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DOPAMINE CONCENTRATIONS IN NUCLEUS ACCUMBENS SUBREGIONS ARE DIFFERENTIALLY AFFECTED BY ETHANOL ADMINISTRATION

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by

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DEDICATION

I dedicate this work to my parents, Melanie Denton & Heiri Gugger, Jim & Debbie Pelky, and Fon Denton, for their unending support. I also dedicate this work to my loving husband, Eddie Howard, for being a continuous source of strength and courage.

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DOPAMINE CONCENTRATIONS IN NUCLEUS ACCUMBENS SUBREGIONS ARE DIFFERENTIALLY AFFECTED BY ETHANOL ADMINISTRATION

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Dopamine increases in the nucleus accumbens after contingent and non-contingent ethanol administration in rats, but the contributions of the core, core-shell border, and shell subregions to this response are unclear. Also, it is not fully understood if increases in dopamine under these circumstances are due to the pharmacological effects of ethanol, stimuli associated with administration, or both. The studies presented in this dissertation were conducted to investigate dopamine's role in each of these accumbal regions during ethanol administration and presentation of associated stimuli. Using microdialysis, ethanol and dopamine concentrations in accumbal subregions were measured every five minutes before, during, and after either experimenter-delivered intravenous ethanol or operant ethanol self-administration. After intravenous ethanol infusions, the increase in dopamine in the shell of the accumbens was significantly higher

than that observed in the core. During operant ethanol self-administration, the core, core-shell border, and shell, all exhibited significant increases in dopamine during transfer of the animal into the operant chamber, with animals trained to drink sucrose + ethanol showing significantly higher increases when compared to those trained to drink sucrose alone. Dopamine increased significantly only in the core-shell border during ethanol consumption, and dopamine levels in the core and shell responded in a similar manner during all phases of the experiment. Together, these results suggest that dopamine responses to intravenous ethanol infusions and operant ethanol self-administration are subregion specific. Also, while increases in dopamine resulting from intravenous ethanol infusions in naïve animals appear to be due to the pharmacological effects of the drug, increases in ethanol-experienced animals during transfer into the operant chamber, and during ethanol consumption, may also be due to stimuli associated with ethanol administration.

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Chapter One: General Introduction

The objectives of this dissertation were (1) to determine the effect of non-contingent intravenous ethanol administration on extracellular dopamine concentrations in the nucleus accumbens core and shell and (2) to determine the effect of operant ethanol self-administration on extracellular dopamine concentrations in the nucleus accumbens core, shell and core-shell border. The subsequent sections of this introduction will review the role of mesolimbic dopamine during ethanol administration and reinforcement, and will serve as the theoretical basis for studying both non-contingent administration and operant self-administration of ethanol.

THE MESOLIMBIC DOPAMINE SYSTEM

The neurotransmitter dopamine has many functions in the brain. Dopamine has been shown to be involved in movement, reward, and reinforcement (Blandini et al., 2000; Horvitz, 2000), and changes in dopamine signaling can result in disruptions of these processes, such as Parkinson's disease and drug abuse and addiction (Robinson and Berridge, 1993; Blandini et al., 2000). The dopamine pathways for movement, such as the nigrostriatal dopamine pathway, appear to be distinct from the pathways for reward and reinforcement, such as the mesolimbic dopamine pathway (Gardner and Ashby, 2000). While the nigrostriatal pathway has been shown to be involved in goal-directed and stimulus-response behaviors (reviewed by Packard and Knowlton, 2002; Yin et

al., 2008) the rest of this introduction will focus on the mesolimbic dopamine system and its role in ethanol reinforcement.

ANATOMY AND CELLULAR PROCESSES OF THE MESOLIMBIC DOPAMINE SYSTEM

The mesolimbic dopamine pathway consists of a group of neurons that project from the midbrain region known as the ventral tegmental area (VTA) (Fallon and Moore, 1978b; Gessa et al., 1985; Ikemoto, 2007). The projections of these cells make up one component of the medial forebrain bundle (MFB), and terminate at the nucleus accumbens (NAcc); however, the VTA also sends projections to the prefrontal cortex, olfactory tubercule, amygdala, and the hippocampus (Fallon and Moore, 1978a; Fallon and Moore, 1978b; Fallon et al., 1978; Swanson, 1982). Major output pathways for the nucleus accumbens include the ventral pallidum, substantia nigra, and the VTA (Heimer et al., 1991; Zahm and Brog, 1992; Zahm, 1999; Zhou et al., 2003). While many studies implicate dopamine in other terminal regions of the mesolimbic pathway in reinforcement (Hodge et al., 1996; Roberts et al., 1996; Samson and Chappell, 2001; Vorel et al., 2001; Melendez et al., 2003), involvement of dopamine in the NAcc is much more established and will be the focus of this introduction.

The nucleus accumbens is comprised mostly, approximately 90% of cells, of medium spiny neurons (MSNs) (O'Donnell and Grace, 1993; Heimer et al., 1997),

GABAergic cells that are bathed in dopamine as a result of innervation by dopaminergic cells from the VTA (Fallon and Moore, 1978b; Brog et al., 1993). The MSN is thought to integrate convergent synaptic signals innervating the NAcc, such as glutamatergic cortical afferents and dopaminergic VTA afferents. The MSN has a single inhibitory output to either the VTA, or pallidal areas, such as the ventral pallidum and entopeduncular nucleus. Then the pallidal structures and VTA project to areas of the thalamus, pre-motor and motor areas (Chang and Kitai, 1985; Heimer et al., 1991; Zahm and Brog, 1992; Zahm, 1999).

Extracellular dopamine concentrations in the NAcc are a result of the balance between exocytotic release of dopamine from the terminals of VTA neurons and uptake by dopamine transporters (Nirenberg et al., 1997). Firing of VTA cells may be a major contributor to the resulting extracellular dopamine concentrations in the NAcc, as it has been shown to increase dopamine transients in this terminal region (Floresco et al., 2003; Sombers et al., 2009). The activity of these cells fluctuates between single-spike action potentials, and periods of rapid bursts. Basal levels of dopamine in the NAcc result from the single-spike, spontaneous, pacemaker-like, VTA firing of approximately 5 Hz, while the transient bursts increase firing to 15-20 Hz (Inoue, 2000; Hyland et al., 2002; Shen, 2003). The location of dopamine transporters (DATs) outside the synapse allow for tonic concentrations of dopamine in the extracellular space of approximately 4 – 10 nM (Parsons and Justice, 1992; Crippens et al., 1993;

Chefer et al., 2003; Tang et al., 2003a). When environmental stimuli are encountered, or drugs of abuse are administered, burst firing of VTA neurons leads to saturation of the DAT from a transient increase in DA concentrations, or a phasic dopamine signal (Gonon, 1988). Gonon (1988) estimated that the phasic signal can lead to a concentration up to 6 times greater than basal levels. The literature indicates that both tonic and phasic dopamine signaling are important for drug related behaviors and reinforcement, and that dopamine levels that occur after bursting may be an important mechanism by which dopamine cells modulate accumbal cell signaling (Rebec et al., 1997; Grace, 2000; Robinson et al., 2001; Robinson et al., 2002b).

Whether dopamine neurons are exhibiting tonic or phasic firing, the signal is relayed in the same way. First, the transmitter is released from vesicles that dock at the cell terminals during exocytosis and diffuses into the synaptic cleft (Garris et al., 1994). Then, dopamine signals are transduced by G-protein coupled receptors: D1-like (D1 and D5) and D2-like (D2, D3, and D4). D1-like receptor activation is excitatory and stimulates adenylate cyclase, whereas D2-like receptors inhibit adenylate cyclase.

Anatomy and cellular processes in the core, core-shell border, and shell

The nucleus accumbens is a heterogeneous structure, and has been described as having many anatomically distinct subregions, including the rostral pole, cone,

core, shell, and more recently the core-shell border or “shore” (Heimer et al., 1991; Zahm and Brog, 1992; Prinssen et al., 1994; Heimer et al., 1997; Rebec et al., 1997; Zahm, 1999; Hipolito et al., 2008). Separation of the nucleus accumbens into subregions was first foreshadowed in the late 1970s, when projections to and from the accumbens were observed to differ depending on accumbal location (Nauta et al., 1978). In 1985, Zoborszky et al. described a “central core of densely packed cells which encircle the anterior commisure” which was “surrounded by an outer shell with somewhat larger and more loosely arranged cells”. In 1994, Prinssen et al. were the first to study the core-shell border as a separate subregion, and coined it the “shore”. These subregions, especially the core and shell, have been since well defined through the use of histochemical markers, behavioral functions, dopamine responses, as well as anatomical connectivity (Zahm and Brog, 1992; Zahm, 1999; Zahm, 2000). For example, the core of the accumbens stains much more darkly for calbindin than the shell (Meredith et al., 1996; Brauer et al., 2000). However, it is accepted that these subregions themselves are not homogeneous and that further divisions may be possible. In support of this idea, dopamine in the lateral and medial shell has been shown to respond differently to administration of drugs (Heimer et al., 1997). The scope of this introduction includes only the core, shell, and core-shell border subregions. The core of the nucleus accumbens is more lateral and dorsal than the shell, which can wrap around the medial and ventral edge of the core

(Figure 1). However, the exact shape and location of these structures depends upon the specific anterior-posterior location.

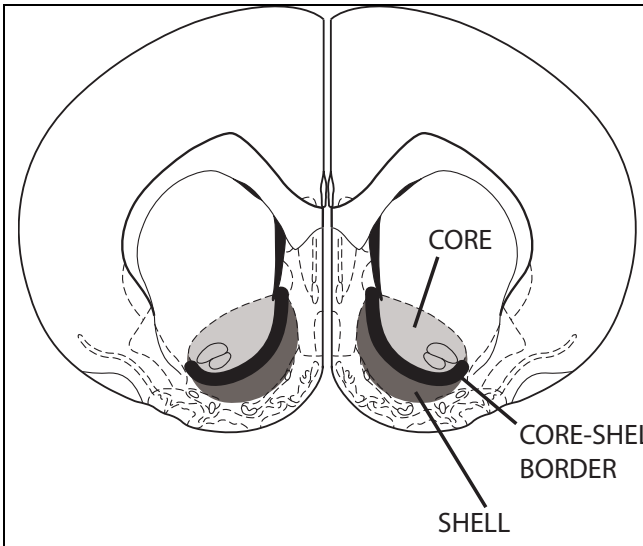

Figure 1. Core, Core-Shell Border and Shell of the Nucleus Accumbens. Core is shaded in light grey, core-shell border is denoted by a thick black line, and shell is shaded in dark grey. These subregions are shown on a coronal slice of a rat brain (Bregma 1.9) in a figure adapted from Paxinos and Watson, 1998.

The core and shell are known to differ in their anatomical connectivity (Zahm and Brog, 1992; Zahm, 2000; Ikemoto, 2007). The core has been described as using "typical" basal ganglia pathways to premotor and motor cortex areas (Zahm and Brog, 1992). The core connects more reciprocally to these areas of the brain that control motor function, like the dorsal striatum, whereas the shell is reciprocally connected to "basal-ganglia like" areas as well as the extended amygdala, an area involved in emotional responses, (Zahm and Brog, 1992; Zahm, 2000), and the lateral hypothalamus, an area reported to play a role in hedonic processing of both natural rewards and drugs of abuse (Befort et al., 2008). While dopamine in the core-shell border has been shown to respond differently from the core and

shell (Rebec et al., 1997), it is not yet known if this area has different anatomical connectivity from these two subregions.

The core and shell also appear to differ in cell morphology, transporter density, and receptor type and distribution. For example, MSNs in the shell are 15 percent smaller and have 25 percent fewer dendrites and dendritic spines than those in the core (Meredith et al., 1992; Meredith et al., 1995). Dopamine transporter density is two times greater in the core than in the shell (Jones et al., 1996). Also, the dopamine type 1 receptor (D1) is expressed more densely (30%) in the shell, while D2 is expressed more (30%) in the core of the accumbens (Lu et al., 1998). The shell stains more for the mu opiate receptor than the core (Heimer et al., 1997), while there are more GABA-A receptors in the core when compared to the shell (Churchill et al., 1992). Characteristics of the core-shell border are less defined, as this is a newly recognized subregion of the nucleus accumbens.

BEHAVIORAL SIGNIFICANCE OF MESOLIMBIC DOPAMINE

The numerous target regions for mesolimbic dopamine signaling, and the web of connections between these areas, suggests that dopamine could be involved in a wide array of behaviors. The following sections on the behavioral significance of mesolimbic dopamine focus on behaviors involved in associative learning processes, such those occurring after incentive, aversive, and novel stimuli, as

these are most relevant to the processes involved in drug administration and reinforcement.

Dopamine responses to salient stimuli

Dopamine neurons in the VTA have been shown to respond for events that derive salience from physical sensory characteristics, such as loud clicks or a bright light flashes (Horvitz et al., 1997). These stimuli are not associated with a reinforcer, and indicate that dopamine can serve as a signal to indicate the importance of an event. Dopamine responses to incentive, aversive, and novel stimuli may be due to the salience of each type of stimulus. Because dopamine increases after salient events, these stimuli could later act as cues if they were repeatedly experienced prior to a reward or reinforcer.

Dopamine responses to incentive stimuli

Neuron firing in the VTA and accumbal extracellular dopamine concentrations have both been measured during presentation of incentive stimuli. However, while dopamine increases in the nucleus accumbens have been shown to result from neuronal activity in the VTA (Sombers et al., 2009), the relationship between VTA neuron firing and accumbal dopamine release has not been defined. Firing rate may not be directly correlated with dopamine release because of many pre-synaptic mechanisms. For example, autoreceptors at dopamine terminals (Kohl et al., 1998), glutamatergic inputs from basolateral

amygdala to the NAcc (Howland et al., 2002), and pre-synaptic opioid and nicotinic receptors (Zhou et al., 2001; Rice and Cragg, 2004; Britt and McGehee, 2008), have all been suggested to modulate release. As a result, neuron firing rate and dopamine release are presented separately below.

The idea that midbrain dopamine neurons are activated by stimuli or cues that predict the availability of an incentive was first put forth by studies that investigated the effect of cues associated with food reward. For example, transient increases were observed in VTA neuron firing when primates were presented with a liquid reinforcer (Schultz et al., 1986). A cue light indicated the availability of the reinforcer, and after repeated training sessions, stimulation of dopamine cell firing occurred at the time of the presentation of the predictive cue and not at the time of the reinforcer. Also, it was shown in another set of experiments that the increase in dopamine cell firing resulting from the liquid reinforcer was related to the reliability of the availability of the reinforcer (Hollerman and Schultz, 1998). In addition, Nishino et al. (1987) showed that VTA neuron firing increased during lever-pressing for food, but returned to baseline when food was presented.

Studies measuring accumbal dopamine concentrations also observe increases during the presentation of incentive stimuli. For example, the experiments of Stuber et al. (2008) support the idea of dopamine playing a role in signaling

impending presentation of a reinforcer. During daily sessions, a cue-light was illuminated before the delivery of a sucrose pellet. After five days, the transient increase in dopamine that was originally observed at the time of the sucrose pellet was now present at the time of the cue light. Roitman et al. (2004) also showed that dopamine transients increased during presentation of a cue predicting food but not when food was available.

It is important to note that the motivational state of the animal influences the dopaminergic responses to incentive stimuli. It stands to reason that the more motivated an animal, the larger the incentive of a reinforcer (Kelley and Berridge, 2002). For example, depriving an animal of food increases their motivational drive and is known to affect mesolimbic dopamine activity (Wilson et al., 1995). Also, oral self-administration of sucrose has been shown to increase extracellular dopamine concentrations in microdialysis studies when an animal is food or water deprived (Hajnal and Norgren, 2001; Hajnal et al., 2004; Genn et al., 2004). However, when animals are allowed access to food and water *ad libitum*, no significant increase in dopamine is observed during consumption of sucrose (Doyon et al., 2005). Fast-scan cyclic voltammetry has also shown that dopamine transients do not increase in unrestricted rats during sucrose consumption in an operant paradigm (Roitman et al., 2004).

Dopamine responses to aversive stimuli

Dopamine has been shown to increase in the accumbens during aversive stimuli (Louilot et al., 1986; Doherty and Gratton, 1997; Bertolucci-D'Angio et al., 1990; Imperato et al., 1991; Sorg and Kalivas, 1991; McCullough and Salamone, 1992; Young et al., 1993; Weiss et al., 1997), and animals may encounter stimuli of this type in the experiments presented in this dissertation. For example, the animals receiving intravenous ethanol infusions may experience pain and cardiovascular stress associated with ethanol administration. In addition, the animals self-administering oral ethanol may experience stress during transfer into the operant chamber and may find the taste and smell of ethanol aversive. Therefore, it is important to account for effects of aversive stimuli while interpreting the results of these experiments.

Dopamine increases in the accumbens have been observed using microdialysis and voltammetry in response to aversive events, such as foot shock, tail shock, tail pinch, restraint stress, and the administration of anxiogenic drugs (Louilot et al., 1986; Doherty and Gratton, 1997; Bertolucci-D'Angio et al., 1990; Imperato et al., 1991; Sorg and Kalivas, 1991; McCullough and Salamone, 1992; Young et al., 1993; Weiss et al., 1997). For example, Young (2004) used one-minute microdialysis samples to show that footshock, and the associated cue, increases dopamine in the NAcc. Also, differences in dopamine responses in the core and shell have been observed after exposure to an aversive stimulus. For example,

microdialysis samples collected after mild footshock elicited increases in dopamine in the shell of the accumbens, but not the core (Kalivas and Duffy, 1995).

Not all studies show an increase in accumbal dopamine in response to aversive stimuli, and methodological differences may explain the contradictory findings. For example, some single-unit recordings of VTA dopamine neurons show increased firing to aversive stimuli (Kiaytkin, 1988), and some single-unit studies do not observe this increase (Mirenowicz and Schultz, 1996). While both microdialysis and voltammetry sample from groups of neurons, single-unit recordings are from single cells. Subsets of cells have been shown to release dopamine differently from one another (Wightman et al., 2007), and dorsal VTA neurons have been shown to respond differently than ventral VTA cells to footshock (Brischoux et al., 2009). Therefore, it is possible that single-unit recordings are picking up different responses from dissimilar cells. The average of the responses of these many types of singe cells may yield the dopamine increase picked up by microdialysis and voltammetry.

Some controversy exists as to whether dopamine increases after aversive events are due to the onset of the stimulus, or the negative reinforcement that occurs at the offset of the aversive stimulus. This idea is supported by findings that rewarding and aversive taste stimuli elicit opposite patterns in dopamine release

during, and for 20 seconds after, an oral infusion, with sucrose increasing and quinine decreasing dopamine transients (Roitman et al., 2008). On the contrary, when aversive conditions are present for an entire microdialysis sampling period (60-120 min), increases in DA concentrations have been observed to increase before offset of the event (Imperato et al., 1991; Bradberry et al., 1991; Puglisi-Allegra et al., 1991). It is possible that the offset of acute aversive stimuli are responsible for increases in DA, while chronic, uninterrupted, aversive events lead to eventual increases in dopamine concentrations.

Dopamine responses to novel stimuli

As previously discussed, dopamine concentrations have been shown to increase when salient stimuli are encountered, so it stands to reason that novel stimuli would have a similar effect. Methods like fast-scan cyclic voltammetry and single-unit recording have been used to show increased firing and dopamine concentrations for brief periods after novel stimuli are encountered (Ljungberg et al., 1992; Rebec et al., 1997; Rebec, 1998). For example, single-unit recordings have shown dopamine responses in animals when a novel compartment door is opened while animals exhibit target-directed saccades. Once this ocular reaction ends, the dopamine response is no longer observed (Ljungberg et al., 1992). Also, increases in dopamine efflux observed using fast-scan cyclic voltammetry in the accumbens shell when animals entered a novel environment were confined to the brief entry period (8 sec) (Rebec et al., 1997). These brief

increases may be large enough to account for increases observed using methods with poor temporal resolution, like microdialysis. For example, when an intraoral chocolate-sucrose solution is delivered for the first time to rats, an increase in dopamine is observed in the nucleus accumbens core and shell (Bassareo et al., 2002). When the same solution is given to a pre-exposed rat, the increase is no longer observed in the shell, but is still observed in the core. The differential roles of dopamine in the nucleus accumbens core and shell will be discussed, and may account for this difference.

THE ROLE OF DOPAMINE IN DRUG REINFORCEMENT

The exact function of increased dopamine signaling in the mesolimbic pathway, specifically increases in accumbal dopamine, is not yet known. Many hypotheses about the role of dopamine exist, including control of movement, reward, motivation, hedonic impact, emotional processing, learning, and attention (Berridge and Robinson, 1998; Gonzales et al., 2004). It has been difficult to define dopamine's functions, in part, because of the great degree of overlap of these concepts. For example, in order to exhibit behaviors associated with motivation, both learning and attention may be employed as well (Berridge and Robinson, 2003).

