sucrose-fading procedure (Samson, 1986). There were 3 rats in this study, and the animals served as their own controls. To increase motivation, the rodents were water deprived for 16-22 hours before each lever press training session, but the water was restored and given to the rats *ad libitum*, once the animals were trained to lever press. The rats had one session with a 10% sucrose solution. After which, a 2% ethanol solution was introduced into the drinking solution. The concentration of ethanol in the drinking solution was gradually increased from 2 to 10% (progression was 2, 5, 10) over a period of 6 days, while the sucrose concentration in the solution was maintained at 10%. The animals were habituated to the apparatus for 15-minutes prior to the presentation of two cues (a chamber light and a fan, both turning on), and this was followed by the presentation of the lever. Completion of the response requirement (4 lever presses) retracted the lever and led to the presentation of the drinking spout for 20 continuous minutes. The spout then retracted for 20-minutes of post-drink time, and the cues were no longer available. On the sixth day of ethanol exposure, we collected behavioral data for the regular spout. On the seventh day of ethanol self-administration, we exposed animals to the placebo spout, and they received 10% sucrose in the 30 ml syringe instead of the 10% ethanol +

10% sucrose solution. One of the animals had an eighth session in which it was given a 10% sucrose solution (regular spout). Licks, milliliters of fluid consumed, and body weights were recorded daily. The spillage caused by the constantly running pump was collected in a small tray and measured. This value was subtracted from the total consumption.

The second placebo spout was tested with a small number of animals (n=3) and had a within subject control. Unfortunately, the results from the behavioral data were not promising. Unlike the previous placebo model, the rats seemed to notice that there was only a small amount of the 10% ethanol + 10% sucrose solution, followed by a 10% sucrose solution for them to drink. A t-test showed that the number of licks between the regular and placebo spout were different ($p = 0.05$ (table 2)). Furthermore, the animal that had the extra session the day after being exposed to the placebo spout, consumed 2.8 times more fluid on the day the placebo spout was used than on the following day, when 10% ethanol + 10% sucrose was self-administered. In addition, the number of licks the animal had with the regular spout the day before and after being exposed to the placebo spout were very similar, 493 and 403 licks, respectively.

Table 2 lickometer parameters on experiment day (Within subject comparison)

Parameter	Regular spout (n=3)	Placebo spout (n=3)
Total licks	1089 ± 450	2321 ± 652
Lick rate (15 sec) [a]	382 ± 36	279 ± 71
Lick rate (30 sec) [a]	312 ± 34	268 ± 43
Lick rate (1 min) [a]	301 ± 27	276 ± 30

[a] Rate is determined by the number of licks divided by time to complete licks.

We cannot say with certainty why the rodents used to test the second placebo did not demonstrate similar drinking behaviors when presented with the regular and the placebo spout. However, we do have some hypotheses on why the second design failed. Since we used a pump, fluid was constantly flowing at a rate faster than the animals could lick. Some of the fluid would roll down the wall, and the animals would lick the wall. This interfered with our lick count. In addition, the droplets of fluid produced by the pump were too big and could have affected the licking behavior, also interfering with the lick count. Furthermore, the self-administration protocol used for this study was different than the one used for the first placebo study, which was successful in providing similar behavioral results between the regular and placebo spouts. The animals in this study had the concentration of ethanol gradually increased (2%, 5%,10%) over a period of 6 days, while the animals in the first placebo study self-administered a constant but high (10%) concentration of ethanol for 5 days. It is possible that the number of days or concentration of ethanol exposure affected the animals' behavior. Although we cannot be sure, the difference in ethanol exposure could have

caused the animals in this placebo study to notice the difference between drinking a small amount of ethanol followed by a 10% sucrose solution and consuming a 10% ethanol + 10% sucrose solution only. Overall, the results demonstrated that the design of the 2nd placebo spout needed improvement, and since the behavioral data was not promising, we did not proceed with a microdialysis experiment.

Conclusions

It should be noted that any future testing of a placebo spout should be performed in the core-shell border of the nucleus accumbens instead of the shell or core subregions. Although we were unable to perform a successful microdialysis procedure due to problems with the design of the placebo spout, when we began this experiment, we aimed for the shell of the nucleus accumbens. A recent study, which was performed and published after we had tested this placebo design, showed that there is no increase in dopamine in the shell subregion of the nucleus accumbens during operant ethanol self-administration, but that instead, the transient increase in dopamine occurs in the core-shell border (Howard et al., 2009). Therefore, the core-shell border should be the region of interest when testing the placebo spout.

In summary, the goal in this experiment was to create a placebo spout that would allow us to determine if the sensory stimuli of ethanol is responsible for the

increase of dopamine in the nucleus accumbens during operant self-administration. We sought to maintain a similar olfactory and gustatory experience for rodents drinking 10% sucrose and rodents drinking 10% ethanol + 10% sucrose by allowing the rats in the placebo group (consuming 10% sucrose) to drink a low concentration of ethanol at the beginning of their self-administration session that would result in a negligible pharmacological dose. However, both placebo designs failed to meet our two main objectives simultaneously, which were: 1. To produce a similar drinking behavior in animals when consuming a sucrose solution as when consuming a solution containing ethanol (10% w/v) mixed with sucrose. 2. To be able to effectively perform a microdialysis procedure to test dopamine levels while using the placebo spout.

In summary, by making a few modifications and merging our two designs, a successful placebo spout can be created. The self-administration protocol used for the first placebo spout design and the method of delivery used for the second placebo spout design seem to only need a few modifications to be effective. In particular, adding a few more days to the training protocol and replacing the 10% ethanol + 10% sucrose drinking solution with a 10% ethanol + 10% polycose gelatin solution would be needed to develop an effective placebo spout; A spout that can be used to study the relationship between the sensory stimuli of ethanol and accumbal dopamine's role during operant ethanol self-administration.

Chapter Three: A 3-day exposure to 10% ethanol with 10% sucrose successfully initiates ethanol self-administration.

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Abstract

The initiation phase of ethanol self-administration is difficult to study using the well-established, sucrose-fading procedure due to the changing concentrations of ethanol in the first few days. The purpose of this experiment was to test whether a modified sucrose-substitution procedure in which rats are initially exposed to high concentrations of ethanol and sucrose for three days would successfully initiate ethanol self-administration. Male Long-Evans rats were trained to lever-press with a 10% sucrose solution in which four or 20 responses allowed 20-min access to the solution. Subsequently, rats were exposed to a 3-day period of operant self-administration of 10% sucrose + 10% ethanol. This constant-concentration exposure was followed by the standard procedure in which sucrose is completely faded out. The establishment of ethanol self-administration was determined by ethanol intake, pre- and post procedure two-bottle choice preference tests, and extinction trials. The mean ethanol intake was 2.2 times

higher on day 2 compared with day 1 on the 10% sucrose + 10% ethanol solution. After fading out the sucrose, the daily intake of 10% ethanol solution over 5 days was stable at approximately 0.57 g/kg. Ethanol preference was approximately threefold higher after the modified sucrose-fading procedure. Responding during a single session extinction test was dramatically increased from 4 to 61 ± 13 or 20 to 112 ± 22 responses in 20 min. Similar to the standard sucrose-fading method, we did not observe a significant relationship between extinction responding and ethanol intake. Blood alcohol concentrations were 4.5 mM 20 min after consumption began. We conclude that initiation and establishment of ethanol self-administration will occur using this modified sucrose-fading procedure.

Introduction

The sucrose-fading procedure has been well established as a method of initiating ethanol self-administration (Samson, 1986; Samson et al., 1999; Schwarz-Stevens et al., 1991). This sucrose-substitution paradigm has been used in previous studies to investigate the effects of pharmacological agents on ethanol self-administration, as well as neurochemical events during operant ethanol self-administration (for reviews, see Gonzales et al., 2004; Samson & Czachowski, 2003). For example, dopamine activity has been measured in the nucleus accumbens during oral consumption of ethanol initiated by sucrose-substitution

(Doyon et al., 2003) or its variant, saccharin-substitution (Weiss et al., 1993). The effects of the dopamine D2 antagonist, remoxipride, on ethanol seeking and drinking behaviors have been studied after ethanol initiation with the sucrose-fading procedure (Czachowski et al., 2002). Rogowski et al. (2003) also used the sucrose-fading procedure to test the potential regulation of ethanol self-administration by dopamine D2 receptors. Also, drinking behaviors of different strains of rats have been compared after sucrose-substitution was used to initiate ethanol self-administration (Samson et al., 1998b; Vacca et al., 2002). In fact, it was shown that in the alcohol-preferring rat, the initiation procedure used affects ethanol reinforced behaviors (Schwarz- Stevens et al., 1991).

Although the sucrose-fading paradigm is useful in establishing stable ethanol self-administration, very little is known about the mechanisms that contribute to the initiation of ethanol self-administration. The varying concentrations of ethanol and sucrose throughout the various phases of the sucrose-fading method make it difficult to determine when the initiation versus maintenance of ethanol self-administration is being expressed. For example, initiation could begin during the early phases of exposure to low concentrations of ethanol, or it could be later in the method during which consumption of high doses of ethanol occurs. One potential critical period in the initiation process could be the earliest times that a rat achieves a dose of ethanol that leads to an intoxicating effect. In the original sucrose-fading method, this may not occur for several days after ethanol has

been introduced because, in general, the highest doses of ethanol self-administered will be when the concentration of sucrose and ethanol are highest. A model using a constant concentration of ethanol (10% wt/vol) and sucrose over several days could enable the study of the earliest phase of initiation. Furthermore, a brief period of exposure to this solution may shorten the entire procedure, because it bypasses the time it takes to fade in the ethanol. In the present study, we tested a modification of the standard sucrose-fading procedure in which the animals are initially exposed to 10% sucrose and 10% ethanol (10S10E) and the ethanol concentration was kept constant throughout the study. Specifically, we tested whether this model would lead to the initiation and maintenance of ethanol self-administration, as with the validated sucrose-substitution procedure. The appetitive strength and preference of ethanol were also estimated using this new model.

Materials and Methods

Animals

We used six cohorts (total n of 52) of ethanol naive male Long-Evans rats (Charles River Laboratories, Wilmington, MA) for this study. Their weight at the start of the study ranged between 237 and 368 g (approximately 50-80 days old). We handled the rats for at least 1 week, and they were housed individually under

a 12-h light/dark cycle (on at 7:00 am and off at 7:00 pm). The animals had food and water ad libitum during the entire experiment except for a brief period during which they were being trained to lever-press (see below). A summary of the cohorts and the various experimental procedures to which they were subjected is shown in Table 3. All procedures complied with guidelines specified by the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and were approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin.

Table 3. Experimental cohorts

Cohort	n[a]	Successful initiation[b]	Unsuccessful initiation	Ethanol preference[c]	RR[d]	Extinction[e]	Prewait[f]	Controls[g]
1	8	6	2	yes	4	6	yes	no
2	6	3	3	no	4	3	yes	no
3	5	5	0	no	4	5	yes	no
4	12	5	1	no	20	11[h]	no	6
5	9	5	0	no	20	9	no	4
6	12	8	4	yes	20	0	no	no

[a] n = the number of rats that started the experiment
[b] Successful initiation indicates the number of rats that met the criterion of consuming at least 0.3 g/kg of 10% ethanol in at least 4 of 5 consecutive days at the end of the initiation procedure.
[c] Ethanol preference indicates whether that cohort underwent the pre- and post-initiation two bottle choice procedure.
[d] RR = the final intended response requirement during the training period
[e] Extinction = the number of rats that went through the extinction procedure at the end of the training.
[f] Pre-wait indicates whether that cohort had an extra 45 min wait period in the chamber before the session began.
[g] Controls = the number of rats that were never exposed to ethanol during the training.
[h] Three controls were not included in the extinction analysis because they were not able to meet consistently the RR of 20.

