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The Effect of Adolescent Alcohol on Dopaminergic and Gabaergic Neurotransmission in the Adult Prefrontal Cortex

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THE EFFECT OF ADOLESCENT ALCOHOL ON DOPAMINERGIC AND GABAERGIC NEUROTRANSMISSION IN THE ADULT PREFRONTAL CORTEX

by

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SAMUEL WILLIAM CENTANNI. The effect of adolescent alcohol on dopaminergic and GABAergic neurotransmission in the adult prefrontal cortex. (Under the direction of L. JUDSON CHANDLER).

The prefrontal cortex (PFC) is thought to play an important role in cognitive processes that are negatively impacted by alcohol exposure. Compared to other brain regions, the neuronal connections of the PFC undergo a critical period of reorganization and refinement during adolescence that coincides with improvements in cognitive control and decision-making. Environmental insults that occur during this period may be particularly damaging to the PFC, resulting in aberrant neurodevelopment along with long-lasting effects on cognitive functioning that negatively impacts decision-making and behavioral control. Experimentation with alcohol typically begins during adolescence when it is often consumed in excessive binge-like episodes resulting in high levels of intoxication followed by a short period of abstinence. This dissertation addresses the hypothesis that binge-like adolescent alcohol (AIE) exposure alters the development of neurotransmitter systems in the prelimbic PFC (PrL-C) and as a result, PFC-dependent cognitive functions are compromised in adulthood. First, the effect of adolescent alcohol abuse on dopaminergic neurotransmission in the adult PrL-C was examined. AIE compromised adult protein expression of the dopamine-related enzymes tyrosine hydroxylase and catechol-O-methyl transferase. Electrophysiology studies revealed a loss of D1 receptor modulation of pyramidal neuron evoked firing in adult layer V PrL-C. The next part of this dissertation focuses on the effect of AIE on development of the PrL-C GABA

system. AIE produced marked reductions in $GABA_A$ receptor-mediated tonic currents in pyramidal neurons in layer V PrL-C. This effect appears to be largely mediated by developmental alterations specifically in $GABA_A$ receptors containing the δ-subunit. The dissertation concludes by assessing the effect of AIE on risky decision-making in adulthood. Furthermore, given the role of GABA in decision-making, exploratory studies sought to enhance tonic GABA currents using the novel δ-$GABA_A$ receptor positive allosteric modulator AA29504 and testing the effect of the drug on risk/reward decision-making. The results suggest that AIE did not alter risk/reward decision-making in adulthood. Moreover, AA29504 administration did not alter decision-making on the probabilistic discounting task. Taken together, this dissertation reveals that AIE exposure results in persistent deficits in both dopaminergic and GABAergic neurotransmission in the adult PrL-C that may contribute to deficits in PFC-dependent behavioral control in adulthood.

CHAPTER 1

BACKGROUND AND SIGNIFICANCE

THE PREFRONTAL CORTEX AND EXECUTIVE FUNCTION

Executive function encompasses a wide range of complex cognitive processes that requires intricate coordination between different systems in the brain to achieve a particular goal. The executive system provides a platform where information about the environmental surroundings of a given situation can be sorted and manipulated to create and execute an appropriate behavioral response. Many common behaviors fall under the umbrella concept of executive function, but working memory is an exemplary system often associated with, and occasionally used synonymously with, executive function. Working memory can be conceptualized as the temporary convergence of temporal and spatial information necessary to complete complex cognitive tasks such as language

comprehension, learning, planning, reasoning, and behavioral flexibility (Baddeley 1992). In essence, working memory is an interface between perception, long-term memory and action. The distinguished neuroscientist Patricia Goldman-Rakic aptly referred to working memory as the “blackboard of the mind” (Goldman-Rakic 1996). The Baddeley model of working memory proposes that this system is composed of five components: a control system, the central executive and three storage systems (Baddeley 2000). The three storage systems, the visuospatial sketchpad, episodic buffer and the phonological loop, work in unison to integrate with external and internal cues, providing visual semantics, episodic long-term memory and language, and “report” this information to the central executive. The central executive collects incoming information from many different systems and formulates the appropriate behavioral response. Working memory, as opposed to short- and long-term memory, allows for the manipulation of transiently stored information, so that behavioral strategies can be rapidly adjusted based on the ever-changing environment in order to accomplish a specific goal.

Behavior flexibility is a critical component of executive function that dramatically influences both current and future behaviors (Unterrainer and Owen 2006; D'Esposito 2007). Testing specific components of working memory such as behavioral flexibility was made possible by the development of simple, translational tasks. One of the most common tasks used to measure working memory and behavioral flexibility in humans is the Wisconsin Card Sorting Test (WCST). This task requires subjects to sort a set of cards based on a specific

rule (i.e. color). The rule is then switched to a new sorting rule (i.e. number) without the subject's knowledge. Thus, sorting the cards by the previous rule no longer yields a correct response (Millner 1963). The time it takes for an individual to learn the new rule is used as a measure of behavioral flexibility, attention and working memory. This task has been widely used in human and non-human primates. Furthermore, rodent analogs of the WCST provide alternatives to studying working memory in primates. Maze-based (Ragozzino et al. 1999; Stefani et al. 2003; Floresco et al. 2006), digging-based (Birrell and Brown 2000) and cognitive-based (Floresco et al. 2008) versions of the WCST are widely used to test cognitive function in rodents.

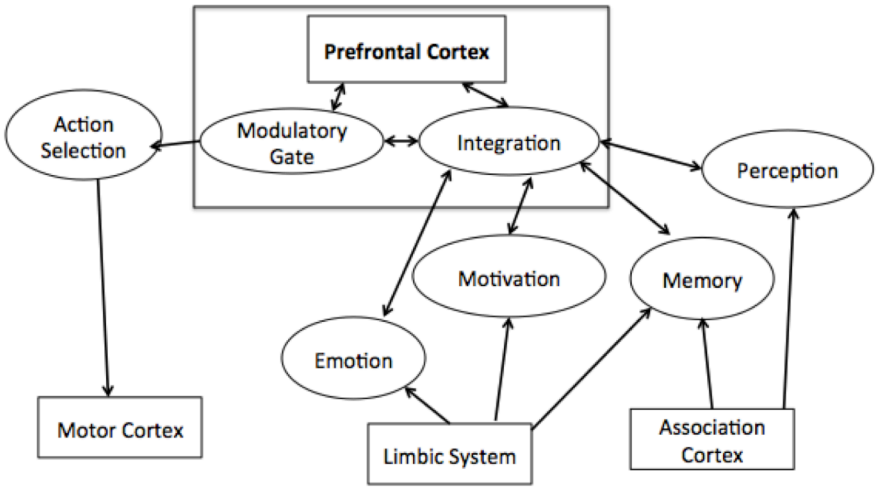

Figure 1. Schematic illustrating the coordination between the prefrontal cortex (PFC) and other components of the executive system. Sensory information from other cortical regions and subcortical regions provide emotional, motivational, long- and short-term memories and external perceptions to the PFC. The PFC coordinates and organizes this information to determine the appropriate course of action. Once an action is determined, outputs to the motor cortex perform the intended behavior.

The prefrontal cortex as the "central executor"

The prefrontal cortex (PFC) is one of the largest and most differentiated neocortical areas of the primate brain. It is considered to be the central executive component for complex cognitive tasks such as working memory and behavioral flexibility. The importance of the PFC for executive function was brought to light in 1848 by the now famous case of Phineas Gage, a man who, while working as a railroad foreman, was inadvertently impaled through the orbital bone by an iron rod. When he miraculously recovered, Gage could still walk, talk, see, hear and remember, and doctors concluded that his brain was not permanently damaged. Gage did, however, experience drastic changes in behavior that led him to be impulsive and, in general, behaviorally inflexible and uninhibited. The case of Phineas Gage shed light on the importance of the PFC for higher order cognitive processing. Research on the PFC and cognitive function advanced with seminal lesion studies conducted in non-human primates in the 1920s and 1930s that provided great insight into the importance of this region (Bianchi 1922; Jacobsen 1935,1936). Further implicating the PFC as the central component of executive function, damage to the PFC, either accidental or through lesion studies, has been shown to dramatically impair behavioral flexibility on the WCST (Millner 1963), planning on the Tower of London Test (Shallice 1982), and various fluency tasks (Jasper 1995). Collectively, these impairments suggest a breakdown in coordinative processes. Subjects are relatively unimpaired on tests focusing on a particular action or task, but when a number of different functions must be coordinated, deficits are exposed. Outside of a laboratory setting, when

attentional demand is high, subjects with damage to the PFC find it difficult to plan out effective daily strategies, whether it is performing basic household chores or multitasking various work duties (Shallice and Evans 1978).

It is now understood that the PFC serves as the interface between the barrage of sensory information from an ever-changing environment and long-term memory stores. Holding incoming sensory information online while simultaneously accessing memories from cortical and subcortical areas provides an intricate system where current and future circumstances converge and guide the appropriate responses to the dynamic environmental conditions (Figure 1). Once the sensory information from cortical and subcortical areas has been integrated with previously formed associations and an appropriate behavioral response has been selected, projections to movement-associated areas then initiate that behavior (Unterrainer and Owen 2006; D'Esposito 2007). To account for this high demand, the PFC has developed into a complex, yet highly organized structure capable of managing the extensive network activity.

PFC ANATOMY AND CONNECTIVITY

In primates, the PFC is located in the anterior pole of the frontal cortex and can be divided into three general subregions, the orbital frontal cortex (OFC), the medial prefrontal cortex (mPFC), and the dorsal lateral prefrontal cortex (dlPFC), all of which play a distinct, yet equally important, role in executive

control of behavior (Goldman-Rakic 1988). Unlike brain regions that have distinct cytoarchitectonic borders, the PFC is divided into three regions based on task-specific information obtained and functional connectivity from imaging studies and then further parcellated based on cytoarchitecture (Figure 2).

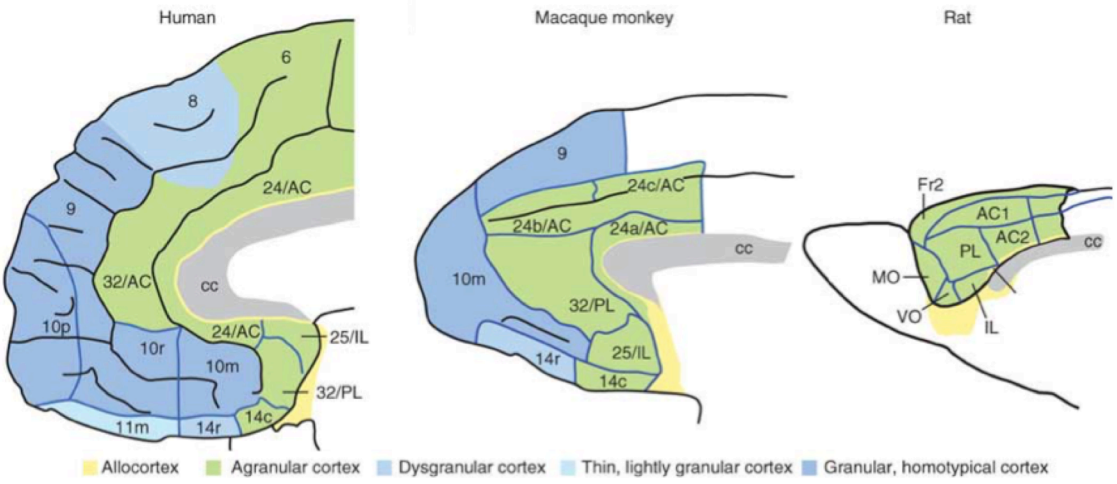

Figure 1-2. Anatomical regions of the human (left), non-human primate (middle) and rodent (right) PFC. Numbers indicate cortical fields in humans and non-human primates. Colors indicate different cortical cytoarchitecture. Abbreviations: AC, anterior cingulate cortex; cc, corpus callosum; IL, infralimbic cortex, MO, medial orbital area; PL, prelimbic cortex; VO, ventral orbital area. Modified from Wallis, 2012

Medial prefrontal cortex

In humans, the mPFC (Brodmann areas 9, 10, 14, 24, 25, 32) is often subdivided into the anterior cingulate cortex (ACC, Brodmann areas 24, 32) and the ventromedial PFC (vmPFC, Brodmann areas 9, 10, 14, 25). This area is involved in early learning and problems solving (particularly error detection and

behavioral flexibility), anticipation, attention, motivation and modulation of emotional responses (Allman et al. 2001; Wallis 2012; Passingham et al. 2013). The mPFC establishes extensive connections with other cortical areas, as well as subcortical regions, namely the amygdala, nucleus accumbens, striatum and hypothalamus. It receives one of the richest dopamine (DA) innervations of any cortical area from the ventral tegmental area (VTA), which is released in the mPFC in response to a reward or reward anticipation (Gaspar et al. 1989; Schultz 1998). It also receives strong inputs from the amygdala, which relays fear related information. In return, the mPFC sends strong glutamatergic projections to the striatum and motor cortex that mediate initiation of behavioral responses (Devinsky et al. 1995). Thus, this region is particularly important in conflict resolution. When the mPFC receives conflicting inputs from subcortical control areas such as the striatum and nucleus accumbens, it is tasked with sorting these inputs and ultimately determining which response to initiate. Inactivation of the mPFC has been shown to impair spontaneous initiation of movements and speech (Cohen et al. 1999). Additionally, individuals with damage to this particular brain region exhibit the inability to suppress externally triggered movements.

Orbital frontal cortex

In humans, the OFC comprises the ventral surface of the PFC (Brodmann areas 10, 11 and 47). The exact function of this subregion in humans is not fully

understood, however it is generally thought of as an important regulator of impulse control and contributing the emotional and affective value associated with cues to assist in the overall decision-making process. Many of the behavioral changes associated with the case of Phineas Gage were later attributed to damage specifically to the OFC. More recent neuroimaging studies suggest that the expected reward value and subjective pleasantness of reinforcers are represented in the OFC (O'Doherty et al. 2000; Gottfried et al. 2003; Kringelbach et al. 2003; Stalnaker et al. 2014). OFC lesions produce significant impairments in response inhibition particularly in the context of changing reinforcement contingencies, social processing, reversal learning, as well as the ability to utilize cues in the environment to predict future rewarding or aversive events (Zald and Andreotti 2010). In addition, the OFC has been shown to be an important component of risky decision-making and gambling. For example, inactivating the OFC causes significant impairments on decision-making tasks such as the Iowa Gambling Task (Iversen and Mishkin 1970; Bechara et al. 1994; Hornak et al. 2004).

The OFC receives inputs from all five sensory modalities: gustatory, olfactory, somatosensory, auditory and visual cortices (Carmichael and Price 1995), and has reciprocal connections with the amygdala, basal ganglia, striatum, hypothalamus, hippocampus and dlPFC (Amaral et al. 1984; Carmichael and Price 1995; Cavada et al. 2000; Ongur and Price 2000). The OFC-amygdala-striatal network is thought to be a particularly important circuit involved in goal-directed and habit-forming behaviors (Kringelbach 2005). Altered

connectivity between the OFC and reward circuitry have been implicated in addiction (Schoenbaum et al. 2006; Schoenbaum et al. 2009; Lucantonio et al. 2012; Jasinska et al. 2014) and impaired OFC function has been shown to relate to both impulsive drug use and the overvaluation placed on drugs of abuse (Levy and Glimcher 2012).

Dorsolateral prefrontal cortex

The dlPFC (Brodmann areas 9 and 46) is thought to be one of the most recently evolved regions of the human brain. It is associated with complicated and high energy demanding behaviors such as planning (i.e. organizing thoughts), decision-making, attention, and motivation (Goldman-Rakic 1995). The dlPFC is a key component of working memory (Barbey et al. 2013), as it actively holds multiple pieces of transitory information "online." For instance, the "what" and "where" information from the temporal and parietal lobes, respectively, is manipulated and organized in the dlPFC to provide a clear picture of an individual's perception of a situation. The dlPFC functions to guide behaviors based on previous experience. Disruption of neurotransmission in this particular subregion has been implicated in several disorders including, but not limited to, schizophrenia (Callicott et al. 2000), depression (Koenigs and Grafman 2009), stress (Qin et al. 2009) and addiction (Kalivas et al. 2005).

Animal correlates of the PFC

The proportion of the brain dedicated to the PFC gradually increases phylogenetically, reaching a maximum in humans. Non-human primates, therefore, are an excellent analogue to humans. They are more genetically and physiologically related to humans than any other species. However, despite the phylogenetic similarities, differences in brain structure and anatomy exist, specifically in the PFC. Comparative imaging and lesion studies in human (injury) and non-human primates (experimental manipulations) provide support for anatomical differences in the human and non-human primate PFC (Wallis 2012). Nonetheless, functionally homologous areas of the non-human primate PFC have been accurately identified based on similar behavioral impairments in the lesioned PFC (Funahashi et al. 1993; Owen 1997; Ploner et al. 1999). Lesions to this area produce very specific cognitive deficits on working memory tasks that differ dramatically from deficits observed with damage to nearby cortical regions (Bechara et al. 1998; Manes et al. 2002; Berlin et al. 2004; Curtis and D'Esposito 2004; Gomez-Beldarrain et al. 2004). The inability to manipulate cortical function, or to closely examine cortical activity (as with electrophysiology) in humans has spawned research directed at modeling the human condition in animals.

Although a strong correlation exists between the human and non-human primate PFC, significant anatomical differences exist between the rodent PFC and the primate PFC, and similarities between primate and rodent PFC remain an area of debate. Much of the discussion stems from differing cytoarchitecture between rodents and primates, however functional similarities certainly exist

between species. As discussed above, analogous versions of several PFC-dependent tasks have been developed to test executive function in rodents. Lesions to specific areas of the rodent PFC produce very similar impairments on working memory tasks compared to those observed in humans, further validating the use of rodents as translational models to study PFC function (Delatour and Gisquet-Verrier 1999; Ragozzino et al. 1999; Delatour and Gisquet-Verrier 2000; Dias and Aggleton 2000; Chudasama et al. 2003; Uylings et al. 2003; Floresco et al. 2008; Seamans et al. 2008).

The commonalities of functional connectivity that exist between rodents and primates support the use of rodent models to study human behavior. The presence of dense reciprocal projections between the PFC and mediodorsal nucleus (MD) of the thalamus is considered a major defining structural feature of the PFC that is applicable to all mammalian species (Fuster 2008). The MD thalamus receives inputs from several cortical and subcortical regions, including the hypothalamus, striatum, limbic system and cerebellum. It organizes the incoming neural processes and efficiently relays information to the cortex (Kolb 1984; Uylings and van Eden 1990; Uylings et al. 2003). These thalamocortical projections are essential for executive function in every mammalian species. In addition, the PFC receives direct inputs from subcortical structures, such as the hippocampus, amygdala and striatum. It also receives dense modulatory inputs from the main nuclei of origin of the major forebrain cholinergic and monoaminergic neurotransmitter systems such as DA, norepinephrine, serotonin and acetylcholine (Daviss and Lewis 1995; Groenewegen et al. 1997; Robbins

2000; Dalley et al. 2004; Arnsten 2011). These projections are essential for emotive behaviors, learning and memory, and the execution of movement in rodents and humans.

The rodent PFC is subdivided into four main regions with each regulating very distinct functions such as working memory, goal-directed behavior, and attention (Goldman-Rakic 1987; Kolb 1990; Goldman-Rakic 1994). The existence of a rodent correlate to the dlPFC is still a topic of debate, however many researchers believe the dlPFC is a product of evolution to accommodate for higher cortical demand. Others have argued that the dlPFC is homologous with the certain areas of the ACC (Passingham et al. 2013). The OFC is much more conserved in rodents, as it exhibits functional and anatomical similarities with primates (Uylings et al. 2003; Wallis 2012). The rodent mPFC can be further subdivided into two regions, the infralimbic (IL-C) and prelimbic prefrontal cortex (PrL-C). Both regions share many similarities in their projections to various limbic brain regions and generally are associated with diverse emotional, cognitive and mnemonic processes (Sesack et al. 1989; Takagishi and Chiba 1991). However, more recent studies suggest a functional dichotomy between the two areas (Hoover and Vertes 2007). The IL-C has been implicated in inhibitory control and habit responding, while the PrL-C is involved in initiation of behavior and goal-directed learning (Coutureau and Killcross 2003; Vidal-Gonzalez et al. 2006; Quirk and Mueller 2008). Lesions to the entire mPFC produce cognitive deficits that are similar to those produced by lesions to the ACC in primates (Goldman-Rakic 1987,1994; Floresco et al. 1997; Groenewegen et al. 1997; Seamans et al.

2008). However, a more recent study by Oualian et al examined the effect of specific IL-C and PrL-C lesions on behavioral flexibility in rats (Oualian and Gisquet-Verrier 2010). This study supported the notion that as a whole, the mPFC is involved in strategy switching, however specific IL-C lesions produced difficulties in using a previously non-rewarding strategy. Once rats selected the correct strategy, the lesion no longer affected the performance. On the other hand, PrL-C lesions did not affect the shift from one strategy to another, but rather it affected the rat's ability to select and maintain the use of the new strategy, particularly when rats had previously learned that this strategy was ineffective. This study exemplifies the individual contributions of the IL-C and PrL-C to cognitive function.

PFC CYTOARCHITECTURE

The PFC is organized into highly specific layers that provide the ideal environment for both inter-cortical communication and coordination with numerous other brain regions. These organized layers, defined by distinct cytoarchitectonic borders and reciprocal anatomical connections with the mediodorsal thalamus, lead to the eventual success of comparative mapping of the PFC between humans, non-human primates, and rodents. In the human PFC, all six layers can be identified with relative ease based on inputs/outputs and cellular composition. The non-human primate PFC is organized in much the same way as humans, with slight differences in size and granulation of the

layers. The rodent PFC contains very organized cellular layers, however it lacks a granule cell layer (Layer IV) (Wallis 2012). The lack of a granule cell layer in the PFC is one of the defining features distinguishing the primates PFC from other mammals.

The PFC contains two major types of cells, pyramidal cells and interneurons (Figure 1-3) (Houser et al. 1983; DeFelipe and Farinas 1992). The majority of cells in the PFC are excitatory pyramidal cells that release glutamate and form local connections with other cortical cells, but also send projections to subcortical regions. Pyramidal neurons in the PFC share similar anatomical, physiological and molecular properties. Earlier studies sub-divided pyramidal neurons into two morphological classes. One type of pyramidal neuron is larger in size and exhibits axons with thick apical dendrites, while the other is smaller and has less apical branching (Lewis et al. 2002). However, advancements in immunohistochemistry, retrograde tracing and *in vivo* electrophysiology have lead to the identification of numerous specialized subtypes of pyramidal neurons based on their specific target region (Dembrow et al. 2010), physiological properties (Zaitsev et al. 2012; Ardid et al. 2015), and neurotransmitter characteristics (Gee et al. 2012). Pyramidal neuron activity is critical for neural network synchronization and behavioral output. For instance, age related changes in working memory performance have been directly linked to age-related changes in intrinsic membrane properties of pyramidal neurons in the primate PFC (Luebke and Amatrudo 2012), thus highlighting a distinct role for pyramidal neurons in neuronal communication and behavioral output.

The remaining neurons in the PFC are inhibitory interneurons that release GABA and only form local connections within the cortex and thus support local inhibitory functions that enhance saliency and contrast of the excitatory patterns of pyramidal neurons (DeFelipe 1997; Tamas et al. 1998). Interneurons as a whole share many common features. Most GABAergic interneurons contain aspiny dendrites, can receive inhibitory and excitatory inputs, and can selectively target different subdomains of neurons (i.e. dendritic regions, soma or axon) to control activity (Markram et al. 2004). The diversity of interneurons make it difficult to generalize GABAergic interneurons into different classes. In the cortex, a common way to divide interneurons is based on where they synapse onto pyramidal neurons (DeFelipe 1993; Tamminga et al. 2004; Huang et al. 2007). Using this approach, interneurons can be divided into 3 classes. One type of interneuron, chandelier cells, synapses to the axon initial segments of neighboring pyramidal neurons that they presumably regulate. These neurons generally express the calcium-binding protein parvalbumin and display fast-spiking properties that allow them to exert a powerful influence over pyramidal cell activity and output (Williams and Stuart 2003). A second type of interneuron, basket cells, target the soma and proximal dendrites of pyramidal neurons (Miles et al. 1996). Basket cells can adjust the gain of the integrated synaptic response and have been implicated in synchronizing the firing of neuronal populations. The third class of interneurons generally targets dendrites. There are several subtypes of dendrite targeting interneurons including neurogliaform cells, Martinotti cells, and double bouquet cells (Markram et al. 2004; Kubota et al.

2007). These neurons can regulate dendritic integration, the back propagation of sodium spikes and the generations of dendritic calcium spikes. A general schematic of interneurons in the cortex is depicted in Figure 1-3. Several different subclasses of interneurons exist within these three categories based on characteristics such as morphology, electrical properties, ion channel composition, and molecular properties (for review see (Markram et al. 2004). Interneuron diversity is crucial for providing sufficient sensitivity, complexity and dynamic range for the inhibitory system to match excitation regardless of the intensity and complexity of the stimulus. Within the PFC, maintenance of information following the removal of a sensory stimulus requires persistent excitation of pyramidal neurons and fine-tuning by interneurons (Goldman-Rakic 1996; Wang et al. 2013). For example, inputs from a diverse group of GABAergic interneurons onto somatodendritic compartments of pyramidal neurons refine spatial and temporal specificity in this system (Goldman-Rakic 1995; Zaitsev et al. 2009). Thus, it is the excitatory/inhibitory tone that gives the PFC the ability to hold information “online” while a task is being performed. Persistent network activity between pyramidal neurons and inhibitory modulation from interneurons has been strongly considered to be the cellular correlate of working memory (Goldman-Rakic 1995).

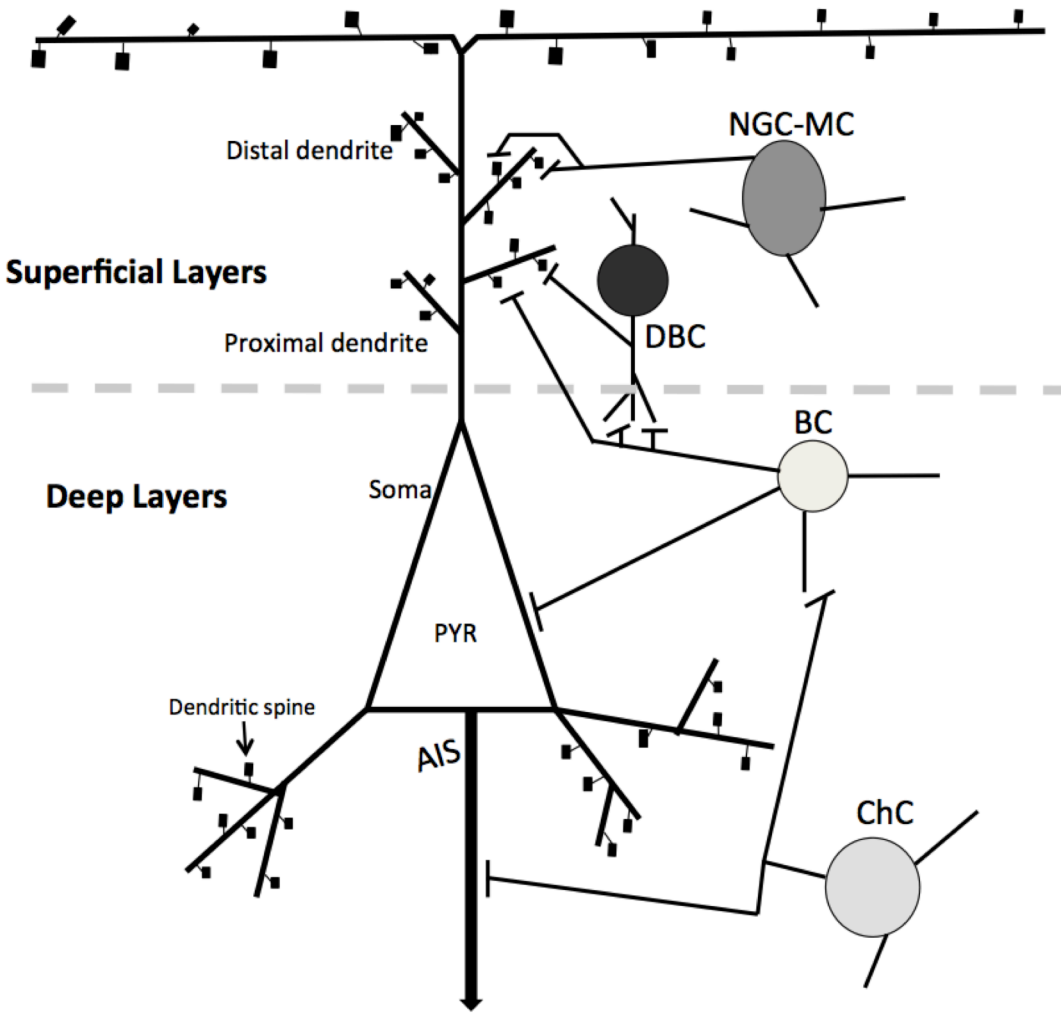

Figure 1-3. Schematic of interneuron modulation of cortical pyramidal neurons in the PFC. The subcellular organization of different classes of GABAergic inhibitory inputs allows for effective regulation of synaptic integration, spike generation, back propagation and plasticity. Interneurons can synapse at the soma, axon initial segment (AIS) or dendrites to exert inhibitory control over inputs to, and outputs from, pyramidal neurons. Abbreviations: AIS, axon initial segment; BC, basket cell; ChC, chandelier cells; DBC, double bouquet cell; NGC-MC, neurogliaform-Martinotti cell; Pyr, pyramidal neuron. Adapted from (Huang et al. 2007).

GABA SIGNALING

GABA is the main inhibitory neurotransmitter in the brain, as it activates anion channels to reduce neuronal activity (Kuffler and Edwards 1958; Krnjevic and Schwartz 1967). GABA is synthesized from glutamate as a substrate of the enzyme glutamic acid decarboxylase and taken up by vesicular GABA transporters, which are embedded in presynaptic vesicular membranes. GABA is then released in the synaptic cleft where it acts on either ionic or metabotropic GABA receptors to facilitate the flow of anions. The removal of GABA from the synaptic cleft involves uptake via GABA transporters and subsequent catabolism via GABA transaminase and succinate semialdehyde dehydrogenase, which convert GABA into intermediates of the Krebs cycle and substrates for new production of glutamate.

As highlighted in the previous section GABAergic interneurons exert an inhibitory influence over glutamatergic signaling, shunting neuronal communication. GABAergic neurotransmission is important for the generation and synchronization of rhythmic activity between neural networks in disparate brain regions such as the PFC and hippocampus (Cobb et al. 1995; Wang and Buzsaki 1996; Howard et al. 2003; Sohal et al. 2009; Uhlhaas et al. 2010). One GABAergic interneuron can synapse onto hundreds of glutamatergic projection neurons, carefully and accurately orchestrating the activity of each neuron (Chandler 2003; Markram et al. 2004; Ascoli et al. 2008; Abernathy et al. 2010). These GABA interneurons precisely adjust the timing and amplitude of the signal to provide optimal synchronization. Synchronized networks represent a means of

encoding information that is independent of firing rate, and can be recognized and interpreted by downstream coincidence detectors (Ainsworth et al. 2012). Small changes in any facet of GABA inhibition can critically alter the way information is transmitted through these cortical networks.

A particularly interesting aspect of GABAergic neurotransmission is that it can function in both phasic and tonic modes (Farrant and Nusser 2005). While both forms of neurotransmission share the same ultimate goal of neuronal inhibition, the distinct mechanisms by which they exert inhibition results in profound differences in control of neural network activity.

Phasic GABAergic neurotransmission

Phasic GABA transmission takes place in a conventional manner; an influx of calcium triggers release of GABA-containing vesicles, releasing thousands of GABA molecules into the synaptic cleft. Opposite of the release site, low-affinity $GABA_A$ receptors are clustered, awaiting activation through binding of the recently released GABA. Once two GABA molecules bind to the receptor, the ion channel is activated and chloride influx occurs, resulting in an inhibitory postsynaptic current (IPSC). This is a transient (i.e. phasic) response. The high concentration of GABA in the synapse quickly dissipates, therefore the receptor is open for only a short period of time before it is deactivated and GABA diffuses away from the synaptic cleft or is taken up through GABA transporters (Mody et al. 1994; Nusser et al. 1997; Cherubini 2012). Diffusion rate and GABA

transporter efficiency are the main variables that dictate receptor occupancy and in turn, activity.

Tonic GABAergic neurotransmission

Tonic GABA currents were first identified in rat cerebellar granule cells after application of the $GABA_A$ receptor antagonists bicuculline and SR-95531 not only blocked spontaneous activity, but also resulted in a decrease in the current needed to hold the cell near its resting membrane potential (Kaneda et al. 1995). Tonic currents had been observed with other forms of neurotransmission, most notably tonic acetylcholine at the neuromuscular junction (Katz and Miledi 1977). The observation of a tonic GABA current in the brain provided the intriguing possibility that, compared to phasic GABA currents, tonic GABA currents might have an equally important, yet distinct role in neuronal inhibition. Neural network synchronization through gamma oscillations is thought to be mediated by phasic GABA neurotransmission, however, tonic activation of $GABA_A$ receptors is now known to play a more direct role in controlling network activity through a process akin to volume neurotransmission (Mann and Mody 2010). Compared to synaptic receptors, extrasynaptic $GABA_A$ receptors have a much higher affinity for GABA and thus can be tonically activated by ambient levels of GABA in the extrasynaptic space. Tonic GABA currents are responsible for a persistent increase in input conductance that, in essence, dampens the excitatory post-synaptic current (EPSC) and decreases the probability of action potential

generation. As opposed to phasic currents, tonic currents efficiently shunt several synapses at once, thereby dampening signals on a much larger scale than phasic currents.

Tonic GABA currents have been observed in several brain regions including the cerebellum (Brickley and Mody 2012), hippocampus (Stell et al. 2003; Fleming et al. 2007), thalamus (Cope et al. 2005), amygdala (Herman et al. 2013), striatum (Santhakumar et al. 2010) and cortex (Nishikawa et al. 2011). The presence of this current in multiple brain regions suggests the tonic current is an important regulator of neural network activity within and between brain regions. The source of ambient GABA is still a topic of debate. Some studies suggest that the tonic current arises primarily from spillover of GABA from the synapse (Wall and Usowicz 1997; Herman et al. 2013), while others suggest there is evidence that non-vesicular GABA (e.g. from astroglia) may also contribute to ambient levels of extrasynaptic GABA (Barbour and Hausser 1997; Kullmann 2000; Telgkamp et al. 2004; Lee et al. 2010). In sum, evidence from multiple studies suggests that the source of tonic current varies widely between brain region and even between cell types.

Specific GABA receptors regulate tonic and phasic currents.

There are two principal types of GABA receptors in the brain, $GABA_A$ and $GABA_B$, that are differentiated based on structure and signaling properties. Metabotropic $GABA_B$ receptors are commonly located presynaptically to function

as autoreceptors that modulate GABA release through G-protein mediated regulation of cell excitability (Bowery et al. 2002). Their associated G-proteins are also coupled to Ca^{2+} and K^{+} channels, which allow them to regulate the activity levels of the cells on which they are expressed. $GABA_B$ receptors are G_i-protein coupled receptors therefore they inhibit adenylate cyclase activity by decreasing the production of cAMP from ATP, which in turn, results in decreased activity of cAMP-dependent protein kinase. $GABA_B$ expression in the brain is widespread and somewhat convoluted, with both presynaptic and postsynaptic components resulting in complex, brain region specific regulation of GABA neurotransmission.

To date, 19 ionotropic heteropentameric $GABA_A$ receptor subunits have been cloned in the mammalian CNS. These receptors are made up of any of the following subunit classes organized into multiple different combinations: α, β, γ, Σ, ε, π, δ, and ρ. Additional variants from alternative splicing have also been identified (Hevers and Luddens 1998; Farrant and Nusser 2005)]. The pentameric organization of $GABA_A$ receptors can vary, but the most common $GABA_A$ receptor stoichiometry is $2\alpha{:}2\beta{:}1\gamma$ or δ subunit. The expression patterns and subunit combinations vary widely between brain regions and even between cell types within a brain region. Studies examining mRNA expression of different $GABA_A$ receptor subunits have comprehensively characterized expression levels throughout the rat brain (Laurie et al. 1992).