A theory proposed in 1978 by Wise focused on dopamine signaling as a mediator of hedonic processes, that dopamine systems mediate the pleasure experienced

after natural and drug rewards; however, many studies dispute this theory. For example, dopamine systems have been shown to be activated in anticipation of a reward (reviewed by Berridge and Robinson, 1998). An argument can be made that the cues associated with the reward become rewarding themselves, but after repeated exposures the increase in dopamine at the time the cue is presented appears larger than the increase at the time of the reward during the initial presentation (Stuber et al., 2008). It seems unlikely that the cue predicting reward could be more pleasurable than the reward itself. Dopamine systems, in some cases, are also activated during acquisition of a rewarding behavior, but not once the behavior is established (Garris et al., 1999). Lastly, mice that cannot synthesize dopamine still show preference for sucrose when compared to water (Cannon and Palmiter, 2003). This indicates that the experience was rewarding despite being severely depleted of dopamine. Taken together, the findings of these studies indicate that dopamine is not directly mediating hedonic processing. Three contemporary theories about the role of accumbal dopamine that have gained prominence are discussed below.

Dopamine and Associative Learning

The first time the pharmacological effects of a drug are experienced, associations between these effects, like euphoria, and the drug start being formed in a process known as associative learning. In this instance, the drug is a powerful reinforcer and increases the probability that an individual will administer the drug

again. Increases in accumbal dopamine may function to form, and later strengthen, the association between the effects of the drug and the stimuli associated with administration (Di Chiara, 2002).

This theory is based on studies that show dopamine in the accumbens shell increases during initial administration of a drug (Pontieri et al., 1995; Pontieri et al., 1996), and that this increase does not habituate after repeated exposures to the same drug (Di Chiara, 2002). In this way, dopamine increases resulting from drugs of abuse, differ from those that result from natural reward (Bassareo and Di Chiara, 1999a; Bassareo et al., 2002). Therefore, if increases in dopamine serve to strengthen associations, those made for drugs of abuse would be much stronger than those resulting from naturally occurring reinforcers. However, it has been argued that an intact dopamine system is not required for these associations to be learned (Berridge and Robinson, 1998). This finding would not refute the associative learning theory, as it is possible that dopamine signaling is involved in, but not required for, associative learning to occur.

Incentive Salience

Another theory about the role of dopamine in drug abuse focuses on brain changes in assigning significance to a stimulus after repeated administration of a drug. The stimulus associated with the drug becomes a salient “incentive” that is sought out much more intensely than those associated with natural reinforcers,

like sleep, food and water. This theory known as “incentive – sensitization” asserts that repeated drug results in a sensitization of the neural systems responsible for assigning salience, or importance, to incentives (Robinson and Berridge, 1993). The authors define drug craving as intensely “wanting” drugs, and separate this motivational state from “liking”, which they define as finding something pleasurable. By separating these concepts, the hedonic value of the drug is not altered by repeated drug exposure, while brain mechanisms, specifically the mesolimbic dopamine pathway, assigning incentive salience are enhanced.

The incentive-sensitization view of drug addiction has strengths and weaknesses. The major strength is that it explains the compulsive drug-taking behavior that occurs in spite of decreasing reward from drugs. However, one weakness is that the results that support this theory are, for the most part, from studies using psychostimulants that have a strong effect on dopamine signaling. It is not clear if these findings are generalizable to other drugs of abuse that do not have as dramatic of an effect on dopamine, like alcohol. Also, the exact neurobiological mechanisms underlying “wanting” and “liking” have not been elucidated, and more research in this area is needed.

Reward Prediction

An alternate view of dopamine's functions in drug abuse focuses on prediction of the availability of a reinforcer. This theory asserts that an individual learns about reward availability by monitoring errors in prediction about availability, and that the mesolimbic dopamine pathway is crucial in this process. Findings from electrophysiological studies of VTA dopamine neurons during exposure to natural rewards were the basis for suggesting that these cells code for prediction of reward (Schultz et al., 1997). While presentation of natural reward increased neuron firing (Mirenowicz and Schultz, 1994; Hollerman and Schultz, 1998), repeated pairing of cues with reward resulted in a change of the timing of VTA dopamine neuron firing. After pairing, neurons fired at the time of the presentation of the cue and not at the time of presentation of the reward (Schultz et al., 1997; Hollerman and Schultz, 1998). Contemporary studies have also corroborated these findings, while proposing that this pairing occurs in as few as five pairings of the cue with the natural reward (Stuber et al., 2008). These results support the theory that dopamine neurons are coding for predictions about rewards. On the first exposure, the reward is unexpected, and dopamine neurons fire to signal an error in prediction. Once the cue is associated with the reward, the reward does not elicit an increase in firing as its availability was correctly predicted. However, the conditioned cue elicits firing as it is now the unpredicted event.

This theory, like the others discussed, has advantages and disadvantages. Application of the reward prediction theory is that it can be directly applied to learning theory and can be used to predict and test quantifiable behaviors (Schultz et al., 1997; Schultz, 2002; McClure et al., 2003). However, the results of the studies that are the basis of this theory are based on cell firing, and do not give any information about dopamine release at cell terminals. Also, 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), which has been shown to change motivational drive and may confound results.

THE ROLE OF DOPAMINE IN THE NUCLEUS ACCUMBENS SUBREGIONS

The role of accumbal dopamine concentrations in drug abuse may differ depending on the subregion of interest. As discussed earlier, the nucleus accumbens core and shell have been shown to have different anatomical connectivity, cell morphology, transporter density, and receptor type and distribution. These differences indicate that dopamine in the core and shell may play different roles in the reinforcing effects of drugs of abuse. While less is known about the core-shell border of the accumbens in terms of anatomy and cellular processes, recent work shows that dopamine in this area responds differently, when compared to the core and shell, to novelty (Rebec et al., 1997). As a result, the role of dopamine in drug abuse should be studied in each of

these subregions separately from one another. Some work has been done to this effect, and will be discussed below.

The role of dopamine in the nucleus accumbens core

As indicated by the anatomical connectivity of the accumbens core with the mesolimbic pathway, including the VTA as well as the dorsal striatum and other brain regions in the basal ganglia, dopamine in the core has been shown to be involved in the motor aspects of motivation, such as the acquisition and expression of instrumental behaviors. For example, a study by Garris et al. (1999) reported that an increase in dopamine in the core was required for acquisition of responding for intracranial stimulation. Also, injections of the neurotoxic agent 6-hydroxydopamine into the core significantly impaired the expression of instrumental responding in an operant paradigm for food reward (Sokolowski and Salamone, 1998).

Dopamine in the core has also been implicated in associative conditioning. Specifically, dopamine in this subregion increases when conditioned stimuli associated with a reinforcer are present. For example, core dopamine responds to cues associated with food or drugs of abuse. Bassareo and Di Chiara (1999a) used microdialysis to show that dopamine increased in the core of animals exposed to the sight and smell of the food reward, and Day et al. (2007) used fast scan cyclic voltammetry to show that dopamine transients increased at the

time conditioned stimuli associated with food reward were presented. Cocaine associated cues have also been shown to increase dopamine in this subregion of the accumbens (Ito et al., 2000).

Core dopamine also responds to initial presentations of a reinforcer. For example, dopamine in the core has been shown to respond during non-contingent administration of ethanol, amphetamine, or cocaine in naïve rodents (Zocchi et al., 2003; Giorgi et al., 2005), and dopamine increases in this subregion during an initial presentation of food reward (Bassareo and Di Chiara, 1999a; Bassareo et al., 2002). However, the methods of these studies may have affected the results. For example, Giorgi et al. (2005) used high- and low-avoidance lines of rats, which may have exhibited responses that would not be generalizable to other strains. In addition, Zocchi et al. (2003) investigated core and shell dopamine using microdialysis in mice, and it is unlikely that these probes sampled exclusively from the subregion of interest. It is also important to note that in many of these studies, the dopamine response in the shell is significantly greater than that in the core (Bassareo and Di Chiara, 1999a; Giorgi et al., 2005), indicating that dopamine in the shell may be more involved than the core in novelty.

The core of the accumbens has been implicated in instrumental behavior and associative conditioning; however, neither of these behavioral processes

explains the increase in this subregion during an initial presentation of a reinforcer. The idea that the core may be involved in both acquisition and expression of instrumental behaviors could explain the increase. The dopamine response may be involved in the formation of associations necessary to express instrumental behaviors and to respond to conditioned stimuli. Also, the increase may be explained by Schultz's theory of reward prediction that accumbal dopamine responds to unpredicted rewards.

The role of dopamine in the nucleus accumbens shell

Studies indicate that dopamine in the nucleus accumbens shell is involved in conditioned stimuli, in a similar manner to that in the core. For example, dopamine in both the shell and core responds to conditioned stimuli (Cheng et al., 2003; Bassareo and Di Chiara, 2007). However, it should be noted that the schematic representations in both these studies indicated that the microdialysis probes appeared to sample from regions above and below the core or shell, such as the olfactory tubercule, ventral pallidum, and dorsal striatum, as well as the core-shell border. As a result, dopamine in these other regions may be contributing to the response. Also in Cheng et al. (2003), increased motivation resulting from food deprivation may be increasing accumbal dopamine responses (Wilson et al., 1995; Bassareo and Di Chiara, 1999b; Carr et al., 2009). In addition, a lack of an increase in dopamine in the shell has been reported during presentation of cocaine-associated cues (Ito et al., 2000). As a result of these

inconsistencies, more studies are needed to further investigate whether dopamine in the shell responds to conditioned stimuli.

Dopamine in the shell has also been shown to respond to the presence of novel stimuli. For example, when entering a novel environment, dopamine transients increase in the shell during the brief period of entry (Rebec et al., 1997). Also, Bassareo and Di Chiara (1999b) reported that dopamine increased in the accumbens shell during consumption of a novel food reward, but not when that food had been consumed 24 hours earlier, indicating that the novel aspect of the reward was responsible for the increase in dopamine.

Many studies investigate dopamine in the core separately from that in the shell during administration of drugs of abuse. However, so far the literature has not shown definitively that dopamine levels in these two subregions are responsible for separate sets of behaviors. This dissertation aims to test whether dopamine in the core plays a different role from that in the shell during ethanol administration.

The role of dopamine in the nucleus accumbens core-shell border

Very little is known about dopamine in the core-shell border (Rebec et al., 1997). The first study to investigate dopamine in the core-shell border observed a difference in oral behavior after microinjections of dopamine receptor agonists into the core and core-shell border when compared to the shell (Prinssen et al.,

1994). However, the injection sites denoted as in the border area are now in an area commonly thought to be the core of the accumbens. More recent papers indicate that dopamine may respond differently in the core-shell border when compared to either the core or shell. For example, in animals entering a novel environment, dopamine responds differently in the core-shell border when compared to the core and shell. Increases in transients in this border region were less rapid and more prolonged than those in the shell, and no change in number of transients occurred in the core (Rebec et al., 1997). Also, opioid agonists have been shown to affect dopamine in these accumbal subregions differently (Hipolito et al., 2008). However, this effect was only observed 20 minutes after the agonists were no longer being perfused, which brings into question whether this was a result of these pharmacological agents or experimental error. These studies indicate that dopamine may play a unique role in the core-shell border; however, more research is needed to define its exact functions.

OPERANT REINFORCEMENT

Reinforcement is defined as an increase in the frequency or probability of a particular behavior (the operant response) after presentation of a given stimulus (Skinner, 1938). While reinforcement can be classified as negative or positive, depending on the stimulus, this introduction will focus on positive reinforcement as drugs of abuse like ethanol have incentive value. Because reinforcement requires a behavioral response that can be measured, it is a concept that can

easily be studied through experimental analysis. These types of experiments are widely used to measure of the incentive value of drugs, most often using an operant procedure. During operant reinforcement, the animal is required to perform a specific behavior, such as a lever-press, nose poke, or the turn of a wheel, in order to gain access to the reinforcer. Behavioral and consumption parameters associated with the operant procedure, such as time it takes to perform the response requirement, the amount of the drug administered, and the number of times the drug is administered, can be used to measure the motivational state of the animal.

Dopaminergic transmission during operant reinforcement

Operant self-administration studies are useful to investigate dopamine's function in reinforcement, and have supported the hypothesis that dopamine is involved in both the reinforcing properties of drugs of abuse and food reward. For example, dopamine increases in the nucleus accumbens during operant self-administration of food reward (Sokolowski et al., 1998), ethanol (Weiss et al., 1993; Weiss et al., 1996; Gonzales and Weiss, 1998; Melendez et al., 2002; Doyon et al., 2003; Doyon et al., 2005), cocaine (Weiss et al., 1992; Hemby et al., 1997), heroin (Caille and Parsons, 2003), and nicotine (Kiianmaa et al., 2000; Cohen et al., 2002). However, increases in dopamine in this paradigm may be due to several features of operant self-administration, such as pharmacological effects of a drug or reinforcing effects of a food reward, or cues associated with administration of

the reinforcer, and work is being done to tease apart the contribution of each of these aspects. This will be discussed further in the operant ethanol self-administration section.

Dopamine during several phases of operant self-administration of a reinforcer has been investigated using microdialysis, including baseline, transitionary, anticipatory, appetitive, and consummatory periods. Modifications in the experimental design allow for each of these phases to be separated out into distinct epochs. For example, samples taken during the baseline period are used as a basis for comparison for dopamine concentrations achieved during other phases, such as responding or consumption. Also, many studies include a wait period after transferring the animal into the operant chamber and before access to the lever (Weiss et al., 1993; Gonzales and Weiss, 1998; Melendez et al., 2002; Doyon et al., 2003; Doyon et al., 2005). This allows researchers to measure dopamine increases that result from handling and anticipation separately from those that result from responding or consumption. In addition, some studies have begun separating out appetitive behaviors, such as responding by lever-pressing, from consummatory behaviors, such as drinking of fluids (Doyon et al., 2003; Doyon et al., 2005). In this way, dopamine responses due to behaviors associated with each phase can be teased apart from one another. For example, many studies have shown that dopamine increases during transfer into the operant chamber (Doyon et al., 2003; Doyon et al., 2005),

anticipation of self-administration (Weiss et al., 1993; Gonzales and Weiss, 1998; Melendez et al., 2002), and consumption of reinforcers (Doyon et al., 2003; Doyon et al., 2005).

MESOLIMBIC DOPAMINE AND ETHANOL

Pathways other than the mesolimbic dopaminergic system have been implicated in ethanol self-administration (Roberts et al., 1996; Thiele et al., 1998). However, many studies implicate dopamine in drug reinforcement in general (Roberts et al., 1980; Bozarth and Wise, 1981), suggesting that dopamine may play a role in the reinforcing properties of ethanol as well. While dopamine in the nucleus accumbens has been implicated in the reinforcing effects of many drugs of abuse, the remainder of this dissertation will focus on ethanol.

Dopamine in the nucleus accumbens has been shown to increase after intraperitoneal ethanol administration (Imperato and Di Chiara, 1986; Carboni et al., 1989; Yoshimoto et al., 1992b; Acquas et al., 1993; Blanchard et al., 1993; Blomqvist et al., 1993; Heidbreder and De Witte, 1993; Kiianmaa et al., 1995; Samson et al., 1997; Tanda and Di Chiara, 1998; Yim et al., 1998; Yan, 1999; Yim et al., 2000). It has also been suggested that this effect is due to an increase in VTA neuron firing (Yim et al., 1998; Brodie et al., 1999). Accumbal dopamine has also been shown to respond to intravenous ethanol and oral self-administration of ethanol, and will be discussed in the sections below.

Pharmacological manipulation of mesolimbic dopamine during ethanol administration

Directly measuring extracellular dopamine concentrations in the nucleus accumbens can yield information about the involvement of dopamine in drug-related behaviors and reinforcement; however, another effective method is to manipulate the mesolimbic dopaminergic system and observe changes in drug-related effects and behaviors. Two common methods of pharmacological manipulation that have been studied are systemic injections and microinjections of dopamine agents. Further clarification of the role of mesolimbic dopamine in ethanol reinforcement can be achieved through manipulations of this kind, and are discussed below.

Systemic injections of dopamine agents have been used to investigate the effect of pharmacological manipulation of the mesolimbic dopamine system as a whole; however, the findings of these studies are often contradictory. For example, systemic administration of direct and indirect agonists, as well as antagonists, has been shown to decrease responding for ethanol (Pfeffer and Samson, 1985; Pfeffer and Samson, 1988; Samson et al., 1993; Rassnick et al., 1993a; Cohen et al., 1998; Files et al., 1998; Cohen et al., 1999; Liu and Weiss, 2002; Price and Middaugh, 2004). Also, systemic injections of dopamine receptor agonists have been shown to significantly increase (D'Souza et al., 2003) and decrease (Cohen et al., 1998; Cohen et al., 1999) self-administration of ethanol.

Microinjections of dopamine agents have been used to test the effects of manipulating dopamine in specific regions that comprise the mesolimbic pathway on ethanol self-administration behaviors. Not surprisingly, the direct pharmacological manipulation in these studies leads to findings that are more in agreement than those in the studies using systemic administration. For example, dopamine agonists in the nucleus accumbens increase responding for alcohol (Hodge et al., 1992), and dopamine antagonists into this region lead to a decrease (Hodge et al., 1997). In the nucleus accumbens, dopamine agonists could increase dopamine signaling and contribute to the enhancement in responding. Dopamine antagonists in this area could result in the opposite effect on responsiveness. Also, microinjections of dopamine agonists into the ventral tegmental area result in dose-dependent decreases in responding (Hodge et al., 1993). This finding is in agreement with the studies of dopamine in the nucleus accumbens, as dopamine agonists in the VTA have been shown to decrease cell firing and could also lead to decreased responding. Microinjections studies indicate that dopamine plays a role in the reinforcing properties of ethanol.

Lesions of mesolimbic dopamine during ethanol administration

Neurotoxin-induced lesions have also shown that manipulations of dopamine in the mesolimbic pathway can affect ethanol reinforcement in contradictory ways. Studies have shown that 6-hydroxydopamine lesions decrease ethanol self-

administration (Myers and Melchior, 1975; Brown and Amit, 1977; Ikemoto et al., 1997), increase ethanol self-administration (Quarfordt et al., 1991), and don't affect ethanol self-administration (Rassnick et al., 1993b; Koistinen et al., 2001; Shoemaker et al., 2002). While lesions are a useful tool to block dopaminergic input to the nucleus accumbens, they may also result in confounds such as effects on other neurotransmitter systems and neural compensatory changes, making the results of these studies difficult to interpret.

Operant ethanol effects on mesolimbic dopamine

Operant ethanol studies are useful to investigate dopamine's function in ethanol reinforcement in a behavioral context, and have supported the hypothesis that dopamine is involved in reinforcement. In general, increases in dopamine concentrations in the nucleus accumbens have been observed during operant ethanol self-administration (Weiss et al., 1993; Weiss et al., 1996; Gonzales and Weiss, 1998; Melendez et al., 2002; Doyon et al., 2003; Doyon et al., 2005), and many studies report that cues associated with administration are responsible for this increase (Doyon et al., 2003; Doyon et al., 2005). However, only one study has tested the effect of operant ethanol self-administration on dopamine in the core, shell, and core-shell border of the nucleus accumbens separately from one another, and will be presented in chapter three of this dissertation.

Operant ethanol self-administration studies often differ in experimental design, and these modifications affect the results. For example, studies differ in strain of rats used, microdialysis probe placements, cues that predict ethanol availability, dialysate sample lengths, concentration of ethanol solutions, ethanol dose administered by the animal, and reinforcement schedules. While these studies agree that dopamine increases in the nucleus accumbens during ethanol self-administration, the magnitude and time point of the response is affected by these variations in design. For example, Melendez et al. (2002) reported a 70% increase in dopamine 30 minutes into access to the drinking solution in alcohol-preferring (P) rats that consumed 1.4 g/kg of 15% ethanol, and Doyon et al. (2005) reported at 20% increase after 5 minutes in Long-Evans rats that consumed 1.6 g/kg of 10% ethanol + 10% sucrose. However, these studies differed in the cues presented, sampling time period, concentration of the ethanol solution, strain of rat, and schedule of reinforcement.

Many operant ethanol self-administration studies have investigated whether increases in accumbal dopamine are due to the pharmacological effects of ethanol or stimuli associated with its administration. Conditioned stimuli associated with administration, such as olfactory cues and illuminated lights have been shown to increase accumbal dopamine (Katner and Weiss, 1999; Melendez et al., 2002). Therefore, it stands to reason that cues directly associated with the ethanol solution, such as taste and smell, may also increase accumbal dopamine

and contribute to the response observed during consumption. This idea is supported by the finding that the increase in dopamine concentrations during drinking is associated with the drinking pattern and not brain ethanol concentrations (Doyon et al., 2003; Doyon et al., 2005).

A major indication of the underlying cause of dopamine responses to ethanol consumption is the time course of the increase in dopamine. For example, if a dopamine peak is observed immediately after the start of consumption before ethanol reaches the brain, it may indicate that this increase is due to conditioned stimuli, such as the taste and smell of the solution. If the peak is observed at a time point when brain ethanol is high, the increase in dopamine may be due to pharmacological effects of the drug. Some studies do report peak dopamine levels 30 minutes after the start of consumption (Weiss et al., 1996; Melendez et al., 2002). However, Weiss et al. (1996) used alcohol preferring rats, and it is not unreasonable that these animals would respond differently from other strains. Lastly, both of these studies used reinforcement schedules that required lever-pressing throughout self-administration. When completion of lever-pressing results in continuous access to the ethanol solution, peak dopamine levels are observed within the first 5 minutes of consumption, a time when brain ethanol is low (Doyon et al., 2003; Doyon et al., 2005).

