ASSESSING THE ADOLESCENT AND ADULT PREFRONTAL CORTEX PROTEOME FOR MECHANISMS OF ENHANCED VULNERABILITY TO ALCOHOLISM

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ABSTRACT

Abigail Elizabeth Agoglia: Assessing the Adolescent and Adult Proteome for Mechanisms of Enhanced Vulnerability to Alcoholism
(Under the Direction of Clyde W. Hodge)

Alcoholism is a debilitating neurobiological disorder affecting millions of people in the United States and around the globe. Treatment options for alcoholism are limited, due in part to incomplete understanding of the development and molecular mechanisms that lead to alcohol addiction. Age of drinking onset is one of the most significant predictors of lifetime alcoholism risk, with individuals initiating alcohol use during adolescence at substantially increased risk for developing alcoholism than those who initiate drinking during early adulthood. However, the specific neurobiological mechanisms responsible for this increased risk remain elusive. Synaptic plasticity is a critical component of adolescent brain development, and evidence suggests that alcohol and other drugs of abuse may induce aberrant plasticity in the brain, leading to long-term changes in the response to these drugs. Thus, the goals of this dissertation were to characterize the development of the adolescent and adult prefrontal cortex, a region known to be involved in adolescent maturation and drug taking behavior, and assess the differences in how the adolescent and adult prefrontal cortex respond to the presence of alcohol at the protein-expression level.
Several of the proteins regulated by age and alcohol are involved in synaptic plasticity, including the protein phosphatase calcineurin (PPP3R1), which displays increased expression during adolescence and reduced expression following alcohol drinking in the adolescent but not adult prefrontal cortex. Systemic inhibition of PPP3R1 with the drug FK506 reduced alcohol drinking more potently in adolescent versus adult mice, suggesting that PPP3R1 may be functionally involved in age differences in alcohol consumption. Next, to evaluate the role of PPP3R1 in long-term vulnerability to alcoholism following adolescent exposure to alcohol, a protocol for adolescent drinking and subsequent adult operant self-administration of alcohol was developed. Mice exposed to alcohol drinking during adolescence exhibited increased operant responding for alcohol during adulthood, and finding that was not evidenced in adult mice exposed to alcohol and subsequent operant self-administration. The increased responding for alcohol in adolescent alcohol exposure was reduced by the PPP3R1 inhibitor FK506. In contrast, operant responding for alcohol was not affected by FK506 in mice exposed to water during adolescence or adult mice exposed to either alcohol or water, demonstrating relative selectivity for the unique pattern of increased intake exhibited by mice exposed to alcohol during adolescence. The role of PPP3R1 in the prefrontal cortex was assessed via site-directed microinjection of FK506, which increased operant responding for alcohol in mice exposed to either alcohol or water during adolescence. Lastly, to examine brain regions known to be both regulated by input from the prefrontal cortex and involved in alcohol reinforcement, the amygdala, dorsal striatum and nucleus accumbens were all assessed for expression and phosphorylation of CaMKII, a protein critical
for synaptic plasticity. Adolescent mice displayed reduced phosphorylation of CaMKII in the amygdala following voluntary alcohol drinking, whereas adult mice exhibited no changes in expression or phosphorylation. Conversely, phosphorylation of the AMPA receptor subunit GluA1 was increased in adult mice following alcohol consumption but reduced in adolescent mice. Finally, systemic administration of tianeptine, an atypical antidepressant that upregulates pGluA1 in a CaMKII-dependent manner, increased alcohol consumption in adolescent mice but decreased alcohol consumption in adult mice. Collectively, these experiments demonstrate that the adolescent and adult brains have noticeably different responses to alcohol consumption. The findings provide evidence that long-lasting alterations in synaptic plasticity may be responsible for the increased alcohol intake observed after adolescent exposure to alcohol.
ACKNOWLEDGEMENTS

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I am also grateful for the advice and comradery of Dr. Sara Faccidomo, without whom I would probably still be struggling to train mice to lever press. From Sara, I have learned that there is always one right way to display data, how to design the most pleasing poster layout, that two doses do not constitute a “curve” no matter how much I might want them to, and that graphs made in Excel deserve nothing but contempt. It has been a privilege to work alongside such a fantastic scientist (with rapier wit.)

All the other members of the Hodge lab have contributed to my education and experimental progress. In particular, Dr. Sarah Holstein taught me everything I know about Western blots, Reggie Cannady has been a true mentor and friend since we first met,
Grant Reid kept the lab running and was a ready source of science commiseration, and Valarie Eastman sacrificed many weekends to assist with my operant behavior experiments. Undergraduates Amanda Small, Jessica Trexler and Briana Saunders also made much of my work possible and allowed me to work on my own mentorship skills, such as they are. Thank you all for your assistance, support and friendship.