Preference testing

After a 7-day adaptation period to their home cage, the first (n=6) and sixth cohorts (n=8) of rats were given a two-bottle choice preference test for 5 days. Two versions of the preference test were used. For the first cohort, the preference test was conducted as described in Samson (1986). Briefly, water and ethanol (5% wt/vol) were continuously available in the home cage from 500-ml bottles. Bottle position was alternated each day to control for side preference. The fluid consumed was recorded and the bottles were refilled at the same time each day (1:00 pm). The animals were also weighed at that time. Control bottles placed on empty cages were also monitored for spillage and evaporation. Ethanol preference was estimated by dividing the amount of ethanol consumed by the total fluid intake. For the sixth cohort, a similar procedure was used except that 10% ethanol (wt/vol) was used during this initial preference test.

A second two-bottle choice preference test was performed immediately following the last operant drinking session in the first and last cohorts. The procedure for this second preference test was the same as described above except that 10% ethanol (wt/vol) was used for both cohorts. In both pre- and post-initiation preference tests, control bottles of ethanol and water were measured daily and refilled at the same time as the bottles on the rat cages.

Behavioral training

Self-administration training took place in standard operant chambers (Med Associates Inc., St. Albans, VT). Each chamber contained a single, retractable lever on the left side (2 cm above the grid floor). After the rat reached the appropriate response requirement (RR), a retractable drinking spout entered the chamber on the right side of the same wall (5 cm above the grid floor). The metal bars that made up the grid floor were connected to the metal spout of the drinking bottle through a lickometer circuit (Med Associates). Each operant chamber was housed in a cubicle with the front doors left open during training. An interior chamber light and a sound-attenuating fan were activated with the start of each operant session. Operant chamber components and acquisition of lickometer data were controlled by PC using software from Med Associates.

After the pre-initiation preference test, rats were given 2-3 days in their home cage environment with a single bottle of water and ad libitum food. Animals were trained to lever-press for 10% (wt/vol) sucrose (10S). To facilitate the acquisition of lever-pressing behavior, animals were fluid deprived for 10-22 h before each training session. Lever-pressing was established in 2-9 days. During all subsequent sessions water was once again available ad libitum for the remainder of the study.

Throughout the initiation procedure, operant training sessions occurred 5-7 days per week. Each cohort of rats underwent the training in sequence, although there were differences in some of the training parameters and tests between the cohorts (summarized in Table 3). The ethanol drinking protocol began with 4 days of responding for 10S while increasing pre-wait period (waiting in the operant chamber before starting the program), wait period (before lever is available for pressing), and RR. The pre-wait period for the first three cohorts was increased incrementally to 45 min; there was no pre-wait period in the subsequent cohorts. The wait period was increased to 15 min, and the RR was increased from 1 to 4 lever-presses for the first three cohorts (n=19). The subsequent cohorts (4-6) had an RR of 20 (n=23) to match studies done using the already established sucrose-substitution procedure (Samson et al., 2001). For all sessions, the subject had 20 min to complete the RR, and completion of the RR allowed access to the bottle for 20 min. The second phase of the protocol was 3 days of access to 10S10E solution. Following this, there was no pre-wait period in the remaining sessions. Over the next 4 days, the sucrose in the solution (10S10E) was decreased to 5% sucrose/10% ethanol (5S10E) ethanol and to 2% sucrose/10% ethanol (2S10E). The rats were given 10% ethanol (wt/vol, 10E) for the remainder of the operant sessions. This final phase of the protocol varied in length from subject to subject. Rats continued the operant sessions until their intake reached >0.30 g/kg for at least four of five consecutive sessions. This took 5-13 days of 10E-sessions to reach this criterion (6.8 ± 0.4

days). Fluid consumption was monitored for each operant session. The fluid intake was calculated by measuring the fluid remaining in the bottle (with a resolution of 0.1 ml) and subtracting the amount of fluid spilled (collected in small weigh boats placed under the spout).

Control groups were included in cohorts 4 and 5 in which the rats were trained to lever-press for sucrose as described above. However, ethanol was not introduced during the sucrose fade-out, and eventually the rats consumed water during the operant session. These controls were matched with rats in the ethanol group so that the total sessions were similar between the two groups, and no additional fluid restriction was performed in these controls.

Extinction tests

After completion of the post-initiation preference test, the first cohort of animals was given an additional 4 days of operant sessions with 10E solution, and intake was reestablished to similar levels achieved before the post-initiation preference test. On day 5, a single extinction test was performed. Because they did not receive a post-initiation preference test, the remaining cohorts performed the extinction test immediately after completion of the initiation procedure. The extinction test consisted of a 15-min wait period followed by 20 min of responding with no access to the drinking bottle. A bottle of 10E was present, but no amount

of responding would trigger its descent into the operant chamber. The number of responses in the 20-min period was recorded.

Blood alcohol concentration

The blood alcohol concentration was determined in the sixth cohort (n=8). The rats were reintroduced to the operant self-administration of 10E for 1-4 days to reestablish normal levels of intake of 10E after completion of the preference test. Four of the rats were allowed to drink 2-3 min before blood samples were collected. The other four rats had access to the 10E solution for the entire operant drinking session (20 min), and blood samples were collected immediately after the session ended. Blood was collected from the saphenous vein while under isoflurane anesthesia. The alcohol content in 10 µl of blood was measured using gas chromatography. The blood sample was immediately mixed with 90 µl of saturated sodium chloride solution, and the sealed sample was placed in a heated autosampler at 40°C. A Varian CP 3800 gas chromatograph with a flame ionization detector and a Varian 8200 headspace autosampler was used to analyze the concentrations of ethanol in the samples. The stationary phase was an HP Innowax capillary column (30m x 0.53 mm x 1.0 µm film thickness) and helium was the mobile phase. Resulting ethanol peaks were recorded using Varian Star Chromatography Workstation software, and calibration was achieved using external standards.

Statistics

Repeated measures analysis of variances were performed to determine if differences existed in (1) intake of 10S10E solution over time and (2) in the number of licks during the last day of 10S consumption and the first three days of consumption of 10S10E. Post hoc t-tests were used for individual comparisons using the Bonferroni correction. T-tests were used to (1) determine the effect of the pre-wait period on 10E consumption, (2) compare extinction responding in the ethanol group with that in the control group, and (3) compare the ethanol preference before and after the initiation procedure. Regression analyses were used to determine if there was a significant relationship between (1) extinction responding and ethanol intake, (2) extinction responding and water intake, and (3) total licks/ session and ethanol intake in g/kg. The criterion for significance was $P < .05$.

Inspection of the lickometer data (total number of licks) during the various phases of the experiment revealed that in some cases the lickometer malfunctioned due to a lack of proper contact between the leads and the metal spout, which produced low lick counts. Therefore, we carried out an analysis of the ratio between licks and ml consumed during the consumption of 10S because the volume consumed was generally high, and the measurement error would be

correspondingly low. Analysis of 185 sessions with the explore command in SPSS indicated extreme outliers (< 85 and > 285 licks/ml), and therefore, licks/ml values falling within this range were further analyzed. Lickometer parameters selected for this study include latency, total licks/session, lick rate, and number of bouts. A bout is defined as a run of at least 25 licks with no more than 2 min between licks. Latency was defined as the time between the last lever-press response and the first lick. Lick rate was analyzed for the first bout (number of licks in the first bout divided by the time between the first and last licks of the first bout) and for the first half of the first bout (number of licks in the first bout divided by two, and this quantity is divided by the time between the first and median licks of the first bout).

Results

We excluded 10 of the 42 rats in the ethanol drinking groups because they did not drink greater than 0.30 g/kg of 10E for at least 4 of 5 consecutive days (Table 3). We used 31 rats for the extinction test. Fourteen rats received a pre- and post-initiation ethanol preference test. There were 10 rats used in the control groups, but three of these were not included in the extinction analysis because they would not consistently meet the response requirement to match the experimental group.

The first three cohorts were trained using a 45-min pre-wait period and had a response requirement of 4. The consumption of 10E over the last 5 days was 0.54 ± 0.04 g/kg (n=14). We decided to delete the pre-wait period in subsequent cohorts because the modified protocol appeared to be successful, and we wanted to reduce the time needed for training. In addition, we increased the response requirement in these groups to 20. The consumption of 10E in the rats trained without the pre-wait period was 0.58 ± 0.06 g/kg (n=18). There was no significant difference in 10E consumption between the two groups ($T(30) = 0.67$, $P > .05$). In addition, we also examined selected lick parameters during the last 5 days of consumption of 10E for these two groups, and no significant differences were observed (data not shown). Therefore, we combined the two groups for subsequent analyses. The ethanol consumption of the 10S10E solution, and the last 5 days of 10E consumption are shown in Figure 9. There is clearly a significant increase in ethanol intake after day 1 [$F(2,62) = 15.1$, $P < .05$]. The ethanol intake increased 2.2 times from day 1 to day 2 [$F(1,62) = 26.1$, $P < .05$]. Although the intake was slightly decreased from day 2 to day 3 of the exposure to 10S10E, this difference was not statistically significant [$F(1,62) = 0.6$, $P > .05$]. For the 5 days of 10E solution, the average ethanol intake was fairly stable (approximately 0.6 g/kg), and there was no significant difference among the 5 days of intake [$F(4,124) = 1.8$, $P > .05$]. The numbers of licks for the last day of 10S, the 3 days of 10S10E, the 2 days of 5S10E, the 2 days of 2S10E, and the last 5 days of 10E are shown in Figure 10. The fluid consumption drops off

dramatically on the first day of exposure to the 10S10E solution [F(1,57) = 86.5, P < .05], but this rebounds on day 2 as described above for the intake data [F(1,57) = 20.2, P < .05].

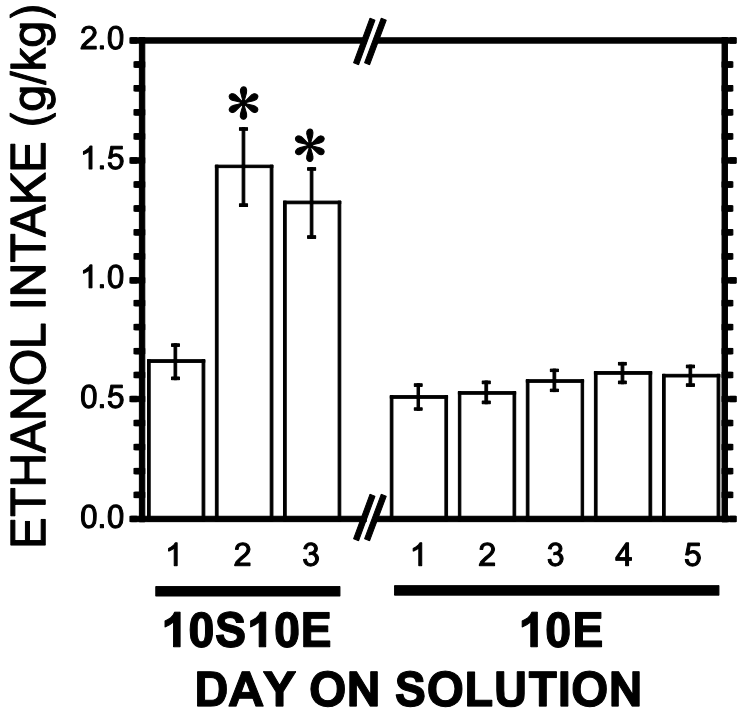

Figure 9. Ethanol intake during first 3 days of operant self-administration of 10% sucrose and 10% ethanol and last 5 days of 10% ethanol. Ethanol intakes (g/kg) are presented as mean ± SEM (n=32). * indicates a statistically significant difference in ethanol intake compared with day 1 (p < .05).