SPECIFIC AIMS

While the nucleus accumbens has been widely studied during ethanol administration, the relative contributions of the core, core-shell border, and shell of the accumbens to dopamine release still need to be clarified. The core and shell have different anatomical connections, and from what little is known about the core-shell border, it seems that the anatomy of the core-shell border may differ from core and shell. As a result, these subregions may play different roles during ethanol administration. For example, the core may be related to instrumental behaviors and the shell may be related to the acknowledgement of novel stimuli. Both of these behaviors are critical during operant ethanol self-administration; a frequently used behavioral model to study ethanol drinking. However, the core, core-shell border, and shell of the accumbens have not been studied individually during ethanol administration. Therefore, the function of dopamine in these subregions during ethanol reinforcement is not yet known.

The following section represents the specific aims of this dissertation:

1. To determine the effect of non-contingent intravenous ethanol on dopamine in the nucleus accumbens core and shell of naïve rats. Five-minute microdialysis samples will be taken before, during, and after an experimenter-delivered bolus intravenous infusion of 0.5, 1.0, or 1.5 g/kg ethanol, or a volume equivalent of saline for controls. Probes will be placed in either the core or shell of the nucleus accumbens. The effect of

repeated non-contingent intravenous ethanol on dopamine in the nucleus accumbens shell will also be investigated. In these experiments, five-minute microdialysis samples will be taken from probes placed in the nucleus accumbens shell before, during, and after four experimenter-delivered bolus intravenous infusions of 1.0 g/kg. One infusion will be given every fifteen minutes, to reach a cumulative dose of 4.0 g/kg ethanol. Dopamine and ethanol concentrations will be measured using High Pressure Liquid Chromatography and Gas Chromatography, respectively.

2. To determine the effect of ethanol on dopamine in the nucleus accumbens core, core-shell border, and shell during operant self-administration of ethanol of ethanol experienced rats. To evaluate the effect of environmental stimuli associated with operant sessions separately from the consumption of the drinking solution, the experiment will be separated into different phases: baseline, transfer and waiting, drink, and post-drink. Male Long-Evans rats (with a guide cannula above either the core or shell of the accumbens) will be trained to lever-press for 20 minutes of access to either 10% sucrose (10S) or 10% sucrose + 10% ethanol (10S10E) using a modified sucrose-fading protocol. We will gradually habituate the rats to a 15 min wait period across training sessions, which will precede presentation of the lever, and a response requirement of 4 lever-presses. On experiment day, five-min microdialysis

samples will be collected from the core, core-shell border, or shell during the four phases of the session. Dopamine and ethanol concentrations will be measured using High Pressure Liquid Chromatography and Gas Chromatography, respectively.

Chapter Two: The shell of the nucleus accumbens has a higher dopamine response compared with the core after non-contingent intravenous ethanol administration

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ABSTRACT

Dopamine increases in the nucleus accumbens after ethanol administration in rats, but the contributions of the core and shell subregions to this response are unclear. The goal of this study was to determine the effect of various doses of intravenous (i.v.) ethanol infusions on dopamine in these two subregions of the nucleus accumbens. Male Long-Evans rats were infused with either acute i.v. ethanol (0.5, 1.0, 1.5 g/kg), repeated i.v. ethanol (four 1.0 g/kg infusions resulting in a cumulative dose of 4.0 g/kg), or saline as a control for each condition. Dopamine and ethanol were measured in dialysate samples from each experiment. The *in vivo* extraction fraction for ethanol of probes was determined using i.v. 4-methylpyrazole, and was used to estimate peak brain ethanol concentrations after the infusions. The peak brain ethanol concentrations after the 0.5, 1.0 and 1.5 g/kg ethanol infusions were estimated to be 20, 49 and 57 mM, respectively. A significant dopamine increase was observed for the 0.5 g/kg

ethanol group when collapsed across subregions. However, both the 1.0 g/kg and 1.5 g/kg ethanol infusions produced significant increases in dopamine levels in the shell that were significantly higher than those in the core. An ethanol dose-response effect on dopamine in the shell was observed when saline controls, 0.5, 1.0, and 1.5 g/kg groups were compared. For the cumulative-dosing study, the first, second, and fourth infusions resulted in significant increases in dopamine in the shell. However, these responses were not significantly different from one another. The results of this study show that the shell has a stronger response than the core to intravenous ethanol, that dopamine in the shell increases in a dose-dependent manner between 0.5 -1.0 g/kg doses, but that the response to higher ethanol doses reaches a plateau.

INTRODUCTION

Many brain regions and pathways may be involved in the reinforcing properties of drugs of abuse; however, the mesolimbic dopaminergic pathway is among the most highly studied. Dopaminergic neurons of this pathway originate in the ventral tegmental area and terminate in the nucleus accumbens and may be activated during the acquisition or expression of ethanol reinforcement (Spanagel and Weiss, 1999; Weiss and Porrino, 2002; Gonzales et al., 2004). It is well established in many animal models that extracellular dopamine levels increase in the nucleus accumbens after ethanol administration (Imperato and Di Chiara, 1986; Yoshimoto et al., 1992a; Yoshimoto et al., 1992b; Blomqvist et al., 1993;

Weiss et al., 1993; Campbell and McBride, 1995; Kohl et al., 1998; Olausson et al., 1998; Yim et al., 2000; Tang et al., 2003b). However, the potential contribution of environmental stimuli associated with ethanol administration, separate from a direct pharmacological effect, to the ethanol-evoked dopamine response is still not clear.

The nucleus accumbens has been described as having anatomically distinct core and shell subregions (Heimer et al., 1991; Zahm and Brog, 1992; Zahm, 1999), and these subregions may have different behavioral functions. The core of the nucleus accumbens has been implicated in associative conditioning and instrumental behaviors (Kelley et al., 1997; Sokolowski and Salamone, 1998; Bassareo and Di Chiara, 1999a; Ito et al., 2000; Day et al., 2007; Gremel and Cunningham, 2008). The shell may be important during exposure to novel stimuli or the acquisition of place preference (Rebec et al., 1997; Bassareo and Di Chiara, 1999a; Di Chiara et al., 2004; Fenu et al., 2006). However, the potential differential response of the core and shell to ethanol has not been well defined.

The ethanol-induced dopamine response in the core and shell of the nucleus accumbens may differ depending on the route of ethanol administration. Animals that self-administer ethanol orally encounter stimulus properties of the drinking solution, and animals that receive an intraperitoneal (i.p.) injection may experience handling stress. Environmental factors associated with ethanol self-

administration, such as a cue lights, may also contribute to the stimulation of accumbal dopamine (Melendez et al., 2002). Intravenous (i.v.) ethanol administration may reduce or eliminate some confounding environmental factors that occur with i.p. or oral administration including handling stress and the stimulus properties of ethanol associated with its taste and smell. The i.v. administration of ethanol has been shown to enhance the firing rate of dopamine neurons in the ventral tegmental area, the origin of the mesolimbic pathway, in anesthestized or paralyzed naïve rats (Gessa et al., 1985; Foddai et al., 2004). However, it is not clear whether these increases in firing rate of dopamine neurons lead to dopamine release in the nucleus accumbens, particularly in freely-moving rats. Here, the i.v. method was used to compare extracellular dopamine in the core and shell in response to acute non-contingent administration of ethanol.

The major goal of this study was to determine whether there is a pharmacological effect of ethanol on dopamine in the core and shell of the nucleus accumbens over a wide range of ethanol doses, and whether this ethanol-evoked dopamine release differs in these subregions. We also estimated the tissue ethanol concentration in the area surrounding the microdialysis probe after the bolus injection of ethanol. This was done by determination of the *in vivo* extraction fraction for ethanol after inhibition of ethanol metabolism with an alcohol dehydrogenase inhibitor.

MATERIALS AND METHODS

Animals

A total of eighty-four male Long-Evans rats (Charles River Laboratories, Wilmington, MA), weighing 250-550 grams on dialysis day, were used for these experiments. Sixty-six were used for the acute ethanol studies, thirteen were used for the cumulative dosing study, and five were used to determine the extraction fraction for ethanol. The rats were housed individually in a temperature (25˚C) and light (12 hour light/12 hour dark) controlled room, and had access to food and water *ad libitum*. The rats were handled and weighed for at least four days prior to surgery. All procedures were carried out in compliance with the guidelines set forth by the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* and the Institutional Animal Care and Use Committee of the University of Texas at Austin.

Surgical Procedures

A jugular catheter was inserted, and a guide cannula was placed over the nucleus accumbens in each rat using a modification of the procedure of Duvauchelle et al. (1998). The jugular catheter was fed subcutaneously to an incision on the head. Intravenous catheters were constructed from silastic tubing (0.30 mm ID, 0.64 mm OD, Fisher Scientific, Hampton, NH), a cannula (22 gauge, Plastics One, Roanoke, VA), and silicon adhesive (DAP Inc., Baltimore, MD). The rats were under isoflurane anesthesia (4.0 % during the induction period and

2.0 % during maintenance) during surgery. The guide cannula used for microdialysis (21 gauge, Plastics One, Roanoke, VA) was implanted above either the core (coordinates in mm relative to Bregma: AP +1.3, ML +1.6, DV -3.5 or -3.2) or shell (AP +2.2, ML +0.7, DV -4.0) of the nucleus accumbens while the animal was in a stereotaxic frame. The DV coordinate represents the bottom of the guide cannula, and the probe extends an additional 4.0 mm below the cannula when seated into the guide. We noted that some of the probes aimed at the core passed through the core and penetrated the ventrolateral shell. To minimize this, the core DV coordinate was changed to -3.2 midway through the study. An obturator was placed in the guide cannula to prevent blockage. Rats were allowed to recover from surgery for 2 (4-methylpyrazole experiments) or at least 4 days (acute and cumulative studies). A longer recovery period was allowed for the ethanol studies because we monitored their behavior following the injection. During the recovery period, the catheter was flushed daily with 0.1 ml of timentin (67 mg/ml; Henry Schein, Inc., Melville, KY) in heparinized saline (American Pharmaceutical Partners, Inc., Los Angeles, CA).

Microdialysis

The evening before the dialysis experiment, a laboratory constructed probe (1.5 mm active membrane length, 270 µm OD, 18,000 molecular weight cut-off) was implanted through the guide cannula and perfused (CMA 100 microinjection pump, Acton, MA) with artificial cerebrospinal fluid (ACSF: 149 mM NaCl, 2.8 mM

KCl, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 0.25 mM ascorbic acid, 5.4 mM D-glucose). The rats were placed in individual chambers with free access to water and food, and the flow rate was lowered to 0.2 µl/min overnight. After a stabilization period of 12-15 hours, the flow rate was increased to 2.0 µl/min, and two hours were allowed at this new flow rate before sample collection commenced using five-minute intervals.

For the ethanol dose-response experiments, four basal samples were taken before the infusion of ethanol (10%, w/v, in saline) or saline. For the 0.5 and 1.0 g/kg ethanol groups, post-infusion samples were taken for 30 minutes. For the 1.5 g/kg ethanol group post-infusion samples were collected for one hour. The i.v. infusions occurred over approximately 30 seconds, one minute, and one and a half minutes for the 0.5, 1.0, and 1.5 g/kg ethanol groups, respectively. For the cumulative dosing study, four basal samples were taken before the first infusion of 1.0 g/kg i.v. ethanol, and fifteen minutes elapsed between each of the remaining infusions. The rate of infusion was 15 seconds per milliliter of solution for all infusions. The volume of the infusions ranged from approximately 1.5 to 6.0 milliliters, depending on dose and body weight. For all groups, every dialysis sample was analyzed for dopamine, and ethanol was determined in the last two basal and the post-infusion samples in the ethanol groups. Upon completion of the experiment, the perfusate was switched to calcium-free ACSF. A five-minute

sample was taken after one and a half hours to verify that dopamine recovered in the experimental samples was due to exocytotic release.

For each microdialysis experiment the behavior of the rat was observed, and sedative effects of ethanol were recorded. Behaviors that indicated sedation included loss of motor coordination during locomotion, or hypnosis.

In Vivo Extraction Fraction for Ethanol

Separate experiments in another group of rats were carried out to determine an in vivo extraction fraction for ethanol for our probes using the method of Robinson et al. (2000). This was done to enable us to estimate the brain tissue concentrations around the probe under our experimental conditions. This method allows us to estimate tissue concentrations after various ethanol doses with the use of a minimal number of animals, unlike that which would be necessary with ethanol analysis from tissue extractions. Briefly, five animals were given an alcohol dehydrogenase inhibitor, 4-methylpyrazole (2.0 mg/kg, i.v.) one hour before being given i.v. 10% ethanol infusions (0.5 g/kg). Alcohol dehydrogenase is the major pathway for ethanol metabolism (Matsumoto et al., 1994), and inhibiting this enzyme allowed blood ethanol concentrations to remain relatively constant. During this “pseudo-steady state”, blood was drawn from the jugular catheter one hour after the ethanol infusion. Then three five-minute dialysate samples were collected, which was followed by another blood draw.

The second ethanol infusion (0.5 g/kg) was given after the second blood draw to bring the animal to a cumulative dose of 1.0 g/kg ethanol. The blood and dialysis sampling was repeated as described for the first ethanol infusion. Samples were analyzed for ethanol using gas chromatography.

The in vivo extraction fraction for ethanol was calculated for each dose by computing the ratio of the dialysate ethanol concentration to the blood ethanol concentration. The peak brain ethanol concentrations for each dose examined in the dose-response experiments was estimated by dividing the dialysate ethanol concentration in the first sample obtained after the infusion by the in vivo extraction fraction.

Histology

The day after dialysis, the animals were overdosed using an i.p. injection of sodium pentobarbital (150 mg/kg). After the animal was perfused intracardially with saline and then 10% formalin, the brain was extracted and placed in 10% formalin overnight. The brains were sectioned (100 µm thick) with a vibratome (Leica, Nussloch, Germany) and then stained with cresyl violet to confirm probe placement. The probe tracks were mapped using the atlas of Paxinos et al. (1999).

High Pressure Liquid Chromatography (HPLC)

Dialysate dopamine was analyzed using HPLC with electrochemical detection. The system used a Polaris 3 µm C18 column (50 x 2 mm, Varian, Lake Forest, CA). The mobile phase consisted of 0.50 g octanesulfonic acid, 0.05 g decanesulfonic acid, 0.13 g ethylenediaminetetraacetic acid, 11.1 g NaH_2PO_4, and 150 ml methanol in 1 liter of deionized water. The mobile phase had a pH equal to 5.6. Seven microliters of the dialysate sample were mixed with ascorbate oxidase at 4° C prior to injection. Dopamine was detected with an electrochemical detector (Model VT03, Antec Leyden, Netherlands) at a potential of + 450 mV (relative to an Ag/AgCl reference). A second system was used for some samples in which the reference was an in situ Ag/AgCl (ISAAC). KCl was added to the mobile phase in appropriate concentrations in this case. The limit of detection was approximately 0.3 nM. The peaks were recorded using EZChrom software, and the concentration of dopamine in each sample was determined using external standards. The signal to noise ratio was determined for an external standard (0.625 nM dopamine) and a basal sample for each animal. Only animals with a signal to noise ratio > 3 for the standard and > 6 for the basal sample were included in the analyses.

Ethanol Analysis by Gas Chromatography

Ethanol was analyzed in 2 µl aliquots that were transferred into 2 ml gas chromatography vials immediately after collection of the microdialysis sample.

Blood alcohol concentration was determined in 10 µl of the blood sample that was immediately added to 90 µl of saturated sodium chloride solution in a gas chromatography vial. Dialysate and blood ethanol concentrations were determined following the method of Doyon et al. (2003). A Varian CP 3800 gas chromatograph with flame ionization detection 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 (30 m 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.

Statistical Analysis – Basal Dopamine Concentrations for Core vs. Shell

The basal dopamine concentrations for core and shell were collapsed across all experiments, and the values were compared using a t-test. Significance was assigned if $p < 0.05$.

Statistical Analysis – Acute Intravenous Ethanol Experiments

A two-way ANOVA (mixed model with a randomized factor and a repeated measures factor) was used for the 0.5 g/kg ethanol experiments. The between-subject variable was subregion which had two levels (core and shell). Time was the within-subject variable. Three-way ANOVAs (mixed-models) were performed for the saline vs. 1.0 g/kg ethanol and the saline vs. 1.5 g/kg ethanol experiments

(dose and subregion were between-subject variables). A two-way ANOVA (repeated measures, time; between-subject variable, dose) was performed for the shell data for the saline, 0.5, 1.0, and 1.5 g/kg ethanol groups to determine if a dose-response effect was present in this subregion. For all ANOVAs, if an interaction between the variables was observed, the simple effects were further analyzed to identify any sources of variation. Significance was assigned if $p < 0.05$. Because of a lack of homogeneity of variance in the between-subject variable for the 0.5 g/kg ethanol group, the 1.5 g/kg ethanol group, and the dose-response analysis, the analyses for these experiments were carried out on log-transformed data.

Statistical Analysis – Cumulative Intravenous Ethanol Experiments

For the cumulative dosing experiment, separate one-way repeated measures ANOVAs were used to analyze for the effect of time in the ethanol group and the saline group. Significance was assigned if $p < 0.05$. Because of a lack of homogeneity of variance the analysis for these experiments was carried out on log-transformed data.

Statistical Analysis – 4-Methylpyrazole Experiments

For the 4-methylpyrazole experiments, repeated measures ANOVA was used to compare the blood ethanol concentration before and after the dialysis sampling period at each of the two ethanol doses. Repeated measures ANOVA was also

used to compare the extraction fraction values obtained after the 0.5 and 1.0 g/kg ethanol doses.

Statistical Analysis – 1.0 g/kg Ethanol-Evoked Dopamine in the Core and Shell

To compare the ability of ethanol to produce a dopamine response in the core and shell, the area under the dopamine response curve (AUC) and ethanol concentration curve was calculated. For the dopamine response AUC the basal values (nM) were subtracted to obtain a net response at each time point, and the sum of the post-infusion samples was obtained for successive samples in which two or more net responses were positive. The ethanol concentration AUC was computed by taking the sum of the post-infusion points. The ratio between the dopamine and ethanol AUC was calculated and a T-test was used to evaluate significance using $p < 0.05$ as the criterion.

RESULTS

Basal Dialysate Dopamine Concentrations in the Core and Shell

In order to compare the basal values of extracellular dopamine in the core and shell, all experiments for each subregion were collapsed across ethanol doses. The overall concentrations were 1.5 ± 0.1 nM (n = 49) for the shell and 1.3 ± 0.1 nM (n = 30) for the core. These values were not significantly different from one another ($T_{73} = -1.0$, NS), and they agree with previously published results

obtained with male Long-Evans rats (Blanchard et al., 1993; Benjamin et al., 1993; Hernandez et al., 2007)

Intravenous Infusion of 0.5 g/kg ethanol

Infusion of 0.5 g/kg ethanol (i.v.) increased dopamine release in the core and shell (Figure 2; $F_{10,150} = 4.5$, $p < 0.05$ for effect of time), but there was no difference between core and shell ($F_{10,150} = 1.4$, NS for time x subregion interaction). Also, the increase in dopamine concentrations in the shell did not differ from that in the core when comparing area under the curves ($T_{15} = 1.7$, NS).

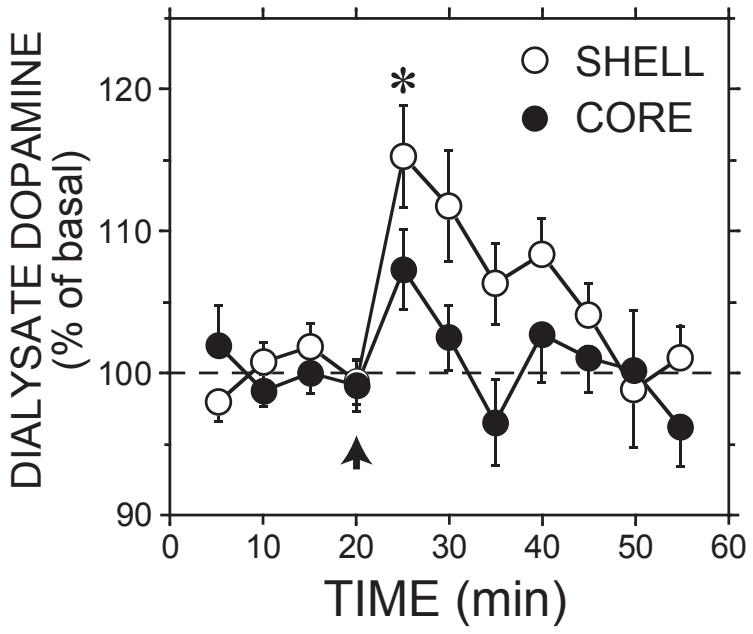

Figure 2. Effect of 0.5 g/kg i.v. ethanol on accumbal core and shell dopamine concentrations. * $p < 0.05$ for core and shell when collapsed across dose and compared to basal. The average basal levels were 1.1 ± 0.2 nM for the core group and 1.5 ± 0.3 nM for the shell group. Arrow indicates bolus injection. Mean ± sem are shown (n = 7-10).