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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>2-AG</td>
<td>2-arachidonoylglycerol</td>
</tr>
<tr>
<td>AEA</td>
<td>Anandamide</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate acid</td>
</tr>
<tr>
<td>AMY</td>
<td>Amygdala</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>AUD</td>
<td>Alcohol Use Disorder</td>
</tr>
<tr>
<td>BAC</td>
<td>Blood Alcohol Concentration</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Ca$^{2+}$/calmodulin-dependent Protein Kinase II</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>CB1</td>
<td>Type 1 Cannabinoid Receptor</td>
</tr>
<tr>
<td>CFL1</td>
<td>Cofilin-1</td>
</tr>
<tr>
<td>CNR1</td>
<td>Cannabinoid Receptor 1 Gene</td>
</tr>
<tr>
<td>CSA</td>
<td>Cyclosporine A</td>
</tr>
<tr>
<td>CTA</td>
<td>Conditioned Taste Aversion</td>
</tr>
<tr>
<td>COMT</td>
<td>Catechol-O-methyl transferase</td>
</tr>
<tr>
<td>D2</td>
<td>Dopamine Receptor 2</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DNM1</td>
<td>Dynamin-1</td>
</tr>
<tr>
<td>DPYSL2</td>
<td>Dihydropyrimidinase-like 2</td>
</tr>
<tr>
<td>DSM-V</td>
<td>Diagnostic and Statistics Manual of Mental Disorders: Fifth Edition</td>
</tr>
<tr>
<td>dSTR</td>
<td>Dorsal Striatum</td>
</tr>
</tbody>
</table>
ERK 1/2 | Extracellular Signal-Regulated Kinase 1/2
---|---
ETOH | Ethyl Alcohol or Ethanol
FASD | Fetal Alcoholism Spectrum Disorder
FDA | Food and Drug Administration
FR | Fixed-Ratio
GABA | gamma-Aminobutyric acid
GABA_ARs | GABA A Receptors
GCP | Global Canonical Pathway
GFA | Global Functional Analysis
GluA1 | GluA1 AMPA Receptor Subunit
GluN2B | NMDA Receptor Subunit
g/kg | Gram Per Kilogram
GWAS | Genome-Wide Association Study
IEF | Isoelectric Focusing
I.P. | Intraperitoneal injection
IP3 | Inositol Triphosphate
IPA | Ingenuity Pathway Analysis
LTD | Long-Term Depression
LTP | Long-Term Potentiation
MALDI-TOF | Matrix-Assisted Laser Desorption/Ionization-Time of Flight
MC | Primary Motor Cortex
mGluR | Metabotropic Glutamate Receptor
mPFC | Medial Prefrontal Cortex
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAc</td>
<td>Nucleus Accumbens</td>
</tr>
<tr>
<td>NIAAA</td>
<td>National Institute on Alcohol Abuse and Alcoholism</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl-D-aspartic Acid</td>
</tr>
<tr>
<td>pCaMKII</td>
<td>Phosphorylated $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>PFC</td>
<td>Prefrontal Cortex</td>
</tr>
<tr>
<td>pGluA1</td>
<td>Phosphorylated GluA1 AMPA Receptor Subunit</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PND</td>
<td>Post-Natal Day</td>
</tr>
<tr>
<td>PPP3</td>
<td>Calcineurin</td>
</tr>
<tr>
<td>PPP3R1</td>
<td>Calcineurin subunit B</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SNP</td>
<td>Single-Nucleotide Polymorphism</td>
</tr>
<tr>
<td>SSRI</td>
<td>Selective Serotonin Reuptake Inhibitors</td>
</tr>
<tr>
<td>STXB1</td>
<td>Syntaxin Binding Protein 1</td>
</tr>
<tr>
<td>TMS</td>
<td>Transcranial Magnetic Stimulation</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral Tegmental Area</td>
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CHAPTER ONE: INTRODUCTION