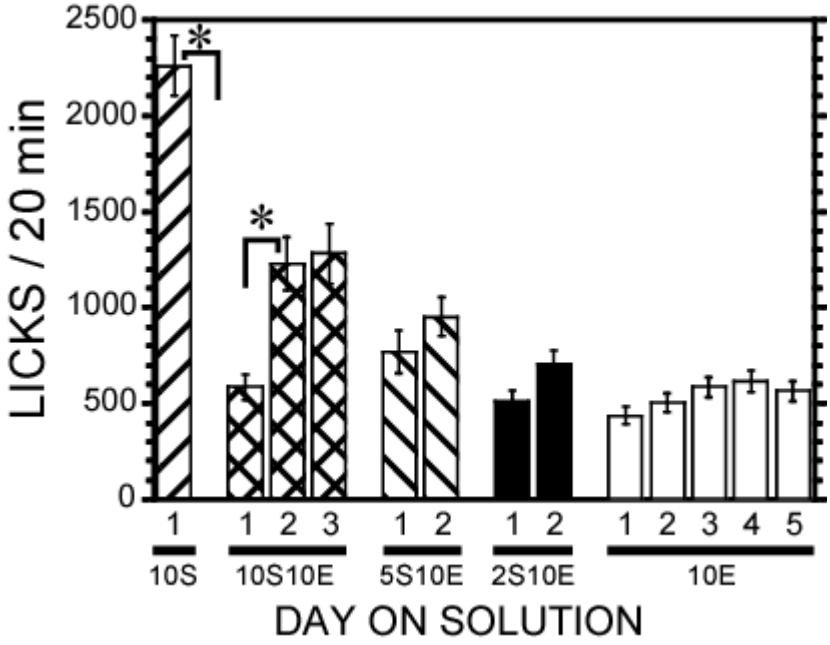

Figure 10. Ethanol intake (licks) during the various stages of the protocol. The licks represent the ethanol consumption during the operant self-administration of the last day of 10% sucrose, the first 3 days of 10% sucrose and 10% ethanol, the 2 days of 5% sucrose and 10% ethanol, the 2 days of 2% sucrose and 10% ethanol, and the last 5 days of 10% ethanol. Ethanol intakes (licks) are presented as mean ± SEM (n=19-22). * indicates a statistically significant difference between the indicated groups by post hoc analysis after significance was found for the overall analysis of variance.

There was a significant correlation between the licks and ethanol intake across the sessions shown in Figures. 9 and 10 ($r^2 = 0.99$, $P < .05$). This confirms that the intake measures obtained by determining the volume of fluid in the bottle before and after the session is due to consumption and is not confounded by spillage. Selected lickometer parameters for the last day of 10E self-administration were compiled to show that the data collected using this new

protocol are similar to data collected using the established sucrose-substitution procedure (Doyon et al., 2003) and are shown in Table 4.

Table 4. Lickometer parameters on last day of 10E self-administration

Parameter[a]	Value (n=26[b])
Latency to begin drinking (min)	0.076 ± 0.033
Number of bouts	1.8 ± 0.2
Total licks	558 ± 48
Initial bout response rate (licks/min)	181 ± 19
Response rate for half of first bout (licks/min)	269 ± 18

[a] See Methods for definition of each parameter and how it was calculated.
[b] Six rats were not included due to malfunction of the lickometer or because the licks/volume measurements were determined to be outliers (see Methods). Values are shown as mean ± SEM. All values are representative of the last 5 days of 10E self-administration except for the latency. The mean latency is almost double that observed for the previous days of 10E self-administration because one rat had a latency of 0.89 min on the last day. Without this value the latency is 0.043 ± 0.005 (n=25).

The effect of the initiation procedure on ethanol preference was measured using a two-bottle choice procedure in two ways. In the first method, the concentration of ethanol during the pre-initiation preference test was 5%, and this was changed to 10% for the post-initiation preference test. The preference for 5% ethanol during the last 3 days of the test was 0.13 ± 0.05 (n=6) before the initiation procedure, and the preference for 10% ethanol was 0.29 ± 0.03 (n=6) after initiation. This suggests that the initiation procedure increased ethanol preference, but we did not do a statistical analysis because the two-bottle choice solutions were different for the pre- and post-initiation tests. To more directly compare ethanol preference before and after the initiation procedure, we replicated the study using 10% ethanol for both preference tests (Table 5). The

ethanol preference during the last 3 days of the post-initiation test was approximately threefold higher than that in ethanol naïve rats ($T(7) = 3.9$, $P < .05$). Although the loss of ethanol solution from the control bottles (due to spillage and evaporation) is high relative to the overall change in volume of ethanol bottles during the pre-initiation test, the spillage values are smaller than those from the drinking bottles indicating that small amounts of ethanol were consumed. The low values obtained during the pre-initiation test period likely reflect the strong aversion for 10% ethanol in naïve Long-Evans rats. Therefore, the results of both methods of assessing ethanol preference confirm that the initiation procedure produced an increase in ethanol preference.

Table 5. Ethanol preference testing before and after initiation of ethanol self-administration.

	Before initiation			After initiation		
	Day 3	Day 4	Day 5	Day 3	Day 4	Day 5
Ethanol (ml)[a]	2.2 ± 0.4	2.3 ± 0.4	2.4 ± 0.4	7.0 ± 0.9	5.9 ± 0.9	7.2 ± 1.4
Water (ml)[b]	37.0 ± 1.5	35.8 ± 1.7	36.2 ± 1.7	35.1 ± 2.0	33.8 ± 2.2	29.8 ± 2.1
Preference[c]	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.17 ± 0.02	0.15 ± 0.03	0.19 ± 0.03
Dose (g/kg/d)	0.50 ± 0.09	0.53 ± 0.09	0.53 ± 0.08	1.15 ± 0.16	0.98 ± 0.17	1.20 ± 0.26

Values shown are mean ± SEM (n=8) for ethanol and water consumption and preference.
[a] Average loss for control ethanol bottle was 2.1 ± 0.4 ml before initiation and 1.5 ± 0.1 ml after initiation.
[b] Average loss for control water bottle was 1.6 ± 0.3 ml before initiation and 1.5 ± 0.1 ml after initiation.
[c] Ethanol preference was significantly higher after initiation compared to that before initiation (paired t-test, $p < 0.05$)

In the first extinction test carried out on rats trained to drink 10E with an RR of 4, the mean responses during the single extinction session were 64 ± 14 lever-presses in 20 min (n=14), a value clearly higher than the four responses required

during training. As shown in Figure 11, the relationship between extinction responding and intake was not significant [$F(1,12) = 2.1$, $P > .05$, $R^2 = 0.15$]. However, because the RR was relatively low, we repeated the extinction test with another group of animals using a RR of 20. In addition, we compared the ethanol group with a control group, which was trained to lever-press for sucrose, but had the sucrose faded out to water during the same period as the ethanol group. Three of the 10 rats in the control group did not maintain the response requirement of 20, and so they were not included in the subsequent extinction analysis. However, the control rats did consistently consume water if the response requirement was met (11.0 ± 2.6 ml, n=7). The correlation between the extinction responding and intake was not significant for the ethanol group ($r^2 = 0.01$, $P > .05$), but it was significant for the control group ($r^2 = 0.75$, $P < .05$). The relatively high level of drinking in the control group and the significant correlation were due to four out of the seven rats that had extremely high intakes that were clustered near the top of the range. The number of lever-presses during the extinction trial under these conditions was 112 ± 22 (n=10) for the ethanol drinking rats and 140 ± 48 (n=7) for the control rats ($T(9) = 0.55$, $P > .05$).

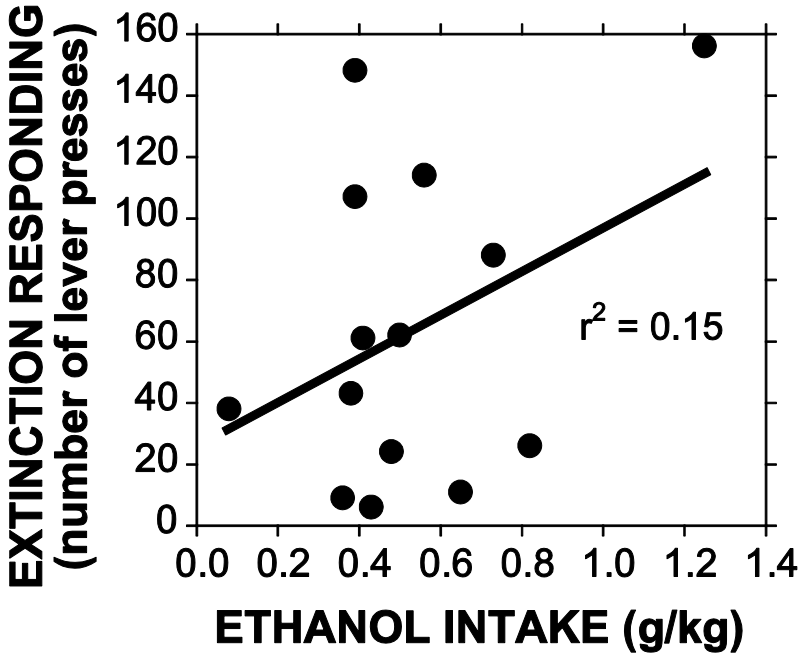

Figure 11. Extinction responding versus prior day ethanol intake. Ethanol intake is shown in g/kg for the last day of access to 10% ethanol for rats that had been trained using an RR of 4 (n=14). The line indicates the linear regression, which was not statistically significant.

We also determined blood alcohol concentration after ethanol intake. The sixth cohort used in the study (Table 3) was trained to self-administer ethanol after performing a response requirement of 20. Two groups were used which differed in the time between the beginning of consumption and the time at which blood samples were taken from the saphenous vein (13.0 ± 2.0 and 27 ± 0.3 min), and gas chromatography was used to determine the blood alcohol concentration. The group with the short delay consumed 0.42 ± 0.05 g/kg (n=4) and had a blood alcohol concentration of 0.77 ± 0.43 mM (3.5 mg/dl). The group with the longer delay consumed 0.71 ± 0.09 g/kg (n=4) and had a blood alcohol concentration of

4.5 mM (21 ± 3 mg/dl). The blood alcohol concentration was almost six fold higher in the group with the longer delay compared with that in the short delay group, although the higher consumption of alcohol likely contributed to this difference also.

Discussion

During the standard sucrose-fading method for initiation of ethanol self-administration, the concentration of ethanol is slowly increased over days from 2% to 10%. Because it is not clear how the changing ethanol concentrations contribute to behavioral changes, this procedure limits the interpretation of experiments that may be designed to investigate mechanisms that promote the initiation of ethanol self-administration behavior. In the present study, we modified the initial phase of the standard sucrose-fading procedure by exposing rats to a constant concentration of 10S10E. Our main findings were that we can get a stable intake and an increase in ethanol preference by using an ethanol initiation procedure with a fixed concentration of ethanol and sucrose for 3 days, followed by a sucrose fade-out. Moreover, the concentration of ethanol was kept constant in this protocol, and this may allow a more detailed analysis of the factors that contribute to the development of ethanol self-administration behavior, especially during the first few days of exposure to ethanol.

