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MODERATE FETAL ALCOHOL EXPOSURE, THE NUCLEUS ACCUMBENS, AND ALCOHOL CONSUMPTION IN ADULTHOOD

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For Samara, Alexander, and Jacob

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Project Abstract

Recent findings using a moderate prenatal alcohol consumption model in rats found that male offspring had reduced dendritic fields in medium spiny neurons of the nucleus accumbens (NAc). These results suggest that moderate fetal alcohol exposure (FAE) leads to long-lasting alterations in brain regions involved in reward processing that could lead to abnormal behavior related to ethanol (EtOH) in adulthood. Here, five experiments were carried out to establish the extent to which moderate FAE leads to increased EtOH consumption in adulthood, how functional and structural alterations in the NAc are related to EtOH consumption, and whether moderate FAE has any effect on EtOH metabolism and general reward learning. Rats exposed to moderate FAE had increased consumption and preference for both a 10% and 20% EtOH solution across several weeks of exposure, and FAE animals also had significant reductions in measures of NAc core and shell dendritic morphology, as well as

reductions in core *Homer1a* immediate-early gene expression. In control animals, measures of shell dendritic morphology and core *Arc* expression served as significant predictors of ethanol consumption while core dendritic morphology predicted EtOH consumption in FAE rats. No significant differences were found between FAE animals and controls for measures of general reward processing in an intra cranial self-stimulation task or for EtOH metabolism. These results suggest that moderate FAE results in structural and functional alterations in the NAc, and that these effects have important implications for reward processing and EtOH consumption in adulthood.

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1. Introduction

1.1 Brain development and alcohol exposure

Since Fetal Alcohol Syndrome (FAS) was first described in systematic detail by Jones and colleagues (1973), a considerable amount of research has been conducted in an attempt to describe the long-term effects of ethanol exposure on the central nervous system during early development. Much of this contemporary research has focused on the effects of heavy, binge-like alcohol exposure on the developing fetus *in utero.* This type of exposure is the most likely to result in a diagnosis of FAS, the worst-case scenario in humans, as well as to establish a model for potential treatments (i.e. Thomas et al., 2007) and the discovery of ethanol consumption biomarkers in pregnant women (i.e. Joya et al., 2012). This research has been valuable in describing the behavioral and neurobiological consequences of FAS, as well as to identify potential treatments and improved diagnostic methods for this disorder. It is important to keep in mind, however, that FAS is only the most extreme outcome in a broad range of deficits that arise as a result of fetal ethanol exposure that fall under the umbrella of Fetal Alcohol Spectrum Disorders (FASDs; May et al., 2009).

In addition to FAS, other developmental deficits that have been described as FASDs include partial fetal alcohol syndrome (PFAS), alcohol related neurodevelopmental disorders (ARND), alcohol related birth defects (ARBD), and fetal alcohol effects (FAE). Although the occurrence of FAS is rare (~1%) in the general population (Abel, 1995), it is estimated that nearly 10-20% more children are exposed to moderate levels of ethanol during gestation that do not meet the

diagnostic criteria for FAS (Day et al., 1993). Before discussing the effects of more moderate levels of ethanol exposure on behavior and related brain circuitry, it is important to examine the more established effects of high levels of exposure that are associated with FAS.

1.1.1 Fetal Alcohol Syndrome (FAS)

In humans, FAS is associated with several physiological and cognitive deficits in children. The most recognized alterations in FAS involve stunted growth and distinctive facial dysmorphologies that serve as an indicator of central nervous system damage as a result of ethanol exposure (Jones and Smith, 1975). These abnormal facial features serve as key diagnostic criteria for FAS, but brain damage may still occur even in cases where they are absent. In addition to physical abnormalities in children with FAS, there are several behavioral and neurobiological deficits that have been identified that appear to be related to ethanol exposure during development. These deficits are also used as criteria for an *ex post facto* diagnosis of FAS in school-age children (Coles et al., 1991).

Based on the information amassed by Stratton et al. at the Institute of Medicine (1996), ethanol exposure *in utero* has been linked to gross reductions in cerebellar and hippocampal volumes that are associated with a myriad of behavioral deficits in children with FAS. These include hyperactivity, deficits in fine motor skills, impaired learning and memory (e.g. classical eye blink conditioning; Jacobson et al., 2008), abnormal social behavior, poor impulse control, and impairments in reward-guided learning. These effects appear to last

throughout the lifespan of the individual and may be related to hyperactivity, predispositions for other mental health diagnoses, and substance abuse issues (Streissguth, 1997). As noted previously, the incidence of FAS is rare. More recent research investigating the effects of more moderate fetal alcohol exposure suggests that, although less severe than the deficits noted in individuals with FAS, there are critical neurobiological and behavioral deficits in children with lower levels of exposure even in situations where physical markers are absent.

1.1.2 Fetal Alcohol Spectrum Disorders (FASD) in humans

In a review of relevant literature by Kodituwakku (2009), children exposed to ethanol during gestation that do not meet clinical criteria for FAS still show a stable pattern of cognitive deficits that fall under the FASD continuum. A consistent theme in this review is that, in comparison to controls, children with FASDs tend to do worse on tasks as the level of complexity increases. That is, while children with FAS are distinctly different from controls on simple tasks, children with FASDs might not. Only when the tasks become more complex do differences between FASD and control children become apparent. FASD children were found to be impaired in language skills, visual processing, social awareness, number processing, as well as slower processing of information and attention deficits. In addition, these children also had lower average IQ scores compared to the general population.

Several recent studies involving FASD children have demonstrated similar findings. Behavioral studies in children with FASDs indicate that these individuals have reduced intelligence, memory impairments, and have reduced scores on

tests of executive functioning and attention. Specifically, FASD children and adolescents have reduced verbal IQ and poor recall for faces and numbers (Rasmussen et al., 2006). It should also be noted that the pattern of cognitive deficits in this study varied based on the ethnicity, gender, and age of the participants. Another study that investigated gender differences and the comorbidity of FASD and attention deficit hyperactivity disorder (ADHD) found that the rate of FASD adolescents diagnosed with ADHD was higher for males than females (68% to 29%), and that males with both FASD and ADHD diagnoses had improved scores of executive function and behavior compared to those with FASD alone. The opposite was true for females, where adolescents with both FASD and ADHD diagnoses were impaired on the same tasks compared to those with only FASD (Herman et al., 2008). From this study it is difficult to determine the extent to which gender plays a role in FASD, due to the high rate of comorbidity between FASD and ADHD and the extent to which ADHD diagnoses may be skewed because males are more likely to be diagnosed with the disorder than females.

Recent studies have been conducted to determine more specifically the behavioral and neurobiological deficits in children and young adults with FASD. In one case, participants were given a neuropsychological battery in order to determine if there might be a fast and convenient way to diagnose individuals with FASD. Using the Cambridge Neuropsychological Tests Automated Battery (CANTAB), it was found that FASD individuals had longer reaction times and took longer to make decisions compared to controls, and that these children were

impaired in tasks that required planning, spatial working memory, and set shifting (Green et al., 2009a). In addition, Green and coworkers (2009b) found sensory processing and motor deficits in FASD children using eye tracking equipment during a pro- and anti-saccade cognitive task. In order to examine changes in the brain that might underlie the behavioral deficits that have been described in children and adolescents with FASD, Willoughby and colleagues (2008) took structural magnetic resonance imaging (MRI) images of children with FASDs and controls before the participants completed tasks involving verbal and spatial memory recall. The results found that FASD children were impaired in both verbal and spatial recall, and that these deficits were related to reductions in the size of certain brain structures. Specifically, FASD children had a smaller left hippocampus compared to controls. Furthermore, the only significant correlation between hippocampal volume and recall performance was in the FASD children, and while controls demonstrated a steady increase in hippocampal volume throughout the study, FASD children did not.

Based on these findings it appears that changes in the brain as a result of even moderate ethanol exposure *in utero* are long lasting. These studies tended to involve individuals from a wide range of ages, from childhood to young adulthood. Although development of the brain (specifically the frontal cortex) is still ongoing during this time (Squire, 2008), given that controls show increases in the size of brain areas related to learning and memory while FASD individuals do not (i.e. Willoughby et al., 2008), it would be reasonable to conclude that these cognitive deficits persist throughout the lifespan. It also appears that

neurobiological deficits related to FASD include both cortical and subcortical structures, as many of the cognitive impairments are related to frontocortical circuitry, as well as subcortical limbic structures involved in learning and memory (i.e. the hippocampus). It is, however, difficult to determine the specific effects of fetal ethanol exposure in humans due to the high variability in the amount and extent of alcohol consumed by the mother, the lack of reliable and valid biomarkers to confirm prenatal ethanol exposure, and potential cofounding variables that might also explain the deficits described above, including poly-drug abuse and socioeconomic status, among others. In this regard, animal models of FASD have become invaluable in the study of ethanol exposure effects on the developing central nervous system.

1.1.3 Moderate Fetal Alcohol Exposure (FAE)

Rodent models of moderate FAE have become important with regard to studying the neurobehavioral effects associated with FASD. This is due to the fact that the amount of ethanol consumed by the pregnant mother and the timing of ethanol exposure during gestation can be explicitly controlled. For example, work by Thomas et al. (2007) involves a rat model of 3rd trimester equivalent binge alcohol consumption in rat pups by way of intra-gastric infusions, while other researchers utilize voluntary drinking during the 1st and 2nd trimester (Savage et al., 2010). Using a rat model of moderate FAE allows us to assess the effects of ethanol exposure on the developing fetus throughout the lifespan without the various confounds associated with human subjects. Several studies

suggest that moderate FAE has an effect on a wide range of behaviors and related brain areas.

Spatial learning

As mentioned above, children with FASD demonstrate deficits in spatial working memory, and that these deficits are correlated with hippocampal volume reductions based on structural MRI measures (Willoughby et al., 2008). In rodent models of moderate FAE, the Morris water task (MWT; Morris, 1981; Morris, 1984) is often used to study spatial memory. Using two versions of the MWT, it was found that moderate FAE rats were impaired in the moving platform version of the task where the platform was moved within the pool every four trials. When the platform was in a single fixed location throughout training, FAE animals performed similarly to controls (Sutherland et al., 2000). The moving platform version of the MWT is considered a more difficult task compared to the fixed platform task, because the rat must learn to extinguish responding to the old platform and learn to navigate to the new location. This effect was also found to be dose dependent based on the amount of ethanol consumed by the mother during pregnancy (Savage et al., 2002). Thus, as with FASD children, moderate FAE impairments in rats become more apparent as the task demands increase.

These results are contrasted with models of heavy prenatal ethanol exposure that result in more profound deficits in the MWT where the escape platform remains in the same location throughout training and measures of persistence during test trials are relative to this single location (Thomas et al., 2007). Binge-like 3rd trimester exposure has also been found to impair initial

acquisition learning of the fixed-platform MWT (Ryan et al., 2008), whereas differences in initial training have not been noted in moderate FAE rats (Sutherland et al., 2000; Savage et al., 2002).

Long-term potentiation

In addition to the behavioral deficits noted in the MWT, moderate FAE has also been found to alter function in the hippocampus, a region of the brain that is involved in spatial learning and memory (Morris et al., 1982). Using *in vivo* electrophysiological recordings in anesthetized rats that were exposed to either 5% ethanol, saccharin, or pair-fed (isocaloric) controls throughout gestation, Sutherland, McDonald, and Savage (1997) describe deficits in long-term potentiation (LTP) in the moderate FAE rats. There were no group differences in evoked responses in the dentate gyrus after stimulation of the perforant path inputs into this region, but when LTP was induced by way of high-frequency stimulation of the perforant path FAE rats had diminished responses compared to controls. Another study found that deficits in LTP as a result of moderate FAE are related to histamine H_3 receptors in the hippocampus, as drugs that antagonize these receptors blocked LTP impairments in FAE rats (Varaschin et al., 2010). In relation to heavier prenatal ethanol exposure paradigms, these results demonstrate a more modest effect compared to prior *in vitro* studies of LTP after prenatal ethanol exposure (Swartzwelder et al., 1988). Other studies, however, have failed to note FAE deficits in LTP in hippocampal slices (Tan et al., 1990).

Although it does appear that moderate FAE results in more subtle effects in LTP compared to heavier doses, it appears that rat strain (Tan et al., 1990)

and electrophysiological protocols for high-frequency stimulation (Varaschin et al., 2010), among other factors, can make interpretations of these results more difficult. In any case, this impairment in cellular mechanisms that are proposed to underlie learning and memory could help explain the deficits noted in spatial learning in FAE rats. In addition to these findings, studies further indicate that moderate FAE also impairs the activity-evoked release of D-aspartate (D-ASP) in the hippocampus in a dose-dependent manner based on prenatal ethanol exposure (Savage et al., 2002). These findings, along with the MRI studies in humans with FASD (Willoughby et al., 2008), suggest that limbic structures, including the hippocampus, are affected by moderate FAE.

Social behavior

Another consistent finding in children with FASD is abnormal social behavior, with a higher than normal diagnosis of ADHD (Herman et al., 2008). Similar deficits in social behavior have been noted in rats as well. Hamilton and coworkers (Hamilton et al., 2010b; Hamilton et al., 2010a) conducted a series of experiments that involved cage mate manipulations in rats exposed to either moderate levels of ethanol (FAE) or saccharin (SAC) throughout gestation. In control animals, rats remained with the same cage mate throughout the study, while the social experience animals experienced a new cage mate every 48 hours. While these cycles of changing cage mates were taking place, initial social interactions between rats were videotaped and specific behaviors of interest were coded. Analyses of these social behaviors found that FAE rats engaged in more aggressive behaviors (i.e. Boxing and Wrestling) than SAC controls

(Hamilton et al., 2010b). A similar, but less pronounced, pattern of behavioral results were found in a situation where FAE and SAC rats were isolated for 24 hours before being reunited with their cage mate (Hamilton et al., 2010a). Although it appears that moderate FAE results in abnormal social behaviors in these animals, the results are somewhat ambiguous. It is open to interpretation the extent to which these behavioral alterations indicate aggressive behavior, poor social memory, or play behavior that would be otherwise expected to stop as the rat entered adulthood. Further research on this topic is underway in our laboratory.

Again, these subtle deficits in social behavior are contrasted to the more severe deficits in social behavior noted in animals that are exposed to higher doses of ethanol prenatally. These deficits include increases in aggressive behaviors (Lugo et al., 2003), immature play behavior in adult rats (Royalty, 1990), significant alterations in sexually distinct play behaviors (Meyer and Riley, 1986), differential responses to social stimuli (Kelly and Dillingham, 1994), as well as deficits in socially acquired food preference and social recognition memory (Kelly and Tran, 1997). This wide array of deficits as a result of heave prenatal ethanol exposure may not be detected in moderate FAE rats.

Dendritic morphology

In order to determine potential neurobiological alterations that could account for the abnormal social behaviors noted above, brain tissue was extracted following the completion of the social experience studies. Neurons from the frontal cortex were stained and traced with a microscope to measure the

complexity of dendritic fields. Results showed that FAE rats had reductions in dendrite length, branching, and spine density in layer 2/3 cortical pyramidal neurons in the agranular insular cortex (AID), a region of the frontal cortex that is functionally analogous to the orbital frontal cortex in humans (Hamilton et al., 2010b). These effects were not found in another region of the medial frontal cortex (Cg3), despite other research reports using a 3rd trimester binge exposure paradigm that found reductions in spine density, but not in dendritic length and branching, in Cg3 (Whitcher and Klintsova, 2008). In another study involving postnatal binge ethanol exposure, Hamilton, Whitcher, and Klintsova (2010c) found a different result: FAE rats had increases in spine density while dendritic complexity was reduced. These differential results suggest that fronto-cortical alterations as a result of ethanol exposure may be dependent on the timing of exposure.

Other studies of alterations in dendritic morphology as a result of FAE suggest that regions of the cortex that process sensory information, including the visual cortex, are also affected (Cui et al., 2010). In a follow up to the reductions described by Hamilton et al (2010b) in AID, Rice et al (2012) found that FAE rats also had reductions in dendritic length and branching in the nucleus accumbens (NAc) shell, but not the NAc core, dorsomedial striatum (DMS), or dorsolateral striatum (DLS). These results are important given that the frontal cortex and striatum are highly interconnected (Voorn et al., 2004).

Functional brain activity

In addition to the structural alterations in the frontal cortex mentioned above, functional alterations in neurons of the frontal cortex are also affected by FAE. These functional changes were measured by immediate early gene (IEG) expression in the frontal cortex. The IEGs of interest included *c-fos*, a protein that is expressed as a general marker of cellular activation, and activity related cytoskeleton (*Arc*) protein, which is related to plasticity functions (i.e. dendrite growth, spine genesis) in the brain (Guzowski et al., 2001). *Arc* and *c-fos* expression were measured after a brief social interaction between cage mates after they had been separated for 24 hours. The results found that FAE blunted the increases in IEG expression found in the frontal cortex (including AID) of social experience SAC rats (Hamilton et al., 2010b). Furthermore, using a comparison of regression slopes, it was found that these changes in IEG expression in the frontal cortex differentially predicted aggressive behaviors in FAE and SAC rats (Hamilton et al., 2010a). More dramatic effects on frontal cortex *c-fos* expression were found in rats exposed to high amounts of ethanol perinatally (Charles Lawrence et al., 2008).

1.1.4 FAE and reward circuitry

There have been relatively few published reports describing the extent to which FAE affects reward-guided learning and related brain circuitry in humans or rodents. One study in children with FAS or FAE was conducted using structural MRI and magnetic resonance spectroscopy imaging (MRSI) to measure the size of the caudate nucleus and the ratio of N-acetyl-aspartate (NAA) to creatine (Cr) in this brain area. Results showed that both FAS and FAE

children had reduced volume in the caudate nucleus, the brain region that includes the NAc. Furthermore, both FAE and FAS children had increased NAA/Cr ratios that appear to be due solely to increases in NAA (Cortese et al., 2006). Although the role of NAA in the CNS is not clear, the authors speculate that it may be related to myelination, apoptosis, and/or dendritic pruning. This could serve as an indicator of altered developmental processes in the caudate, which may lead to disruptions in reward-guided learning throughout the lifespan. A more recent study in children exposed to heavy alcohol exposure prenatally found significant reductions in caudate nucleus volume in alcohol-exposed children, as well as significant impairments in cognitive control and verbal learning and memory in these individuals. Importantly, in comparison to other brain regions measured, the caudate nucleus was the most consistent predictor of behavioral performance in the alcohol-exposed group (Fryer et al., 2012).

Based on these findings in humans and the reductions in NAc shell dendritic morphology observed in FAE rats (Rice et al., 2012), as well as the importance of this brain region in reward-guided learning and addiction (Yin et al., 2008), further research into the effects of ethanol exposure during early brain development on the NAc is prudent.

1.2 Nucleus accumbens (NAc) circuitry

In rodents, the striatum is divided into ventral and dorsal regions, with the NAc making up the ventral aspect (Voorn et al., 2004). The NAc itself is made up of distinct regions, with the NAc shell surrounds the medial, lateral, and ventral regions of the core (Figure 1).

The primary inputs into the NAc involve dopamine (DA) neurons projecting from the midbrain, a collection of fiber tracts commonly referred to as the mesolimbic dopamine pathway (Pierce and Kumaresan, 2006). These DA neurons originate in the substantia nigra (SN) and the ventral tegmental area (VTA). The VTA predominantly projects to the NAc, while the SN primarily projects to the dorsal striatum. Everitt and colleagues (2008) have shown that activity within the striatum alters DA activity in the SN and VTA, and can thus indirectly alter function in other striatal regions. Specifically, the NAc shell receives input from the medial VTA, while also projecting back to the lateral VTA. This affects VTA input into the NAc core, which sends projections to the SN. Given the connectivity between the SN and the dorsal striatum, activity in the NAc can thus alter downstream activation of the NAc core and the dorsal striatum (see Figure 1). This ascending pathway from the NAc to the dorsal striatum (Haber et al., 2000) forms the basis of information processing within the striatum, and is also one proposed mechanism for the consolidation of procedural learning and addictive behaviors (Everitt et al., 2008; Yin et al., 2008). Projections from other brain areas outside the well-established mesolimbic dopamine pathway also play an important role in NAc function. The NAc receives cortical and subcortical inputs from several areas- most notably the frontal cortex, hippocampus, and amygdala. These projections into the NAc are excitatory glutamatergic (Glu) inputs that have been found to modulate DA input from the

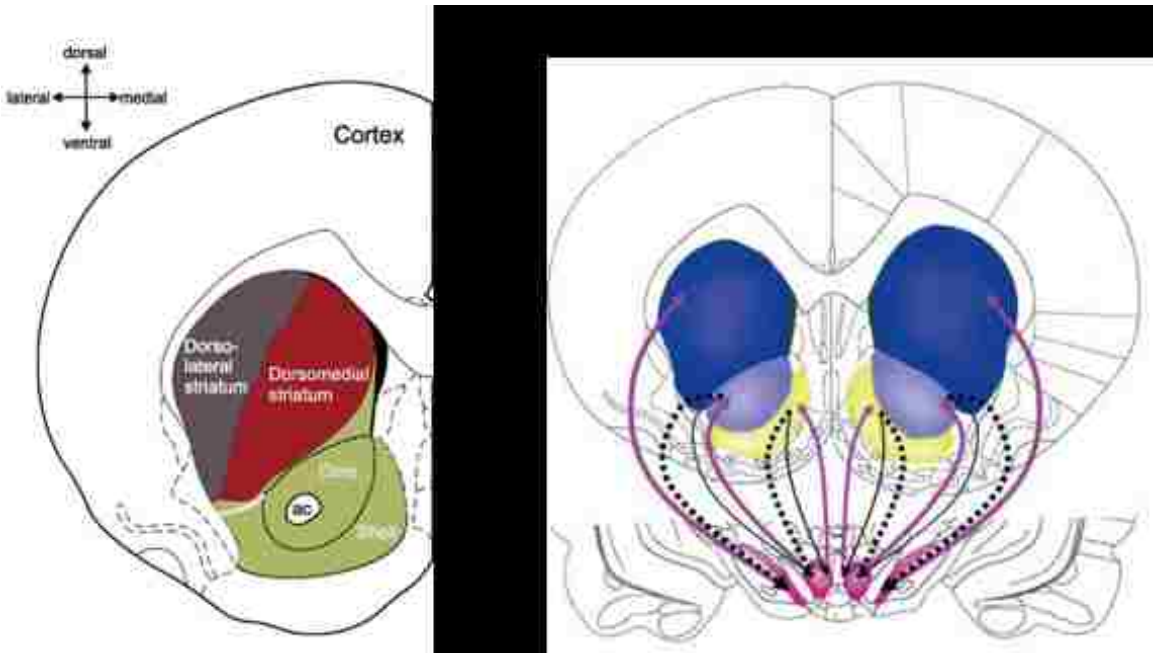

Figure 1. Anatomical locations of the rat striatum (caudate nucleus). The image on the left is a coronal section with color-coding to indicate the different regions of the striatum, including the DLS in grey, the DMS in red, and the NAc (core and shell) in green. The anterior commissure is abbreviated (ac). On the right is a coronal section highlighting the midbrain dopaminergic pathways involved in progression of the ascending striatal chain. The VTA (medial pink structures) and SN (lateral pink structures) project DA into the striatum (pink arrows). The NAc core (in purple) and shell (in gold) feed back onto the SN and VTA (dashed arrows). The end of the spiral is the dorsal striatum (in blue). From (Everitt et al., 2008).

SN and VTA (Humphries and Prescott, 2010). In turn, the NAc sends GABA-ergic projections to the ventral pallidum (globus palladus), which has also been implicated in reward processing (Berridge et al., 2009; Smith et al., 2009). The ventral pallidum sends projections to the thalamus, which then projects to the frontal cortex and other regions of the striatum. Given the inputs and outputs of the NAc and the dorsal striatum, these structures form the basis of proposed cortico-striatal-thalamocortical loops (Yin et al., 2008).

1.2.1 Theories of NAc function

There are several prominent theories that have attempted to summarize the general function of the NAc. While most of these theories are based on the role of the NAc in addiction processes, more recent reports have attempted to

integrate other natural reward functions as well (e.g. feeding behavior). The three theories that have received the most attention concerning the role of the neurotransmitter dopamine (DA) in the NAc include the dopamine-hedonic hypothesis, the learning-reward hypothesis, and the incentive-salience hypothesis (Berridge, 2007).

Dopamine-hedonic theory

The dopamine-hedonic hypothesis is one of the oldest theories regarding the role of the NAc in reward processing. This theory suggests that DA acts as a "hedonic neurotransmitter" in the NAc (Wise, 1980), based on research demonstrating that DA increases in the NAc are positively associated with increased drug consumption in animals. It is believed that the DA increase in the NAc is what reinforces the behavior associated with the rewarding stimuli. Many actions that animals take that are rewarding result in the release of DA into the NAc, including food, sex, and drugs of abuse (Everitt and Robbins, 2005). This hypothesis has received renewed interest as a result of advances in human neuroimaging, with studies demonstrating that subjective ratings of pleasure for food are correlated with dopamine receptor activation (Small et al., 2003), and that reduction in D2 receptor binding correlates with subjective measures of anhedonia in humans (Wang et al., 2001).

There are several problems with the dopamine hedonic theory. Most importantly, there is a growing body of evidence that questions the role of DA in hedonic responding in general. Recent research suggests endogenous opioids, not DA, released in the NAc and ventral pallidum serve as the driving force

between stimuli and the subjective feeling of “liking”. Berridge (2007) reports the results of several studies that show that depletion of DA neurons in the ascending striatal pathway, as well as blockade of DA receptors in the NAc, do not reduce “liking” reactions in rats. Likewise, increased DA release as a result of amphetamine infusions into the NAc did not induce greater levels of “liking” in rats (Wyvell and Berridge, 2001). Thus, it may not necessarily be the case that DA is the “hedonic neurotransmitter”. Similar results have been reported in human studies, where a series of experiments involving subjects with Parkinson’s disease (PD) found that these individuals had similar ratings of subjective pleasure for sweet foods like sucrose, chocolate milk, and vanilla milk as matched controls (Sienkiewicz-Jarosz et al., 2005). Since PD is associated with depletion of DA neurons in the SN, it is clear that DA is not the primary (or only) neurotransmitter involved in measures of hedonic value.

Learning-reward theory

The learning-reward hypothesis states that the function of DA is to “stamp in” the associations between stimuli and reward, so as to guide future behavior. That is, DA serves to strengthen stimulus-stimulus (S-S) and stimulus-response (S-R) associations in the striatum (Berridge, 2007). This theory is particularly relevant with respect to computational models of reward learning, as these models employ algorithms in which DA signals are computed to compare expected vs. achieved outcomes. One such algorithm is known as the temporal-difference reinforcement-learning (TDRL) model (Redish, 2004). This model posits that the NAc acts as a “learning” node, and DA acts as a “teaching” signal.