Alcoholism

Alcoholism is a chronic neuropsychiatric disorder involving excessive consumption of alcohol leading to deficits in the patient’s social, personal and professional life. The Diagnostic and Statistical Manual of Mental Disorders maintained by the American Psychiatric Association lists 11 criteria for Alcohol Use Disorder (AUD), focusing primarily on excessive consumption of alcohol (1-4), persistence of excessive drinking despite negative consequences (5-8), and physiological effects of alcohol including blackouts, tolerance and withdrawal (9-11) [1]. Patients exhibiting two to three of the 11 criteria are classified as having mild AUD, patients with four to five are considered moderate, and patients with six or more criteria are designated severe. Individuals suffering from alcoholism face many negative health risks, including withdrawal, seizures, memory loss, anxiety, depression, brain damage and death [2]. A key feature of alcohol addiction is increased salience of alcohol and related cues over non-drug aspects of life, leading to the neglect of career, social life and health [3, 4]. The alterations in alcohol cue responsivity may represent a unique challenge of alcohol addiction treatment, since unlike most other drugs of abuse alcohol is freely available for purchase throughout the United States and indeed has an important cultural and social role in societies
throughout the world. Completely removing oneself from the influence of alcohol is very difficult in a culture that readily promotes its use.

The prevalence of AUD in the United States is relatively high, with recent estimates at 15.1 million adults (6% of total population) and an additional 600,000 youths (3% of total population) meeting the criteria for an AUD diagnosis [SAMHSA, 5]. Globally, alcohol consumption is the fifth leading risk factor for premature death and disability [6], and contributes to more than 3 million deaths worldwide each year [WHO, 7]. The economic burden on American society is also significant; in 2010, excessive alcohol consumption resulted in nearly $290 billion in expenses across the U.S., and in the state of North Carolina alone over $7 billion [8].

Despite the pressing public health consequences of excessive drinking, treatment options for patients with AUD remain limited and often ineffective. Currently, only three medications are FDA-approved to treat alcoholism (disulfiram/Antabuse, naltrexone/Trexan, and acamprosate/Campral), and all three medications have suffered from inconsistent clinical outcomes [9] and under-prescription by physicians [10]. Outpatient treatment options, including individual therapy, substance abuse counseling and 12-Step programs such as Alcoholics Anonymous, have shown variable success rates, with some studies reporting 1–year sobriety levels of 30-60% [11-14], but other reports finding sobriety at 1–year follow up to be as low as 16-20% [15, 16]. Inpatient treatment centers have generally been found to produce similar results to outpatient treatment [17, 18]. There is an urgent need for improved therapeutic interventions for alcoholism, including more effective pharmacotherapies, better targeting of treatments to individual patients, and
preventative strategies to identify at-risk individuals and provide intervention before problem drinking develops.

**Use of Animal Models in Alcoholism Research**

The consumption of alcohol is an endogenous behavior for several disparate species in addition to humans. Non-human primates have been observed to eat fermented fruit containing significant alcohol content (up to 5% by volume) in the wild [19]. Indeed, genetic sequencing suggests that a common ancestor of human and non-human primates began to display a more efficient form of alcohol dehydrogenase 4 (ADH4), an enzyme responsible for alcohol metabolism, about 10 million years ago [20]. This finding suggests evolutionary pressure favoring better metabolism of alcohol, which may be indicative of increased alcohol intake among primates at that time. Fruit flies are attracted to fermented fruit in a similar fashion and can achieve doses of alcohol that induce intoxication-like behavior under normal conditions [21]. Animal models of alcohol intake and dependence are therefore not far removed from the behavior of wild animals in their natural habitat. Non-human primates and fruit flies have been used to model alcoholism and provided important insight into the behavioral and genetic underpinnings of alcohol consumption, respectively [22, 23]. Work in rodent models of alcoholism, however, provides more behavioral complexity than invertebrate models combined with a more efficient means to evaluate genetic and molecular underpinnings of addiction than models using relatively long-lived primates. These features have made rodent models of alcoholism attractive to researchers for decades.
Rodent Models of Alcoholism

Both rats and mice readily consume alcohol, although the amount of intake and degree of preference can vary wildly by strain. Notably, several inbred strains of rodents have been selected for both high and low alcohol intake. In rats, selective breeding has created five lines with increased alcohol intake and preference: ALKO/Alcohol (AA), alcohol-preferring (P), high alcohol-drinking (HAD-1 and HAD-2), and Sardinian alcohol-preferring (sP), all of which have been used to model alcoholism and related behaviors. [24]. In mice, intake and preference of alcohol varies markedly across strain [25]. Notably, the inbred C57BL/6 strain displays high alcohol intake and preference [26]. These behavioral characteristics have made P rats and C57 mice attractive as models for alcoholism due to their ready self-administration of alcohol under normal conditions. In particular, C57 mice can achieve intoxicating doses of alcohol through simple home-cage self-administration, with intake in excess of 20 g/kg per day and blood alcohol levels in excess of 100 mg/dL [27]. C57 mice show strong motivation to consume alcohol, as evidenced by their willingness to work for access to alcohol solutions in operant self-administration procedures [28]. Finally, direct assessment of brain reward via intracranial self-administration has demonstrated that alcohol potentiates brain stimulation reward in C57 mice [29]. Together, these findings demonstrate the appropriateness of mouse models for the rewarding and consumatory aspects of alcohol drinking behavior.