$GABA_A$-ρ receptors are a subclass of $GABA_A$ receptor containing only the ρ subunit. (Olsen and Sieghart). $GABA_A$-ρ receptors form pentameric ligand gated ion channels that contain any combination of the three different ρ subunits (ρ1/2/3) and to form homo-pentamers or hetero-pentamers with one other ρ subunit. These subunit combinations give $GABA_A$-ρ receptors a unique pharmacological profile. Typical $GABA_A$ receptor modulators such as neurosteroids, anesthetics, barbiturates or benzodiazepines do not bind to these receptors. Because of this, $GABA_A$-ρ receptors are sometimes considered an entirely separate class of $GABA_A$ receptors. In the CNS, these receptors are expressed primarily in the retina and other brain regions involved in the visual pathway. While the exact role of these receptors is unclear, studies have suggested that alterations in $GABA_A$-ρ receptors may underlie autosomal recessive retinitis pigmentosa (Marcos et al. 2000). More recent studies have identified small amount of $GABA_A$-ρ in the hippocampus, cerebellum and amygdala, although the role these receptors play in GABAergic neurotransmission in these brain regions, if any, is relatively unclear (Martinez-Delgado et al. 2010).

The regulated expression and localization of $GABA_A$ receptors in the membrane is critical to both tonic and phasic $GABA_A$ receptor-mediated neurotransmission. The membrane localization of the heteromeric receptor is highly dependent upon its subunit composition. Synaptic $GABA_A$ receptors generate the rapidly desensitizing phasic GABA currents. Again, the subunit

combination of these receptors varies between brain regions, but in general the stoichiometry for a synaptic $GABA_A$ receptor is $2\alpha{:}2\beta{:}1\gamma$. The two α-subunits are typically $\alpha1$, $\alpha2$ or $\alpha3$. These receptors have a low affinity for GABA and are therefore activated by large action potential dependent release of presynaptic GABA into the synaptic cleft. Furthermore, several $GABA_A$ receptor subunits possess distinct sIPSC kinetics (Okada et al. 2000; Bacci et al. 2003). For example, the $\alpha1$ subunit is associated with faster sIPSC whereas the $\alpha2$ and $\gamma2$ subunits produce slower decay times (Dixon et al. 2014).

In contrast, extrasynaptic $GABA_A$ receptors generate the non-desensitizing tonic current. The general conformation of extrasynaptic $GABA_A$ receptor is $\alpha_{4/6}\beta_x\delta$ or $\alpha_5\beta_x\gamma_2$ (Moss and Smart 2001). The different mechanisms and properties regulating synaptic versus extrasynaptic localization remains relatively unclear, however the presence of the δ-subunit and $\alpha5$-subunit are unique to extrasynaptic $GABA_A$ receptors. These two subunits are exclusively involved in mediating the tonic GABA current (Nishikawa et al. 2011). Research also suggests that the presence of the $\gamma2$ subunit likely promotes synaptic localization (Jacob et al. 2005; Thomas et al. 2005; Tretter and Moss 2008; Petrini et al. 2014). $\gamma2$ has been shown to interact with the $GABA_A$ receptor anchoring protein gephryin, which is exclusively expressed at GABA synapses (Kneussel and Loebrich 2007). However, when $\gamma2$ is coupled with an $\alpha5$ subunit, $\gamma2$-driven synaptic localization appears to be superseded, and those receptors localize exclusively extrasynaptic. Why this occurs is not known, however evidence

suggests that the protein radixin, which is found exclusively outside of the synapse and interacts with the α5 subunit, may play an important role in preventing migration to the synapse and anchoring $GABA_A$ receptors to the perisynaptic and extrasynaptic space (Loebrich et al. 2006).

$GABA_A$ receptors containing the δ subunit exhibit the highest affinity for GABA (EC_{50} in the nM range), suggesting that this subunit is likely the main driving force behind producing tonic GABA currents in many brain regions (Zheleznova et al. 2009). Studies of tonic GABA current in mice with a genetic deletion of the δ subunit or pharmacologically targeting the δ subunit demonstrate a necessity of this subunit to generate tonic current (Wei et al. 2003; Cope et al. 2005; Farrant and Nusser 2005; Drasbek and Jensen 2006; Santhakumar et al. 2010; Herman et al. 2013). In addition to α, γ and δ subunits, β subunits appear to play a unique role in regulating GABA currents. Beta subunits are particularly important for proper functioning and expression of both synaptic and extrasynaptic receptors. Beta subunits appear to be sensitive to modulation by intracellular kinase pathways involving PKA, PKC and possibly PLC. PKA mediated phosphorylation of the β1 and β3 subunits of $GABA_A$ receptors reduce and increase, respectively, channel conductance without altering surface expression, while β2 phosphorylation appears to be more important in regulating surface expression via PKA activation (McDonald et al. 1998; Brandon et al. 2002). Taken together, the diversity in subunit combination and localization of

ionotropic GABA receptors allows for precise inhibitory modulation of multiple cell types in multiple brain regions.

While distinct classes of synaptic and extrasynaptic GABA receptors have been well characterized, researchers are still just beginning to understand the role these receptors play in modulating GABA currents. Even minor alterations to a specific $GABA_A$ receptor subunit can have an attenuating effect on GABA-mediated neurotransmission. The most notable class of drugs that bind to $GABA_A$ receptors and strongly influence GABA currents are the benzodiazepines. Benzodiazepines do not bind to the same site as GABA (between α and β) but rather this class of drugs has a separate binding site between the α and γ subunit (Sigel and Buhr 1997). The specific effect of benzodiazepines depends on which α subunit it binds. For example, $GABA_A$ receptors that contain the $\alpha 1\beta\gamma_x$ subunit mediate the sedative-hypnotic effects of benzodiazepines, whereas the anxiolytic effects of benzodiazepines arise from the presence of the $\alpha 2\beta\gamma_x$, $\alpha 3\beta\gamma_x$ and $\alpha 5\beta\gamma_x$ subunits. In contrast, $GABA_A$ receptors that contain an $\alpha 4$ or $\alpha 6$ subunit are insensitive to benzodiazepines due to the absence of the γ subunit (Rudolph et al. 1999; Low et al. 2000; McKernan et al. 2000; Follesa et al. 2004; Yu et al. 2006). In addition to high potency, low rate of desensitization, and low efficacy to GABA, δ-$GABA_A$ receptors exhibit very low sensitivity to benzodiazepines and are highly sensitive to low concentrations of alcohol (Mohler 2006), further underscoring the unique pharmacological profile of these receptors. δ-$GABA_A$ receptors are powerfully modulated by neurosteroids such as allopregnanolone

and pregnanolone (Kuver et al. 2012). Fluctuations in endogenous steroid levels have been observed during pregnancy, the ovarian cycle and prolonged periods of stress (Herd et al. 2007). These changes dramatically alter expression levels of δ-$GABA_A$ receptors, and consequently tonic GABA currents (Maguire et al. 2005).

DOPAMINE SIGNALING

Dopaminergic neurons produce the modulatory neurotransmitter DA from the amino acid tyrosine. Tyrosine is converted to L-DOPA by the enzyme tyrosine hydroxylase (TH), and L-DOPA is then further converted to DA by the enzyme aromatic L-amino acid decarboxylase (AADC). DA is a unique neurotransmitter in that it is a precursor for the synthesis of two other monoamine neurotransmitters norepinephrine and epinephrine. DA neurons are localized to two main areas of the brain: the substantia nigra pars compacta (SN), and the ventral tegmental area (VTA). Three major pathways arise from these terminals: DA neurons originating in the SN project to the striatal brain regions (nigrostriatal DA system), VTA and SN DA neurons project to the limbic brain regions (mesolimbic DA system) and DA projections from the VTA and SN to the cortical areas of the brain (mesocortical DA system) (Thierry, Blanc, et al. 1973; Thierry, Stinus, et al. 1973). DA is conventionally released into the synaptic cleft. Postsynaptic targets of DA neurons contain different types of DA receptors that can have varying modulatory effects on neuronal activity. There are five types of

DA receptors in the brain divided into two families based on similar pharmacological profiles: D1-like receptors (D1, and D5) and D2-like receptors (D2, D3 and D4) (Brown and Makman 1972; Greengard et al. 1972; Kebabian et al. 1972). All DA receptors are metabotropic, but the two families of receptors are distinguishable by different metabotropic pathways. D1-like receptors are coupled to the G protein G_s or G_q, which activates adenylyl cyclase (AC) thereby increasing the intracellular concentration of the second messenger cyclic AMP and stimulating calcium channel activity and inhibiting potassium channels. In contrast, D2-like receptors signal through the G protein G_i or G_o signaling pathway, which inhibits AC and results in an inhibitory effect on downstream signaling through decreased calcium channel activity and increased potassium channel activity (Ohara et al. 1988). The remaining DA in the synaptic cleft is taken up by monoamine transporters such as the DA transporter (DAT) and norepinephrine transporter (NET) or degraded by the enzymes monoamine oxidase (MAO) or catechol-O-methyl transferase (COMT).

Expression levels of DA receptors vary widely between brain regions, but in general the D1 and D2 receptors are more highly expressed in the CNS relative to the other DA receptors subtypes (Weiner et al. 1991; Levey et al. 1993). Other DA receptor subtypes appear to have more restricted expression patterns. For example, mRNA encoding D5 receptors, appears to be restricted to specific thalamic and hypothalamic nuclei, cells of the hippocampus and the cortex (Khan et al. 2000). In contrast, D3 mRNA expression is highest in the olfactory bulb and the nucleus accumbens, while D4 mRNA is expressed mainly

in the frontal cortex and brain stem (Meador-Woodruff et al. 1989; Bouthenet et al. 1991). In the PFC, autoradiographical and immunoblot experiments have identified mRNA expression of all 5 DA receptor subtypes (Laurier et al. 1994; Meador-Woodruff et al. 1996; Lidow et al. 1998; Tarazi and Baldessarini 2000). However, the relative expression of each subunit is still a topic of debate. A more recent study examined layer specific, and cell-type specific differences in D1/D2 expression in the mPFC (Santana et al. 2009). This study demonstrates that D1 mRNA expression is greater than D2 mRNA expression in the mPFC, however layers II/III and VI drive this difference. In layer V of the mPFC, the percentage of neurons expressing D1 or D2 receptors is equal (Santana et al. 2009). Further highlighting the detailed influence of DA on PFC neurotransmission, specific DA receptors within Layer V of the mPFC exclusively localize to different populations of interneurons and pyramidal neurons (de Almeida and Mengod 2010; Gee et al. 2012). This may play an important role in maintaining the optimal balance of D1/D2 receptor activity necessary for specific cognitive functions (Floresco 2013).

Together with GABAergic interneurons, DA inputs into the PFC are responsible for stabilizing and modulating neural network activity. Manipulation of DA transmission in the PFC leads to changes in working memory and executive function via dopaminergic effects on synaptic glutamate and GABA neurotransmission (Wang et al. 2002; Wang et al. 2003; Zhang et al. 2004; de Almeida and Mengod 2010; Yuen et al. 2010; Yuen and Yan 2011; Gee et al. 2012). As aforementioned, the mesocortical DA system projects from the VTA, in

large part to different areas of the PFC. A major portion of DA projections from the VTA go specifically to the dlPFC, thus influencing dlPFC-dependent behaviors like working memory and behavioral flexibility (Floresco and Magyar 2006). The influence of DA depends on which receptors, D1-like or D2-like, are present on the postsynaptic neurons. Proper cognitive functioning relies on an optimal balance of activation of both D1- and D2-like receptors (Arnsten et al. 1994; Gao and Goldman-Rakic 2003; Galvan et al. 2006). Specifically, it has long been proposed that PFC D1 and D2 receptor modulation of working memory follows an “inverted U” shaped curve (Arnsten 1997; Zahrt et al. 1997). In this model, D1 receptor activation favors focus and attention, while activation of D2 receptors favors higher flexibility of the PFC network. This notion, while highly relevant to working memory, does not take into account other PFC dependent tasks such as behavioral flexibility or decision-making. This theory has recently been expanded to suggest that optimal levels of D1 and D2 modulation are task dependent, and that not all tasks follow an inverted U shaped curve (for review see (Floresco 2013)). It is widely accepted that an optimal concentration of DA is required to perform PFC-dependent behaviors. The dramatic effect of variations from optimal DA receptor modulation has long been a focus of research on several disorders that effect cognition such as schizophrenia, ADHD and depression.

DEVELOPMENT OF THE PFC

Adolescence is often characterized as a transitional period during which there is increased risk-taking, impulsive decision-making and overall drive for independence and social acceptance. The adolescent brain undergoes widespread changes in form and function, both within individual regions and in the connections between them. Throughout adolescence, the brain continues to undergo critical maturational changes that occur in a caudal to anterior direction, which allows for greater plasticity of the cortex during development. A simple study involving toddlers highlights the impulsivity and lack of cognitive control in early development (Mischel et al. 1989). In this highly replicated study, 4 year olds were tested to see how long they could resist immediate reward (a cookie) in favor of a larger, delayed reward (two cookies). Although variable, subjects had difficulties resisting the smaller, immediate reward in favor of the larger, delayed reward. This study highlights the sensation-seeking behaviors, lack of impulse control, and an overall hypersensitive reward system in early development. Interestingly, the individual outcome of this study was predictive of cognitive and social competency in adolescence in these subjects (as measured by scholastic performance) and in a separate study (Davidson et al. 2006). Cognitive control gradually improves with age. In late childhood/early adolescence, motivational cues of potential reward become particularly salient and can lead to active suppression of cognitive control. Sensation seeking and risky/impulsive behaviors generally increase as adolescence progresses,

peaking during middle/late adolescence before slowly declining during the latter stages of adolescence and into adulthood (Steinberg 2005).

There are several theories that attempt to explain why adolescents exhibit risky and impulsive behaviors during this particular window of development. One well-accepted theory argues that differential development of subcortical structures relative to the PFC leads to subcortically driven behaviors during adolescence (Figure 1-4) (Somerville and Casey 2010). Cognitive control greatly improves throughout adolescence, and in the fully developed adult, the PFC exerts top-down modulation of the subcortical systems (Davidson et al. 2006; Hare et al. 2008). The PFC exhibits both prolonged anatomical and cognitive development, and as such, the underdeveloped PFC may contribute to several adolescent behavioral phenotypes. Imaging (Sowell et al. 2003; Gogtay et al. 2004; Galvan et al. 2006), as well as postmortem studies (Huttenlocher 1979; Rakic et al. 1994) have suggested that greater cognitive control is positively correlated with structural and functional development. A recent report compared results from two separate studies, one examining self-reported measures of impulsivity and another measuring fMRI activity throughout adolescence and into adulthood. Collectively, these studies demonstrate that impulsivity and fMRI activity both gradually decline at the same rate as age increases (Casey and Jones 2010), supporting a relationship between functional PFC development and maturation of adolescent behaviors. On the structural level, studies have shown that a reduction in cortical gray matter begins in preadolescence and continues into the mid-20s, possibly reflecting a normal pruning process (Blakemore and

Robbins 2012). Furthermore, the number of excitatory synapses declines greatly via experience-dependent pruning of dendritic spines during adolescence, which likely accounts for the decline in grey matter (Petanjek et al. 2011). This refinement occurs in conjunction with the fine-tuning and maturation of PFC-dependent behaviors. Equally compelling are data showing that white matter volume increases over the course of adolescence, presumably reflecting connectivity changes, including axonal extension and myelination.

Adolescent behavioral phenotypes exist in most mammalian species and are considered evolutionarily advantageous. These changes contribute to the ultimate goal of species reproduction, while avoiding genetic inbreeding. During adolescence, individuals become attracted to novelty and manifest a desire to move away from the safe familial nest, whereas at the same time, acceptance by social peers becomes an essential determinant of behavior. These two factors collectively drive risky, rebellious, impulsive and novel seeking behaviors. The plasticity of the adolescent PFC allows it to undergo experience-dependent modifications that are critical to its development. However, because of the extensive maturational changes, the developing adolescent brain may be especially vulnerable to the deleterious effects of exogenous agents, including drugs and alcohol, both of which are commonly abused during this critical period of development. In addition, many disorders that have a neurodevelopmental component can arise from alterations in the maturation and refinement of the circuitry the PFC during this critical period of development and lead to persistent impairments in cognitive control (Spear 2000; Uhlhaas et al. 2010; Uhlhaas and

Singer 2010). Both the environmental and behavioral experiences during this early period of synaptic production can alter the developmental trajectory of the PFC and shape adult behaviors. The ability to identify individuals prone to neurodevelopmental disorders or risky, impulsive decision-making is an important goal of developmental neuroscience research. Understanding the normal development of neurotransmitter systems in the PFC and the impact of exogenous insults on PFC development will provide clues that may allow for earlier pharmacological or behavioral intervention to curb improper PFC development.

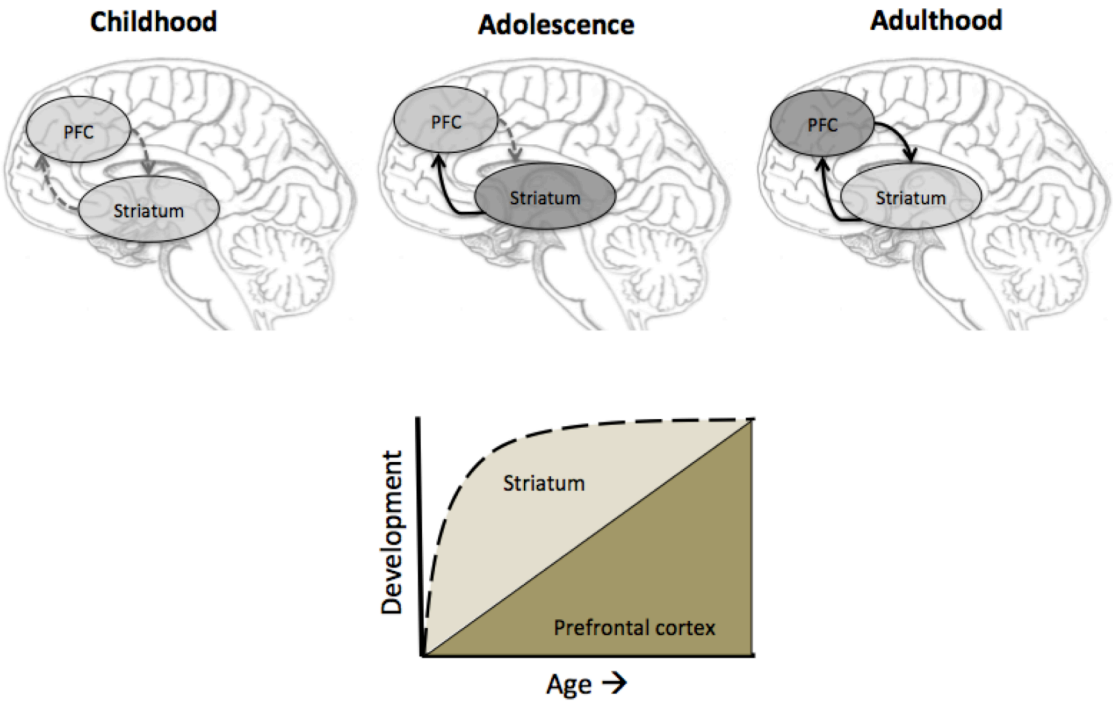

Figure 1-4. Development of the PFC relative to the striatum. The striatum is almost completely developed by the onset of adolescence and therefore influences the underdeveloped PFC. Once the PFC is fully developed in adulthood, the PFC exert top-down inhibitory control over striatum. The net result is striatal driven behaviors during adolescence, and PFC driven behaviors during adulthood. Adapted from Somerville and Casey, 2011.

$GABA_A$ receptor development

Early in development (before postnatal day 7), GABA is depolarizing and mostly excitatory due to the high intracellular chloride concentration. It plays a key role in regulating a number of processes including migration, morphological

maturation and differentiation (Ben-Ari et al. 2007; Wang and Kriegstein 2009). Recent studies have measured the mRNA expression of $GABA_A$ receptor subunits underlying GABAergic neurotransmission in the dlPFC at different developmental time points from early childhood to adulthood (Hoftman and Lewis 2011). Protein markers for GABA neurons that specifically synapse at the AIS of pyramidal neurons revealed that the maturation of these synapses progressed well into adulthood. This study further demonstrated that pyramidal neuron AIS immunoreactive for GABA-associated protein markers undergo substantial changes in relative density, laminar distribution, and/or length from early childhood to adulthood in macaque monkeys. More recent findings suggest that mRNA expression of the α1, α2, α4, and δ subunits in the dlPFC dramatically changes from birth to adulthood in macaque monkeys (Hoftman and Lewis 2011). In all of these subunits, a majority of the change in expression occurs during childhood and adolescence (3 months to 3 years). For example, in adolescence α1 and δ subunit expression increases and reaches maximal levels of mRNA expression in early adulthood (Figure 1-5). In contrast, α2 and α4 subunits exhibit maximal mRNA levels at birth followed by a steady decline throughout adolescence and reach stable levels in early adulthood. Furthermore, Datta et al used precise laser microdissections and PCR to identify varying $GABA_A$ receptor subunit developmental patterns between mPFC layers in macaque monkeys (Datta et al. 2014). In this study, an increase in expression of the δ-$GABA_A$ receptor in layer V of the mPFC was observed throughout postnatal development, with a particularly significant increase occurring during

adolescence. Interestingly, there was no evidence of a change in δ-$GABA_A$ receptor mRNA layer III expression, highlighting the unique, layer-specific changes in δ-$GABA_A$ receptor expression during adolescence. The extensive developmental changes occurring in PFC $GABA_A$ receptor subunit expression during adolescence implicate these receptors as particularly vulnerable to environmental insult during adolescence. Alteration in the GABA system during adolescence could impact its developmental trajectory. For example, repeated cocaine use during adolescence has been shown to elicit a state of mPFC disinhibition resulting from a functional impairment of the local prefrontal GABAergic network that endures through adulthood (Cass et al. 2013). Drug and alcohol abuse during adolescence is a major social issue and studies such as this support the notion that insult to the developing PFC can result in persistent, long-lasting changes to neurotransmission in the adult PFC.

Development of the DA system

PFC function relies heavily on the balance of excitatory and inhibitory neurotransmission, and as outlined above, many aspects of this circuitry are rapidly developing during adolescence. Growing evidence suggests that DA modulates the neural networks responsible for many PFC-dependent behaviors (Andersen et al. 2000; Costa 2007; Brenhouse et al. 2008). Midbrain DA neurons begin to develop and migrate through the striatum to cortical regions early in gestation (Olson and Seiger 1972). The developmental increase of cortical DA

innervation continues until ~PD60 in rats, at which point the density and topography of dopaminergic neurons begins to stabilize (Kalsbeek et al. 1988). Other components of dopaminergic neurotransmission including DAT, COMT, DA receptors D1, D2 and D4, and whole brain tissue DA levels all undergo similar developmental profiles (Figure 1-5), progressively emerging and increasing expression levels throughout the majority of adolescence before undergoing significant refinement and not fully stabilizing until adulthood (Noisin and Thomas 1988; Tarazi et al. 1998; Andersen et al. 2000; Moll et al. 2000; Tarazi and Baldessarini 2000; Tunbridge et al. 2007). Moreover, the developmental patterns of DA innervations to the PFC, striatum and nucleus accumbens (Bjorklund et al. 2008; Lammel et al. 2008) parallel the maturation of cognitive functions, outlining an important role for this system during adolescence (Naneix et al. 2012). In sum, strong supporting evidence suggests that DA has a critical role in modulating neurodevelopment and functional circuit formation.

Alterations in DA neurotransmission in the developing PFC can have detrimental and lasting effects on behaviors that rely on an optimal balance of excitatory and inhibitory tone. Disturbances in D1, D2 and/or D4 development in the PFC have been implicated in neuropsychiatric disorders such as schizophrenia. Schizophrenics exhibit deficits in PFC-dependent behaviors such as working memory, attention, and decision-making (i.e. negative symptoms) and this has been linked to altered DA neurotransmission (Davis et al. 1991; Okubo et al. 1997; Abi-Dargham 2003). Supporting the link between DA and neuropsychiatric disorders, many atypical antipsychotics either act directly or

indirectly at DA receptors (Van Tol et al. 1991; Kapur and Remington 2001,2001). Although the cause of schizophrenia is still commonly debated on a wider spectrum, building evidence suggests that insult (i.e. from drugs and/or alcohol) to the developing PFC may have lasting effects on cognition and PFC-dependent behaviors in adulthood (Crews et al. 2007).

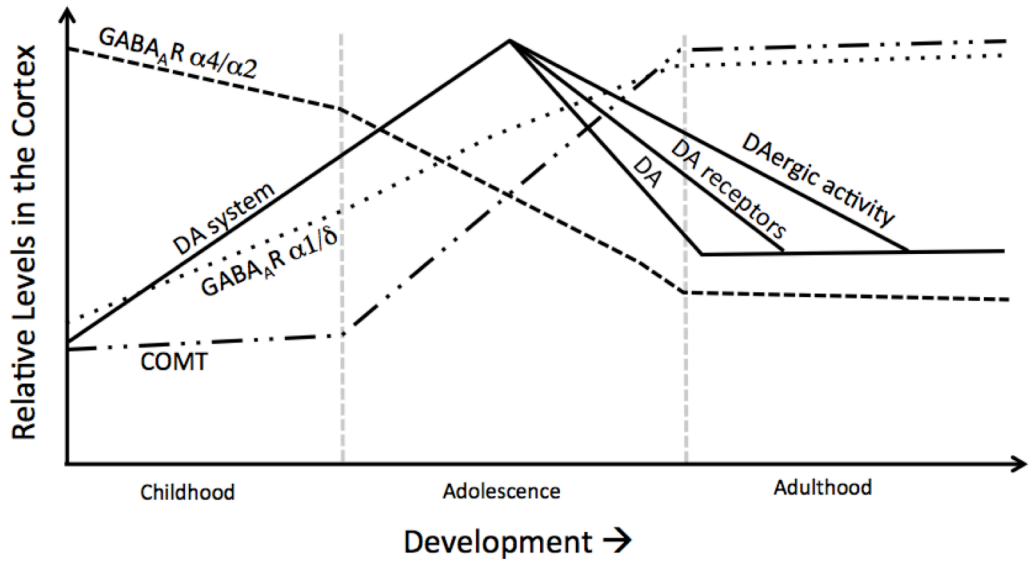

Figure 1-5. Examples of GABAergic and dopaminergic developmental profiles in the PFC. $GABA_A$ receptors undergo differential developmental patterns. α4 and α2 containing $GABA_A$ receptors exhibit a gradual decease in expression throughout adolescence and into adulthood while α1 and δ containing $GABA_A$ receptors gradually increase expression through adolescence before stabilizing in adulthood. The DA system peaks during adolescence before undergoing extensive refinement during late adolescence and adulthood. COMT expression increases through adolescence before stabilizing in adulthood. Adapted from (Tunbridge et al. 2007; Hoftman and Lewis 2011; Suri et al. 2015).

ALCOHOL AND NEUROTRANSMISSION

Effect of acute alcohol on the GABA system

It is well known that acute consumption of alcohol has a general suppressive effect on the nervous system. Alcohol consumption initially leads to decreased attention, alterations in memory, drowsiness and impaired motor coordination. Excessive alcohol consumption can result in confusion, amnesia, difficulty breathing, severely impaired motor skills, loss of consciousness and even death (Avdulov et al. 1995; Valenzuela 1997). The inhibitory, depressant effects of alcohol can be attributed to an imbalance of excitatory/inhibitory neurotransmission in the brain. $GABA_A$ receptors represent one of several pharmacological targets of ethanol, resulting in enhanced agonist-mediated chloride flux and therefore enhancing GABA-mediated neurotransmission (Allan and Harris 1986; Mehta and Ticku 1988; Weiner et al. 1994; Lovinger 1997; Harris et al. 1998). $GABA_A$ receptor agonists such as benzodiazepines, sedatives and anesthetics, and anticonvulsants all produce behavioral affects that, to certain degrees, produce behavioral impairments similar to acute alcohol. In support of this relationship, combining GABA agonists with alcohol has additive effects (White et al. 1997; Breese et al. 2006) and cross-tolerance can develop between alcohol and drugs that target $GABA_A$ receptors (Martz et al. 1983; Mihic 1999). Technical advances in whole-cell patch clamp provided a means to directly study an ethanol-GABA interaction *in vitro.* Namely, the ability to specifically isolate the $GABA_A$ receptors component was made possible. These advances provided an abundance of tools that could be used to answer

questions about the effect of acute ethanol on GABAergic neurotransmission. The hippocampal slice was the first in vitro brain tissue preparation to gain widespread use in electrophysiological studies. This allowed for the examination of alcohol's effect on the $GABA_A$ receptors in the organized hippocampal microcircuitry (Durand et al. 1981; Carlen et al. 1982). Even within this microcircuitry, diverse effects of alcohol were observed. In the dentate gyrus, ethanol inhibited long-term potentiation but had no effect on sIPSC (Morrisett and Swartzwelder 1993), whereas in hippocampal CA1 neurons, ethanol potentiated sIPSC (Weiner et al. 1994; Wan et al. 1996). Beyond the hippocampus, neocortical neurons exhibited enhanced hyperpolarization after acute ethanol (Marszalec et al. 1998; Soldo et al. 1998), while in acute amygdalar slices, ethanol increased the amplitude of sIPSC and the frequency of mIPSC suggesting that ethanol alters the release of GABA in this brain region (Roberto et al. 2003). Another brain region that plays an integral role in controlling motor systems is the cerebellum (Carta et al. 2004). Cerebellar function is impaired by both acute and chronic ethanol. This effect is largely believed to be a product of ethanol's modulatory action at $GABA_A$ receptor neurotransmission in the cerebellum (Botta et al. 2007). In the cerebellum ethanol impairs the flow of information by increasing $GABA_A$ receptor mediated neurotransmission in both Purkinje cells and granule cells. Ethanol does not affect release of GABA, as was previously observed in other brain regions, but rather it increases the GABAergic interneuron tone onto the excitatory granule cells (Palmer and Hoffer 1990; Valenzuela and Jotty 2015). Taken together, early studies identified a general

interaction between GABA and ethanol. However, it became increasingly clear that these interactions varied widely between brain regions and even between different cell types and $GABA_A$ receptor subtypes. Subsequent studies have focused on the effect of alcohol on GABAergic neurotransmission in specific brain regions and on specific subtypes of $GABA_A$ receptors.

It is worth noting, however, that many of the early studies testing the effects of ethanol on GABAergic neurotransmission were observed using high concentrations of ethanol. The effect of ethanol on $GABA_A$ receptors became more relevant with the observation that extrasynaptic $GABA_A$ receptors, which have a high affinity for GABA and modulate tonic GABA currents, are also activated/enhanced by comparatively lower doses of ethanol (Sundstrom-Poromaa et al. 2002; Wallner et al. 2003; Hanchar et al. 2005; Glykys et al. 2007). These findings with recombinant receptors in expression systems were later extended to the acute slice preparation. Extrasynaptic $GABA_A$ receptors containing δ and/or $\alpha 4$ subunits are particularly sensitive to the facilitatory effect of ethanol on chloride conductance (Fleming et al. 2007; Chandra et al. 2008; Liang et al. 2008; Fleming et al. 2012; Olsen and Spigelman 2012; Herman et al. 2013). These receptors are therefore viable candidates for mediating the behavioral effects of pharmacologically relevant doses of alcohol that cause intoxication in humans. However, the inconsistent effects of ethanol on different brain regions, coupled with the variability in expression of extrasynaptic $GABA_A$ receptors, highlights the need to better understand the variability in $GABA_A$ receptor subunit composition, functionality and microcircuitry between brain

regions and cell types and furthermore, the differential effects of ethanol on $GABA_A$ receptors in different brain regions.

Similar to other brain regions, the direct effect of alcohol on $GABA_A$ receptors in the mPFC is relatively unclear to date. Earlier studies from Weitlauf et al examined the effect of acute ethanol on pharmacologically isolated GABA currents in layer V of the rat mPFC (Weitlauf and Woodward 2008). These studies revealed no effect of high concentrations of ethanol on frequency or amplitude of sIPSC or the paired pulse ratio. In addition, no evidence of a tonic GABA-mediated current or any effect of ethanol on the holding current was observed. However, these studies, while informative towards characterizing the effects of ethanol in deep-layer mPFC neurons, were conducted in younger rats. As aforementioned, the δ-$GABA_A$ receptor, which is considered a key contributor to the tonic current in the PFC, undergoes a gradual increase in mRNA expression throughout adolescence and into adulthood (Datta et al. 2014). Thus, it is possible that tonic currents in the mPFC emerge later in development.

Effects of acute alcohol on the DA system

It is generally accepted that virtually all drugs of abuse act within the mesocortical DA system and that changes in the neuronal circuitry of this system underlie the development of addictive behaviors. Unlike other psychostimulants that directly target the DA system, alcohol appears to indirectly affect DA neurotransmission. DA is thought to elevate the reinforcing and rewarding

properties of alcohol that, after prolonged use, may lead to addiction. Several studies used *in vivo* microdialysis to examine DA levels throughout the brain in response to alcohol. These studies demonstrate an acute alcohol-induced increase in DA release specifically to the mesolimbic DA system, which in turn leads to an increase in extracellular DA in target areas (Imperato and Di Chiara 1986; Weiss et al. 1993; Doyon et al. 2003). The nucleus accumbens, a major component of the reward system, has been shown to be particularly sensitive to ethanol related increases in extracellular DA. Ethanol-induced increases in extracellular DA in the nucleus accumbens have been demonstrated in several studies using various doses, species and routes of administration (for review see (Gonzales et al. 2004)). Another major component of the DA reward system, the VTA, also appears to be sensitive to the effects of ethanol. Acute ethanol increases the firing frequency of DA neurons in the VTA both *in vitro* (Gessa et al. 1985) and *in vivo* (Brodie et al. 1990). Furthermore, this effect is mediated by both increased postsynaptic $GABA_A$ receptor sensitivity and enhanced GABA release onto VTA DA neurons, consequently increasing firing frequency of VTA DA neurons.

DA neurons in the VTA also send dense projections to the mPFC. Acute ethanol administration in humans is known to produce deficits in PFC-dependent behaviors such as spatial recognition, planning, working memory and decision-making on a gambling task (Weissenborn and Duka 2003; George et al. 2005; Saults et al. 2007). All of these tasks are highly dependent on optimal levels of DA and DA receptor modulation therefore studies were conducted to directly

examine the effect of acute alcohol on the PFC DA system. One study used *in vitro* co-cultures containing the mPFC, hippocampus, and VTA and found that co-perfusion of a D1 antagonist with acute ethanol produced marked inhibition of persistent activity that was greater than that seen in neurons exposed to 50mM ethanol alone (Tu et al. 2007). In contrast to the nucleus accumbens, the neurochemical effect of ethanol on extracellular DA levels in the mPFC has been controversial. Early studies indicated that systemic injection of ethanol failed to increase extracellular levels of DA in the mPFC (Hegarty and Vogel 1993; Engleman et al. 2006). However, a recent study demonstrated that passive ethanol administration increased extracellular DA levels in the mPFC (Schier et al. 2013). Acute alcohol-induced alterations in DA receptor modulation of the GABA system in the PFC may underlie some of the deficits in cognition, working memory and executive function observed following ethanol intake. In sum, acute alcohol has unique, yet overlapping effects on both the GABA system and the DA system that likely underlie many of the behavioral effects of acute alcohol exposure.

ALCOHOL ABUSE

Chronic alcohol abuse

Chronic alcohol abuse leads to a wide range of changes in the brain that, depending on severity of abuse, can persist for days, months, weeks and even years. Prolonged alcohol abuse can damage the function of many organs

including the liver, kidneys, heart and brain. Tolerance to alcohol exponentially exacerbates the negative effects and a 'downward spiral' toward alcohol dependence can occur. Addiction is a chronic relapsing disorder and drug-seeking behavior that progresses from impulsive use to compulsive use in three stages: binge/intoxication, withdrawal/negative affect, and preoccupation/anticipation (Koob and Le Moal 1997). After prolonged alcohol use, the brain begins to reestablish homeostasis to account for the new 'normal.' Rapid cessation of drugs and alcohol unmasks this state of adaptation causing changes in the opposite direction to those produced acutely by the drug (Himmelsbach 1941). Alcohol withdrawal symptoms can manifest anywhere from 6 hours to a few days after the last drink. The severity of symptoms varies widely based on length of use and amount of alcohol consumed. They can range from mild in certain individuals to life threatening in extreme cases. In order to be clinically diagnosed with alcohol withdrawal two or more of the following criteria must be met: insomnia, autonomic symptoms (sweating, increased heart rate), nausea/vomiting, restlessness, anxiety, seizures and/or hallucinations (Peer et al. 2013).