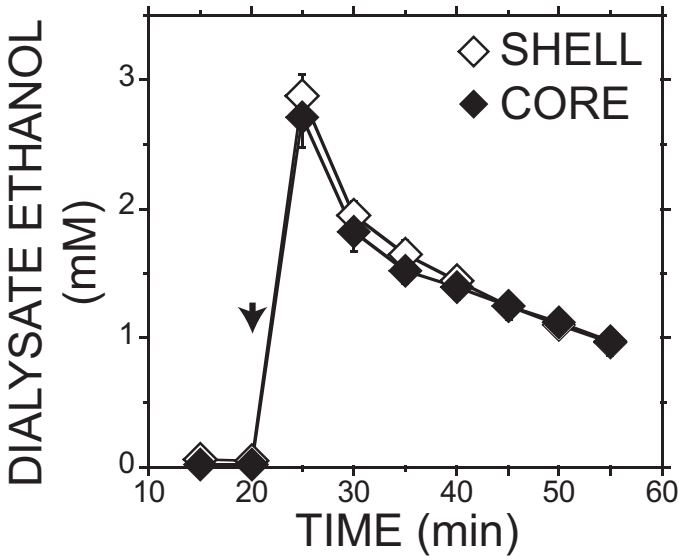

Figure 3. Core and shell ethanol concentrations after 0.5 g/kg i.v. ethanol infusion. Dialysate ethanol concentrations are indicated (mean ± sem, n = 7-10). Arrow indicates bolus injection.

One shell animal was given an equivalent volume of saline, and no increase in dopamine was observed (data not shown). A more complete saline control was not conducted because higher volumes of saline in the control experiments for the higher ethanol doses did not produce any significant increase in dopamine (see below). The peak dialysate ethanol concentration was obtained 5 minutes after the injection, and no difference was observed between core and shell time courses (Figure 3; $F_{6,90} = 1.2$, NS, for the interaction between time and subregion).

Intravenous Infusion of 1.0 g/kg ethanol

The i.v. infusion of 1.0 g/kg ethanol increased dialysate dopamine compared with saline (Figure 4). The ethanol-evoked dopamine response was significantly

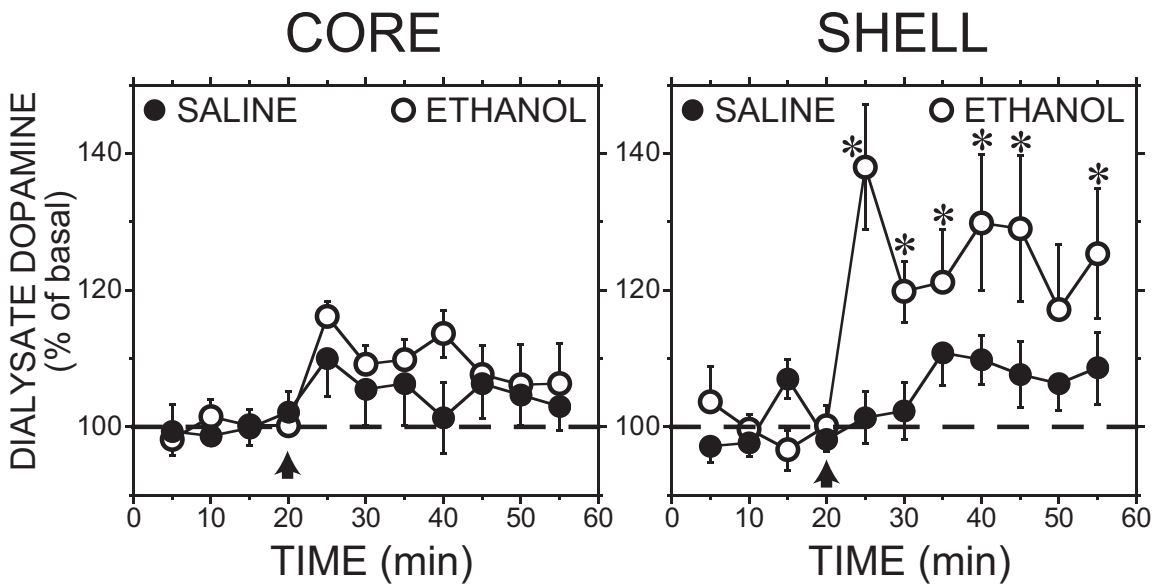

Figure 4. Effect of 1.0 g/kg ethanol and saline infusion (i.v.) on dopamine concentrations in the core (left) and shell (right). * $p < 0.05$ for ethanol when compared to basal. The average basal levels were 1.7 ± 0.4 nM for the core ethanol group, 1.4 ± 0.2 nM for the core saline group, 1.7 ± 0.3 nM for the shell ethanol group, and 1.4 ± 0.2 nM for the shell saline group. Arrow indicates bolus injection (n = 5-9).

greater in the shell compared with the core ($F_{10,230} = 1.9$, $p < 0.05$, for subregion x time interaction). Furthermore, the dopamine response was significantly above basal in the shell but not the core (Figure 4; $F_{10,230} = 4.5$, $p < 0.05$ for the shell, and $F_{10,230} = 0.6$, NS for the core). The peak dialysate ethanol concentration was again obtained 5 minutes after the injection, and ANOVA revealed a significant difference in ethanol time courses between the core and shell (Figure 5; $F_{6,54} = 3.5$, $p < 0.05$, for the interaction between time and subregion). However, post hoc analyses did not show a difference between the subregions at any individual time point ($F_{1,9} \leq 6.2$, NS). The overall difference in dialysate ethanol concentrations between the core and shell prompted us to examine whether the

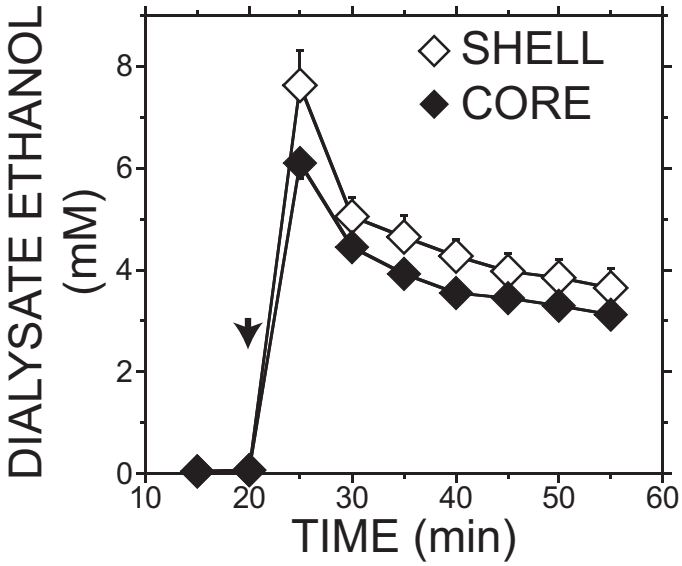

Figure 5. Core and shell ethanol concentrations after 1.0 g/kg i.v. ethanol infusion. Dialysate ethanol concentrations are indicated (mean ± sem, n = 5-6). Arrow indicates bolus injection.

difference in dopamine response could be influenced by the concentration of ethanol reaching the sites from which dopamine is being sampled. Therefore, the ability of ethanol to produce a dopamine response in each subregion was calculated. First, the area under the curves (AUC) for both the dopamine concentration (nM) versus time and ethanol concentration (mM) versus time plots was computed for each rat. The ratio of the dopamine AUC to the ethanol AUC was 0.04 ± 0.01 for the core and 0.08 ± 0.02 for the shell ($T_5 = -2.1$, $p < 0.05$), suggesting that the small difference in ethanol concentration between the subregions does not account for the differential dopamine response to ethanol.

Intravenous Infusion of 1.5 g/kg ethanol

The i.v. infusion of 1.5 g/kg ethanol increased dialysate dopamine differentially in the core and the shell when compared to saline for each subregion (Figure 6; $F_{17,306} = 1.87$, $p < 0.05$, for interaction between dose, subregion, and time). Again, the response was significantly larger in the shell relative to the core ($F_{17,306} = 2.2$, $p < 0.05$, for interaction between subregion and time). The peak dialysate ethanol concentration was also obtained 5 minutes after the injection, and the overall ethanol time course did not differ between core and shell (Figure 7; $F_{13,130} = 1.4$, NS).

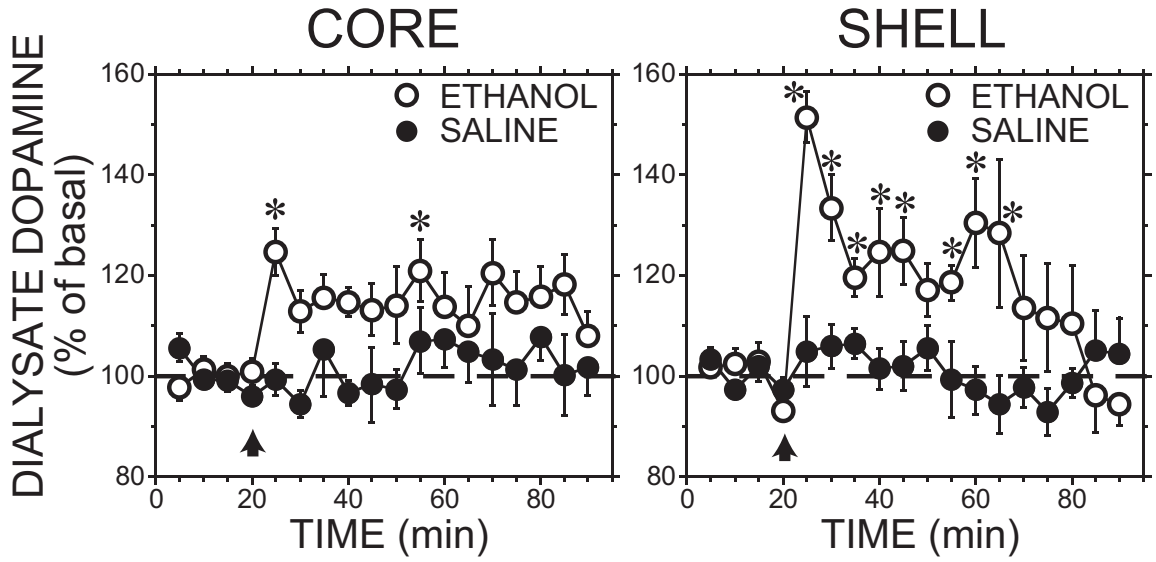

Figure 6. Effect of 1.5 g/kg ethanol and saline infusion (i.v.) on dopamine concentrations in the core (left) and shell (right). * $p < 0.05$ for ethanol when compared to basal. The average basal levels were 1.4 ± 0.2 nM for the core ethanol group, 1.1 ± 0.3 nM for the core saline group, 2.2 ± 0.4 nM for the shell ethanol group, and 1.5 ± 0.3 nM for the shell saline group. Arrow indicates bolus injection (n = 4-6).

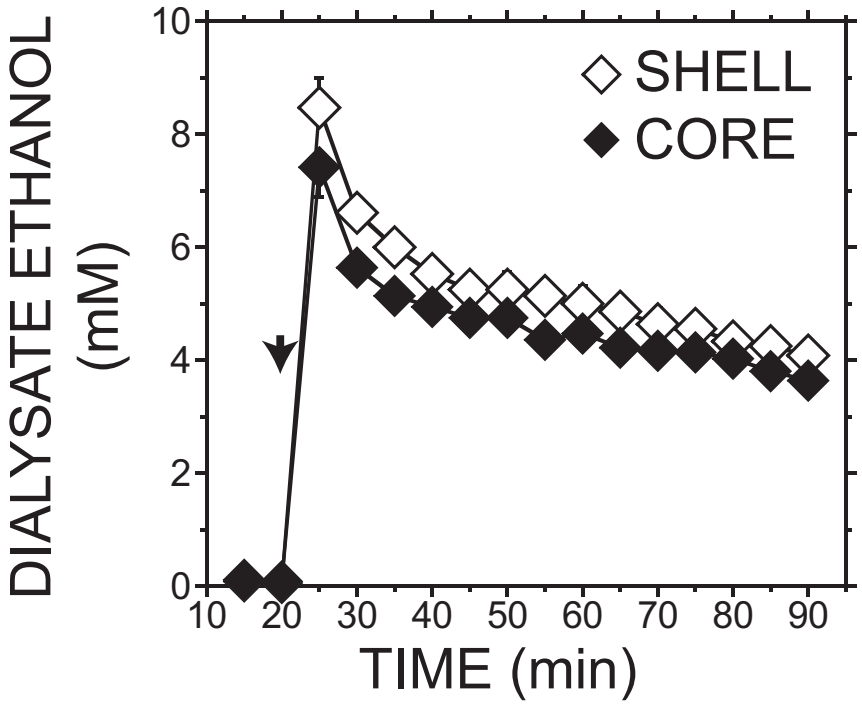

Figure 7. Core and shell ethanol concentrations after 1.5 g/kg i.v. ethanol infusion. Dialysate ethanol concentrations are indicated (mean ± sem, n = 6 for each group). Arrow indicates bolus injection.

Dose Response Effect in the Shell of the Nucleus Accumbens

A significant difference in dialysate dopamine from the shell after infusion of saline, 0.5, 1.0, or 1.5 g/kg ethanol was observed ($F_{30,320} = 4.1$, $p < 0.05$, for the dose x time interaction). Post-hoc analyses revealed that the saline and 0.5 g/kg ethanol groups individually differed from the 1.0 and 1.5 g/kg groups ($F_{10,320} \geq 3.3$, $p < 0.05$), but that the saline and 0.5 g/kg groups, and the 1.0 and 1.5 g/kg groups did not differ from each other ($F_{10,320} = \leq 2.0$, NS). Also, a significant effect of dose was observed for the ethanol concentrations across groups ($F_{12,108} = 13.0$, $p < 0.05$). Post-hoc analyses showed that the ethanol concentrations resulting from 0.5 g/kg intravenous infusions significantly differed from the 1.0

and 1.5 g/kg infusions ($F_{6,108}$ = ≥12.9, $p < 0.05$), however the 1.0 and 1.5 g/kg infusions did not result in concentrations that significantly differed from one another ($F_{6,108}$ = 1.6, NS)

Cumulative Intravenous Ethanol Experiments

In the ethanol group, a significant increase in dialysate dopamine in the shell of the nucleus accumbens was observed (Figure 8; $F_{17,136}$ = 3.9, $p < 0.05$, for effect of time). In contrast, no effect was observed in the group receiving repeated saline infusions (data not shown. $F_{17,51}$ = 1.1, NS). The first, second, and fourth i.v. ethanol infusions of 1.0 g/kg significantly increased dialysate dopamine concentrations compared with basal values ($F_{4,135}$ = ≥6.1, $p < 0.05$). No significant difference was observed between the dopamine increases following the four 1.0 g/kg ethanol infusions ($F_{3,135}$ = 0.9, NS). Each peak dialysate ethanol concentration was observed 5 minutes after each infusion, and were all significantly different from baseline and from one another (Figure 9; $F_{15,120}$ = 628.7, $p < 0.05$, for effect of time; $F_{3,120}$ = 485.1, $p < 0.05$, for comparison of the four injection time points).

Behavioral Analysis

Behavioral observations were recorded for the first 5 minutes after each ethanol infusion. We focused on this period because ethanol concentrations peaked during this time in all experiments. Peak dopamine responses generally occurred

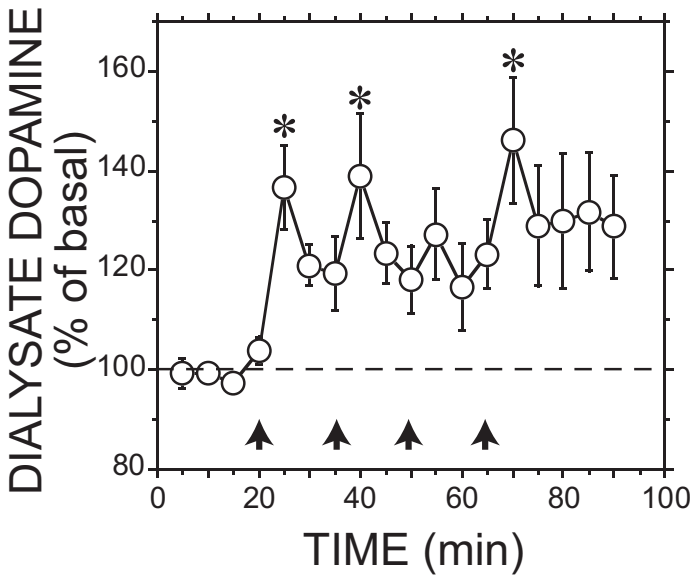

Figure 8. Effect of cumulative 1.0 g/kg ethanol infusions on dopamine in the nucleus accumbens shell. * $p < 0.05$ for ethanol when compared to basal. The average basal level was 1.3 ± 0.2 nM. Arrows indicate bolus injections (n = 9).

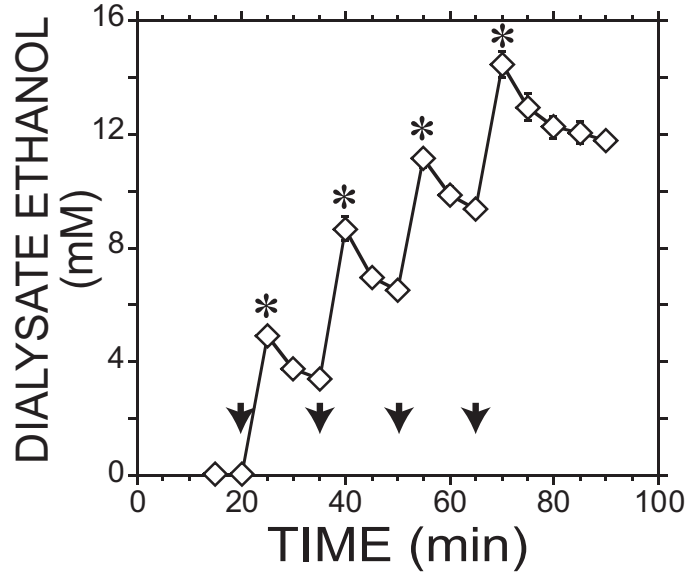

Figure 9. Shell ethanol concentrations after cumulative 1.0 g/kg ethanol infusions. * Indicates the ethanol dose differs from all other doses. Dialysate ethanol concentrations are indicated (mean ± sem, n = 9). Arrows indicate bolus injections.

during this period as well. For the 0.5 g/kg i.v ethanol group, 0 of 5 core animals and 2 of 7 shell animals showed signs of sedation during the first 5 min post-infusion, corresponding to the peak ethanol concentrations. For the 1.0 g/kg acute i.v. ethanol group, 5 of 8 core and 5 of 7 shell animals showed signs of sedation. For the 1.5 acute i.v. ethanol group, 4 of 6 core and 6 of 7 shell animals showed signs of sedation. For the cumulative ethanol dosing group (all shell animals), 6 of 9 animals after the first 1.0 g/kg ethanol infusion and 9 of 9 animals after each of the additional three infusions showed signs of sedation in the 5-min period post-infusion.

4-Methylpyrazole Experiment: In vivo Extraction Fraction for Ethanol

A separate group of rats was used to determine the in vivo extraction fraction for ethanol for the probes used in this study. The major metabolic pathway of ethanol was blocked by inhibiting alcohol dehydrogenase, and this produced "pseudo-steady state" blood ethanol levels, and presumably, the brain ethanol concentrations were also relatively stable during this period (Gonzales et al., 2002; Robinson et al., 2000). Table 1 shows the blood ethanol concentration, dialysate ethanol concentration, decrease in blood ethanol concentration during the sampling period, and the extraction fraction after 0.5 and 1.0 g/kg ethanol (i.v.) doses. The slight decrease in blood ethanol concentrations during the dialysis sampling period was significant during each sampling period ($F_{1,4} = 12.9$, $p < 0.05$ for 0.5 g/kg; $F_{1,4} = 20.9$, $p < 0.05$ for 1.0 g/kg). This small drop in blood

ethanol concentrations reflects the contribution of catalase and cytochrome P450 to the metabolism (Zimatkin and Buben, 2007; Zimatkin et al., 2006). The extraction fractions obtained for the 0.5 and 1.0 g/kg ethanol doses were not significantly different ($F_{1,4} = 2.2$, NS), and therefore, a mean extraction fraction was calculated (0.14). The peak brain ethanol concentrations were estimated for each of the ethanol doses used previously by dividing the peak dialysate concentration by the mean extraction fraction. This was also done for the 1.5 g/kg dose also because the in vivo extraction fraction for ethanol is independent of the concentration. These estimates yielded peak concentrations of 20, 49, and 57 mM for the 0.5, 1.0, and 1.5 g/kg acute i.v. infusion experiments, respectively. For the cumulative dosing study, we estimate that the peak tissue concentrations after each infusion to be 35, 62, 79, and 103 mM, respectively.