When a rewarding experience occurs, the initial expectancy is near zero, due to the novelty of the situation. Thus the high reward signal compared to the low expectancy in the NAc results in a large DA response, in order to “stamp in” the association. As the stimuli are encountered in future situations, the difference between the expected and actual outcomes becomes smaller, and the DA signal is reduced. Theoretically there is a point at which there is no DA signal at all, as the expected and actual outcomes are equal. It has been suggested that addiction results from the continued release of DA during drug consumption even in the case where there is no difference between expected and actual outcomes (Redish, 2004). This result is often cited as support for the learning-reward hypothesis.

This theory is also supported by studies demonstrating that D1 receptor activation is necessary for the induction and maintenance of LTP in the striatum (Yin et al., 2007). In addition, it has also been shown that DA signals that initially signal reward delivery will eventually shift to the stimuli that signal the availability of reward (CSs; Ito and Doya, 2009) over time. In a similar vein, this theory is also applied to the process of habit formation. Recent findings implicate the dorsal striatum, and in particular the DLS, is critical for the consolidation and expression of S-R habit learning (Yin and Knowlton, 2006).

The main criticism of this theory is the extent to which DA is sufficient, and for that matter, necessary for learning. Important studies utilizing DA deficient “knockout” mice indicate that DA is not necessary for reinforcement learning. Cannon and Palmiter (2003) used mice that were unable to produce DA as a

result of deletion of the tyrosine hydroxylase gene. Although these mice had severe PD-like symptoms, they were still able to learn to discriminate between, and show a preference for, a water bottle that contained sucrose compared to one that did not. Based on this theory it would also be predicted that increased DA activity would enhance S-S and S-R associations related to reward based on the learning-reward hypothesis. Again using “knockout” mice deficient in the dopamine transporter (DAT) gene, it has been found that these hyperdopamanergic mice did not differ from controls in acquiring or maintaining specific S-S or S-R associations compared to controls (Yin et al., 2006). These findings indicate that DA activation in the NAc is not necessary for the formation of meaningful reward associations. Even though DA activity is correlated with the cues that predict reward (and the reward delivery itself), this activity does not appear to be the primary cause of reward learning.

Incentive-salience theory

This theory hypothesizes that DA is important to reward-guided learning, but only for the “wanting” aspect, not “liking”. The other aspects of “liking” and learning are not reliant on DA function in the NAc. The idea here is that encounters with stimuli that are associated with reward will lead to a motivational state that integrates “wanting”, “liking”, and prior learning. Thus stimuli associated with highly rewarding outcomes work to create a memory trace that increases the salience of said stimuli, and increases the incentive to acquire the associated reward. The identification of “hedonic hotspots” in the ventral pallidum (Smith et al., 2009) and NAc (Pecina, 2005) that are related to endogenous opioid and

endocannabinoid (Mahler et al., 2007) activation lend support to this theory. With these “hedonic hotspots” acting as a neural substrate to “liking” and DA activity in the NAc functioning as a signal for “wanting”, other inputs from the cortex, hippocampus, and amygdala form the basis of reward-guided learning.

The greatest amount of criticism of the incentive-salience hypothesis has been directed to the fact that it is too general with respect to what is deemed rewarding. Di Chiara (2004) has proposed that different circuits in the NAc integrate endogenous vs. exogenous reward. Indeed, studies have found that food reward is processed in a different manner than other rewards (e.g. drugs of abuse). Even studies involving “hedonic hotspots” in the NAc have found regions of activation specifically related to drugs of abuse and different regions that are functionally related to feeding behavior. For example, in mapping the opioid related “hotspot” in the NAc shell, Pecina (2005) noted a smaller region near the drug “hotspot” that was highly reactive to pleasurable food, and general activation throughout the striatum was found when the animal was feeding compared to when drugs of abuse where present. Other studies have also shown that specific pharmacological manipulations in the NAc can also alter responses to drugs, but not feeding behavior. A recent study found that specific GABA receptor subunits were critical for the moderate consumption of ethanol in rats. Reductions in the α4-subunit GABA receptors resulted in reductions of voluntary ethanol consumption, but did not alter water consumption (Rewal et al., 2009). With this in mind, updated theories must take into account the specific types of reward that may be involved.

Cortico-striatal loops and reward-guided learning

More recent theories have attempted to integrate other aspects of NAc function within the greater framework of cortico-striatal-thalamocortical loops. Of particular interest in this theory of striatal loops is the involvement of the DLS and DMS in reward-guided learning. Here the divide between the NAc and dorsal striatum is based on differential roles in classical conditioning and instrumental behavior. Yin and coworkers (2008) propose that the NAc is critical for Pavlovian approach and avoidance behavior, while the DMS and DLS are critical for instrumental behavior. Evidence for hypothesized functional roles lies in the finding that the NAc is not necessary for instrumental learning, based on tests of outcome devaluation and instrumental contingency degradation (Corbit et al., 2001). It is proposed here that the function of the NAc core is to form stimulus-outcome (S-O) associations based on preparatory conditioned responses (CRs) to guide anticipatory approach to goals. The shell, on the other hand, is critical for the formation of S-O associations for consummatory CRs and hedonic unconditioned responses (URs; Yin et al., 2008). The functions of the NAc within this framework are summarized in the left panel of Figure 2.

Striatal loop theory is unique in that it also takes into account recent findings involving the DLS and DMS. The main findings of interest are that the DMS is critical for the formation of R-O associations, while the DLS is critical for the formation of S-R associations (Balleine et al., 2009). This is part of the striatal circuit that is critical for habit formation (Yin and Knowlton, 2006), and it has been demonstrated that skill learning is initially dependent on the function on the DMS,

but after asymptotic performance is reached, dependent on DLS function (Yin et al., 2009). It is concluded from these results that the NAc is important for Pavlovian approach/avoidance behaviors, and that these associations are then integrated into the DMS to initially evaluate R-O contingencies, and eventually to the DLS for S-R habit formation (Figure 2).

Another important aspect of this theory is the involvement of cortical input from different regions of the frontal cortex on DA activity from the VTA and SN. The bigger picture here is that the striatal regions (NAc core, shell, DLS, and DMS) function in parallel, and that processes related to reward-guided learning shift from one region to another in a serial manner over time based on interactions between the striatum, the VTA, SN, and frontal cortex. These interactions are presented in the right panel of Figure 2. Theoretically, information about stimuli related to reward is initially processed in the NAc shell, which is altered by DA input from the medial VTA and Glu input from the orbital

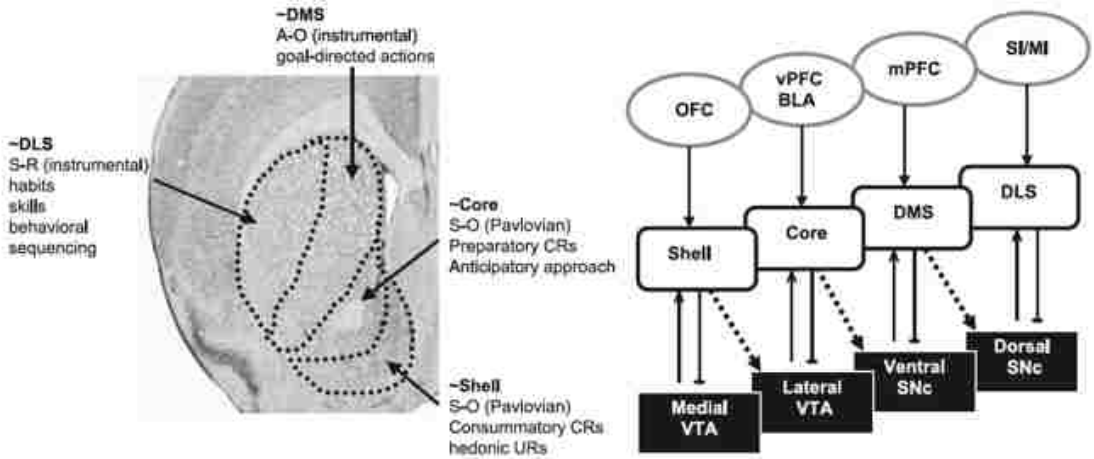

Figure 2. Proposed functions of the NAc core, shell, DLS, and DMS in reward-guided learning are presented in the left panel**.** The proposed interactions of these striatal regions within proposed cortico-striatal-thalamocortical loops, integrating DA from the VTA and SN and Executive functions from regions of the frontal cortex. From (Yin et al., 2008).

frontal cortex (OFC).

A practical example of this theory involves drug addiction, where cues associated with the drug are initially processed in the NAc to form Pavlovian conditioned associations. This could, in part, explain the early stages of drug addiction, where the context and specific cues are associated with the rewarding aspects of the drug. At the same time, the DMS is active in processing the consequences of drug taking behavior based on working memory circuits in the mPFC. Over time, as drug taking behavior becomes compulsive, the DLS takes over and habit formation is the driving force for drug-seeking behavior. This may explain why addicts are unable to stop seeking out drugs, even in the face of devalued outcomes.

Although the striatal loop theory is useful in that it takes into account other brain areas in striatal function, there are several valid aspects of other theories and research that appear to be ignored. For example, there is clear evidence that opioid receptors and related “hedonic hotspots” are an important aspect of reward learning (Pecina, 2005). Also missing is an explanation of the importance of inputs from other brain regions, specifically spatial information from the hippocampal formation (Sargolini, 2003; De Leonibus, 2005; Ito et al., 2008; Humphries and Prescott, 2010). Given the importance of spatial information in drug seeking behavior (i.e. Chaudhri et al., 2009), it is important to consider how this might fit into the functions of striatal loops. Although there are many aspects of this theory to be worked out in future studies, this work stands out for including

the entire striatum, as well as related cortical and subcortical structures in reward-guided learning.

In sum, these theories of NAc function differ with respect to their explanation of how reward is processed in the brain and the extent to which this is related to overt behaviors and addiction. In any case, it is clear from the extensive research conducted based on these theories that the NAc is an important bran region involved in reward-guided learning, and that alterations in this region as a result of fetal ethanol exposure is an important experimental target for future research.

1.2.2 NAc shell and core dissociations

Although the NAc core and shell are similar in terms of cellular composition and location, there are distinct neurochemical differences that have been reported that help to dissociate these regions functionally. Approximately 90-95% of the NAc is made up of medium spiny neurons (MSNs; Meredith, 1999). Large cholinergic interneurons (Kuzmin et al., 2008), fast-spiking medium aspiny neurons, and low-threshold spiking medium aspiny neurons account for the remaining 5-10% of neurons (Humphries and Prescott, 2010). Many MSNs within the NAc contain receptors for the endogenous opioid peptides enkephalin and dynorphin (Meredith, 1999), which have been found to be critical for hedonic "liking" properties of natural rewards and drugs of abuse (Peciña, 2008). In spite of these similarities between the NAc core and shell, there are several differences concerning inputs and neurotransmitter receptors that have come to define these regions of the ventral striatum (Heimer et al., 1997). Both the core

and shell receive inputs from the brain regions described above, but the specific amount of input varies across the NAc. Specifically, the shell has been found to receive more extensive input from the hippocampus, while the core receives more input from the amygdala (Voorn et al., 2004). It has also been shown that regions of the frontal cortex also differentially project to the NAc, with medial frontal areas projecting predominantly to the shell and orbital frontal areas projecting predominately to the core (Berendse et al., 1992).

In addition to these differences in input, the NAc shell is unique in that 1) this region sends projections outside of the striatum, most notably to the lateral hypothalamus (Usuda et al., 1998), and 2) there are distinct opioid- (Pecina, 2005) and endocannabinoid-rich (Mahler et al., 2007) regions within the shell deemed “hedonic hotspots” that are highly active in the presence of exogenous rewarding stimuli (e.g. sweetness) and during normal feeding or drinking behavior, respectively.

Behavioral dissociations

With these neurobiological differences between the core and shell in mind, several studies have attempted to establish behavioral dissociations related to NAc function. Much of the research related to functional dissociations in the shell and core involve variations of Pavlovian (classical) conditioning. Blaiss and Janak (2009) found that reversible inactivation of the core impaired the expression, but not the initial consolidation, of a specific conditioned stimulus-unconditioned stimulus (CS-US) association using a Pavlovian conditioned approach paradigm. Inactivation of the shell also reduced responding to the

conditioned stimulus (CS+), but also increased responding in the absence of the stimulus (CS-). The authors conclude that the core is critical for approach behavior related to reward-associated stimuli, while the shell is critical for the inhibition of approach to non-reward stimuli.

More detailed analysis of the role of the NAc in instrumental learning indicate that rats with neurotoxic lesions of the shell are impaired in Pavlovian instrumental transfer, a task that requires the animal to utilize learned associations based on classical conditioning in a instrumental behavior paradigm. Lesions of the core, on the other hand, rendered the rat insensitive to reinforcer devaluation in which the animal was satiated on one of the two rewards that had been previously associated with a specific lever. Core lesioned rats were further impaired in the initial acquisition of the lever-pressing reward association (Corbit et al., 2001). These results suggest that the shell is functionally distinct from the core, and from the rest of the striatum, given that the effects of core lesions are similar to the effects of lesions and pharmacological inactivations in the dorsal regions of the striatum (Balleine et al., 2009). Additional studies have identified shell vs. core distinctions based on DA (Hara and Pickel, 2005; Pattij et al., 2006; Lex and Hauber, 2008) and Glu (Di Ciano and Everitt, 2001) receptor activation in various instrumental tasks.

Functional properties of the NAc core

Although it is often suggested that the core region of the NAc is functionally similar to the dorsal striatum (Humphries and Prescott, 2010), there are unique properties of this region that are noteworthy, including the role of the

core in egocentric representations related to rewarding stimuli. Haluk and Floresco (2009) investigated set shifting and reversal learning and found that D1 receptor blockade in the core impaired new learning related to shifts in instrumental responding. D2 receptor blockade resulted in impairments in reversal learning, while D2 agonists increased errors of perseveration. The authors suggest that the impairments involving new learning in the core are related to the inability to integrate egocentric representations.

Similar results have been found with respect to spatial navigation. Using the MWT, researchers have shown that manipulations of the NAc can alter strategy and impair memory for egocentric spatial information. Blockade of NMDA receptors in the core after training resulted in impairments in memory for the platform location during test trials for both distal and proximal cue versions of the water task (De Leonibus, 2005). Given the established involvement of the dorsal striatum in spatial learning, especially for cue-guided egocentric navigation (Devan et al., 1999), it would make sense that the NAc core would also be involved in this behavior. One possible explanation is that the core is important for the integration of cue information leading to motor output in the dorsal striatum (Redish and Touretzky, 1997).

Physiological processes within the core have also been found to be critically involved in the early stages of drug addiction. In a recent report, Kasanetz and colleagues (2010) trained mice to self administer cocaine with a progressive ratio schedule of reinforcement. Approximately 20% of the mice developed symptoms that are commonly associated with cocaine addiction,

including responding in the absence of the drug and continuing to respond to the drug in the face of negative consequences. These “addicted” rats were found to have impairments in long-term depression (LTD), a process associated with the weakening of synapses that is thought to be related to forgetting (Malenka and Bear, 2004). LTD is an important homeostatic process that acts in contrast to LTP. Deficits in LTD could thus impair the processing of new information, and thus reduce the ability of the animal to adjust to new situations in a flexible manner. *In vivo* studies of NAc activity have also reported pauses in activation in core MSNs (as well as those in the shell) prior to water consumption in rats (Krause et al., 2010). This pause appears to facilitate approach behavior to the target, because mild stimulation that inactivates these MSNs reduced drinking behavior. Taken together, these results support the idea that the core is critical for integrating new information (indirectly from the shell) and comparing that with expected outcomes based on prior learning/experience. This would be a critical lower-order step before such information could be processed by downstream regions of the dorsal striatum, which are associated with response-outcome (R-O) and stimulus-response (S-R) learning (Yin et al., 2008; Balleine et al., 2009).

Functions of the NAc shell

Overall, the prevailing theory is that NAc shell is distinct from other striatal regions, owing to specific inputs and outputs not noted in the core (Humphries and Prescott, 2010). In accordance with these unique properties in the shell, recent theories of NAc function suggest that this region is involved in the initial aspects of reward-guided learning (Figure 2; Yin et al., 2008).

Several studies have been executed to describe the specific functions of the NAc shell. Ito and coworkers (2008) report that rats with lesions of the shell were disrupted in a CPP task involving a sucrose solution, suggesting the importance of hippocampal input into the shell in processing spatial information. In other words, the spatial information originating in the hippocampus is important for NAc shell processing of reward-location associations. Similar results were found in another study involving EtOH as a rewarding stimulus. It was found that inactivation of the shell resulted in impairments in responding to an EtOH-conditioned cue in an environment that was not previously associated with EtOH delivery (Chaudhri et al., 2009). In both cases, the importance of the NAc shell in the processing of contextual aspects of reward serves as an important component of drug addiction and relapse (Janak and Chaudhri, 2010). Aversive learning has also been found to involve NAc shell processing. Bradfield and McNally (2010) investigated the effects of shell lesions in Pavlovian fear conditioning and found that lesions had no effect on initial fear learning, but did disrupt learning about future neutral stimuli. Again, this serves as evidence for the importance of the shell in new reward-related learning.

There are several mechanisms that have been identified that could underlie these behavioral differences in the NAc shell. Given the large representation of MSNs in the NAc, alterations in different GABA receptor subtypes have been implicated in relation to reward processing. Specifically, the targeted reduction of α4-containing $GABA_A$ receptors via viral RNA infusions in the NAc shell reduced moderate EtOH consumption in rats. This effect was not

found in other striatal regions, and was specific to EtOH, as no differences were found for consumption of a sucrose solution (Rewal et al., 2009). DA activity has also been found to be different in the shell compared to the core. Suto et al. (2010) found greater release of DA into the shell compared to the core after cocaine self-administration. An examination of the role of D1 and D2 receptors in the NAc shell on conditioned flavor preference found that antagonizing both DA receptor types did not alter acquisition, but did result in reduced preferences for the CS+ compared to the CS-. DA antagonism also hastened the extinction of conditioned flavor preference (Bernal et al., 2008).

Alterations in other NAc shell neurotransmitters have also been identified in reward processing, including serotonin (5-HT) and opioids. Viral vectors injected in to the shell to increase the number of 5-$HT1_B$ receptors resulted in increased EtOH consumption in both voluntary and forced consumption paradigms. Specific analyses of drinking behavior found that up-regulation of 5-$HT1_B$ receptors in the shell increased high-frequency bouts of EtOH consumption compared to controls, and that this effect was specific to the acquisition, but not the maintenance of EtOH consumption (Furay et al., 2011). Opioid receptor systems in the shell have also been previously discussed, but it is important to point out that hedonic “hotspots” related to reward liking have been well defined in the NAc shell (Pecina, 2005; Peciña, 2008). Additional “hotspots” related to endocannabinoid function have also been identified in the shell, and agonists of cannabinoid CB1 receptors have been found to increase liking for reward in rats (Mahler et al., 2007). μ-opioid receptors in the NAc shell have been proposed to

regulate inhibitory control, but not impulsiveness induced by amphetamine sensitization (Wiskerke et al., 2011), and antagonism of μ-opioid receptors in the shell using naloxone has been reported to block the increasing palatability of sucrose normally induced by food deprivation (Wassum et al., 2009).

Studies involving genetic models of high EtOH consumption and fetal EtOH exposure have independently reported selective deficits in dendritic morphology in the NAc shell, which serves as an important mechanism for increased sensitivity for drug seeking behavior. In alcohol preferring P rats, research has shown that these animals will readily self-administer an EtOH solution directly into the NAc shell but not the core (Engleman et al., 2009). Zhou and colleagues (2007) have also reported alterations in NAc shell dendritic morphology as a result of chronic EtOH exposure in alcohol preferring P rats. Chronic-continuous exposure in these rats resulted in reduced dendritic spine density and up regulation in the NR1-containing (GluN1) NMDA receptors in the shell. Chronic-intermittent exposure also resulted in reduced dendritic spine density in the shell while also increasing the occurrence of large and multi-headed spines, and indication of altered signaling specific to the shell. Recently our laboratory has found deficits in dendritic length and branching in MSNs specifically in the NAc shell of rats exposed to moderate levels of EtOH *in utero* (Rice et al., 2012), as well as increased voluntary consumption of 10% and 20% EtOH solutions in another set of FAE rats (Rice et al., 2011).

In establishing the importance of the NAc shell in processing of new learning related to rewarding stimuli, two recent studies are worth mentioning

here. As LTP and LTD are hypothesized to be involved in learning and forgetting, respectively (Malenka and Bear, 2004), impairments in these processes in the NAc shell could explain maladaptive behavior towards drugs of abuse. *In vitro* and *ex vivo* physiology in rat NAc shell slices indicate that exposure to EtOH in a chronic-intermittent paradigm blunted LTP induction at low doses, and reversed LTP to LTD at the highest dose. In addition, LTP and LTD were completely blocked during withdrawal from EtOH exposure (Jeanes et al., 2010).

Finally, two other unique properties of the NAc shell have implications for theories of reward learning. The first involves the connectivity of the shell with the lateral hypothalamus. Millan and coworkers (2010) were interested in how activity in the shell after extinction to EtOH is related to downstream activation of the lateral hypothalamus. Inactivation of the shell after EtOH extinction increased EtOH seeking during a reinstatement period, and this was associated with increased expression of the immediate-early gene *c-fos* in the lateral hypothalamus. Furthermore, inactivation of the lateral hypothalamus counteracted the effect of shell inactivation in EtOH relapse. The authors conclude that the NAc shell-lateral hypothalamus pathway is critical for extinction learning, where satiety signals in the lateral hypothalamus reduce the rewarding aspects of the consumed stimulus (in this case EtOH). Second, DA input into the shell also has been shown to involve the release of Glu into this brain area as well. Stuber and coworkers (2010) used sophisticated neurotransmitter labeling techniques to measure DA and Glu co-released into the NAc shell and DMS. They found that DA terminals in the shell, but not the DMS released both DA and

Glu. These results are important for the temporal aspects of spatial learning that is proposed to underlie reward-related contextual learning in the NAc shell.

1.2.3 Relation to alcohol (EtOH) consumption

EtOH is classified as a depressant because of the overall reduction in activity it has on the central nervous system. It has been well documented that EtOH blocks NMDA receptors as a non-competitive antagonist, which reduces NMDA receptor activity and reduces excitation. It has also been shown that EtOH binds GABA receptors, which induces a net inhibition in neuronal activity. More recent studies have found that EtOH also has acute effects on opioid, serotonin, and cannabinoid receptors as well (Julien, 2010). Despite its overall depressive actions, EtOH is still an addictive substance that involves similar processing in the NAc. With this in mind it is understandable that aspects of reward circuitry might be affected differently by EtOH compared to other drugs of abuse.

Naive rats

There have been several published reports investigating the role of the NAc in the consumption and learning related to EtOH exposure. One interesting finding is that EtOH has been shown to induce conditioned place preference (CPP) in spite of its depressant effects. CPP is assessed by giving the animal access to two distinct environments, with one environment serving as the location of drug administration and the other serving as a neutral control. The animal learns to associate the environment related to EtOH exposure to the rewarding aspects of EtOH consumption, and will show a conditioned preference for this location. To elucidate the neural substrates of CPP to EtOH, Gremel and

Cunningham (2008) examined the effects of blocking DA input from the amygdala and blocking Glu in the NAc core. D1, D2, And D3 receptors were antagonized in the amygdala, and this abolished CPP to EtOH. This effect was specific to the basolateral region of the amygdala, as there were no significant effects in the central extended amygdala. The infusion of the NMDA antagonist AP-5 into the NAc core similarly blocked EtOH-associated CPP. This effect of DA blockade was not found in the core, suggesting that CPP to EtOH involves DA input from the basolateral amygdala and Glu activity in the NAc core. This also provides evidence for the cooperation between the amygdala and the core, an input that is less represented in the shell region.

With respect to reward processing, several studies have attempted to describe the effects of EtOH exposure on the NAc. A recent study reported that deep-brain stimulation of the NAc resulted in reduced EtOH self-administration, as well as reduced responding to EtOH-associated cues in a relapse paradigm. Rats were implanted with electrodes aimed at the NAc that could be stimulated at low levels by the experimenter while the animal was awake. After recovery from surgery, rats were trained to lever press to deliver EtOH reward. Prior to test sessions and relapse sessions, rats received stimulation of the electrodes in the NAc, a way of temporarily inactivating this brain area. Both the shell and core were affected by this stimulation paradigm, but the effect of stimulation was an overall reduction in EtOH self-administration and reduced responding when re-introduced to the EtOH-associated environment after a period of abstinence. It is also important to note that no significant effects on water consumption were

found in the stimulated rats, which rules out any possible deficits in general motor function (Knapp et al., 2009). To further link NAc function with EtOH consumption, Chaudhri and coworkers (2008) tested the effects of selective NAc core or shell inactivation on operant responding to EtOH. Inactivation was achieved via GABA antagonist infusion directly into the core or shell of the NAc. All rats readily learned to respond for EtOH reward. After training, rats were subjected to extinction training where lever presses did not result in delivery of EtOH, and then relapse training where EtOH was again made available in the operant environment. Inactivation of the core had no effect on extinction processing, but did result in reduced responding during relapse training. Shell inactivation, on the other hand, did not impair responding during relapse, but result in impairments in responding during extinction. These studies provide strong evidence for the importance of the NAc in responding to EtOH.

Studies involving the physiological behavior of striatal neurons in the presence of EtOH have also been important for the understanding of the neurobiology of reward processing. *In vivo* recording of NAc neurons while rats lever press for the delivery of EtOH or water found that there were specific subsets of neurons in both the core and shell regions that responded either to water or EtOH delivery. Nearly 85% of the cells recorded differentiated between EtOH and water, and these neurons had become spatially tuned to the position of the levers that delivered each outcome (EtOH or water). The pattern of firing for these cells also occurred for distinct aspects of the operant behavior. For cells that fired in response to EtOH, some cells increased in activity immediately after

EtOH delivery. Another set of cells decreased activity immediately after EtOH delivery. A third class of cells fired immediately after the delivery of water. No significant differences were found between the NAc core and shell regions for any of these three cell types (Robinson and Carelli, 2008). That separate sets of neurons in the NAc code for EtOH versus water consumption would fit with the idea that there are hedonic "hotspots" in the NAc that are specifically related to drug reward, but not consumption of natural reinforcers like water (Mahler et al., 2007; Peciña, 2008).