As the length of abstinence increases, many withdrawal symptoms dissipate. However, some of the effects of heavy alcohol abuse can persist long into abstinence. The long-term effects of chronic alcohol abuse include, but are not limited to, deficits in cerebellar function (motor coordination), the limbic system (memory and emotion) and the cerebral cortex (cognitive function). Imaging studies show that alcoholics have reduced ventricle size, and in many

subjects, this reduction was prevalent 6-9 months after entering rehabilitation programs (Wobrock et al. 2009). Mounting evidence has made it increasingly clear that exposure to alcohol has significant effects on the functional and structural composition of the PFC (Jernigan et al. 1991; Fein et al. 2002; Makris et al. 2008; Rando et al. 2011). Given the key role of this brain region in the integration, manipulation and assessment of incoming sensory and cognitive information, chronic alcohol-induced deficits in executive function, decision-making and problem solving are common. Well into prolonged abstinence (2 months - 7 years), deficits in cognitive tasks such as nonverbal abstract reasoning, mental flexibility, nonverbal short-term memory and visuospatial abilities have been observed (Brandt et al. 1983). For example, alcoholics exhibit deficits on the WCST (Adams et al. 1993) and the Iowa Gambling Task (Bechara et al. 2001), both of which rely heavily on the PFC. In many tasks the poor performance of alcoholics is similar to patients with lesions of the ventromedial PFC (Bechara et al. 2001). Human imaging studies demonstrate that alcoholics have reduced PFC gray matter, white matter and overall reduced PFC volume compared to controls (Rando et al. 2011). Furthermore, this same study suggests that cortical volume positively correlates with relapse probability. How long these deficits last is still debated, however Fein and colleagues suggest that many of the effects of chronic alcohol abuse on cognition can last upwards of 7 years (Fein and McGillivray 2007).

Animal Models of Alcohol Exposure

Several animal models have been developed to mimic typical human drinking patterns including both chronic and intermittent binge-like models (Kokka et al. 1993). These models provide the ability to examine long-term consequences of ethanol exposure on the brain, and also the ability to focus on key characteristics of chronic alcohol consumption like withdrawal and tolerance, in a well-controlled environment. In order to closely mimic human alcohol consumption, several different routes of administration have been developed and employed in both rodents and non-human primates. It is nearly impossible to create an animal model that identically mimics human consumption; therefore different models are utilized based on how accurate the model is at answering the intended research question. Each model has strengths and weaknesses, with some models better suited for identifying certain possible adaptations. All animal models of alcohol consumption fall under two categories: passive, involuntary alcohol exposure and active, voluntary alcohol exposure. Active alcohol exposure models involve simply providing the animal with free access to alcohol in its home cage with or without the option of water (Sarles et al. 1971; Rossi and Zucoloto 1977). This mode is physiologically relevant for testing voluntary consumption of alcohol that resembles human behavior. The disadvantage of this model is the lack of control over the amount of ethanol intake and consequently, the animal will often not reach substantial levels of intoxication.

Involuntary alcohol exposure models allow for precise control over the amount of exposure and timing, eliminating much of the between-animal

variability associated with voluntary consumption. Several models of involuntary alcohol exposure have also been developed. One method used for chronic ethanol administration in animals is intragastric alcohol administration (IG). This model involves administering ethanol via temporary intubation of the esophagus or stomach (Lieber et al. 1989; Siegmund et al. 2003). IG is advantageous in that it mimics the gastrointestinal absorption route alcohol takes in humans. Moreover, IG allows for exact control over the administered ethanol dose. This method is, however, stressful and is more suitable for acute alcohol administration than it is for chronic administration.

Another common technique used for alcohol administration is intraperitoneal ethanol injection. This model involves directly administering ethanol into blood circulation via syringe injection (Seitz et al. 1990; Siegmund et al. 2003). This is the preferred method for studying the acute effects of ethanol on metabolic changes and behavior in animals because the injection dose can be tightly regulated. The avoidance of first-pass-metabolism removes much of the between-animal variability in metabolic rates that can confound results. Intraperitoneal alcohol administration is not ideal for chronic ethanol administration and similar to other models, it lacks strong face validity. In humans, alcohol is typically not injected directly into the bloodstream.

A newer animal model of ethanol administration that is becoming increasingly popular is ethanol vapor inhalation. This method involves placing an animal into an enclosed cage that is saturated with ethanol vapor (Rogers et al. 1979; Gilpin et al. 2008). Alcohol vapor inhalation is a relatively stress free, non-

invasive procedure that allows for precise control of the dose, duration and pattern of exposure. Vapor levels can be accurately modified to accommodate for tolerance and/or desired blood alcohol levels. This method is more suitable for chronic or intermittent exposure to alcohol and is not ideal for acute ethanol administration. However, although inhalation of alcohol has been reported in humans, this method lacks physiological relevance. In sum, while several negatives and positives exist within each animal model, they all share the same intention of providing a well-controlled model of alcohol exposure.

Neuroadaptive changes in response to chronic ethanol

Alcohol withdrawal is a major contributing factor of relapse. Research in both humans and animal models is being conducted to identify the neural mechanisms underlying these symptoms. The intended goal of these studies is to pharmacologically target GABA, glutamate, monoamines and/or neuropeptides to alleviate withdrawal symptoms. On the cellular level, homeostatic changes in $GABA_A$ receptor, glutamate receptors, monoamines and neuropeptides decrease the effects of alcohol resulting in the need for an increase in alcohol dose to achieve the previous level of intoxication (Tabakoff and Hoffman 1996; Valenzuela 1997). The compensatory changes in neurotransmission lead to physical dependence on alcohol that is observed as a hyperexcitability syndrome upon withdrawal. With regards to the glutamate system, NMDA receptors appear to be particularly sensitive to alcohol compared to other neurotransmitter

receptors. The affects of chronic alcohol exposure vary widely between brain regions, but in general, acute alcohol administration attenuates NMDA receptor activity (Samson and Harris 1992); therefore the expected neuroadaptive response of the brain to chronic alcohol is increased NMDA receptor expression/function. This idea has indeed been supported by numerous studies showing that chronic alcohol leads to an upregulation of NMDA receptors as well as changes in NMDA receptor-associated genes and proteins (Littleton 1998; Kliethermes 2005; Nagy 2008).

While observations of changes in the GABA system as a result of chronic alcohol abuse vary widely between brain regions, some generalizations can be made. When the GABA system is first introduced to alcohol, a large potentiation in GABA neurotransmission occurs. After prolonged use, however, multiple molecular mechanisms are thought to counteract the acute effects of alcohol that could be considered within-system neuroadaptations. The system adapts to the constant presence of alcohol by decreasing GABA neurotransmission via downregulation of $GABA_A$ receptors and decreased receptor function (Littleton 1998; Kliethermes 2005; Nagy 2008). Furthermore, many of the short-term and long-term effects of chronic alcohol abuse can be linked to specific $GABA_A$ receptor subunits and subunit combinations, resulting in varying degrees of neuroadaptations (Cagetti et al. 2003). For example, it has been hypothesized that chronic ethanol decreases $GABA_A$ receptor function through a downregulation of the $\alpha 1$ subunit (Mhatre and Ticku 1993; Devaud et al. 1996). Cagetti et al. used hippocampal homogenates to examine expression levels of

various subunits after two days of withdrawal from chronic alcohol treatment and observed an increase in $\alpha 4$-$GABA_A$ receptors and $\gamma 2$-$GABA_A$ receptors as well as a decrease in $\alpha 1$-$GABA_A$ receptors and δ-$GABA_A$ receptors (Cagetti et al. 2003). These results were supported by studies using whole cell electrophysiology, which revealed decreases in amplitude, frequency and decay of TTX-resistant miniature IPSC (mIPSC) (Liang et al. 2006). In addition, chronic ethanol decreased sensitivity to diazepam and neurosteroids, drugs specific to $\alpha 1$-$GABA_A$ receptors and δ-$GABA_A$ receptors, respectively (Khisti et al. 2002). Taken together, alcohol specifically acts at particular $GABA_A$ receptor subunits, suggesting a unique role for subunit in modulating GABA currents. Unlike the hippocampus, in the amygdala, chronic ethanol exposure has been reported to act through increased presynaptic GABA release (Roberto et al. 2004). In the cerebellum, chronic exposure to alcohol reduced mRNA levels of the $\alpha 1$-$GABA_A$ receptor, while increasing mRNA levels of $\alpha 6$-$GABA_A$ receptor (Mhatre and Ticku 1992; Morrow et al. 1992; Vekovischeva et al. 2000). In the cerebral cortex, levels of $\alpha 1$-$GABA_A$ receptors were decreased and $\alpha 4$-$GABA_A$ receptor and $\gamma 1$-$GABA_A$ receptor levels were increased (Mhatre and Ticku 1992). These are just a few examples of the many different changes occurring to specific $GABA_A$ receptor subunits after chronic alcohol abuse. Taken together, these findings underscore the complex actions of alcohol on different GABA systems in the brain, highlighting the need to avoid overgeneralization of the effects of chronic alcohol on the GABA system.

DA modulation of GABA adds another level of complexity to identifying the underlying effects of chronic alcohol abuse. Physiological studies demonstrate that repeated ethanol exposure is associated with decreased GABA inhibition of VTA DA neurons (Brodie 2002). Moreover, persistent reductions in the bioavailability of DA and DA neuron activity are observed during alcohol withdrawal, which can be alleviated by alcohol administration (Martinez et al. 2005). In addition, VTA neuron firing rate is coupled to D2 receptor modulation, and chronic ethanol has been shown to compromise D2 enhancement of mid-brain GABA neuron firing (Ludlow et al. 2009). Reduced D2 levels are frequently associated with a variety of drugs of abuse (Koob and Volkow 2010). In general, reductions in D2 receptor levels persist across abstinence. It has been proposed that decreased D2 receptor levels could be a critical adaptation that promotes relapse in the abstinent addict. In alcoholics, lower D2 receptor availability in the nucleus accumbens and PFC is associated with severity of ethanol craving and greater cue-induced activation of several subregions of the PFC (Heinz et al. 2004). Partial recovery of DA receptor function across early abstinence is associated with decreased risk of relapse (Markianos et al. 2001; Heinz et al. 2004). Furthermore, reduced DA in these regions is thought to contribute to the aversive/anhedonic properties of the withdrawal/negative affect state of addiction (Koob and Volkow 2010). Using a rodent model of chronic ethanol exposure, studies from our lab suggest that chronic exposure to alcohol results in a complete loss of D2 receptor modulation of pyramidal cell firing and neurotransmission in layer V of the PrL-C (Trantham-Davidson et al. 2014). DA

receptor modulation of inhibition/excitation greatly influences neuronal communication and neuronal circuitry, and in turn neuroadaptations in the DA system may be a major underlying factor for the compulsive, and impulsive behaviors associated with alcohol addiction.

Adolescent alcohol abuse

Adolescence is a unique period that includes rapid and dramatic maturation of behavioral, cognitive, psychosocial and hormonal systems (Blakemore and Choudhury 2006; Crone and Dahl 2012). As highlighted above, neurotransmitter systems in several brain regions undergo fine-tuning during this critical period of brain development. Because of the extensive maturational changes, the developing adolescent brain may be especially vulnerable to the deleterious effects of exogenous agents, including alcohol, which is commonly abused during this critical period of development Alcohol is among the most commonly used intoxicating substances during adolescence, with nearly half of adolescents between 12 and 18 reporting past-year alcohol use and nearly 25% reporting past year intoxication (Johnston 2013). These rates dramatically increase, and by the end of high school (~18 years old) nearly 70% of adolescents report alcohol consumption, with nearly a quarter reporting binge episodic drinking (5 or more drinks on one occasion) in the past 2 weeks (Johnston 2013). Alcohol consumption in this age group typically occurs in these excessive binge-like patterns that lead to very high levels of intoxication (Patrick

and Schulenberg 2010; NIAAA 2012; Johnston et al. 2014). These high rates of heavy alcohol use during adolescence are concerning, as the adolescent brain undergoes extensive morphological and functional maturation. Alcohol abuse during adolescence may put an individual at risk for heavy alcohol and drug use in adulthood, and may even result in early manifestation of psychiatric illnesses (Tapert and Schweinsburg 2005; Nixon and McClain 2010; Silveri 2014). Why do adolescents partake in binge-like alcohol consumption despite perpetually being told it is harmful on so many different levels? This popular question cannot be directly answered, however, it is well known that adolescents have an innate drive to explore, take risks, and experiment with novel activities (Blakemore and Robbins 2012). Compounded within these innate characteristics, the effects of alcohol on the adolescent brain differ than those on the adult brain, as adolescents have been found to be hyposensitive to the negative effects of alcohol. Adolescents who drink alcohol are less responsive to alcohol-induced sedation and motor impairments than adults. In addition, these individuals display less severity of acute withdrawal symptoms ("hangover"), compared to adults that consumed the same amount of alcohol (based on body weight) (Hollstedt et al. 1980; Little et al. 1996; Silveri and Spear 1999). This hyposensitivity to the aversive effects of alcohol, coupled with the innate risky and impulsive characteristics, are major factors contributing to alcohol abuse during adolescence.

Adolescents also exhibit *hypersensitivity* to some of the effects of alcohol when compared to adults. Many of the hypersensitive effects of alcohol involve

cognition and behavior. Alcohol abuse during adolescence leads to changes in overall mood, declines in scholastic or athletic performance, memory impairments, and/or increased risky decision-making and impulsivity (Chambers et al. 2003; Oscar-Berman and Marinkovic 2003; White and Swartzwelder 2005; Masten et al. 2008). Brain regions that undergo significant neurodevelopment during adolescence (PFC, limbic system and cerebellum) all participate, to some degree, in these aforementioned behaviors. The hippocampus has been an area of interest with regards to the effect of adolescent alcohol abuse on memory consolidation (White and Swartzwelder 2005). It was reported that adolescents diagnosed with an alcohol use disorder had smaller hippocampal volumes relative to age-matched non-drinkers, and this effect was greater with earlier age of alcohol use (De Bellis et al. 2000; Nagel et al. 2005). Furthermore, animal studies examining the effects of adolescent alcohol abuse on spatial memory using a Morris Water Maze task and a discrimination task demonstrate that impairments in spatial memory persist well beyond the termination of repeated alcohol treatments in adolescence (Markwiese et al. 1998; Land and Spear 2004; Sircar and Sircar 2005). Moreover, in humans, individuals in their early 20s are more sensitive to alcohol's effect on verbal and figural memory tasks than in their late 20s (Acheson et al. 1998). These findings are underscored by *in vitro* studies indicating that acute alcohol impairs the induction of long-term potentiation (LTP) in hippocampal slices from adolescent rats compared to those from adults (Swartzwelder et al. 1995). These observations collectively indicate that both memory formation and memory-related hippocampal function are more sensitive

to the effects of acute ethanol during adolescence than adulthood. The common acceptance that adolescent alcohol abuse is concurrent with a critical period of brain development and the heightened vulnerability highlighted above, suggests that the normal maturation in many of the developing brain regions may be impacted. In the rat hippocampus, adolescent exposure to alcohol has been shown to reduce tonic $GABA_A$ receptor currents in hippocampal formation in adulthood (Fleming et al. 2012; Fleming et al. 2013) as well as reductions in δ-$GABA_A$ receptor protein expression (Centanni et al. 2014). These observations were unique to the exposure period, as the effect was not evident in adult rats chronically exposed to alcohol further suggesting that adolescence represents a distinctively vulnerable period of such effects.

The PFC is a particularly unique brain region in part due to the delayed development and refinement occurring during the typical period of adolescent alcohol abuse. Similar to other brain regions, it is hypothesized that exposure to alcohol during adolescence, when a majority of the synaptic refinement and structural remodeling is taking place, alters the function/structure of the adult brain and impair behavioral control in the adult (Spear 2000). For instance, cross-sectional studies using structural MRI have reported smaller PFC volumes in heavy-drinking compared with nondrinking teens (Squeglia et al. 2009). Furthermore, longitudinal studies demonstrate that adults who abused alcohol during adolescence showed accelerated cortical thinning and overall smaller prefrontal grey and white matter volumes relative to age-matched controls (De Bellis et al. 2005; Squeglia et al. 2014), suggesting lasting effects of adolescent

alcohol abuse. Normal cortical thinning in adolescence is reflective of better performance on tests of learning and memory, visuospatial functioning, spatial planning and problem solving (Squeglia et al. 2013), however accelerated thinning is suggested to reflect premature cortical gray matter decline similar to that seen in normal again and in adult alcoholics (Squeglia et al. 2015). Furthermore, reduced prefrontal volume has been associated with deficits in inhibitory control, poor decision-making, and increased propensity to engage in risky behaviors (Crews and Boettiger 2009). Using a tightly controlled animal model of adolescent binge-like alcohol exposure, studies from our lab demonstrate that adolescent alcohol exposure leads to reduced behavioral flexibility, reduced anxiety and/or increased exploratory drive, and increased resistance to extinction of ethanol-seeking behavior in adulthood (Gass et al. 2014). Among the several neurotransmitter systems undergoing postnatal maturation in the PFC, converging evidence suggests the GABAergic and dopaminergic systems experience profound changes during adolescence (Vincent et al. 1995; Benes et al. 1996; Tseng and O'Donnell 2007; Kilb et al. 2011). Drinking during adolescence could result in altered PFC-dependent behavior in adulthood caused by compromised development of the GABA and DA systems. For example, a recent study examined the effect of administering positive or negative modulators of $GABA_A$ receptors throughout adolescence and found that targeting $GABA_A$ receptors during adolescence led to increased drinking in adulthood (Hulin et al. 2012). This study highlights the vulnerability of

the GABA system during adolescence and how alterations in neurotransmission during development can affect adult behavior.

All of the studies discussed thus far have greatly contributed to our knowledge of alcohol and the GABA and DA systems, however with this knowledge, several new questions have surfaced. The interaction between ethanol and $GABA_A$ receptors is evident, as is the effect of ethanol on DA neurotransmission. However, the discrepancies observed between brain regions and between exposure models serve as a reminder that much more research needs to be done to uncover the specific effect of adolescent alcohol on the GABA and DA systems in the cortex. Studies have begun to address the effect of alcohol on the immature and mature GABA and DA systems in the cerebral cortex (Mehta and Ticku 2005; Yu et al. 2006; Crews et al. 2007; Hulin et al. 2012), yet a major question remains: what are the effects of *adolescent* alcohol abuse on the mature cerebral cortex, and more specifically, the mPFC? Addressing this issue will provide valuable insight into an area of research that is understudied, yet extremely relevant given the prevalence of adolescent alcohol abuse. The studies contained in this dissertation will address this gap in our knowledge.

SUMMARY

The PFC mediates executive functions such as inhibitory control, behavioral flexibility and informed decision-making. Unlike most other brain

regions that are fully developed by the onset of adolescence, the PFC exhibits a delayed period of development that continues into early adulthood. The neurocircuitry of the PFC undergoes significant synaptic changes during adolescence that occurs in parallel with refinement of its cognitive function. While the adolescent PFC is highly plastic and adaptable, this property may render it especially vulnerable to environmental insults that can negatively impact its development and maturation.

Alcohol consumption typically begins during adolescence when it is most often consumed in heavy binge-like episodic patterns. Increasing evidence suggests that adolescent alcohol abuse may result in deficits in behavioral control and decision-making that can persist well into adulthood. Adolescent alcohol abuse can be conceptualized as a neurodevelopmental disorder in which repeated binge-like alcohol exposure result in alterations in normal brain development.

STATEMENT OF PROBLEM AND SPECIFIC AIMS

While adults who abused alcohol during adolescence exhibit behaviors such as increased risk-taking and impulsivity, the question of whether alcohol exposure produces these deficits or whether they represent a pre-existing phenotype is not clear. Therefore, well-controlled studies in animal models are needed to more fully understand the impact of adolescent alcohol abuse on decision-making and behavioral control in the adult.

Although the excitatory activity of deep-layer pyramidal neurons underlies information flow in the PFC, inhibitory GABAergic neurotransmission and modulatory dopaminergic neurotransmission are particularly important in tuning and synchronizing prefrontal network activity. Both dopaminergic and GABAergic neurotransmission in the PFC undergoes extensive changes during adolescence and alterations in cortical both neurotransmitter systems during this critical period have been implicated in neurodevelopmental disorders such as schizophrenia. However, little is known about the impact of adolescent alcohol abuse on these neurotransmitter systems in the adult PFC.

In summary, the work presented in this dissertation aims to answer the overarching question: does binge-like alcohol exposure during adolescence negatively affect development of the PFC and in turn, alter cognitive functioning and neurotransmission in the adult PFC? A rat model of adolescent intermittent ethanol (AIE) exposure by vapor inhalation (Figure 1-6) was used in the dissertation to begin to address this gap in our knowledge. The GABA, glutamate and DA systems were all assessed in the PrL-C after adolescent exposure to alcohol. The major hypothesis being tested was that ***binge-like alcohol exposure during adolescence results in improper development of the neurotransmitter systems in the PrL-C.*** This hypothesis was tested through the following three specific aims:

SPECIFIC AIM 1: TEST THE HYPOTHESIS THAT AIE EXPOSURE ALTERS THE DEVELOPMENT OF DOPAMINERGIC NEUROTRANSMISSION IN THE PRELIMBIC CORTEX (PRL-C).

The delayed development of the PFC is thought to underlie adolescent behaviors such as impulsivity, inhibitory control, and poor decision-making. During adolescence, the PFC undergoes extensive refinement and development that is thought to render it particularly vulnerable to drugs and alcohol. The DA system in the PFC also undergoes extensive development during adolescence. The hypothesis of this aim was that binge-like alcohol exposure during this critical period of development alters adult DAergic neurotransmission.

SPECIFIC AIM 2: TEST THE HYPOTHESIS THAT AIE EXPOSURE ALTERS THE DEVELOPMENT OF TONIC $GABA_A$ RECEPTOR-MEDIATED CURRENTS IN THE PRELIMBIC CORTEX (PRL-C).

In addition to the DA system, the GABA system similarly undergoes extensive development in the adolescent PFC. GABAergic tone tightly regulates network activity and circuit formation in the PFC throughout development. More specifically, tonic GABA currents are thought to play a critical role in neural network oscillations. Preliminary data suggested that tonic GABA currents gradually emerge throughout adolescence and into adulthood. Therefore in this aim, the hypothesis that adolescent binge-like exposure to alcohol compromises proper development of tonic GABA currents leading to attenuated currents in adulthood was tested.

SPECIFIC AIM 3: TEST THE HYPOTHESIS THAT AIE-INDUCED COGNITIVE DEFICITS CAN BE REVERSED USING A δ-$GABA_A$ RECEPTOR POSITIVE ALLOSTERIC MODULATOR (PAM).

Previous studies from our lab and others demonstrate that AIE results in extensive deficits in adult PFC-dependent cognitive performance (Nasrallah et al. 2009; Boutros et al. 2014; Gass et al. 2014). The goal of this aim was to assess the effect of AIE on risk/reward decision-making using a probabilistic discounting task. Recent studies suggest that alteration in GABA neurotransmission in the PrL-C affect risky decision-making (Paine et al. 2015). Given the observed reduction in d-$GABA_A$ receptor-mediated neurotransmission after AIE, an exploratory set of studies focused on the effects of acute administration of a novel δ-$GABA_A$ receptor PAM on risk/reward decision-making.

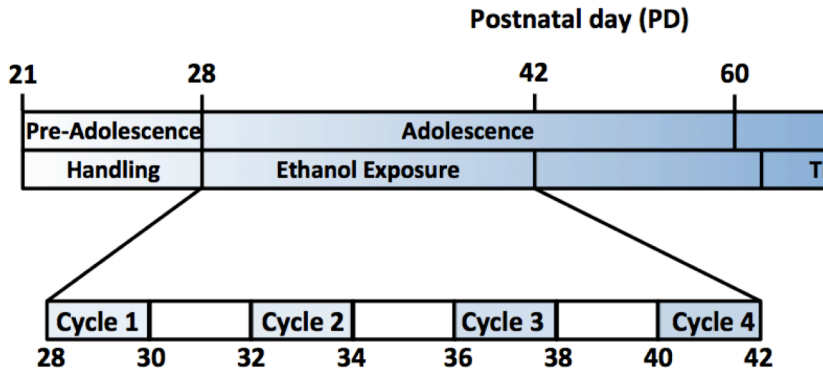

Figure 1-6. Overview of the AIE experimental design. Adolescent male Long-Evans rats were exposed to four intermittent cycles of vapor alcohol from postnatal day (PD) 28-42.

CHAPTER 2

EXPOSURE TO ALCOHOL DURING ADOLESCENCE ALTERS DOPAMINERGIC NEUROTRANSMISSION IN THE ADULT PRELIMBIC CORTEX

BACKGROUND AND SIGNIFICANCE

DA critically modulates the neural networks that mediate cognitive processes in the PFC. The cognitive deficits associated with alcoholism have been linked to imbalances in the dorsolateral PFC DA system (Volkow et al. 1996; Volkow et al. 2002). Using a rat model of chronic alcohol exposure in adult rats, we recently identified alcohol-induced deficits in behavioral flexibility using an operant set shifting task (Trantham-Davidson et al. 2014). The important role of DA modulation of PFC-dependent behaviors led to the hypothesis that

alterations in dopaminergic neurotransmission may contribute to the observed deficits in behavioral flexibility.

The development of the DA system in the PFC parallels the maturation of cognitive functions, outlining an important role for this system throughout adolescence (Naneix et al. 2012). Both D1- and D2-like DA receptors exhibit gradual increases in mRNA expression throughout adolescence, and peak mRNA levels are not observed until adulthood (Tarazi and Baldessarini 2000). In order to properly function, the PFC requires an optimal balance of activation of both D1- and D2-like DA receptors (Arnsten 1997; Gao and Goldman-Rakic 2003). Improper development of DA receptors in the PFC has been linked to a number of developmental disorders such as ADHD and schizophrenia. In addition, using the AIE model described in Chapter 1, studies from our lab also demonstrate decreased behavioral flexibility in adulthood (Gass et al. 2014). Interestingly, these findings closely resemble findings from experiments in adult rats exposed to chronic alcohol (Trantham-Davidson et al. 2014). The AIE model follows a binge-like intermittent design that closely resembles typical adolescent drinking patterns, whereas the chronic ethanol exposure model mimics daily alcohol intake, which is typified in adult alcoholics. Although many of the behavioral phenotypes are similar between the two models, the mechanism underlying these deficits may be different. It is evident that the circuitry underlying the cognitive deficits associated with adolescent alcohol abuse need to be further elucidated to better understand the long-term effects, therefore the present studies utilized the AIE model to examine how adolescent alcohol

exposure alters the cellular and neurophysiological processes in the PFC, and how alcohol administration during adolescence may contribute to deficits in behavioral control in the adult.

This work was performed in collaboration with several other members of our lab and with members of the Neurobiology of Adolescent Drinking in Adulthood (NADIA) consortium. My specific contributions to this project focused on identifying biochemical and physiological changes to the glutamate and DA system in the adult mPFC that are brought about by binge-like exposure to alcohol during adolescence.

MATERIALS AND METHODS

Animals

Male Long-Evans rats were bred in house and weaned at post-natal day 21. Rats were then divided into control and experimental groups and pair housed in standard polycarbonate cages. Pairing was always done within litter and there was always a within litter control and experimental group (e.g., a litter size must have had a minimum of 4 male rats to be used in the study). Access to food and water in the home cage was continuous throughout the experiment. The animal colony room was maintained on a reverse 12:12 light-dark cycle with lights off at 08:00. All experimental procedures were conducted with the approval of the Institutional Animal Care and Use Committee at the Medical University of South

Carolina, and within guidelines set forth by the National Research Council's Guideline for the Care and Use of Mammals in Neuroscience and Behavioral Research (2003).

Adolescent intermittent ethanol vapor exposure and measurement of blood ethanol content

The AIE exposure model used in this dissertation involved intermittent binge-like exposure to alcohol by vapor inhalation as previously described (Gass et al. 2014). In brief, AIE exposure was carried out during early to middle adolescence (PD28-42) and involved 4 cycles of 2 consecutive episodes of alcohol vapor inhalation, with each exposure consisting of 14-hours in the vapor chambers followed by 10-hours out of the chambers. Rats were exposed to alcohol on PD28 & 29 (cycle 1), PD32 & 33 (cycle 2), PD36 & 37 (cycle 3), and PD40 & 41 (cycle 4). A 5-point behavioral intoxication rating scale was used (Nixon and Crews 2002; Gass et al. 2014) to provide an index of the level of intoxication that was achieved during each of the exposure cycles. The rats were scored according to the following behaviors: 1 = No signs of intoxication; 2 = Slightly intoxicated (slight motor impairment); 3 = Moderately intoxicated (obvious motor impairment but able to walk); 4 = Highly intoxicated (severe motor impairments, loss of righting reflex is what the scoring sheet says); 5 = Extremely intoxicated (loss of righting reflex (30 sec) and loss of eye blink reflex). A target level of slight to moderate intoxication was chosen, which corresponded to an

intoxication rating of 2 to 3, respectively. In addition to providing a measure of the level of intoxication, the rating also provided immediate information that could be used to make adjustments in the level of ethanol vapor in the chambers.

Measurement of blood ethanol content (BEC)

Tail vein blood was obtained at the end of each of the 2-day ethanol vapor exposure cycles. Immediately following the collection of blood from the tail vein, the blood was centrifuged at 13,000 X *g* for 10 minutes to obtain a plasma supernatant, which was then stored at 4 °C for a maximum of 24 hours. Next, 10 µl of plasma was used for determination of ethanol levels using a colorimetric enzymatic assay as described previously (Prencipe et al. 1987).

Dendritic spine imaging and 3D analysis

Other members of the lab performed dendritic spine imaging and 3D analysis, and the results of these studies were incorporated into the present study. Diolistic labeling of slices obtained from fixed brains was used to assess the effects of AIE exposure on dendritic spine morphology in the mPFC as previously described (Kroener et al. 2012). In brief, adult AIE-exposed and control rats (~PD90) were anesthetized and perfused with 0.1 M phosphate buffer followed by 1.5% paraformaldehyde (PFA) in phosphate buffer and post-fixed in 1.5% PFA for 30 min before coronal sections (150 µm) were prepared on a vibratome. Tungsten particles (1.3 µm diameter) coated with DiI were delivered

diolistically using a Helios Gene Gun (Bio-Rad) fitted with a polycarbonate filter (3.0 μm pore size; BD Biosciences). DiI was allowed to diffuse overnight at 4°C. The slices were then post-fixed in 4% PFA for 1 hour prior to mounting. Images of the basal dendrites (50–60 μm) of layer V pyramidal neurons in the PrL-C region were collected in the Z-plane and used to create a deconvolved 3-D image. A filament of the dendritic shaft and spines was then created using Imaris XT (Bitplane, Zurich, Switzerland). Dendritic spines were classified into 4 categories (long, mushroom, stubby, or filopodia) based on their length and neck and head width, where *L* is spine length, *WH* is spine head width, and *WN* is spine neck width. Long spines were identified as having a $L \geq 0.75$ μm and <3 μm, mushroom spines had a $L < 3.5$ μm, $WH > 0.35$ μm and a $WH > WN$, stubby spines had a $L < 0.75$ μm, and filopodia were identified as having a $L \geq 3$ μm.

Western blotting

The effects of AIE on expression of a select group of proteins in a membrane-enriched fraction were quantified using standard immunoblot procedures as previously described (Kroener et al. 2012). In brief, rats were lightly anesthetized by being placed into a bell jar containing isolfurane. They were euthanized by decapitation, the brains rapidly removed and immediately immersed for 1-2 min in ice-cold phosphate buffered saline (pH 7.4). The brain was then sectioned on ice into 1 mm thick coronal slices using an adult rat brain matrix (ASI Instruments, Warren, MI). Punches containing the PrL-C region were

obtained from the slices (Figure 2-1) on an ice-cold dissecting plate and the tissue temporarily stored at -80°C. Subsequently, a detergent soluble and detergent resistant membrane fraction was prepared as previously described (Mulholland et al. 2011). An aliquot of each sample was diluted with NuPAGE 4X LDS sample loading buffer (Invitrogen Corp., Carlsbad, CA; pH 8.5) containing 500 mM dithiothreitol, and samples were denatured for 10 min at 70°C. Five µg of each sample was separated using the Bis-Tris (375 mM resolving buffer and 125 mM stacking buffer, pH 6.4; 7.5% acrylamide) discontinuous buffer system with MOPS electrophoresis buffer (50 mM MOPS, 50 mM Tris, 0.1% SDS, 1 mM EDTA, pH 7.7). Protein was then transferred to Immobilon-P PVDF membranes (Millipore, Bedford, MA) using a semi-dry transfer apparatus (Bio-Rad Laboratories, Hercules, CA). After transfer, blots were washed with phosphate-buffered saline containing 0.1% Tween 20 (PBST) and then blocked with PBST containing 5% nonfat dried milk (NFDM) for 1 hour at room temperature with agitation. The membranes were then incubated overnight at 4°C with primary antibodies directed against GluN1 (1:4000; BD Transduction Laboratories, San Jose, CA; Catalog #556308), GluN2A (1:2000; Millipore Corp., Billerica, MA; Catalog # AB07-632), GluN2B (1:2000; NeuroMab, Antibodies, Inc. & UC Davis, Davis, CA; Catalog # 75-097), GluA1 (1:2000; Millipore Corp., Billerica, MA; Catalog # AB1504), GluA2 (NeuroMab, Antibodies, Inc. & UC Davis, Davis, CA; Catalog # 75-002), PSD-95 (1:5000; NeuroMab, Antibodies, Inc. & UC Davis, Davis, CA; Catalog # 75-028) or COMT (1:1000; BD Transduction Laboratories, San Jose, CA; Catalog # 611970) diluted in PBST containing 0.5% NFDM and

washed in PBST prior to 1 hour incubation at room temperature with horseradish peroxidase conjugated secondary antibodies diluted 1:2000 in PBST. Membranes received a final wash in PBST and the antigen-antibody complex was detected by enhanced chemiluminescence.

Catecholamine HPLC

The catecholamine HPLC assay was done in collaboration with Vanderbilt University Neurochemistry Core. In brief, control and AIE-exposed rats were lightly anesthetized by being placed into a bell jar containing isoflurane. Rats were then euthanized by decapitation and the brain was removed and tissue punches containing the PrL-C obtained exactly as describe above (Figure 2-1). PrL-C containing punches were immediately flash frozen on dry ice and shipped to the Vanderbilt University Neurochemistry Core for further HPLC analysis.

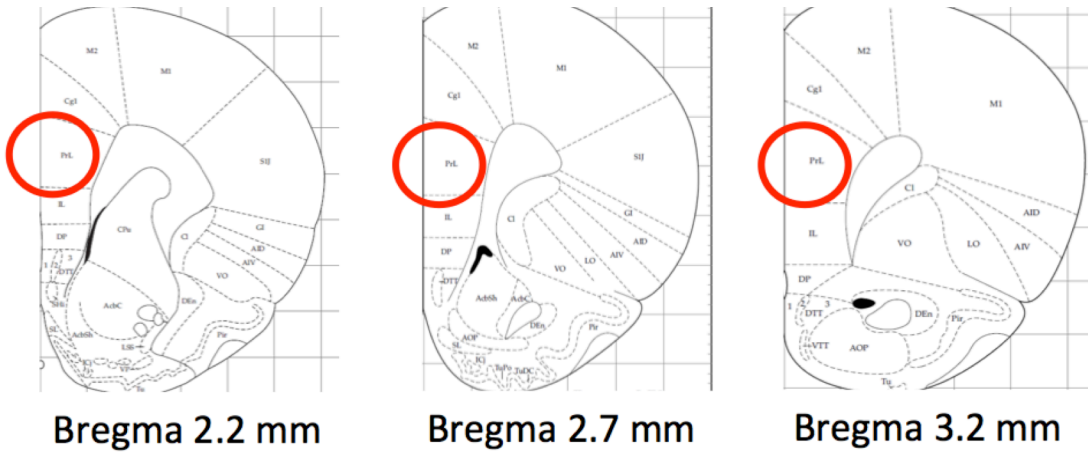

Figure 2-1. Schematic of mPFC tissue punch location. Tissue punches were taken from the entire PrL-C using a 2 mm tissue punch (red). Modified from Paxinos and Watson 2005

Immunohistochemistry

Immunohistochemistry studies were conducted in collaboration with Fulton Crews at the University of North Carolina Chapel Hill. Control and AIE-exposed rats were lightly anesthetized by being placed into a bell jar containing isoflurane. Rats were euthanized by decapitation and the brain was removed and immediately flash-frozen on dry ice and shipped to UNC Chapel Hill for further analysis. To examine tyrosine hydroxylase (TH) immunoreactivity (IR) in the mPFC, 40 µm thick slices were obtained from fixed brains, pretreated with 2% Triton-X for 5 minutes, and incubated with a rabbit polyclonal antibody against TH (1:500, Millipore, AB152) overnight at 4 °C. The sections were then rinsed and incubated with a biotinylated goat anti-rabbit for 1 hour at room temperature. The sections were then rinsed and incubated for 1 hour with avidin-biotin complex reagents (Vector ABC kit, Vector Laboratories). Diaminobenzidine (DAB, Sigma) was used as a chromagen to develop the reaction with 0.05% of a 3% stock of H_2O_2, and nickel ammonium sulfate (2.5%, Sigma) was used to enhance the reaction. Each step was separated by three 10 min washes in PBS. Sections were mounted on glass slides and cover-slipped with DPX mounting media. TH-IR was quantified as previously described (Liu et al. 2008) using Bioquant Nova Advanced Image Analysis (R&M Biometric, Nashville, TN). Images were captured using an Olympus BX50 Microscope and Sony DXC-390 video camera. For TH positive IR (Th+IR), the PrL-C and IL-C (bregma 3.2 to 2.2 mm), (Paxinos and Watson 2005), were outlined and pixel density was measured from the outlined area. From each section, data was obtained from both the left

and right hemisphere from at least four to five sections for each brain, and the average value were used and reported as the mean of at those sections.