Table 1. Blood and dialysate ethanol concentrations, change in blood concentration, and derived in vivo extraction fraction values for ethanol at two doses after i.v. administration.

i.v. Ethanol Dose	Blood Ethanol (mM)[a]	Dialysate Ethanol (mM) [b]	Decrease in Blood Ethanol during Sampling Period (mM)[c]	Extraction Fraction[d]
0.5 g/kg	11.2 ± 0.7	1.5 ± 0.1	1.1 ± 0.3	0.14 ± 0.01
1.0 g/kg	18.6 ± 1.1	2.7 ± 0.2	2.4 ± 0.5	0.15 ± 0.02

[a]The mean ethanol concentration (± sem) from the blood taken before and after the dialysis sampling period
[b]The mean dialysate ethanol concentration (± sem) in three samples taken at five minute intervals
[c]Calculated as the concentration difference between the pre- and post- dialysis sample blood draws
[d]The extraction fraction was derived from the ratio of the dialysate ethanol concentration to the blood ethanol concentration

Probe Placement Verification

Figure 10 shows the representation of the probe placements for the single dose ethanol experiments. None of the probes aimed at the shell overlapped with the core, although some of the core probes sampled from the ventral shell to a limited degree. For the core analysis we only included animals with probes that penetrated the shell by no more than 30% of the active dialysis membrane length. For the cumulative experiments, all probes were placed in the shell (Figure 11), and for 4-methylpyrazole experiments, all probes were placed on the medial border of the shell (Figure 12).

DISCUSSION

Two major findings are reported in the present study. First, the results show a larger dopamine response to ethanol in the shell subregion of the nucleus accumbens compared with the core in naïve Long-Evans rats. Increases in dialysate dopamine were seen after infusions of 0.5 g/kg ethanol when compared to saline; however, there was no significant difference between the effect in the core (7% above baseline) and the effect in the shell (15% above baseline). In contrast, the 16-38% increase observed after an infusion of 1.0 g/kg ethanol was statistically different between the two subregions. The 25-51% increase in dialysate dopamine observed following the 1.5 g/kg ethanol infusion was also significantly different between core and shell. The second major finding is the concentration-dependence of the accumbal dopamine response after i.v. ethanol

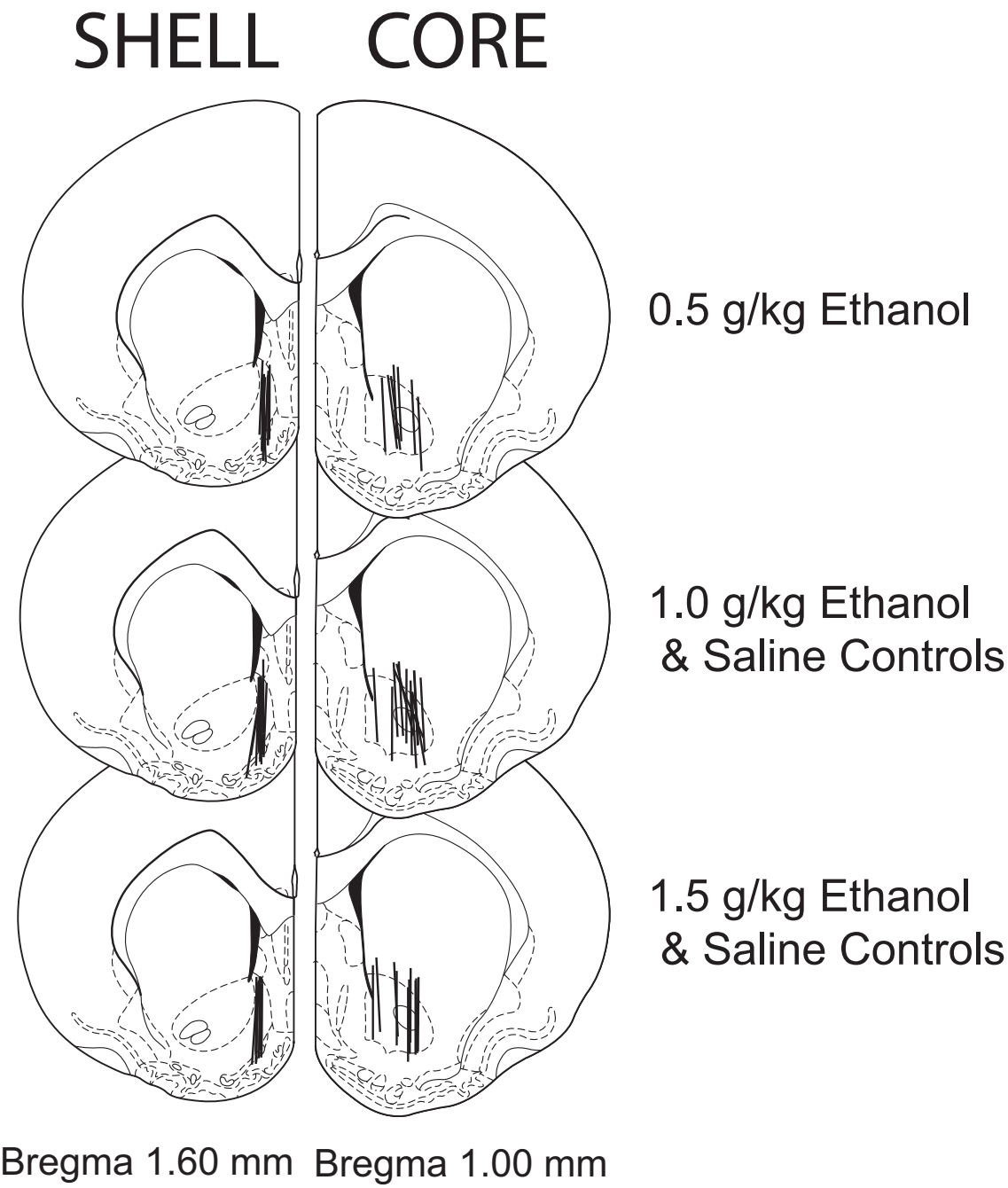

Figure 10. Coronal sections indicating microdialysis probe placements in the core and shell of the nucleus accumbens for the 0.5 g/kg (n = 7 for the core, n = 10 for the shell), 1.0 g/kg and saline control (n = 13 for the core, n = 14 for the shell), and 1.5 g/kg and saline control (n = 10 for the core, n = 12 for the shell) groups. This figure was adapted from Paxinos and Watson (1998) and is representative of all probes collapsed onto one slice for either the core or shell.

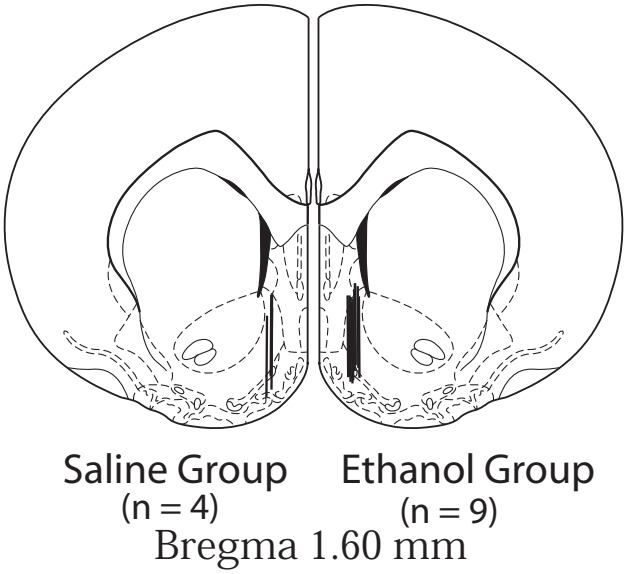

Figure 11. Coronal sections indicating microdialysis probe placements in the shell of the nucleus accumbens for the cumulative dosing experiments using intravenous saline (right) or ethanol (left). This figure was adapted from Paxinos and Watson (1998) and is representative of all probes collapsed onto one slice.

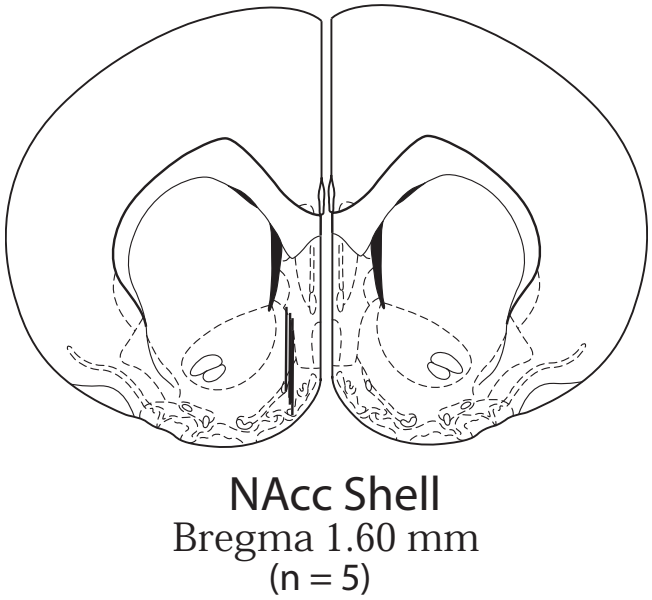

Figure 12. Coronal sections indicating microdialysis probe placements in the shell of the nucleus accumbens for the 4-methylpyrazole experiments. This figure was adapted from Paxinos and Watson (1998) and is representative of all probes collapsed onto one slice.

administration. The 0.5 g/kg infusion of ethanol produced a peak accumbal ethanol concentration of 20 mM, and a modest dopamine response was observed. The 20 mM concentration produces moderate intoxication in non-tolerant humans (equivalent to a blood alcohol concentration of 0.9 mg/ml) and would be achieved after consumption of approximately 4 standard drinks within 60 min (Duarte et al., 2008; Schweizer et al., 2006; Brasser et al., 2004; Erickson, 2007). In contrast, the 1.0 g/kg and 1.5 g/kg ethanol infusions produced a robust dopamine response in the shell at estimated peak brain concentrations of 49 mM and 57 mM. These concentrations would produce severe intoxication in humans accompanied by motor incoordination and hypnosis (blood alcohol concentration of 2.3-2.7 mg/ml; Erickson, 2007. The concentration-dependence of the ethanol-stimulated dopamine response reported in the present study is not consistent with the idea that concentrations of ethanol that are associated with low to moderate intoxication strongly activate the mesolimbic dopamine system.

During the cumulative dosing study, 1.0 g/kg i.v. ethanol infusions were administered every fifteen minutes to reach a dose of 4.0 g/kg. While the first infusion resulted in an increase in dopamine in the shell (37%) similar to our 1.0 g/kg ethanol shell group in the acute study (38%), the additional three infusions did not increase dopamine release in a dose-dependent manner. Therefore, this experiment indicates that the effect of ethanol on dopamine release in the shell is relatively constant over a three-fold concentration range starting with an initial

dose of 1.0 g/kg. This is in marked contrast to the steep dose-dependence of dopamine release in the shell observed between 0.5 and 1.0 g/kg which produces peak ethanol concentrations in the range of 20-49 mM. It is important to note that acute tolerance to ethanol during these repeated infusions may account for the plateau of the dopamine responses. Also, because these experiments measured dopamine from the shell of the accumbens, the dopamine response may signal the novelty of the increasing intoxication resulting from the repeated ethanol infusions.

The core and shell subregions of the nucleus accumbens may be differentially activated by ethanol exposure depending on the environmental context. For example, Bassareo et al. (2003) showed that administration of ethanol through an intraoral catheter produced a transient dopamine response in the shell in naïve rats, but not in rats that had been exposed to ethanol the previous day. This is consistent with the proposed role of the shell in the recognition of novel stimuli (Rebec et al., 1997; Di Chiara et al., 2004). On the other hand, the core may show a more robust dopaminergic response after the formation of associations between ethanol and conditioned stimuli during behaviors that require instrumental responses or in operant conditioning (Kelley et al., 1997; Sokolowski and Salamone, 1998; Bassareo and Di Chiara, 1999a; Ito et al., 2000). In the present study the ethanol was administered to naïve rats, so the larger dopamine response we observed in the shell is consistent with the idea

that the dopamine signaling in the shell may code for a novel experience. However, we found that the dopamine response in the core was also significantly enhanced in the 1.5 g/kg ethanol group, and it may be possible that the core also plays this role, although not to the same extent as the shell.

Our conclusion that the shell has a higher dopamine response to ethanol than the core is tempered by two issues. First, we found that the peak ethanol concentration is slightly higher in the shell than the core after the 1.0 g/kg dose, a finding that was not replicated at a lower or higher dose. To account for the difference in ethanol concentrations we compared the ratio of the dopamine AUC to the ethanol AUC in both subregions. This analysis showed that a significantly greater dopamine response was produced in the shell compared with the core after the 1.0 g/kg dose, in agreement with the 1.5 g/kg dose. A second issue that should be considered is the large volume of fluid that we infused, particularly for the 1.0 and 1.5 g/kg doses. It is likely that physiological changes in cardiovascular parameters occur after these infusions. However, volume-matched saline infusions did not alter accumbal dopamine release demonstrating that the accumbal dopamine response is specific to ethanol. However, we can not rule out the possibility that the physiological changes in cardiovascular function may interact with the pharmacological effects of ethanol to contribute to the overall responses we measured. Also, these large volume infusions may have an osmotic effect on cells as the bolus infusion moves through the system.

However, we did not observe a significant increase in dopamine after saline infusions, and would expect the effects of the ethanol infusions to be similar because the osmotic stress caused by ethanol should be low as it readily moves across cell membranes. In any case, these issues do not invalidate our conclusion that the shell responds to ethanol with a greater magnitude than the core.

To our knowledge, the present study is the first to specifically compare dialysate dopamine in core versus shell after ethanol administration in rats. However, the present results contradict a previous study of ethanol-evoked dopamine release in the core and shell in which no difference was reported (Zocchi et al., 2003). This discrepancy could be due, in part, to the use of mice by Zocchi et al. (2003). Mice have smaller core and shell subregions compared with rats, and it may be more difficult to place probes exclusively in the core or shell subregions in the mouse model. Alternatively, the core-shell difference in ethanol response we report may be found in rats but not mice.

The measurement of the in vivo extraction fraction for ethanol allowed us to clearly define the concentration-dependence of the ethanol-evoked accumbal dopamine response. Relatively high concentrations of ethanol (~20 mM) yield modest dopamine release (7-15%) in the nucleus accumbens after a bolus i.v. infusion of 0.5 g/kg. This effect is lower in magnitude to that previously reported

after a higher dose (1.0 g/kg) given i.p. Previous work has consistently shown that i.p. administration of 1.0 g/kg ethanol elicits a more robust accumbal dopamine response (40-200% above baseline) (Carboni et al., 1989; Yoshimoto et al., 1992a; Acquas et al., 1993; Heidbreder and De Witte, 1993; Kiianmaa et al., 1995; Yim et al., 2000). Although these doses and routes of administration differ (0.5 g/kg, i.v. vs. 1.0 g/kg, i.p.), both produce similar peak brain concentrations (Nurmi et al., 1994). Based on Nurmi's work using one minute microdialysis samples, a 1.0 g/kg ethanol injection i.p. should produce a peak ethanol brain concentration around 35 mM. Although the estimated peak brain concentration for the 0.5 g/kg ethanol group was 20 mM, this value was determined from the average brain concentration during the first five minutes whereas the peak is actually much higher within 1-2 minutes after the injection and is more likely to be in the range of 35 mM (Robinson et al., 2002a). Preliminary data in our lab also indicates that a 0.5 g/kg i.v. infusion and 1.0 g/kg i.p. infusion produce similar peak concentrations of ethanol in the brain (dialysate concentration of 3.0 mM) and yet yield very different increases in dopamine (16% increase in dopamine above baseline after i.v. infusion and 44% increase above baseline after the i.p. injection). These considerations lead us to suggest that the accumbal dopamine response previously reported after i.p. injection of low to moderate doses of ethanol is due, in part, to physiological mechanisms, in addition to, a direct pharmacological action on the mesolimbic system.

The reasons for this discrepancy between the results of this study and previous studies are not clear, but several potential explanations can be offered. The differing routes of administration could contribute because the i.p. route involves handling of the animal, while in the present study the i.v. route eliminated handling-induced stress. The stress associated with handling and saline injection has been demonstrated to increase accumbal dopamine in some (Barrot et al., 2000; Tang et al., 2003b), but not all previous studies (Yoshimoto et al., 1992a; Acquas et al., 1993; Heidbreder and De Witte, 1993; Kiianmaa et al., 1995; Yan, 1999). Intraperitoneal injection-induced stress may interact with ethanol to enhance the effect of ethanol on the accumbal dopamine response reported in previous studies (Imperato and Di Chiara, 1986; Carboni et al., 1989; Yoshimoto et al., 1992a; Acquas et al., 1993; Blanchard et al., 1993; Heidbreder and De Witte, 1993; Kiianmaa et al., 1995; Samson et al., 1997; Tanda and Di Chiara, 1998; Yan, 1999). In addition, it is possible that i.p. administration of ethanol produces a unique sensation in the peritoneum of the animal after injection. For example, many previous studies used 15-20% ethanol, and this concentration may dehydrate the peritoneal lining to produce a visceral sensation. This novel sensation may contribute to an increase in dopamine in the shell of the accumbens (Rebec et al.,1997; Di Chiara et al., 2004).

Another issue to consider is the strain of rat used in this experiment (Long-Evans). Blanchard et al. (1993) reported that a low dose of i.p. ethanol (0.25

g/kg) stimulates accumbal dopamine release in male Long-Evans rats, but that higher doses (0.5 and 1.0 g/kg, i.p.) were ineffective, and the highest dose used (2.0 g/kg, i.p.) inhibited dopamine release. However, Samson et al. (1997) observed that 1.0 g/kg ethanol (i.p.) stimulated accumbal dopamine release in this strain. Thus, there is no clear consensus in the literature regarding the dose-dependence of ethanol-stimulated accumbal dopamine release in male Long-Evans rats when given by the i.p. route. Our results using the i.v. route of administration match those of Samson et al. (1997), although caution should be maintained in comparing results from an i.v. study with an i.p. study. Many other investigators have reported that 1-2 g/kg doses of ethanol given i.p. stimulate accumbal dopamine release in Sprague-Dawley and Wistar rats (Imperato and Di Chiara, 1986; Acquas et al., 1993; Yim et al., 1998; Yim et al., 2000; Campbell and McBride, 1995; Kohl et al., 1998). Because the present study is the first to report the effect of i.v. ethanol administration on dialysate accumbal dopamine concentrations, it is not clear whether results obtained in Long-Evans rats will generalize to other rat strains.

The results of this study can also be compared to previous behavioral studies such as ethanol self-administration or ethanol-induced conditioned place preference using peak tissue concentration estimates. The present data show that the concentrations of ethanol that produce a robust accumbal dopamine response (20-50%) are considerably higher than those in rats that orally self-

administer ethanol (Weiss et al., 1993; Weiss et al., 1996; Gonzales and Weiss, 1998; Melendez et al., 2002; Doyon et al., 2003; Doyon et al., 2005). Although tissue concentrations weren't measured in these previous studies, based on the amounts of ethanol consumed (0.5 -1.4 g/kg), and the dialysate ethanol levels reported when dopamine peaks, it is unlikely that concentrations higher than those of the 0.5 g/kg i.v. ethanol group in this study would have been reached. The 0.5 g/kg i.v. ethanol group only showed dopamine responses of 7% and 15% above basal in the core and shell, respectively. Therefore, this adds further support to the suggestion that accumbal dopamine is not regulated by ethanol through only a pharmacological mechanism during self-administration (Doyon et al., 2005), but rather also through physiological processes associated with ethanol consumption. For example, during self-administration animals are exposed to stimulus cues from the drinking solution, and over time the animal may associate these cues with ethanol's rewarding effects. Eventually, these cues may be responsible for producing the change in dopamine (Schultz et al., 1997).

Another method of assessing the rewarding properties of ethanol is conditioned place preference (Cunningham et al., 2003). Most studies with rats have used the i.p. route of administration, although one report showed that 0.6 g/kg (i.v.) ethanol did not produce place conditioning after four conditioning trials (van der Kooy et al., 1983). In contrast, ethanol-induced conditioned place preference

was demonstrated by several groups after prolonged place conditioning (at least 14 pairing trials). Unfortunately, the dose-dependence of this effect has not been firmly established with one group showing that 0.5 g/kg (i.p.) ethanol did not produce place conditioning (Biala and Kotlinska, 1999) whereas others have reported success using this dose and route of administration (Bozarth, 1990; Zhu et al., 2007). The peak brain concentrations after 0.5 g/kg ethanol (i.p.) should be approximately 17 mM (Nurmi et al., 1994), and our present data suggest that this concentration will produce a modest, threshold effect on dopamine release. Taken together, it is tempting to speculate that ethanol-induced conditioned place preference in rats is largely independent of an accumbal dopamine response. However, a major caveat with this speculation is that the present data are from naïve rats, and conditioned place preference requires repeated exposure to ethanol, similar to what is required to establish ethanol self-administration. It can be noted that that repeated exposure to ethanol may induce sensitization of the dopamine response (Szumlinski et al., 2005), although this finding has not been reported in all studies (Zapata et al., 2006). Further research is needed to define the mechanisms that are responsible for the stimulation of accumbal dopamine activity in behavioral contexts in which the rewarding properties of ethanol are apparent.