In vitro recordings of NAc brain slices has also found interesting effects of EtOH on plasticity-related events in the striatum. Jeanes and colleagues (2010) reported the effects of various EtOH bath concentrations on plasticity in the NAc using patch clamp recordings. Whole cell MSNs from the shell of mice were voltage-clamped, and AMPA-mediated excitatory post-synaptic currents (EPSCs) were recorded during LTP stimulation in the presence of EtOH. Increasing doses of acute EtOH resulted in dose-dependent impairments in LTP, with the highest doses actually reversing LTP to LTD. *In vivo* recordings in NAc shell slices taken from mice exposed to EtOH in a chronic-intermittent fashion (two separate four-day exposures) resulted in abolished LTD, but did not have any significant effects on LTP. Finally, both LTP and LTD were abolished in NAc shell slices taken from rats that had been withdrawn from EtOH for 72 hours after three days of continuous EtOH exposure. These results are important for two reasons: 1) the demonstration of plasticity deficits in the NAc shell after EtOH exposure would fit with the idea that this aspect of the striatum is critical for establishing new reward

associations, and 2) withdrawal is also an important phenomenon that affects NAc processing. These results also compliment the aforementioned findings in the NAc core (Kasanetz et al., 2010) and DMS (Yin et al., 2007) involving plasticity changes in the striatum as a result of drug exposure.

Epigenetic alterations

Several laboratories have developed lines of rats (i.e. alcohol-preferring P rats; Besheer et al., 2010) or mice to selectively increase alcohol preference and/or consumption. In addition to these genetic modifications, epigenetic changes based on alcohol exposure can also provide insight into neurogenetic and functional bases of ethanol exposure. Several laboratories have reported increased EtOH (and stimulant) consumption in rats exposed to EtOH during development. These studies involve EtOH exposure during gestation and/or early postnatal periods. Using a paradigm that involved moderate to high EtOH exposure during both gestation and lactation periods, Barbier et al. (2008; 2009) have reported increased tolerance and sensitivity to various drugs in rats exposed to EtOH compared to controls. Initial reports found that EtOH-exposed rats consumed more of an EtOH solution later in life and demonstrated increased measures of CPP to cocaine. EtOH-exposed rats also had increased sensitivity to the anxiolytic effects of EtOH consumption, and these rats were behaviorally sensitized to cocaine and amphetamine as well. These behavioral deficits coincided with decreases in D1 and D2 receptor binding in the striatum of EtOH-exposed rats, suggesting an overall increase in addictive potential in these animals (Barbier et al., 2008). In a follow up to these results, it was again found

that EtOH-exposed animals had increased behavioral responses to EtOH, cocaine, amphetamine, and apomorphine, a DA agonist. EtOH consumption in these rats was also increased, and CPP to EtOH was also higher compared to controls. EtOH-exposed rats demonstrate reduced sedative responses after subsequent EtOH exposure, and these behavioral effects were correlated with reductions in the DA transporter DAT (Barbier et al., 2009). Although these effects were not quantified specifically in the NAc, much less the core or shell specifically, it is clear that early exposure to EtOH alters processing in the striatum, and that this can affect responding to drugs of abuse later in life.

It has been suggested that increased EtOH consumption in rats exposed to EtOH during development is due to alterations in sensory processing (i.e. taste, smell) in these animals. Youngentob and Glendinning (2009) investigated the effects of fetal EtOH exposure on sensory reactivity in adult rats. They found that fetal EtOH exposure resulted in increased preference and consumption of both an EtOH and quinine solution, which have similar taste qualities, but not for a sucrose solution. In addition, EtOH odor priming increased consumption of an EtOH solution in fetal exposed animals compared to controls. Here it appears that sensory, as well as reward, circuitry can be affected by EtOH exposure during early development. The EtOH exposure paradigms employed in these studies involved forced exposure to EtOH, or resulted in higher doses that are more closely related to the diagnoses of fetal alcohol syndrome (FAS) in humans.

Using a moderate fetal EtOH paradigm, our laboratory has found reduced measures of dendritic length and branching in the NAc shell, but not the NAc core, DMS, or DLS in rats exposed to moderate EtOH *in utero* (Rice et al., 2012). In another set of rats exposed to moderate EtOH during gestation, it was found that these rats had a higher preference for, and consumed more of, a 10% and 20% EtOH solution in adulthood (Rice et al., 2011). Interestingly, fetal EtOH-exposed animals in this experiment did not consume more of a sucrose or quinine solution compared to controls, in contrast to the previously mentioned findings of Youngentob and Glendinning (2009). In sum, it appears that exposure to various levels of EtOH during development can have profound effects on the striatum, and in particular, the NAc. These results point to the NAc shell as an important brain region in the processing of reward-related stimuli, including EtOH. The purpose of this proposal is to establish a link between the deficits observed in the NAc of rats moderately exposed to EtOH throughout gestation and the increased consumption of ethanol in adulthood in these animals. It is also important to establish the extent to which these alterations are specific to the NAc core and shell, in order to better understand the functional and structural dissociations between these regions with respect to EtOH consumption and reward processing.

2. Rationale, research questions, and specific aims

2.1 Rationale

Exposure to moderate levels of ethanol during gestation has been shown to result in long lasting deficits in behavior and related central nervous system function. Although deficits in several brain regions related to social behavior and learning and memory have been identified in animals exposed to moderate levels of ethanol *in utero,* few studies have investigated these effects on brain circuitry related to reward learning and addiction. Furthermore, how these deficits in reward circuitry may impact behavior in adulthood is also poorly understood. Using a rodent model of moderate prenatal ethanol exposure, we have identified the nucleus accumbens and the basal forebrain as brain regions of interest with respect to deficits as a result of both prenatal ethanol exposure and ethanol consumption in adulthood. The results of these experiments will lead to a better understanding of how learning and memory related to alcohol are impacted by early development insults, and provide a therapeutic target for future research and treatments.

2.2 Research questions

2.2.1 Do fetal alcohol exposed rats drink more alcohol in adulthood than saccharin exposed controls?

2.2.2 What are the effects of fetal alcohol exposure on nucleus accumbens dendritic morphology, and are these alterations predictive of voluntary alcohol consumption?

2.2.3 Are there functional differences within the nucleus accumbens that are related to alcohol consumption in fetal alcohol exposed rats?

2.2.4 Are there differences between fetal alcohol exposed and saccharin exposed rats regarding general reward processing?

2.2.5 Are there different rates of alcohol metabolism for fetal alcohol exposed rats compared to saccharin-exposed controls?

2.3 Working hypothesis

Moderate fetal alcohol exposure results in increased ethanol consumption in adulthood. This increased ethanol consumption will not be due to altered ethanol metabolism or deficits in general reward learning, but rather will be related to reductions in measures of dendritic morphology and immediate-early gene expression in the NAc core and shell.

2.4 Specific Aims

This working hypothesis will be approached in four specific aims:

2.4.1 Specific aim 1

To characterize structural and functional alterations in the nucleus accumbens (NAc) as a result of moderate fetal alcohol exposure.

Aim 1a

To examine the effects of moderate fetal alcohol exposure on voluntary ethanol consumption in adulthood.

Additional control: Differences in ethanol metabolism between fetal ethanol exposed animals and controls.

Aim 1b: Altered reward responding to intra cranial self-stimulation as a result of fetal alcohol exposure.

2.4.2 Aim 2

To predict voluntary ethanol consumption based on alterations in the NAc core and shell.

2.4.3 Aim 3

To evaluate engagement of the NAc core and shell in relation to voluntary ethanol consumption.

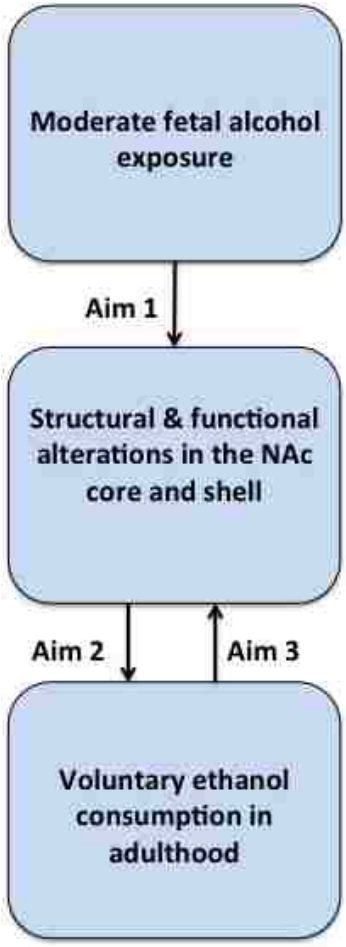

Figure 3. A schematic representation of the specific aims to be investigated in this dissertation in establishing a link between fetal ethanol exposure, structural and functional alteration in the nucleus accumbens, and voluntary ethanol consumption in adulthood.

3. Experimental Design

3.1 Experiment 1: Dendritic morphology and EtOH drinking behavior

3.1.1 Background

Our laboratory has recently reported that moderate fetal alcohol exposure (FAE) results in reductions in dendritic morphology in the NAc shell, but not in the core or the medial and lateral regions of the dorsal striatum (Rice et al., 2012). Recent published reports in naïve (Rewal et al., 2009) and alcohol preferring P rats (Zhou et al., 2007; Engleman et al., 2009), suggest that the NAc shell plays an important role in voluntary EtOH consumption and serves as a critical first step in reward learning in the striatum. With this in mind, Experiment 1 was intended to describe the extent to which moderate FAE affects EtOH consumption in adulthood. Adult SAC and FAE rats were given access to a 10% EtOH solution in the home cage on 3 nonconsecutive days a week for 6 weeks. After this initial EtOH consumption phase, rats were given access to a 2% sucrose solution on one day and a 0.2% quinine solution on another day to measure appetitive and aversive flavor preference, respectively. After the taste reactivity test, rats were then given access to a 20% EtOH solution for an additional 3 weeks to observe changes in drinking behavior to a stronger concentration of EtOH.

3.1.2 Hypothesis

It was hypothesized that FAE rats would drink more of, and show a greater preference for, a 10% and 20% EtOH solution compared to a control bottle that only contained tap water. Also, there would be no group differences for

measures of consumption and preference for either the sucrose or quinine solutions during the taste reactivity test.

3.1.3 Subjects

Subjects included 12 (6 SAC and 6 FAE) male Long-Evans rats (Harlan Industries, Indianapolis, IN), bred at the University of New Mexico Health Sciences Center Animal Resource Facility (HSC-ARF) exposed to saccharin or ethanol *in utero* (described below). At approximately 6 months of age, animals were transferred to the Psychology Department ARF where they were individually housed in standard plastic cages with food and water available ad libitum, and maintained on a reverse 12-hour light/dark cycle with lights off at 1000. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of New Mexico and were conducted in accordance with the NIH Guide for Care and Use of Laboratory Animals.

3.1.4 Moderate fetal alcohol exposure

All breeding procedures were conducted in the University of New Mexico HSC-ARF. Three to four-month-old Long-Evans rat breeders (Harlan Industries, Indianapolis, IN) were single-housed in plastic cages at 22°C and kept on a reverse 12-hour light/dark schedule (lights on from 2100 to 0900 hours) with Purina Breeder Block rat chow and tap water ad libitum. After at least one week of acclimation to the animal facility, all female rats were provided 0.066% saccharin in tap water for four hours each day from 1000 to 1400 hours. On Days 1 and 2, the saccharin water contained 0% ethanol, on Days 3 and 4, the saccharin water contained 2.5% ethanol (vol/vol). On Day 5 and thereafter, the

saccharin water contained 5% ethanol (vol/vol). Daily four-hour consumption of ethanol was monitored for at least two weeks and average daily ethanol consumption was determined for each female breeder. Following two weeks of daily ethanol consumption females that drank less than one standard deviation below the mean of the entire group were removed from the study. The remaining females were assigned to either a saccharin control or 5% ethanol drinking group and were matched such that the mean pre-pregnancy ethanol consumption by each group was comparable.

Subsequently, females were placed with proven male breeders until pregnant as evidenced by the presence of a vaginal plug. Female rats did not consume ethanol during the breeding procedure. Beginning on Gestational Day 1, rat dams were provided saccharin water containing either 0% or 5% ethanol for four hours a day, beginning precisely at 1000 hours (1 hour following the onset of the dark cycle). The volume of 0% ethanol saccharin water provided to the controls was matched to the mean volume of 5% ethanol in saccharin water consumed by the ethanol-drinking group, which has been found to be relatively consistent at 16mL per four-hour drinking period over multiple breeding rounds. Rat chow was available ad libitum during both the drinking and non-drinking periods. Maternal weight gain during pregnancy and offspring birth-weight was not expected to differ based on prenatal diet (Hamilton et al., 2010b; Savage et al., 2010). Daily four-hour ethanol consumption was recorded for each dam. Ethanol consumption ceased birth, and all litters were weighed and culled to 10 pups. Offspring were weaned at 24 days of age and transferred from the HSC-

ARF to the Psychology ARF as described in the subjects section. No more than two offspring from a single breeding pair were assigned to any particular experimental condition. In order to estimate maternal BACs produced with this voluntary drinking protocol, maternal serum ethanol levels were determined in 12 rat dams concurrent with the breeding round for offspring used in the present study. Due to potential stress effects associated with blood collection all measurements were performed in a separate set of dams that were not used to generate experimental offspring. The detailed methods for quantification of blood ethanol concentrations for daily 4-hour consumption have been described previously (Hamilton et al., 2010b; Savage et al., 2010).

3.1.5 EtOH consumption

Figure 4 summarizes the timeline of experimental procedures carried out in Experiment 1. After a minimum of two weeks of acclimation, rats were given access to an EtOH solution for 24 hours in the home cage in the Psychology department ARF. At the start of the dark cycle (1000 hours) rats had their water bottle removed, and two new water bottles of the same size were inserted into the left and right side of the cage top. One bottle contained tap water, the other a 10% ethanol solution in tap water. The bottle location (left vs. right) was randomized for each animal. All bottles were weighed at the beginning and at 4-hour and 24-hour time points in order to determine liquid volume consumption. An empty cage in the same room had two bottles inserted into it as well and served as a control for spilled liquid during insertion and retraction of the bottles,

and to account for evaporation. Measures of consumption were calculated based on the weight change in the bottles minus the values from the control cage.

At the end of the 24-hour drinking session, the bottles were removed and a fresh water bottle was placed in the cage top for 24 hours. On the following day, fresh 10% EtOH and tap water bottles were again inserted into the cages, with the locations of the bottles alternated across sessions. This chronic-intermittent 24-hours access to EtOH was carried out 3 days a week (Monday-Wednesday-Friday) for six weeks. After this initial exposure to 10% EtOH, taste reactivity was tested by inserting a bottle with a 2% sucrose solution into the home cage, with another bottle again containing tap water. As with the EtOH solution, measures of sucrose consumption and preference were taken at 4- and 24-hours. A week later, a similar test was carried out with a 0.1% quinine solution. A week after the quinine solution test, rats were again given access to a EtOH solution, but this time the concentration was increased to 20%. As with the 10% solution, access to 20% EtOH was given for 24-hours, 3 days a week for a total of three weeks. Again, 4- and 24-hour measures of consumption and preference were recorded.

3.1.6 Expected outcomes

It was expected that FAE rats would drink more of the 10% and 20% EtOH

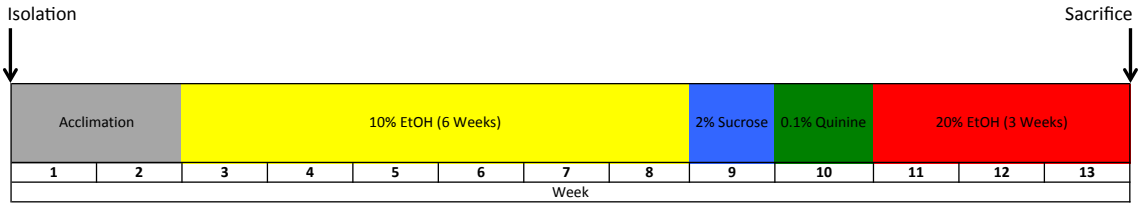

Figure 4. Experimental timeline for 10% EtOH consumption, taste reactivity testing, and 20% EtOH consumption for Experiment 1.

solutions than SAC controls, and that FAE rats would also show a higher preference for the EtOH bottle as well. No significant differences were expected between groups for sucrose or quinine consumption, suggesting no differences in the sensory processing of FAE rats.

3.2 Experiment 2: EtOH clearance

3.2.1 Background

It is possible that any increased EtOH consumption in FAE rats observed in Experiment 1 might be the result of differences in EtOH metabolism in these animals. Other studies have found no differences between FAE rats and controls with respect to ethanol clearance (Barbier et al., 2009). However, it is important to confirm this in the moderate FAE paradigm used in Experiment 1. This was carried out in Experiment 2 by testing blood ethanol concentrations in naïve FAE and SAC rats after specific concentrations of ethanol are administered. Groups were compared using a within-subjects design at various time points post EtOH exposure to address whether these groups differ on ethanol clearance, thus providing further support for our hypothesis that these affects are a result of neurobiological processes in the NAc.

3.2.2 Hypothesis

Given that prior research suggests that there is not difference between FAE and control rats on EtOH clearance, the hypothesis to be tested in this experiment is that there will be no differences between groups, nor any significant group by time point interactions for blood ethanol concentrations.

3.2.3 Subjects & moderate fetal alcohol exposure

Ten male Long Evans rats (5 FAE and 5 SAC) served as subjects for Experiment 2. These subjects will be obtained and housed as described in Experiment 1. The moderate fetal alcohol exposure paradigm described in Experiment 1 will again be utilized for this Experiment.

3.2.4 Alcohol Exposure

On five nonconsecutive days, rats were injected with a specific dose (20% vol./vol.) of alcohol (2.5g/Kg of body weight, i.p.). Each test day rats had blood drawn from the ventral surface of the tail either 1, 2.5, 4, 5.5 or 7 hours post injection. A within subjects design was utilized so that each rat received five injections across all testing days and blood was sampled at each possible time point post injection. Injections sites and tail bleed locations were rotated to avoid issues or scarring and blood clotting.

3.2.5 Blood alcohol concentrations

Approximately 150μL of blood was collected from the tail bleeds on each exposure day at the assigned time point. These samples were centrifuged at 13,000 revolutions per minute, at which point blood serum was removed and stored at -80°C for analysis on an Analox AM1 alcohol analyzer (Analox Instruments, Lunenburg, MA).

3.2.6 Expected results

No differences between FAE and SAC rats were expected for BAC measures for any of the time points post EtOH injection, which would support the

hypothesis that increased voluntary consumption of EtOH in FAE rats is not a result of changes in EtOH metabolism.

3.3 Experiment 3: Dendritic morphology and EtOH drinking behavior

3.3.1 Background

In order to further elucidate the relationship between alterations in NAc dendritic morphology and voluntary EtOH consumption in FAE rats, Experiment 3 involved FAE rats and controls given brief (4 hour) access to an EtOH solution. At the completion of the drinking session, rats were immediately sacrificed. Blood was collected to measure blood alcohol concentrations, and brains were removed and stained in order to examine changes in dendritic length, branching, and spine density in MSNs in the NAc core and shell. These measures were used as predictors of EtOH consumption in a regression model. In order to compare FAE rats with controls, an analysis of heterogeneity of regression slopes (ANCOHET) procedure was utilized (Maxwell and Delaney, 2004), where significant differences between regression slopes were used as an indication of a significant group difference.

3.3.2 Hypothesis

FAE rats will have significant reductions in dendritic morphology in the NAc shell, but not the core, compared to SAC control rats. Furthermore, these measures of dendritic length and branching in the shell, but not the core, will predict voluntary ethanol consumption in FAE and control rats. This relationship is hypothesized to be negative, with reductions in NAc shell dendritic morphology

being associated with increased ethanol consumption. It is expected that this effect will be significantly greater in FAE rats when compared to controls.

3.3.3 Subjects, moderate fetal alcohol exposure, and EtOH consumption

Subjects included 12 (6 SAC and 6 FAE) male Long-Evans rats. These subjects were obtained and housed as described in Experiment 1. The moderate fetal alcohol exposure paradigm described in Experiment 1 was again be used for Experiment 3. EtOH consumption was carried out using the same paradigm as in Experiment 1. Briefly, rats were given access to a 20% EtOH solution in tap water in the home cage for a single 4-hour session, while another water bottle containing only tap water was available on the other side of the cage. Drinking sessions were videotaped with an infrared camera to observe approach behavior, drinking, and time spent at each bottle.

3.3.4 Golgi-Cox staining and dendritic morphology analysis

At the completion of the 4-hour drinking session, rats were immediately anesthetized with an overdose of sodium pentobarbital and perfused transcardially with 0.9% (wt/vol) saline, resulting in exsanguination. The brains were extracted, weighed, and immersed in Golgi-Cox solution (Glaser and Van der Loos, 1981) for 14 days and subsequently immersed in 30% (wt/vol) sucrose for at least 3 days. The brains were then cut into coronal sections (200μm thick) on a vibrating microtome, mounted on slides, and stained according to the procedures described by Gibb and Kolb (1998).

Medium spiny neurons from the nucleus accumbens (shell and core) were selected for analysis (See Figure 5 for an example NAc MSN and analysis

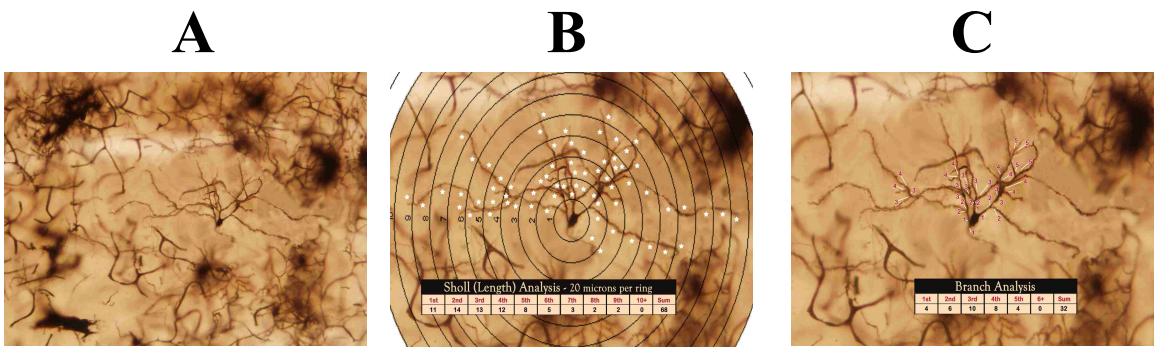

Figure 5. Representative medium spiny neuron visualized in the NAc (A) using the *camera lucida* technique (200X magnification). Scholl analyses were carried out using concentric circles and counting the number of dendrite segments that crossed each subsequent ring (B) to get a measure of dendritic length. Branches were counted at each bifurcation of the dendrite for 1st through 6th (and higher) order and summed for a measure of total branches (C).

procedures). The brain regions of interest were first identified at low power (100X magnification), and neurons were traced at 250X (final magnification) using the *camera lucida* technique on an Olympus light microscope (Model BX51) equipped with a drawing attachment. Sampling for all regions included sections ranging from 1.0-1.7 mm anterior to Bregma. Selection was limited to neurons that were not obscured by stain precipitate, blood vessels, astrocytes, or other artifacts, and had intact dendritic fields that were well impregnated and visible within a single section. Ten neurons were drawn for each region of interest (5 per hemisphere) for each animal. Outcome measures were averaged for each rat and collapsed across hemispheres.

Dendritic branching was measured by counting bifurcations for each dendrite (Coleman and Riesen, 1968). Dendritic segments prior to the first bifurcation from the soma were designated as first-order branches and branch order was incremented by 1 for each subsequent bifurcation on a given dendritic branch. The number of first- through sixth-order (and higher) branches was quantified and an estimate of total branches was determined from these values

(Figure 5C). Dendritic length was measured using a Sholl analysis of ring intersections (Sholl, 1981). A series of concentric rings at 20μm increments (calibrated to the final magnification of 250X) printed on a transparency was centered over the soma (Figure 5B). The total number of intersections between each ring and dendritic branches were counted and converted to estimates of dendritic length as a function of distance from the soma (i.e., for each 20μm segment) and overall dendritic length. Spine density was measured by tracing a dendritic terminal tip (>20μm in length) at high power (2000X final magnification). Total spine density per 10μm was calculated from these values. Spine density was quantified on five terminal segments for each hemisphere on third-order (or greater) branches. In addition, specific spine morphologies were also quantified by counting the number of mushroom, thin, stubby, filopodia, and multi-headed spine types for each segment. Figure 6 shows a representative dendrite segment from the NAc with examples of the spine morphologies counted in Experiment 3.

3.3.5 Blood alcohol concentrations

Approximately 150μL of blood was collected at the same time as the transcardial perfusion. These samples were centrifuged at 13,000 revolutions per minute, at which point blood serum was removed and stored at -80°C for analysis on an Analox AM1 alcohol analyzer (Analox Instruments, Lunenburg, MA).

3.3.6 Expected results

It was expected that dendritic morphology in the NAc shell, but not the core, would significantly predict ethanol consumption in both FAE and control animals. This was expected to be a negative relationship, with reductions in

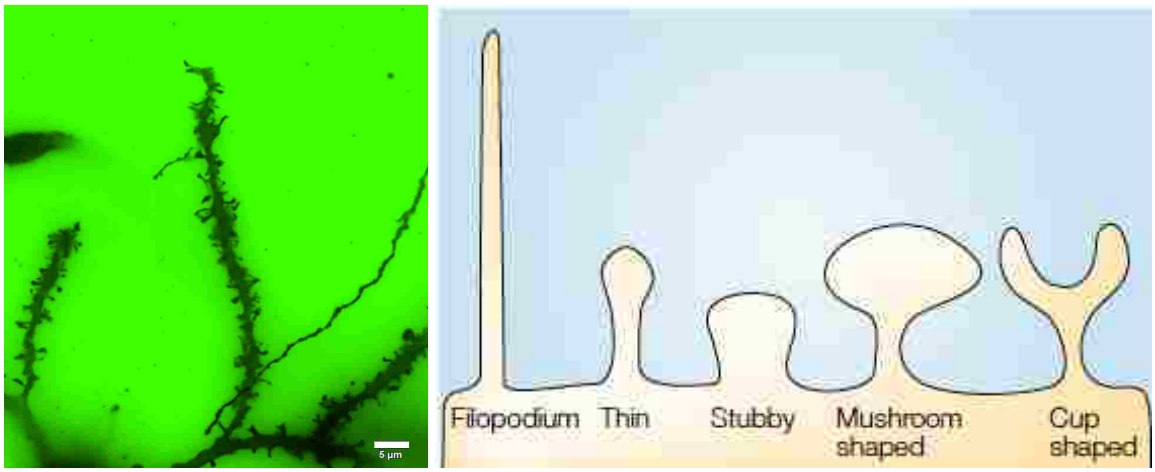

Figure 6. Representative dendrite segment (2000X magnification) that was analyzed for measures of spine density and type in the NAc (A). Examples of different spine morphologies that were counted for spine density measures, (B) adapted from Hering & Sheng (2001).

dendritic morphology associated with increases in ethanol consumption. Furthermore, it was expected that the regression slopes for FAE rats would significantly greater (steeper) than the slopes for controls. We did not expect to observe significant effects of ethanol consumption on dendritic morphology in the shell or core with the low level and short duration of ethanol consumption.