In previous studies of ethanol self-administration using the sucrose-fading procedure, preference for ethanol was clearly increased after the sucrose-fading procedure as measured by an unlimited access two-bottle choice method (Files et al., 1996; Samson, 1986; Samson et al., 1989; Tolliver et al., 1988). For example, in the studies cited above the preference before the sucrose-fading procedure was in the range of 5-24%, and this increased to approximately 42-70%. The modified method we used also increased ethanol preference several fold, similar to the previous work. Two issues should be considered here. First, in the initial preference test the concentration of ethanol used for the post-initiation test was higher (10%) than the pre-initiation test (5%), and we did not do a statistical analysis of the change in preference because of this. However, rats have a lower preference for 10E compared with 5% ethanol (Almeida et al., 1998; Shoaib & Almeida, 1996). Our data indicated that in naive rats the preference for 5% ethanol was 0.11-0.15. After the initiation procedure the preference for 10E was 0.27-0.32. This is clearly higher than the original pre-initiation preference for 5% ethanol. However, we also replicated the preference test using 10% ethanol for both the pre- and post-initiation tests, and in this case we also observed a statistically significant 3.2-fold increase in ethanol preference (Table 5). It should be noted that there are several methodological differences between the previous studies and the present one. However, it is unlikely that these differences influence the preference measurement since the results are similar. Overall, the results of the preference test support the idea that the

modification we introduced produced a similar behavioral change compared with the traditional sucrose-fading method.

This modified protocol successfully initiated ethanol self-administration in 76% of the rats. The overall level of ethanol intake and the pattern of ethanol consumption during the 20-min access period (measured by a lickometer) were similar to previous data using the well-established sucrose- fading protocol (Table 4) (Czachowski et al., 2001;Doyon et al., 2003). Moreover, we did not observe a significant relationship between ethanol intake in the session prior to the extinction test and the number of lever-presses during the extinction trial (Figure 11), which is also similar to previous studies (Samson et al., 2001, 2003). Thus, the change in the procedure that we introduced early in the training period did not significantly alter the eventual establishment of stable intake patterns of 10E solutions or appetitive responding for ethanol.

It has been suggested by Samson et al. (2003) that measures of ethanol intake alone may not accurately describe the appetitive strength of ethanol. To further examine this issue, extinction tests were performed in two groups of rats that were trained using two different RRs: 4 and 20. In both cases the extinction responding was much higher than the original RR (15-fold increase and 5.6-fold increase for the RRs of 4 and 20, respectively), results consistent with the idea that reliable ethanol self-administration had been established using our modified

protocol. In addition, we also examined a control group of rats which were trained to drink 10S with an RR of 20 and never had access to ethanol. The extinction responses were higher (although not significantly) in the control group that was trained to lever- press for sucrose, but then had the sucrose faded out similar to the ethanol group. This level of extinction responding in the control group has been previously reported for rats trained with the standard sucrose-fading protocol (Samson et al., 2003). These findings suggest that the exposure to sucrose during training has a long lasting influence on appetitive responding. However, the results of this control experiment also raise the possibility that the extinction responding in the ethanol group is influenced by the previous exposure to sucrose, and this potential confound makes it difficult, if not impossible, to relate the level of responding in the ethanol group to the level of ethanol reinforcement that had been established. In any case, these findings emphasize the importance of using ‘‘alternate fluid’’ control groups and the continued investigation of differences between fluid and ethanol-reinforced responding (e.g., Doyon et al., 2003, 2004, 2005).

It is interesting to examine the first few days of consumption of the ethanol-containing solution. The consumption of fluid dramatically declines during the initial exposure to 10S10E compared with the previous day consumption of 10S. However, this rebounds on the second and third day of exposure to the ethanol-containing solution (Figures. 9 and 10). The reasons for these changes in fluid

consumption behavior are not clear. The drop in consumption during the first day exposure to ethanol may be due to factors such as the novelty of the solution and natural aversion to the relatively high concentration of ethanol. Comparison of the lick rates for the first 30 seconds of fluid consumption revealed that the rate was significantly lower during the first day of 10S10E (127 ± 12, n=20) than that of the previous day (196 ± 5) ($T(19) = 5.5$, $P < .05$, paired t-test). This suggests that palatability of the solution affected the consummatory behavior (Davis & Smith, 1988). The rebound on days 2 and 3 may be due to factors such as habituation to the aversive flavor, the natural preference for the calories provided by both the sucrose and ethanol, and the reinforcing properties of the sucrose and ethanol. Further studies are required to determine the potential involvement of these factors.

In summary, we tested a modification of a well-established method for initiating ethanol self-administration in which rats are initially exposed to a relatively high and constant concentration of ethanol (10%) along with 10S for three days. This modified protocol produced similar patterns of intake and ethanol-seeking behavior and successfully initiated measurable ethanol self-administration in a large majority of rats. The consumption of 10E solutions produced reasonable blood alcohol concentrations. The findings from this study provide the necessary foundation for future studies of the processes that regulate the initiation of ethanol drinking that will not be confounded by changing ethanol concentrations.

Chapter Four A single exposure to voluntary ethanol self-administration produces adaptations in ethanol consumption and accumbal dopamine signaling

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Pending Publication

Abstract

In well trained animals, accumbal dopamine release is stimulated during operant ethanol self-administration, but the time course of development of this dopaminergic response, particularly during the acquisition of drinking behavior, remains unknown. In order to examine this, we trained male Long-Evans rats to self-administer 10% ethanol + 10% sucrose, using a self-administration protocol in which the concentration of ethanol was kept constant through the study. The animals were required to press the lever 4 times to gain continuous access to the drinking solution for 20 minutes, and microdialysis was performed on either the first or second day of 10% ethanol + 10% sucrose self-administration or 10% sucrose as controls. Ethanol and dopamine were both analyzed in the dialysates. All groups (day 1 and 2 ethanol and their corresponding sucrose controls) showed an increase in accumbal dopamine during the transfer from the home cage into the operant chamber. Our main finding was an increase in dopamine in

the nucleus accumbens during the first 5 minutes of consumption on the second day but not the first day of ethanol self-administration. This study is the first to describe the time course of the dopaminergic response observed during the acquisition of ethanol self-administration. Our results suggest that a single exposure to a 10% ethanol + 10% sucrose drinking solution may be sufficient to learn the association between ethanol cues and its rewarding properties.

Introduction

Mesolimbic dopamine transmission is implicated in various aspects of reward processing, including the formation of associations between a reward and the stimulus cues that predict the reward. If an animal is exposed to an unexpected reward, the firing rate of dopamine neurons is enhanced and extracellular dopamine concentrations are elevated in terminal regions, such as the nucleus accumbens (Day et al., 2007; Schultz 1998, 2007). However, in Pavlovian and instrumental conditioning experiments, repeated pairing of a cue with a receipt of a food reward produces an increase in dopamine neuronal activity at the time at which the cue is presented (Day et al., 2007; Roitman et al., 2004; Schultz 2010). Similarly, the association between a cue and drug reward or intracranial self-stimulation is accompanied by a temporal shift in accumbal dopamine concentrations, as assessed by fast-scan cyclic voltammetry (Aragona et al., 2009; Owesson-White et al., 2008). The number of trials required to produce the

temporal shift in dopamine activity during operant conditioning is typically at least 30, and the shift has been demonstrated to take place within one day (Aragona et al., 2009; Stuber et al., 2008). However, no one has investigated the time course of the association between reward and cues for ethanol self-administration or potential adaptations in accumbal dopamine signaling during the initial stages of ethanol reinforcement.

Previous work from our lab showed that extracellular accumbal dopamine increases transiently (~5 min) at the commencement of consumption of ethanol in a limited-access operant self-administration model after extensive (months) experience with ethanol (Doyon et al., 2003). We later found that this dopamine response pattern is apparent after only 6 days of ethanol intake (Doyon et al., 2005). Interestingly, the increase in dopamine occurs before peak brain ethanol concentrations have been achieved. The accumbal dopamine response during ethanol consumption is instead consistent with the idea that the sensory stimuli of ethanol (taste and smell) serve as cues that predict the intoxicating effects of ethanol. These results show that mesolimbic dopamine signaling is enhanced during ethanol consumption in rats that have experienced the intoxicating and presumably the rewarding effects of ethanol compared with control rats that consume sucrose or water. However, no one has studied the development of the dopaminergic response or its relationship with changes in ethanol consumption that occur during the initiation of ethanol self-administration.

To further define the time course of adaptations in mesolimbic dopamine function that accompany the development of ethanol reinforcement, we used a novel ethanol self-administration protocol in which rats are switched from a 10% sucrose solution to one containing 10% sucrose + 10% ethanol (Carrillo et al., 2008). Ethanol intake increases significantly after the first day of ethanol exposure (Carrillo et al., 2008). Therefore we hypothesized that neuroadaptations in dopamine signaling within the nucleus accumbens, would also occur during this period. Here, we show that a single exposure to ethanol in a drinking solution is sufficient to produce both an enhanced accumbal dopamine response and enhanced ethanol consumption 24 hrs later.

Materials and methods

Animals

We used forty male Long-Evans rats (Charles River Laboratories, Wilmington, MA, USA) for this study. The animals were handled and weighed for a minimum of 5 days before performing surgery on them. Each rat was housed individually in a temperature (25°C) and light (12 hours light／12 hours dark) controlled environment. The rats had food and water available ad libitum in their home cage. All procedures complied with guidelines specified by the National Institutes

of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin.

Behavioral Apparatus

Operant chambers (Medical Associates Inc., St. Albans, VT, USA) modified for microdialysis were used for self-administration training and microdialysis testing. A retractable lever was located on the left side of one of the wall, (2 cm above the floor) which triggered the entry of a retractable drinking spout on the right side of the same wall (5 cm above the grid floor) when pressed. The opposite wall contained an interior chamber light. The floor was composed of parallel stainless steel bars, which connected with the spout of the drinking bottle to form a lickometer circuit (Medical Associates Inc.). The operant chamber was housed within a sound-attenuating chamber with a fan, but the doors were removed to facilitate training and microdialysis. The interior light and sound-attenuating fan were activated at the beginning of each operant session. Operant chamber function and acquisition of lickometer data were controlled by PC using software from Med Associates.

Surgery

We surgically inserted a stainless steel guide cannula (21 gauge; Plastics One Inc., Roanoke, VA, USA) above the left nucleus accumbens core–shell border of the rats in the study using a stereotaxic device. The coordinates were (in mm relative to bregma): +1.7 antero-posterior, +1.0 or 1.1 lateral, - 4.0 or -3.8 ventral to the skull surface (Paxinos et al., 1999). The surgical procedure used in our lab has been previously described (Doyon et al., 2005). The rats were given a week to recover from surgery before starting their operant training.

Self-administration training

Operant sessions occurred once a day for 7 days/week. Animals were trained to lever-press for access to a 10% sucrose (w/v) solution. Rats were water deprived (15-22 h/d) prior to each training session to facilitate learning of the operant response. A reliable lever-pressing response for the drinking solution occurred within 1 to 3 days and once established, the rats were given water *ad libitum* for the remaining sessions. The rats were then trained to lever-press for either 10% sucrose or 10% ethanol (w/v) + 10% sucrose (w/v) as previously described by Carrillo et al. (2008). Briefly, all rats had 4 sessions of 10% sucrose self-administration. During the first 4 sessions, we gradually habituated the animals to a 15-min wait period, which preceded access to the lever and drinking solution.

The lever pressing requirement was progressively increased to 4 during the first 4 sessions as well. When the response requirement was completed, the lever was retracted, and the rats had access to the drinking solution for 20 min. Following this period the drinking spout retracted, and the rats remained in their operant chambers for another 20 min post-drink period without the lever or drinking solution. After the fourth session, the ethanol groups were switched to a 10% ethanol + 10% sucrose drinking solution, and microdialysis occurred on either the first or second day of ethanol exposure. Sucrose controls continued to self-administer 10% sucrose throughout the study and microdialysis was performed on equivalent days to their ethanol counterparts. Consumption of the drinking solutions was monitored throughout the self-administration protocol by a lickometer circuit and by measuring the volume of liquid in the drinking bottle before and after the session, accounting for spillage. Body weights were recorded every day.