In conclusion, the results of this study indicate that dopamine release in the shell is higher than the core in response to acute i.v. ethanol administration. Also,

tissue concentrations near 20 mM only produce modest dopamine increases in these subregions. The results also indicate that dopamine in the shell increases in a dose-dependent manner between 0.5 -1.0 g/kg doses, but higher doses result in a plateau of the response. These findings imply that the robust accumbal dopamine response observed in previous studies using i.p. administration or oral self-administration in which similar concentrations have been achieved may be due to stimulus cues associated with ethanol or an interaction between ethanol and handling stress.

Chapter Three: The dopamine response in the nucleus accumbens core-shell border differs from that in the core and shell during operant ethanol self-administration

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ABSTRACT

Ethanol self-administration has been shown to increase dopamine in the nucleus accumbens; however, dopamine levels in the accumbal subregions (core, shell, and core-shell border) have not yet been measured separately in this paradigm. The present study was designed to determine if dopamine responses during operant ethanol self-administration are similar in the core, core-shell border and shell, particularly during transfer from the home cage to the operant chamber and during consumption of the drinking solution. Six groups of male Long-Evans rats were trained to lever-press for either 10% sucrose (10S) or 10% sucrose + 10% ethanol (10S10E) (with a guide cannula above the core, core-shell border, or shell of the accumbens). On experiment day, five-min microdialysis samples were collected from the core, core-shell border, or shell before, during, and after drinking. Dopamine and ethanol concentrations were analyzed in these samples.

A significant increase in dopamine occurred during transfer of the rats from the home-cage into the operant chamber in all six groups, with those trained to drink 10S10E exhibiting a significantly higher increase than those trained to drink 10S in the core and shell. No significant increases were observed during drinking of either solution in the core or shell. A significant increase in dopamine was observed during consumption of ethanol in the core-shell border. We conclude that dopamine responses to operant ethanol self-administration are subregion specific. After operant training, accumbal dopamine responses in the core and shell occur when cues that predict ethanol availability are presented and not when the reinforcer is consumed. However, core-shell border dopamine responses occur at the time of the cue and consumption of the reinforcer.

INTRODUCTION

Extracellular dopamine concentrations in the nucleus accumbens increase during operant ethanol self-administration, but the precise function of the stimulation of accumbal dopamine signaling during ethanol reinforced behavior is still debated (Doyon et al., 2005; Doyon et al., 2003; Gonzales and Weiss, 1998; Melendez et al., 2002; Weiss et al., 1993). The core and shell subregions of the nucleus accumbens are anatomically distinct from one another (Heimer et al., 1991; Zahm and Brog, 1992; Zahm, 1999), but it is unclear whether core or shell dopamine contributes to the observed response during ethanol self-administration. Furthermore, dopamine in the nucleus accumbens core and shell

may have different behavioral functions. For example, dopamine in the core of the nucleus accumbens has been implicated in associative conditioning and instrumental behaviors (Bassareo and Di Chiara, 1999a; Day et al., 2007; Ito et al., 2000; Sokolowski and Salamone, 1998), whereas dopamine in the shell may be important during exposure to novel or conditioned stimuli or the acquisition of place preference (Bassareo and Di Chiara, 1999b; Cheng et al., 2003; Fenu et al., 2006; Rebec et al., 1997). In addition, dopamine signaling in the core and shell differs during reinstatement of heroin self-administration (Bossert et al., 2007), operant responding for food reward (Sokolowski et al., 1998), and acute non-contingent intravenous ethanol administration (Howard et al., 2008). However, the potential differential changes in dopamine levels in the core and shell during operant ethanol self-administration have not been defined.

More recently, studies have started to investigate dopamine in the border between the core and shell subregions, known as the “shore”. Little is known about the behavioral functions of dopamine in the core-shell border of the accumbens and the anatomical connections of this area. However, dopamine in this area has been reported to differ from that in the core and shell during novelty and opioid agonists (Hipolito et al., 2008; Rebec et al., 1997). No studies investigating ethanol’s effects on dopamine in the core-shell border have been published, and more work is needed to define the role of dopamine in this area in ethanol reinforcement.

It is also unclear if the dopamine increase observed in the accumbens during operant ethanol self-administration is due to the pharmacological effects of ethanol or environmental cues associated with its administration. It has been well established that dopamine increases in the accumbens during acute ethanol administration in naïve animals (Blomqvist et al., 1993; Campbell and McBride, 1995; Howard et al., 2008; Imperato and Di Chiara, 1986; Kohl et al., 1998; Tang et al., 2003b; Yim et al., 2000; Yoshimoto et al., 1992a; Yoshimoto et al., 1992b). In contrast, previous studies from our lab indicated that the time course of the increase in accumbal dopamine did not match that of brain ethanol suggesting that the increase in dopamine was more physiological rather than pharmacological (Doyon et al., 2003). After associative conditioning between a cue and reinforcer, the cues that predict a reinforcer may increase dopamine in the accumbens. For example, Schultz et al. (1997) showed that after repeated training sessions, stimulation of dopamine cell firing rate changed from the presentation of the unpredicted reward to the presentation of the cue that predicted the subsequent reward availability. Also, Stuber et al. (2008) reported that cue-evoked dopamine release in the nucleus accumbens developed over the course of the learning of the association between the cue and the subsequent reward. However, these studies did not compare the dopamine responses in core, core-shell border, and shell of the accumbens. In order to further define the role of dopamine in ethanol reinforcement, it is important to investigate whether

dopamine levels increase as a result of pharmacological effects or environmental cues, and if this response is the same in the accumbal subregions.

The major goal of this study was to determine whether dopamine in the core and shell of the nucleus accumbens would respond in a similar manner during operant ethanol self-administration, and if dopamine in the core-shell border would respond in a similar manner to the core or shell. A secondary goal was to investigate dopamine levels in these three accumbal subregions during transfer from the home-cage into the operant chamber, when environmental stimuli associated with operant ethanol self-administration are introduced.

MATERIALS AND METHODS

Subjects

Forty-seven male Long-Evans rats (Charles River Laboratories, Wilmington, MA) were included in the analyses for these experiments. The rats were housed individually in a temperature (25˚C) and light (12 hour light/12 hour dark) controlled room, and had access to food and water *ad libitum*. The rats were handled and weighed for at least five days prior to surgery. All procedures were carried out in compliance with the guidelines set forth by the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin.

Surgery

Prior to operant training and testing, a stainless steel guide cannula (21 gauge, Plastics One Inc., Roanoke, VA) was placed in each rat above either the left nucleus accumbens core (coordinates in mm relative to bregma: AP +1.3, ML +1.6, DV -3.2), core-shell border (AP +1.7, ML +1.2, DV -3.8), or shell (AP +2.2, ML +0.7, DV -3.8) while the animal was in a stereotaxic frame. The DV coordinate represents the bottom of the guide cannula, and the microdialysis probe extended an additional 4.0 mm below the cannula when seated into the guide. The guide cannula and a single steel bolt used for tethering the animal during microdialysis were cemented to the skull using dental cement (Plastics One Inc.). An obturator was placed in the guide cannula to prevent blockage during training. The rats were under isoflurane anesthesia (4.0 % during the induction period and 2.0 % during maintenance) during surgery. Rats were allowed to recover from surgery for at least one week before training.

Behavioral Apparatus

Operant chambers (Med Associates Inc., St. Albans, VT) modified for microdialysis were used for self-administration training and testing. One wall of each chamber contained a retractable lever on the left side (2 cm above stainless steel bar floor) and a retractable drinking spout on the right side (5 cm above floor), while the opposite wall contained an interior chamber light. The bars which

made up the floor were connected to the metal spout via a lickometer circuit (Med Associates). The operant chamber was housed within a sound-attenuating chamber with a fan; however, the doors were removed to facilitate training and microdialysis. At the start of each operant session, the interior light and sound-attenuating fan were activated. Computer software (Med Associates) controlled operant chamber function and acquisition of lickometer data.

Self-Administration Training

Operant sessions occurred once a day for 5 days per week. Rats were trained to lever-press for access to 10S (w/v). Animals were water deprived (<22 hr / day) prior to each session (30 – 45 min) to facilitate learning of this operant response. Reliable lever-pressing occurred within 2 – 4 days, and for the remainder of the study, rats were given water *ad libitum*. After the rats were trained to lever-press, half of the subjects were trained to self-administer 10% ethanol plus 10% sucrose (10S10E) using a modified sucrose fading procedure in which the sucrose was not faded out (Samson, 1986). We increased the concentration of ethanol (w/v) in the drinking solution across sessions (2-10% over 6 days), but did not remove the sucrose from the drinking solution (Table 2). The other half of subjects self-administered 10% sucrose (10S) over the same number of days as the 10S10E group. During the modified sucrose fading procedure leading up to the dialysis experiment, we also gradually habituated the rats to a 15 min wait period, which preceded presentation of the lever, and a response requirement of

4 lever-presses (Table 2). For each session, completion of the response requirement retracted the lever and led to presentation of the drinking spout for 20 min. The spout then retracted for 20 min of post-drinking time. In previous studies (Doyon et al., 2005; Doyon et al., 2003), some rats developed an ethanol aversion after drinking large doses when the ethanol concentration in the drinking solution reached 5 or 10%. In the present study, after one rat developed an aversion to ethanol, we tried to prevent this by limiting the volume of ethanol consumed during the training to 15 ml for 5% ethanol and 7 ml for 10% ethanol. However, during the day of microdialysis, the rats were not limited and had access to 20 ml. The 10S groups were never exposed to ethanol. Self-administration parameters were monitored during training and microdialysis by a lickometer circuit. Milliliters of drinking solution consumed and body weights were recorded each day.

Table 2. Operant training procedure for drinking solution self-administration.

Day	Drink solution^	Pre-drink wait (min)	Response requirement
1	10S	2	2
2	10S2E	4	2
3	10S2E	6	2
4	10S5E	8	2
5	10S5E	10	4
6	10S10E	12	4
7	10S10E	15	4
8*	10S10E	15	4

The sucrose control group followed the same schedule except that ethanol was not faded into the drink solution.
^ S stands for sucrose and E stands for ethanol. Numeral represents percentage (w/v for sucrose; w/v for ethanol).
*Dialysis session for all groups.

Microdialysis

Habituation to the microdialysis tethering apparatus occurred during the two days prior to microdialysis. Rats were tethered using brief (~5 min) 2% isoflurane anesthesia and left overnight in the testing room, with continued tethering during the operant session the following day. Immediately after this session, animals were again briefly anesthetized (10-15 min) using 2% isoflurane to allow the probe to be slowly placed in the guide cannula.

The probes were constructed in the laboratory according to the methods of Pettit and Justice (1991) (1.5 mm active membrane length, 270 µm OD, 18,000 molecular weight cut-off) and perfused (CMA 100 microinjection pump, Acton, MA) with artificial cerebrospinal fluid (ACSF: 149 mM NaCl, 2.8 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 0.2 mM ascorbic acid, 5.4 mM D-glucose). After probe implantation, rats were placed in individual cages with free access to water and food, and the flow rate was lowered to 0.2 µl/min overnight. After a stabilization period of 12-15 hours, the flow rate was increased to 2.0 µl/min, and two hours were allowed at this new flow rate before sample collection commenced using five-min intervals. Samples were manually changed, 2 µl were pipetted into a 2 ml glass vial for ethanol analysis if the animal was consuming 10S10E, and then immediately frozen on dry ice. The samples were stored at -80˚ C until analyzed.

Experimental Timeline

Dialysis samples were taken every five minutes except for the last wait-period sample as indicated below. Four basal samples were collected while the animal was still in the home-cage placed beside the operant chamber. One sample was

collected after the animal was transferred from the home-cage to the chamber. The operant program started after the transfer period sample was taken. Three samples were then taken prior to presentation of the drinking spout: two samples during the wait period, and the third including the variable lever-pressing time (approximately 5.2 minutes total). When the drinking spout entered the chamber, the first five-minute drink sample began. In this way, any increases in dopamine resulting from responding were including in the third wait sample, and increases due to consumption started in the first drink sample. Completion of the response requirement was followed by a 20 min drinking period (four samples) and then a 20 min post-drinking period (four samples). At the end of the operant program, the rat was transferred back to the home-cage. Then the perfusate was switched to calcium-free ACSF. A five-minute sample was taken after one and a half hours to verify that dopamine recovered in the experimental samples was due to exocytotic release.

Histology

The day after dialysis, the animals were overdosed using an i.p. injection of sodium pentobarbital (150 mg/kg). After the animal was perfused intracardially with saline and then 10% formalin, the brain was extracted and placed in 10% formalin overnight. The brains were sectioned (100 µm thick) with a vibratome (Leica, Nussloch, Germany) and then stained with cresyl violet to confirm probe placement. The probe tracks were mapped using the atlas of Paxinos et al. (1999).

Dopamine Analysis by High Pressure Liquid Chromatography (HPLC)

Dialysate dopamine was analyzed using HPLC with electrochemical detection. The system used a Polaris 3 µm C18 column (50 x 2 mm, Varian, Lake Forest, CA). The mobile phase consisted of 0.50 g octanesulfonic acid, 0.05 g decanesulfonic acid, 0.13 g ethylenediaminetetraacetic acid, 11.08 g NaH_2PO_4, and 150 ml methanol in 1 liter of deionized water. The mobile phase had a pH equal to 5.6. Seven microliters of the dialysate sample were mixed with ascorbate oxidase at 4° C prior to injection. Dopamine was detected with an electrochemical detector (Model VT03, Antec Leyden, Netherlands) at a potential of + 450 mV (relative to an Ag/AgCl reference). A second system was used for some samples in which the reference was an in situ Ag/AgCl (ISAAC). KCl was added to the mobile phase in appropriate concentrations in this case. The limit of detection was approximately 0.3 nM. The peaks were recorded using EZChrom software, and the concentration of dopamine in each sample was determined using external standards. The signal to noise ratios were calculated and recorded for all samples. Only animals with ratios of 3 or higher for the 0.625 nM dopamine standard and 7 or higher for the first basal sample were included in the study.

Ethanol Analysis by Gas Chromatography (GC)

Ethanol analysis was conducted according to the methods described by Doyon et al. (2003). Briefly, ethanol was analyzed in 2 µl aliquots that were transferred into 2 ml gas chromatography vials immediately after collection of the microdialysis sample. A Varian CP 3800 gas chromatograph with flame ionization detection 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 (30.0 m x 0.5 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.

Statistical Analyses Performed on Data Collected from the Core and Shell of the Accumbens

Three-way analysis of variance (ANOVA) with repeated measures was used for dialysate dopamine concentrations (nM) during transfer from the home-cage to the operant chamber and during consumption of drinking solutions. The four home-cage samples served as the baseline with which the transfer and wait samples were compared, while the three wait samples served as the baseline for the drinking and post-drinking samples. For these analyses, time was the within-subject variable, and both subregion (two levels: core and shell) and drinking solution (two levels: 10S10E or 10S) were between-subject variables. Dialysate ethanol levels (mM) were analyzed using a two-way ANOVA with repeated measures. Time was the within-subject variable, and subregion was the between-subject variable. For the analysis of the time course of drinking, the percent of total licks in each of the four five-min time bins in the drink period was analyzed using a three-way ANOVA, with drinking solution and subregion as between subject variables. Behavioral parameters during operant self-administration were analyzed using multivariate ANOVA. The body weights for each of the four groups were compared using a two-way ANOVA. The between-subject variables were subregion and drinking solution. For all ANOVAs, if an

interaction between the variables was observed, the simple effects were further analyzed to identify any sources of variation. The basal dopamine concentrations for core and shell were collapsed across drinking solutions, and the values were compared using a t-test. Significance for all analyses was assigned if $p < 0.05$. Post hoc tests were Bonferroni corrected.

Statistical Analyses Performed on Data Collected from the Core-Shell Border of the Accumbens

Core-shell border experiments were conducted post-hoc, and the data collected from this subregion was analyzed separately from core and shell groups. A two-way ANOVA with repeated measures was used for dopamine concentrations (time x drinking solution). Also, in order to verify that all groups experienced similar conditions basal dopamine levels, dialysate ethanol concentrations, ethanol dose consumed, behavioral parameters, and body weights were compared across core, core-shell border, and shell. In order to compare the dopamine responses in core, core-shell border, and shell, an ANOVA was conducted on the dopamine data for all three subregions.

RESULTS

Body Weights and Basal Dialysate Dopamine Concentrations for Core and Shell Groups

The body weights of the rats measured on the day of the microdialysis session varied widely (300 – 460 g). To make sure that the neurochemical and behavioral analyses were not influenced by differences in body weight among the

experimental groups, we analyzed the distribution of weights across groups. ANOVA indicated no significant differences in body weights between groups (core, shell, ethanol + sucrose drinkers, sucrose drinkers), because there was no significant interaction between subregion and drinking solution ($F_{1,30} = 3.6$, NS). Therefore, variation in body weight did not affect the subsequent analyses.

Dialysate dopamine concentrations in the basal samples taken in the home cage before the operant session were 1.4 ± 0.2 nM (n = 15) for the shell and 1.6 ± 0.2 nM (n = 19) for the core. These values were not significantly different from one another ($T_{32} = 0.7$, NS).

Dialysate Dopamine Concentrations after Transfer from the Home-Cage into the Operant Chamber for Core and Shell Groups

Accumbal extracellular dopamine was stimulated during the transfer from the home cage into the operant chamber in all experimental groups, and the enhanced dialysate dopamine concentration was sustained during the waiting period (Figure 13). The dialysate dopamine concentration in the first sample taken after the transfer increased over the home cage baseline with a significantly larger increase observed in the rats trained to drink ethanol + sucrose (33%) compared to those trained to drink sucrose (14-21%; $F_{6,180} = 2.2$, $p < 0.05$ for the drinking solution x time interaction). However, this dopamine response was similar in both core and shell subregions ($F_{6,180} = 0.3$, NS for subregion x time interaction).

Dialysate Dopamine Concentrations during Liquid Consumption for Core and Shell Groups

The dialysate dopamine concentrations during the wait and lever-press periods were used as a new baseline to analyze potential changes in accumbal dopamine during the drinking and post-drink periods. Overall, small, nonsignificant, increases in dialysate dopamine during the drinking period were observed in all groups (5-8% above baseline) (Figure 14; $F_{10,300} = 1.0$, NS for the drinking solution x subregion x time interaction).

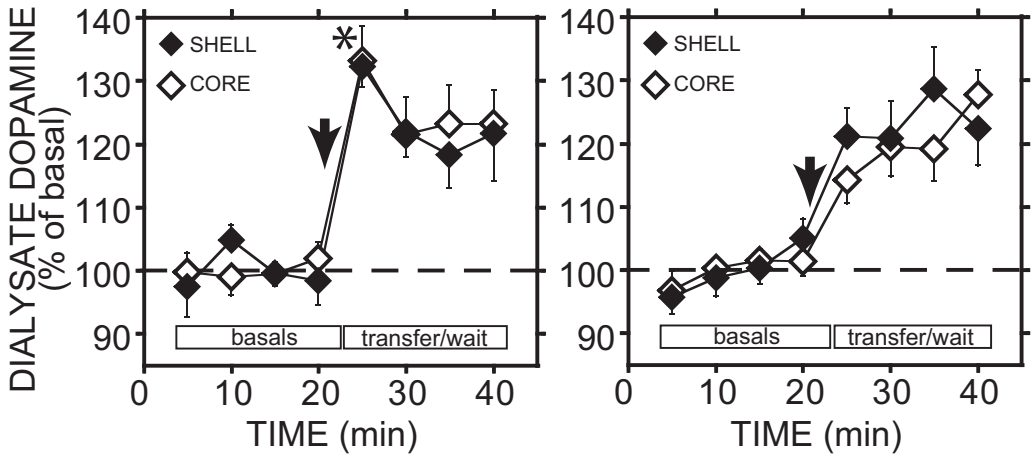

Figure 13. Dialysate dopamine from the core and shell of the nucleus accumbens during transfer from home-cage into the operant chamber. * indicates a significantly larger dopamine response for the 10S10E trained rats (left) compared with the 10S trained rats (right). For rats drinking 10S10E, basal dopamine concentrations were 1.6 ± 0.2 nM for core (n = 9), and 1.5 ± 0.3 nM for shell (n = 7), and for rats drinking 10S, basal dopamine concentrations are 1.6 ± 0.2 nM for core (n = 10) and 1.4 ± 0.2 nM for shell (n = 8). Arrows indicate time of transfer of rat from home-cage into operant chamber. Mean ± sem are shown.