3.4 Experiment 4: Immediate early gene expression and EtOH drinking behavior

3.4.1 Background

Experiment 3 was intended to measure structural changes in the NAc as a function of FAE and adult EtOH exposure, and the extent to which these alterations could serve as predictors of voluntary EtOH consumption. The purpose of Experiment 4 was to follow up the findings in Experiment 3 using an analysis of functional activity in the NAc by processing brain tissue for immediate early gene (IEG) expression after EtOH consumption.

The genes of interest for the current experiment included *c-fos, Arc,* and *Homer 1a.* Previous studies in our laboratory have shown that moderate fetal alcohol exposure blunted the increases in frontocortical *c-fos* and *Arc* IEG expression found in saccharin exposed rats that were involved in social interactions with alternating cage mates (Hamilton et al., 2010b; Hamilton et al., 2010a). With this in mind, it is of interest to investigate changes in *c-fos* and *Arc* expression in the NAc, a brain region that is downstream of the frontal cortex (Voorn et al., 2004). *Homer 1a* is another IEG that is related to metabotropic glutamate receptor (MGluR) function. Prior research in alcohol-preferring P rats found that the MGluR5 antagonist MPEPy administered into the NAc dose-dependently reduced voluntary ethanol consumption (Besheer et al., 2010). Homer 1a expression is critically involved in LTD and reductions in dendritic spine function. This is thought to be a neural counterpart to LTP and may be related to forgetting (Sala et al., 2003).

3.4.2 Hypothesis

As in Experiment 3, it was hypothesized that FAE rats would have significant reductions in measures functional activity in the NAc. Specifically, reductions in *c-fos* and *Arc* and significant increases in *Homer 1a* expression in the NAc shell, but not the core, were expected in FAE rats compared to SAC rats. Also, these measures of IEG expression in the shell, but not the core, would serve as a significant predictor of voluntary ethanol consumption in FAE and control rats. This relationship was hypothesized to be negative for *c-fos* and *Arc*, with reductions in NAc shell IEG expression being associated with increased

ethanol consumption. For *Homer 1a,* it was expected that the relationship between IEG expression and ethanol consumption would be positive. It was expected that these effects would be significantly greater in FAE rats when compared to controls.

3.4.3 Subjects

Subjects were 16 male Long Evans rats, 8 FAE and 8 SAC. These subjects were obtained and housed as described in Experiment 1.

3.4.4 Moderate fetal alcohol exposure, EtOH consumption, and BAC measures

All procedures for moderate fetal alcohol exposure, voluntary EtOH consumption, and blood alcohol concentration measures were exactly as described in Experiment 1.

3.4.5 RT-PCR

Immediately after the 4-hour EtOH drinking session, rats were deeply anesthetized with isoflurane in an enclosed chamber and decapitated. Brains were rapidly removed and 14-gauge punches of the NAc shell and core were collected. RT-PCR was carried out using a TaqMan assay kit (Invitrogen, Carlsbad, CA).

3.4.6 Expected results

Significant decreases in *c-fos* and *Arc* expression in the NAc shell of FAE rats were expected. This is based on the pattern of results noted in the frontal cortex in prior studies (Hamilton et al., 2010b; Hamilton et al., 2010a). Given that increased *Homer 1a* expression is related to reductions in dendritic morphology (Sala et al., 2003), increases were expected in FAE rats. In all cases, IEG in the

NAc shell, but not the core, was expected to serve as a significant predictor of voluntary ethanol consumption, and that the slopes for FAE and SAC rats would be significantly different.

3.5 Experiment 5: Intra-cranial self stimulation

3.5.1 Background

Several studies have shown that FAE exposure results in increased consumption of ethanol (and other drugs) later in adolescence (Williams et al., 2009) and adulthood (Vetter et al., 2007; Barbier et al., 2008; Barbier et al., 2009) in rats. It has also been shown that down regulation of alpha-4 subunit containing $GABA_A$ receptors in the NAc shell is associated with increased ethanol consumption in adult rats (Rewal et al., 2009). Although these results are similar to the deficits we have found in the NAc shell in FAE rats, it is important to establish how other brain regions in the mesolimbic reward circuit might also be impacted by fetal ethanol exposure. Rats will reliably self-administer ethanol into the posterior VTA (Rodd-Henricks et al., 2000) and will self-administer ethanol and other drugs into the NAc (Shin et al., 2008; Engleman et al., 2009) which is one of the primary targets of VTA efferents. Rats have also been shown to self-stimulate when electrodes are placed in the NAc (Beyene et al., 2010) and the MFB, as the MFB is a major afferent/efferent from the VTA and the NAc (Hernandez et al., 2006). The purpose of these experiments is to provide the first account of operant responses in FAE rats to ICSS of the MFB. If alterations in brain reward circuitry including changes in NAc MSN morphology result in

increased responding for reinforcement in FAE rats then fetal-ethanol-related alterations in MFB self-stimulation should be observed.

3.5.2 Hypothesis

It was expected that the minimum current necessary to maintain steady responding and frequency-rate curves for stimulus intensity and operant responses will be shifted to the left in FAE rats compared to SAC controls. This would indicate a potency shift in FAE rats, suggesting a greater response to reward stimuli like EtOH.

3.5.3 Subjects & moderate fetal alcohol exposure

Twenty male Long Evans rats (10 FAE, 10 SAC) served as subjects for this Experiment. These subjects were obtained and housed as described in Experiment 1. The moderate fetal alcohol exposure paradigm described in Experiment 1 will again be utilized for Experiment 5.

3.5.4 Surgery

After transfer and acclimation to the Psychology Department ARF, equal numbers of FAE and SAC rats were surgically implanted with a monopolar electrode aimed at the MFB. Survival surgeries were carried out under isoflurane anesthesia (2-4% in oxygen; Phoenix Pharmaceuticals, St. Joseph, MO). Anesthetized rats were mounted in a stereotaxic frame (David Kopf Instruments, Tujunga, CA), and a small incision was made along the midline of the scalp. Flat skull stereotaxic coordinates for the MFB were derived from Paxinos & Watson (2005) using Bregma of the skull surface as the reference point. A Dremel tool equipped with a dental bur was used to produce a 1mm hole in the skull above

the MFB. Electrodes (monopolar, stainless steel; 0.25 mm in diameter) were coated with polyamide insulation except for 1mm from the tip. The coordinates for electrode placement for the left MFB at the level of the lateral hypothalamus (in millimeters) are as follows: anterior/posterior -2.8, medial/lateral -1.7, dorsal/ventral -7.8 (Pereira Do Carmo et al., 2009). Jeweler's screws were also inserted into the skull and served as the return component of the stimulation circuit. Dental cement was applied to the electrodes, screws, and the skull, with the screws serving as an anchor to the implant. Sutures were applied to close any open wounds around the implant, and rats were given Buprenorphine (0.1mL) for two days for post-operative analgesia. Rats were single housed after surgery and allowed a minimum of 14 days to recover from surgery before behavioral testing began.

3.5.5 Operant conditioning

ICSS experiments were conducted in operant chambers (Coulbourn, Whitehall, PA) equipped with two response levers (only one lever is active) and an isolated stimulus generator (A-M Systems, Poulsbo, WA). Electrodes were connected to the stimulator via a swivel connector (Model 2L2C, Plastics One, Roanoke, VA). The stimulator was controlled by an in house computer software program and an I/O interface (National Instruments, Austin, TX) that also controlled all programming parameters and data collection. After shaping the animals to readily lever press for self-stimulation, rats were trained under a continuous reinforcement schedule (CR-1) with each lever press resulting in the delivery of a 0.5-second train of square wave cathodal pulses (0.1-millisecond

pulse duration). Responses during the 0.5-second stimulation period did not result in additional stimulation. Initially, the frequency of stimulation was held constant at 141 Hz, and the stimulation current was gradually reduced from 500 μA to the lowest value that sustained a rate of responding greater than or equal to 20 lever presses per minute. This descending pattern of current values continued for 4 sixteen-minute trials in order to sustain a consistent pattern of responding in each rat. This current value was used to compare differences between FAE and SAC rats on minimal response currents, and was held constant for the reminder of the experiment for each animal.

From this point on, ICSS training consisted of sequential 8-minute components. During each component, a descending series of 8 current frequencies (141-28 Hz in 0.1 log increments) were presented, with a single 2-minute trial at each frequency. Each frequency trial is initiated by a 5-second priming phase, during which rats received stimulation not dependent on lever pressing, followed by a 115-second response phase, where stimulation occurs under the CR-1 schedule. The time line of procedures for Experiment 5 is summarized in Figure 7.

The primary dependent variable was response rate (responses/min)

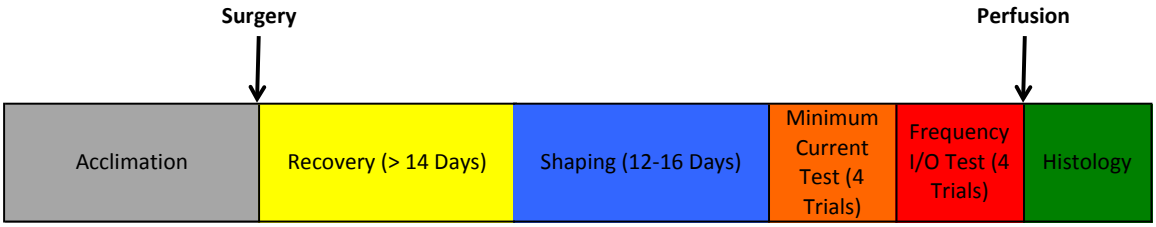

Figure 7. Experimental timeline for surgery, shaping, and test trials carried out during ICSS training for all animals in Experiment 5.

during each frequency trial. Data will be normalized by converting raw scores to Percent Maximum Control Rate (%MCR), with the MCR defined as the mean of the maximal rates observed during any frequency trial of that session. Thus, %MCR values for each trial were calculated as (Response Rate During a Frequency Trial/Maximal Response Rate) x 100. ICSS frequency-rate curves were constructed for each rat during each component by plotting %MCR as a function of log frequency.

3.5.6 Histology

At the completion of behavioral testing, all rats were given an overdose of sodium pentobarbital and the location of the terminal stimulation site was marked by passing a direct anodal current of 1.0 mA for 5 seconds at the electrode tip. Brains were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde, and brains were extracted and stained with methyl green pyronin to confirm electrode placement.

3.5.7 Expected results

A lower minimum current to maintain responding and a significant leftward shift in frequency-rate curves for FAE rats compared to SAC rats at each intensity level was expected. This would indicate an increase in potency for brain stimulation reward in FAE rats compared to controls.

4. Results

4.1 Experiment 1: FAE and EtOH drinking behavior

4.1.1 Data analysis

Measures of EtOH consumption (g/Kg of body weight) and preference (EtOH consumption/total fluid consumption X 100) were calculated for 4- and 24-hour time points for each animal and averages were computed for SAC and FAE groups. Data were averaged for each week (three 24-hour drinking days, every other day) resulting in 6 drinking sessions for 10% EtOH and 3 drinking sessions for 20% EtOH. Data were also collapsed across sessions to compare overall mean 4- and 24-hour EtOH consumption and preference for 10% and 20% concentrations. Measures of consumption and preference were similarly calculated for 2% sucrose and 0.1% quinine solutions in the taste reactivity experiment. A type I error rate of 0.05 was adopted for all significance tests and estimates of effect size (partial η^2 and R^2, where appropriate) are reported for all effects. All analyses were performed using SPSS (version 21 for Macintosh).

4.1.2 10% EtOH consumption and preference

Average (+SEM) measures for 4- and 24-hour consumption and preference for a 10% EtOH solution for each drinking week are presented in Figure 8. A 2-way ANOVA was carried out with prenatal diet condition (SAC or FAE) as a between-subjects factor and drinking session as a within-subjects factor. For daily 4-hour consumption (Figure 8A), there was no significant main effect of diet [$F(1,8) = 0.89$, $p = 0.37$, $\eta^2 = 0.10$], but there was a significant main effect of session, with overall EtOH consumption increasing across weeks

[$F(5,40) = 6.55$, $\eta^2 = 0.45$]. There was also a significant diet X session interaction [$F(5,40) = 2.47$, $\eta^2 = 0.24$]. Planned comparisons at each drinking session revealed a significant increase in 10% EtOH consumption during the first week for FAE rats [$F(1,8) = 6.41$, $\eta^2 = 0.45$], as well as a trend towards an increase in FAE rats for the third week [$F(1,8) = 3.88$, $p = 0.084$, $\eta^2 = 0.33$]. All comparisons at the other sessions were not significant (all p's > 0.72).

A similar analysis was carried put for 4-hour preference for the 10% EtOH solution (Figure 8B). There was no significant effect of diet [$F(1,8) = 0.32$, $p = 0.59$, $\eta^2 = 0.04$], but there was a main effect of session [$F(5,40) = 8.84$, $\eta^2 = 0.53$] and a significant diet X session interaction [$F(5,40) = 2.86$, $\eta^2 = 0.26$]. Planned comparisons found a significant increase in 10% EtOH preference for FAE animals compared to SAC controls during the first week [$F(1,8) = 4.30$, $\eta^2 = 0.35$], for all other sessions preference scores were not significantly different between groups (all p's > 0.48).

For measures of 24-hour EtOH consumption (Figure 8C), there was a significant main effect of session [$F(5,40) = 6.03$, $\eta^2 = 0.43$], the main effect of diet approached significance [$F(1,8) = 4.01$, $p = 0.08$, $\eta^2 = 0.33$], and the diet X session interaction failed to reach significance [$F(5,40) = 1.83$, $p = 0.13$, $\eta^2 = 0.19$]. Planned comparisons at each session did reveal increased EtOH consumption in FAE rats compared to SAC controls during the first [$F(1,8) = 15.69$, $\eta^2 = 0.66$] and third weeks [$F(1,8) = 5.93$, $\eta^2 = 0.43$], as well as a trend for increased consumption during the second week [$F(1,8) = 3.89$, $p = 0.084$, $\eta^2 =$

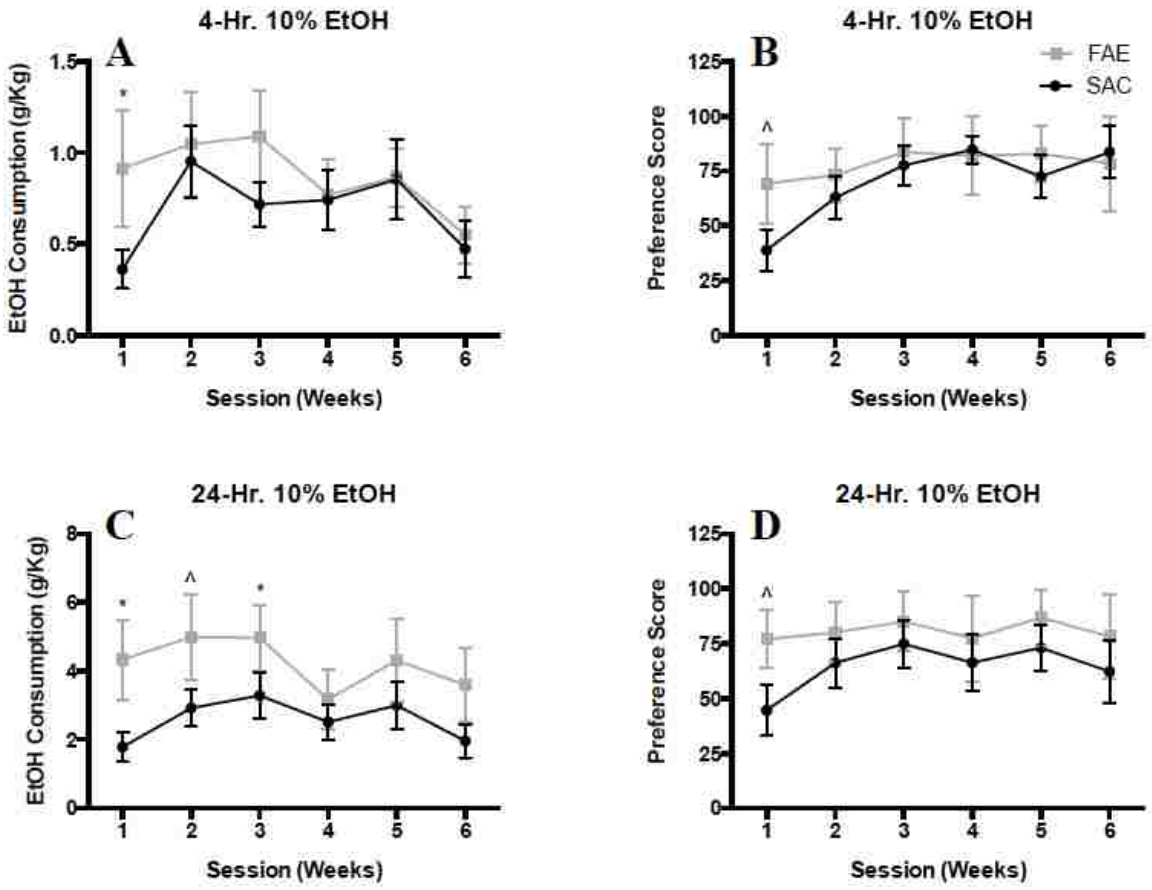

Figure 8. Mean (+SEM) 4- and 24- hour 10% EtOH consumption (A,C) and preference scores (B,D), respectively, for SAC (n = 6) and FAE (n = 4) rats. * indicates significant between-group effect ($p < 0.05$), ^ indicates $p < 0.10$.

0.33]. For sessions 4-6, no group differences were found for EtOH consumption (all p's > 0.14).

An analysis of 24-hour preference for 10% EtOH (Figure 8D) again found a significant effect of session [$F(5,40) = 5.47$, $\eta^2 = 0.41$], but the main effect of diet [$F(1,8) = 0.82$, $p = 0.39$, $\eta^2 = 0.09$] and the diet X session interaction were not found to be significant [$F(5,40) = 1.81$, $p = 0.20$, $\eta^2 = 0.19$]. Comparisons between groups at each session did find a trend towards increased preference for EtOH in the FAE group compared to SAC controls [$F(1,8) = 4.29$, $p = 0.072$, $\eta^2 = 0.35$], but all other comparisons were not significant (all p's > 0.42).

4.1.3 20% EtOH consumption and preference

Mean (+SEM) 4- and 24-hour consumption and preference for a 20% EtOH solution for each drinking week are presented in Figure 9. Again separate ANOVAs for 4- and 24-hour consumption and preference were carried out with prenatal diet condition as a between-subjects factor and the 3 drinking sessions as a within-subjects factor. For 4-hour consumption (Figure 9A), There was a significant main effect of diet, with FAE rats drinking more EtOH than SAC rats across all sessions [$F(1,8) = 6.72$, $\eta^2 = 0.46$]. There was also a trend for main effect of session, with overall drinking increasing across the 3 weeks [$F(2,16) = 3.46$, $p = 0.056$, $\eta^2 = 0.30$]. The diet X session interaction failed to reach significance [$F(2,16) = 2.62$, $p = 0.104$, $\eta^2 = 0.25$]. Planned comparisons between the SAC and FAE groups at each session found that FAE rats drank significantly more EtOH during the first [$F(1,8) = 10.99$, $\eta^2 = 0.58$] and third [$F(1,8) = 6.19$, $\eta^2 = 0.44$] sessions, but this difference during the second session was not significant [$F(1,8) = 2.97$, $p = 0.12$, $\eta^2 = 0.27$].

For 4-hour preference (Figure 9B), the main effect of diet approached significance [$F(1,8) = 4.31$, $p = 0.072$, $\eta^2 = 0.35$], but the main effect of session [$F(2,16) = 0.17$, $p = 0.85$, $\eta^2 = 0.02$] and the diet X session interaction were not significant [$F(2,16) = 0.42$, $p = 0.66$, $\eta^2 = 0.05$]. Planned compassions within each session found a significant increase for EtOH preference during the first week for FAE rats compared to controls [$F(1,8) = 6.59$, $\eta^2 = 0.45$], this effect was not found to be significant for sessions 2 and 3 (all p's > 0.09).

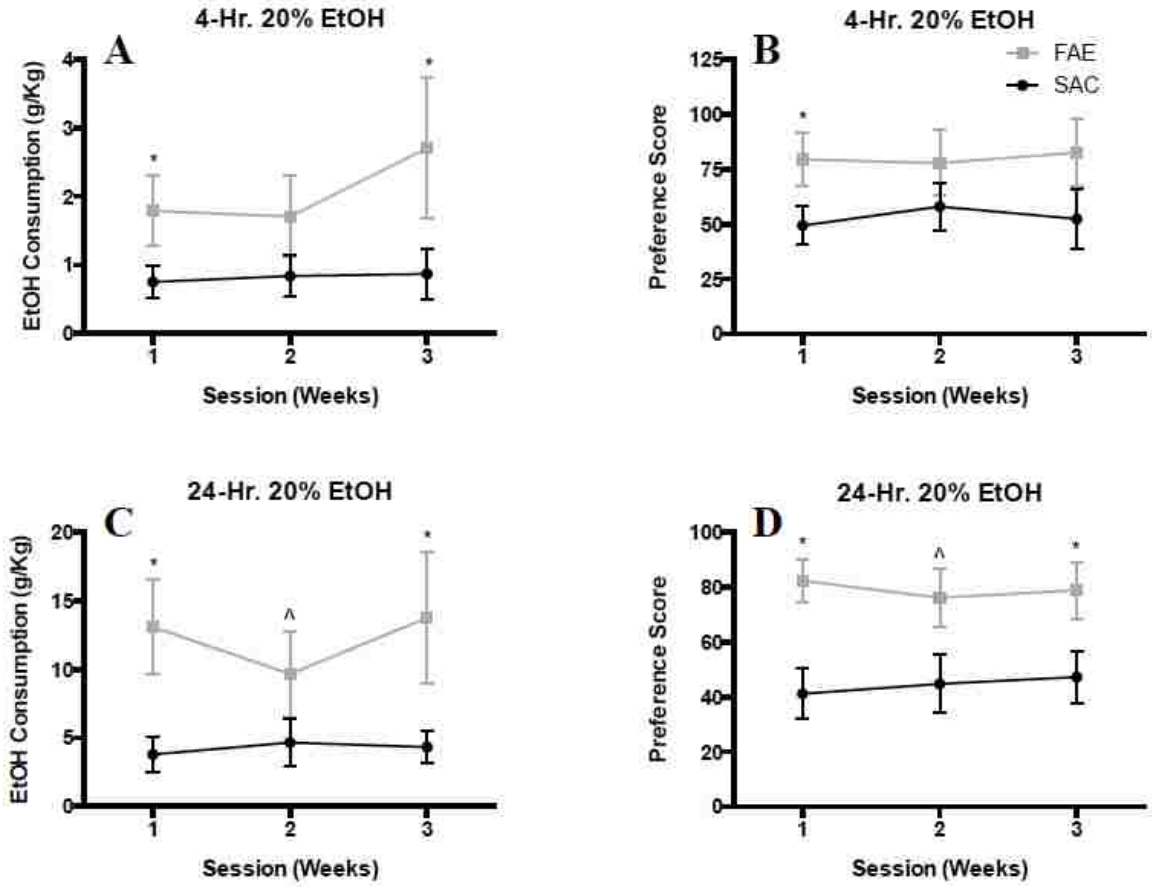

Figure 9. Mean (+SEM) 4- and 24- hour 20% EtOH consumption (A,C) and preference scores (B,D), respectively, for SAC (n = 6) and FAE (n = 4) rats. * indicates significant between-group effect ($p < 0.05$), ^ indicates $p < 0.10$.

The analysis for 24-hour consumption (Figure 9C) found a significant main effect of diet, with FAE rats consuming significantly more of a 20% EtOH solution than SAC controls [$F(1,8) = 9.39$, $\eta^2 = 0.54$]. The main effect of session was not significant [$F(2,16) = 1.05$, $p = 0.38$, $\eta^2 = 0.12$], nor was the diet X session interaction [$F(2,16) = 1.80$, $p = 0.20$, $\eta^2 = 0.18$]. Analyzing each session separately, FAE rats drank significantly more EtOH during the first [$F(1,8) = 17.05$, $\eta^2 = 0.68$] and third sessions [$F(1,8) = 5.98$, $\eta^2 = 0.31$], and this difference approached significance during the second session [$F(1,8) = 3.53$, $p = 0.097$, $\eta^2 = 0.31$].

For 24-hour preference (Figure 9D), again there was a significant main effect of diet with FAE rats having higher preferences for the EtOH bottle compared to SAC rats [$F(1,8) = 8.48$, $\eta^2 = 0.51$]. The main effect of session [$F(2,16) = 0.27$, $p = 0.77$, $\eta^2 = 0.03$] and the diet X session interaction were not significant [$F(2,16) = 1.25$, $p = 0.31$, $\eta^2 = 0.14$]. Planned comparisons within each session found significant increases in EtOH preference for FAE rats during the first [$F(1,8) = 14.51$, $\eta^2 = 0.65$] and third session [$F(1,8) = 6.21$, $\eta^2 = 0.44$], with this difference approaching significance during the second session [$F(1,8) = 4.97$, $p = 0.056$, $\eta^2 = 0.38$].