MB-COMT promoter methylation

The MB-COMT promoter methylation assay was done in collaboration with Subhash Pandey at the University of Illinois Chicago. Tissue punches containing the PrL-C obtained exactly as describe above for western blotting and temporarily stored at -80 °C. Samples were then shipped to Subhash Pandey and further analysis was conducted. Genomic DNA was obtained using the QIAGEN DNeasy Blood & Tissue kit (Cat. #69504, QIAGEN Inc., Valencia, CA) following the manufacturers protocol. The isolated DNA was sonicated to 200bp fragments and subjected to the MethylMiner™ methylated DNA enrichment (Cat. #ME10025, Life Technologies, Carlsbad, CA) also according to the protocol provided by the manufacturer. This method that uses MBD-Biotin molecules to pull down methylated fragments. A single high salt (2000mM NaCl) elution was then carried out and the resulting DNA amplified by Real-Time quantitative PCR using the RT2 SYBR Green ROX qPCR Mastermix (Cat. #330522, QIAGEN Inc., Valencia, CA). Primers were obtained from IDT (Integrated DNA Technologies Inc., Coralville, Iowa) and were designed to be specific to two regions the promoters of the rat COMT exons I and II. Primer sequences for exon I were CCAGAACACTGGTCTCGTGATA (forward) and TGTGGGAGTGTCCACAGG (reverse), and the sequences for Exon II were GAAGGGCACAAGACACACAG

(forward) and GGAGACCCAATGAGACTGCA (reverse). Input genomic DNA that was not subjected to the methylation enrichment was used as a control and fold change was calculated using the 2−ΔΔCT.

Electrophysiological recordings

Acute slices were obtained for electrophysiological recordings from AIE-exposed and air-exposed control adult rats (PD 90-120). Rats were anesthetized with isoflurane, rapidly decapitated and the brain was immediately removed and placed in an ice-cold ACSF dissection solution containing (in mM): 125 sodium chloride (NaCl), 2.5 KCl, 1.25 monobasic sodium phosphate ($NaHPO_4$), 25 $NaHCO_3$, 4 $MgCl_2$, 1 $CaCl_2$, 10 d-glucose, 15 sucrose, 0.4 ascorbic acid. Slices were incubated at 34°C for at least 1 hour before recordings in continuously aerated (5% carbon dioxide/95% oxygen) incubation ACSF containing (in mM): 125 sodium chloride (NaCl), 2.5 KCl, 1.25 monobasic sodium phosphate ($NaHPO_4$), 25 $NaHCO_3$, 4 $MgCl_2$, 1 $CaCl_2$, 10 d-glucose, 15 sucrose, 0.4 ascorbic acid, and 2 kynurenic acid. After incubation, slices were transferred to a submerged recording chamber, held at 34 °C, and bathed with oxygenated recording ACSF containing (in mM): 125 NaCl, 2.5 KCl, 25 $NaHCO_3$, 2 $CaCl_2$, 1.3 $MgCl_2$, 10 glucose, 0.4 ascorbic acid. The pH of all solutions listed above was adjusted to 7.3 using NaOH and osmolarity was measured to be approximately 300 mOsm.

Recordings were made with a Multiclamp 700B amplifier (Axon Instruments, Union City, California), connected to a computer running Windows XP and Axograph X software and later analyzed offline. All recordings were obtained from pyramidal neurons in Layer V of the PrL-C region (Paxinos and Watson 2005) and were identified visually with infrared-differential interference contrast optics and video-microscopy. For current-clamp experiments, recording electrodes (4-6 MΩ resistance) were filled with a solution containing (in mM): 125 potassium gluconate, 20 KCl, 10 HEPES, 1 EGTA, 2 $MgCl_2$, 2 NaATP, and 0.3 Tris-GTP, 10 phosphocreatine. The pH was adjusted to 7.3 using KOH and osmolarity was measured to be approximately 285 mOsmols. To analyze firing characteristics and intrinsic properties, current steps were applied for 1 s and increased in 20 pA increments from -100 pA until the cell fired 8-10 action potentials for pyramidal neurons. For voltage-clamp experiments, recording electrodes (4-6MΩ resistance) were filled with a solution containing (in mM): 135 CsCl, 2 $MgCl_2$, 10 HEPES, 1 EGTA, 4 NaCl, 2 NaATP, and 0.3 Tris-GTP, 10 phosphocreatine. QX-314 Cl- (1 mM) was added to patch pipettes to block voltage-sensitive sodium channels, thus preventing generation of action potentials. The pH was adjusted to 7.3 using KOH and osmolarity was measured to be approximately 285 mOsmols. Series resistances (<20 MΩ) and input resistances were continually monitored throughout the experiment via a -1mV (100 ms) hyperpolarizing pulse. Excitatory postsynaptic currents were evoked using a tungsten bipolar stimulating electrode placed in layer V within 200 μM of

the cell being recorded. The stimulating electrode was connected to a Grass S88 stimulator and stimulation isolation unit. An input-output relationship was obtained by varying the intensity of stimulation from an amount that produced no response up to an amount that produced a response of maximal amplitude regardless of further increases in stimulus intensity. The stimulus intensity was then reduced to elicit a response that was approximately 75% of the maximal amplitude, and evoked responses were then measured at holding potentials from -80 mV to +40 mV in 10 mV increments. Evoked NMDA (eNMDA) and evoked AMPA (eAMPA) currents were elicited by focal electrical stimulation in the presence of 100 μM picrotoxin and either CNQX (10 μM) or dl-APV (50 μM), respectively.

Data analysis and statistics

Dendritic spine analysis was performed on basal dendrites beginning ~75 μm distal to the soma. Spine data n = 10/group; 75 dendritic sections (2-9 sections/rat); 6875 spines) were averaged for each dendritic section and then were analyzed as a general linear mixed model (SAS PROC MIXED). Westerns blots analysis involved identifying the band corresponding to the appropriate sized protein and quantifying the mean optical density using computer-assisted densitometry with ImageJ v1.41 (National Institutes of Health, USA). Electrophysiology recordings were measured using Axograph X software. Intrinsic firing experiments were analyzed using a two-way repeated measure

ANOVA, followed by a Sidak's Multiple Comparisons test. All other data was analyzed using Student's t-tests. Statistical analysis was performed in Prism (GraphPad Software) or SAS statistics program. Statistical significance level was $p < 0.05$, and data are presented as mean ± SEM.

RESULTS

The rats used in these studies (n = 111) were scored on a 1-5 intoxication rating scale during the AIE procedure as previously described (Nixon and Crews 2002; Gass et al. 2014). Intoxication scores measured at the end of each 14-hour ethanol vapor exposure period were averaged across all 4 cycles for all rats and the grand average was 2.31 ± 0.04. Tail-vein blood drawn at the end of each of the 2-day ethanol vapor exposure cycles revealed that the average BEC for each of the 4 cycles was (in mg%) 298.04 ± 14.41, 334.41 ± 10.31, 299.66 ± 10.39, and 230.79 ± 11.32, respectively, with a grand average across all 4 cycles of 290.73 ± 6.1 mg% (Figure 2-2).

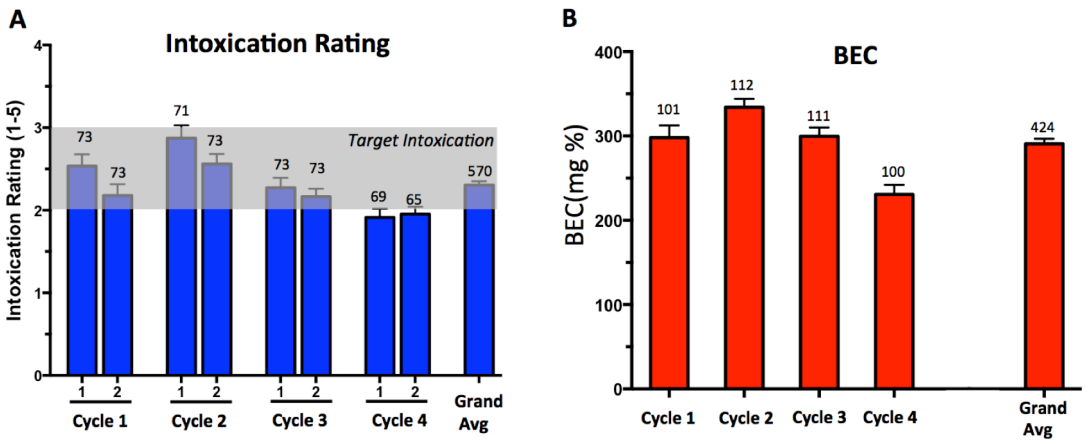

Figure 2-2. Intoxication ratings and blood ethanol levels for the rats used in the current study. A) Average intoxication rating for each exposure day. The target intoxication rating of 2-3 (gray) was determined for each cycle (total n = 570 measurements). **B)** Tail vein blood was drawn from each rat at the end of each cycle and blood ethanol content was determined (total n = 424 measurements).

In addition to the observed effects of AIE on PFC-dependent cognitive flexibility (Gass et al. 2014), other members of our lab used diolistic labeling coupled with confocal imaging and 3D image analysis to examine the effect of AIE on spine morphology in layer V pyramidal neurons in the PrL-C of adult rats. AIE significantly increased total spine density in the apical dendrites of layer V pyramidal neurons in the adult PrL-C ($F_{(1,18)} = 4.64$, $p = 0.045$; Figure 2-3D). Classification of spine density based upon morphology (Figure 2-3B) revealed that the increase in overall spine density was due to a significant increase in the density of long spines, with no change in the density of stubby, mushroom or filopodia ($F_{(3,54)} = 5.01$, $p = 0.004$; post-hoc, $p < 0.001$). This suggests that AIE-exposed adult rats exhibit a larger number of immature plasticity-associated long spines in the PrL-C.

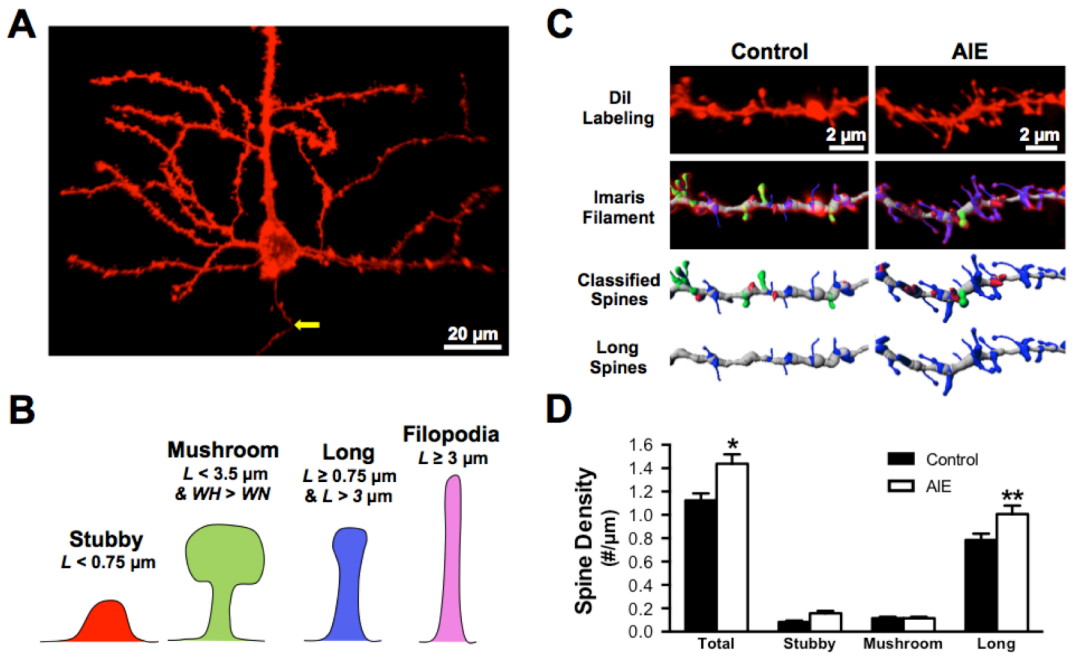

Figure 2-3. Adolescent intermittent ethanol exposure increases the density of dendritic spines in layer V PrL-C pyramidal neurons. A) Representative image of the basal dendrites of layer V pyramidal neurons in the mPFC (yellow arrow identifies the axon stemming from the base of the soma). B) Illustration of the common morphological classifications of dendritic spines and the basic parameters that were used to segregate spines into these 4 subclasses (L = length; WH = spine head width; WN = spine neck width; see methods for full parameters). C) Representative image of diolistic labeling of basal dendrites from control and AIE exposed rats. Also shown is the automated filament detection and classification of the dendritic shaft (grey) and spines (n=10 rats/group; stubby (red), long (blue), mushroom (green), filopodia (pink)) D) Total spine density was significantly increased in adulthood by AIE exposure (*$p < 0.05$), an effect that was mediated by an increase in the density of long spines (**$p < 0.001$). (Data collected by Natasha New)

As a follow-up to examining the structural changes in dendritic spines of glutamatergic synapses, changes in NMDA and AMPA receptor expression and function as a result of AIE exposure were measured in adult rats. First, a PSD-enriched, detergent-resistant membrane (DRM) fraction was prepared from tissue punches taken from PrL-C (Figure 2-1) and subjected to immunoblot analysis. As shown in Figure 2-4, the purity of this fraction was confirmed by the selective recovery of PSD95 in the DRM fraction. Analysis of AMPA and NMDA subunit expression revealed that all of the subunits with the exception of GluA1 were detected almost exclusively in the DRM fraction. Also as shown in Figure 2-4, there were no differences in the expression of AMPA and NMDA receptors or PSD95 in the AIE-exposed adult compared to controls. The next set of studies examined the effect of AIE on AMPA and NMDA currents in the layer V pyramidal neurons in the adult PrL-C. As shown in Figure 2-5, AIE had no effect on evoked AMPA EPSC ($t_{(21)}$ = 1.1, p = 0.298) or evoked NMDA EPSC ($t_{(20)}$ = 1.2, p = 0.235). These observations suggest that while AIE exposure increased the density of long spines, this was not accompanied by significant changes in the expression or function of synaptic ionotropic glutamate receptors.

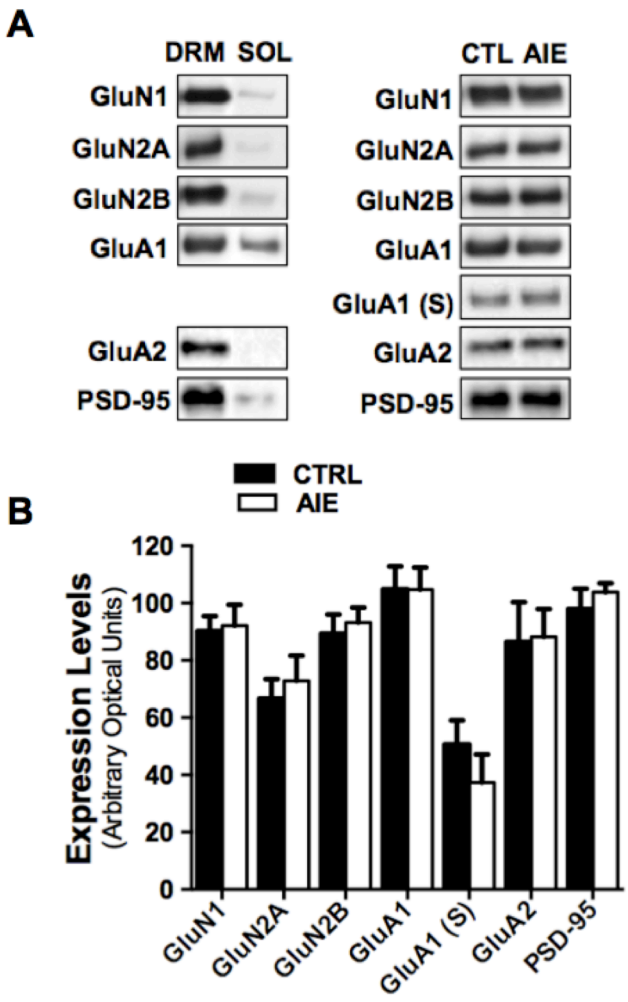

Figure 2-4. Adolescent intermittent ethanol exposure does not alter glutamate receptor protein expression in the adult PrL-C. A) Representative blots of NMDA and AMPA receptor subunits in fractionated tissue from control and AIE exposed rats. (DRM = triton X100 resistant fraction, Sol = Triton X100 soluble fraction). NMDA and AMPA receptor subunits were detected solely in the DRM fraction except for GluA1, which was detected in the Sol (GluA1 (S)). Expression of GluA1 (S) was therefore quantified. **B)** AIE has no effect on AMPA or NMDA receptor subunit protein expression in the adult mPFC (n = 5-7 animals/group).

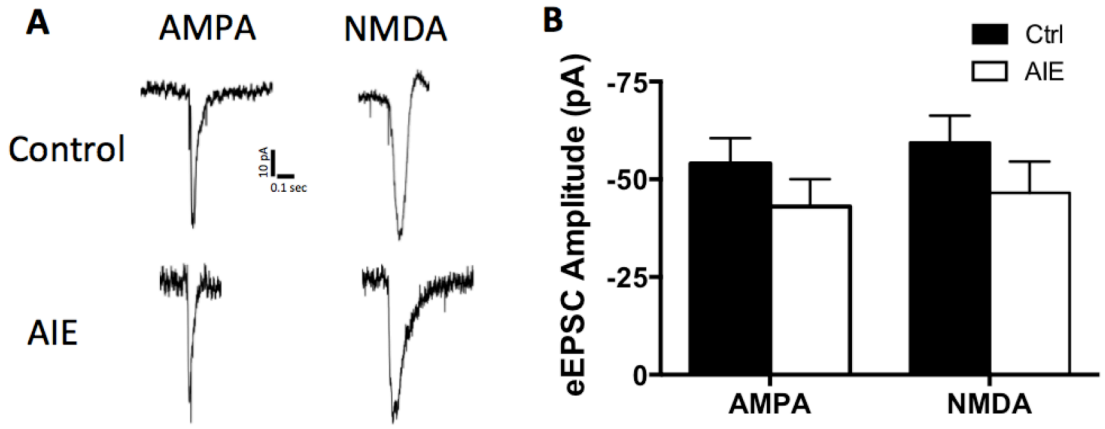

Figure 2-5. Adolescent intermittent ethanol exposure does not alter evoked EPSC (eEPSC) in layer V pyramidal neurons in adult PrL-C. A) Representative trace showing eEPSC AMPA (left) and eEPSC NMDA (right). **B)** Baseline amplitude of eEPSC AMPA (left) and eEPSC NMDA (right) were similar in pyramidal neurons from acute slices layer V PrL-C of AIE rats relative to control rats. (Courtesy of Heather Trantham-Davidson)

DA plays a major role in modulating network activity and the cognitive function of the PFC. Since dopaminergic innervation of the PrL-C undergoes extensive developmental changes during the transition from adolescence to adulthood, the next set of studies examined the effect of AIE exposure on dopaminergic neurotransmission in the adult PrL-C. Tyrosine hydroxylase (TH) is the rate-limiting enzyme responsible for converting the amino acid tyrosine to DA and is a useful marker of dopaminergic innervation. Immunohistochemistry assays were conducted to address whether AIE has an effect on TH levels in the PrL-C. This work was done in collaboration with Fulton Crews, at the core IHC

facilities within the NADIA consortium. As shown in Figure 2-6A-B, AIE exposure was associated with a significant reduction in TH staining in the adult PrL-C ($F_{(1,40)} = 10.35$, $p = 0.003$). A similar decrease was observed in the infralimbic cortex (IL-C), but this was not statistically different ($t_{(20)} = 1.6$, $p = 0.122$; control = 2.33 ± 0.36, AIE = 1.53 ± 0.34).

Due to the sparse expression of DA transporters in the PFC, membrane bound COMT (MB-COMT) plays a critical role in regulating extracellular concentrations of DA. Immunoblot analysis was used to examine the effect of AIE exposure on the expression of MB-COMT in PrL-C. As shown in Figure 2-6, AIE resulted in a significant reduction in the levels of the 28 kDa, MB-COMT in the adult (PD90) PrL-C compared to that of control rats ($t_{(13)} = 2.3$, $p = 0.038$). This reduction was persistent, as MB-COMT was also significantly reduced in 6-month-old (PD180) AIE-exposed rats compared to age-matched controls ($t_{(13)} = 2.3$, $p = 0.039$; Figure 2-6C-D).

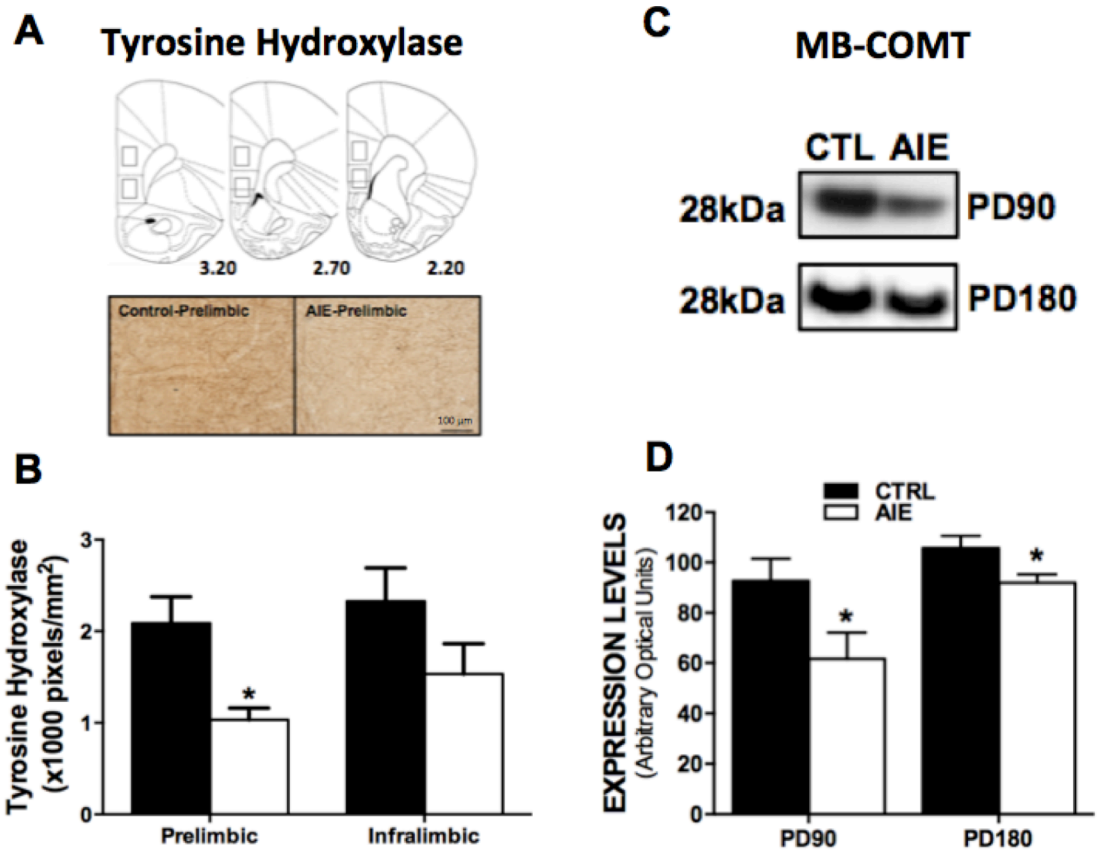

Figure 2-6. Adolescent intermittent ethanol exposure compromises expression of dopamine-related enzymes in the adult PrL-C. **A)** Schematic of prelimbic and infralimbic area analyzed (top) and representative image of TH positive immunoreactivity in the PrL-C of control and AIE-exposed rats. **B)** The pixel density of TH positive immunoreactivity is significantly decreased in the PrL-C after ethanol treatment (*$p < 0.05$). This work was done in collaboration with Fulton Crews **C)** Representative Western Blot of the 28 kDa band membrane bound (MB) COMT band. **D)** AIE exposure significantly reduced MB-COMT protein expression at PD90 and at PD180 (n = 7-9 rats/group; *$p < 0.05$).

Epigenetic modifications via methylation of the COMT promoter have been shown to regulate COMT expression (Ursini et al. 2011). MB-COMT expression is highly regulated by changes in methylation of its promoter region (Murphy et al. 2005; Abdolmaleky et al. 2006), and an increase in methylation typically decreases transcription. Follow-up experiments were performed in collaboration with another member of the NADIA consortium, Subhash Pandey, to determine whether the observed reduction in MB-COMT expression in AIE-exposed adult might relate to alterations in methylation. DNA was extracted from PrL-C tissue punches obtained from AIE-exposed and control rats (PD90) and the methylation at CpG sites of the MB-COMT promoter was examined. Two methylation sites in the promoter region were analyzed: a CpG-rich site in exon I, and an evolutionarily conserved CpG site in exon II that has been linked to neurodevelopmental disorders. As shown in Figure 2-7, methylation analysis revealed that AIE exposure was associated with a significant increase in methylation of the conserved CpG site of exon II ($t_{(15)} = 2.8$, $p = 0.013$) but no change in methylation of the CPG rich site of exon I ($t_{(15)} = 0.43$, $p = 0.671$).

The observed changes in DA metabolic enzyme expression suggest that AIE may alter DA levels in the PrL-C. As a follow up experiments, the effect of AIE on total tissue levels of catecholamines in the mPFC was assessed. PrL-C tissue was dissected from acute brain slices and sent to Vanderbilt University Neurochemistry Core, and subsequent HPLC analysis was conducted. This analysis revealed that AIE had no effect on protein levels of any of the catecholamines tested (Figure 2-8).

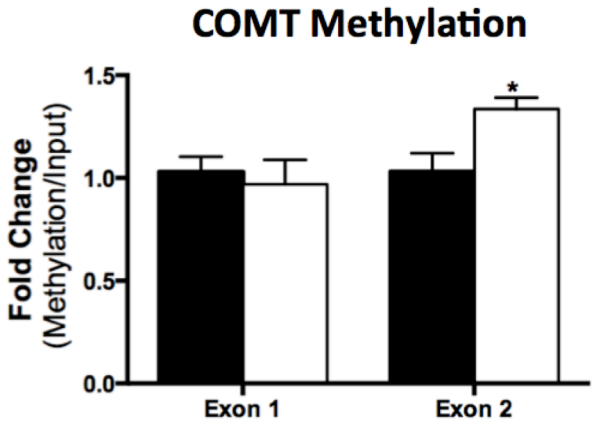

Figure 2-7. Adolescent intermittent ethanol exposure increases COMT methylation in the adult PrL-C. AIE exposure significantly increased methylation at the Exon 2 promoter but not at the Exon 1 promoter (n = 9 animals/group; *p < 0.05). This work was done in collaboration with Subhash Pandey.

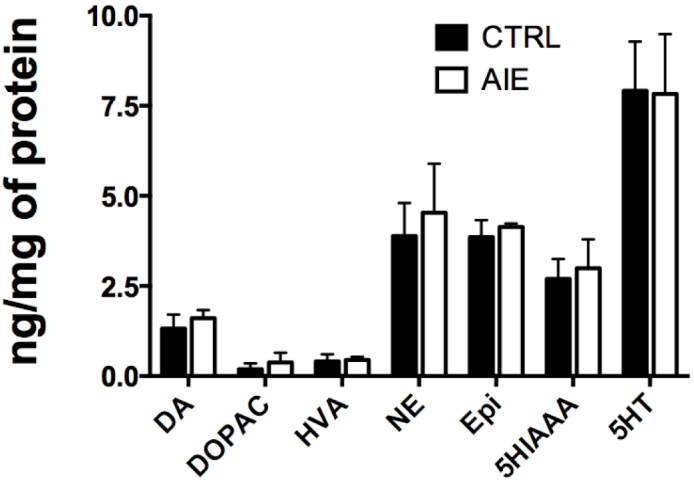

Figure 2-8. Adolescent ethanol exposure has no effect on whole-tissue catecholamine levels in the PrL-C. Tissue levels of dopamine (DA), DOPAC, HVA, norepinephrine (NE), epinephrine (Epi), 5HIAAA and serotonin (5HT) were all assayed. Tissue levels of all catecholamines and associated enzymes in the PrL-C were similar between control and AIE-exposed adult rats (Analyzed by Vanderbilt Neurochemistry Core).

DA plays an integral role in providing either an inhibitory or stimulatory influence on neuronal communication, and alterations in DA modulation specifically within the PFC have been shown to significantly alter PFC-dependent behaviors. Furthermore, DA receptor expression in the PFC has been observed to undergo dramatic developmental changes in the PFC (Tarazi and Baldessarini 2000). An initial set of studies from our group revealed a developmental increase in the percentage of layer V PrL-C pyramidal neurons that responded to D1 and D2 stimulation, which is consistent the observations of Tarazi and colleagues. Together, evidence suggests extensive development and refinement occurs during adolescent alcohol exposure. Therefore the next series of studies used patch-clamp electrophysiology in the acute slice preparation to examine the effect of AIE on D1, D2 and D4 receptor modulation of pyramidal neurons in layer V in the adult PrL-C. As shown in Figure 2-9, the enhancement of evoked firing observed in control slices in response to stimulation of D1 receptors by SKF38393 (5 μM; $t_{(9)} = 2.7$, $p = 0.024$; Figure 2-9) was absent in PrL-C slices obtained from AIE-exposed adult rats (96.5 ± 5.72% of baseline, control versus AIE interaction $F_{(1,15)} = 5.4$, $p = 0.034$, control post-hoc $p < 0.05$; Figure 2-7B). In contrast, the attenuation of firing normally observed with stimulation of D2 receptors by quinpirole (10 μM) and D4 receptors with PD168077 (40 μM) was similar in AIE-exposed adults and controls (quinpirole; $F_{(1,15)} = 22.8$, $p = 0.0002$, post-hoc $p < 0.01$ for controls, $p < 0.05$ for AIE, PD168077: $F_{(1,12)} = 30.9$, $p = 0.0001$, post-hoc $p < 0.01$ for control and AIE; Figure 2-9B). Therefore, these observations reveal an AIE-induced attenuation of the function of D1, but not

D2/D4, receptors in the adult PrL-C. The effect of AIE was specific to D1 modulation of evoked firing, as AIE had no effect on other intrinsic properties of layer V PrL-C pyramidal neurons (Table 2-1).

Table 2-1. Intrinsic properties of layer V PrL-C pyramidal neurons.

	I_h (sag+rebound ADP)*	V_{rest} (mV) #	R_{in} (MOhms)&
Controls	2.5 ± 0.4	-66.2 ± 2.2	123.4 ± 12.6
AIE	2.4 ± 0.3	-64.3 ± 2.4	118.9 ± 7.9

*I_h current

#Resting membrane potential (V_{rest})

&Input resistance (R_{in})

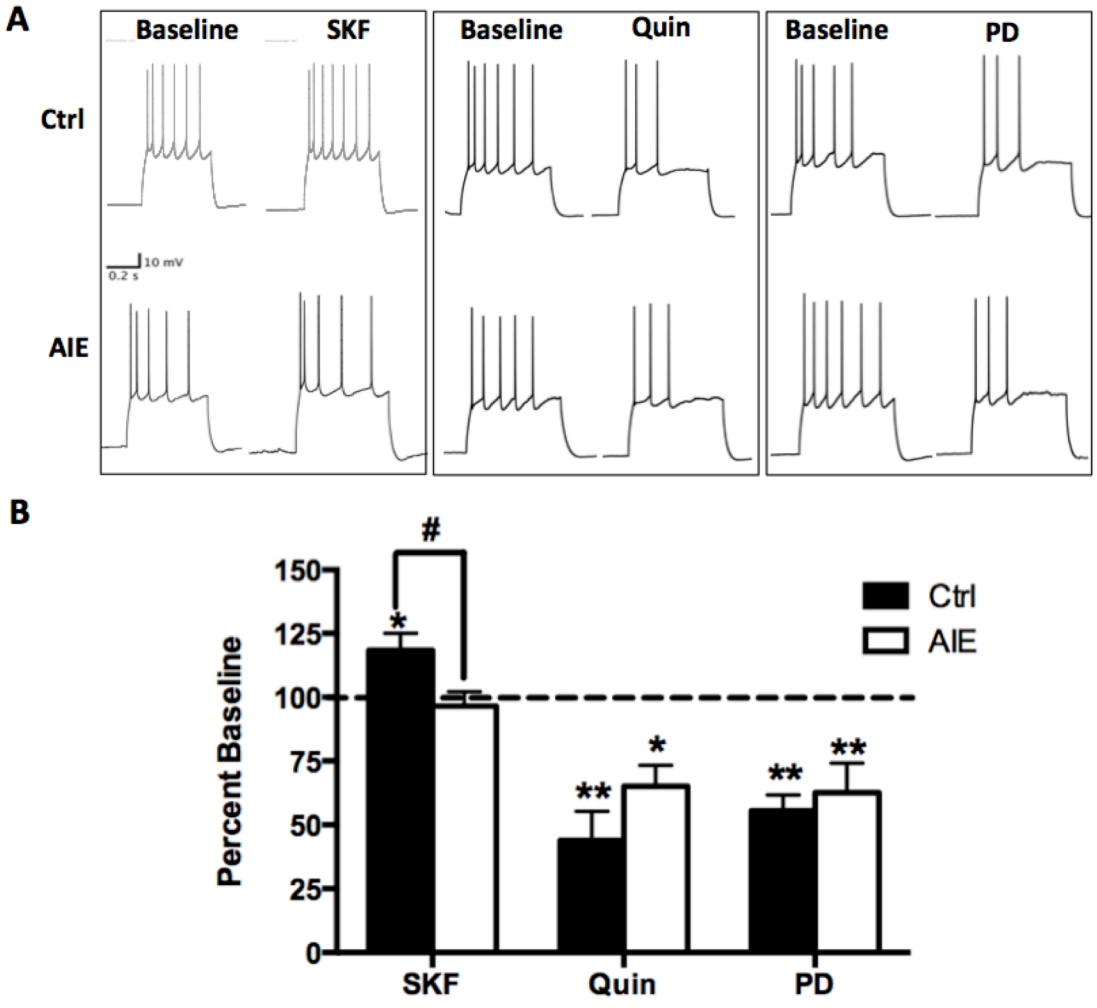

Figure 2-9. Adolescent intermittent ethanol exposure results in a loss of D1 receptor modulation of firing frequency in the adult PrL-C. A) Representative traces from recordings of evoked firing frequency before and after D1 (SKF38393 5 μM), D2 (Quinpirole, 10 μM) or D4 (PD168077, 40 μM) receptor stimulation. Top panel represents cells obtained from control rats. Bottom panel represents cells obtained from AIE-exposed rats. B) AIE exposure results in a loss of D1 receptor modulation of firing frequency in the adult PrL-C ($^{\#}p < 0.05$, Ctrl vs AIE; $^{*}p < 0.05$ and $^{**}p < 0.01$, drug versus its respective baseline; n = 6-10 cells/group).