Microdialysis, experimental timeline, and histology

The microdialysis probes were constructed according to the methods described by Pettit and Justice (1991) (2.0 mm active membrane length, 270 mm OD, 18,000 molecular weight cut-off). The procedures for microdialysis and the experimental timeline were formerly described by Doyon et al. (2005) (Figure 12). At the end of the operant session, the artificial cerebral spinal fluid was switched

to one without calcium and left running for 1-1.5 hr. Two samples were then collected, to verify that the dopamine in our sample tubes was due to exocytotic release. Histologies were performed as in Howard et al. (2009) using Paxinos et al. (1999).

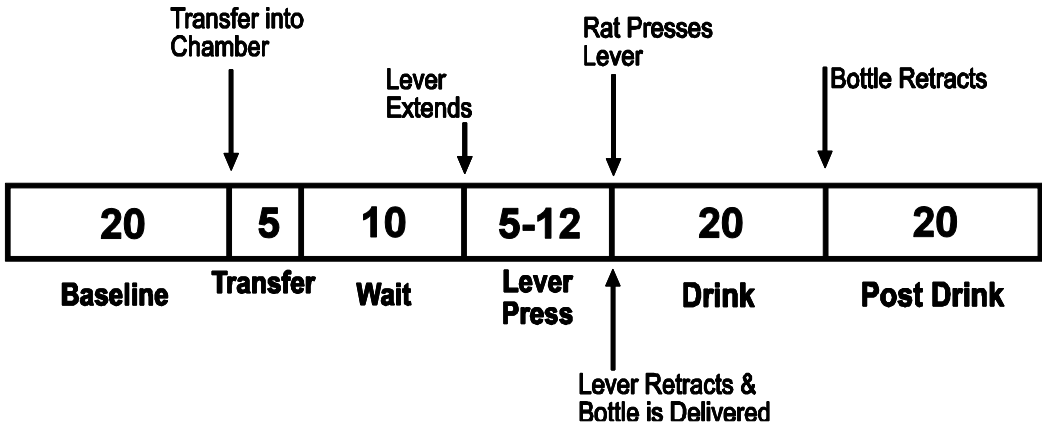

Figure 12. Experimental timeline for microdialysis session. Phases include: baseline, transfer, wait, wait including lever press, drink, and post-drink. Numbers indicate the time in minutes for each phase.

Dopamine analysis by high pressure liquid chromatography

Reversed-phase high performance liquid chromatography with electrochemical detection was used to analyze dialysate dopamine. Seven µl of the dialysate were mixed with 1.5 µl of ascorbate oxidase prior to injection, and 5 - 7 µl of this mixture was injected into the system with an 8125 manual injector (Rheodyne, Cotati, CA). Separation of dopamine occurred with one of several C18 columns used during the course of these experiments (Luna 50 x 1.0 mm, 3-µm particle size, Phenomenex, Torrance CA; 50 x 0.5 mm column, Higgins analytical,

Mountain View, CA; or 50 x 2.0 mm column; Varian; Palo Alto, CA). Detection of dopamine took place with either a VT-03 flow cell with a 2 or 0.7 mm diameter glassy carbon working electrode (ISAAC reference, Antec Leyden, Netherlands) or a SenCell flow cell with a 2 mm electrode. Mobile phase had a pH of 5.6 and consisted of 0.50 g octanesulfonic acid, 0.05 g decanesulfonic acid, 0.13 g ethylenediaminetetraacetic acid, 11.08 g NaH_2PO_4, 4.47 g KCl, and 150 ml methanol in 1 liter of deionized water. EZChrom software (Scientific Software Inc., Pleasanton, CA) was used to record and analyze chromatograms. External dopamine standards were used to determine the concentration of dopamine in each sample. The signal to noise ratios were calculated and recorded for all samples, and only dopamine peaks with a signal to noise ratio above 10 were included in the analyses.

Ethanol analysis by gas chromatography

A detailed description of the methods used for ethanol analysis is described by Doyon et al. (2003). Briefly, during ethanol self-administration, 2 µl of dialysate collected from each sample was put into a glass vial and used for ethanol analysis. This was conducted using a gas chromatograph (Varian CP 3800; Varian, Walnut Creek, CA, USA) with flame ionization detection (220°C).

Statistics

Two phases of the experiment were analyzed separately: effect of transfer into the operant chamber and effect of consumption of ethanol. Initially we analyzed the home cage dialysate dopamine concentrations for stability using the criterion that the relative standard deviation had to be less than 0.2. One rat did not meet this criterion and was excluded from the transfer effect analysis. Another rat was excluded because the increase in dopamine during the transfer period was an outlier (Q-test). A third rat was excluded due to lost samples during the baseline and wait phases of the experiment. Dialysate dopamine levels (nM) were log transformed to attain homogeneity of variance and were then analyzed using a three-way ANOVA with repeated measures. To perform these analyses, we used time as the within subject factor and drinking solution and day as between subject factors. If significant interactions were found, post hoc tests were used to compare individual time points to baseline samples within that group. Dialysate ethanol levels (mM) were analyzed using t-test. Behavioral measures were analyzed using Univariate ANOVAs, and if an interaction was observed, post hoc tests were run to determine the source of variation. Three behavioral parameters (number of licks, milliliters consumed, and licks during initial bout response rate) were log transformed to achieve homogeneity of variance. The number of bouts, a period of at least 25 licks with no more than 2 min between licks, was analyzed using the non-parametric Mann U Whitney Test. Significance for all analyses was

determined when $p < 0.05$, and Bonferroni corrections were used for post hoc tests.

Results

The consumption of ethanol was significantly greater on the second day of exposure to the 10% ethanol + 10% sucrose solution (0.38 g/kg ± 0.06 for the first day, 1.3 g/kg ± 0.15 for the second day, $p < 0.05$ by t-test). The increase in consumption was confirmed by measurement of peak dialysate ethanol concentrations which were significantly higher ($p < 0.05$, t-test) in the day 2 ethanol group (1.7 ± 0.3 mM) compared with the day 1 ethanol group (0.4 ± 0.3 mM). However, ethanol concentrations peaked in the brain at approximately the same time in both groups, about 30 minutes after ethanol consumption had commenced (Figure 13 D). The volume of 10% sucrose consumed was similar across these two corresponding days for the control group (Table 6). The increased consumption of the ethanol solution during the second day of exposure compared with the first was also corroborated by analysis of the lickometer data, which indicated higher values for total number of licks during the session, number of licks during the initial bout, initial bout response rate, and number of bouts (Table 6). A large majority of licking was observed in the first 5 minutes of fluid consumption for all groups (day 1 and 2 ethanol and the sucrose controls). For the day 2 ethanol group the total licks within the first 5 minutes of self-

administration were 73 ± 7%, and this dropped to 16 ± 6%, 6 ± 3%, and 4 ± 2% in the next three 5-min periods. The day 1 ethanol group and the two sucrose control groups had similar licking patterns (day 1 ethanol: 78 ± 7% in the first 5 minutes and 9 ± 5%, 4 ± 2%, and 9 ± 5% in the following three 5-min periods; day 2 sucrose: 72 ± 5% in the first 5 minutes and 22 ± 6%, 6 ± 3%, and 0.2 ± 0.2% for the next three 5-min periods; day 1 sucrose: 68 ± 5% for the first 5 minutes and 26 ± 4%, 2 ± 1%, and 0.2 ± 0.2% in the subsequent three 5-min periods). Also, lever-press behavior during the microdialysis session was not significantly different among the two ethanol drinking days or the corresponding sucrose drinking days (Table 6).

Table 6. Lickometer parameters on microdialysis day

Parameter	Day 1		Day 2	
	Sucrose	EtOH + Sucrose	Sucrose	EtOH + Sucrose
Latency to begin drinking (min)	0.15 ± 0.04	0.33 ± 0.08	0.26 ± 0.13	0.17 ± 0.08
No. of bouts	1.10 ± 0.10	1.67 ± 0.19 [a]	1.38 ± 0.18	1.65 ± 0.18
Total licks	2339 ± 209	362 ± 66 [b]	2027 ± 236	1414 ± 224
Initial bout response rate (licks/min)	280 ± 19	100 ± 23 [b]	250 ± 20	178 ± 21
Licks during initial bout	2325 ± 206	265 ± 44 [b]	1948 ± 263	1291 ± 240
Milliliters consumed	12.0 ± 1.0	1.8 ± 0.3 [b]	10.3 ± 1.1	6.3 ± 0.8
Time to complete lever pressing requirement (min)	2.69 ± 1.24	1.77 ± 0.53	1.42 ± 0.39	1.96 ± 0.76

Values are shown as mean ± SEM.
A bout is a period of at least 25 licks, with no more than 2 min between licks.
[a] Significantly different from Day 1 sucrose by univariate ANOVA ($p < 0.05$)
[b] Significantly different from Day 2 ethanol and Day 1 sucrose by univariate ANOVA ($p < 0.05$)

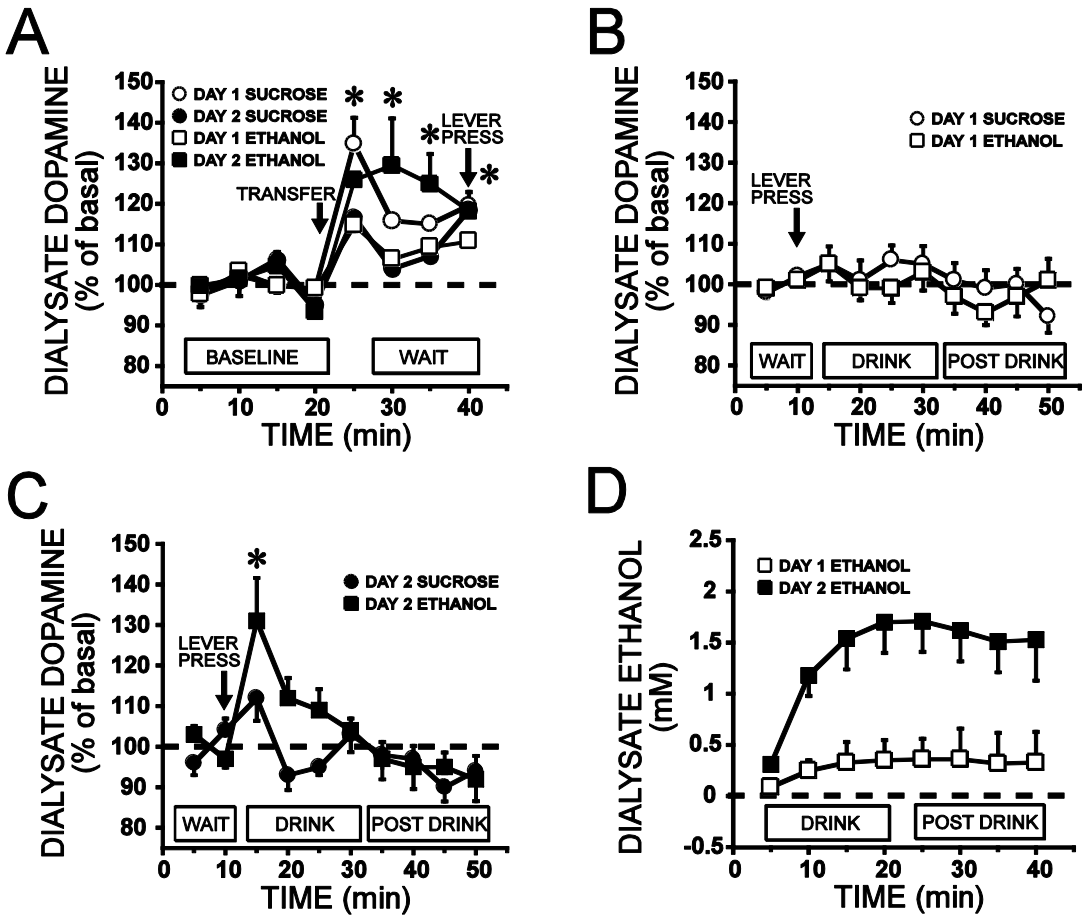

Figure 13. Accumbal dopamine during operant ethanol or sucrose self-administration. A. Dialysate dopamine from the nucleus accumbens during home-cage baseline, transfer from home cage into the operant chamber, and the following waiting period. There was a significant increase in the dopamine response during the transfer period for all groups: both days of 10% ethanol + 10% sucrose or 10% sucrose (n = 37). Arrows indicate the time of transfer from home cage into operant chamber and time during which the rat had access to the lever. For clarity only the largest error bar for each time period is shown. Asterisks indicate $p < 0.05$ for dopamine when compared to the home cage baseline when all groups are collapsed. B. Dialysate dopamine from the nucleus accumbens during the wait, drink, and post-drink periods on the second day of exposure to ethanol (n=10) and corresponding sucrose controls (n=8). The arrow indicates the time during which the animal had access to the lever. Mean ± SEM are shown. The asterisk indicates a significant increase $p < 0.05$ for dopamine during the first drink period, when compared to the wait period (new baseline). C. Data collected from the first day of 10% ethanol + 10% sucrose (n=12) and the corresponding day for the 10% sucrose group (n=10). D. Dialysate ethanol levels from the nucleus accumbens during the drink and post-drink periods on the first (n=12) and second days of ethanol self-administration (n=10).