4.1.4 10% and 20% EtOH consumption and preference summary

Figure 10 summarizes the mean values (+SEM) for 10% and 20% EtOH consumption and preference collapsing across all drinking sessions. Repeated measures ANOVAs with prenatal diet condition as a between-subjects factor and time points as a within subjects factor were carried out for 10% and 20% EtOH concentrations separately. For 10% EtOH consumption (Figure 10A), the main effect of time point was significant as expected, with rats drinking more during the 24-hour than the 4-hour time point [$F(1,8) = 66.82$, $\eta^2 = 0.89$]. The main effect of diet was not significant [$F(1,8) = 3.29$, $p = 0.11$, $\eta^2 = 0.29$], but the diet X time point interaction approached significance [$F(1,8) = 5.21$, $p = 0.052$, $\eta^2 = 0.39$]. Planned comparisons between diet conditions at each time point found no significant differences in EtOH consumption at 4-hours [$F(1,8) = 0.89$, $p = 0.37$, $\eta^2 = 0.10$], but there was a trend for increased EtOH consumption in FAE rats

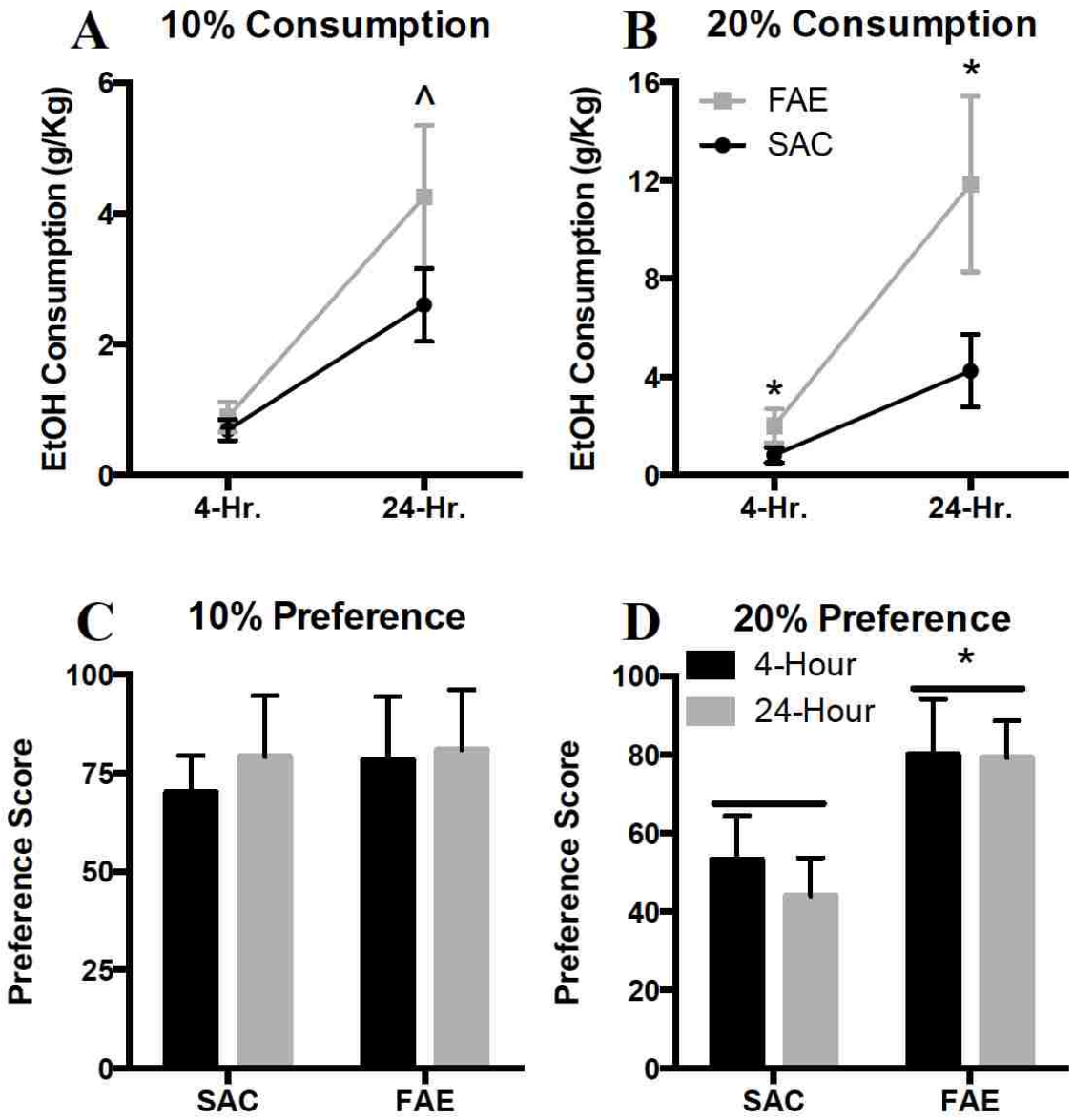

Figure 10. Mean (+SEM) 4- and 24-hour 10% (A,C) and 20% (B,D) EtOH consumption and preference for SAC (n = 6) and FAE (n = 4) rats averaged across all drinking sessions. * indicates significant between-groups effects ($p < 0.05$), ^ indicates $p < 0.10$.

compared to controls at the 24-hour time point [$F(1,8) = 6.56$, $p = 0.08$, $\eta^2 = 0.33$]. The same analysis was carried out for 20% EtOH consumption (Figure 10B) and found significant main effects of diet [$F(1,8) = 9.10$, $\eta^2 = 0.53$], time point [$F(1,8) = 40.26$, $\eta^2 = 0.83$], as well as a significant diet X time point interaction [$F(1,8) = 9.71$, $\eta^2 = 0.55$]. Analyses of the simple effects indicate an

increase in EtOH consumption for FAE rats compared to controls at both the 4-hour [$F(1,8) = 6.72$, $\eta^2 = 0.46$] and 24-hour time points [$F(1,8) = 9.39$, $\eta^2 = 0.54$].

The analysis of 10% EtOH preference scores (Figure 10C) found no main effect of diet [$F(1,8) = 0.58$, $p = 0.47$, $\eta^2 = 0.07$], or time point [$F(1,8) = 0.24$, $p = 0.64$, $\eta^2 = 0.46$], and there was no significant diet X time point interaction [$F(1,8) = 1.86$, $p = 0.21$, $\eta^2 = 0.19$]. These results suggest that there was no difference between SAC and FAE rats for 10% EtOH preference at either the 4- or 24-hour time points across 6 weeks of drinking. For 20% EtOH (Figure 10D), there was a significant main effect of diet, with FAE rats having higher overall preference for the EtOH bottle at both time points compared to SAC controls [$F(1,8) = 6.33$, $\eta^2 = 0.44$]. The main effect of time point approached significance with EtOH preference being slightly increased at the 4-hour time point compared to the 24-hour time point for all groups [$F(1,8) = 4.84$, $p = 0.059$, $\eta^2 = 0.38$]. The diet X time point was not significant, indicating similar within-group preference scores at each time point [$F(1,8) = 3.28$, $p = 0.11$, $\eta^2 = 0.29$].

4.1.5 Taste reactivity

Average (+SEM) 24-hour consumption of, and preference for, a 2% and 0.1% quinine solution are presented in Figure 12. Separate ANOVAs found no significant differences between SAC and FAE rats for measures of 2% sucrose consumption [$F(1,8) = 0.02$, $p = 0.89$, $\eta^2 = 0.002$] or preference [$F(1,8) = 0.459$, $p = 0.52$, $\eta^2 = 0.05$]. There was a trend for increased consumption [$F(1,9) = 2.89$, $p = 0.12$, $\eta^2 = 0.24$] and preference [$F(1,9) = 2.60$, $p = 0.14$, $\eta^2 = 0.22$] for a 0.1%

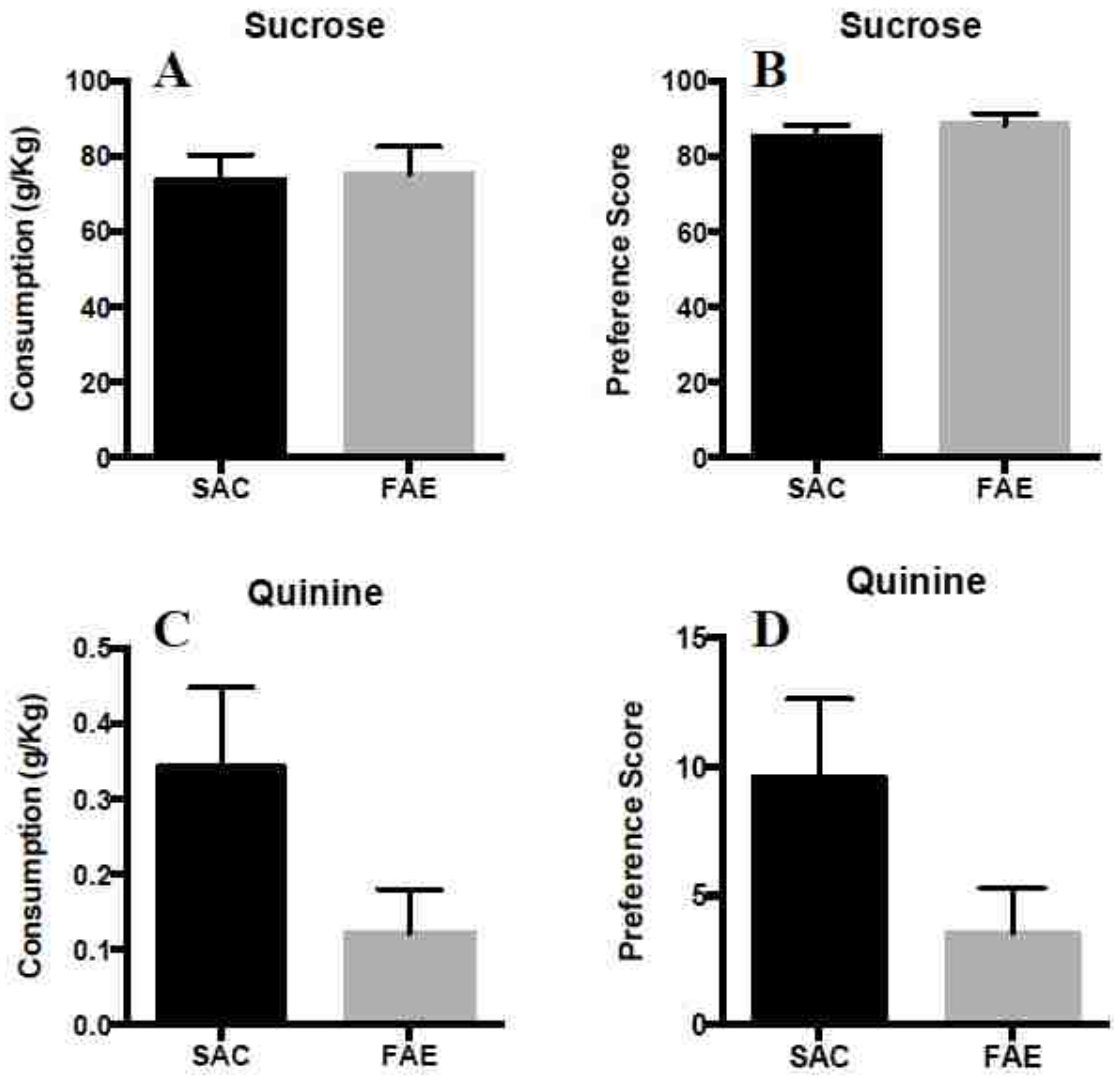

Figure 11. Mean (+SEM) measures of 2% sucrose (A,B) and 0.1% quinine solution consumption and preference for SAC (n = 6) and FAE (n = 5) rats for the taste reactivity test in Experiment 1.

quinine solution in SAC rats compared to FAE rats, but these results failed to reach significance.

4.1.6 Discussion

The results of experiment 1 demonstrate a significant increase in EtOH consumption in rats exposed to moderate levels of alcohol throughout gestation compared to controls. This increase in both 10% and 20% EtOH solutions does not appear to be due to the taste or smell of EtOH or sensory processing as a

result of moderate FAE, as test sessions found no significant differences between SAC and FAE animals for either consumption or preference of a 2% or 0.1% quinine solution. After being isolated, rats were given access to a 10% EtOH solution (as well as a bottle containing only tap water) for 24 hours. Measures of consumption were taken at 4 and 24-hours, and after the session was completed, rats were withdrawn for a period of 24-48 hours. These sessions occurred three times a week, and averages were taken for each week.

Interestingly, the effect of increased consumption and preference for EtOH in FAE rats was consistently higher during the first week of exposure. During the subsequent 5 weeks for the 10% EtOH solution, the differences between groups were reduced to the point that after the last week of exposure, both groups were drinking similar amounts of EtOH and had similar preference scores for the EtOH bottle compared to the water bottle. When the concentration of EtOH was increased to 20%, FAE rats again had initial elevated levels of consumption and preference, but unlike with the 10% EtOH solution, this increase in consumption and preference in FAE rats persisted throughout the three weeks of drinking. As the concentration of EtOH doubled, the SAC rats appeared to respond by reducing their fluid intake from the EtOH bottle. This did not appear to be the case for FAE rats, as the fluid amounts consumed remained about the same regardless of EtOH concentration. This suggests that FAE rats may have been insensitive to changes in EtOH magnitude, at least from a 10% to a 20% concentration. Preference values seem to confirm this, as SAC rats demonstrated a decrease in preference for the EtOH bottle as the concentration

increased, while FAE rats had similar preference scores for both 10% and 20% EtOH. There were no significant differences between groups for water consumption, this is important because there is no reason to suspect that moderate FAE affects motor responses to the bottle, or that differences in general drinking behavior are in any way different as a result of FAE.

The results of the taste reactivity test are important because previous research has found that elevated EtOH consumption in rats exposed to higher does of EtOH prenatally may be due to these animals liking the taste and smell of EtOH. Youngentob and Glendinning (2009) showed that FAE rats drank more of a quinine solution and had elevated preferences for the taste and smell of quinine, which has similar sensory properties as alcohol, suggesting possible alterations in sensory processing as a result of heavy FAE. Here we do not see such differences in quinine consumption or preference using a moderate FAE paradigm. In fact, there was a trend for increased quinine consumption and preference in SAC animals. The results for sucrose preference and consumption are also important in terms of the specificity or reward learning affects, as there results suggest normal reward learning for an appetitive outcome (sucrose) for both Sac and FAE rats. This suggests that general reward processing may not be altered by moderate FAE, and that the increased consumption of, and preference for, 10% and 20% EtOH noted in Experiment 1 are the result of specific deficits in reward learning related to EtOH consumption.

4.2 Experiment 2: EtOH clearance

4.2.1 Data analysis

Rats were given i.p. injections of a known dose of EtOH (2.5 g/Kg; 20% EtOH) and had blood drawn at 1, 2.5, 4, 5.5, and 7 hours post injection. Across multiple non-consecutive days, each rat was injected and tested at all possible time points. A two-way repeated measures ANOVA was used with prenatal diet condition as a between-subjects factor and time point post injection as a within-subjects factor. Rates of elimination were also calculated for SAC and FAE rats to compare between groups as well as previous research reports.

4.2.2 Blood alcohol concentrations

Results for blood alcohol concentrations for SAC and FAE rats at the five time points tested are summarized in Figure 12. There was no significant main effect for prenatal diet condition [$p = 0.62$, $\eta^2 = 0.03$] suggesting that, across all time points, there was no difference in EtOH clearance between SAC and FAE rats. There was a significant main effect for time point post injection [$F(4,32) = 26.87$, $\eta^2 = 0.77$], indicating an near-linear reduction of blood alcohol concentrations over the 7-hour period observed, as expected. Importantly, the prenatal diet X time point interaction was not significant [$p = 0.34$, $\eta^2 = 0.13$] which supports the claim that there are no differences between FAE and SAC rats for measures of blood alcohol concentrations at any of the specific time points tested here.

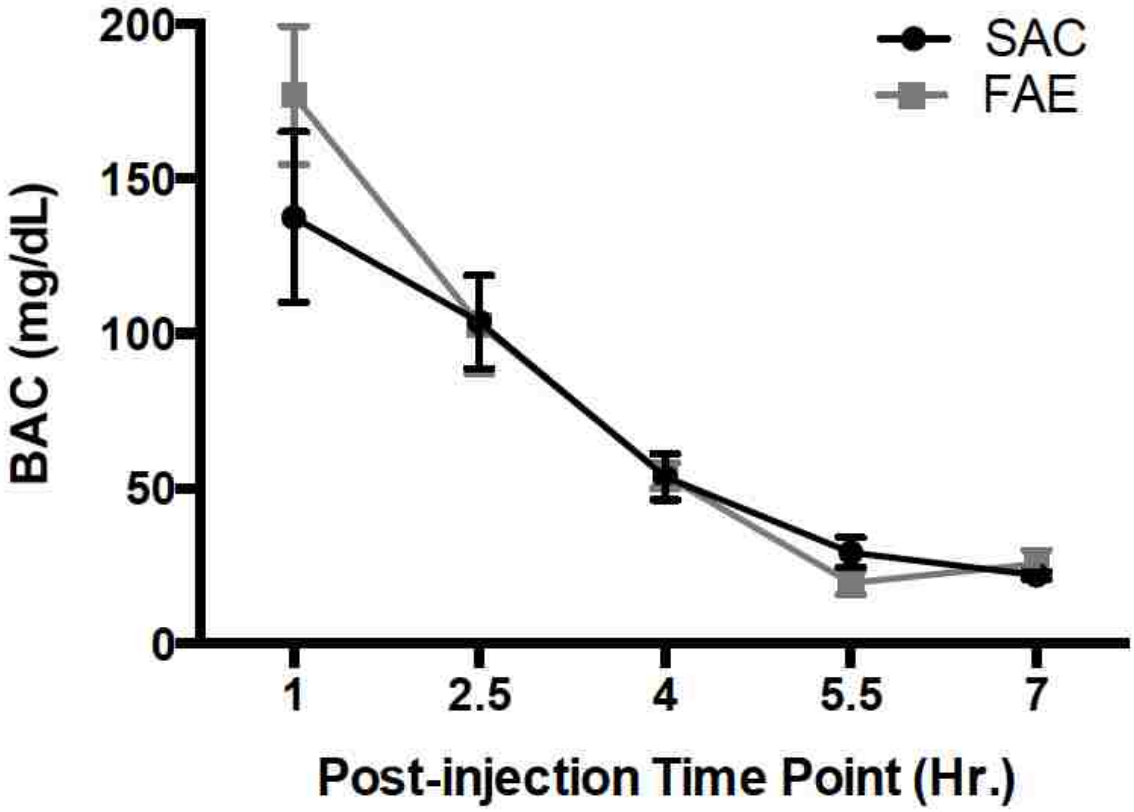

Figure 12. Mean (+SEM) measures of blood alcohol concentrations for SAC (n = 5) and FAE (n = 5) rats across the 5 time points tested in Experiment 2.

4.2.3 Rate of EtOH elimination

Rate of elimination was calculated for SAC and FAE rats at the group level; this was calculated based on the slope of a linear regression equation computed for each group. For SAC rats, the zero-order rate of elimination was 30.614 mg/Kg/Hr. For FAE rats, the rate of elimination was found to be 38.619 mg/Kg/Hr. These values are similar to results noted in previous research reports based on the age of the rats (~6 months old) used in the current study (Kim et al., 2003).

4.2.4 Discussion

These results suggest that there is no difference in the metabolism of EtOH between FAE and SAC rats. Similar levels of blood alcohol concentrations were seen at all time points post EtOH injection, from peak levels (1-2.5 hours post injection) to the time at which EtOH had been almost completely metabolized (5.5-7 hours post injection). Blood-alcohol concentrations were measured at a wide range of time points post exposure because differences observed both early on and later after exposure could explain the elevated consumption of EtOH in FAE rats described in Experiment 1. Both SAC and FAE rats demonstrated a fairly linear metabolism of EtOH; this is a characteristic that is unique to EtOH due to the zero-order nature of EtOH elimination (Julien, 2010).

One factor that was not taken into account in this experiment is the first-pass metabolism of EtOH that occurs in the stomach and can be contingent on several factors. In the present experiment, EtOH was injected i.p. so first-pass metabolism is bypassed entirely. In previous experiments, our outcome of interest was EtOH consumption, which is based on the actual drinking of EtOH, which is subject to first-pass metabolism. Differences in levels of alcohol dehydrogenase (ADH) in the stomach alter the amount of EtOH that is absorbed into the blood stream, and this could affect blood alcohol concentration measures. ADH can also change as a function of EtOH experience and can also be affected by stomach contents at the time of EtOH consumption (Julien, 2010). It may be the case that there are inherent differences between SAC and FAE rats

on levels of ADH in the stomach, future studies need to be carried out to address this possibility. Otherwise it does not appear that EtOH metabolism is affected by moderate prenatal EtOH exposure.

The observed power for this particular experiment was low, and it may be the case that we have committed a Type II error in concluding that there is no difference in EtOH metabolism between groups. Even though such an effect is not expected, further studies that utilized a between-subjects design or a more systematic approach with more subjects might provide a more definitive result.

4.3 Experiment 3: Dendritic morphology and EtOH drinking behavior

4.3.1 Data analysis

Results for EtOH consumption and dendritic morphology are reported as percent control to compare FAE and SAC prenatal diet conditions. One-way analysis of variance (ANOVAs) were used to compare groups on dependent measures for amount of EtOH consumed, EtOH preference scores, dendritic length, dendritic branching, and dendritic spine density. Correlations and regression equations were also computed to determine what measures of dendritic morphology serve as significant predictors of EtOH consumption in both diet conditions, and the ANCOHET method was used to determine significant differences between prenatal diet regression slopes.

4.3.2 20% EtOH consumption

Results for EtOH consumption and preference scores (Mean and SEM) expressed as a percentage of SAC animals are presented in Figure 13. Although FAE rats drank on average 32% more EtOH than SAC rats, this difference failed

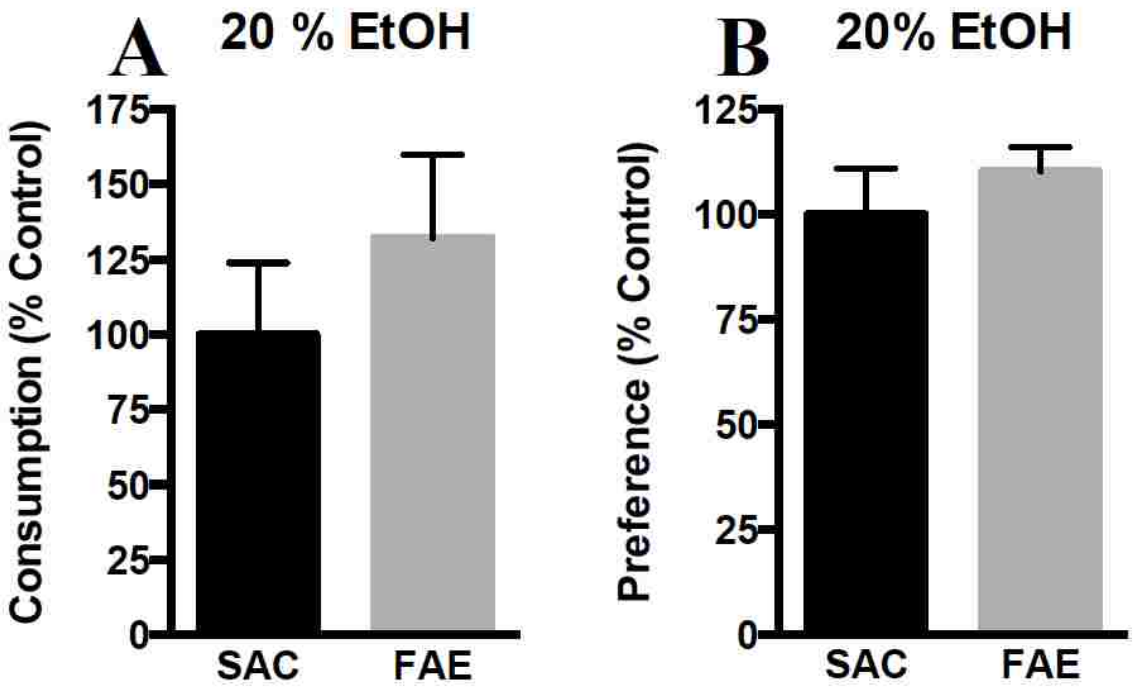

Figure 13. Mean (+ SEM) amount of 20% EtOH consumed (A) and average preference cores (B) for the EtOH bottle for FAE (n = 6) and SAC (n = 6) rats in Experiment 1. Values are presented as percent of SAC controls.

to reach significance [$F(1,10) = 0.77$, $p = 0.40$, $\eta^2 = 0.07$]. FAE rats also had a slightly higher preference for the EtOH bottle versus the water bottle compared to controls (10%), but this difference also was not significant [$F(1,10) = 0.70$, $p = 0.42$, $\eta^2 = 0.07$]. Because of the small sample sizes reported here, Mann-Whitney U tests were also carried out for these data. As with the parametric analyses, no significant effects were found for 20% EtOH consumption [$U = 11$, $p = 0.31$] or preference [$U = 12.5$, $p = 0.412$].

4.3.3 NAc dendritic length and branching

Measures of total number of branches and overall dendritic length were computed for each animal based on the average value of 5 medium spiny neurons drawn per hemisphere from both the NAc core and shell. Results for total branching and length in the core and shell expressed as a percentage of

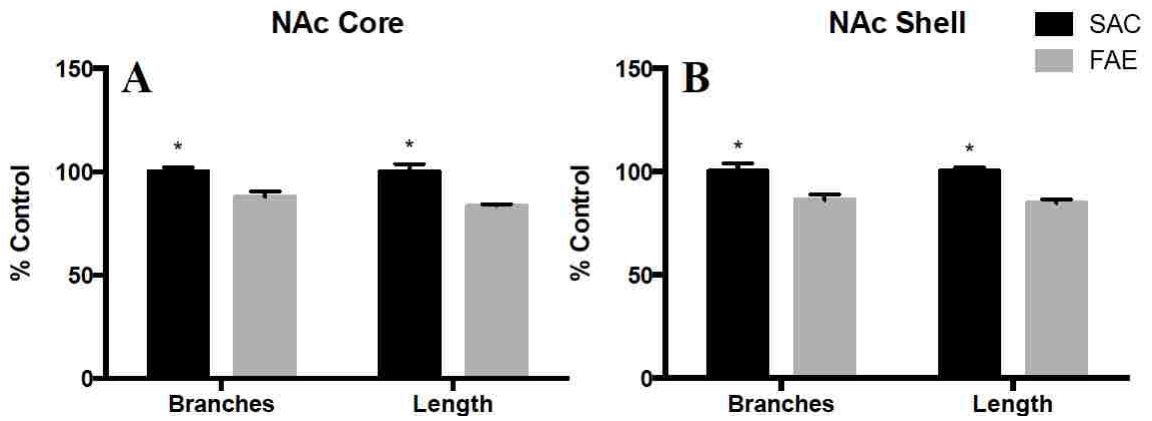

Figure 14. Measures of dendritic branching and length (mean + SEM) for FAE (n = 6) and SAC (n = 6) rats in the NAc core (A) and Shell (B). * indicates a significant between-diet effect ($p < 0.05$).

SAC animals are summarized in Figure 14. In general, FAE animals had 12-16% reductions for both MSN dendritic branching and length in both the NAc core and shell. Significant reductions in FAE animals compared to SAC controls were found for core branching [$F(1,10) = 12.83$, partial $\eta^2 = 0.56$], core length [$F(1,10) = 17.3$, partial $\eta^2 = 0.63$], shell branching [$F(1,10) = 7.88$, partial $\eta^2 = 0.44$], and shell length [$F(1,10) = 9.35$, partial $\eta^2 = 0.48$]. These results suggest that FAE rats had smaller and less complex MSN dendritic fields compared to SAC rats in both regions sampled in the NAc. In order to control for variability in EtOH consumption, the same analyses were carried out using analysis of covariance (ANCOVA) with EtOH consumption entered as a covariate. In each case, results were similar to those found using ANOVA for the effect of prenatal diet condition on core branching [$F(1,9) = 10.35$, partial $\eta^2 = 0.54$], core length [$F(1,9) = 14.44$, partial $\eta^2 = 0.62$], shell branching [$F(1,9) = 7.0$, partial $\eta^2 = 0.44$], and shell length [$F(1,9) = 7.17$, partial $\eta^2 = 0.44$].

4.3.4 NAc dendritic spine density

Measures of spine density were calculated in the NAc core and shell for total spines by averaging five segments from the left and right hemisphere for each region of interest. In order to examine the effects of both prenatal and adult EtOH on spine morphology, additional measures of spine density were computed for mushroom, stubby, thin, filopodium, and multi-headed spines from the dendritic segments that were drawn. Results for all measures of spine density in the core and shell for SAC and FAE rats are presented in Figure 15. There was an trend towards increased stubby spine density in the core of FAE rats that approached significance [$F(1,10) = 4.8$, $p = 0.053$, $\eta^2 = 0.32$] but no significant differences in spine density were found between FAE and SAC rats for other measures of specific spine morphologies in either the core or shell (all p's > 0.052).