DISCUSSION

The major finding in this chapter is that binge-like alcohol exposure during adolescence results in alterations in dopaminergic neurotransmission in the PrL-C of the adult. The observed AIE-induced increase in spine density could reflect impaired pruning and altered synaptic connectivity that could relate, at least in part, to alterations in dopaminergic neurotransmission in AIE-exposed adult PrL-C. AIE resulted in a persistent reduction in expression of MB-COMT and TH that are both important regulators of DA neurotransmission in the mPFC. Furthermore, AIE resulted in a loss of D1 receptor modulation of pyramidal cell firing. In light of the critical role that D1 receptor signaling plays in the organization of cognitively-modulated neural networks in the PFC, deficits in D1 receptor signaling in the PrL-C of the AIE-exposed adult may contribute to impairments in top-down cognitive control of behavior.

Compared to the adult PFC, the adolescent PFC has a higher density of dendritic spines and a larger population of immature spines that are thought to represent unstable and malleable synaptic connections (Arnsten et al. 2012). During the transition to adulthood, synapses in the PFC are formed into more stable, mushroom type spines or are pruned back as the cortical circuitry and neural networks are refined (Dumitriu et al. 2010; Petanjek et al. 2011). In the present study, AIE exposure resulted an increase in the density of dendritic spines of layer 5 pyramidal neurons in the adult PrL-C. Further analysis revealed this increase was due to an increase in the number of long thin type of spines. Adolescence is a critical period of dendritic pruning in the PFC in which the

number of excitatory synapses and spines is significantly reduced and the neurocircuitry undergoes experience-dependent refinement. An increase in spine density of long thin spines in the AIE exposed adult PrL-C may reflect an impairment of the normal pruning process and altered structural plasticity. Computer modeling studies indicate that network synchronization is critically dependent on the number of mature, excitatory synapses (Gourley et al. 2012) and the observation of an increase in the relative density of unstable and immature spines in the AIE-exposed adult would be expected to impact cortical function.

Efficient information processing by neural networks of the mPFC is critically dependent upon the extracellular concentration of DA. By modulating intrinsic cellular excitability and synaptic NMDA, AMPA and $GABA_A$ currents, DA controls the balance between inhibitory and excitatory neurotransmission via D1 and D2 receptors to optimally tune neural networks (Zheng et al. 1999; Gonzalez-Islas and Hablitz 2003; Trantham-Davidson et al. 2004; Tseng and O'Donnell 2004; Borgers et al. 2012). Unlike the striatum, where extracellular DA is primarily regulated by reuptake through the DA transporter (DAT), the mPFC expresses very little DAT. Instead, the concentration of extracellular DA in the mPFC is controlled equally by reuptake through the norepinephrine transporter and metabolism by MB-COMT (Sun et al. 2005). A previous study examining changes in mRNA in whole brain demonstrates that levels of COMT mRNA in adult AIE-exposed rats were approximately one-half of that found in naive adult rats (Kaenmaki et al. 2010; Coleman et al. 2011). We measured the levels MB-

COMT protein and, consistent with observations of mRNA in whole brain, AIE-exposure induced a similar reduction in MB-COMT expression that persisted for at least 6 months of age. MB-COMT expression is highly regulated by changes in methylation of its promoter region (Murphy et al. 2005; Abdolmaleky et al. 2006), and an increase in methylation typically decreases transcription. Interestingly, methylation of Exon II of the MB-COMT promoter was enhanced in the adult PrL-C of AIE-exposed rats compared to litter-matched controls. Altered methylation at this site has been linked to cognitive impairments observed in schizophrenia (Coleman et al. 2011).

In addition to the above findings, in collaboration with the NADIA consortium immunohistochemistry core at the University of North Carolina Chapel Hill, TH levels in the PrL-C were examined. These studies revealed an AIE-induced reduction TH expression in the PrL-C of adult AIE-exposed rats compared to control rats. Since TH is the rate-limiting enzyme in DA synthesis, a reduction in TH expression in the PrL-C of the AIE-exposed adult rat could reflect reduced DA synthesis. However, while changes in MB-COMT and TH staining strongly suggest that AIE exposure altered dopaminergic innervation of the mPFC, how this impacts extracellular DA is not clear. A reduction in MB-COMT would be expected to result in reduced degredation of released DA, and thus increased levels of extracellular DA. A reduction of TH expression could have an opposing effect by reducing DA synthesis and release. Although speculative, the data suggest that the reduction of TH expression may represent a compensatory down-regulation in response to increased extracellular DA associated with

reduced expression of MB-COMT. To begin to address this question, total biogenic amine levels in whole tissue extracts from mPFC were examined. Although AIE had no effect on total tissue levels of the catecholamines tested, potential changes in the basal level of extracellular DA is not known.

During adolescence, there is a marked increase in behaviors that are considered to be markers of the adolescent phenotype that include increased impulsivity and enhanced reinforcement learning (van der Knaap et al. 2014). DA inputs to the mPFC drastically increase during adolescence (Lambe et al. 2000; Spear 2000) and D1 receptor expression increases in mPFC neurons that project to the nucleus accumbens (Andersen et al. 2000; Lewis and Gonzalez-Burgos 2000). Previous studies examining DA receptor mRNA expression revealed that D1-, D2-, and D4- DA receptors in the PFC significantly increase during adolescence (Brenhouse et al. 2008), and unpublished data from our lab (Centanni et al., in prep) suggests that the number of pyramidal neurons in the mPFC that can be modulated by D1 and D2 receptors also dramatically increases during adolescence. This observation is consistent with the suggestion that developmental changes in the dopaminergic system in the PFC during adolescence render it especially susceptible to the adverse effects of excessive alcohol exposure that result in protracted deficits in dopaminergic function in adulthood.

Consistent with the suggestion that AIE exposure disrupts development of dopaminergic neurotransmission in the mPFC was the observation of a reduction of PrL-C D1 receptor function. A reduction in both MB-COMT and D1 receptor

function are consistent with human studies showing that COMT activity is positively correlated with D1 receptor availability. This positive correlation was observed in the dorsolateral PFC, but there is no correlation in the striatum where DAT, and not COMT, is responsible for removal of extracellular DA (Tarazi and Baldessarini 2000). Early studies of D1 receptor modulation of cognitive function indicated that there is an “inverted U” shaped dose-response relationship for the effects of D1 stimulation on working memory such that both high and low levels of stimulation negatively impact performance (Arnsten 1997; Zahrt et al. 1997; Slifstein et al. 2008). More recent studies highlight the importance of D1 receptor stimulation on other aspects of cognition such as behavioral set shifting and decision-making. However, the dose-response function for these other measures does not conform to the traditional U-shaped model that was initially conceptualized for working memory function (Druzin et al. 2000; Floresco and Magyar 2006; Williams and Castner 2006). The commonality between these studies is that D1 receptor activity is critical for optimal executive functioning that may contribute to the impairments we observed in adult rats exposed to alcohol during adolescence.

Taken together, the results demonstrate that adolescent binge-like exposure to ethanol results in compromised dopaminergic function in the adult mPFC. These studies identified structural, biochemical, epigenetic, physiological, and cognitive changes in the DA system in the PrL-C that can disrupt dopaminergic tone, network synchronization, and executive function. These findings shed light on potential lasting effects of adolescent binge drinking and

highlight the need to better understand the damaging effects that accompany adolescent alcohol abuse. The observed effects of AIE on the DA system in the PFC and the extensive modulatory role DA has on the balance of excitation/inhibition, coupled with the lack of AIE effect on glutamate receptor expression and function suggest that AIE may alter GABAergic neurotransmission in the PrL-C. Therefore subsequent studies examined the effects of AIE on the inhibitory GABA system in the PFC.

CHAPTER 3

ADOLESCENT ALCOHOL EXPOSURE PRODUCES DEFICITS IN DELTA $GABA_A$ RECEPTOR MEDIATED TONIC CURRENTS IN THE ADULT PRELIMBIC CORTEX

BACKGROUND AND SIGNIFICANCE

Stimulation of $GABA_A$ receptors generates two forms of neurotransmission: a rapidly desensitizing phasic current that is principally associated with activation of synaptic receptors, and a non-desensitizing tonic current that is associated with activation of extrasynaptic receptors (Farrant and Nusser 2005). Accumulating evidence indicates that the tonic current is especially important in modulating neurodevelopment and plasticity (Ben-Ari et al. 2007; Wang and Kriegstein 2009). The δ-subunit, which typically partners with an α4- and β-subunit, is localized almost exclusively to the extrasynaptic

membrane and mediates the bulk of the tonic current (Moss and Smart 2001; Drasbek and Jensen 2006; Zheleznova et al. 2009; Nishikawa et al. 2011). The non-desensitizing nature and high affinity for GABA allows δ-subunit containing receptors to be activated by ambient levels of neurotransmitter present in the extrasynaptic space (Barbour and Hausser 1997; Kullmann 2000; Telgkamp et al. 2004).

Recent studies from our lab and others have demonstrated that adolescent alcohol exposure results in long-lasting deficits in many behaviors including working memory, behavioral flexibility, decision-making, and learning and memory (Oscar-Berman and Marinkovic 2003; Chambers et al. 2004; White and Swartzwelder 2005; Masten et al. 2008; Gass et al. 2014). However, the cellular mechanisms that underlie these cognitive deficits are poorly understood. In the studies described in Chapter 2, AIE resulted in changes to several components of the DA system in the PFC, without altering the glutamate system. In addition to dopaminergic modulation of neuronal activity, GABAergic inhibitory neurotransmission plays a critical role in regulation of neuronal function and behavioral control. Furthermore, $GABA_A$ currents are important in neurodevelopment and neuroplasticity and the high sensitivity of the developing PFC to environmental insults. The observed effects of AIE on the DA system, led to the hypothesis that AIE exposure alters the development of GABAergic neurotransmission is the PFC. Thus, the present study investigated the impact of binge-like adolescent alcohol exposure on GABAergic neurotransmission in the PFC.

MATERIALS AND METHODS

Animals and adolescent intermittent ethanol exposure

Long-Evans rats were obtained from our in house breeding colony (breeder stock was obtained from Charles River). Rats were weaned, housed and handled as described in Chapter 2. Unless otherwise noted, all studies were conducted in male rats and a litter size consisted of at least 4 males in order to be included in the study. The AIE exposure model used in this chapter was the same as the model described in detail in the previous chapter and in previous publications (Gass et al. 2014).

Electrophysiological recordings

Acute brain slices (300 μm thickness) containing the medial PFC (mPFC) were obtained at different ages (as indicated in the results section) for electrophysiological recordings as previously described (Gass et al. 2014). In brief, the brain was immediately removed following isoflurane-induced anesthesia and rapid decapitation, and subsequently placed in ice-cold artificial cerebrospinal fluid (ACSF) dissection buffer containing (in mM): 125 sodium chloride (NaCl), 2.5 KCl, 1.25 monobasic sodium phosphate ($NaHPO_4$), 25 $NaHCO_3$, 4 $MgCl_2$, 1 $CaCl_2$, 10 d-glucose, 0.4 ascorbic acid, and 2 kynurenic acid. Slices were maintained in this solution at 34-36 °C and continuously aerated with 5% carbon dioxide/95% oxygen before being transferred to the

recording chamber. Following at least 1 hour of recovery, slices were transferred to a submerged recording chamber and bathed with oxygenated recording ACSF containing (in mM): 125 NaCl, 2.5 KCl, 25 $NaHCO_3$, 2 $CaCl_2$, 1.3 $MgCl_2$, 10 glucose, 0.4 ascorbic acid. The pH of all solutions listed above was adjusted to 7.4 and osmolarity was measured to be approximately 300 mOsm.

Recordings were made with a Multiclamp 700B amplifier (Molecular Devices, Union City, California), connected to a computer running Windows 7 and Axograph X software (Axograph, Sydney, Australia) and later analyzed offline. Unless otherwise specified, all recordings were obtained from pyramidal neurons in layer V of the prelimbic region of PFC (PrL-C) (Paxinos and Watson 2005) identified visually with infrared-differential interference contrast optics and video-microscopy. For voltage-clamp experiments, recording electrodes (3-6 MΩ resistance) were filled with an internal solution containing (in mM): 135 CsCl, 2 $MgCl_2$, 10 HEPES, 1 EGTA, 4 NaCl, 2 NaATP, 0.3 Tris-GTP, 10 phosphocreatine, and 5 QX-314 Cl. The inclusion of QX-314 Cl in the internal solution was used to block both voltage-sensitive Na^+ channels and $GABA_B$ receptor stimulation of K^+ channels (Nathan et al. 1990). For all experiments, dl-APV (50 μM) and CNQX (10 μM) were added to the recording ACSF to block NMDA and AMPA currents, respectively. The pH was adjusted to 7.4 using KOH and osmolarity was ~285 mOsmols. Series resistances (<20 MΩ) and input resistances were continually monitored throughout the experiment via a 1 mV (100 ms) hyperpolarizing pulse and cells with a change of more than 20% were discarded from our analysis. IPSCs were evoked using a tungsten bipolar

stimulating electrode placed in layer V within 200 μM of the soma of the pyramidal neuron being recorded. The stimulating electrode was connected to a Grass S88 stimulator and stimulus isolation unit. An input-output relationship was obtained by varying the intensity of stimulation from an amount that produced no response up to an amount that produced a response of maximal amplitude regardless of further increases in stimulus intensity. The stimulus intensity was then reduced to elicit a response that was approximately 75% of the maximal amplitude, and evoked responses were then measured at holding potentials of -70 mV.

Western Blotting

The expression of $GABA_A$ subunits in tissue obtained from punches of the PrL-C of control and AIE exposed rats were determined by western blotting as previously described in Chapter 2 and previous studies (Trantham-Davidson et al. 2014). In brief, rats were lightly anesthetized using isoflurane followed by rapid decapitation at which point brains were immediately removed and immersed for 1-2 min in ice-cold phosphate buffered saline (pH 7.4). Brains were subsequently sectioned into 1 mm thick coronal slices using an adult rat brain matrix (ASI Instruments, Warren, MI) and punches were taken from the dorsal mPFC, which contains the PrL-C region (Figure 2-1). Tissue punches were then placed into ice-cold 2% lithium dodecyl sulfate (LDS) detergent, solubilized via sonication and stored at -80 °C until ready for processing. Protein concentration

was determined using a standard Bovine serum albumin (BSA) protein assay and analyzed using an iMark Microplate Absorbance Reader (Bio-Rad Laboratories, Hercules, CA). Once protein concentration of the sample was determined, an aliquot of each sample was diluted with NuPAGE 4X LDS sample loading buffer (Invitrogen Corp., Carlsbad, CA; pH 8.5) containing 500 mM dithiothreitol, and samples were then incubated for 10 min at 80 °C. The denatured proteins were then separated by gel electrophoresis using the Bis-Tris (375 mM resolving buffer and 125 mM stacking buffer, pH 6.4; 7.5% acrylamide) discontinuous buffer system with MOPS electrophoresis buffer (50 mM MOPS, 50 mM Tris, 0.1% SDS, 1 mM EDTA, pH 7.7). Protein was then transferred to Immobilon-P PVDF membranes (Millipore, Bedford, MA) using a semi-dry transfer apparatus (Bio-Rad Laboratories, Hercules, CA). After transfer, blots were washed with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBST) and then blocked with PBST containing 5% nonfat dried milk (NFDM) for 1 hour at room temperature with agitation. The membranes were then incubated overnight at 4 °C, and washed in PBST prior to 1 hour incubation at room temperature with horseradish peroxidase-conjugated secondary antibodies diluted 1:2000 in PBST. Membranes received a final wash in PBST and the antigen-antibody complex was detected by enhanced chemiluminescence (Immun-Star WesternC Chemiluminescence Kit, Bio-Rad Laboratories, Hercules, CA; Catalog # 170-5070) using a ChemiDoc MP Imaging system (Bio-Rad Laboratories, Hercules, CA). Antibodies targeting the $GABA_A$ subunits included α1 (1:2000; NeuroMab Antibodies, CA; Catalog # 75-136), α4 (1:1000; Millipore

Corp., Billerica, MA; Catalog # AB5457), α5 (1:1000; PhosphoSolutions, Aurora, CO; Catalog # 846-GA5C), γ2 (1:1000; Millipore Corp., Billerica, MA; Catalog # AB5559) and δ (1:1000; PhosphoSolutions, Aurora, CO; Catalog # 868-GDN).

Surface expression of $GABA_A$ receptors containing the δ-subunit was assayed as previously described (Grosshans et al. 2002). Brains were sectioned and punches of mPFC were dissected as described above, except that tissue from each hemisphere was pooled. Tissue punches were then cut into 100 μm cubes using a McIlwain tissue chopper (Campden Instruments). One half of the tissue cubes were set aside for immunoblot analysis of total expression of $GABA_A$ subunits as described above, and the other half were used for measurement of surface expression of the δ-$GABA_A$ receptors. For these experiments, the tissue cubes were immediately transferred to ice cold ACSF containing the membrane impermeable cross-linking reagent bis(sulfosuccinimidyl)suberate (BS^3, Pierce #21850; 2.5 mM) and then incubated with gentle agitation for 30 min at 4 °C. Following this, the cubes were incubated for 10 minutes with ice-cold ACSF containing 20 mM glycine to quench the cross-linking reaction. The tissue was then rinsed three times with ACSF, solubilized in 2% LDS with brief sonication, and processed for immunoblot analysis as described above.

Immunohistochemistry

Layer-specific expression of the δ-subunit was determined in the PrL-C of slices prepared from control and AIE exposed adult rats following previously described methods (Trantham-Davidson et al. 2014) with minor modification. These studies were designed in collaboration with another member of our lab and conducted by said member. In brief, rats were transcardially perfused with PBS (pH 7.4) followed by perfusion with 4% paraformaldehyde. The brain was then rapidly removed from the skull and immediately immersed in 4% paraformaldehyde overnight followed by immersion in 30% sucrose for 48-72 hour. Brains were subsequently frozen on dry ice and sectioned coronally at 40 µm using a cryostat maintained at -22 °C. Free floating sections were stored in cryoprotectant at -20 °C until ready for immunohistochemical processing. Sections containing the PrL-C were processed for δ-$GABA_A$ receptor labeling using standard avidin-biotin complex (ABC) methods. Tissue was first rinsed in PBS followed by incubation in 1% H_2O_2 for 1 hour to inhibit endogenous peroxidases. Non-specific binding was blocked by incubation in PBS containing 0.4% Triton-X and 5% normal donkey serum for 1 hour. The tissue was then incubated overnight at room temperature in the same solution with the addition of the δ-$GABA_A$ receptor primary antibody (rabbit polyclonal, 1:500; PhosphoSolutions, Aurora, CO) followed by incubation in biotinylated donkey anti-rabbit secondary antibody (1:1000; Jackson ImmunoResearch) for 30 minutes. The antigen was then visualized by incubation in ABC (Vectastain Elite Kit; Vector Laboratories) for 1 hour followed by incubation in 0.05% 3,3-

diaminobenzidine, 0.05% ammonium nickel(II) sulfate hexahydrate and 0.015% hydrogen peroxide for 20 min. Sections were then mounted on Frost Plus slides, dried, dehydrated, and coverslipped in preparation for analysis. For each rat, optical density of δ–subunit immunolabeling was measured in four sections spanning the rostrocaudal extent of the PrL-C spaced 480 μm apart.

Data Analysis and Statistics

Westerns blots analysis involved identifying the band corresponding to the appropriate sized protein and quantifying the mean optical density using computer-assisted densitometry with ImageJ v1.41 (National Institutes of Health, USA). Immunohistochemistry images of δ-$GABA_A$ receptor staining intensity were obtained on a Leica EZ4C microscope and staining intensity from two 800 x 250 μm^2 regions of interest across layers I/II and VVI analyzed using ImageJ (NIH). Measurements within each subject were averaged across sections to obtain a single measure of staining intensity for layers I/II and layers V/VI. A two-way repeated-measures ANOVA (Treatment and Layer) was used to determine statistical significance ($p < 0.05$). Electrophysiology recordings were measured using Axograph X software. Spontaneous IPSC data was analyzed using MiniAnalysis software and subsequent Student's t-tests to determine statistical significance. Studies involving multiple developmental time points or multiple doses of a drug were analyzed using a two-way repeated measure ANOVA, followed by a Sidak's Multiple Comparisons test. For tonic current studies, a cell

was considered to have a tonic current if the change in holding current (I_{hold}) in response to picrotoxin was ≥ 5 pA. I_{hold} was determined by creating 5 pA bins of all data points, determining the mean and filtering out data points > 75 pA away from the mean (to eliminate large sIPSC contribution to the I_{hold}) All statistical analysis was performed in Prism (GraphPad Software) or SAS statistics program. Statistical significance level was $p < 0.05$, and data are presented as mean ± SEM.

RESULTS

In order to provide a quantitative measure of the level of intoxication achieved during the AIE procedure, a previously described 1-5 point intoxication rating scale was used (Gass et al. 2014). Intoxication scores measured at the end of each 14-hour ethanol exposure period and averaged across all 4 cycles for all rats (n = 98) revealed an overall level of intoxication of 2.67 ± 0.04 (rating of 2 = slight intoxication, 3 = moderate intoxication). Tail-vein blood drawn at the end of each exposure cycle revealed that the average BEC for each of the cycles was (in mg%) Cycle 1, 303.69 ± 12.47; Cycle 2, 290.56 ± 14.98; Cycle 3, 333.13 ± 16.69; and Cycle 4, 264.09 ± 13.4, and a grand average across all 4 cycles of 297.43 ± 7.22. Both the average intoxication rating and average BEC level were very similar to those obtained in the previous chapter (Figure 2-2).

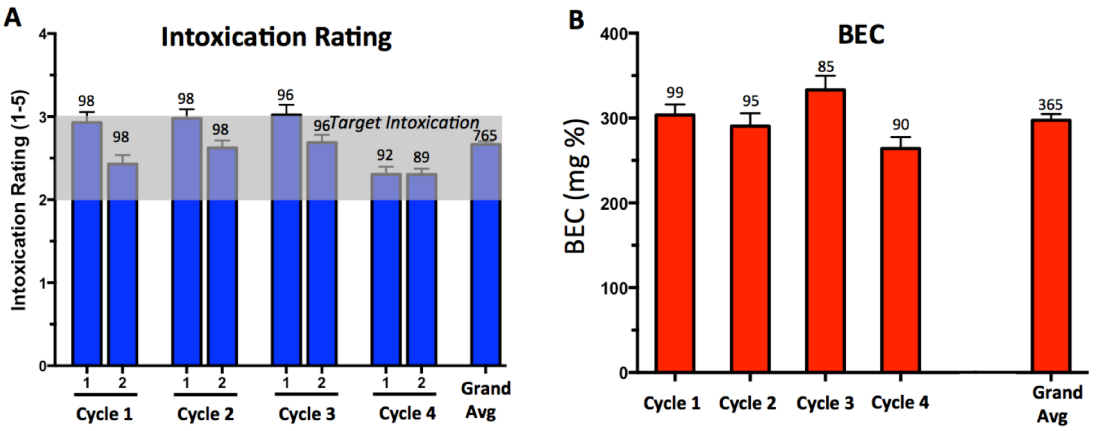

Figure 3-1. Intoxication ratings and blood ethanol levels for the rats used in the current chapter. A) Average intoxication rating for each exposure day. The target intoxication rating of 2-3 (gray) was determined for each cycle (total n = 765 measurements). **B)** Tail vein blood was drawn from each rat at the end of each cycle and blood ethanol content was determined (total n = 365 measurements).

Preliminary studies conducted in our lab examined the developmental changes in GABAergic neurotransmission in layer V pyramidal neurons of the PrL-C using acute slice patch-clamp electrophysiology. A typical voltage clamp (-70 mV clamp) recording consisted of a 15-minute baseline period followed by 20 minutes of bath application of the $GABA_A$ receptor antagonist picrotoxin (100 μM) in order to determine the amount of tonic current (Figure 3-2A). As shown in Figure 3-2B, both $GABA_A$ sIPSC's and $GABA_A$ receptor-mediated tonic currents were of similar amplitude when measured in slices obtained from rats across development and adulthood (age blocks = PD16-30, PD31-45, PD46-60, PD 61-75, and >PD75). There was, however, a change in the number of pyramidal neurons that exhibited a tonic current across development. As shown in Figure 3-2C, the percentage of neurons that exhibited a change in holding current in

response to picrotoxin was very low early in development (~20% at PD16-45). This percentage progressively increased during the transition from adolescence to adulthood such that virtually all cells recorded in slices obtained from fully mature adults exhibited a picrotoxin sensitive tonic current ($\chi^2_{(5)}$ = 19.6, p = 0.002). In contrast to changes in the tonic current, all cells recorded from across all age groups exhibited similar sIPSC amplitude and frequency and thus, unlike the tonic current, there were no developmental changes in synaptic $GABA_A$ currents.

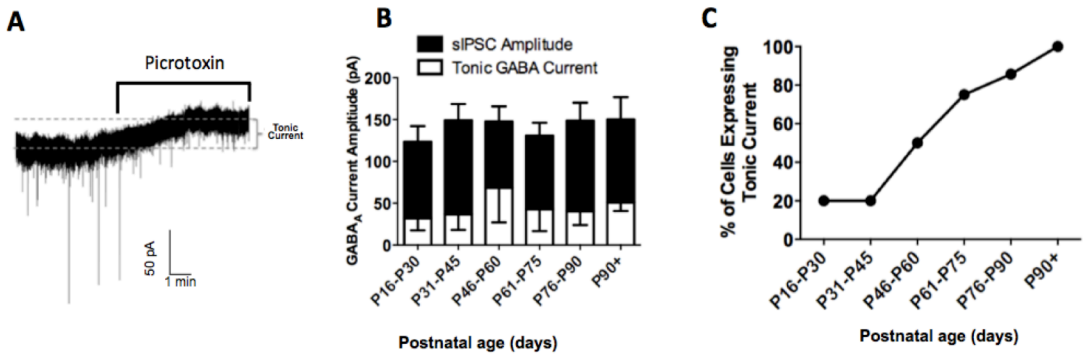

Figure 3-2. Expression of the $GABA_A$ tonic current is developmentally regulated in layer V pyramidal neurons of the PrL-C. A) Representative trace showing the measurement of tonic current as a change in holding current (I_{hold}) after bath application of the $GABA_A$ antagonist picrotoxin (100 μM, gray dashed line). **B)** Phasic (black) and tonic (white) $GABA_A$ currents measured at specific developmental time-points (PD: postnatal day) revealed that the amplitude of both tonic and phasic GABA currents remained stable throughout development. **C)** Although there was no developmental change in tonic current amplitude, the percentage of cells expressing a tonic current exhibited a dramatic developmental increase (p = 0.002) (Courtesy of Heather Trantham-Davidson).

The next set of studies examined the effect of AIE exposure on both phasic and tonic GABAergic neurotransmission. Whole-cell patch-clamp analysis revealed that the frequency of the sIPSC's in control and AIE-exposed adult rats was not significantly different ($t_{(30)}$ = 0.06, p = 0.948), suggesting that AIE did not alter synaptic GABA release (Figure 3-3A-B). Consistent with this, neither the paired-pulse ratio ($t_{(16)}$ = 0.9586, p = 0.352; Figure 3-3C-D), nor the frequency of miniature IPSCs measured in the presence of TTX (1 μM) were affected by AIE exposure ($t_{(15)}$ = 0.333, p = 0.7438; Figure 3-3E-F). In contrast to the lack of an effect of AIE on presynaptic GABAergic neurotransmission, AIE significantly attenuated the amplitude of the sIPSC ($t_{(39)}$ = 3.1, p = 0.004) (Figure 3-3A-B), potentially indicating a reduction in the postsynaptic response to the action potential generated synaptic release of GABA. However, this reduction in amplitude of sIPSC's was not reflected by a similar reduction in the amplitude of miniature IPSCs ($t_{(15)}$ = 1.051, p = 0.3098; Figure 3-3C-D). As supported by additional experiments described below, this effect most likely reflects reduced activation of perisynaptic $GABA_A$ receptors during spillover of action potential dependent synaptic release of GABA. Other characteristics of the IPSCs were analyzed including rise time, decay time and area (Table 3-1). sIPSC area was significantly reduced in slices from AIE-exposed rats, which is likely attributed to the observed reduction in sIPSC amplitude.

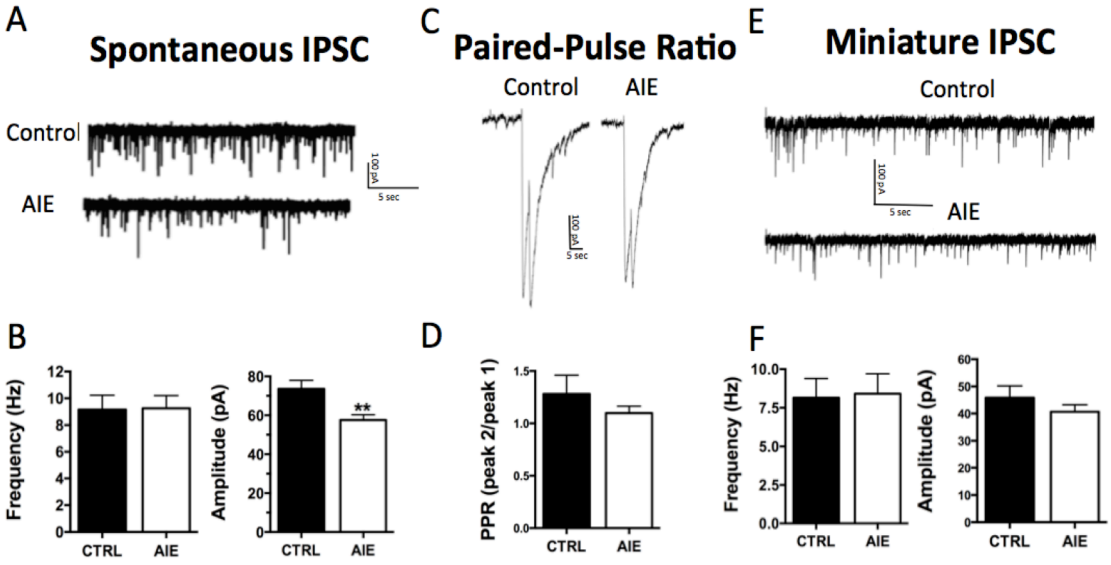

Figure 3-3. Effect of adolescent ethanol exposure on phasic $GABA_A$ currents in layer V pyramidal neurons of the adult PrL-C. A) Representative traces of IPSCs from adult PrL-C slice obtained from control and AIE exposed rats. Cells were voltage-clamped at -70mV. **B)** While the frequency was similar in slices from both control and AIE treated rats, spontaneous IPSC (sIPSC) amplitude was significantly reduced in slices from AIE-exposed rats (n = 21-22 cells/group; **p = 0.004). **C)** Representative paired-pulse traces of evoked IPSC (eIPSC) currents from adult PrL-C slices obtained from control and AIE treated rats. **D)** The paired-pulse ratio (PPR) was not altered by AIE exposure (n = 8-9 cells/group). **E)** Representative traces of miniature IPSCs (mIPSC) from adult PrL-C slices obtained from control and AIE exposure rats. **F)** AIE did not alter either the amplitude or frequency of mIPSC's (n = 8-9 cells/group).

Table 3-1 Summary of characteristics of spontaneous IPSC (sIPSC) and miniature IPSC (mIPSC) in control versus AIE-exposed adult PrL-C slices.

	sIPSC			mIPSC		
	Ctrl	**AIE**	**P Value**	**Control**	**AIE**	**P Value**
Amplitude (pA)	73.56 ± 4.38	57.49 ± 2.76	0.004**	45.89 ± 4.35	40.66 ± 2.67	0.31
Frequency (Hz)	9.16 ± 1.08	9.25 ± 0.96	0.948	6.02 ± 0.82	6.56 ± 1.34	0.743
Rise (ms)	1.79 ± 0.08	1.82 ± 0.07	0.921	2.54 ± 0.23	2.33 ± 0.09	0.393
Decay (ms)	3.73 ± 0.22	3.57 ± 0.19	0.593	5.37 ± 0.38	5.32 ± 0.25	0.923
Area (pAms)	286.8 ± 23.8	207.0 ± 21.6	0.026*	229.2 ± 22.4	204.5 ± 18.6	0.406

*p < 0.05, **p < 0.01 on an unpaired Student's t-test

To determine whether AIE altered the tonic $GABA_A$ current, changes in holding current were tested in response to bath application of picrotoxin in slices from rats of different developmental ages. A Gaussian distribution was fit to all-point histograms for each cell to determine the tonic current by subtracting the mean baseline holding current (I_{hold}) from the mean I_{hold} after 100 μM picrotoxin (Figure 3-4). Consistent with the data presented in Figure 3-2, of the cells that expressed a tonic current, there were no significant differences in the amplitude of the tonic current as a function of age ($F_{(3,22)}$ = 0.74, p = 0.538; Figure 3-4C). However, as shown in Figure 3-4, there was a significant difference in tonic current amplitude between control and AIE-exposed rats ($F_{(1,37)}$ = 32.2, p <

0.0001) at all three ages tested (PD45 ± 3 days, $t_{(9)}$ = 2.7, p = 0.025; PD60 ± 3 days, $t_{(5.415)}$ = 3.2, p = 0.021; PD90 ± 6 days, $t_{(8.421)}$ = 3.2, p = 0.012). Furthermore, AIE exposure also significantly attenuated tonic GABA currents in female rats ($t_{(13)}$ = 4.9, p = 0.0003; Figure 3-4D), suggesting that the observed AIE-induced reduction in tonic GABA current is not sex specific. Together, these data demonstrate that AIE exposure results in the blunting of $GABA_A$ receptor-mediated tonic current in layer V PrL-C pyramidal neurons that persists into adulthood.

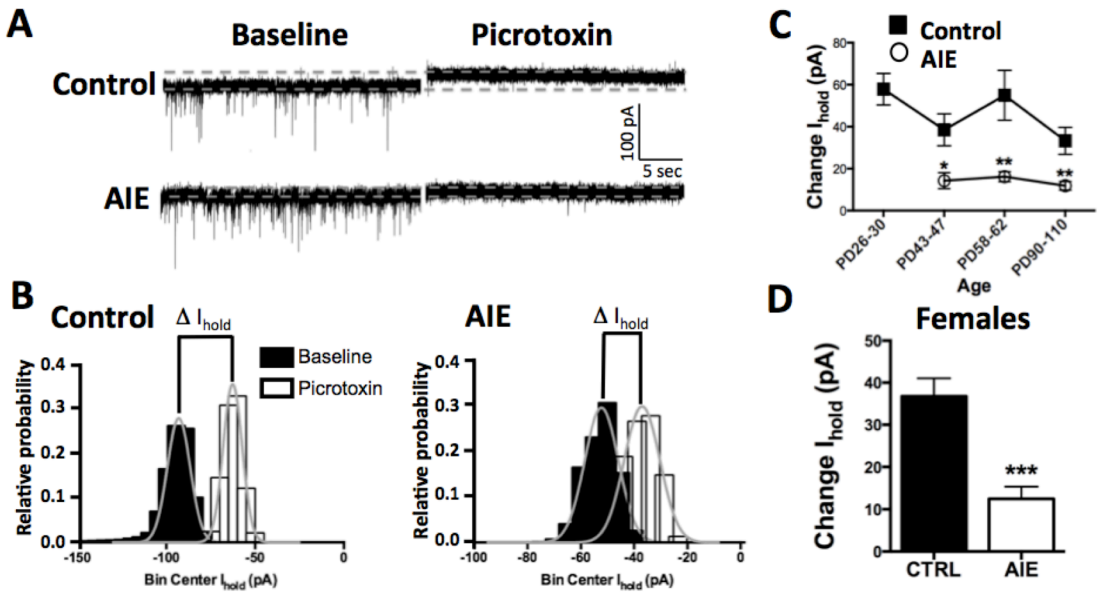

Figure 3-4. Adolescent ethanol exposure results in a reduction in the amplitude of the $GABA_A$-mediated tonic current. A) Representative trace showing tonic GABA current, which is measured as the magnitude of change in holding current that occurs after bath application of the $GABA_A$ antagonist picrotoxin (100 μM) in PrL-C slices obtained from control and AIE exposed rats. **B)** Representative all-point histograms and fitted Gaussian functions for the whole-cell current data showing the peak I_{hold} before and after picrotoxin in slices from control (left panel) and AIE (middle panel) rats. **C)** Slices obtained from male control rats revealed that the amplitude of the tonic $GABA_A$ current remained stable throughout development and AIE exposure significantly attenuated tonic GABA current at all time-points examined, including shortly after exposure (PD 45 ± 3 days; n = 5-6 cells/group; *p < 0.05), in early adulthood (PD 60 ± 3 days; n = 6-8 cells/group; **p < 0.01), and in the fully mature adult (PD90 ± 6 days; n = 8-10 cells/group; **p < 0.01). **D)** AIE exposure significantly attenuated tonic GABA current in layer V pyramidal neurons from adult female rats (n = 7-8 cells/group, ***p < 0.001).