Microdialysis samples from the nucleus accumbens core-shell border were taken on the first or second day of ethanol consumption and in the sucrose controls corresponding to these days. Dialysate dopamine concentrations taken during the baseline period were not statistically different among any of the groups (1.1 ± 0.2, 1.1 ± 0.1 nM for the 2 days of sucrose consumption; 0.9 ± 0.1, 1.4 ± 0.2 nM for the ethanol groups). Accumbal dopamine was significantly higher during transfer from the home cages into the operant chambers (transfer period) and during the wait period compared with baseline for all groups (Figure 13 A) ($F_{7,226} = 17.8$, $p < 0.05$). The time course of the increase in dopamine significantly varied across microdialysis day and drinking solution ($F_{7,226} = 2.4$, $p < 0.05$), and post hoc analysis revealed a difference between the day 2 ethanol group and the day 1 ethanol and two sucrose control groups ($F_{7,226} = 2.3$, $p < 0.05$).

The last two samples in the wait period were used to establish a new dialysate dopamine baseline for the analysis of accumbal dopamine during the drink and post-drink periods. Dialysate dopamine concentrations during this new baseline were not significantly different between groups (1.1 ± 0.1, 1.2 ± 0.1 nM for the two days of sucrose consumption; 0.9 ± 0.1, 1.6 ± 0.3 nM for the two days of ethanol consumption; $F_{1,35} = 2.7$, $p > 0.05$). Dialysate dopamine was significantly elevated above baseline in the rats that drank ethanol on the second day of exposure, but not in any of the other groups of rats (Figure 13 B-C) ($F_{8,283} =$

10.3, $p < 0.05$). Post hoc tests showed a statistically significant dopamine increase during the first drink sample compared with baseline ($F_{1,283} = 27.5$, $p < 0.05$) in the day 2 ethanol group.

Calcium dependency of dialysate dopamine was 79 ± 2% (n=40). All of the subjects had probes placed through the nucleus accumbens with the probes spanning the core and shell subregions, but with 53% of the animals having at least 45% of the probe active area in the core-shell border of the nucleus accumbens (Figure 14). However, since dopamine in the shell or core does not appear to increase during the operant self-administration of 10 % ethanol + 10 % sucrose or 10% sucrose (Howard, 2009), we do not think this should be a concern.

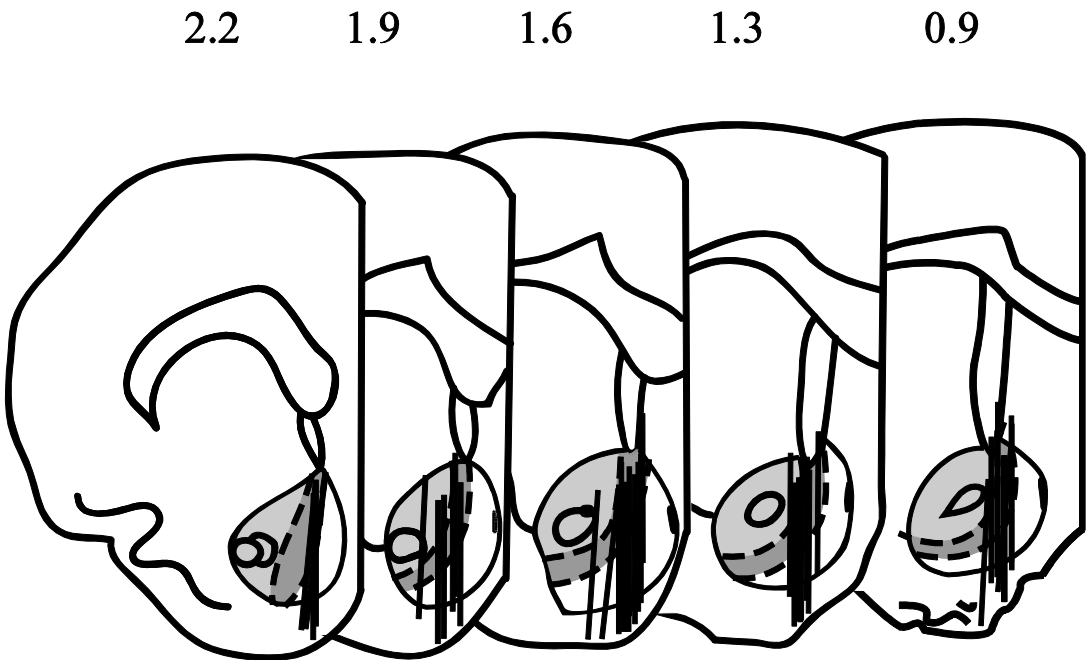

Figure 14. Schematic representation of probe placements in the nucleus accumbens for all groups. Groups include: day 2 and day 1 ethanol self-administration and the corresponding sucrose groups. Numbers above slices denote the location in millimeters from bregma. This figure was based on the rat brain atlas of Paxinos et al. (1999). The light grey shading indicates the core. The dark grey shading indicates the core-shell border, and the white area of the accumbens indicates the shell subregion.

Discussion

This is the first study to investigate adaptations in accumbal dopamine signaling during the first two days of acquisition of operant ethanol self-administration. We approached this by first training rats to lever-press for a solution of 10% sucrose. This was followed by exposure to either one or two consecutive days of a solution containing 10% ethanol + 10% sucrose. On the second day of ethanol exposure, the rats more than tripled their intake compared to the rats which

received ethanol on the first day, which suggests that an increase in the reward value of the solution occurred. In addition, dopamine activity in the core-shell border of the nucleus accumbens was significantly enhanced during consumption of the ethanol solution on the second day, but not the first day or during sucrose consumption (Figure 13 B-C). Therefore, a single trial of ethanol exposure is sufficient to produce neuroadaptations in accumbal dopamine signaling that are apparent 24 hrs after the trial.

The mechanisms that underlie the increase in ethanol consumption from day 1 to day 2 are unclear, but we propose two possibilities. Perhaps the rats became habituated to the novel aversive taste that the rat had previously experienced on day 1 of exposure to the ethanol + sucrose solution. Analysis of the licking behavior support this idea, since the rats had a lower lick rate during the first ethanol drinking bout than the rats previously exposed to ethanol or the sucrose controls. A previous study showed that habituation to aversive fluids occurs to a limited degree in Long-Evans rats, but this can only explain about 25% of the greater than 300% increase in ethanol consumption we observed (Gartside and Laycock, 1987). In conjunction with habituation, it is likely that the change in drinking behavior across the two days was also caused by a mild intoxication on the first day of ethanol consumption. An association between the rewarding effects of ethanol and the cues present during consumption of the novel ethanol solution may have been formed. Thus, the increased ethanol intake on day 2

may in part be due to the enhanced motivation to seek the ethanol solution because of the newly formed association. The dopamine response may reflect the reward-prediction role of mesolimbic dopamine that developed after the single pairing between the stimulus cues of ethanol that are present during consumption and the subsequent reward produced by ethanol after a sufficient concentration has reached the brain (Horvitz, 2000; Schultz 1998, 2007; Stuber et al., 2008). Our data are the first to show that a single exposure to voluntary ethanol self-administration is sufficient to produce adaptations in accumbal dopamine signaling.

It may be argued that part of the reason for the appearance of the accumbal dopamine response on the second day of exposure is simply due to the larger intake of ethanol along with greater brain concentrations of ethanol on that day compared to the first day. Higher brain ethanol concentrations would produce stronger and more long-lasting pharmacological effects that produce intoxication. However, in this and in previous studies, the transient dopamine response occurs before appreciable ethanol has reached the brain, and this argues against the idea that the pharmacological effects of ethanol contribute to the response that appears on day 2. Instead, the dopamine response occurs during the period of time when the rat is consuming the majority of ethanol on the basis of the lickometer data and when the stimulus cues for ethanol are strongest (Doyon et al 2003, 2005; Howard et al., 2009).

An increase in dialysate dopamine within the nucleus accumbens core-shell border was also observed during the transfer from the home cage into the operant chamber (transfer period), and the time course of this effect varied with respect to the day of ethanol exposure and the drinking solution. The dialysate dopamine concentration increased above baseline followed by a drop for the next 10 min during the wait period for 3 of the groups (both days of sucrose consumption and day 1 of ethanol consumption). A different temporal pattern was observed for the day 2 ethanol group in which the initial enhancement in dopamine was sustained for the next 10 min (Figure 13 A). Further examination revealed a significant difference between the patterns of dopamine signaling that occurred in the day 2 ethanol group and the other 3 groups (day 1 ethanol group and the two sucrose control groups). Overall the present data provide evidence that dopamine signaling is beginning to change in ethanol-exposed rats even during exposure to the cues associated with the operant chamber after a single day of exposure to ethanol, but not in control rats. Additional work will be required to verify this suggestion.

In summary, the main finding of this study is that there is a transient increase in dopamine within the nucleus accumbens the day after a single exposure to ethanol. This study gives us new insight that the development of the neurochemical and behavioral changes during the acquisition of ethanol self-

administration occurs quite rapidly after a single pairing trial. More specifically, these results are consistent with the proposed role of accumbal dopamine as a reward-prediction signal during the development of voluntary ethanol consumption, furthering our understanding of the relationship between mesolimbic dopamine and ethanol reinforcement.

Chapter Five: General Discussion

There were three major outcomes of these studies: (1) we developed an operant training protocol, in which a constant concentration of ethanol (10%) is used throughout training, (2) with this training protocol, rodents showed a rapid increase in ethanol consumption after only one day of ethanol self-administration, (3) this increase in ethanol consumption corresponded to an increase in accumbal dopamine that was observed after a single trial of ethanol self-administration.

We developed a self-administration protocol that kept the concentration of ethanol (10%, w/v) constant, while at the same time producing sufficient ethanol intake levels. Most protocols for ethanol self-administration use variations of the sucrose fading procedure (Samson, 1986) in which ethanol is gradually added into the drinking solution over several days and a sweetener is gradually faded out. With the protocol we created, there is no variability in the ethanol concentration that may confound the interpretation of studies investigating the mechanisms involved in the acquisition of ethanol drinking. Thus, we can study the early stages of ethanol self-administration and the neurochemical changes that occur during this period. While testing the protocol, though, we found a couple of interesting things.