A separate analysis of covariance (ANCOVA) was carried out with measures of spine density as dependent variables and 4-hour EtOH consumption entered as a covariate. Total spine density across both regions of the NAc was reduced in FAE animals compared to SAC controls [Figure 6A; $F(1,9) = 5.21$, $\eta^2 = 0.37$]. There was also a significant increase in FAE rats for multi-headed spines in the shell [$F(1,9) = 5.24$, $\eta^2 = 0.37$]. Although there were no significant differences between diet conditions on any measures of specific spine morphologies (all p's > 0.057), as Figures 16B and 16C indicate there were trends for reductions in mushroom spines for FAE rats compared to SAC rats in both the shell [$F(1,9) = 4.68$, $p = 0.059$, $\eta^2 = 0.34$] and in the shell

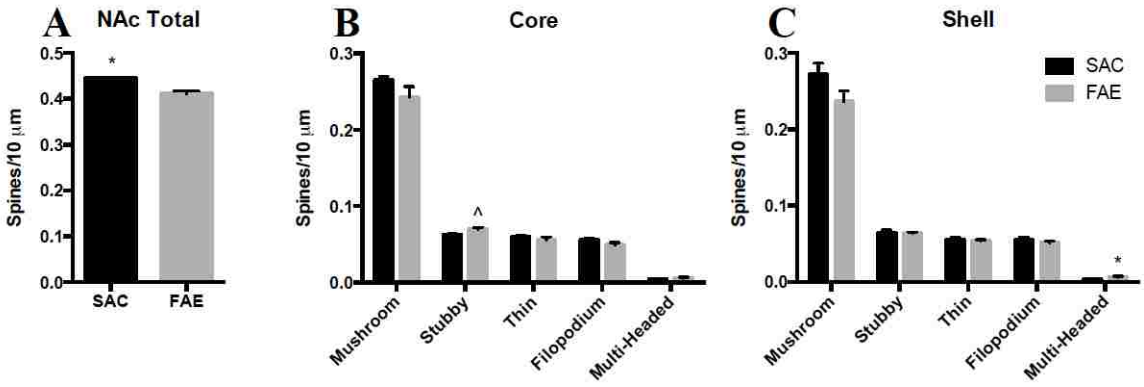

Figure 15. Mean (+ SEM) total spine density (A), as well as different spine morphologies, in the NAc core (B) and shell (C) for SAC (n = 6) and FAE (n = 6) rats. * indicates a significant effect ($p < 0.05$) when controlling for EtOH consumption; ^ indicates $p = 0.053$.

and core combined [$F(1,9) = 4.76$, $p = 0.057$, $\eta^2 = 0.35$]. These trends, along with the lack of significant for increases (e.g. stubby, multi-headed) for other spine morphologies, suggests that the overall reduction is spine density in the NAc of FAE rats is driven by reductions in mushroom shaped spines.

4.3.5 NAc morphology as a predictor of EtOH consumption

Regression equations were calculated with measures of dendritic morphology and spine density entered as predictors of EtOH consumption, either individually or in combination to determine specific alterations both within and between diet conditions that might predict voluntary EtOH consumption. Results for measures of dendritic morphology as predictors of EtOH consumption with separates regression slopes for each diet condition are presented in Figure 16. The number of branches in the NAc shell across both diet conditions [$F(1,10) = 6.49$, $R^2 = 0.393$] and the number of branches in the core for FAE rats [$F(1,3) = 10.53$, $R^2 = 0.78$] were found to be significant predictors of EtOH consumption. The relationship between shell branching and EtOH consumption was negative ($r = -0.63$), indicating that reductions in NAc shell branching were associated with

increased EtOH consumption. While the overall relationship between core branching and EtOH consumption was negative [r = -0.19, p = 0.58], the slopes were positive for SAC [r = 0.36, p = 0.49] and FAE rats [r = 0.88, p = 0.048] individually. The significant positive correlation in FAE rats suggests that increased core branching in these animals was associated with increased EtOH consumption. All other regression equations that included measures of dendritic morphology and spine density in the core, shell, or the entire NAc as predictors of EtOH consumption in both prenatal diet conditions separately or combined were not significant (all p's ≥ 0.07). A summary of these correlations and effect sizes are included in Table 1.

4.3.6 Discussion

Although FAE rats, on average, consumed more of a 20% EtOH solution during the 4-hour drinking session compared to SAC controls, this difference was not found to be statistically significant even though the reported effect sizes are relatively large. Even though the effects were not quantitatively different, it should be noted that the trends are qualitatively similar to the results of Experiment 1.

There are several potential explanations for the high variability in EtOH consumption that may have occluded any significant differences in EtOH consumption between SAC and FAE rats. First, the data collected for 20% EtOH consumption in Experiment 1 involved long term chronic-intermittent drinking sessions lasting 24 hours, three times a week where measures were taken at the 4- and 24- hour time points. Here rats were only given access to 20% EtOH for a single 4-hour session. Second, the data in Experiment 1 were collected in rats

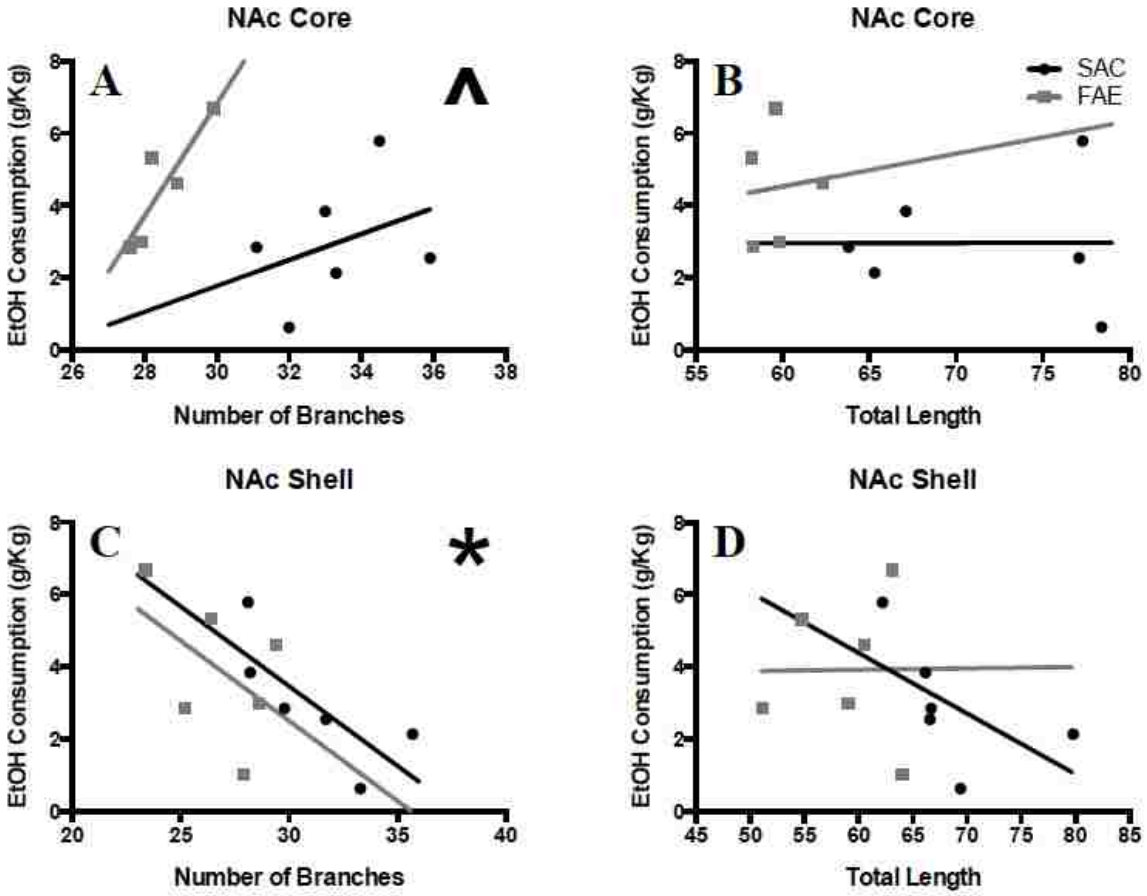

Figure 16. Measures of dendritic branching in the core (A) and shell (C) and dendritic length in the core (B) and shell (D) as predictors of EtOH consumption. Separate regression slopes are shown for FAE and SAC rats. * significant relationship for SAC + FAE combined ($p < 0.05$), ^ significant relationship for FAE rats ($p < 0.05$).

that were first exposed to a 10% EtOH solution for 6 weeks before having the dose increased to 20% for an additional period of 3 weeks. Perhaps the initial 10% exposure made the EtOH solution more palatable in these animals when the dose was increased, and the rats in Experiment 4 found the 20% EtOH solution aversive. It might also be the case that SAC control rats drank more due to the novelty of the EtOH solution, where SAC rats in Experiment 1 had learned to avoid or reduce drinking EtOH after multiple drinking sessions and potential adverse affects associated with EtOH consumption and/or withdrawal symptoms. Lastly, elevated EtOH consumption in FAE rats might be the result of drinking

behavior increasing during the light cycle where SAC rats are more likely to be asleep. Several studies have reported abnormal circadian rhythms and disrupted sleep/wake cycles in rats exposed to EtOH prenatally (Rosett et al., 1979; Stone et al., 1996). In any case, these results did not support our initial hypotheses.

For measures of dendritic morphology, significant reductions for total length and number of branches were found in FAE rats compared to SAC controls in both the NAc core and shell. The effects in the shell were similar to previous findings reported from our laboratory in FAE rats that did not drink EtOH, and these results support our hypothesis (Rice et al., 2012). The effects in the core, however, were not expected as our previous report failed to detect significant differences in the core in FAE rats that did not drink EtOH. It seems unlikely that a single 4-hour EtOH drinking session would be enough to induce such dramatic changes in core MSN morphology, but it may be the case that the low variability in these measures reported here might have given the current experiment more power to detect significant group differences than our prior study. When analyzing the relationship between measures of dendritic morphology and EtOH consumption, changes in total branches in the NAc, specifically the number of branches for both SAC and FAE rats in the shell and for FAE rats in the core, were significant predictors of EtOH consumption. This result suggests that structural changes in the NAc are related to increased EtOH consumption, which lends some support to our hypothesis that changes in the shell and to some extent in the core of FAE rats drive initial EtOH drinking behavior. Since we have previously shown that moderate FAE results in

		SAC (n = 6)			FAE (n = 5/6)			SAC+FAE (N = 11/12)		
Region	**Morphology**	**r**	**p value**	**R^2**	**r**	**p value**	**R^2**	**r**	**p value**	**R^2**
Core	Dendritic Branching	0.357	0.488	0.12745	**0.882**	**0.431***	**0.77792**	-0.186	0.584	0.0346
	Dendritic Length	0.001	0.998	> 0.001	0.093	0.882	0.00865	-0.343	0.302	0.11765
	Total Spines	0.446	0.376	0.19892	0.537	0.272	0.28837	0.304	0.337	0.09242
	Mushroom Spines	0.414	0.414	0.1714	0.429	0.397	0.18404	0.227	0.478	0.05153
	Stubby Spines	0.21	0.689	0.0441	-0.273	0.601	0.07453	0.059	0.856	0.00348
	Thin Spines	-0.194	0.713	0.03764	0.635	0.175	0.40323	0.254	0.425	0.06452
	Filopodium Spines	0.166	0.753	0.02756	0.545	0.263	0.29703	0.208	0.517	0.04326
	Multi-headed Spines	0.365	0.476	0.13323	0.133	0.802	0.01769	0.276	0.386	0.07618
Shell	Dendritic Branching	**-0.766**	**0.075#**	**0.58676**	-0.495	0.318	0.24503	**-0.627**	**0.029***	**0.39313**
	Dendritic Length	-0.58	0.227	0.3364	0.009	0.986	> 0.001	-0.384	0.2178	0.14746
	Total Spines	0.384	0.452	0.14746	0.438	0.385	0.19184	0.214	0.504	0.0458
	Mushroom Spines	0.513	0.298	0.26317	0.405	0.426	0.16403	0.257	0.419	0.06605
	Stubby Spines	-0.466	0.351	0.21716	0.119	0.822	0.01416	-0.243	0.446	0.05905
	Thin Spines	0.42	0.407	0.1764	0.385	0.452	0.14823	0.317	0.315	0.10049
	Filopodium Spines	-0.198	0.707	0.0392	0.204	0.699	0.04162	-0.101	0.756	0.0102
	Multi-headed Spines	-0.42	0.407	0.1764	-0.179	0.802	0.03204	-0.043	0.893	0.00185
Core + Shell	Dendritic Branching	-0.499	0.314	0.249	-0.61	0.198	0.3721	**-0.53**	**0.076#**	**0.2809**
	Dendritic Length	-0.506	0.306	0.25604	0.002	0.997	4E-06	-0.348	0.267	0.1211
	Total Spines	0.42	0.407	0.1764	0.517	0.293	0.26729	0.272	0.393	0.07398
	Mushroom Spines	0.512	0.299	0.26214	0.425	0.401	0.18063	0.256	0.422	0.06554
	Stubby Spines	-0.316	0.542	0.09986	-0.153	0.773	0.02341	-0.123	0.704	0.01513
	Thin Spines	0.233	0.657	0.05429	**0.741**	**0.092#**	**0.54908**	0.405	0.191	0.16403
	Filopodium Spines	-0.066	0.9901	0.00436	0.474	0.343	0.22468	0.061	0.85	0.00372
	Multi-headed Spines	0.072	0.892	0.00518	-0.016	0.976	0.00026	0.129	0.688	0.01664

Table 1. Correlations (r), p values, and effect sizes (R^2) for measures of dendritic morphology and spine density as predictors of EtOH consumption. * indicates a significant correlation ($p < 0.05$), # indicates a trending association ($p < 0.10$).

reductions in NAc shell dendritic morphology (Rice et al., 2012), it is unlikely that the changes in the shell found in the current study are a result of EtOH consumption in adulthood. As for the significant reductions in the core, it is possible that the 4-hour EtOH drinking session had enough of an effect on structural plasticity in this region to exasperate the trends noted in our previously published results.

When controlling for 4-Hour EtOH consumption, there was an overall reduction in spine density in FAE rats in the NAc, but there were no differences between diet conditions for measures of spine density when the core and shell were analyzed separately, and this effect was not found when EtOH consumption was excluded as a covariate. This suggests that the overall reduction in spine density in the NAc of FAE rats (Figure 15A) was not related to EtOH

consumption in adulthood but rather an effect of prenatal ethanol exposure. Although this effect was not found to be significant in our previously published research (Rice et al., 2012), it is qualitatively similar.

We also examined the densities of several different spine types in the NAc in SAC and FAE rats as well. It has been shown that different spine morphologies can be related to dendrite maturity, development, and dysfunction (Hering and Sheng, 2001). In the current study, we found no significant differences for mushroom, stubby, thin, or filopodium spine densities in either the NAc core or shell between SAC and FAE rats. There was a significant increase in multi-headed spines in FAE rats in the shell region, a finding similar to reports in the shell of alcohol-preferring P rats (Zhou et al., 2007). Across both regions of the NAc, there was around a 10% reduction in mushroom shaped spine density for FAE rats. Although this decrease was not statistically significant, it is the largest decrease among the various spine morphologies counted in the current study, and would appear to be the primary factor in the significant decrease in over NAc spine density. In the core, there was a trend for increased stubby spine density in FAE rats. This transient increase, along with significant increases in multi-headed spines in the shell of FAE rats noted earlier may be countered by the reductions noted in other spine types (see Figure 15). It could be that stubby spine formation in the NAc core and multi-headed spine formation in the shell might be a result of EtOH consumption in FAE animals, and that these changes might underlie maladaptive reward processing and EtOH consumption that have noted been in FAE animals in Experiment 1 and elsewhere (Barbier et al., 2008).

Future studies on this hypothesis seem warranted given our results in a limited number of animals. Also, future studies that could examine changes in dendrite and spine densities and morphologies on a smaller temporal scale, or in real time, would lead to greater insight into how EtOH exposure effects NAc dendritic fields.

4.4 Experiment 4: Immediate early gene expression and EtOH drinking behavior

4.4.1 Data analysis

As in Experiment 3, data for 20% EtOH consumption and immediate-early gene (IEG) mRNA expression are expressed as percent to SAC controls. Measures of IEG were also used as individual predictors of EtOH consumption using multiple regression and ANCOHET procedures were used to determine significant slope differences between prenatal diet conditions.

4.4.2 20% EtOH consumption

Results for 20% EtOH consumption and preference expressed as percent of SAC control animals are summarized in Figure 17. FAE rats, on average, drank 56% more EtOH during the 4-hour drinking session than SAC rats. However, this difference failed to reach significance due to individual variability in EtOH consumption [$F(1,14) = 1.09$, $p = 0.31$, $\eta^2 = 0.07$]. Preference scores were also not significantly different between FAE ($M_{FAE} = 70.28$, $SEM_{FAE} = 10.68$) and

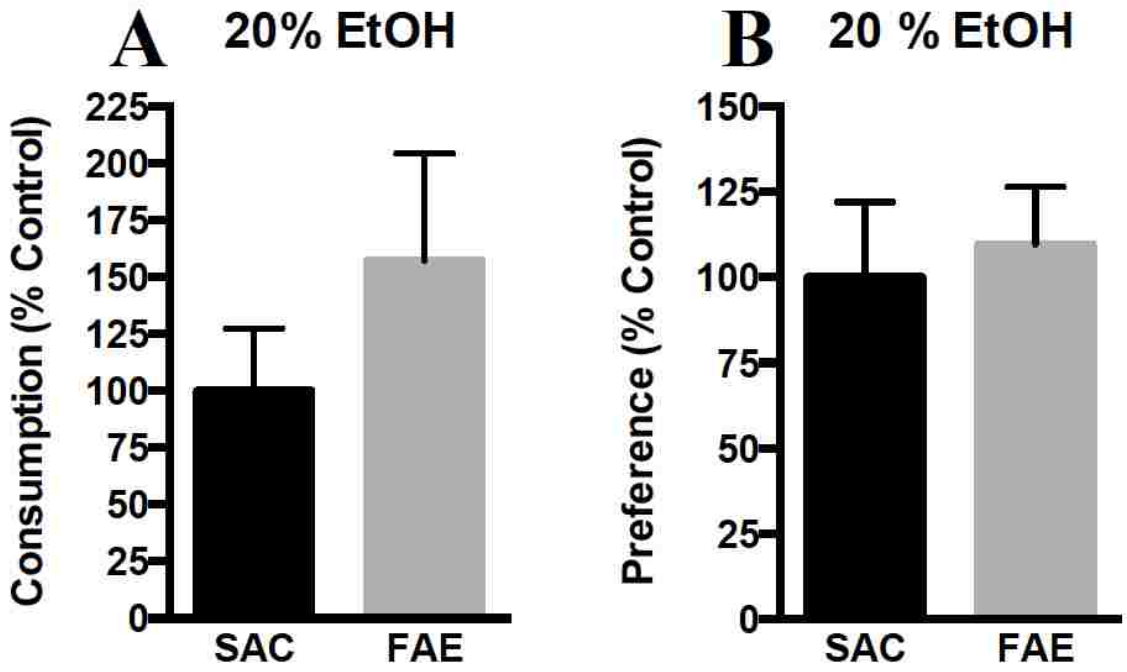

Figure 17. Mean (+ SEM) EtOH consumption and preference scores for SAC (n = 8) and FAE (n = 8) rats as a percentage of SAC controls for the 4-hour 20% EtOH consumption in Experiment 2.

SAC (M_{SAC} = 64.05, SEM_{SAC} = 14.1) rats [$F(1,14) = 0.12$, $p = 0.73$, $\eta^2 = 0.01$]. Again, non-parametric analyses were carried out to account for the small sample sizes; no significant differences were found for consumption [$U = 27$, $p = 0.64$] or preference [$U = 29$, $p = 0.80$].

4.4.3 Drinking behavior

In order to examine specific approach and avoidance behavior to the EtOH bottle during the 4-hour drinking session, video footage of SAC and FAE rats from Experiment 3 and Experiment 4 was digitized and coded for time spent attending to the EtOH bottle, the water bottle, or neither bottle once per second. Rats form each prenatal diet condition were rank ordered for time spent at the EtOH bottle; these results are presented in Figure 18. From a qualitative standpoint, rats from both groups had similar measures of time spent at the EtOH bottle for the highest and lowest rankings. Where differences between SAC and

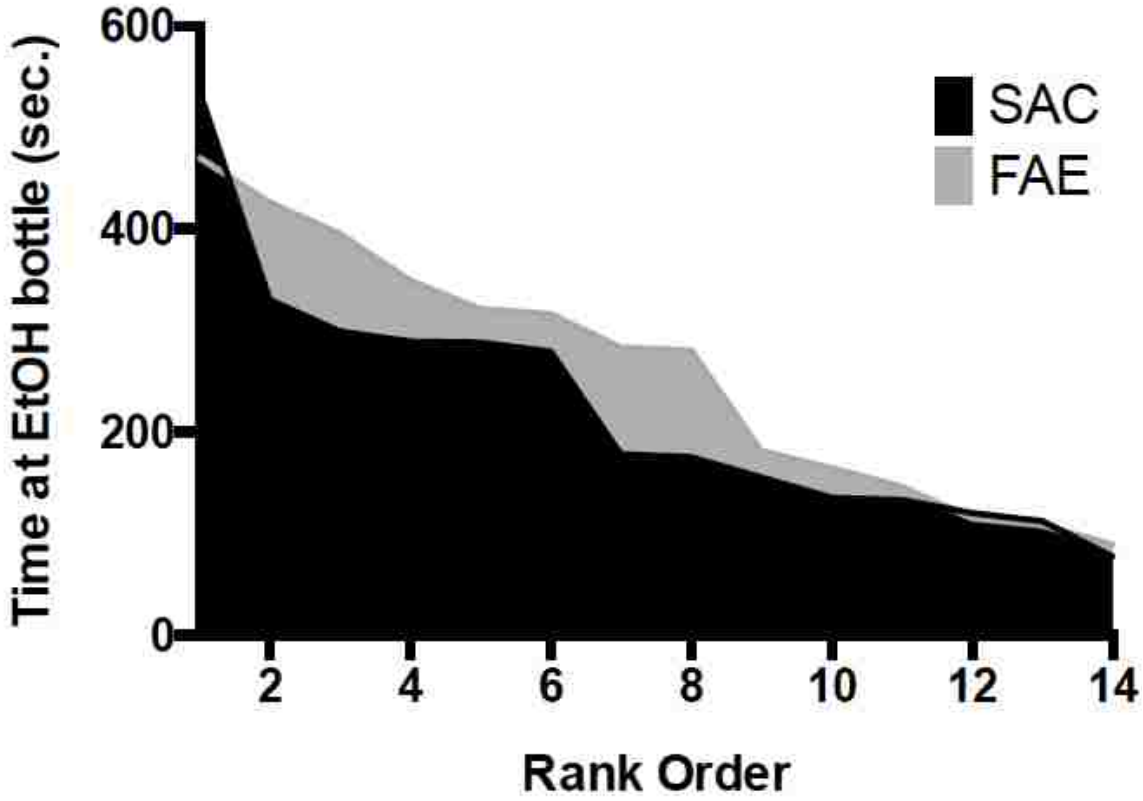

Figure 18. Time spent at or near the EtOH bottle for all rats, ranked from highest to lowest for each diet condition.

FAE rats become apparent is near the middle of the rankings, where FAE rats spent consistently more time attending to the EtOH bottle at the 150 to 400 second range, corresponding to the rankings of 2-12 (out of 14 ranks per group). These results suggest that, excluding extreme EtOH drinkers and non-drinkers from both groups, the FAE rats around the median approach and spend more time at the EtOH bottle than similarly ranked SAC rats.

4.4.4 RT-PCR mRNA expression

A*rc, c-fos,* and *Homer1a* expression in the NAc core and shell were analyzed using separate one-way ANOVAs. Results for IEG in each NAc region expressed as percent to SAC controls are presented in Figure 19. In the shell, no significant differences were found between SAC and FAE rats for mRNA

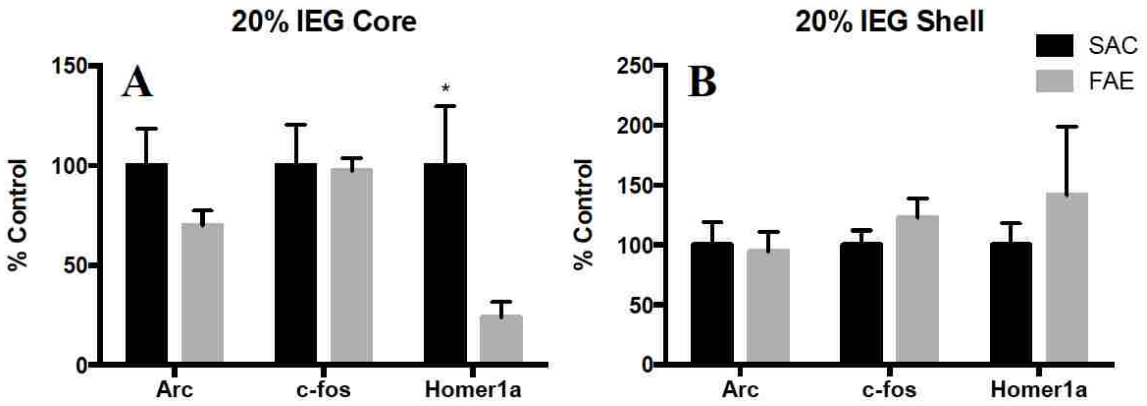

Figure 19. Mean (+ SEM) values for *Arc, c-fos,* and *Homer1a* expression in the NAc. Measures were computed for the shell and core for SAC (n = 8) and FAE (n = 8) rats.* indicates a significant between diet effect ($p < 0.05$).

expression of *Arc, c-fos,* or *Homer1a* (all p's > 0.28). Results were similar even when EtOH consumption was entered as a covariate using ANCOVA (all p's > 0.19). In the core, there was a significant reduction (73%) in *Homer1a* expression for FAE rats compared to SAC rats [$F(1,14) = 6.19$, $\eta^2 = 0.31$]. No significant differences between SAC and FAE rats were found in the core for mRNA expression of *Arc* [$F(1,14) = 2.31$, $p = 0.15$, $\eta^2 = 0.14$] or *c-fos* [$F(1,14) = 0.02$, $p = 0.9$, $\eta^2 = 0.63$]. Again, a similar pattern of results emerged when EtOH consumption was controlled for using ANCOVA for *Homer1a* [$F(1,14) = 6.05$, $\eta^2 = 0.32$], *Arc* [$F(1,14) = 1.43$, $p = 0.25$, $\eta^2 = 0.10$], and *c-fos* [$F(1,14) = 0.01$, $p = 0.94$, $\eta^2 < 0.01$] expression.