One potential explanation for the observed AIE-induced reduction in tonic $GABA_A$ receptor-mediated current is a reduction in basal levels of extracellular GABA that could result in reduced tone on extrasynaptic $GABA_A$ receptors. To examine this possibility, $GABA_A$ receptor-mediated currents were measured following bath application of the GABA reuptake inhibitor nipecotic acid (500 μM). As shown in Figure 3-5, this resulted in a very large hyperpolarizing current that presumably reflected activation of all GABA receptors (i.e. synaptic and extrasynaptic). To determine the contribution of the $GABA_A$ component of the nipecotic acid induced current, picrotoxin was applied in combination with nipecotic acid. Once again, recordings taken from AIE exposed rats exhibited a significant reduction ($t_{(13)}$ = 2.3, p = 0.041) in the amplitude of the $GABA_A$ tonic current suggesting that AIE-induced attenuation of the tonic $GABA_A$ current was not the result of reduced levels of ambient GABA, and instead most likely reflects altered function and/or availability of extrasynaptic $GABA_A$ receptors.

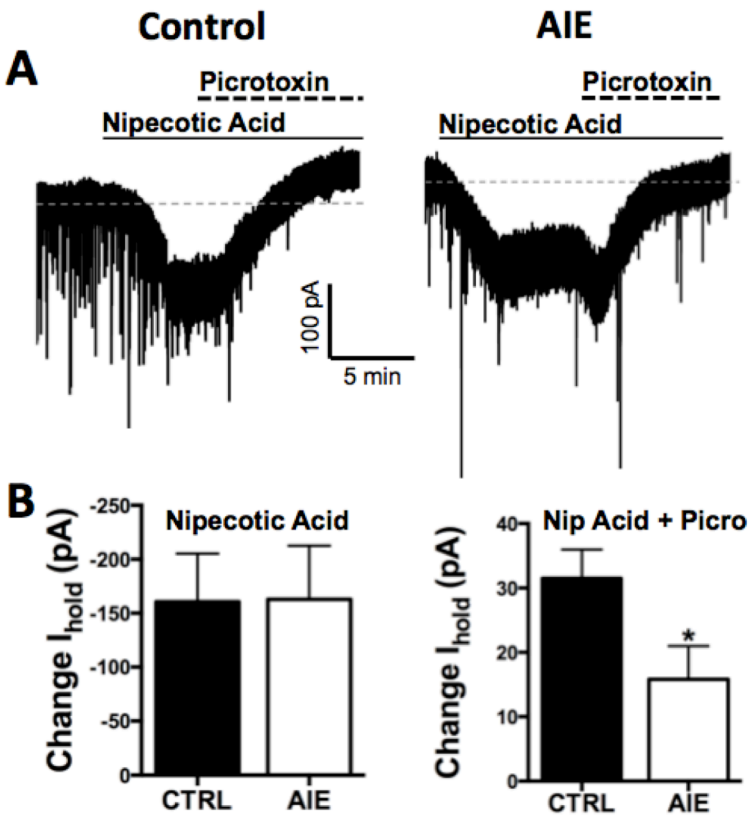

Figure 3-5. Blocking GABA reuptake does not reverse AIE-associated reduction in tonic GABA currents. A) Representative trace showing changes in holding current that occur following bath application of nipecotic acid in slices from the adult PrL-C. After establishing a stable baseline holding current, nipecotic acid (500 μM) was bath applied for 5 minutes. Next, picrotoxin (100 μM) was also added to the bath. The tonic current was measured as the change in I_{hold} from baseline (gray line) to that observed after picrotoxin. **B)** AIE exposure resulted in a significant decrease in the amplitude of the $GABA_A$ tonic current amplitude compared to slices from control rats (n = 6-7 cells/group; *$p < 0.05$).

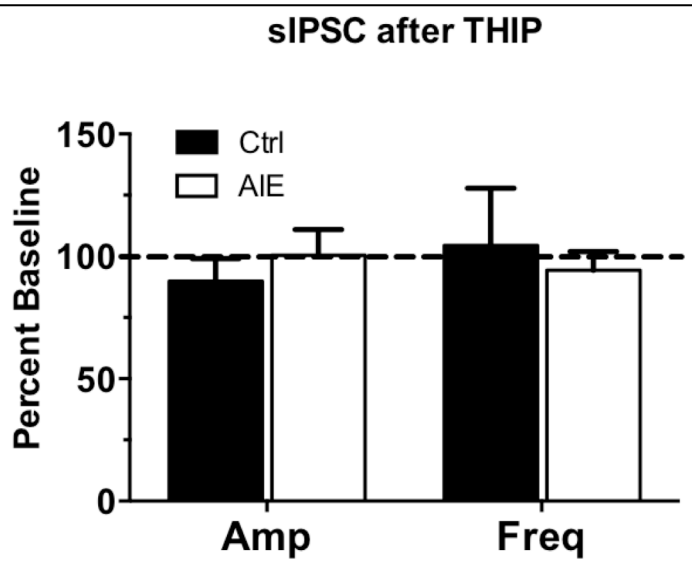

Figure 3-6. THIP acts exclusively on extrasynaptic $GABA_A$ receptors. The δ-$GABA_A$ receptor agonist THIP did not affect spontaneous IPSC (sIPSC) amplitude or frequency in slices from control and AIE-exposed rats, providing evidence for the selectivity of the drug for extrasynaptic δ-$GABA_A$ receptors.

Delta-subunit containing $GABA_A$ receptors are thought to be localized almost exclusively to the extrasynaptic membrane. Together with α5-containing extrasynaptic $GABA_A$ receptors, these receptors mediate the majority of the GABA receptor-mediated tonic current in layer V neurons in the mPFC (Moss and Smart 2001; Drasbek and Jensen 2006; Zheleznova et al. 2009; Nishikawa et al. 2011). To determine whether the AIE-induced attenuation of tonic current reflected a reduction in δ-$GABA_A$ receptor-mediated currents specifically, the δ-$GABA_A$ receptor agonist 4,5,6,7-tetrahydroisoxazolo[5,4-*c*] pyridin-3-ol hydrochloride (THIP, 1 μM) was bath applied to induce tonic currents in slices obtained from both control and AIE-exposed rats across development. THIP also

has affinity for non δ-containing α4-$GABA_A$ receptors that can be located near the synapse and contribute to the tonic current (Liang et al. 2007). To confirm specificity of THIP for extrasynaptic $GABA_A$ receptors, sIPSC's were examined before and after bath application of THIP. The results verified that THIP has no effect on either sIPSC amplitude ($F_{(1,8)}$ = 1.68, p = 0.2310) or frequency ($F_{(1,8)}$ = 0.26, p = 0.625; Figure 3-6), confirming its selectivity for extrasynaptic δ-$GABA_A$ receptors.

Gaussian distributions were fit to all-point histograms for each cell and used to determine THIP-mediated current by subtracting the mean baseline I_{hold} from the mean I_{hold} after 1 μM THIP (Figure 3-7). In slices obtained from control rats, progressive increase in the amplitude of THIP-mediated currents was observed from PD28 to PD90 ($F_{(3,22)}$ = 4.362, p = 0.015). This is in contrast to the studies shown in Figure 3-4 in which the amplitude of the $GABA_A$ (picrotoxin sensitive) tonic current did not change across development. Examination of the effect of AIE on the developmental time-course of δ-$GABA_A$ currents revealed a significant age-dependent effect. No between group differences were observed in the amplitude of δ-$GABA_A$ tonic current in slices obtained shortly after the last cycle of ethanol exposure (PD43-PD47; $t_{(8)}$ = 0.29, p = 0.783) or at the onset of adulthood (PD58-PD62; $t_{(12)}$ = 1.35, p = 0.203; Figure 3-7). In contrast, in fully mature adult AIE-exposed rats, the amplitude of the δ-$GABA_A$ current was significantly attenuated compared to controls (PD90-PD100; $t_{(17)}$ = 2.21, p = 0.041). These data indicate that early in development, GABA mediated tonic

current is facilitated by $GABA_A$ receptors that do not contain the δ-subunit (e.g. containing α5), whereas later in development and into adulthood a shift occurs towards increasing involvement of δ-subunit containing $GABA_A$ receptors. Importantly, this developmental emergence of the δ-$GABA_A$ receptor-mediated tonic current is significantly attenuated by AIE exposure. Because levels of $GABA_A$ receptors, specifically those that mediate the tonic current, have been shown to be hormonally regulated and can fluctuate throughout the estrous cycle, the next set of studies tested whether or not the effect of AIE on δ-$GABA_A$ receptor-mediated tonic currents was sex specific. Thus, THIP-mediated currents were measured in slices from control and AIE-exposed adult female rats. As shown in Figure 3-7D, AIE significantly attenuated THIP-mediated currents in AIE-exposed adult female rats ($t_{(13)}$ = 4.0, p = 0.002), indicating that the effect of AIE on THIP-induced tonic $GABA_A$ currents is not sex specific.

Table 3-2. Developmental expression of tonic and δ-$GABA_A$ receptor currents in layer V pyramidal neurons in the PrL-C

	Cells expressing a tonic current (%)		Cells expressing a δ-$GABA_A$ current (%)	
	Control	**AIE**	**Control**	**AIE**
PD45	50	55.56	83.33	83.33
PD60	60	66.67	80	77.78
PD90+	93.33	90.91	90.91	91.67

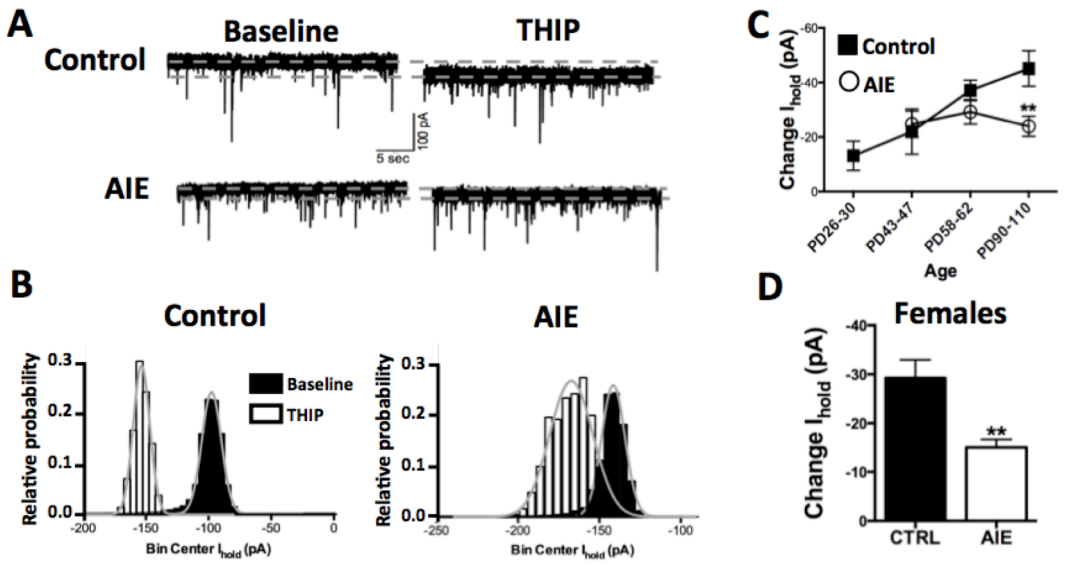

Figure 3-7. The decrease in the tonic $GABA_A$ current following adolescent ethanol exposure reflects attenuation of current mediated by δ-subunit containing receptors. A) Representative trace showing the effect of bath application of the selective δ-$GABA_A$ receptor agonist THIP**.** After stable baseline was obtained, a low dose of the $GABA_A$ antagonist gabazine (0.5 μM) was used to block activation of synaptic $GABA_A$ receptors in order to isolate extrasynaptic $GABA_A$ currents (not shown in trace). Following the application of gabazine, THIP (1 μM) was bath applied. **B)** Representative all-point histogram and Gaussian function that were used to measure the effect of THIP as a change in the I_{hold} for each individual cell. **C)** THIP-mediated change in the I_{hold} in PrL-C slices obtained from control and AIE exposed rats at different developmental ages. In slices from rats prior to initiation of AIE exposure (PD28, n = 5 cells/group), shortly after the last cycle of AIE exposure (PD45, n = 5 cells/group), and in early adulthood (PD60, n = 7 cells/group) there were no significant differences in THIP modulation of holding current. However, in slices from adult rats, there was a clear reduction in the THIP mediated current (n = 9-10 cells/group, *p = 0.04). **D)** AIE exposure significantly attenuated THIP-mediated current in layer V pyramidal neurons from adult female rats (n = 6-9 cells/group **p < 0.01).

In agreement with figure 3-2, the developmental expression of tonic currents gradually increased from PD45-PD90 (Table 3-2). Interestingly in control slices, the percentage of cells expression a δ-$GABA_A$ receptor-mediated current did not significantly differ from PD45-PD90, suggesting these receptors are present and can be activated during adolescence, however their contribution to the tonic current is not fully evident until adulthood. The percentage of cells expressing a tonic current and a δ-$GABA_A$ receptor current was similar in control and AIE-exposed slices (Table 3-2).

Recent studies have shown that not only are there developmental changes in $GABA_A$ subunit expression within the PFC but also that these changes are layer specific, with different cortical layers exhibiting unique developmental $GABA_A$ receptor subunit profiles (Datta et al. 2014). Therefore the next set of studies examined the layer-specific effects of AIE on tonic $GABA_A$ receptor-mediated currents by recording from layer II/III pyramidal neurons in slices obtained from control and AIE exposed adult rats. As shown in Figure 3-8, slices obtained from both groups exhibited similar tonic current amplitudes ($t_{(8)}$ = 0.5, p = 0.655). In addition, in experiments using THIP to activate δ-$GABA_A$ receptors, AIE exposure had no effect on the amplitude of the tonic current ($t_{(9)}$ = 0.6, p = 0.571; Figure 3-8). Taken together, these data demonstrate that the AIE-induced reduction of $GABA_A$ receptor-mediated tonic currents is specific to layer V PrL-C pyramidal neurons.

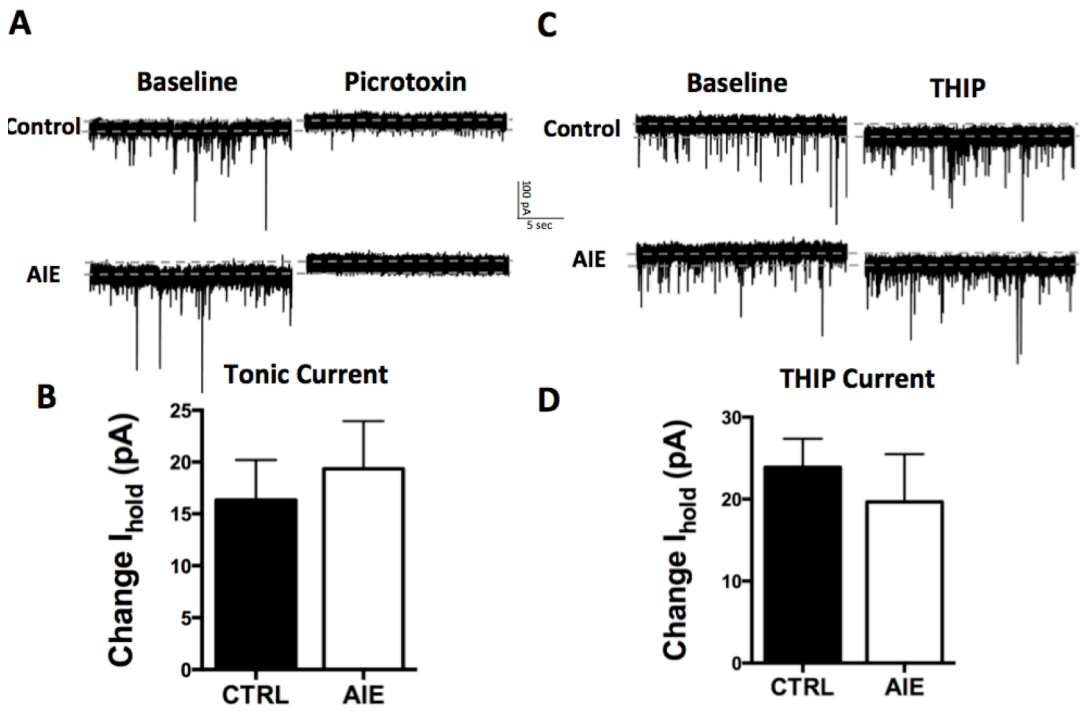

Figure 3-8. Adolescent ethanol exposure has no effect on the tonic $GABA_A$ current of pyramidal neurons in layer II/III of the adult PrL-C. A) Representative traces showing the effect of picrotoxin (100 μM) on $GABA_A$ tonic currents in layer II/III pyramidal neurons. **B)** AIE had no effect on picrotoxin-sensitive $GABA_A$ tonic current in pyramidal neurons of layer II/III (n = 4-6 cells/group). **C)** Representative traces showing current mediated by δ-subunit containing $GABA_A$ receptors in layer II/III pyramidal neurons measured using the δ-$GABA_A$ agonist THIP (1 μM). **D)** THIP-mediated changes in the holding current revealed that AIE had no effect on δ-$GABA_A$ current amplitude in layer II/III of PrL-C (n= 5-6 cells/group).

Acute ethanol enhances extrasynaptic $GABA_A$ tonic currents, and evidence suggests that this sensitivity to ethanol is imparted by the presence of the δ-subunit (Wallner et al. 2003; Breese et al. 2006). To determine whether the AIE-induced reduction in δ-subunit containing $GABA_A$ receptor-mediated tonic current was associated with a change in the acute sensitivity to ethanol, the next experiments determined the effect of bath application of ethanol on the $GABA_A$ tonic current in layer V PrL-C slices obtained from control and AIE-exposed adult rats. As expected, bath application of ethanol (10-100 mM) produced a dose-dependent facilitation of I_{hold} amplitude in slices from both control ($F_{(1.8,10.7)}$ = 33.1, $p < 0.0001$; Figure 3-9) and AIE-exposed rats ($F_{(1.5,10.5)}$ = 21.51, $p = 0.0004$). Post-hoc analysis revealed a significant reduction in I_{hold} in slices from AIE-exposed rats at 50 mM ethanol (Student's t-test; $t_{(14)}$ = 2.5, $p = 0.026$) and 100 mM ethanol (Student's t-test; $t_{(13)}$ = 3.3, $p = 0.005$), but not 10 mM ethanol (Student's t-test; $t_{(13)}$ = 0.87, $p = 0.399$; Figure 3-9) . As shown in Figure 3-9C, when the amplitude of the ethanol-facilitated current was expressed as a percentage of the amplitude of the average THIP-induced current (data from Figure 3-7), the percentage of the ethanol-facilitated increase was similar in control and AIE slices (Figure 3-9C). Confirming that the effect of ethanol was specific to tonic GABA currents, ethanol had no effect on sIPSC amplitude ($F_{(3,36)}$ = 0.57, $p = 0.637$) or frequency ($F_{(3,36)}$ = 0.39, $p = 0.395$; Figure 3-10) at any dose.

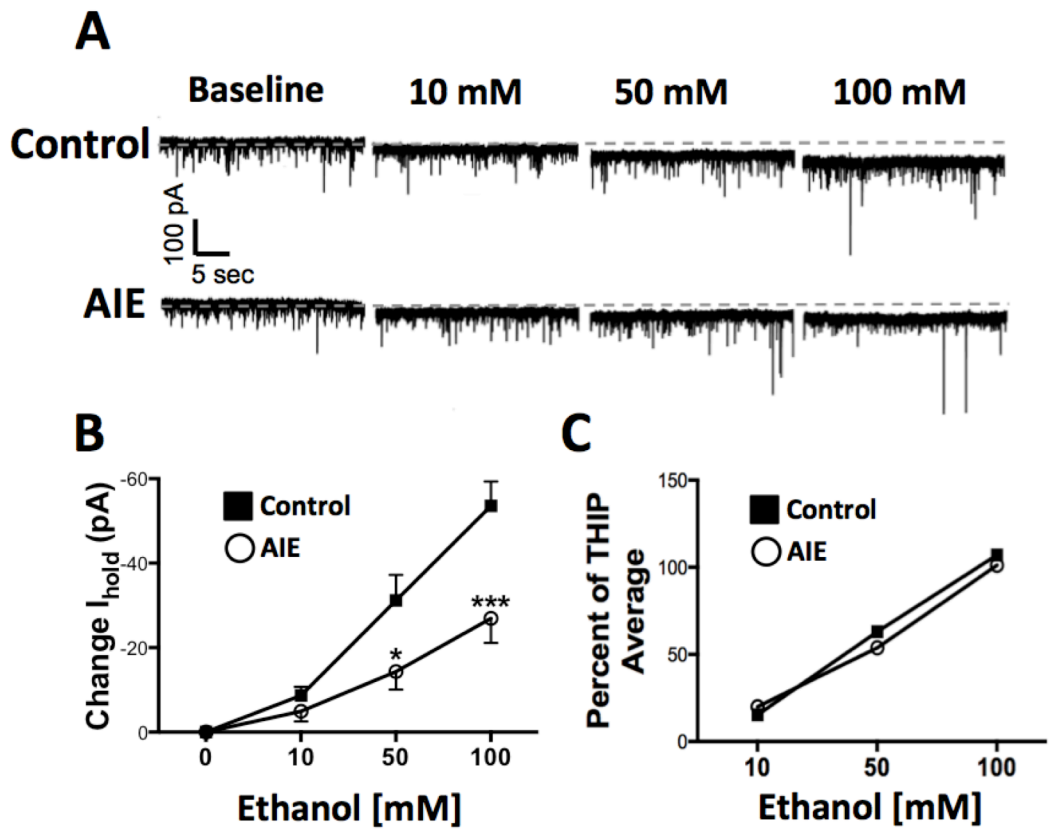

Figure 3-9. Adolescent ethanol exposure significantly decreases ethanol-induced facilitation of the tonic current in adult PrL-C. A) Representative traces showing the change in I_{hold} in layer V pyramidal neurons in response to bath application of ethanol (10 mM, 50 mM and 100 mM) in PrL-C slices obtained from control and AIE exposed rats. **B)** Application of ethanol resulted in a dose-dependent facilitation of the tonic current, especially at higher doses (n = 15 cells/dose, $p < 0.0001$). The enhancement of tonic current that occurred with bath application of 50 and 100 mM ethanol was significantly attenuated in slices obtained from AIE exposed rats compared to slices from control rats (n = 6-10 cells/group for each dose; *$p < 0.05$, ***$p < 0.001$). **C)** Comparison of ethanol facilitation of the tonic current to the average THIP current in control and AIE exposed conditions indicates that AIE did not alter the affinity of the δ-$GABA_A$ receptor to acute alcohol and instead reduced the efficacy of facilitation of tonic currents.

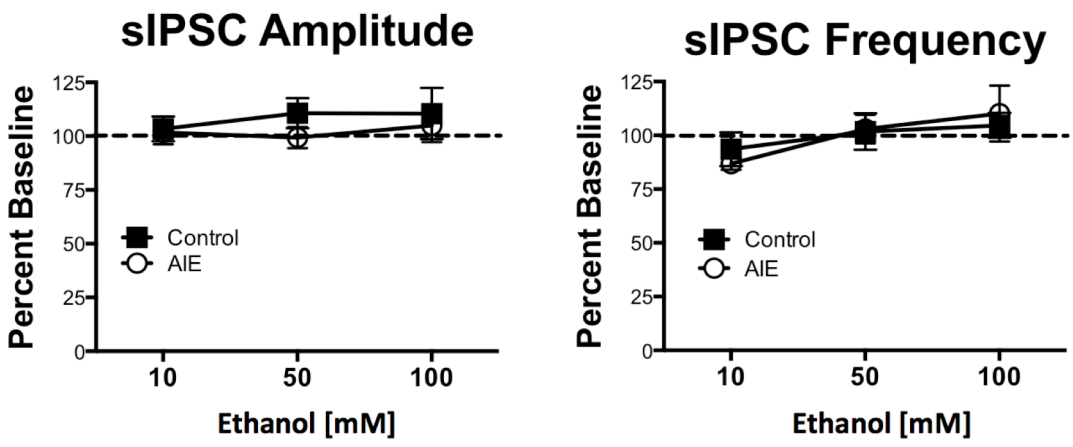

Figure 3-10. Ethanol does not alter phasic $GABA_A$ receptor mediated phasic currents. Application of increasing concentrations of ethanol did no affect spontaneous IPSC (sIPSC) amplitude (right) or frequency (left) in slices from control or AIE-exposed rats, suggesting that acute ethanol preferentially acts at extrasynaptic $GABA_A$ receptors that mediate the tonic current.

Since $GABA_A$ tonic currents are also sensitive to neurosteroids, which like alcohol also appear to act as positive allosteric modulators of δ-$GABA_A$ receptors, the effects of bath application of allopregnanolone (0.1 and 1.0 μM) on the tonic current was tested. As expected, allopregnanolone enhanced I_{hold} in slices from control ($F_{(1.2,11.08)}$ = 36.5, $p < 0.0001$) and AIE-exposed ($F_{(1.3,9.3)}$ = 10.7, $p < 0.007$) adult rats (Figure 3-11). Similar to the effect of AIE on ethanol-facilitated tonic current, AIE also significantly attenuated allopregnanolone-facilitated tonic current at both low and high concentrations (0.1 μM $t_{(13.6)}$ = 2.2, $p = 0.048$; 1.0 μM $t_{(16)}$ = 2.25, $p = 0.039$). Again, when expressed as a percentage of the average amplitude of THIP-activated current, no significant between group

differences were observed (Figure 3-7). While the above observations further demonstrate that AIE exposure results in reduced δ-$GABA_A$ receptor-mediated tonic currents (observed as a reduced efficacy of both ethanol and allopregnanolone to facilitate channel conductance), importantly, the sensitivity of δ-$GABA_A$ receptors to the facilitatory effect of either compound does not appear to be affected.

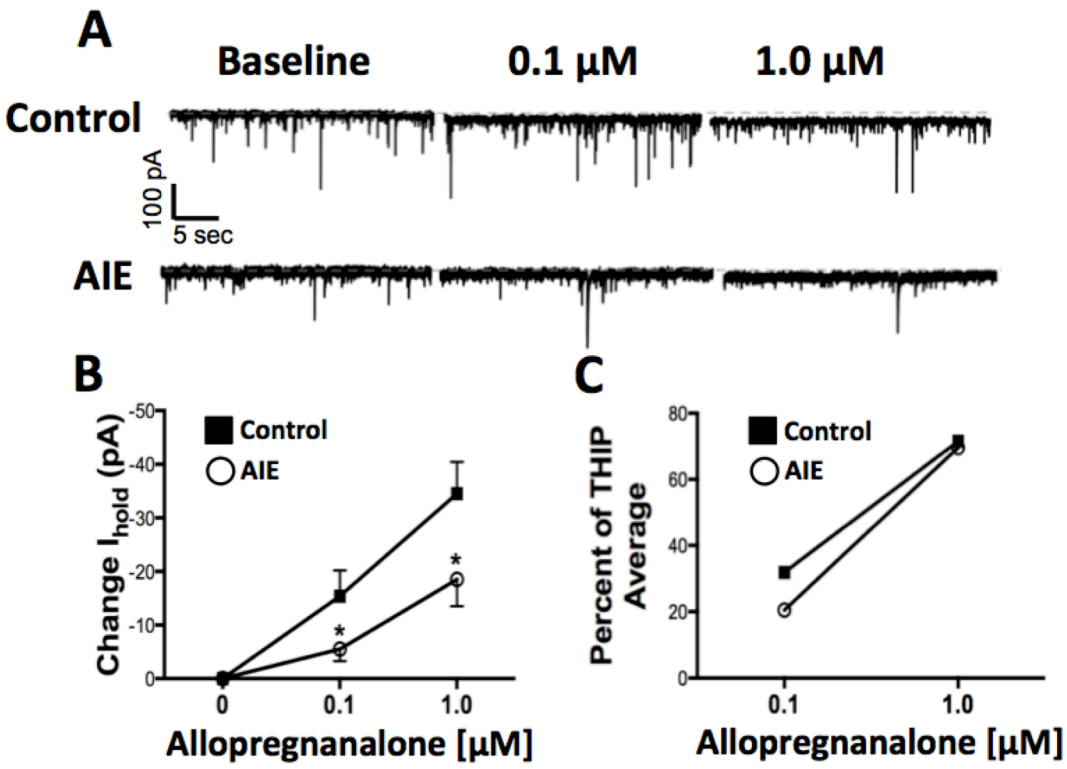

Figure 3-11. Adolescent ethanol exposure significantly decreases allopregnanolone-induced facilitation of the tonic current in adult PrL-C. **A)** Representative traces showing the change in I_{hold} in layer V pyramidal neurons in response to bath application of a low (0.1 µM) and high dose (1.0 µM) of the allopregnanolone in PrL-C slices obtained from control and AIE exposed adult rats **B)** Allopregnanolone dose-dependently facilitated the tonic current (n = 18 cells/dose, ***p = 0.0009), and this enhancement was significantly attenuated in slices obtained from AIE exposed rats compared to slices from control rats (n = 8-9 cells/group for each dose, *p < 0.05). **C)** Comparison of allopregnanolone facilitation of the tonic current to the average THIP current in control and AIE exposed conditions indicates that AIE did not alter the affinity of the δ-$GABA_A$ tonic current to acute allopregnanolone and instead reduced the efficacy of facilitation.

One possible explanation for the AIE-induced attenuation of the tonic current is a reduction in the expression of δ-$GABA_A$ receptors. To investigate this possibility, tissue punches were obtained from the PrL-C of control and AIE-exposed rats. Immunoblot analysis of the isolated membrane fraction revealed similar protein expression levels of α1, α4, α5, δ or γ2 GABA receptor subunits (Figure 3-12A). Since the reduction in the δ-mediated tonic current may have resulted from in reduction in their surface expression that was offset by an increase in their internal expression, the BS^3 crosslinking procedure was utilized to specifically measure surface protein expression. Immunoblot analysis of the total and intracellular (non-cross-linked) levels of the δ-subunit again showed no significant difference between control and AIE-exposed rats (Figure 3-12B). Because the slice electrophysiology studies revealed layer-specific effects of AIE on δ-$GABA_A$ receptor-mediated tonic currents, it is possible that the immunoblot procedures were unable to detect any changes in $GABA_A$ receptor subunit expression because the analysis was performed using tissue punches that contained all cortical layers within the PrL-C. In collaboration with another member of our lab (Liz Burnett), a follow-up experiment involved using immunohistochemistry to examine layer-specific expression of the δ-subunit in the PrL-C of both control and AIE-exposed adult rats. Consistent with previous reports regarding δ-subunit distribution, immunohistochemistry revealed high levels of expression in the granule cell layer of the cerebellum, the dentate gyrus of the hippocampus and the anterodorsal and anteroventral nuclei of the thalamus (not shown), with lower levels of cortical expression (Figure 3-13A-B)

(Pirker et al. 2000). Also in agreement with previous reports, marked differences in δ-subunit expression across the cortical layers were observed, with superficial layers (I and II/III) exhibiting significantly higher levels of δ-subunit expression compared to the deep layers (layer V/VI) ($F_{(1,8)}$ = 182.1, p < 0.0001). However, consistent with the immunoblotting experiments, we did not observe a significant difference between control and AIE-exposed rats in the staining intensity of the δ-subunit in either the superficial or deep layers of the PrL-C.

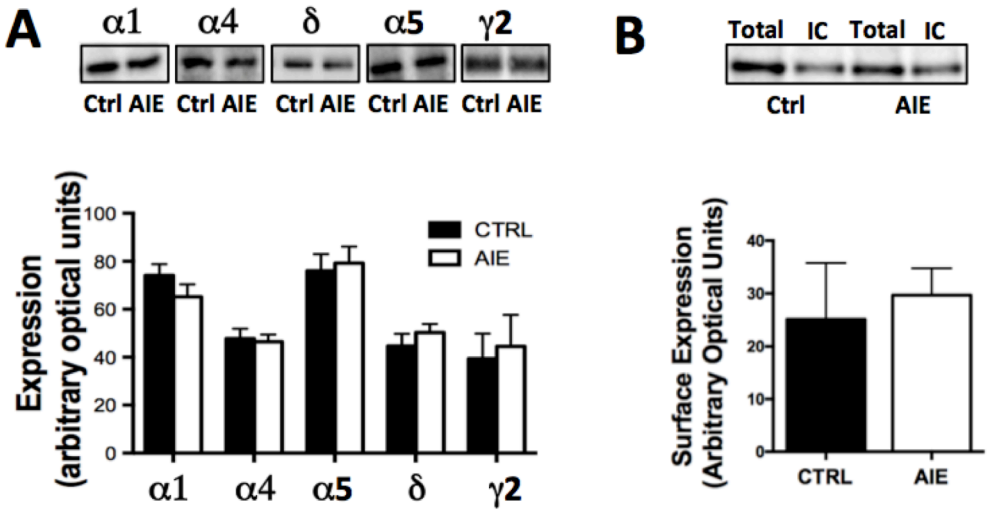

Figure 3-12. Adolescent ethanol exposure has no effect on expression of $GABA_A$ subunits that contribute to the tonic current. A) Protein expression of $GABA_A$ receptor subunit in the PrL-C of AIE exposed adult rats measured in total tissue extracts (n = 8-9 rats/group). **B)** Surface expression of δ-$GABA_A$ receptors using BS^3 cross-linking of surface membrane proteins in acute slices. Quantification of δ-subunit surface expression represents the difference between the non-cross-linked intracellular and the total subunit expression (n = 5-6 rats/group).

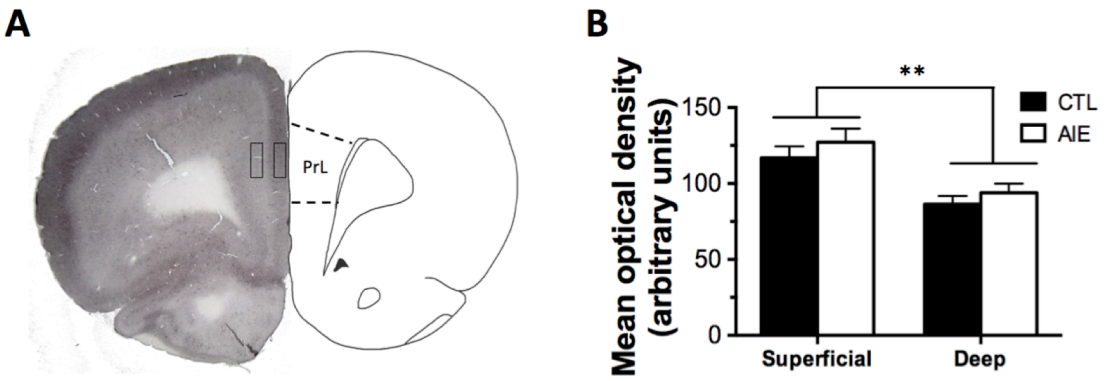

Figure 3-13. Adolescent ethanol exposure has no effect on layer-specific δ-GABA$_A$ expression in the adult PrL-C. A) Representative images of immunohistochemical labeling for the δ-subunit showing differential expression in superficial and deep layers of the cortex. **B)** Quantification of the boxed area in C revealed significantly greater δ-subunit expression in superficial versus deep layers of the PrL-C (**p < 0.001) but a significant effect of AIE exposure was not observed. (Courtesy of Liz Burnett)

DISCUSSION

The present chapter demonstrates that layer V pyramidal neurons in the PrL-C of the rat undergo a developmental increase in tonic GABA$_A$ receptor-mediated inhibition. The developmental emergence of this current was observed as a change in the number of pyramidal neurons that expressed a measurable tonic GABA$_A$ current and a concomitant increase in current mediated by δ-subunit containing GABA$_A$ receptors. Importantly, intermittent exposure to alcohol during the early to middle period of adolescence (PD28-42) resulted in a

reduction in the amplitude of this tonic current. This reduction was observed shortly after the last period of AIE exposure (i.e., PD43-47) and persisted well into adulthood (i.e., >PD90). This effect was not sex specific as AIE exposure attenuated the tonic current to a similar extent in both male and female adult rats. In contrast to these observations in layer V pyramidal neurons, AIE exposure had no effect on the tonic $GABA_A$ current in layers II/III. Taken together, these observations demonstrate that AIE exposure results in a remarkably protracted and layer-specific deficit in tonic $GABA_A$ currents in PrL-C. The reduced inhibitory tone of layer V projection neurons likely contributes, at least in part, to recent demonstrations of AIE-induced deficits in decision-making and behavioral control in adulthood (Gass et al. 2014).