We observed an initial decrease in fluid consumption when the solution was switched from 10% sucrose to 10% ethanol + 10% sucrose and that was followed by a two-fold increase in ethanol intake on day 2 of 10% ethanol + 10% sucrose compared to day 1. These results suggested that a mechanism was functioning to increase ethanol intake as the reinforcing properties of ethanol were learned. The decrease in fluid consumption during the first exposure to 10% ethanol could have been caused by an initial aversion to ethanol's taste and smell, to the novelty of the new solution, or to a combination of the two. In fact, the aversion that rats seem to have for alcohol is the primary reason why most ethanol drinking protocols make use of the sucrose fading procedure. However, the reason for the dramatic increase in 10% ethanol + 10% sucrose consumption from day 1 to day 2 was less clear. Although there could have been a rapid habituation to the aversive properties of ethanol, it is more likely that there was some form of learning involved in which the animals adapted their behavior to the presence of a reward or at the very least perceived the solution to be of no harm.

Once we determined the success of the training protocol in establishing and maintaining ethanol self-administration, we used a shorter version of the protocol to test the hypothesis that a second, but not a first time, exposure to 10% ethanol + 10% sucrose would have an effect on dopamine signals in the core-shell border of the nucleus accumbens (Chapter 4). The study presented in Chapter 4 was the first to investigate the developmental adaptations in accumbal dopamine

during the early stages of ethanol reinforcement. Ethanol-naïve male Long Evans rats were used for the study. The rodents received a novel solution of 10% ethanol + 10% sucrose for either one or two consecutive sessions. The animals in the control groups were never exposed to ethanol and received a 10% sucrose solution for the same number of days as their ethanol consuming counterparts.

We examined dopamine signaling in the core-shell border of the nucleus accumbens instead of the core or shell because recent evidence suggests it is a unique subregion (Fabbricatore et al., 2009; Hipolito et al., 2008; Howard et al., 2009; Rebec et al., 1997). Although very little is known about the core-shell border, it is possible that the differences seen between dopamine release in this subregion versus the core or shell is caused by some of the different anatomical connections between subregions. The core-shell border is in between the core and shell, and it is therefore possible that it is a “hot spot” that receives and sends projections to areas that both, the shell and core do, like the lateral hypothalamus or medial and lateral VTA. Perhaps the core-shell border combines the limbic and motor aspects that describe the shell and the core subregions, respectively. However, it is also possible that this overlap in connections between the core and the shell are causing an additive effect of the core and the shell in the core-shell border. In fact, it has been suggested that some accumbal subregions may not be well defined and that this can lead to a controversy of whether or not a particular region is a unique subregion (Zahm,

1999). However, this idea of an additive effect in the core-shell border seems unlikely, since a recent study in our lab showed that there is an increase in dopamine during operant ethanol self-administration in the core-shell border of the nucleus accumbens, but not in the core or shell subregions (Howard et al., 2009).

The core-shell border has not been well defined. In fact, even the studies that have been performed in this region do not agree with where the region begins and ends. To better define the core-shell border, we used Photoshop to overlap three images and Illustrator to define the core-shell border subregion. We did this for each of the 5 bregmas we used (bregma: 0.9, 1.3, 1.6, 1.9, and 2.2). The first image was from a subject's brain section (stained with cresyl violet) and the other two images were from the atlas of Paxinos et al. (1999), one stained with calbindin and the other with Nissl. We used a brain image from one of our own rats because we use Long-Evans rats in our study and the atlas consists of brain sections from Wistar rats. Although the atlas is highly reliable, we noticed that the rat brain pictures in the atlas did not match our own rats' brains. For example, some of our probe tracks were mapped right in between the anterior commissure and the islands of calleja, but in the atlas created by Paxinos and colleagues, the probe tracks looked closer to the islands of calleja (1999). To more accurately represent our probe placements, we used the image of one of the Long-Evans rats in our study and overlapped it with the calbindin and Nissl images from the

Paxinos et al brain atlas (1999). The calbindin images were used because calbindin stains darker for the core than the shell subregion, and this particular stain therefore allowed us to see the separation between the two subregions more clearly. The Nissl stain allowed us to better see other landmarks, like the islands of calleja, which helped us to match the three images. We imported the 3 images into Photoshop and outlined the images using the landmarks and borders created by the different stains to create a single image. We then imported the new figure into Illustrator and drew a line through the core-shell border. We then measured the distances between the anterior commissure and the islands of calleja and divided the distance by three to represent the size of the core, shell, and the core-shell border. However, since the subregions are not the same size throughout the nucleus accumbens, this measurement was only used for the midsection of the accumbens. The size of the core- shell border was adjusted as we moved away from the midsection of the nucleus accumbens, but was distributed evenly from both sides of the line we had drawn to represent the border of the core and shell. We followed this procedure for bregmas 0.9, 1.3, 1.9, and 2.2, but not for bregma 1.6 because the core-shell border subregion was clearly smaller in the midsection than in the more ventral and dorsal areas of the accumbens. Once we defined the core-shell border subregion, we mapped our probe tracks.

The behavioral results for the day 2 ethanol group were similar to those seen while testing the ethanol self-administration protocol (chapter 3), which showed that the animals drank more of the ethanol solution on day 2 than on day 1, but not for the animals self-administering ethanol for the first time. The animals used to test the new training protocol consumed 0.66 g/kg ± 0.07 of ethanol on the first day of ethanol self-administration and 1.47 g/kg ± 0.16 on day 2. Similarly, the animals in the day 2 ethanol group consumed 0.63 g/kg ± 0.07 of ethanol during the first ethanol self-administration session and 1.3 g/kg ± 0.15 on the second day of ethanol exposure. However, the rodents in the day 1 ethanol group consumed lower amounts of the ethanol drinking solution (0.38 g/kg ± 0.06) than the rats used to test the self-administration protocol (0.66 g/kg ± 0.07). This could be because of a combination of consuming a naturally aversive solution (ethanol) and having microdialysis performed on the same day. Although two days before microdialysis we used a spring to habituate the animals to the feeling of having something on their heads, the microdialysis procedure could have still been more stressful to the animals, since a probe is actually inside their brain.

The increase in ethanol consumption seen on the second day of ethanol exposure corresponded to an increase in accumbal dopamine that occurred during the first 5 minutes of ethanol access. We did not observe a rise in dopamine levels in animals that self-administered 10% sucrose. Similar to studies done using the standard ethanol self-administration protocol (Samson,

1986), we showed that the time course of ethanol and dopamine dialysate concentrations did not match (Doyon et al., 2003, 2005; Howard et al., 2009). Dopamine levels in the brain peak within 5 minutes of ethanol access, while the brain ethanol levels continued to rise and did not reach peak concentrations until about 30 minutes after the drinking period commenced. This transient increase in dopamine during ethanol self-administration occurred while the animal consumed the majority of the total fluid intake, measured by licks and volume. The scent of the solution was in close proximity and the animals could also taste the ethanol. Thus, the increase in dopamine appears to coincide with the period in which ethanol's sensory cues were strongest.

These findings were also consistent with an increase in dopamine levels seen on two animals on the first day of ethanol consumption during the first and last drink samples of the drink period. We examined the licking patterns and noticed that the increases in accumbal dopamine coincided with the times during which these animals were consuming ethanol and when ethanol's sensory cues were strongest. Interestingly, although their drinking patterns were similar, they consumed different amounts of ethanol, 0.73 g/kg and 0.29 g/kg, and ethanol did not reach peak concentrations in the brain until 35 and 30 minutes, respectively, after the drinking period had started. The data showed that the increase in accumbal dopamine on day 1 corresponded to licking behavior and not to brain concentrations of ethanol. The increase in accumbal dopamine appeared to be

independent of dose. Although no solid conclusions can be made from these data (n = 2), it matches the findings from the day 2 ethanol self-administration group; There was a mismatch between dopamine and ethanol concentrations in the brain and a correlation between licks and the rise in dopamine seen during ethanol self-administration. Thus, the data from these two animals on day 1 of ethanol exposure also suggests that ethanol's sensory cues are responsible for the increase in accumbal dopamine seen during operant ethanol self-administration.

In addition, it is possible that the TRP channel of vanilloid type 1 (TRPV1) is responsible for at least part of the sensory stimuli of ethanol (taste) and that the activation of TRPV1 is perceived as a cue to the rewarding effects of ethanol by the rats. It has been shown that TRPV1 is activated by ethanol (Lyall et al., 2005), and there are TRPV1 channels in taste receptor cells (Lyall et al., 2005). Although the taste of ethanol may activate other gustatory and non-gustatory sensations and therefore other receptor mechanisms, the burning sensation caused by the oral consumption of ethanol is likely caused by the activation of the TRPV1 channel. In fact, rats have a natural aversion for ethanol and studies with another rodent, mice, have shown that TRPV1 knockouts have a higher ethanol preference and consumption (Blednov and Harris, 2009), as well as a decrease in aversive orosensory responses to alcohol (Ellingson et al., 2009). It is therefore possible that the TRPV1 channels in taste receptor cells are

responsible for the aversion rats seem to have for ethanol during a first exposure. However, if the animals learned to associate this burning sensation with the rewarding effects of ethanol, the rats would consume more ethanol the following day and we would also see an increase in accumbal dopamine during this cue (taste) and not during the time that ethanol peaks in the brain, which is what we found. It is important to note, though, that although we have shown evidence that suggests that the sensory stimuli of ethanol are the cues responsible for the increase in accumbal dopamine, we did not directly test whether or not the increase in dopamine was caused by the taste and smell of ethanol, so we cannot dismiss other possibilities.

Overall, our results indicate that dopamine responds to salient cues that signal a reward (Di Chiara, 1995; Horvitz, 2000; Schultz 1998, 2007; Stuber et al., 2008). The present work is the first to show that a single session of ethanol consumption is enough to learn the association between ethanol's rewarding properties and cues. These findings fit into the framework of contemporary theories of dopamine function based on the idea that dopamine signaling contributes to reward-related learning (Schultz, 2002). This is also the first study to observe an increase in accumbal dopamine during ethanol self-administration after a single trial and to investigate the role of dopamine in the core-shell border of the nucleus accumbens during the early stages of ethanol reinforcement. The present work supports previous findings by suggesting that the transient increase in accumbal

dopamine is not due to the pharmacological effects of ethanol but to the sensory stimuli, which may serve as cues.

It has been shown that the dopamine signal shifts from the reward to the cue predicting the reward after repeated pairings between a reward and a cue in Pavlovian and instrumental paradigms (Aragona et al., 2009; Schultz et al., 1997; Schultz 1998, 2007), and although our data strongly suggest this, it should be noted that we did not see an actual shift in dopamine signaling from reward to cue. Our data suggest that the transient increase in accumbal dopamine observed during a second self-administration of ethanol is caused by cues associated with ethanol's intoxicating effects that are experienced the previous day. However, since we did not observe an increase in dopamine during a first exposure to ethanol, we do not show an actual shift in the dopamine signal from reward (ethanol) to cue (sensory stimuli). It is possible, though, that there was transient dopamine response that lasted a few seconds during the drink or post drink period in the day 1 ethanol group, after ethanol had reached peak levels in the brain, and that we were unable to see it with our microdialysis technique. Using microdialysis, we take 5 minute dopamine samples throughout the self-administration session. It is likely that if there is a transient dopamine response that lasts only seconds, we would be unable to measure it with our sampling time. Perhaps if we used fast-scan cyclic voltammetry, which can measure dopamine at a sub-second time scale, we would be able to see a phasic

dopamine release produced by a first time exposure to ethanol. In fact, several studies have shown that there are rapid changes in dopamine concentrations that last only seconds and in response to a variety of events that are detected through the use of fast-scan cyclic voltammetry (Aragona et al., 2009, Day et al., 2007; Owesson-White et al., 2008; Roitman et al. 2004; Schultz, 2007). Thus, if dopamine is increasing and coming down to baseline levels in a matter of seconds, the signal would have gone undetected in our samples, but we would likely see it if we used fast-scan cyclic voltammetry. Another possibility is that there is a dopamine signal during the first day of ethanol self-administration, but that due to the novelty of the drinking solution it is only seen in the shell subregion instead of the core-shell border. Howard et al reported that there was no significant increase in dopamine during ethanol self-administration in the core or shell subregions (2009). However, the animals in the study by Howard and colleagues had been self-administering ethanol for about a week before microdialysis was performed (2009). Studies have consistently showed that the shell is involved in novelty (Bassareo and Di Chiara, 1999; Hernandez et al., 2002; Rebec, 1997). The rodents in the day 1 ethanol group in the present study were ethanol naïve. It is therefore possible that the novel solution is causing an increase in dopamine in the shell instead of in the core-shell border during the first exposure to ethanol and that the dopamine signal is present in the core-shell borer instead of in the shell after a single exposure to ethanol (day 2 of ethanol self-administration). Our behavioral data supports this possibility, since the

animals fluid intake drops dramatically on the first day of ethanol self-administration, but after a single exposure to ethanol the animals double their ethanol intake. It could be interpreted that the animals only perceive the ethanol solution as novel on day 1 of ethanol exposure but not on day 2.