4.4.5 IEG expression predicting EtOH consumption

Measures of *Arc, c-fos,* and *Homer1a* in the NAc core and shell were used as individual predictors of 20% EtOH consumption in separate regression equations, with outliers removed separately for each particular IEG. Results of these regression analyses for SAC and FAE animals in each region of the NAc are presented in Figure 20. When *Arc, c-fos,* and *Homer1a* expression in the

NAc were analyzed with both SAC and FAE rats combined, *Arc* expression was a significant predictor of EtOH consumption [$F(1,14) = 4.72$, $R^2 = 0.25$], but not *c-fos* [$p = 0.39$, $R^2 = 0.05$] or *Homer1a* [$p = 0.66$, $R^2 = 0.01$]. This relationship between NAc *Arc* expression and EtOH consumption was negative, indicating that increased EtOH consumption was associated with reduced *Arc* expression for both diet conditions. Analyzing prenatal diet conditions separately, *Arc* significantly predicted EtOH consumption in SAC [$F(1,6) = 6.02$, $R^2 = 0.50$], but not FAE [$p = 0.32$, $R^2 = 0.16$] rats. This relationship was negative, in that increased EtOH consumption was associated with reduced *Arc* expression. When NAc *c-fos* and *Homer1a* expression were entered separately for SAC and FAE rats, neither predicted EtOH consumption (all p's > 0.35).

Next, *Arc, c-fos,* and *Homer1a* mRNA expression in the NAc were entered as predictors of EtOH consumption by region (core vs. shell). When collapsing across diet condition *Arc, c-fos,* and *Homer1a* in either the core or shell failed to predict EtOH consumption (all p's > 0.11). When these analyses were carried out separately in SAC and FAE rats, *Arc, c-fos,* and *Homer1a* expression in FAE rats did not predict EtOH consumption in these animals (all p's > 0.28) in either the core or shell. For SAC rats, *Arc* expression in the core was a significant predictor of EtOH consumption [$F(1,6) = 6.93$, $R^2 = 0.54$]. Again, this relationship was negative in SAC rats with increased EtOH consumption being associated with decreased *Arc* expression in the core. *Arc* expression in the shell and *c-fos* and *Homer1a* expression in both the core and shell did not significantly predict EtOH consumption in SAC rats (all p's > 0.25).

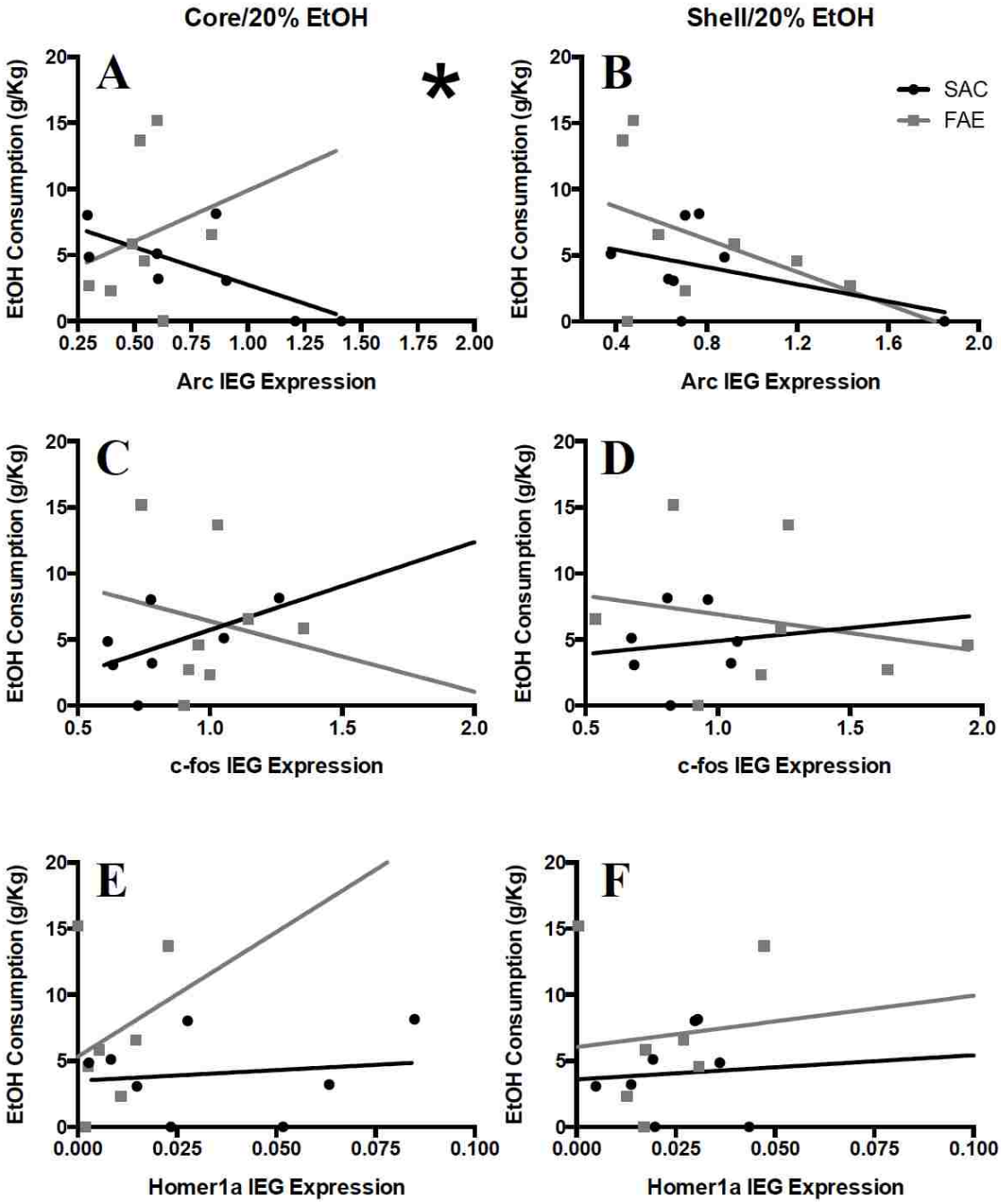

Figure 20. Mean IEG Expression in the core (A, C, E) and shell (B, D, F) as predictors of 4-Hour 20% EtOH consumption in SAC and FAE rats. * significant correlation for SAC and SAC + FAE rats combined ($p < 0.05$).

Finally, when *Arc, c-fos,* and *Homer1a* in either the core or shell were entered simultaneously to combine mRNA expression in these regions, no

significant effects were found for SAC or FAE rats separately or collapsing across prenatal diet conditions (all p's > 0.15).

4.4.6 Discussion

As in Experiment 3, no significant differences were found for 4-hour EtOH consumption between FAE and SAC rats, even though FAE rats, on average, drank 56% more EtOH than SAC rats. Again, it is important to note that the trend for increased EtOH consumption in FAE rats is consistent with our findings in Experiment 1. The lack of significant results can be partially explained by the high variability within groups for EtOH consumption, especially among the FAE animals. It may be the case that a longer period of EtOH availability, or an intermittent exposure paradigm might be necessary to observe EtOH consumption differences that we have found previously. Other paradigms that encourage EtOH consumption, i.e. reducing EtOH availability to one hour (Rewal et al., 2009) or vapor exposure (Bernier et al., 2011) could also lead to significant differences between SAC and FAE rats.

For IEG expression in the NAc, there was a significant effect found after 20% EtOH exposure for a reduction in *Homer1a* expression in the core of the NAc of FAE rats. This was the opposite of our original hypothesis, in which increases in *Homer1a* were expected after EtOH consumption. Prior research has shown that alterations in MGluR5 receptors alters voluntary EtOH consumption in alcohol-preferring P rats (Besheer et al., 2010), and that *Homer2* and MGluR5 are both upregulated in mice that binge drink EtOH using a drinking in the dark (DID) procedure (Cozzoli et al., 2009). Thus we expected to see an

		SAC (n = 7/8)			FAE (n = 7/8)			SAC+FAE (N = 15/16)		
Region	**Morphology**	**r**	**p value**	**R^2**	**r**	**p value**	**R^2**	**r**	**p value**	**R^2**
Core	***Arc***	**-0.732**	**0.039***	**0.53582**	0.229	0.585	0.05244	-0.339	0.199	0.11492
	c-fos	-0.317	0.444	0.10049	-0.18	0.67	0.0324	-0.199	0.46	0.0396
	Homer1a	0.149	0.725	0.0222	0.276	0.55	0.07618	-0.067	0.814	0.00449
Shell	***Arc***	-0.457	0.255	0.20885	-0.428	0.29	0.18318	-0.414	0.111	0.1714
	c-fos	-0.422	0.298	0.17808	-0.233	0.578	0.05429	-0.189	0.484	0.03572
	Homer1a	0.073	0.863	0.00533	0.103	0.825	0.01061	0.049	0.862	0.0024
Core + Shell	***Arc***	**-0.708**	**0.05***	**0.50126**	-0.401	0.489	0.1608	**-0.502**	**0.047***	0.252
	c-fos	-0.375	0.36	0.14063	-0.288	0.489	0.08294	-0.232	0.388	0.05382
	Homer1a	0.17	0.687	0.0289	0.175	0.707	0.03063	-0.034	0.904	0.00116

Table 2. Correlations (r), p values, and effect sizes (R^2) for measures of IEG expression as predictors of EtOH consumption. * indicates a significant correlation ($p < 0.05$).

increase in *Homer1a* expression that might indicate an increase in MGluR5 activity, especially in FAE rats. However, this was not the case. It could be that *Homer1a* and *Homer2* compete for the same resources when both are expressed (Shiraishi-Yamaguchi and Furuichi, 2007), and thus the *Homer2* expression increases with EtOH consumption and *Homer1a* expression is reduced in a compensatory manner. Future studies of the other *Homer* family of genes would be necessary to determine if this is the case. Likewise, specific analysis of MGluR5 receptor density and function in FAE rats also would be important.

Although there were no differences found for *Arc* IEG expression in either the NAc core or shell between SAC and FAE rats, *Arc* expression did serve as a significant predictor of EtOH consumption. This is important to results of Experiment 3, as *Arc* has been shown to be an important aspect of signaling cascades involved in dendritic outgrowth and spine formation (Lyford et al., 1995). Although not significant, it is possible that reduced *Arc* expression in in the NAc core in FAE animals might, in part, explain the reductions in dendritic morphology reported in Experiment 3. It is also interesting to note that in Experiment 3, structural alterations in the NAc shell (for all animals) and core (for

FAE rats) were statistically related to voluntary EtOH consumption, where as in Experiment 4 only functional changes in the NAc core were related to EtOH consumption. *Arc, c-fos*, and *Homer1a* IEG measures are occurring on a more rapid time scale than changes in dendritic morphology, but it is expected that these structural and functional processes are related. It could be that acute EtOH exposure has an effect on IEG expression in the core, or that structural changes in the shell alter functional processing downstream in the core. It would be important to establish the extent to which there are baseline differences in IEG expression for SAC and FAE rats in future studies. There were rats in both prenatal diet conditions that did not consume EtOH during the drinking session. Although it is not enough of a sample to address the extent to which there are baseline differences in IEG expression, it is important to include animals that did not drink in the analysis to include this variability in EtOH consumption and how it relates to IEG expression in the NAc.

4.5 Experiment 5: Intra-cranial self stimulation

4.5.1 Data analysis

For the first phase of Experiment 5, rats were given 8 consecutive trials of descending currents that were delivered when they pressed the active lever from 500 µA to 150 µA in 50 µA increments, with the frequency set at 141 Hz. This was carried out in three additional cycles so that each rat was measured for response rate four times at each possible current. A minimum current to maintain responding at 20 lever presses per minute was calculated for each rat based on interpolated values produced by results of a linear regression equation. These

minimum current values were averaged across diet conditions and analyzed using a one-way ANOVA.

For the second phase, rats were given a similar set of trials, but with the current held constant and the frequency reduced from 141 Hz to 28 Hz in 0.1 log increments. Response rates were measured at each possible frequency 4 times total, and these rates were used to compute percent maximal response (%MR) rates. This was calculated by dividing the average response rate at each frequency by the maximum response rate observed during any individual trial. These %MR rates were computed for each rat and averaged across diet condition and entered into a 2-way repeated measures ANOVA with prenatal diet as a between-subjects factor and frequency as a within-subjects factor.

4.5.2 Histology

Representative images of stained tissue for SAC and FAE rats, as well as approximate locations of electrode tips for the animals that completed the study are presented in Figure 21. Five SAC and 5 FAE rats were exclude from the analyses reported below, either because of improper electrode placement, non-responding to brain stimulation reward, or faulty implants that failed to complete a complete circuit. This resulted in a final group size of 10 (5 SAC, 5 FAE).

4.5.3 Shaping behavior

The number of days necessary for the rats to readily lever press for brain stimulation reward was recorded (Figure 22A). On average, SAC animals took 12.8 (SD = 0.84) days to reliably press at a rate of 20+ responses per minute.

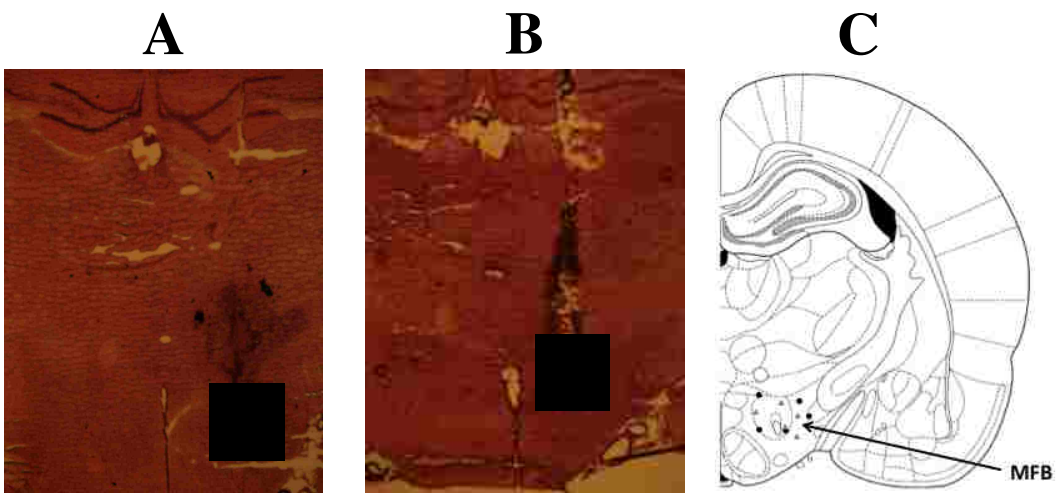

Figure 21. Representative electrode tip locations (black circles) for SAC (A) and FAE (B) rats. Approximate electrode tip locations for the animals included in Experiment 5 are presented in panel C. SAC rats (n = 5) are represented by filled circles, FAE rats (n = 5) are represented by grey triangles. Sections in A and B are slightly elongated and compressed medially compared to the sections illustrated by Paxinos and Watson (2005) used in C.

For FAE rats, the mean number of days to criterion was 14.2 days (SD = 0.84). This difference was found to be significantly different [$F(1,8) = 7.0$, $\eta^2 = 0.47$].

4.5.4 Minimum current response rates

The results for the minimum current to maintain responding at 20 lever presses per minute in SAC and FAE animals are summarized in Figure 22B. Although FAE rats, on average, had higher minimum currents to maintain lever pressing at 20 presses per minute compared to SAC controls, this difference failed to reach significance [$F(1,9) = 1.34$, $p = 0.28$, $\eta^2 = 0.14$]. A non-parametric analysis was carried out o these data due to the small sample size. A Mann-Whitney test failed to detect a significant difference between SAC and FAE rats for the minimum current measure [$U = 7$, $p = 0.31$].

4.5.5 Frequency response I/O curves

Results for the frequency/response curves for the second phase of Experiment 3 are presented in Figure 23. A 2-way ANOVA reveled a significant

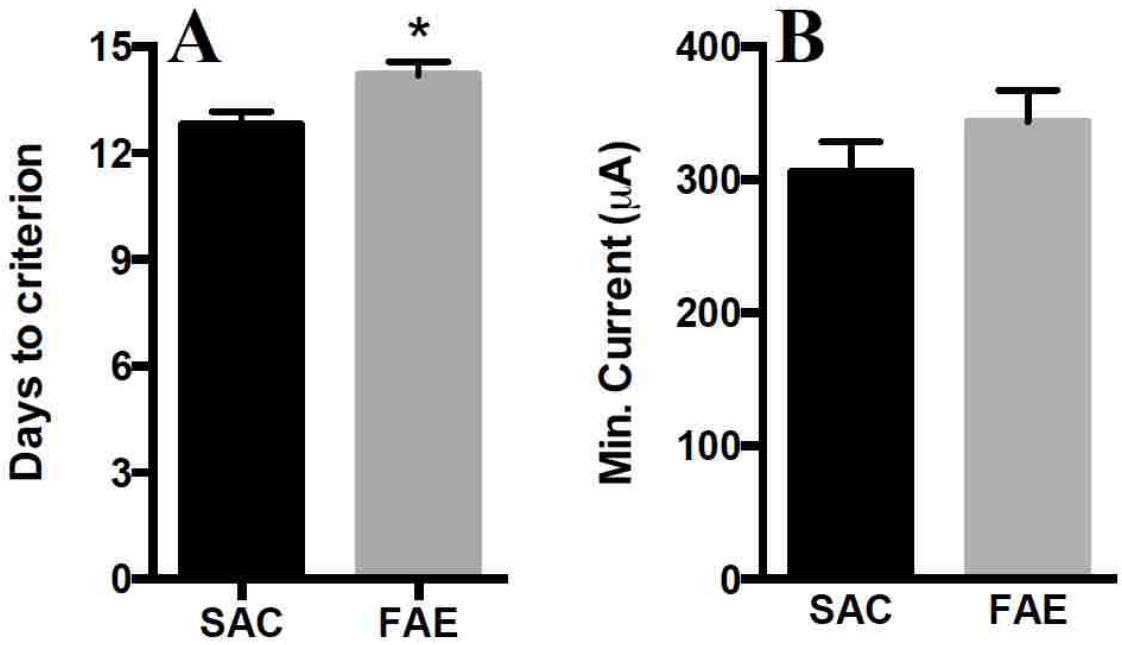

Figure 22. Mean (+ SEM) measures for days of shaping to meet criterion (A) and the minimum current required to maintain responding in the first phase of Experiment 5 (B) for r SAC (n = 5) and FAE (n = 5) rats. * indicates a significant between-group effect ($p < 0.05$).

main effect of frequency, with all animals responding more at the higher frequencies than the lower frequencies [$F(7,56) = 215.44$, $\eta^2 = 0.96$]. There was no significant main effect of prenatal diet condition [$F(1,8) = 0.6$, $p = 0.46$, $\eta^2 = 0.07$] and, although FAE rats tended to show reduced responding compared to controls at all but the highest frequencies, the diet by frequency interaction was also not significant [$F(7,56) = 1.12$, $p = 0.36$, $\eta^2 = 0.12$]. Non-parametric analyses were carried out to compare SAC and FAE rats at each frequency, these analyses all failed to reach significance as well (smallest $U = 6$, $p = 0.22$).

4.5.6 Discussion

The results of Experiment 5 found no significant differences between prenatal diet conditions on ICSS behavior related to reward processing. Although FAE rats took longer to acquire a stable baseline for lever pressing to deliver brain stimulation reward, SAC and FAE rats did not differ on the minimum reward

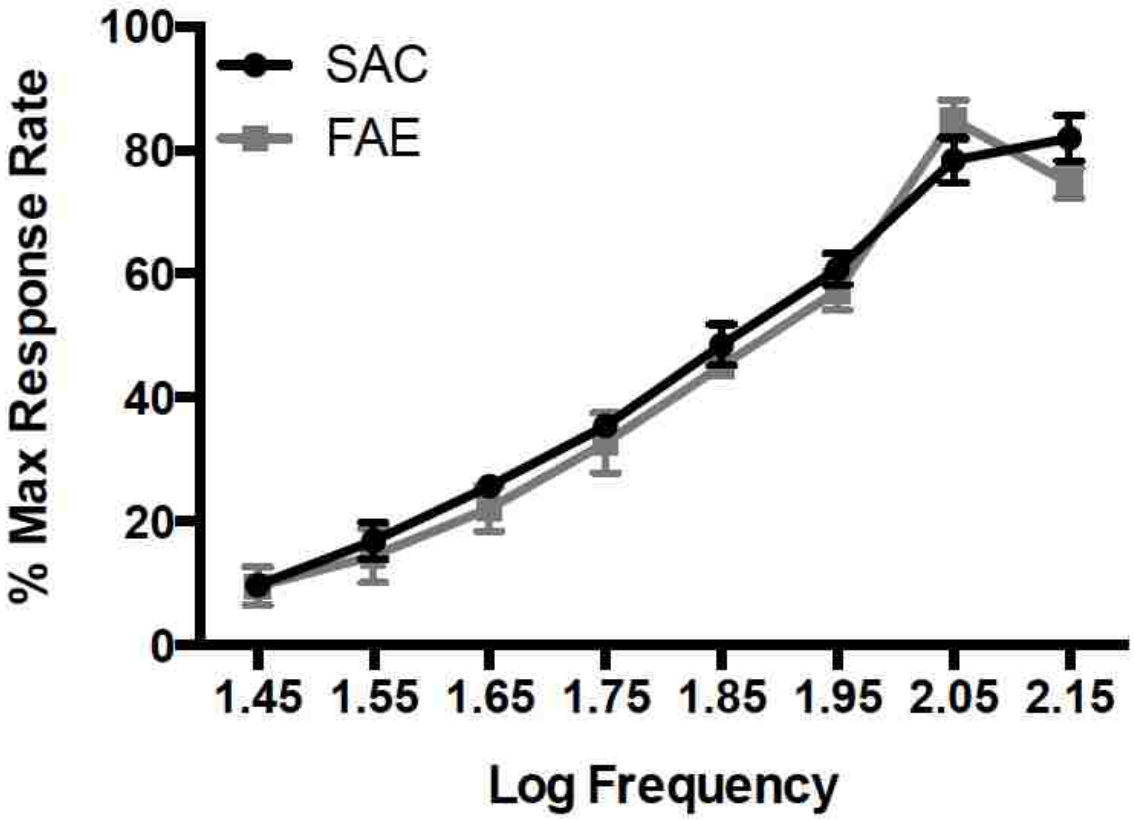

Figure 23. The average (+SEM) % maximal response rate (+ SEM) at each frequency for the second phase of Experiment 5 for SAC (n = 5) and FAE (n = 5) rats.

current that sustained a pre-determined response rate in the first test phase, nor was there any shift in response rates between groups when the current was held constant while the frequency of reward stimulation was altered. FAE rats did demonstrate a trend for higher currents needed to maintain a response rate of 20 lever presses per minute and also had a slight rightward shift in their frequency response curve compared to controls; these results are consistent with a decreased sensitivity to reward in other ICSS manipulations. For example, experiments that involve measuring ICSS thresholds while drugs of abuse (i.e. cocaine) are administered, ICSS thresholds tend to increase during initial drug

exposure, then decrease during withdrawal periods over repeated trials (Carlezon and Chartoff, 2007).

Given the small sample size that was utilized in this study and the subsequent variability in the acquisition of ICSS responding, it is difficult to say with any confidence that there is no difference between SAC and FAE rats on basic reward processing. For one thing, the medial forebrain bundle (MFB) targeted in the current study is made up or DA axons projecting to the NAc from the midbrain VTA. Other neurotransmitter systems that are part of the limbic circuit might also be involved, including GABA-ergic projections from the internal capsule (IC). These IC GABA inputs have been found to be far more sensitive to EtOH exposure and might serve as an interesting target for future studies using FAE animals (Lassen et al., 2007).

5. General Discussion

5.1 Summary of results

The results of the experiments presented here demonstrate that moderate fetal alcohol exposure results in increased EtOH consumption in adulthood as well as structural and functional deficits in the NAc, which has been shown to be important for reward learning and action selection. Furthermore, alterations in the NAc were found to be significant predictors of voluntary EtOH consumption in both SAC and FAE rats. Experiment 3 found that rats with moderate FAE had significant reductions in dendritic branching and length in medium spiny neurons in both the NAc core and shell. There was also a significant reduction in overall NAc spine density in FAE animals when controlling for the amount of 20% EtOH solution consumed during a 4-hour voluntary drinking session. These results suggest that moderate FAE results in smaller and less complex medium spiny neuron (MSN) dendritic fields with fewer synaptic connections in the NAc. Measures of dendritic branching in the NAc shell also served as a significant predictor of voluntary EtOH consumption for SAC and FAE rats combined. This association was significant for SAC rats alone, but was not in FAE rats. In the core, increased dendritic branching was associated with increased EtOH consumption in FAE, but not SAC, animals. In Experiment 4, FAE rats had reduced expression of the IEG *Homer1a* in the NAc core, and *Arc* expression in the NAc also significantly predicted EtOH consumption for SAC and FAE rats combined. As with the measures of dendritic morphology in Experiment 3, this relationship was stronger in SAC rats compared to FAE rats.

We also found that increased EtOH consumption in FAE rats does not appear to be related to alterations in general reward processing or alcohol metabolism. In Experiment 5, no significant differences were found between SAC and FAE rats on operant response rates for brain stimulation reward in an intra cranial self-stimulation (ICSS) paradigm. Finally, when rats were given a known dose of EtOH via injection in Experiment 2, we found no significant differences between SAC and FAE rats for blood alcohol concentrations at various time points post injection.

5.2 FAE effects on EtOH consumption in adulthood

It was hypothesized that FAE rats would drink more of a 10% and 20% EtOH solution than SAC controls. In Experiment 1, chronic-intermittent access to EtOH for several weeks did produce marked increases in both EtOH consumption and preference for a 10%, and to a greater extent, a 20% EtOH solution. Although elevated levels of EtOH consumption in FAE rats were also observed in Experiment 3 and Experiment 4, these results were not statistically significant. It should be noted that in Experiment 1, significant differences in EtOH consumption did not become apparent during the initial 4-hour dinking period, rather consumption was elevated in FAE rats over the course of 24 hours, and primarily during the first Week of access to EtOH. In addition, when rats in Experiment 1 were transitioned from 10% to 20% EtOH solutions, the greatest differences between SAC and FAE rats for EtOH consumption emerged. That is why a 20% EtOH solution was selected for Experiments 3 and 4. The alterations in dendritic morphology and IEG expression reported in Experiments 3 and 4

also serve as a potential mechanism by which increased EtOH consumption during the subsequent 20 hours might occur. With this in mind, the values for EtOH consumption observed in Experiments 3 and 4, while not significant, are qualitatively similar to our previous reports.