Tonic GABA currents principally arise from ambient GABA in the extrasynaptic space that activates high affinity peri-/extrasynaptic $GABA_A$ receptors. The persistent activation of these receptors induces a strong inhibitory action on the cell by shunting the electrical signal. This mode of GABA neurotransmission is increasingly recognized as playing a critical role in the pathophysiology of various CNS and neurodevelopmental disorders (Egawa and Fukuda 2013; Fukuda and Wang 2013; Whissell et al. 2015). While tonic GABA currents have been observed in a number of brain regions, several studies have reported that acute slices from the mPFC lack a tonic GABA current (Drasbek and Jensen 2006; Weitlauf and Woodward 2008; Hoestgaard-Jensen et al. 2010). In the present study, in slices from young rats (PD16-45), only a small percentage of neurons exhibited a measureable tonic $GABA_A$ current. However,

as development progressed, the tonic current emerged such that in the fully mature adult (i.e., >PD90), virtually all layer V pyramidal neurons recorded from exhibited a robust tonic current. This observation is consistent with recent findings in layer V of the dorsolateral PFC of the rhesus monkey that demonstrated a developmental increase in $GABA_A$ subunits that are thought to contribute to the tonic current (i.e., δ, $\gamma 2$, and $\alpha 4$) (Hoftman and Lewis 2011; Datta et al. 2014). Exposure of rats to intermittent ethanol during adolescence also prevented subsequent developmental increases in the expression of the tonic current. Importantly, this AIE-induced reduction in tonic current was still observed in adulthood (e.g. >PD90) several weeks after the last period of exposure to alcohol. This indicates that AIE did not simply shift the time-course of the developmental trajectory of the tonic current but instead produced a long-lasting, potentially permanent, reduction in tonic GABAergic inhibition of layer V PrL-C pyramidal neurons.

The cerebral cortex has a distinct laminar organization, and there is increasing appreciation that the different cortical layers make distinct contributions to information processing within the mPFC. For example, afferent projections from limbic and contralateral cortical regions to the mPFC mainly target neurons in layers I and II/III, while efferent projections from pyramidal neurons in layers V/VI provide information to subcortical structures. A particularly interesting observation was that the AIE-induced reduction in tonic inhibition was layer specific, with attenuation of the tonic current in layer V but no change in layers II/III. Thus, protracted reduction in tonic inhibition associated with AIE

exposure may be selective for pyramidal neurons of the PrL-C that provide output to subcortical structures. Recent studies from our lab demonstrated that AIE exposure results in deficits in behavioral control in the adult that are consistent with PFC dysfunction (Gass et al. 2014). Although it is likely that alterations in the activity of other neurotransmitters and signaling systems contribute to these cognitive deficits, it is reasonable to suggest that the reduction in tonic inhibition we observed may be of particular importance to how the PFC processes information and directs behavior. In the cortex, GABAergic inhibition tunes cortical responses and can either increase temporal precision or de-correlate activity depending on the state of the network. While the density of GABAergic interneurons in the PFC is sparse, they are known to play a critical role in controlling network activity. Although the role that the tonic $GABA_A$ current plays in modulating network organization in the PFC is not clear, alterations in the level of tonic inhibition would be expected to significantly alter the input-output relationship and impact information processing within the mPFC.

While the observation of attenuated tonic current amplitude is consistent with a reduction in the number/function of extrasynaptic $GABA_A$ receptors, it was also possible that the decrease in tonic current results from a reduction of the level of ambient GABA that persistently activates extrasynaptic $GABA_A$ receptors. However, the fact that changes in the sIPSC and mIPSC frequency, or in PPR, were not observed strongly suggests that AIE exposure did not alter presynaptic release of GABA. To further examine whether the AIE-induced reduction in tonic current might reflect a reduction in the level of ambient GABA, the GABA

reuptake inhibitor was bath applied in order to maximize activation of extrasynaptic receptors, and then assessed the amplitude of the tonic current following selective blockade of synaptic $GABA_A$ receptors. These observations again revealed an AIE-induced reduction in δ-$GABA_A$ tonic currents indicating that alterations in ambient GABA did not underlie the AIE-induced reduction in tonic current. In addition, the fact that attenuation of current amplitude was still observed under conditions of elevated extracellular GABA further argues against an AIE-induced reduction in the affinity for activation of the extrasynaptic currents by GABA.

Previous studies suggest that AIE resulted in reduced tonic GABA current in the adult dentate gyrus (Fleming et al. 2013). Follow up studies revealed that the reduced tonic current reflected decreased δ-$GABA_A$ receptor protein expression in adult AIE-exposed rats (Centanni et al. 2014). Based upon these previous findings and the results of our electrophysiological studies, the possibility that the observed AIE-induced reduction in tonic current reflected a reduction in the expression of extrasynaptic δ–subunit containing $GABA_A$ receptors was tested. Contrary to expectations, immunoblot analysis of tissue punches of the adult PrL-C revealed that AIE exposure did not alter the expression of any of the subunits examined ($\alpha 1$, $\alpha 4$, δ, $\alpha 5$, $\gamma 2$). Since AIE-induced attenuation of the tonic current was observed only for layer V pyramidal neurons with no alteration of tonic currents in layers II/III, it is possible that an AIE-induced reduction in δ-subunit expression specifically in layer V could have been

obscured by subunit expression in other layers as a result of the tissue punch procedure that included all cortical layers. However, follow-up immunohistochemistry studies revealed that this did not appear to be the case. Since neither the immunoblot nor immunohistochemistry procedures differentiated surface from non-surface receptors, the possibility remained that a reduction in the expression of surface $GABA_A$ receptors could have been offset by an increase in their intracellular expression. Follow-up cross-linking studies showed that AIE exposure did not alter the surface expression of the δ-$GABA_A$ subunit. Taken together, these data strongly suggest that the AIE-induced reduction in tonic current is not the result of reduced δ-$GABA_A$ receptor expression but instead may be related to a change in receptor efficacy.

As noted previously, there is significant evidence that both ethanol and neurosteroids act as high affinity positive allosteric modulators of δ-$GABA_A$ extrasynaptic receptors. The AIE-induced reduction in the amplitude of the tonic current led to the hypothesis that PrL-C neurons in AIE-exposed adult rats would exhibit reduced sensitivity to acute ethanol and neurosteroids. As expected, bath application of either ethanol or allopregnanolone resulted in a dose-dependent facilitation of the tonic current. In slices from AIE exposed rats, the amplitude of facilitation of the tonic current by either ethanol or allopregnanolone was significantly reduced. However, expression of these results as a percentage of the amplitude of the THIP-activated tonic current obtained in the respective control and AIE conditions revealed no difference in facilitation by either ethanol or allopregnanolone.

Taken together, these observations indicate that AIE exposure results in reduced efficacy but not affinity of the $GABA_A$ tonic current to either ethanol or allopregnanolone. At least in the case of the acute effects of alcohol, these observations are consistent with behavioral studies showing that adolescents are less sensitive to the physiological effects of alcohol when similar doses are administered (Hollstedt et al. 1980; Ramirez and Spear 2010). In addition, exposure to alcohol during adolescence alters the efficacy of acute ethanol in the adult dentate gyrus of the hippocampus (Fleming et al. 2007). It has been suggested that adolescent alcohol abuse can result in a "lock-in" of certain cellular and behavioral aspects of the adolescent phenotype in the adult. The results of the present study are in agreement with this construct and further suggest that this lock-in may include reduced GABAergic activity of the adolescent PFC that persists into adulthood.

CHAPTER 4

ADOLESCENT ALCOHOL EXPOSURE DOES NOT ALTER RISK/REWARD DECISION MAKING IN ADULT RATS

BACKGROUND AND SIGNIFICANCE

The PFC is thought of as the central executor of many behaviors including planning, working memory, inhibitory control, behavioral flexibility and decision-making. Decision-making is a particularly important PFC-dependent behavior that regularly impacts our daily lives. Some decisions are determined by habit and the expectation of immediate fulfillment, however many other decisions are largely the product of logical reasoning and long-term planning. A decision is determined by a number of factors, most notably the amount and timing of potential reward, and the degree of potential risk. Because of the uncertainty of rewards and risks, the decision is then the result of probabilistic estimates of both reward and risk.

Human and non-human primate studies have examined the effect of PFC damage on cognitive behaviors such as decision-making. These studies provide evidence for increased risky, disadvantageous decision-making in patients with damage to the ventromedial and orbital PFC (Bechara et al. 1994; Bechara et al. 1998; Bechara et al. 1999). Subsequent research has provided further evidence for the involvement of the dlPFC, OFC, ACC and insular cortex in risk/reward decision-making (Rogers et al. 1999; Manes et al. 2002; Clark et al. 2008; Lawrence et al. 2009). In the laboratory, operant tasks using rodents have been designed to simulate real-life decisions in terms of uncertainty, reward and punishment. These tasks provide a way to examine the specific involvement of PFC neural circuits and neurotransmitter systems in decision-making. Studies have implicated the glutamate, GABA, and DA systems in the PFC as necessary components for accurate performance on PFC-dependent cognitive tasks (Floresco and Magyar 2006; Floresco et al. 2006; St Onge and Floresco 2010; Floresco 2013; Tse et al. 2014).

Recent studies from our lab and others have demonstrated that adolescent alcohol exposure results in long-lasting deficits in executive function (Chambers et al. 2003; Oscar-Berman and Marinkovic 2003; White and Swartzwelder 2005; Masten et al. 2008; Gass et al. 2014). Using a rat probabilistic decision-making task, previous studies reported that adolescent exposure to alcohol results in increased risky decision-making in adulthood (Nasrallah et al. 2009; Nasrallah et al. 2011; Boutros et al. 2014). The goal of the studies presented in this chapter was to determine, using the vapor inhalation

model, whether AIE exposure results in increased altered decision-making during probabilistic discounting.

The δ-$GABA_A$ receptor is thought to mediate the majority of the tonic GABA current in layer V neurons in the PrL-C (Moss and Smart 2001; Drasbek and Jensen 2006; Zheleznova et al. 2009; Nishikawa et al. 2011). Compounds that selectively target extrasynaptic $GABA_A$ receptor subunits including the α5 and δ-subunit have gained increasing attention as potential treatments for disorders such as insomnia, anxiety and depression, and also as cognitive enhancing agents (Rudolph and Knoflach 2011). THIP is a direct acting agonist of δ-$GABA_A$ receptors and therefore activates all extrasynaptic δ-$GABA_A$ receptors independent of the presence of GABA. Compounds that act as positive allosteric modulators (PAMs) have significant advantage over direct acting agonists because they require binding of endogenous ligand in order to promote activity, and thus exhibit greater specificity and fewer side effects (Ebert et al. 2008; Tan et al. 2011). Recent studies in rodents demonstrate that subcutaneous injections of the novel δ-$GABA_A$ receptor PAM AA29504 dose-dependently enhances δ-$GABA_A$ receptor-mediated neurotransmission *in vitro* and reduces anxiety and stress *in vivo* without causing sedation (Hoestgaard-Jensen et al. 2010; Vardya et al. 2012).

In Chapter 3, data is presented that demonstrates that AIE produces deficits in δ-$GABA_A$ receptor-mediated tonic current in adult rats. These results, coupled with previous findings showing that AIE increased risky decision-making

in adulthood (Boutros et al. 2014), led us to hypothesize that enhancing δ-$GABA_A$ receptor-mediated tonic currents using a δ-$GABA_A$ receptor-specific PAM *in vivo* would restore AIE-induced impairments in decision-making on a probabilistic discounting task. The probabilistic discounting task was chosen over other tasks such as set-shifting procedures, because it is more conducive for these experiments, as it allows for testing the effects of multiple doses of a drug over the course of multiple days. In contrast, in the operant set-shifting task, only a single drug administration can be tested before the rule shift occurs.

MATERIALS AND METHODS

Animals and adolescent intermittent ethanol exposure

Long-Evans rats were obtained from our in house breeding colony (breeder stock was obtained from Charles River). Rats were weaned, housed and handle as described in Chapter 2. All studies in this chapter were conducted in male rats and a litter size consisted of at least 4 males in order to be included in the study. The AIE exposure model used in this chapter was the same as the model described in detail in the Chapter 2 and in previous publications (Gass et al. 2014).

Operant Lever Press Training

Beginning on ~PD80, a group of control and AIE-treated rats were trained (over a period of 5-7 days) to press retractable levers within 10 seconds of their insertion into the chamber. The training protocol was performed as previously described (St Onge and Floresco 2010). In brief, on the day before their first exposure to the operant chamber, rats were given approximately 25 ml of 20% sweetened condensed milk in their home cage. On the following day, they were trained under a fixed ratio one (FR1) schedule to a criterion of 60 presses in 30 minutes, first for one lever and then repeated on the other lever. They were then trained on a simplified version of the full task. These 90-trial sessions began with the levers retracted in the operant chamber with the lights out. Every 40 seconds, a trial was initiated with illumination of the house light and insertion of one of the two levers into the chamber (randomized in pairs). If the rat responded within 10 seconds, the lever was retracted and a small reward (25 µl of 20% sweetened condensed milk) was delivered to the receptacle. Failure to respond resulted in lever retraction and termination of the house light until the next trial (omission). After rats met the criterion of 80 or more successful trials (<10 omissions), side preference was determined and the “risky” lever was assigned as the lever opposite of the one they preferred.

Probabilistic Discounting Task

Following completion of the lever press-training phase, rats began training on a probabilistic discounting task as previously described (St Onge and Floresco 2010; St Onge et al. 2012). Rats received daily training sessions 6-7 days/week, consisting of 90 trials that were separated into 5 blocks of 18 trials. Each 60-minute session began in darkness with both levers retracted (the intertrial state). Trials began every 40 seconds with house-light illumination and insertion of one or both levers after 3 seconds. One lever was designated the large/risky lever, the other the small/certain lever, and this remained consistent throughout training (counterbalanced left/right). No response within 10 seconds of lever insertion reset the chamber to the intertrial state until the next trial (omission). Choosing either lever resulted in retraction of both levers. Choice of the small/certain lever always delivered a smaller reward (25 µl of 20% sweetened condensed milk) with 100% probability; choice of the large/risky lever delivered a larger reward (100 µl of 20% sweetened condensed milk) but with a probability that changed across the 5 trial blocks. Blocks were comprised of 8 forced-choice trials (4 trials for each lever, randomized in pairs), followed by 10 free-choice trials, where both levers were presented. The probability of obtaining 100 µl of 20% sweetened condensed milk after selecting the large/risky option decreased in a systematic manner across blocks (100%, 50%, 25%, 12.5%, 6.25%). The main dependent variable of interest was the proportion of choices of the large risky option over each of the blocks. Rats were trained on this task

for 20 sessions (6-7 days/week), and for the analysis, we compared risky choice between groups over the last 4 days of training.

We also assessed changes in reward and negative feedback sensitivity by calculating the proportion of trials where rats chose the risky option after obtaining the large/risky reward on the previous trial (win-stay behavior) and trials where they switched to the small/certain option after non-rewarded risky choices (lose-shift behavior)(St Onge and Floresco 2010; St Onge et al. 2012). For these analyses, the total number of win-stay and lose-shift responses were divided by the total number of rewarded/non-rewarded risky choices, respectively. Latencies to choose were also recorded.

Solutions and drugs

AA29504 was purchased from Tocris Bioscience (Bristol, United Kingdom; Catalog # 3972). For all experiments, AA29504 was prepared in a vehicle consisting of 50% dimethyl sulfoxide (DMSO) and 50% 0.9% saline and a final concentration of 4 mg/ml was prepared. A dose volume of 0.25 ml/kg and 1 ml/kg and a final drug dose of 1 mg/kg and 4 mg/kg, respectively. AA29504 was administered through subcutaneous injection 30 minutes prior to experimentation as previously reported (Hoestgaard-Jensen et al. 2010).

Locomotor Activity

A separate group of rats were used to determine the effects of AA29504 on locomotor activity. Adult rats (PD90) were placed into an open field box equipped with IC Capture video software and EthoVision tracking software to measure distance traveled and velocity. Rats were given a subcutaneous injection of 50% DMSO vehicle 30 minutes prior to a 1-hour habituation period in the locomotor box. This was repeated 24 hours later. On day 3, each rat received a subcutaneous injection of either 50% DMSO vehicle, 1 mg/kg AA29504 or 4 mg/kg AA29504 30 minutes prior to being placed in the locomotor box. Two additional days of testing occurred to assure that each rat was tested after a vehicle injection and the low and high dose of AA29504 in a counterbalanced manner.

Pharmacological Manipulations

A within-subject design was used to test the effects of AA29504 on risky decision-making. After the initial 20 training sessions on the probabilistic discounting task, the additional drug test period commenced. Each test consisted of a two-day sequence in which animals received subcutaneous vehicle injection (day 1) and then dose 1 of AA29504 (either 1mg/kg or 4mg/kg) (day 2) 30 minutes prior to the training session. Following a drug test day, rats were retrained for 3 days, after which a second sequence of drug tests was administered consisting of vehicle on day 1 and the second dose on day 2.

Data Analysis and Statistics

The primary dependent measure of interest on the probabilistic discounting task was the percentage of choices directed toward the large/risky lever for each block of free-choice trials factoring in trial omissions. For each block, this was calculated by dividing the number of choices of the large/risky lever by total number of successful trials. The choice data were analyzed using two-way within-subjects ANOVAs, with Treatment and Trial Block as the within-subjects factors. Learning of the task was determined using the same within-subject two-way ANOVA design followed by a Tukey's Multiple Comparison test to determined statistical significance between probability blocks. Statistical analysis was performed in Prism (GraphPad Software) or SAS statistics program. Statistical significance level was $p < 0.05$, and data are presented as mean ± SEM.

RESULTS

To provide a quantitative measure of the level of intoxication achieved during the AIE procedure, a previously described 1-5 point intoxication rating scale was used (Gass et al. 2014). Intoxication scores measured at the end of each 14-hour ethanol exposure period and averaged across all 4 cycles for all rats (n = 15) revealed an overall level of intoxication of 2.73 ± 0.06 (rating of 2 = slight intoxication, 3 = moderate intoxication). Tail-vein blood drawn at the end of each exposure cycle revealed that the average BEC for each of the cycles was

(in mg%) 294 ± 34.02, 277 ± 17.12, 352.53 ± 27.34, and 241.36 ± 24.60, respectively, with a grand average across all 4 cycles of 292.37 ± 13.90 mg% (Figure 4-1). Both the average intoxication rating and BEC level were very similar to those obtained in the previous chapters (Figure 2-2, Figure 3-1).

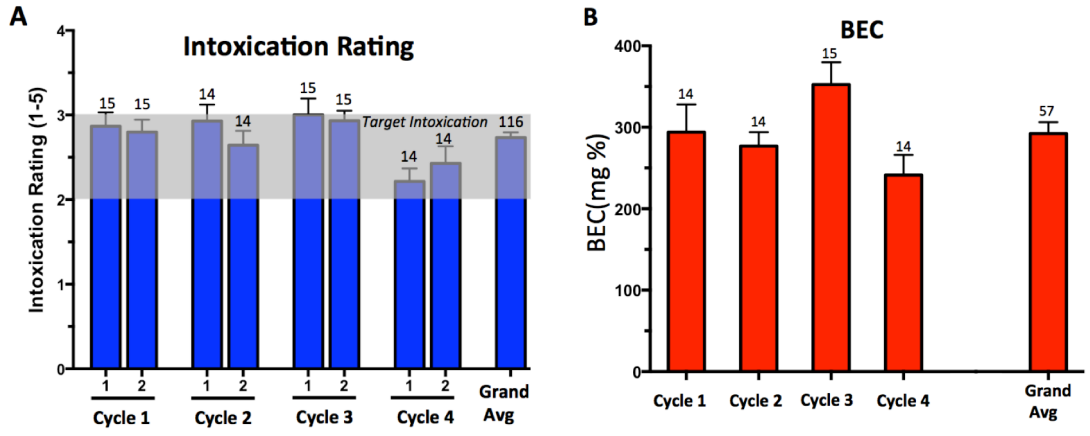

Figure 4-1. Intoxication ratings and blood ethanol levels for the rats used in the behavioral studies. A) Average intoxication rating for each exposure day. The target intoxication rating of 2-3 (gray) was determined for each cycle (total n = 116 measurements). **B)** Tail vein blood was drawn from each rat at the end of each cycle and blood ethanol content was determined (total n = 57 measurements).

In the first set of experiments, differences in risk/reward decision-making were investigated using a probabilistic discounting task (St Onge and Floresco 2010; Boutros et al. 2014), wherein rats chose between smaller, certain rewards and larger rewards delivered with probabilities that varied over the course of a session (100 - 6.25%). Adjusting choice biases in these situations is critically dependent on the mPFC and modulated by GABA and DA signaling in this region

(St Onge and Floresco 2010).

Separate groups of control (n = 14) and AIE (n = 15) rats were trained on the task for 20 days. Choice data were averaged into five blocks of four days for each group. Learning of the task was assessed by measuring differences in discounting rates over the course of the entire training period for all rats combined. Each probability block was compared to the 100% probability block (e.g. 50% versus 100%, 25% versus 100%, etc.), and also to the previous block (e.g. 12.5% versus 25%, 6.25% versus 12.5%, etc.). As shown in Figure 4-2, during the first block of training, rats were unable to differentiate the difference in probability of receiving the large reward during the first four probability blocks. However, rats chose the high-risk lever significantly less during the 6.25% probability block than they did during the 100% probability block suggesting that the rats were able to discriminate the difference in reward probabilities between these blocks. By the second block of training, all rats began to display similar discounting rates (risky choice in a particular block versus the previous block), indicative of progressive learning of the task. By the end of training (sessions 17-20), all animals in this study were able to effectively discriminate between all probability blocks (with the exception of the 100% probability block versus the 50% probability block).

Both control and AIE exhibited nearly identical learning patterns suggesting AIE did not alter learning on the probabilistic discounting task (Figure 4-2). Surprisingly, there were no differences in overall risky choice between the groups over the entire training period ($F_{(4, 135)} = 0.69$, $p = 0.601$). In particular,

while deficits in risk/reward decision-making would likely be observed towards the end of training, when all rats have successfully learned the task, both groups displayed similar patterns of choice across all five probability blocks (main effect of group - $F_{(1, 27)} = 0.01$, $p = 0.92$; group x block interaction - $F_{(4, 108)} = 0.89$, $p = 0.470$; Figure 4-2) suggesting that AIE did not alter probabilistic decision-making on this task.

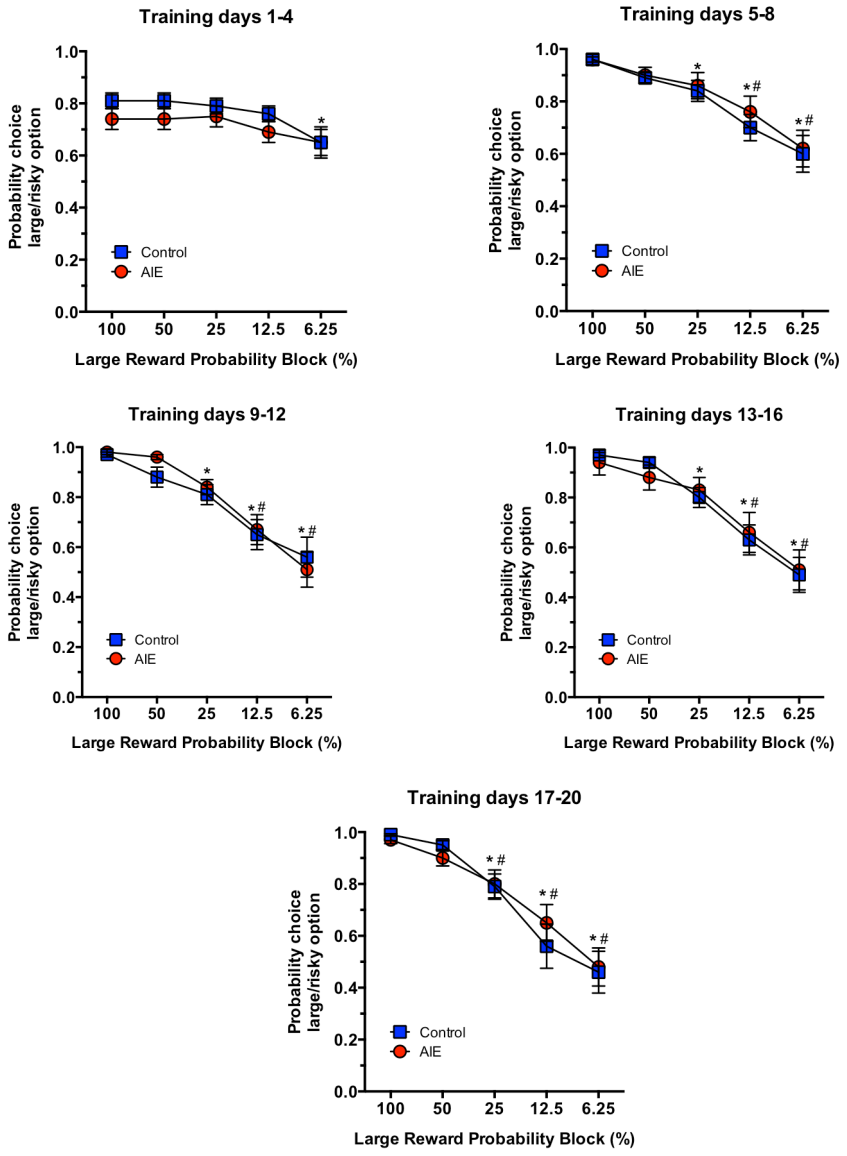

Figure 4-2. AIE does not alter risk/reward decision-making in adulthood using a probabilistic discounting task. Data were binned into five blocks of four days each and differences between control and AIE-exposed rats were tested. Data is plotted as percent choice of the Large/Risky lever during free choice trials by probability block. All rats successfully learned the task by the final 4 days (sessions 17-20), and both groups learned the discounting task at the same rate (n = 14-15 rats/group; *p < 0.05 for *x* probability block compared to the 100% probability block; #p < 0.05 compared to the previous block (e.g. 12.5% versus 6.25%)). Risk/reward decision-making did not differ between control and AIE rats.

In addition to risk/reward decision-making, this task provides insight into positive and negative feedback sensitivity. Choice strategies within the trial after a 'win' (i.e. choosing the risky choice option and receiving a reward) or a 'loss' (i.e. choosing the risky choice option and failing to receive a reward) were assessed in control and AIE-exposed rats. As shown in figure 4-3, both groups displayed similar win-stay/lose-shift strategies suggesting negative feedback sensitivity was unaffected by AIE (Win-stay: $t_{(27)} = 0.09$, $p = 0.928$; Lose-shift: $t_{(27)} = 1.01$, $p = 0.323$; Figure 4-3). Notably, AIE did not alter choice latency ($t_{(27)} = 0.39$, $p = 0.700$; Figure 4-4) or the average number of trial omissions ($t_{(27)} = 0.63$, $p = 0.545$; Figure 4-4) indicating that AIE did not result in any non-specific impairments in motivational processes.

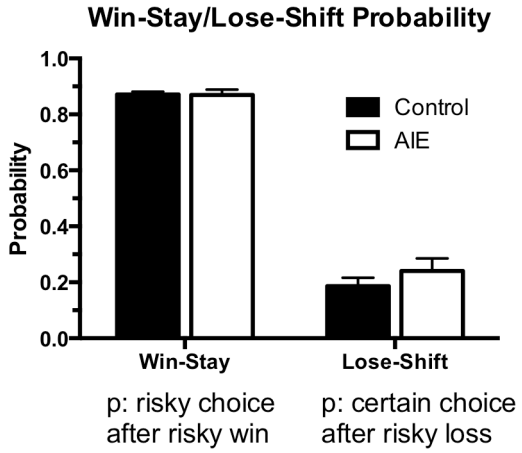

Figure 4-3. Adolescent ethanol exposure did not alter choice strategy. As indexed by the lose-shift behavior (the proportion (p) of choices on the small/certain lever following unrewarded risky choice on the preceding trial, right), AIE did not affect negative feedback sensitivity. Similarly, win-stay behavior (proportion (p) of choices on the large/risky lever following a rewarded risky choice on the preceding trial, left) did not differ significantly between groups.

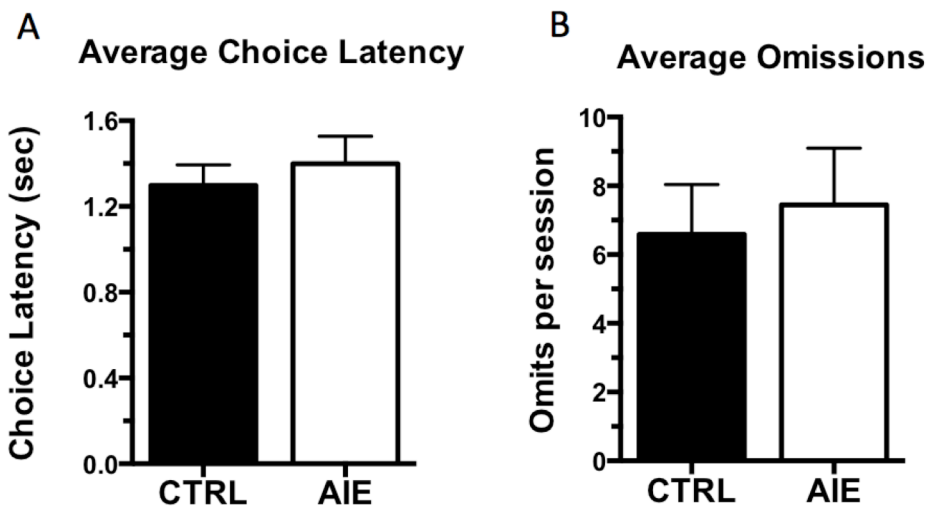

Figure 4-4. Adolescent alcohol exposure did not alter motivational processes. A) AIE did not alter the amount of time the rat took to make a choice once the levers were presented. **B)** Similarly, AIE had no effect on the average omissions per session.

GABA neurotransmission in the PrL-C is critical for decision-making. A recent study by Paine and colleagues demonstrate that blockade of $GABA_A$ receptors in the rodent mPFC altered risk/reward decision-making (Paine et al. 2015). Pilot studies were therefore conducted to test the effect of the novel δ-$GABA_A$ receptor-specific PAM AA29504 on the probabilistic decision-making task. $GABA_A$ receptor-targeting drugs can produce sedative, hypnotic, and/or anesthetic side effects that may impair motor coordination and impede performance on an operant task. Initial experiments characterized the effect of AA29504 on locomotor activity. One mg/kg and 4 mg/kg, administered subcutaneously have been shown to produce pharmacologically relevant blood

plasma and brain levels of the drug that reduce anxiety and stress-induced hypothermia without impairing motor coordination in Sprague-Dawley rats (Hoestgaard-Jensen et al. 2010). To insure these doses did not impair motor coordination the Long Evans rats, 1 mg/kg and 4 mg/kg AA29504 were tested in a locomotor activity experiment. As shown in figure 4-5, neither 1 mg/kg nor 4 mg/kg AA29504 had any effect on distance traveled ($F_{(1.313, 3.938)}$ = 2.1, p = 0.226) or average velocity ($F_{(1.301, 3.905)}$ = 2.2, p = 0.226 Figure 4-5B) suggesting that AA29504 does not produce sedation at these doses, consistent with the previous study. Thus, subsequent experiments tested the effects of both doses of AA29504 on performance on the probabilistic decision-making task.

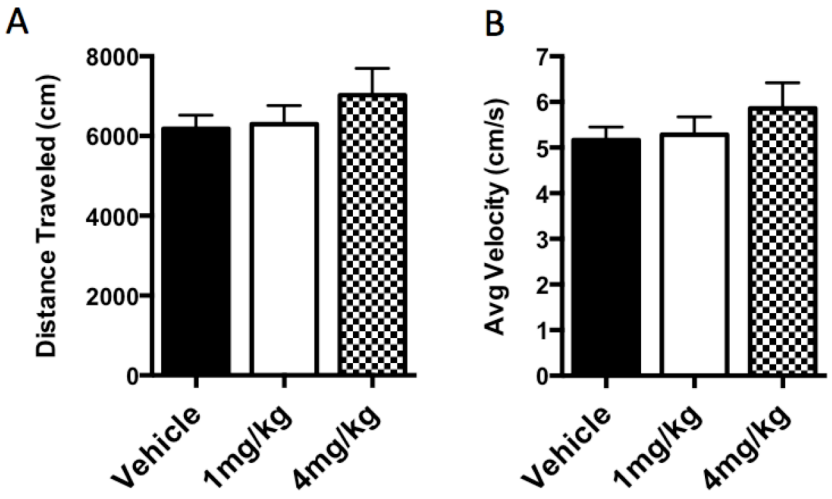

Figure 4-5. AA29504 has no affect on locomotor activity. Rats received a subcutaneous injection of vehicle, 1mg/kg AA29504 or 4 mg/kg AA29504. Thirty minutes later rats were placed in an open field activity box and locomotor activity was measured for 1 hour. **A)** Neither the 1 mg/kg dose nor the 4 mg/kg dose of AA29504 had an effect on distance traveled. **B)** Neither dose of AA29504 had an effect on average velocity (n = 5 rats).

Immediately following completion of the 20 training sessions, a 7-day drug treatment was conducted to test the effect of the δ-$GABA_A$ receptor-specific PAM AA29504 on the probabilistic decision-making task. A vehicle, 1 mg/kg or 4 mg/kg dose of AA29504 was administered subcutaneously 30 minutes prior to the training session. In order to ensure stability in baseline performance on the task, and to identify any possible enduring effects of the drug, the drug treatment paradigm included three "off days" between drug tests. In agreement with the observations prior to drug treatment (training days 17-20; Figure 4-2), both groups performed similarly on the task during the three-day inter drug test training period ($F_{(4, 108)} = 0.59$, $p = 0.674$; Figure 4-6).

Analysis of the choice data across the drug challenge days in control and AIE rats revealed that treatment with neither the 1 mg/kg nor the 4 mg/kg dose affected performance on the probabilistic discounting task in either group. Specifically, there was no group x dose interaction ($F_{(2, 54)} = 2.28$, $p = 0.116$) or group x dose x block interaction ($F_{(8, 216)} = 1.33$, $p = 0.228$; Figure 4-7A-B).

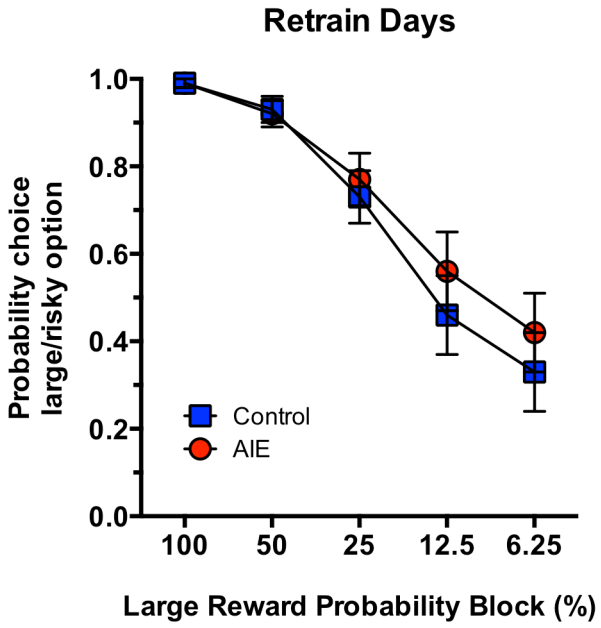

Figure 4-6. Adolescent alcohol exposure did not alter probabilistic decision-making between drug treatments. Rats were retrained on the probabilistic decision-making task for three days in between drug treatment. Control and AIE-exposed rats showed similar choice patterns during all five of the probability blocks.