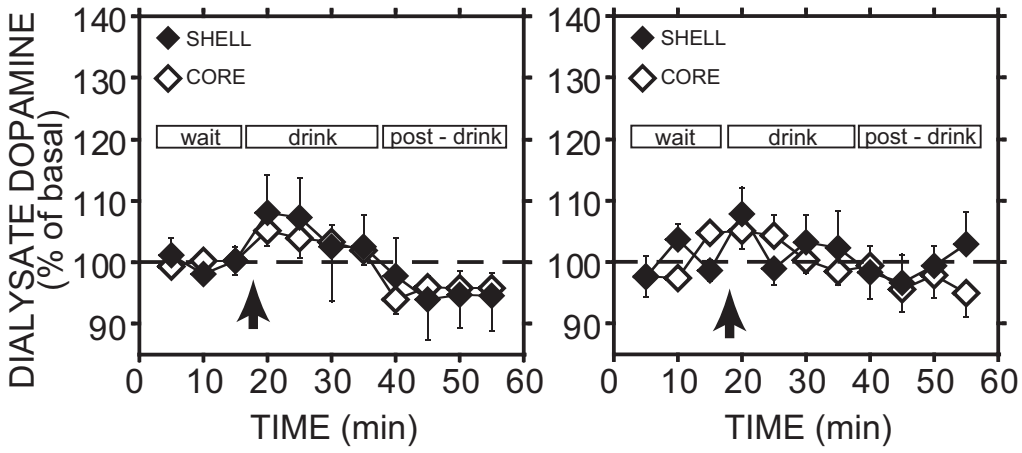

Figure 14. Dialysate dopamine from the core and shell of the nucleus accumbens during the wait, drink, and post-drink periods. For rats drinking 10S10E (left), basal dopamine concentrations were 1.6 ± 0.2 nM for core (n = 9), and 1.5 ± 0.3 nM for shell (n = 7), and for rats drinking 10S (right), basal dopamine concentrations are 1.6 ± 0.2 nM for core (n = 10) and 1.4 ± 0.2 nM for the shell (n = 8). Arrows indicate when the bottle was lowered into the operant chamber. Mean ± sem are shown.

Dialysate Ethanol Concentrations for Core and Shell Groups

Ethanol appeared in the dialysate within 5 min of ethanol availability for all but one of the rats that drank 10S10E. Dialysate ethanol concentrations increased over the entire course of the drink and post-drink periods, with the peak ethanol concentration reaching 2.0 ± 0.3 mM for the shell group and 1.6 ± 0.3 for the core group. The ethanol time course did not differ between core and shell (Figure 15; $F_{7,98}$ = 0.4, NS for subregion x time).

Behavioral Analysis for Core and Shell Groups

The analysis of several behavioral parameters that were determined during the operant self-administration session on microdialysis day shows that the animals consuming 10S drank more than those consuming 10S10E. As a result, four

consumption parameters were significantly lower for the 10S10E groups when compared to the 10S groups (collapsed across subregion): total number of licks, the duration of the first bout, the number of licks in the first bout, and the total milliliters consumed ($F_{1,30} \geq 6.4$, $p < 0.05$ for drinking solution, Table 3). Also, the 10S10E animals drank most during the first five minutes of access to the solution, while the 10S animals continued into the second five minute period. The percentage of licks in each five minute bin of the drink period differed significantly by drinking solution when collapsed across subregion (Figure 16; $F_{3,90} = 12.8$, $p < 0.05$ for drinking solution x time). Four parameters were not significantly different when compared across subregion and drinking solution: time to complete lever-press requirement, time between last lever-press and first lick, number of bouts, and the rate of licking in bout 1 ($F_{1,30} \leq 1.9$, NS for subregion x drinking solution, Table 3). Within the 10S10E drinkers, the shell group consumed 1.9 ± 0.2 g/kg ethanol, and the core group consumed 1.7 ± 0.2 g/kg, and these doses were not significantly different from one another ($T_{14} = 0.7$, NS).

Additional Analyses for Core-Shell Border Groups and Comparison to Core and Shell Groups

Because the dopamine increase observed during ethanol consumption in past studies (Doyon et al., 2005; Doyon et al., 2003) was not seen in the present core and shell groups, we performed additional experiments with probes placed on the core-shell border. A re-examination of probe placements revealed that most probes in these previous studies passed through this area, and sampled from the core-shell border as well as from the shell. Also, two missed placements from our core and shell groups that collected from the core-shell border indicated that

Table 3. Behavioral parameters for rats with microdialysis probe in the core, shell, or core-shell border while self-administering ethanol plus sucrose (10S10E) or sucrose (10S).

	Core		Shell		Core-Shell Border	
Parameter[b]	**10S10E n=9**	**10S n=10**	**10S10E n=7**	**10S n=8**	**10S10E n=7**	**10S n=6**
lever-pressing time (min)	0.08 ± 0.02	0.27 ± 0.15	0.09 ± 0.02	0.33 ± 0.25	0.21 ± 0.06	0.24 ± 0.11
latency to begin drinking (min)	0.11 ± 0.03	0.22 ± 0.10	0.10 ± 0.03	0.05 ± 0.01	0.17 ± 0.06	0.06 ± 0.02
number of bouts	1.2 ± 0.2	1.4 ± 0.2	1.4 ± 0.3	1.4 ± 0.2	1.3 ± 0.2	1.6 ± 0.2
initial bout duration (min)	5.2 ± 0.7	8.9 ± 1.3 [a]	5.9 ± 0.7	10.8 ± 1.0 [a]	7.2 ± 1.1	8.1 ± 1.6[a]
total licks	1439 ± 129	2124 ± 219 [a]	1699 ± 198	2595 ± 172[a]	1744 ± 271	2202 ± 211[a]
licks during initial bout	1343 ± 172	2005 ± 264 [a]	1645 ± 174	2481 ± 147 [a]	1652 ± 222	2071 ± 260[a]
initial bout response rate (licks/min)	277 ± 22	245 ± 31	283 ± 23	243 ± 25	235 ± 17	276 ± 28
milliliters consumed	8.2 ± 0.9	10.9 ± 1.3 [a]	9.4 ± 1.0	12.2 ± 0.7 [a]	9.9 ± 1.4	12.3 ± 0.8[a]

Values shown as mean ± sem

a - Significantly different from the ethanol group (core, shell, and core-shell border combined) by multivariate ANOVA ($p < 0.05$)

b – Parameters are defined as:

lever pressing time: time needed to complete response requirement (RR)

latency to begin drinking: time between completion of RR and first spout lick

bouts: period of at least 25 licks with no more than 2 min between licks

initial bout duration: time needed to complete initial bout licks

total licks: number of licks per session

initial bout licks: number of licks during initial bout

initial bout response rate: initial bout licks divided by initial bout duration

milliliters consumed: volume consumed during session

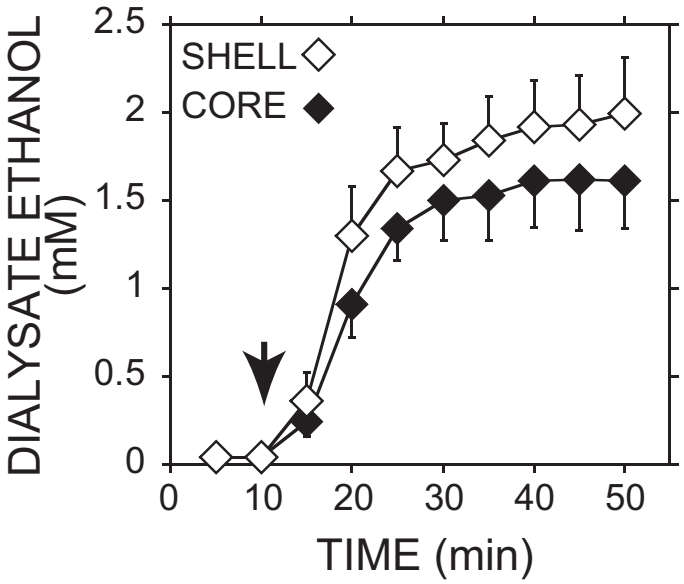

Figure 15. Dialysate ethanol from the nucleus accumbens core and shell during drinking and post-drinking periods. Dialysate ethanol concentrations are indicated (mean ± sem). Arrow indicates presentation of 10S10E drinking solution.

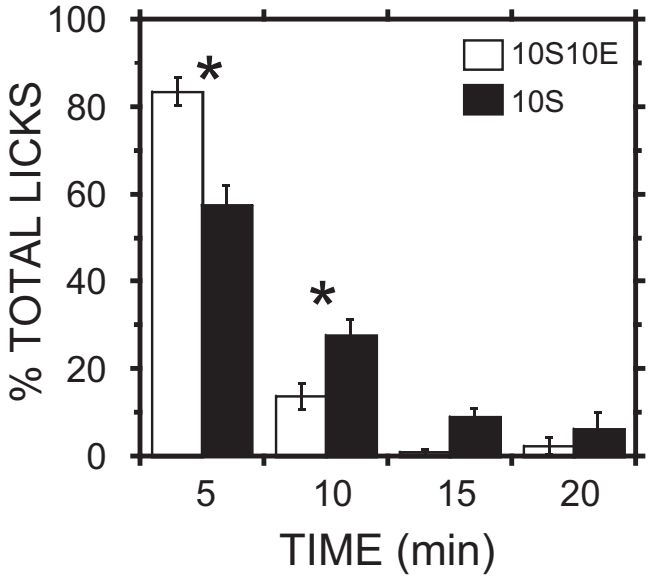

Figure 16. Percentage of total licks during drinking period in 5 minute increments for core and shell animals drinking 10S or 10S10E. The open bars represent 10S10E drinkers and the filled bars represent 10S drinkers. * indicates a significant difference in percentage of licks for the 10S10E groups than the 10S group. Mean ± sem are shown.

dopamine in this subregion was increasing during consumption of ethanol (33% and 59% above baseline).

The body weights and basal dopamine levels in animals in the core-shell border groups were similar to those in the core and shell groups. The body weights measured on the day of the microdialysis were 320-440g for core-shell border animals. ANOVA showed no significant differences in body weights between all six groups (core, core-shell border, shell, ethanol + sucrose drinkers, sucrose drinkers) ($F_{2,41} = 1.9$, NS). Basal dopamine levels for core-shell border groups were 1.1 ± 0.2 nM (n = 7) for 10S10E drinkers and 1.2 ± 0.2 nM (n = 6) for 10S drinkers, and were not significantly different from basal levels in the core and shell ($F_{2,41} = 0.1$, NS).

The increase in dopamine concentration in the core-shell border during transfer of the animal into the operant chamber was similar to that observed in the core and shell groups; however, dopamine in the core-shell border during consumption responded differently from these other two areas. For core-shell border groups, the increase observed during transfer into the operant chamber in rats trained to drink ethanol + sucrose (41%) was not statistically larger than the increase observed in rats trained to drink sucrose (20%) (Figure 17; $F_{9,99} = 1.0$, NS for drinking solution x time interaction), despite the similarity of these percentages to those in the core and shell. In contrast to the core and shell groups, a significant increase in dopamine in the core-shell border was observed during consumption of either solution (Figure 18; $F_{10,110} \geq 2.3$, $p < 0.05$). However, the time course of the dopamine response differed between 10S10E and 10S

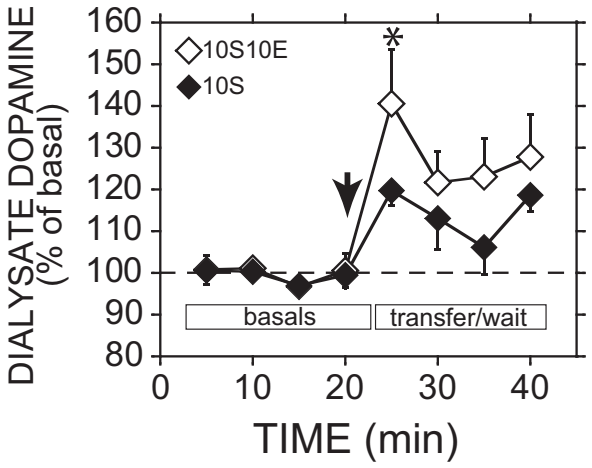

Figure 17. Dialysate dopamine from the core-shell border of the nucleus accumbens during transfer from home-cage into the operant chamber. * indicates a significantly larger dopamine response when compared to the wait period (collapsed across 10S10E and 10S). Basal dopamine concentrations were 1.1 ± 0.2 nM for rats drinking 10S10E (n = 7), and 1.2 ± 0.2 nM for rats drinking 10S (n = 6). Arrows indicate time of transfer of rat from home-cage into operant chamber. Mean ± sem are shown.

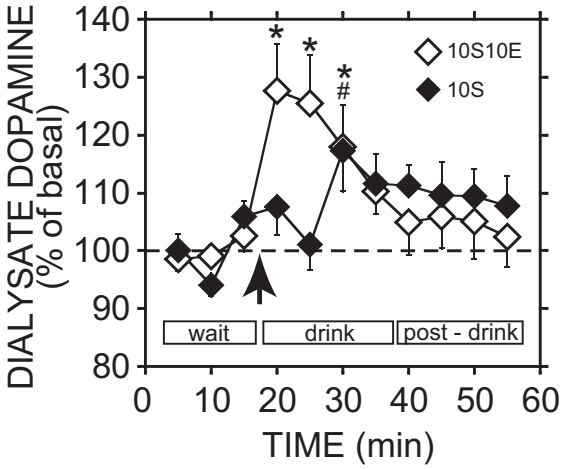

Figure 18. Dialysate dopamine from the core-shell border of the nucleus accumbens during wait, drink, and post-drink periods. * indicates a significantly larger dopamine response for the 10S10E trained rats when compared to the wait period. # indicates a significantly larger dopamine response for the 10S trained rats when compared to the wait period. Basal dopamine concentrations were 1.1 ± 0.2 nM for rats drinking 10S10E (n = 7), and 1.2 ± 0.2 nM for rats drinking 10S (n = 6). Arrows indicate time of transfer of rat from home-cage into operant chamber. Mean ± sem are shown.

drinkers ($F_{10,110} = 2.9$, $p < 0.05$, for the drinking solution x time interaction). For 10S10E drinkers, the first three samples in the drink period were significantly greater than the wait period ($F_{3,110} \geq 4.9$, $p < 0.05$), and for the 10S drinkers, only the third sample was significantly greater than the wait period ($F_{3,110} = 5.3$, $p < 0.05$).

The increase in dopamine in the core-shell border during consumption was not due to greater ethanol concentrations reaching this area. The peak dialysate ethanol level in the core-shell border, 2.2 ± 0.5 mM, was similar to levels in the core and shell (Figure 19). The ethanol time course did not differ between core, core-shell border, and shell ($F_{14,140} = 0.4$, NS for subregion x time).

The overall ANOVA comparing dopamine concentrations across core, core-shell border, and shell, confirmed that animals trained to drink 10S10E exhibited significantly larger dopamine increases than those trained to drink 10S (data not shown, $F_{8,328} = 2.0$, $p < 0.05$ for drinking solution x time effect), with no differences between subregions for this effect ($F_{16,328} = 0.7$, NS for region x time effect). Also, the addition of the core-shell border data to the ANOVA revealed a significant increase in 10S10E animals during ethanol consumption that was not observed in 10S animals (data not shown, $F_{1,40} = 2.1$, $p < 0.05$ for drinking solution x time effect).

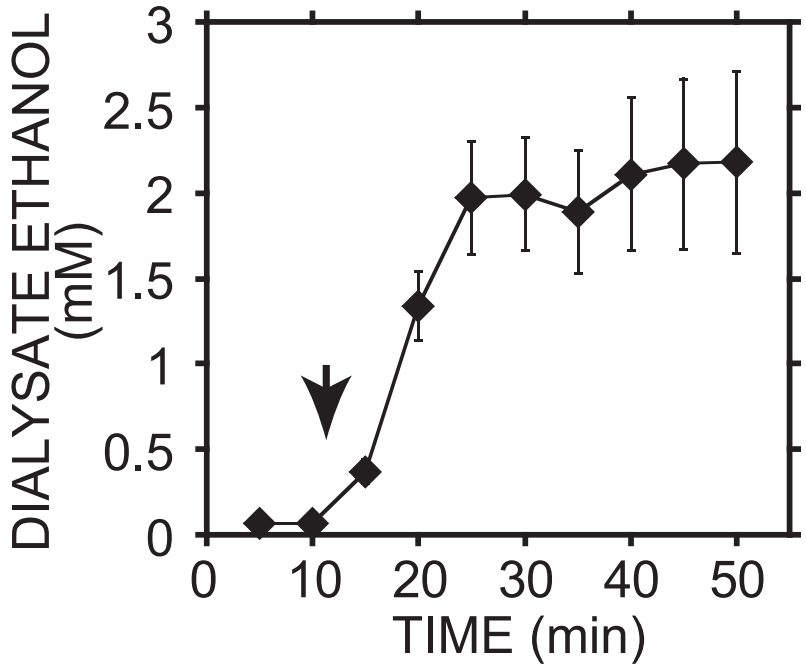

Figure 19. Dialysate ethanol from the nucleus accumbens core-shell border during drinking and post-drinking periods. Dialysate ethanol concentrations are indicated (mean ± sem). Arrow indicates presentation of 10S10E drinking solution.

The behavioral data for the core-shell border groups was very similar to the data collected from the core and shell groups. When this data was included in the analysis, the same four consumption parameters were significantly lower for the 10S10E groups when compared to the 10S groups (collapsed across subregion): total number of licks, the duration of the first bout, the number of licks in the first bout, and the total milliliters consumed ($F_{1,40} \geq 6.6$, $p < 0.05$ for drinking solution, Table 3). Also, the percentage of licks in each five minute bin of the drink period differed significantly by drinking solution (data not shown; $F_{3,120} = 13.3$, $p < 0.05$ for drinking solution x time). Finally, this group consumed 1.9 ± 0.2 g/kg ethanol, and this dose was not significantly different from core and shell groups ($F_{2,20} = 0.4$, NS).

Histological Analysis and Calcium Dependence of Dialysate Dopamine for All Groups

Figure 20 shows the representation of the probe placements. For probes measuring from the core or shell of the accumbens, only animals with at least 80% of the probe sampling from the subregion of interest were included in the analyses. As shown in the figure, in most places the shell wraps around the core. Because of this, the shell is medial to the core and below the core. None of the probes aimed at the shell overlapped with the core, although some of the core probes dipped into the shell below the core to a limited degree. For the core analyses we only included animals with probes that penetrated the shell or core-shell border by no more than 20% of the active dialysis membrane length. In order to keep conditions similar between groups, we continued to use 1.5 mm probes for the core-shell border groups. As a result, less of the probe was within the area of interest, and our criterion for this subregion was that the probe be at least 50% within the core-shell border. For these probes, the remaining percentage sampled from the shell. Overall, the dialysate samples for all groups showed a calcium dependency of 80 ± 1 %.

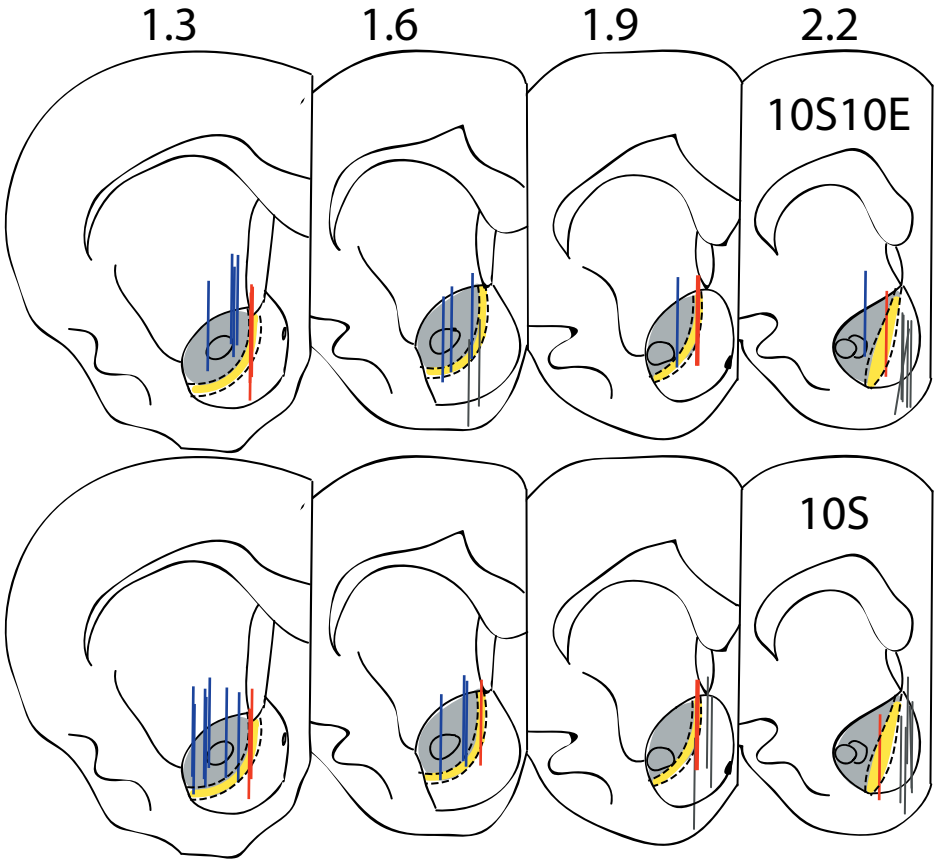

Figure 20. Coronal sections indicating microdialysis probe placements in the core (light grey), core-shell border (yellow), and shell (not shaded) of the nucleus accumbens for the 10S (n = 10 for the core, n = 6 for the core-shell border, n = 8 for the shell) and 10S10E drinking groups (n = 9 for the core, n = 7 for the core-shell border, n = 7 for the shell). Core probes are shown in blue, core-shell border probes are shown in red, and shell probes are shown in dark grey. Numbers above slices denote the location in millimeters from bregma. This figure was adapted from Paxinos et al. (1999).