There are many factors that could contribute to our inability to detect significant differences in EtOH consumption in Experiment 3 and 4. One important aspect is the high variability in drinking behavior. Because rats had only one 4-hour drinking session, the novelty of the EtOH solution could elicit a wide range of behavioral responses. Indeed, prior studies that intended to encourage increased EtOH consumption in rodents often involve restrictions on access to EtOH over multiple sessions (Rewal et al., 2009) or vapor exposure to EtOH between drinking sessions (Jeanes et al., 2010). In Experiment 1, we measured drinking at 4 and 24-hour time points. It was found that the greatest differences emerged over the course of 24-hours; this suggests that FAE rats are drinking more in the latter 20-hour drinking period compared to SAC rats. In each of the studies presented here, all animals were maintained on a 12-hour light/dark cycle with access to EtOH starting at the begging of the dark cycle when they would be expected to be most active. It would appear that FAE rats, then, consume more EtOH during the light phase and that this is where differences in drinking behavior emerge. Several research reports have shown that FAE does alter sleep/wake cycles in rodents and related brain areas that are important for maintaining circadian rhythms (Rosett et al., 1979; Stone et al., 1996).

Interestingly, SAC rats reduced drinking in response to the increased concentration, while FAE rats appeared to drink the same amount of fluid, regardless of weather it was a 10% or 20% EtOH solution. One possibility is that FAE rats might be insensitive to changes in reward magnitude. The amygdala, another limbic structure with strong connections to the NAc (especially the core), has been found to be critically involved in pattern separation for reward magnitude. Gilbert and Kesner (2002) tested rats in an anticipatory contrast paradigm with different concentrations of a sucrose solution. Lesions of the amygdala, but not the hippocampus, disrupted the rats' ability to discriminate between a 2% and 16% sucrose solution. It has also been found that prenatal ethanol exposure reduces DNA concentrations in the amygdala in male, but not female rats (Kelly and Dillingham, 1994). Taken together, it is possible that moderate FAE results in deficits in amygdala processing that are important for the discrimination between 10% and 20% EtOH solutions. This could account for the apparent insensitivity to the change in EtOH concentrations that we have previously observed. Future studies need to be carried out to address this hypothesis.

It may also be the case that moderate FAE alters habit formation, which is thought to be related to compulsive drug-seeking behavior (Everitt and Robbins, 2005; Robbins et al., 2008). An important aspect of habit formation is the fact that responses by an organism become independent of outcome; that is, the animal continues to respond to stimuli even when the outcome has become devalued or degraded (Yin and Knowlton, 2006). In the context of our research findings, it

may be the case that FAE rats have altered habit formation as a result of changes in the NAc. Thus when the concentration of EtOH was increased from 10% to 20%, FAE rats were responding habitually to the EtOH bottle regardless of the percentage of EtOH within. Although previous findings have failed to detect effects of outcome- dependent and outcome-independent learning as a result of NAc lesions (Corbit et al., 2001), it is possible that moderate FAE alters downstream processing in the dorsal striatum in addition to the affects we have noted in the NAc. How FAE alters response-outcome (R-O) and stimulus-response (S-R) learning, as well as the transition from R-O to S-R responding (Yin et al., 2009), will be an important focus of future studies. In any case, it does appear that moderate FAE leads to increases in EtOH consumption in adulthood.

5.3 Effects of FAE on NAc structure and function

5.3.1 Dendritic morphology

Reductions in NAc shell dendritic morphology as a result of moderate FAE are consistent with our prior report (Rice et al., 2012). In Experiment 3 we also found significant reductions in the core, as well as a significant reduction in overall NAc spine density when controlling for EtOH consumption. These reductions were not found by Rice and coworkers (2012) previously, but are similar in terms of magnitude and direction. It may be that the voluntary EtOH consumption in the experiments reported here caused reductions in dendritic morphology in the core and reduced spine density across both regions of the NAc. This seems unlikely given the fact that both effects were significant when controlling for the amount of EtOH consumed during the 4-hour drinking session

by SAC and FAE rats. In fact, the reduction in spine density failed to reach significance when EtOH consumption was not entered as a covariate. It is possible that alterations in dendrites and spine morphology can occur in less than 30 minutes (Hering and Sheng, 2001). In fact, significant alterations in dendritic spine density have been previously reported in the medial amygdala one hour after EtOH exposure (Pandey et al., 2008). The extent to which this may be what is driving the results in the core we report here is unclear, and requires independent study. It could be argued that these reductions are related to withdrawal (e.g. FAE rats started and stopped drinking early in the drinking session, inducing a withdrawal state later on), but an analysis of moment-to-moment drinking behavior did not find any differences between SAC and FAE rats with regard to when the rats started and stopped drinking EtOH. With this in mind, it is more likely that moderate FAE accounts for these reductions regardless of adult EtOH consumption.

When examining the relationship between measures of dendritic morphology and EtOH consumption separately in SAC and FAE rats, changes in the NAc shell significantly predicted voluntary EtOH consumption in SAC rats, while changes in the core predicted EtOH consumption in FAE rats. For SAC rats this relationship was negative, with reductions in dendritic branching in the shell being associated with increases in 4-hour EtOH consumption. For FAE rats, on the other hand, this relationship was positive, with increases in dendritic branching being associated with increased EtOH consumption. This dissociation suggests that the deficits observed in FAE rats that did not drink EtOH (Rice et

al., 2012) might have effects in NAc core processing downstream of the shell, establishing a pattern of altered reward processing that throughout the lifespan. This finding is also in accordance with several published reports that have found alterations in the shell that are related to the consumption of, and/or exposure to, EtOH in normal rats (Rewal et al., 2009; Jeanes et al., 2010; Millan et al., 2010; Furay et al., 2011) and alcohol-preferring P rats (Engleman et al., 2009; Besheer et al., 2010). Given the importance in NAc shell processing in the early stages of reward learning and habit formation (Haber et al., 2000; Everitt and Robbins, 2005; Yin et al., 2008), deficits in the shell might trigger increased responding to alcohol (as in Experiment 1) and other drugs of abuse (Barbier et al., 2008; Barbier et al., 2009) as a result of FAE.

5.3.2 Immediate-early gene expression

In Experiment 4, SAC and FAE rats were given access to a 20% EtOH solution, after which tissue was processed from the NAc to observe changes in immediate-early gene (IEG) expression for *Arc, c-fos,* and *Homer1a.* We expected to see reductions in *Arc* and *c-fos* expression in the shell, but not the core, with increases in shell *Homer1a* expression. In fact, no significant changes for any of the genes of interest were noted in the shell. There was a large reduction in *Homer1a* expression in the core of the FAE rats compared to controls, which was not expected. This reduction in *Homer1a* has implications for the function of metabotropic glutamate 5 (MGluR5) receptors in the core of the NAc, as *Homer1a* expression is part of the signaling cascade that increases MGluR5 expression and function (Shiraishi-Yamaguchi and Furuichi, 2007). This

reduction in *Homer1a* signaling is also interesting because expression of this IEG is associated with reductions in dendritic spine density and spine shape, as well as impairments in NMDAR and AMPAR post-synaptic currents (Sala et al., 2003). One possibility is that *Homer1a* competes with other *Homer* family proteins (Shiraishi-Yamaguchi and Furuichi, 2007). Studies have shown that mice that have been trained to binge drink EtOH also show elevated *Homer2* signaling in the NAc following drinking sessions (Cozzoli et al., 2009), so it may be that voluntary EtOH consumption increases *Homer2* at the expense of *Homer1a*. It might also be that *Homer1a* is an important aspect in forgetting about cues that might predict EtOH consumption, and that reduced signaling in this cascade might be maladaptive in FAE rats, leading to increased EtOH consumption. This possibility is unclear given our findings of a lack of a significant association between *Homer1a* expression and 20% EtOH consumption (See Table 2). It is also unclear whether the reduced *Homer1a* expression in the core of FAE rats compared to controls is a result of EtOH consumption at all, or rather a pre-existing difference resulting from moderate FAE. Nevertheless, given the importance of MGluR5 function and alcohol consumption in rodents (Adams et al., 2008; Bird et al., 2008; Cozzoli et al., 2009; Besheer et al., 2010), future studies of these receptors in the NAc, and how they might be altered by FAE, seems prudent.

Although *Arc* expression was not significantly different between prenatal diet conditions in the NAc, it did significantly predict EtOH consumption, with increased EtOH consumption being associated with reduced *Arc* expression. In

breaking down this relationship for the core and shell for FAE and SAC rats, *Arc* expression in the core significantly predicted EtOH consumption for SAC rats, but not FAE rats. This relationship between EtOH consumption and core *Arc* expression coincides with a non-significant, but relatively large (~30%) decrease in *Arc* expression in FAE rats in the NAc core. This effect appears to be related to adult EtOH consumption, as opposed to a long-term aftereffect of moderate FAE, and might partially explain the reductions in NAc core dendritic morphology and spine density noted in Experiment 1. *Arc* IEG expression is part of an important signaling cascade that is involved in dendrite growth and spine formation (Lyford et al., 1995), and over time it seems likely that reductions in this signaling would reduce MSN dendritic fields, while also potentially disrupting reward learning in the NAc. Since both *Arc* (to some extent) and *Homer1a* are reduced in the core of FAE rats, it appears as if learning about, and forgetting, of new information in the core might both be reduced as a consequence of moderate FAE and adult EtOH consumption.

In analyzing the relationship between these alterations in the NAc and voluntary EtOH consumption, it is important to consider the extent to which the changes in NAc structure and function contribute to EtOH consumption and vice versa. Because our previous reports found baseline differences in NAc shell dendritic morphology, but not core morphology or NAc spine density in FAE rats, it does appear that voluntary EtOH does have effects on dendritic morphology in the NAc. Overall, measures of dendritic morphology in the shell served as the best predictor of EtOH consumption, regardless of prenatal diet condition. It

would be expected that the structural changes in the NAc, and particularly the shell, would have the biggest impact on reward processing, as this is the area of the striatal circuit that is initially engaged during drug exposure. Changes in the core, especially for IEG expression, might be more transient or sensitive to the most recent experience (e.g. the 4-hour drinking session utilized in Experiments 3 and 4). The results for measures of dendritic morphology and IEG expression reported here are nevertheless confounded by EtOH consumption. Even though we included rats that did not consume EtOH in our regression analyses, further studies would need to be carried out to tease apart the unique aspects of fetal versus acute EtOH exposure that result in reductions in NAc structure and function.

Comparing the effect sizes for the relationship between alterations in the NAc and EtOH consumption suggest that structural changes in dendritic morphology and spine density might serve as a better indicator of whether there is an increased sensitivity to EtOH in FAE rats compared to SAC controls. This may be due to the more transient nature of IEG expression in the NAc, or may be the result of mRNA expression as a measure of IEG activity, as opposed to protein expression analysis. It could also be argued that structural changes are a more permanent result of changes in IEG, and that IEG represents only one aspect of signaling cascades that contribute to overall changes in dendrite and spine morphology. There are several other IEGs not included in the current study that may yield better predictors of differential sensitivity to EtOH in FAE and SAC rats

5.4 FAE and general reward learning

The results of Experiment 5 suggest that moderate FAE did not alter operant responding for brain stimulation reward using an ICSS paradigm. There was a trend for FAE rats to require higher levels of initial stimulation to maintain a set level of responding to controls, as well as a trend for reduced responding to a series of set frequencies compared to SAC controls. These trends are comparable to animals that have reduced sensitivity to reward, most often associated with withdrawal symptoms to drugs of abuse (Carlezon and Chartoff, 2007) and response to chronic pain (Pereira Do Carmo et al., 2009). Although we did not detect a significant effect in the present study, these results are meaningful and suggest that moderate FAE results in a general decrease in sensitivity to rewarding stimuli. This might explain the increased responding to drugs of abuse found in FAE animals (Barbier et al., 2008; Barbier et al., 2009), as more of the drug would be required to get the same effects as controls. A more detailed set of experiments would be necessary to determine if this is indeed the case. In the current study, the low sample size combined with variability in electrode placement made it difficult to detect significant differences.

It is not surprising, however, that differences in ICSS responding in FAE rats were not found, as our previous data did not find any differences between prenatal diet conditions on responding to a 2% sucrose (appetitive) and a 0.1% quinine (aversive) solutions (see Appendix 2). Other research reports have found increases in consumption and preference for quinine between rats prenatally exposed to alcohol compared to controls. Since quinine and ethanol have similar

taste and odor qualities, this has been taken as a possible explanation for increased EtOH consumption in FAE rats (Youngentob and Glendinning, 2009). Thus it is possible that a higher level of prenatal alcohol exposure might result in more striking differences in responding to rewarding stimulation in ICSS, the moderate FAE paradigm utilized here results in too subtle of an effect to be detected by general reward processing paradigms like ICSS or sucrose consumption.

The experimental design used in the current study was adapted from Carlezon and Chartoff (2007) and involved electrodes implanted into the lateral hypothalamus in order to stimulate the medial forebrain bundle (MFB). The MFB is the major pathway associated with the mesolimbic dopamine projection from the ventral tegmental area (VTA) to the NAc (Squire, 2008). It is possible that the lack of a significant difference in ICSS responding between prenatal diet conditions observed here are is due to the fact that there are no differences in DA activity in the NAc as a result of moderate FAE. Data collected in our laboratory did not find any significant alterations in D1, D2, or dopamine transporter (DAT) binding in the NAc core or shell of FAE rats using autoradiography (unpublished observations). Even though DA activity may not be altered by the moderate FAE paradigm utilized in our research, other neurotransmitter systems may be affected, and provide an interesting line of research for future studies.

One such target is GABA, with GABA-ergic projections onto the VTA being much more sensitive to the effects of EtOH than dopaminergic pathways in

the MFB (Steffensen et al., 2001). It would be interesting to find out if ICSS responding in FAE rats would be different using stimulation of the internal capsule (IC), which would target GABA projections based on the paradigm used by Lassen and colleagues (2007). Another neurotransmitter system of interest is glutamate (Glu) based on the findings that DA neurons in the NAc co-release Glu, which is important because of the speed of Glu processing at NMDA and AMPA receptors would be necessary for the rapid encoding of information in the NAc (Stuber et al., 2010). Recent theories of NAc function in relation to reward processing have also pointed out the importance of glutamatergic input from the frontal cortex and other limbic structures, including the amygdala (Yin et al., 2008; Humphries and Prescott, 2010). Alterations in regions of the frontal cortex might then be driving the effects in the NAc, both structural and functional, that we have described here. Previous work in our laboratory has found reductions in dendritic morphology and spine density, as well as reductions in *Arc* and *c-fos* signaling, in the agranular insular cortex (AID), a region that is functionally similar to the orbital frontal cortex in humans that sends strong projections to the NAc shell (Hamilton et al., 2010b; Hamilton et al., 2010a). Glu function in the NAc may also be altered by moderate FAE in relation to MGluR activation based on the results from Experiment 2 where a significant reduction in *Homer1a* expression in the core of FAE rats was found.

5.5 FAE and EtOH metabolism

It does not appear that moderate FAE alters EtOH metabolism based on the results of Experiment 2. Using a within-subjects design with a known dose of

EtOH, we examined blood-alcohol concentrations (BACs) at 5 different time points (1-7 hours) post injection. Although there was some variability, including slight increases at the 1-hour time point for FAE rats, none of the differences overall or within specific time points were statistically significant. Although differences in EtOH metabolism were not expected based on prior findings using rodent models of FAE (Barbier et al., 2009), it is important to establish that differences in EtOH metabolism are not contributing to increased EtOH consumption in FAE rats. Blood samples were also analyzed for rats that voluntarily drank EtOH in Experiments 1,3, and 4; in line with the findings in Experiment 2, there was a strong positive correlation between EtOH consumption and BAC (data not shown). Furthermore, both SAC and FAE rats had similar slopes for this relationship, again supporting the hypothesis that moderate FAE does not alter EtOH metabolism in adulthood. Although a within-subjects design was utilized to increase power and decrease the number of animals necessary to complete this experiment, a between-subjects design might be able to provide definitive support for this particular hypothesis. The dose of EtOH administered was meant to mimic the vales that were being achieved by rats that were voluntarily consuming EtOH. It may be that higher or lower doses would result in significant differences between prenatal diet conditions.

5.6 Implications for NAc function in FAE rats

Based on the results of these experiments, the effects of FAE and/or adult EtOH consumption on the NAc are clear and have strong implications for reward-guided learning and addiction processes in these individuals. Recent theories on

NAc function posit a serial progression of activity in striatal circuitry that shifts from one region to another as experience with cues related to goal-directed actions continues (Everitt et al., 2008; Yin et al., 2008). Specifically, the NAc shell is engaged first, followed by the core; learning then shifts to instrumental behavior in the dorsomedial striatum (DMS), and finally to habit formation governed by the dorsolateral striatum (DLS; See Figure 1). Deficits in the NAc shell could have important implications on learning related to the other downstream regions of the striatum. Studies have shown that the shell is critical for contextual learning related to reward, while the core is critical for specific cues related to reward, including alcohol (Chaudhri et al., 2009). The DMS is engaged during R-O learning, where instrumental responding is dependent on the outcome, while the DLS is critically involved in habit formation (Yin and Knowlton, 2006). Deficits in the shell, as we have reported here and elsewhere (Rice et al., 2012) as a result of moderate FAE could induce deficits in contextual and cue learning about rewarding events, as well as instrumental responding related to reward, which might explain the increased responding to stimulants of abuse and other drugs in rats (Barbier et al., 2008; Barbier et al., 2009) and humans (Streissguth, 1997). Other behaviors related to addiction, like impulsivity, might also be affected by reductions in the NAc core. Lesions of the core result in increased impulsivity in rats, where smaller, more immediate rewards are favored over greater rewards that required a longer waiting period (Cardinal et al., 2001).

Given the structural and functional changes found in the core after EtOH consumption in FAE rats that were not noted in FAE rats that were EtOH naïve in

our prior studies, it would appear that structural deficits in the shell do lead to deficits in other striatal regions after EtOH exposure throughout the lifespan. This presents a “two hit” effect of moderate FAE when EtOH is encountered in adulthood, as deficits in both aspects of the NAc emerge. Since the core and shell are well documented to be related to consumption and relapse to EtOH after periods of abstinence (Chaudhri et al., 2008; Janak and Chaudhri, 2010), moderate FAE might result in a significant predisposition for addiction to alcohol and other drugs of abuse even in the absence of the overt physical and cognitive deficits associated with full blown FAS. EtOH exposure in normal rats has also been found to affect mechanisms related to plasticity in the striatum. Low to moderate doses of EtOH have been found to blunt LTP, and high doses have even been found to reverse LTP to LTD in the NAc (Jeanes et al., 2010) and DMS (Yin et al., 2007). Abnormal LTD in the core of the NAc is involved in the transition from normal behavior to addictive responding in mice (Kasanetz et al., 2010). Moderate FAE may have similar effects on cellular mechanisms thought to underlie learning and memory in the NAc and dorsal striatum.

Reductions in NAc dendritic structure and function would also have important implications for the generalizability and specificity of reward learning as well. For example, voluntary EtOH consumption is unique in that drinking is an important behavior related to consumption and survival, and is thus a rewarding behavior. NAc activity is involved in water consumption, and recordings from neurons in freely moving rats have shown that a pause in MSN firing in the NAc is important for the initiation of drinking behavior (Krause et al., 2010). Hedonic

"hot spots" that have been described in the NAc also respond to natural reinforcers like food and water, and tend to be located in regions of the NAc not associated with drug reward (Pecina, 2005; Mahler et al., 2007). Single-unit recordings in the NAc core and shell have also found that roughly 85% of the cells recorded during lever presses for water and EtOH discriminated between the two levers (Robinson and Carelli, 2008). That is, certain neurons fired in response to lever pressing for water, and others for EtOH. This would suggest that MSN activity in the NAc is highly specific to different behaviors related to rewarding stimuli. It might be possible that reductions in the dendritic fields of these neurons might reduce this discriminability, resulting in more generalized behavior. This could lead to abnormal intake of EtOH.

One mechanism by which this generalization might occur is lateral inhibition. This is where activation of one neuron results in reduced activity in nearby neurons, leading to greater specificity in activation of an ensemble of cells. *In vitro* recordings of MSNs from the NAc have demonstrated that D1 receptors modulate lateral inhibition in the NAc (Taverna, 2004). This would be an important aspect of the hedonic hot spots (Pecina, 2005) and how different hot spots are related to natural versus artificial rewards, and how neurons show such distinct firing patterns for water versus EtOH (Robinson and Carelli, 2008). In relation to the current study, reductions in NAc structure and function as a result of moderate FAE might lead to reduced lateral inhibition in the NAc, which results in more generalizability of rewarding stimuli and eventually leading to

increased EtOH consumption. Further research into this phenomenon is necessary.

Many of the non-significant results for 4-hour EtOH consumption and responding for brain stimulation reward appeared to be related to increased variability within groups, especially for FAE rats. This does not appear to be the case based on significance tests for invariance. For all of the dependent measures taken in the experiments reported here, there were isolated instances of a lack of homogeneity of variance between SAC and FAE rats (data not shown), but this effect did not occur in any systematic way, nor was it specific to any single experimental manipulation or variable.

5.7 Limitations

The lack of a significant difference for drinking behavior between prenatal diet conditions makes it difficult to evaluate the relationship between alterations in the NAc and drinking behavior. Although there were significant correlations between 20% EtOH consumption and measures of dendritic morphology and IEG expression, these were driven by significant associations in SAC animals. With such high variability in EtOH consumption values and small sample sizes, the ability to detect significant effects and associations is hampered. This was also an issue for ICSS experiments, as variability in electrode placement may have contributed to alterations in lever pressing for brain stimulation reward. For many rats, there seemed to be a general reduction in responding that was not related to moderate FAE or stimulation parameters. Additional subjects and or test sessions might help address these limitations. Also with regard to ICSS, targeting

the MFB as opposed to other regions (i.e. internal capsule) may have limited our ability to detect an effect of FAE. In addition, levers were used as the operant response and animals took many trials to shape lever-pressing behavior. Using a more natural response, like nose poking, could reduce the potential confound of time spend shaping the behavior.

Although we were interested in addressing changes in the NAc as a function of initial exposure to EtOH, the single 4-hour drinking session may not have been enough time to observe noticeable differences in drinking behavior. Longer sessions or multiple shorter sessions would help reduce variability, but at the cost of understanding IEG expression in the NAc when the animal first experiences the drug. The use of RT-PCR allowed for rapid measures of RNA expression for the IEGs of interest, but measures of protein expression would be more meaningful in relation to our hypothesis. Perhaps other immunohistochemistry methods that analyze protein, rather than mRNA, of IEGs might result in a more meaningful and insightful analysis. Similarly, our method for drawing cells using the *camera lucida* technique results in a 2-dimensional image of the dendrites of interest. Although there is no reason to think that the missing information from a 2-D image would confound our findings, more recent technologies for 3-D dendritic and spine analysis could result in reduced error and better representation of cellular morphology. Lastly, although we attempted to limit our search for MSNs to draw in the NAc to very specific aspects of the core and shell, there are areas of overlap, and cellular size differences within these sub-regions that could influence our results. Given the low variability

reported here, this does not seem to be an issue, but should be taken into account.

5.8 Future directions

As the results here suggest that moderate FAE results in reductions in the early processing centers of the striatum based on the proposed function of cortico-striatal loops in reward learning by Yin, Ostlund, and Balleine (2008), and that EtOH consumption also appears to impact these areas of the striatum as well, further studies on how FAE and/or adult exposure to EtOH affect instrumental responding are important. Studies have shown that habit formation to EtOH occurs at a faster rate than for sucrose reward (Dickinson et al., 2002), and that inactivation of the DMS or DLS can shift operant responding for EtOH to be habitual or susceptible to outcome devaluation, respectively (Corbit et al., 2012). The extent to which R-O behavior and related function in the DMS, or S-R habit and related function in the DLS, are affected by moderate FAE is one area of consideration. This can be studied by biasing animals toward one response type or the other based on schedules of reinforcement. The rates of learning for each type of behavior would be relevant, as well as the transition from one to the other (Yin et al., 2009). This could involve natural reinforcers like food, or EtOH, which would be more interesting based on our findings of increased EtOH consumption, but not other rewarding stimuli (e.g. sucrose solution, ICSS). How these processes are altered by EtOH exposure (and withdrawal) would also be interesting.

Pavlovian to instrumental transfer (PIT) is another phenomenon that would be relevant to the results presented here. In PIT, the organism initially learns a classically conditioned association (tone-food pellet), and then uses this association to guide operant responding in the instrumental phase (tone-press lever-food pellet). Corbit and Janak (2007) conducted an experiment where EtOH-cue associations were learned and then compared to non-EtOH-cue associations in the PIT test. EtOH-cue associations facilitated PIT compared to non-EtOH cues, suggesting that EtOH has an excitatory effect resulting in enhanced learning for cues that predicted EtOH delivery. Since PIT fits in nicely with the idea that the early stages involve classical conditioning thought to be related to NAc function while later the instrumental aspect is dependent on the dorsal striatum, it would be interesting to see how moderate FAE alters PIT performance, and how this might relate to neuronal activity in the NAc, DMS, and DLS. On a more global level, PIT is also related to the basic arguments of the role of the striatum in reward learning and addiction (Balleine et al., 2009) in that shifts from learning to action to habit would follow the PIT phenomenon.

It does appear that moderate FAE does lead to increases in EtOH consumption in adulthood. The mechanisms by which this occurs still need to be addressed. One possibility, as our ICSS data might suggest, is that FAE rats show a behavioral profile of a normal animal in withdrawal. This would be an interesting line of research to pursue, as a withdrawn state might explain the increased EtOH exposure in these animals. ICSS would be an ideal method to address this, with measures of ICSS taken within subjects as the animals are

exposed to EtOH, and then again after a period (or multiple periods) of withdrawal. How the response curves are shifted for FAE rats compared to controls would help to address this question. Finally, it would also be interesting to know how potential postnatal treatments that have been shown to reduce learning and memory deficits that are associated with FAE later in life, such as choline supplementation postnatally (Thomas et al., 2007; Thomas et al., 2010), might help to mitigate increased EtOH consumption and the related deficits in the NAc.

5.9 Conclusion

In summary, four experiments were conducted to determine the extent to which moderate FAE alters structure and function in the NAc, and how these alterations are related to EtOH consumption in adulthood. It was found that FAE rats had reduced measures of dendritic length, branching, and spine density in both the NAc core and shell, as well as reduced expression of the IEG *Homer1a* in the core. Measures of dendritic morphology in the shell, and *Arc* IEG expression in the core were significant predictors of EtOH consumption in SAC, but not FAE rats. Due to the duration of the drinking session, no significant increase in 20% EtOH consumption was found, but there was a trend for FAE rats to drink more than SAC rats. The moderate FAE paradigm used in these studies does not appear to have any significant effect on general reward processing based on ICSS responding, or on EtOH metabolism. These results point to the NAc as a brain region susceptible to the effects of FAE, and that

changes in this brain region can have long-lasting implications on reward learning and abnormal EtOH drinking behavior.

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