Although it is not likely, an alternative explanation to the increase in dopamine observed after a single exposure to ethanol is sensitization. Robinson and colleagues have shown that sensitization to a rotational behavior can occur after a single exposure to amphetamine or cocaine (Robinson, 1984, Robinson et al 1982, Lin-Chu et al., 1985). In addition, an vitro study, using striatal tissue from female rats, showed an enhancement of dopamine release due to amphetamine in rats that had been previously exposed to a single amphetamine injection (Robinson et al., 1984). However, ethanol seems to require more than a single exposure to produce sensitization. For example, a study by Ding et al showed that rats that were treated with ethanol microinjections in the posterior VTA for five days, but not 3 days, had a significantly greater increase in dopamine when exposed to a challenge injection (2009). Thus, although we cannot rule out the possibility of sensitization, it is unlikely that the increase in dopamine we observed during a second exposure to ethanol is due to sensitization.

It is also plausible that this increase in dopamine is not due to an association between cues and ethanol, but to an association formed between the cues and

acetaldehyde. Studies have shown that acetaldehyde activates dopamine neuron firing in the VTA (Enrico et al., 2009; Foddai et al., 2004). Furthermore, acetaldehyde, similar to ethanol, has been reported to produce place preference and it can be prevented by using a D1 receptor antagonist (Spina et al., 2010). However, the role of acetaldehyde in ethanol reinforcement is still unclear (Quertemont and Tambour, 2004). A more direct test to examine acetaldehyde levels in the brain must be performed.

In addition to investigating dopamine in the core-shell border during ethanol and sucrose self-administration (Chapter 4), we also looked at accumbal dopamine during the transfer period. All four groups in the study (day 1 and day 2 ethanol plus their sucrose counterparts) showed a transient increase in dopamine levels within the core-shell border during the transfer from the home cage into the operant chamber. However, post hoc tests revealed a statistical difference between the pattern of dopamine signaling in the day 2 ethanol group and the other three groups (day 1 ethanol and the two sucrose controls). Although there was a significant increase in accumbal dopamine in the day1 ethanol and the two sucrose control groups during the transfer period, dopamine levels began to drop immediately afterwards. The day 2 ethanol group, however, showed a statistically significant rise in dopamine during the transfer period that was maintained for the following 10 minutes. These findings suggest that dopamine signaling is starting to undergo neuronal adaptations during the presentation of cues associated with

ethanol consumption, after only a single day of ethanol self-administration. However, more research is needed to elucidate the significance of this rise in accumbal dopamine during the transfer and wait periods of operant self-administration.

It should be noted that studies in our lab have shown an increase in accumbal dopamine during the transfer of the animals from the home cage to the operant chamber, but that there are some differences between studies. Doyon and colleagues suggested that the transient increase in dopamine during the transfer period may be nonspecific (2003, 2005). A similar increase in accumbal dopamine occurred during the transfer period in animals trained for self-administration (i.e., water, sucrose, or ethanol) and in animals not trained whatsoever for self-administration, that were simply picked up and transferred into the operant chamber (handling control) (Doyon et al., 2003, 2005). These studies suggest that the rise in dopamine does not depend on the anticipation of ethanol, but rather it is related to the physical handling of the animals or the change of environment (Doyon et al., 2003). Recently Howard et al. (2009) reported a significant difference in accumbal dopamine activity during the transfer period between the ethanol and sucrose groups. The present work showed that there was a significant difference between the pattern of dopamine signaling that occurred in the day 2 ethanol group and the other groups (day 1 ethanol and the

sucrose counterparts), but all four groups had a significant increase in accumbal dopamine during the transfer period.

There are some important points to consider that may help explain the apparent discrepancies, though. The study by Howard et al. had a much larger number of rats per group than this study and the studies performed by Doyon and colleagues. It is possible that a larger number of subjects is needed to see a significant difference in the dopamine increase seen during the transfer period between the ethanol and control groups (water, sucrose, or handling). Furthermore, the present study kept the concentration of ethanol constant at 10% throughout the training protocol, while the studies by Doyon et al (2003, 2005) and Howard et al (2009) introduced ethanol into the drinking solution gradually. It is possible that the varying concentrations of ethanol in the self-administration protocols used in the studies by Howard et al (2009) and Doyon et al (2003, 2005) introduced variability to the results. In addition, the rodents in the ethanol groups from the present study were either ethanol naïve or only self administered ethanol once before microdialysis. On the other hand, the ethanol exposed rodents in the studies by Doyon et al (2005) and Howard et al (2009) self-administered ethanol for about a week before microdialysis was performed, and the animals in the 2003 study performed by Doyon and colleagues were exposed to ethanol for about 7 weeks. Perhaps the animals in the day 2 ethanol group in the present study show elevated dopamine levels throughout the wait period

because they were learning. No one has ever looked at the beginning stages of ethanol self-administration, so the changes that the dopamine signals undergo during such an early stage in ethanol reinforcement are unknown. Together, these results indicate the need for further studies to determine the role of dopamine during the periods preceding ethanol access.

In light that the transfer and wait periods are an important part of the self-administration session and that the role that dopamine plays during these periods is not yet clear, we decided to investigate if there was a correlation between accumbal dopamine release during the transfer period and the amount of fluid consumed during the day of microdialysis (mls/kg). We performed a correlation test for all four groups (day 1 ethanol, day 2 ethanol, and the two sucrose control groups). However, we did not find a significant correlation between dopamine in the transfer period and fluid intake in any of the groups (day 1 ethanol: $r^2 = -0.24$, $P > .05$; day 1 sucrose: $r^2 = -0.26$, $P > .05$; day 2 ethanol: $r^2 = - 0.31$, $P > .05$; day 2 sucrose: $r^2 = 0.27$, $P > .05$).

In summary, we have shown that animals can successfully establish and maintain ethanol self-administration with a protocol in which we keep the concentration of ethanol (10%) constant throughout the study. Using this self-administration protocol we were able to study dopamine signaling in the core-shell border of the nucleus accumbens during the early stages of operant ethanol

self-administration and found a transient increase in dopamine during the first 5 minutes of ethanol exposure. Our findings are the first to show that a single exposure to ethanol may be sufficient to form an association between ethanol's rewarding properties and its cues. Furthermore, these results suggest that it is the sensory stimuli of ethanol that serve as cues and cause the transient increase in dopamine seen in the nucleus accumbens during operant ethanol self-administration, even during the early stages of voluntary ethanol consumption. Our findings also indicate a need to further study the relationship between accumbal dopamine and the cues present during the transfer of the rodents into the operant chamber from their home cage. Overall, the studies presented in this dissertation can help us better comprehend mesolimbic dopamine's role in the development of ethanol reinforcement.

Future directions

Our results raise interesting questions that can be investigated with future studies. For example, using microdialysis, we report a transient increase in accumbal dopamine that happens within the first 5 minutes of ethanol self-administration upon a second, but not a first, exposure to ethanol. However, although microdialysis is a practical and useful technique, we cannot say with precision when during those 5 minutes the change in dopamine is occurring. In order to further explore this issue, we would need to use a technique like fast-

scan voltammetry, which would allow us to analyze dopamine at a sub-second time scale. It would be interesting to determine at what time-scale dopamine increases occur during day 2 of ethanol exposure and if there is a response during the first day of ethanol self-administration that occurs too rapidly to be seen with microdialysis.

In addition, as discussed above, there are some discrepancies between some of the studies in our lab regarding the increase in dopamine observed during the transfer of the animal from the home cage into the operant chamber and the following wait period in the operant chamber before having access to the drinking solution. This question can be addressed by doing two things. One, the new self-administration protocol (chapter 3) should be used to examine the dopamine signal during the transfer and wait periods in three groups: an ethanol, a water, and a handling group. Second, the modified version of the new self-administration protocol, which is used and described in chapter 4 of this dissertation, should be used to look at the dopamine response in three groups: day 2 ethanol, day 2 sucrose, and handling group. A larger number of subjects per group (i.e., n=15) should also be used to increase the statistical power. This would allow us to see if the number of subjects per group or the varying concentrations of ethanol are interfering with the results.

Previous findings in our lab and the work presented in this dissertation suggest that it is not the pharmacology of ethanol but its sensory stimuli that cause the transient increase in accumbal dopamine during ethanol intake. We attempted to test this hypothesis by developing a placebo spout (Chapter 2) that would allow us to study the relationship between dopamine and the taste and smell of ethanol, but were unable to create an effective design. Using the data we collected, a new and improved placebo spout can be designed that can be used to test if the increase in accumbal dopamine observed during voluntary ethanol consumption is caused by the smell and taste of ethanol.

The 1st placebo spout was not suitable for microdialysis, since we planned for several steps to be performed within a second or two. The 2nd placebo spout was not effective in the behavioral aspect of the study. The animals did not have similar drinking behaviors while consuming 10% ethanol + 10% sucrose versus 10% sucrose. The two designs could be merged and improved to create a potentially successful placebo spout. We could modify two things: the training protocol and the presentation of the ethanol solution.

The rodents may have needed a few more days of ethanol exposure in their training protocol. Human studies have shown that moderate drinkers have reported to be and acted intoxicated when given a drink that only contained traces of alcohol but that the subjects believed to be an alcoholic beverage

(Gilbertson et al., 2009; Corbin et al., 2008). We could apply the training protocol used for the first placebo spout design (Carrillo et al., 2008) and simply extend the number of days of ethanol self-administration so that the rats can be "moderate" drinkers. The rodents would be exposed to a single concentration of ethanol and be given more days to experience the pharmacological effects of ethanol.

We could also improve the delivery method of the ethanol solution. Rats have been known to self-administer gelatin shots made from 10% ethanol (w/w) and 10% polycose (Peris et al., 2006; Li et al., 2008, 2010). We could replace the 10% ethanol + 10% sucrose solution with 10% ethanol + 10% polycose gelatin shots. After the animals meet the required response, gelatin would be delivered, instead of a drinking solution. If we allow the gelatin to be more liquid than solid, we could deliver it following the same procedure we used for the second placebo spout, using a syringe and a pump. Gelatin is heavier than the 10% ethanol + 10% sucrose solution so it would not flow as fast through the pump, limiting the overflow. The consistency of the gelatin is also different than that of the 10% ethanol + 10% sucrose solution, and it might be able to better disguise the removal of ethanol from the gelatin.

Furthermore, if the placebo spout is successful, we can directly test if it is the taste and smell of ethanol that are serving as cues and causing the increase in

accumbal dopamine that is seen during the first 5 minutes of ethanol self-administration. If our hypothesis is correct, we could also use voltammetry to study the time course of the dopamine response and investigate if there is a shift in the dopamine signaling from reward (ethanol) to cue (sensory stimuli). We could potentially help tease out how cue-induced dopamine release works.

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