DISCUSSION

This is the first study to investigate extracellular dopamine in the core, core-shell border, and shell of the nucleus accumbens during voluntary ethanol self-administration. Three major findings are reported in the present study. First, the results show that accumbal dopamine responses to operant ethanol self-administration are subregion specific. While the core, core-shell border, and shell exhibited dopamine responses during transfer from the home-cage into the operant chamber, only dopamine levels in the core-shell border increased during consumption of ethanol. The second major finding is that rats trained to drink 10S10E exhibit a significantly larger increase in accumbal dopamine in the core and shell during transfer from the home-cage into the operant chamber when compared with those trained to drink 10S. Increases in dialysate dopamine, compared with home-cage baseline values, were seen during the twenty minutes following transfer of the animal into the operant chamber, and the increase in the first five minutes was significantly higher in the 10S10E (33% above baseline) group when compared to the 10S (17% above baseline) group. The core-shell border 10S10E and 10S groups exhibited similar increases (41 and 20%, respectively). The third finding is the similarity of dopamine responses between the core and shell during all phases of this experiment. For the 10S groups dopamine increased 14-21% in the core and shell during the transfer from the home cage to the operant chamber, while in the 10S10E groups the dopamine increased by 33% in both subregions during the transfer period. When fluid consumption started, the small change in dialysate dopamine was 5 and 8% in

the first sample, in the core and shell respectively, for either consumption of 10S or 10S10E.

While the core and shell subregions have been reported to respond differently after administration of drugs of abuse, including alcohol, and receipt of food reward (Bassareo and Di Chiara, 1999a; Bossert et al., 2007; Howard et al., 2008; Sokolowski et al., 1998), we did not observe a difference in dopamine response between core and shell of the nucleus accumbens during operant ethanol self-administration. However, previous publications indicate that dopamine in both these subregions responds to the cues associated with a reinforcer. For example, dopamine in the core has been shown to respond to conditioned stimuli (Bassareo and Di Chiara, 1999a; Day et al., 2007; Ito et al., 2000), as has dopamine in the shell (Bassareo and Di Chiara, 1999b; Cheng et al., 2003). The results of the present study agree with these findings that dopamine in both subregions responds to conditioned stimuli. However, from our results we cannot conclude whether dopamine in the core and shell plays the same role during operant ethanol self-administration, or if these subregions coincidentally exhibit similar dopamine responses that have different functions.

Because the core-shell border is a newly defined subregion of the accumbens, the behavioral functions of dopamine in this area are not yet known. Dopamine in this subregion has previously been shown to respond to novelty (Rebec et al., 1997), with increases in dopamine transients being more delayed and more long-lasting than those in the shell, and no change occurring in the core. However, in the present study animals are exposed to drinking solutions and predictive cues

for over a week, and therefore novelty is unlikely to contribute to the dopamine responses we observed in the core-shell border region.

This study is the first to show that rats trained to drink 10S10E have a significantly higher accumbal dopamine response when transferred from the home cage into the operant chamber compared with those trained to drink 10S. Melendez et al. (2002) reported a similar difference in accumbal dopamine between ethanol and saccharin drinkers during the waiting period before self-administration. The results of the present study agree with previous findings from our lab that enhancement of accumbal dopamine activity occurs during this phase of the experiment (Doyon et al., 2005; Doyon et al., 2003), although we previously did not observe a significant difference between the 10S and 10S10E groups. It is possible that the group sizes may not have been large enough (n=8-11) to reveal a difference in the previous reports. In the present study we did not find a significant interaction between drinking solution and subregion. However, we did find a main effect of drinking solution, and the group sizes were 15 and 19. Thus, the larger groups may have increased the statistical power of this analysis. It is important to note that the results of this study cannot rule out the possibility that animals that regularly self-administer ethanol are more sensitive to salient stimuli, such as handling and transfer into the chamber, in general.

A number of studies have previously reported an increase in accumbal dopamine in trained rats after the presentation of cues that predict future ethanol availability, such as transfer of the animal into the operant chamber or an illuminated light inside the chamber, as well as the cues experienced during ethanol consumption

(Doyon et al., 2005; Doyon et al., 2003; Gonzales and Weiss, 1998; Melendez et al., 2002; Nurmi et al., 1998). A previous study from our lab reported significant dopamine responses in the accumbens during transfer into the operant chamber and during consumption that were similar to those observed in the core-shell border in the present study (Doyon et al., 2005). The authors attributed the increase during consumption to stimuli associated with the drinking solution because of a dissociation between the ethanol and dopamine time-courses. The finding that dopamine responds to ethanol predictive cues is consistent with numerous studies reporting that repeated exposures to a reinforcer, such as food reward or drugs of abuse, produce a temporal shift of both activation of dopamine neurons and increases in dopamine transients (Nishino et al., 1987; Roitman et al., 2004; Schultz et al., 1997; Stuber et al., 2008). The present finding of an increase in dopamine in the core-shell border during transfer into the chamber confirms these previous studies, and reveals for the first time that the response during consumption is restricted to this subregion.

The temporal shift of dopamine neuron activation from the time of the reinforcer to the time of the predictive cue is consistent with an idea put forth by numerous studies that mesolimbic dopamine, specifically in the accumbens, is more important for the appetitive phase than the consummatory phase of motivated behaviors (Blackburn et al., 1989; Ikemoto and Panksepp, 1996; Salamone et al., 1991). For example, disruption of dopamine signaling has been shown to affect responding for, but not consumption of, ethanol (Czachowski et al., 2002). However, these studies did not distinguish the accumbens core, core-shell border, and shell from one another. While the data from the core and shell in the

present study confirm previous reports showing that dopamine in the accumbens is more important for the appetitive phase than the consummatory phase, the data from the core-shell border indicates that there is regional specificity to dopamine's role during the consumption phase of ethanol administration.

Although cues are likely to play a role in the dopamine response observed during transfer into the operant chamber, other factors may also contribute. For example, handling control groups that did not receive operant training in previous studies exhibited dopamine increases (20-30% above baseline) during transfer into the chamber, similar to those observed in groups drinking sucrose or water (Doyon et al., 2005; Doyon et al., 2003). The dopamine response in handling control groups could be caused by physical handling, change in environment, or a combination of the two, and indicates that much of the dopamine increase during transfer of animals trained to drink ethanol into the operant chamber may not be due to cues associated with the liquid reinforcer.

The reasons behind the larger accumbal dopamine response when first transferred into the operant chamber in 10S10E drinkers, when compared to 10S drinkers, are not known. However, it has been shown that dopamine responses to natural rewards, such as food, rapidly habituate, while those responses to drugs of abuse are persistent in nature (Bassareo and Di Chiara, 1999b; Di Chiara, 2002). It has also been suggested that dopamine responses mediate the formation of associations between cues and reinforcers (Di Chiara, 2002). Therefore, it is possible that the larger response in the ethanol drinkers reflects

the formation of stronger associations formed between cues and ethanol, particularly compared to sucrose consumption.

Peak ethanol concentrations observed in the present study lead to levels of intoxication often observed in humans. The concentrations reaching the core and shell are not significantly different, with peak tissue levels achieved estimated to be 11.4 and 14.2 mM in the core and shell respectively (Howard et al., 2008). The peak core-shell border ethanol level is estimated to be 15.6 mM, and is very similar to shell levels. These levels would lead to mild intoxication in non-tolerant humans (equivalent to a blood alcohol concentration of 0.5 – 0.7 mg/ml), and would be achieved after consumption of approximately 2 - 3 standard drinks within 60 min (Brasser et al., 2004; Duarte et al., 2008; Erickson, 2007; Schweizer et al., 2006).

The 10S10E group differed from the 10S group in their drinking pattern in several ways that agree with a past study from our laboratory (Doyon et al., 2005). While the 10S10E group drank mostly in the first five minutes, the 10S animals continued drinking into the second five minutes. The total number of licks, number of licks in first bout, duration of first bout, and milliliters consumed were all significantly lower in the 10S10E groups, which could be due to the intoxicating nature or the aversive taste and smell of the ethanol solution. These parameters were also lower for the 10S10E group of animals with probes sampling from the core-shell border. It is also important to note that we did not observe any differences between core, core-shell border, and shell groups for any of these lickometer parameters, indicating that probe placements did not

influence these behaviors. Also, dopamine levels in the core and shell were not affected by these consumption behaviors as an increase in dopamine was not observed in either subregion during consumption of either of these drinking solutions.

In conclusion, the results of this study indicate that dopamine responses to ethanol self-administration are specific to accumbal subregions. Dopamine levels increase in a similar manner in the core and shell subregions, with increases occurring during the transfer of the rat to the operant chamber but not during drinking. However, dopamine in the core-shell border responds to transfer of the rat as well as during drinking. The results of this study also indicate that dopamine increases during transfer to the chamber are greater in animals expecting ethanol. Overall, these findings provide new details of accumbal dopamine function after the first week of ethanol self-administration.

Chapter Four: General Discussion

Four major findings are reported in these studies. First, that dopamine responds differently in the core and shell of the accumbens during non-contingent ethanol administration in naïve animals. Second, the dopamine response to operant ethanol self-administration is regionally specific. While the core, core-shell border, and shell exhibit significant increases in dopamine during transfer of the animal into the operant chamber, only dopamine in the core-shell border responds during consumption of ethanol. Third, animals trained to drink ethanol show significantly higher increases in dopamine during transfer into the operant chamber when compared to those trained to drink sucrose. Lastly, the dopamine responses in the core and shell are similar during operant ethanol self-administration.

The findings of these studies may have been affected by the type of ethanol administration, which differed between the two groups of experiments. The acute intravenous studies used non-contingent (response-independent) administration, and the operant studies used contingent (response-dependent) administration. It has been shown for cocaine that the accumbal dopamine response to response-dependent administration is significantly greater than that during response-independent administration (Hemby et al., 1997). Also, animals that were exposed to non-contingent ethanol for 3 weeks exhibited a smaller dopamine response to 1.0 g/kg i.p. ethanol than those exposed to contingent ethanol for the

same length of time (Nurmi et al., 1996). While the results indicate that the type of administration may affect dopamine responses to ethanol, it is important to note that the animals in this previous ethanol study were either alcohol-preferring or alcohol-avoiding, and the results may not be generalizable to other strains. Unfortunately, the results of the non-contingent ethanol administration study in this dissertation cannot be directly compared to the one using contingent administration to clarify the dopamine response under these two conditions, because they represent different time points of ethanol exposure.

The duration of ethanol exposure may have also affected how accumbal dopamine responded to ethanol. For example, when naïve animals were first exposed to intravenous ethanol in the experiments presented in chapter two, the infusions lead to increases in dopamine in both the core and shell. However, in animals that had a week of daily operant sessions, dopamine did not respond in either of these subregions to pharmacologically relevant doses of ethanol achieved in the brain in the forty minutes after consumption of an oral ethanol solution (Chapter Three). Under these circumstances, the lack of an increase in dopamine after consumption could be due to a temporal shift in the dopamine signal from the time of the reinforcer to the time of the predictive cues, much like the findings of studies of food reward and predictive cues (Nishino et al., 1987; Roitman et al., 2004; Schultz et al., 1997; Stuber et al., 2008).

The findings of the operant ethanol self-administration experiments show that the dopamine response to ethanol in experienced animals may not be due to the pharmacological effects of ethanol. The dissociation between dopamine and ethanol time courses observed in the core-shell border (Figure 21), in which high accumbal dopamine levels correspond with low accumbal ethanol levels, as well as a similar dissociation previously observed in rats that are self-administering ethanol in an operant paradigm (Doyon et al., 2005) supports this idea. The

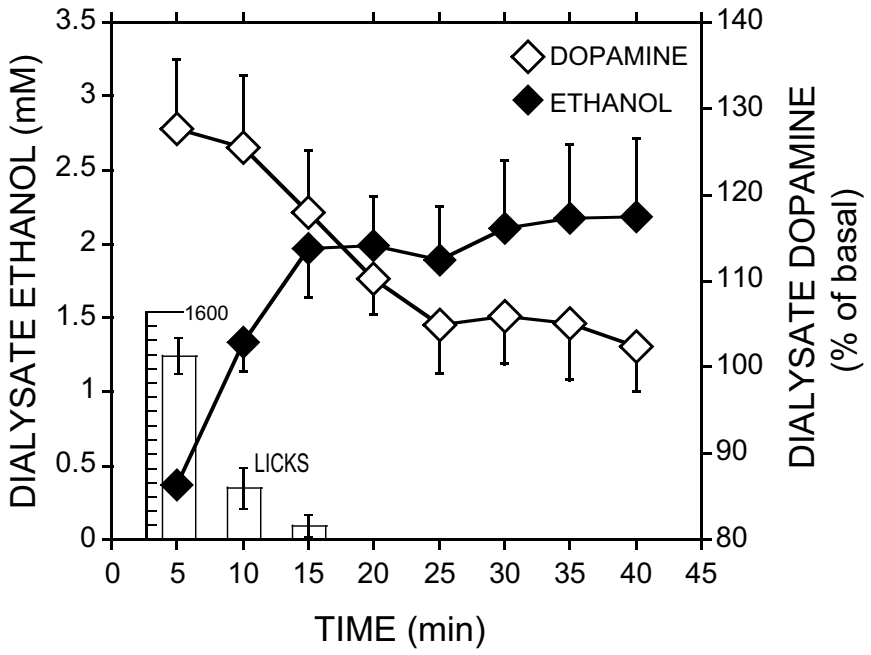

Figure 21. Dissociation of dopamine and ethanol time courses in core-shell border (n = 7).

increases in dopamine observed in both studies were closely associated with the licking behavior of the animals, a time when taste and smell stimuli are encountered. As a result, one explanation of the dissociation is that the increase in dopamine concentrations is due to stimuli associated with administration. The

present data does differ from the previous study because the dopamine response in the core shell border is significant during the first fifteen minutes of the drinking phase of the experiment, while in the previous study this was only true for the first five minutes. However, this is likely due to the fact that the longer probes in the earlier study were also sampling from areas that did not exhibit a response.

The results reported in the present studies agree with the incentive-salience theory of dopamine's role in drug reinforcement. The dopamine response observed in all three accumbal subregions during transfer of an animal trained to drink ethanol into the operant chamber may be due to the incentive stimuli that have been strengthened over repeated ethanol administration. While the present data are not enough to confirm that repeated ethanol administration results in the sensitization of the associated environmental stimuli, they do not refute this theory. Also, because animals only administer ethanol for 8 days, the data collected in the drinking phase of the present study probably does not test drug craving, that the authors term 'wanting', and more likely represents experiencing pleasure, termed 'liking', or a phase of drug-seeking that is between the two.

The findings of this dissertation also support the reward prediction theory of dopamine and drug abuse. In the operant ethanol experiments, transfer of the animal into the chamber was an unexpected event, and led to increases in dopamine in all three subregions. While it is not known if dopamine would

increase during ethanol consumption earlier in training, in the present studies increases were not observed in the core and shell of the accumbens during drinking. This is in agreement with the reward prediction theory, as ethanol availability was predicted by transfer into the operant chamber. However, dopamine responded to consumption of ethanol in the core-shell border of the accumbens, indicating that regional specificity should also be taken into account when interpreting this theory of dopamine's role in drug reinforcement.

The associative learning view of dopamine and drug reinforcement is also supported by the results of the present operant experiments. The dopamine response to transfer into the operant chamber, that is larger increase in animals trained to drink ethanol when compared to those trained to drink sucrose, suggests that associative conditioning between environmental cues and the reinforcer may have been stronger for animals drinking ethanol than those drinking sucrose. However, dopamine in the shell does not increase during consumption of ethanol in the present studies, which does not agree with Di Chiara's associative learning theory that dopamine in the shell is persistent for drugs of abuse (reviewed by Di Chiara et al., 2004).

The findings of the present studies confirm many aspects of the contemporary theories of dopamine's role in drug reinforcement, as well extending them to include that this role may differ depending on regional specificity within the

accumbens. In particular, in our hands, dopamine in the core plays a similar role to that in the shell in ethanol self-administration, but dopamine in the core-shell border plays a different role. While the associative learning theory specifically focuses on dopamine in the shell, the other previous theories do not account for subregion differences.

It is important to note that the regional specificity of dopamine's response to ethanol consumption that we observed may be unique to this time point during ethanol exposure. As drug-seeking behavior becomes habit, control over this process seems to shift from the ventral to the dorsal striatum (Everitt and Robbins, 2005). Also, there appears to be "spiraling" connections between the nucleus accumbens and dorsal striatum regions may allow communication between areas that mediate this shift (Belin and Everitt, 2008). Because our operant experiments test ethanol administration that is not yet habit, but also no longer novel, it is reasonable that the dopamine response we observed may be changing over time.

Because these subregions have only recently been studied separately from one another, the methods used in the present experiments for defining the core, core-shell border, and shell of the accumbens were designed in our lab. First, brains were sliced and stained. Once the slice with the microdialysis probe tract was identified, it was compared to an atlas picturing calbindin immunoreactivity

(Paxinos et al., 1999). The core stains much more intensely for calbindin than the shell (Meredith et al., 1996; Brauer et al., 2000), and the atlas allowed us to calculate how much of the probe was within the region of interest. Assigning core-shell border placements was more difficult, as little is known about how this area differs anatomically from core and shell. We chose to include probes that were within 50 microns of either side of the border between core and shell. However, as more is learned about these accumbal subregions, it may be necessary to redefine these methods.

The data presented in this dissertation does not explain why the increase in dopamine concentrations in the core-shell border differs from those in the core and the shell during ethanol reinforcement. However, when the current findings are considered in conjunction with several other previous publications, a few speculations can be made. For example, anatomy and cellular processes specific to the core-shell border may account for the difference in dopamine responses. More specifically, the lateral VTA has been shown to project to the core and the medial VTA to the shell, and it can be speculated that the core-shell border region receives an overlap from both medial and lateral (reviewed by Ikemoto, 2007). In addition, it has been reported that the area of the core-shell border directly below the lateral ventricle contains a co-localization of vesicular glutamate transporters and calbindin containing cells that is unique to this region (Hartig et al., 2003). Lastly, the dorsal core-shell border defined in these

experiments has also been identified as a “hedonic hotspot” for opioids and endocannibinoids (Pecina and Berridge, 2005; Mahler et al., 2007; Smith and Berridge, 2007). The nucleus accumbens has been thought of as a limbic-motor interface, and these characteristics unique to the core-shell border may allow dopamine in this area to respond differently to ethanol than the core and shell, and function as the accumbal region responsible for the integration of limbic and motor signals. However, there is no data to support these claims yet, and more work is needed to define the role of dopamine in the core shell border in ethanol reinforcement.

In conclusion, the work presented in this dissertation indicates that the role of dopamine in ethanol reinforcement may be regionally specific, and that future investigations should not overlook the heterogeneity of the accumbens. Trying to summarize the role of dopamine in broad strokes does the field a disservice, as it depends upon accumbal subregion, duration of ethanol exposure, involvement of drug-seeking behaviors, and many other variables. However, by recognizing these complications the findings of these studies further our knowledge of ethanol’s neurochemical effects, and may ultimately translate into better treatments for ethanol dependence.

Future Directions

The experiments in chapters two and three investigate ethanol’s effects on accumbal dopamine using non-contingent ethanol administration on naïve

animals and contingent administration in experienced animals. Because both the duration of ethanol exposure and type of ethanol administration differ, it is difficult to compare results between these two studies. Future experiments could investigate the effects of repeated non-contingent intravenous ethanol infusions, and of the first day of operant ethanol self-administration, on dopamine in the core, core-shell border, and shell of the accumbens. Also, it would be useful to measure dopamine in the core-shell border during intravenous ethanol administration in naïve animals, as this was not done in the study presented in chapter two.

The findings of the experiments presented in this dissertation also raise questions that could be addressed by subsequent studies. For example, does accumbal dopamine in the core and shell shift from responding to the pharmacological effects of ethanol to the stimuli associated with its administration, and if so, when? Measuring dopamine in the core and shell at several time points during operant training could provide insight into this issue. Also, what is the function of the increase in dopamine in the core-shell border during ethanol consumption in experienced animals? This could be addressed by blocking dopamine signaling in the core-shell border, with pharmacological agents, and observing the effects on drinking behavior in an operant paradigm. In addition, could the larger increase in dopamine during transfer of an animal trained to drink ethanol +sucrose into the operant chamber, when compared to those

trained to drink sucrose alone, be due to a greater sensitivity to salient stimuli in general? Administering ethanol daily, or water only for controls, to animals in the home cage while they participate in daily operant sucrose self-administration, and then measuring accumbal dopamine during transfer into the operant chamber in both groups could help answer this question.

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VITA

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