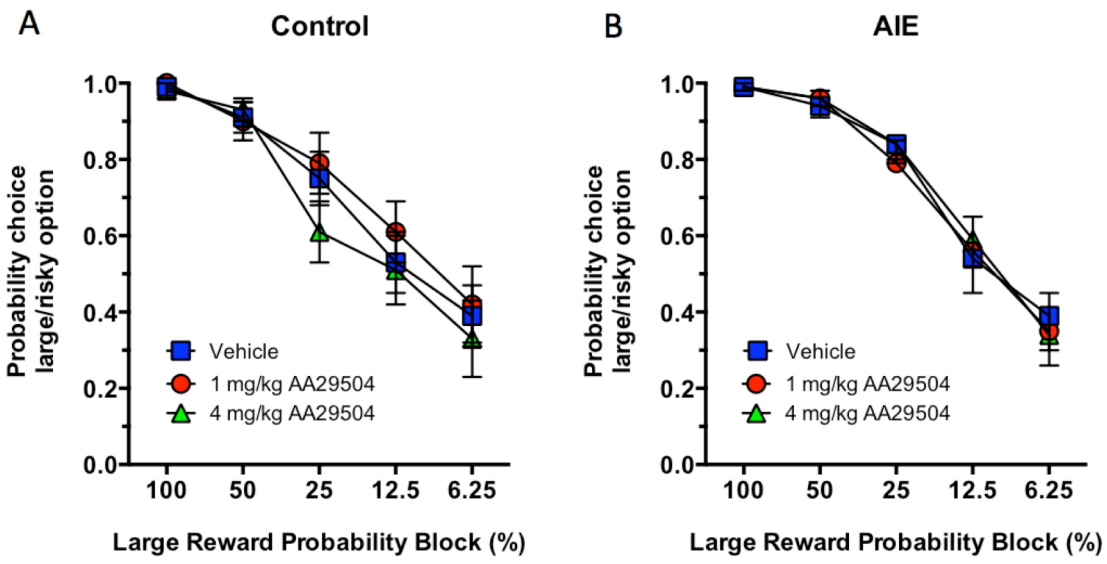

Figure 4-7. AA29504 did not alter performance on the probabilistic decision-making task. A) Control rats treated with an acute subcutaneous injection of 1 mg/kg (red) or 4 mg/kg (green) AA29504 exhibited similar choice patterns when compared to vehicle treatment. **B)** Similar to control rats, AIE-exposed rats did not exhibit changes in choice pattern after 1 mg/kg or 4 mg/kg AA29504.

DISCUSSION

The results of this set of behavioral studies confirmed that rats successfully learn the probabilistic discounting task. By the end of the 20 training sessions, rats were able to accurately adjust their choice strategy to account for the decreasing probability of receiving a large reward. These results are consistent with previous findings using the probabilistic discounting task (St Onge and Floresco 2010; St Onge et al. 2012; Boutros et al. 2014; Schindler et al.

2014). Similar to the results from previous studies, rats exhibited a progressive decrease in choice of the large, uncertain reward option as the probability of receiving the large, uncertain reward decreases.

Using a vapor chamber model of ethanol exposure, the effect of AIE on probabilistic decision-making was assessed. Surprisingly, these studies did not reveal any effect of AIE on probabilistic decision-making when tested in adulthood. AIE rats learned the task at the same rate, and displayed the same choice strategy as control rats. These results are in contrast to recent studies that reported an AIE-induced increase in risky decision-making in adulthood. Nasrallah and colleagues adopted an alcohol gelatin model of voluntary ethanol intake during adolescence. In adulthood, rats were tested on a probabilistic decision-making task that involved separate training sessions for each probability block (Nasrallah et al. 2009; Nasrallah et al. 2011). The results of these studies revealed that AIE rats exhibited preference for the large, uncertain reward option compared to control rats, suggesting increased risky decision-making. Another more recent study conducted by Boutros and colleagues used an adolescent intermittent intragastric oral ethanol gavage (IG) model to assess risk/reward decision-making in adulthood. Using a probabilistic discounting protocol that was similar to the one used in the present study, Boutros et al show that AIE caused an increase in risky decision-making on the probabilistic discounting task in adulthood.

In addition to assessing the effect of AIE on probabilistic decision-making, an exploratory set of experiments were included in the present study to test the

effect of the novel δ-$GABA_A$ receptor PAM AA29504 on decision-making on the probabilistic discounting task. The observation in Chapter 3 that AIE leads to a reduction in δ-$GABA_A$ receptor-mediated current in the adult PrL-C provided the rationale for these experiments. Alterations in tonic GABA currents may contribute to cognitive deficits associated with adolescent alcohol exposure. Therefore we tested the hypothesis that enhancing the tonic current using a δ-$GABA_A$ receptor PAM would improve performance on the probabilistic discounting task. The results of these studies revealed that AA29504 had no effect on risk/reward decision-making in either control or AIE-exposed rats.

As mentioned above, the findings in the present chapter differ from those previously published. As discussed below, there are a number of experimental differences that may have contributed to the inconsistencies between studies.

Probabilistic discounting protocol

While the probabilistic discounting task is an excellent model for studying cognitive function in rodents, the complex nature of the task results in subtle methodological differences that could have potentially contributed to the differences in observations. These include the following parameters.

Experimental design

Variances in the experimental design may have contributed to different effects of AIE on probabilistic decision-making. In earlier studies (Nasrallah et al.

2009; Nasrallah et al. 2011), increases in risky choice were apparent during the initial learning of the reinforcement contingencies associated with different options occurring during the single session. In the present study, the probability of receiving a large reward decreased over the course of a session. Rats were thus familiarized with the changes in risk/reward contingencies over an extended training period.

In the Boutros study, the total length of behavioral testing was substantially longer than the present study (18 total trials/block versus 26 total trials/block). Furthermore, the number of trial omissions during the task was not reported in the previous study, as the procedure used provides an opportunity for the rat to omit from certain trials and develop a choice strategy within each block with no consequences. The experiment continued until the rat responded on all 130 trials (or 180 minutes elapsed). It is possible that in the current procedure, both control and AIE rats are content with receiving a small certain reward throughout the experiment, and thus are equally unwilling to shift choice strategies. Extending the length of each block (time and trials) and eliminating negative consequences for a trial omission could have altered the reward value and/or choice strategy. Furthermore, it has also been shown that the economic value of a reward can change depending on the amount of reinforcer and length of time (O'Doherty 2014). In the present study, the addition of a group that underwent the discounting task in order of ascending probabilities may reveal an AIE-induced increase in risky decision-making earlier in the task when reward value is presumably at its highest.

It should be noted that this task, although heavily dependent on proper PFC-function, also relies on a coordinated system that requires contributions from several other brain regions. For instance, PrL-C does not appear to regulate choice between large/risky versus small/certain rewards when probabilities are static. Instead, it appears that AIE-induced increases in risky choice during initial reinforcement learning may arise from alterations in neurotransmission within other brain regions such as the nucleus accumbens (St Onge and Floresco 2010; Schindler et al. 2014).

Reinforcer

The present study used liquid sweetened condensed milk as the reinforcer while previous studies used sucrose pellets (Nasrallah et al. 2009; Boutros et al. 2014). One possible difference between these studies is that reward motivation for sweetened condensed milk may be higher than for sucrose pellets. Both control and AIE rats may value sweetened condensed milk equally and are unwilling to risk the small certain reward, whereas the value of a sucrose pellet may differ between groups, motivating AIE-exposed adult rats towards riskier choice patterns. Separate studies from our group and others suggest that reward motivation is not likely the cause for discrepancies in the experimental outcome, as AIE did not alter response rate or total number of reinforcers earned for sweetened condensed milk (Gass et al. 2014) or sucrose pellets (Risher et al. 2013) in adulthood. Moreover, the fact that the discounting rates observed in the

present study were similar to those reported by Boutros and colleagues further suggest that the specific reinforce used is unlikely to explain the differences in these results.

In both the current study and previous studies, rats were food restricted to 90% of their free-feeding weight throughout the experiment. Another variable that may have contributed to differences in the results is potential differences in satiation between sweetened condensed milk and sucrose pellets. Sweetened condensed milk contains added fat and caloric value compared to sucrose pellets, and satiation may occur quicker using sweetened condensed milk than sucrose pellets. Reversing the task to examine risk/reward choice on an ascending probability version of this task may provide further insight into this possibility.

Modes of operant conditioning

Different modes of operant conditioning were used in the present study and the study conducted by Boutros and colleagues (lever press versus nosepoke). The acquisition of nose-poke response has been shown to occur much more rapidly than that of lever pressing (Schindler et al. 1993). One possible explanation for these differences is that lever press training requires increased motivation and learning. As mentioned above, previous studies from our lab suggest that motivation for reward did not differ between control and AIE-exposed rats (Gass et al. 2014). Although speculative, it is possible that the

increased work required to obtain a reward through lever pressing may have masked an AIE-induced increase in risk/reward decision-making. Conducting an experiment using a progressive ratio schedule reinforcer with nose-poke instead of lever-press to assess reward motivation in AIE-exposed adults may provide evidence for AIE-induced differences in reward motivation using nose-poke.

Alcohol exposure model

There are a number of important differences between the ethanol exposure model used in the present study and the model used in previous studies. The differences discussed below may have critically impacted the effect of AIE on adult probabilistic decision-making in the studies using a non-vapor model.

Route of ethanol administration

Boutros et al used an intragastric oral ethanol gavage (IG) model for their studies, which is in contrast to the ethanol vapor exposure model used in this dissertation. The IG model is a form of involuntary, forced ethanol administration that likely produces a significant amount of stress. In contrast, vapor inhalation of ethanol represents a passive, relatively low-stress mode of ethanol administration. It is now understood that both acute and chronic stress alters prefrontal development, and furthermore, stress during adolescence has been shown to significantly alter cognitive behaviors in adulthood (Lupien et al. 2009).

The PFC is particularly sensitive to stress during adolescence. Even mild stressors have been shown to impair PFC-dependent cognitive functions such as decision-making (Shafiei et al. 2012). The development and refinement of the stress system (mediated by the hypothalamus-pituitary-adrenal axis) produces a hyporesponsive stress period during adolescence (Insel et al. 1988). The addition of alcohol to this developing system may magnify these stress-induced PFC deficits. Taken together, the additional stress component associated with forced IG administration may be a contributing factor to the increase in risky decision-making in AIE-exposed adult rats reported by Boutros et al.

Housing conditions

Nasrallah and colleagues used a palatable ethanol gelatin matrix to induce voluntary consumption of alcohol during adolescence (Nasrallah et al. 2009). The housing conditions required to properly execute this model likely produces alterations in development that can impact adult behavior. In contrast to the pair-housed rats used in the present study, the rats used in these studies were individually housed upon weaning, and were therefore socially isolated throughout the entire period of adolescence. Social interaction during adolescence is an essential component of rodent development. Isolation during adolescence has been shown to have dramatic effects on adult neurochemistry and behavior (Hall 1998). Alcohol exposure during adolescence may exacerbate the long-term effects of social isolation. Therefore the added confound of

protracted social isolation during adolescence may have promoted an AIE-environmental interaction that collectively resulted in increased risky decision-making in adulthood.

Length and timing of ethanol exposure

The studies in this dissertation focused on the early to middle adolescence period (PD28-PD42), whereas the model used by Boutros and colleagues involved a much longer exposure period that included both early and late adolescence (PD28-53). Extensive refinement of the PFC is thought to occur throughout the entirety of adolescence (Blakemore and Choudhury 2006), and the total length of ethanol exposure may relate to the degree of cognitive dysfunction in adulthood. Longer ethanol exposure periods that include the entire period of adolescence may produce more pronounced effects on PFC development that subsequently uncover deficits in adult risk/reward decision-making. In contrast, shorter periods of ethanol exposure may be sufficient to disrupt PFC-dependent behaviors in adulthood such as behavioral flexibility, exploratory drive and ethanol self-administration and resistance to extinction (Gass et al. 2014). Emerging evidence suggests that the timing of ethanol exposure may also impact the effect on adult behavior. Recent studies suggest that early to middle adolescence represents a specific period of vulnerability that is likely to influence certain social/affective and cognitive behaviors whereas exposure to alcohol during the later stages of adolescence and emerging

adulthood may influence cognitive tasks dependent on neural substrates that are still undergoing maturation at this time (Spear 2015). Therefore it is possible that a longer and/or later ethanol exposure period is needed to produce deficits in risk/reward decision-making.

Rat strain

Male Long Evans rats were used in the present study to assess the effects of AIE on probabilistic decision-making in adulthood. The previous studies of the effect of AIE on risk used male Wistar (Boutros et al. 2014) and Sprague-Dawley rats (Nasrallah et al. 2009). The present study is, to our knowledge, the first to assess the effects of AIE on probabilistic decision-making in adulthood using male Long Evans rats. Numerous studies have highlighted strain differences in cognitive function (for review see (Kacew et al. 1998)). It is therefore likely that different rat strains are differentially affected by AIE in a way that alters performance on the probabilistic decision-making task.

AA29504

The novel δ-$GABA_A$ receptor PAM AA29504 had not been previously tested on cognitive behavioral tasks. A set of exploratory experiments was conducted to determine the effect of this compound on probabilistic decision-making. These studies revealed that neither 1 mg/kg nor 4 mg/kg AA29504 had an effect on locomotor activity. These doses were previously shown to reduce

anxiety and stress-induced hypothermia without altering motor coordination (Hoestgaard-Jensen et al. 2010) therefore they were chosen for testing on the probabilistic discounting task. A 7-day drug treatment period occurred immediately after completion of the initial 20 training sessions (training days 21-28). Neither dose of AA20504 altered performance on the probabilistic decision-making task in control rats. Similarly, 1 mg/kg and 4 mg/kg AA29504 had no effect adult AIE rats tested on the same task. These results suggest that, at least at these doses tested, AA29504 did not alter risk/reward decision-making. The results of these experiments were somewhat surprising given that previous studies identified several behavioral changes associated with this compound. One possible explanation for the lack of effect of the drug is that mode of drug delivery did not produce significant brain and blood concentrations of AA29504 to impact behavior. Previous studies injected the drug subcutaneously and measured pharmacologically relevant doses in the blood and brain (Hoestgaard-Jensen et al. 2010), however subcutaneous drug delivery often produces variability in blood and brain concentrations. Hoestgaard-Jensen et al also identified impairments in motor coordination at a 10 mg/kg dose. In hindsight, a higher dose of AA29504 and/or intraperitoneal injection could have been tested on the locomotor experiments to confirm that the drug was having a pharmacological effect. However, the cost of this compound made additional studies with higher doses difficult. Furthermore, preliminary studies should have replicated the AA29504-induced positive modulation of THIP-induced currents in order to confirm the effect of this compound on layer V pyramidal neurons in the

PrL-C. It should also be noted that the synergistic effect of AA29504 and THIP might still be compromised by AIE, therefore future studies could examine the effect of AIE on AA29504 modulation of THIP-induced tonic currents.

In conclusion, the results of the present chapter did not support the original hypothesis that was based upon published reports. AIE did not alter risk/reward decision-making in adult rats. The lack of effect observed here, combined with previous reports that have described alterations in risk/reward decision making following AIE suggest that subtle differences in training procedure, ethanol exposure and rat strain may have contributed to these previously reported effects. Furthermore, 1 mg/kg and 4 mg/kg AA29504 did not affect locomotor activity or performance on the probabilistic discounting task. However, these negative data are the first to show the use of this novel compound on a cognitive task and are therefore informative to future studies that seek to use this novel compound.

CHAPTER 5

DISCUSSION AND FUTURE DIRECTIONS

The complex neural circuitry involved in optimal cognitive performance requires extensive coordination between the PFC and many other brain regions. The PFC is tasked with accurately interpreting the internal and external environment, and providing detailed feedback for an appropriate behavioral response. Understanding PFC circuitry at the level of neurotransmitter and biochemical systems holds the promise of progressing the field of neuroscience towards identifying pathologies specific to this region, and may pave the way for development of better therapeutics aimed at targeting specific circuits. This

dissertation uses biochemical, physiological and behavioral approaches to uncover lasting neurochemical changes in the PFC that occur after binge-like alcohol abuse during adolescence. Striking differences in both the DA and GABA systems were identified in PrL-C of adult rats exposed to alcohol during adolescence. These alterations may relate, at least in part to, disruptions in development and refinement of the PFC. Both the GABA and DA systems have been shown to undergo extensive refinement and maturation that coincides with the maturation of PFC-dependent cognitive behaviors (Blakemore and Choudhury 2006; Tunbridge et al. 2007; Hoftman and Lewis 2011; Blakemore and Robbins 2012; Datta et al. 2014; Suri et al. 2015). Furthermore, it is generally accepted that acute alcohol directly alters both GABA and DA neurotransmission in the PFC. Taken together, it is possible that exposure to alcohol during adolescence will alter the development trajectory of the DA and GABA systems. In support of this, the effects of adolescent alcohol demonstrated in this dissertation persist months after the exposure period. Many of the findings are specific to alcohol exposure during adolescence. For instance, chronic intermittent ethanol exposure in adulthood does not alter D1 receptor modulation of evoked firing in pyramidal neurons of the PrL-C (Trantham-Davidson et al. 2014), which is in contrast to the findings presented in Chapter 2. Moreover, chronic intermittent ethanol exposure in adulthood does not have an effect on COMT levels (unpublished observation), whereas adolescent ethanol exposure resulted in significantly reduced COMT levels in adulthood. These examples highlight the importance of exposure period and the differential effects alcohol

has on the developing adolescent PFC and the mature adult PFC. However, it should be noted that it is common for human to consume alcohol during adolescence and continue alcohol consumption in adulthood. Although speculative, it is possible that continued exposure from adolescence through adulthood might have a synergistic effect on dopaminergic neurotransmission in the adult PrL-C, which may underlie provide a common mechanism linking many of the behavioral deficits associated with both adolescent and adult alcohol abuse.

AIE ALTERS DOPAMINERGIC NEUROTRANSMISSION IN THE ADULT PrL-C

Findings from Chapter 2 demonstrate that exposure to alcohol during adolescence compromises the development and refinement of the PFC DA system. AIE resulted in reduced TH and MB-COMT expression in the adult PrL-C, suggesting compromised production and/or clearance of DA. Consistent with the suggestion that AIE exposure disrupts development of dopaminergic neurotransmission in the PrL-C was the observation that AIE produced a loss of D1 receptor modulation of pyramidal neurons in layer V PrL-C. A recent study demonstrates that decreased DA levels brought about by increased COMT levels produce an upregulation of D1 receptors (Slifstein et al. 2008). This positive correlation was observed in the dorsolateral PFC, but there is no correlation in the striatum where DAT, and not COMT, is responsible for removal of extracellular DA (Tarazi et al. 1998). These studies are in agreement with the

observed reduction in MB-COMT and reduced D1 receptor modulation of pyramidal neurons presented in Chapter 2. Early studies of D1 receptor modulation of cognitive function indicated that there is an “inverted U” shaped dose-response relationship for the effects of D1 stimulation on working memory such that both high and low levels of stimulation negatively impact performance on working memory tasks (Arnsten 1997; Zahrt et al. 1997; Slifstein et al. 2008). The observed AIE-induced reduction in TH and MB-COMT in adult rats exposed to AIE suggests that AIE may alter extracellular DA levels in the PFC. Previous studies determined that an alcohol-induced increase in DA release occurs in the mesolimbic DA system, which in turn leads to an increase in extracellular DA in target areas (Imperato and Di Chiara 1986; Weiss et al. 1993; Doyon et al. 2003). Alcohol-induced increases in extracellular DA levels during adolescence can be detrimental to the developing DA system in the mPFC. Possible scenarios for the effect of AIE on extracellular DA levels are discussed below.

Scenario 1: The primary response to AIE is decreased TH and DA release.

In this scenario, the primary response to AIE is decreased TH levels that persist into adulthood, resulting in reduced DA production and/or release in the adult mPFC. Although speculative, normal levels of MB-COMT may no longer be needed to clear the reduced amount of DA in the synaptic cleft, which would result in downregulation of the enzyme. The observed AIE-induced decrease in MB-COMT protein expression is therefore a compensatory response to the AIE-

induced decrease in DA release. Two possible outcomes can arise from this scenario. The adult mPFC DA system may have successfully adapted to the reduced protein levels of TH and MB-COMT, thus reestablishing homeostatic extracellular DA. The other outcome of this scenario is that compensation is incomplete, and thus extracellular DA in the mPFC is still reduced in adult AIE-exposed rats.

Scenario 2: The primary response to AIE is decreased MB-COMT

A second possibility is that the primary response to AIE is reduced levels of MB-COMT in the adult mPFC. The reduction in TH levels, and likely reduction in DA production/release, could be interpreted as a compensatory response to the decreased MB-COMT. The mPFC DA system may adequately adapt to the reduced DA-related enzymes after AIE and in turn, acclimate to these changes, and work to reestablish the same homeostatic level of extracellular DA. It is also possible that the adult mPFC DA system is unable to compensate for the reduction in TH and MB-COMT, and the net result is increased extracellular DA levels in the mPFC of adult rats exposed to AIE.

Future studies

Future studies involving the use no-net flux microdialysis could be utilized to directly test the effect of AIE exposure on extracellular DA levels in the adult mPFC. Studies may be designed to measure the effect of AIE on evoked DA

levels in the mPFC. For example, previous studies from Lapish and colleagues show that acute injections of the MB-COMT inhibitor tolcapone enhance PFC DA efflux associated with anticipation and consumption of food (Lapish et al. 2009). The observation that AIE results in reduced MB-COMT levels in the adult mPFC provides a foundation for future studies may involve the use the food anticipation assay to examine the effect of AIE on tolcapone-induced increase in extracellular DA levels in the mPFC. It is possible that the compensatory changes in the mPFC DA system normalize basal levels of DA, but reduced MB-COMT would lead to increased extracellular DA during salient stimuli. In addition, studies demonstrate that reverse dialysis of the DA-norepinephrine reuptake inhibitor methylphenidate can enhance extracellular DA levels (Schmeichel and Berridge 2013), providing another approach for studying the effects of AIE on extracellular DA levels in the mPFC during salient stimuli.

AIE ALTERS GABAERGIC NEUROTRANSMISSION IN THE ADULT PRL-C

Findings from Chapter 3 demonstrate that exposure to alcohol during adolescence affects GABAergic neurotransmission in the adult PrL-C. AIE caused a reduction in sIPSC amplitude, but not frequency. Interestingly, when action potentials were blocked using 1 μM TTX, mIPSC amplitude was not altered by AIE exposure. Although AIE did not alter IPSC frequency, it is still possible that the observed reduction in sIPSC amplitude may be attributed to alterations in presynaptic GABA release. For instance, different classes of

voltage-dependent calcium channels can activate the release mechanisms of distinct pools of neurotransmitters (Kavalali 2015). This suggests the possibility that AIE may specifically alter the presynaptic release mechanism responsible for action potential dependent release of GABA (i.e. sIPSC), while the mechanism responsible for action potential independent release of GABA (i.e. mIPSC) is unaffected. Future studies may examine the effect of AIE on presynaptic voltage-dependent calcium channels and different SNARE proteins responsible for presynaptic GABA release.

In addition, AIE resulted in reduced tonic GABA currents in layer V (but not II/III) pyramidal neurons in the adult PrL-C. Furthermore, the data suggest that the inhibitory conductance mediated by δ-$GABA_A$ receptors is likely responsible for the AIE-induced reduction in tonic current. Another interesting observation made during these studies was the differences in developmental expression of the tonic current and the specific THIP-mediated currents. Although the number of cells expressing a tonic current gradually increased throughout development, the total amplitude of the tonic current elicited by picrotoxin remained constant in control slices from PD28 through PD90. In contrast, there was a progressive increase in currents mediated by the δ-$GABA_A$ receptor from PD28 to PD90, suggesting a developmental emergence of these currents. These data also suggest that early in development, δ-$GABA_A$ receptors are not the sole contributors to the tonic current. $GABA_A$ receptors containing the α5 subunit are viable candidates for mediating the bulk of tonic current prior to

the completion of δ-$GABA_A$ receptors development in the PrL-C. For example, in the hippocampus, α5-$GABA_A$ receptors protein expression has been shown to gradually decrease from adolescence to adulthood (Centanni et al. 2014). Furthermore, α5-$GABA_A$ receptors are thought to mediate at least a portion of tonic inhibitory currents and play a critical role in spatial memory (Prenosil et al. 2006). Partial knockout of α5-$GABA_A$ receptors in mice significantly improves spatial learning, providing the possibility that these receptors may represent a valuable target for cognitive-enhancing drugs (Collinson et al. 2002). In contrast to δ-$GABA_A$ receptors, α5-$GABA_A$ receptor mRNA expression in layer V of the mPFC reaches maximal levels before the onset of adolescence. As adolescence progresses, mRNA levels gradually decrease before reaching stable levels in late adolescence (Datta et al. 2014). Future studies designed to examine the development of α5-$GABA_A$ receptors would provide insight into this subunit's contribution to the tonic GABA current in the PrL-C. To date, full agonists or antagonists acting exclusively at α5-$GABA_A$ receptors are not commercially available, confounding experiments designed to examine α5-$GABA_A$ receptor-mediated currents.

Our study also revealed that AIE exposure resulted in reduced tonic GABA currents shortly after exposure (~PD45), in early adulthood (~PD60) and adulthood (PD90+). In contrast, only in fully mature adult AIE-exposed rats was the amplitude of δ-$GABA_A$ receptor-mediated current attenuated relative to controls. This is consistent with attenuated development of δ-$GABA_A$ receptor-

mediated currents in AIE-exposed rats. Collectively, these data suggest that the δ-$GABA_A$ receptor is not likely the underlying subunit responsible for the AIE-induced reduction in tonic current prior to adulthood. Therefore it is possible that AIE results in reduced α5-$GABA_A$ receptor-mediated currents during AIE exposure, but as development progresses, the normal emergence of the δ subunit does not occur.

In addition to exhibiting high ethanol sensitivity, δ-$GABA_A$ receptors are especially sensitive to modulation by inhibitory neurosteroids such as allopregnanolone, which act as PAMs at δ-$GABA_A$ receptors to enhance the efficacy of GABA (Smith et al. 2007). Neurosteroid levels increase dramatically during puberty (Fadalti et al. 1999), suggesting a possible role for this system in adolescent development. Furthermore, previous studies from VanDoren and colleagues identified a direct interaction between ethanol and the inhibitory neurosteroid allopregnanolone (VanDoren et al. 2000). These studies found that systemic alcohol administration significantly elevated allopregnanolone levels in the cerebral cortex. In data presented in Chapter 3, AIE resulted in decreased allopregnanolone-induced facilitation of tonic current in acute slices from adult PrL-C. Although speculative, exposure to alcohol during adolescence (when neurosteroid levels dramatically elevate) may alter the developmental trajectory of inhibitory neurosteroids that positively modulate $GABA_A$ receptors and furthermore, their ability to properly regulate function of δ-$GABA_A$ receptor. Future studies examining the effects of AIE on inhibitory neurosteroids in the PrL-

C may provide an underlying mechanism for the observed AIE-induced reduction in tonic GABA currents.

ALTERNATIVE BEHAVIORAL EXPERIMENTS

In light of robust changes in dopaminergic and GABAergic neurotransmission in adult AIE-exposed rats, as well as previous studies from our lab and others showing AIE-induced deficits in cognitive function (Chambers et al. 2003; Oscar-Berman and Marinkovic 2003; White and Swartzwelder 2005; Masten et al. 2008; Gass et al. 2014), the negative findings presented in the Chapter 4 were unexpected. Possible explanations for why our observation of a lack of effect of AIE on probabilistic decision-making differs from previous studies (Nasrallah et al. 2011; Boutros et al. 2014) were discussed in Chapter 4. Data from our lab provides strong evidence for AIE-induced deficits in behavioral flexibility using an operant set-shifting task (Gass et al. 2014). In this same study, Gass and colleagues also demonstrated that AIE results in resistance to extinction in adulthood using a self-administration protocol. Future studies may seek to reverse the observed AIE-induced deficits on these tasks by administering the δ-$GABA_A$ receptor PAM AA29504. Additionally, Working memory can be accurately measured with experiments designed using maze-based tasks such as a T-maze with an added variable delay component. These tasks are also conducive for multiple drug administrations. Therefore future

studies could test the effect of different doses of AA29504 on performance on a T-maze.

WORKING MODEL UNIFYING AIE-INDUCED DEFICITS IN GABA AND DA SYSTEMS IN THE ADULT PRL-C

In Chapter 2, studies demonstrated that AIE alters dopaminergic neurotransmission, while the studies in Chapter 3 revealed alterations in tonic GABAergic neurotransmission. The following section will present a proposed mechanistic model linking these two seemingly desperate observations.

As was investigated in Chapter 3, a logical explanation for the observed reduction in δ-$GABA_A$ receptor-mediated currents would be a reduction of expression of δ-$GABA_A$ receptor subunits. However, these studies revealed there were no differences in δ-$GABA_A$ receptor total protein expression or surface expression in the PrL-C. Furthermore, immunohistochemistry studies examining δ-$GABA_A$ receptor expression specifically in layer V PrL-C similarly revealed no effect of AIE on δ-$GABA_A$ receptor expression. Taken together, these data strongly suggest that the AIE-induced reduction in tonic current is not the result of reduced δ-$GABA_A$ receptor expression, but rather may be related to a change in receptor kinetics. Of particular interest are observations that PKC mediated phosphorylation of the β3 subunit of $GABA_A$ receptors increases channel conductance (Brandon et al. 2002). Interestingly, Choi et al demonstrated both anatomical and biochemical links between the PKC isozyme PKCδ and the δ-

$GABA_A$ subunit (Choi et al. 2008). Furthermore, ethanol failed to potentiate tonic currents recorded from PKCδ knockout mice, which is in agreement with our observation that AIE reduced ethanol facilitation of the tonic GABA current. Additional studies demonstrate that PKA mediated phosphorylation of the β1 and β3 subunits of $GABA_A$ receptors reduce and enhance, respectively, channel conductance without altering surface expression (Kittler and Moss 2003; Connelly et al. 2013). Although speculative, the AIE-induced reduction of tonic current amplitude may relate to either an increase in the basal level of PKA-mediated phosphorylation of β1 and/or a decrease in phosphorylation of β3 subunits of extrasynaptic $GABA_A$ receptors. Interestingly, previous studies demonstrated that stimulation of D1 receptors enhanced tonic GABA currents in D1-positive striatal medium spiny neurons in the striatum, while stimulation of D2 receptors reduced tonic current in D2-positive neurons (Janssen et al. 2009). Moreover, PKA infusion enhanced the tonic current in D1-positive medium spiny neurons, while it inhibited current in D2-positive neurons (McDonald et al. 1998). These studies provide a potential link between the AIE-induced deficits observed in the PrL-C DA and GABA systems. A proposed mechanism relating these findings is depicted in figure 5-1. D1 receptors are positively coupled to G_s signaling cascade that activates adenylate cyclase, cyclic AMP and PKA. The AIE-induced reduction in D1 receptor modulation of pyramidal neurons may cause decreased activation of PKA, which in turn leads to reduced PKA phosphorylation of the β3 subunit of α4β3δ $GABA_A$ receptors. The net result is reduced tonic GABA current presumably mediated in large part by δ-$GABA_A$

receptors. Furthermore, this mechanism also fits with observed reduction in MB-COMT. A reduction in both MB-COMT and D1 receptor function are consistent with human studies showing that MB-COMT activity is positively correlated with D1 receptor availability (e.g. reduced MB-COMT results in elevated extracellular DA. The enhanced activation of D1 receptors in turn promoted desensitization or downregulation of D1 receptors). This positive correlation was observed in the dorsolateral PFC, but there is no correlation in the striatum where DAT, and not MB-COMT, is responsible for removal of extracellular DA (Slifstein et al. 2008). As mentioned above, the exact effect of reduced TH expression and reduced MB-COMT on extracellular DA levels is unknown, however, these observations may relate to alterations in the signaling pathway responsible for phosphorylating the β3 subunit. Future studies could directly examine the effect of AIE on phosphorylation of the β3 subunit using β3 subunit-selective antibodies. Additionally, the β2/3 agonist loreclezole may be utilized to examine the function of β2/3 receptors.

In conclusion, the work presented in this dissertation uncovered biochemical and physiological changes in the mPFC that occur after binge-like exposure to alcohol during adolescence. The aim of these studies was to identify alcohol-induced changes that alter the developmental trajectory of the mPFC. This project identified long-lasting changes in both the DA and GABA systems in

the adult mPFC. Therapeutic approaches that selectively target these systems have the potential to reverse the cognitive deficits associated with adolescent alcohol abuse. In addition, this work provides further evidence that abusing alcohol during the critical period of adolescent development can have damaging, and possibly permanent effects on PFC-dependent cognitive function in adulthood.

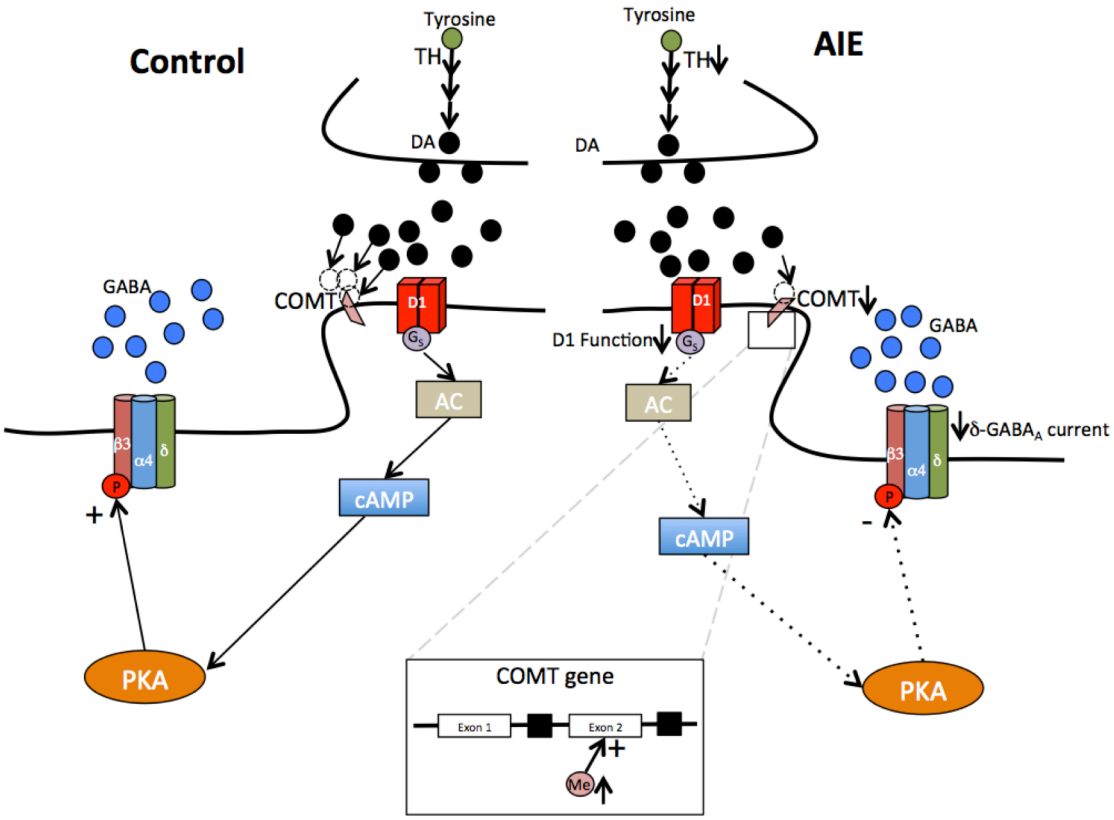

Figure 5-1. Proposed mechanism for AIE-induced deficits in the DA and GABA systems in adult PrL-C. Under normal conditions (left), PKA is activated by metabotropic G-protein coupled receptors (such as D1) that activate the G_S signaling cascade. PKA is one of the major kinases responsible for phosphorylating the β3 subunit, which enhances δ-$GABA_A$ receptor-mediated channel conductance. In AIE-exposed adults (right), reduced COMT levels (from increased COMT methylation (inset)) and TH levels coupled with reduced D1 signaling leads to decreased G_S activation of PKA, which in turn causes decreased phosphorylation of the β3 subunit. The end result is reduced δ-$GABA_A$ receptor-mediated channel conductance. Abbreviations: AC, adenylate cyclase; cAMP, cyclic adenosine monophosphate; COMT, catechol-O-methyl transferase; DA, dopamine; Me, methylation; PKA, protein kinase A; TH, tyrosine hydroxylase.

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