In addition to the positive, rewarding effects of the drug, an important behavioral feature of alcohol addiction is the tendency to relapse, or begin drinking again following a period of sobriety. Rodent models for relapse-like behavior have
made use of operant self-administration procedures to evaluate the degree of alcohol-seeking a rat or mouse engages in following a period of forced abstinence [30]. After training to lever press for the delivery of an alcohol solution, the subject is put through a series of extinction sessions to reduce or eliminate the operant responding. Following extinction, when rats and mice are exposed to an alcohol-related cue (such as the scent of alcohol, a brief period of alcohol access, or the presentation of a neutral stimulus previously paired with alcohol), operant responding for alcohol has been reliably shown to reinstate, even in the absence of alcohol delivery in response to lever pressing. The reinstatement of operant responding for alcohol has been used as a model for relapse, and can reliably be produced through mechanisms known to promote relapse in human alcoholics, such as exposure to alcohol or alcohol-related cues and stress [31]. C57 mice also exhibit cue-induced reinstatement of alcohol responding in a manner comparable to work done in rat models [32, 33]

Finally, human alcoholics display important behavioral and physiological changes as a result of the disease, including anxiety, depression, anhedonia, tolerance and withdrawal [34]. Rodents also readily display tolerance and withdrawal after repeated exposure to alcohol. Indeed, over time rats and mice that freely self-administer alcohol to the point of tolerance/withdrawal become dependent upon it in much the same way as human alcoholics do [35, 36]. Mouse models of chronic alcohol consumption and exposure have also demonstrated increases in anxiety-like and depressive behavior when deprived of alcohol [37, 38]. Thus, the important behavioral characteristics and many of the clinical features of alcoholism
can be accurately reflected in rodent models of alcohol intake.

**Neurobiology of Alcoholism**

The use of both animal models and human participants has elucidated a broad range of neurotransmitter systems impacted by alcohol. A small, lipid-soluble molecule, ethanol readily crosses the blood-brain barrier and can interact with a host of receptors, subcellular targets, and genetic materials [39]. These diverse effects complicate the process of identifying a neurobiological substrate responsible for the physiological and behavioral effects of alcohol in patients with AUD and animal models of alcoholism.

**GABA**

\(\gamma\)-aminobutyric acid (GABA) is an inhibitory neurotransmitter that is critical in regulating synaptic activity throughout the mammalian brain. Alcohol serves as a positive allosteric modulator of GABA A receptors (GABA\(\alpha\)Rs), and has been shown to enhance GABA\(\alpha\)R activity at physiologically relevant concentrations as low as 3 mM [40]. Animal models have demonstrated the importance of GABA\(\alpha\)Rs for several properties of alcohol intoxication, including subjective effects [41], locomotor stimulation [42], sedation/hypnosis [43], and reward [44]. In human studies, genetic variation at the GABA\(\alpha\) \(\alpha\)2 subunit has been associated with increased risk for alcoholism [45, 46]. Whereas acute alcohol enhances GABA signaling in vivo, chronic exposure to alcohol has been shown to suppress GABA activity, reducing normal inhibitory neurotransmission in the absence of alcohol [47]. This suppression
may be related to some of the serious symptoms of alcohol withdrawal, including anxiety/depression and seizures. Further, brain imaging studies have identified alcohol activation of GABA\(_{\text{A}}\)R as a target of alcohol’s acute and chronic effects in humans [48].

**Monoamines**

A common feature of natural reinforcers and nearly all drugs of abuse is their ability to potentiate the release of dopamine into the nucleus accumbens [49]. Alcohol has been shown to acutely enhance accumbal dopamine in rodents, primates and humans [50]. Further, animal models have shown that dopamine activity in the nucleus accumbens is required for the rewarding effects of alcohol [51]. Pharmacological inhibition [52] or systemic knockout [53] of dopamine receptors have both been shown to reduce alcohol intake in rodent models. In human populations, variations in the gene for the monoamine transporter catechol-O-methyl transferase (COMT) have been associated with increased risk for alcoholism and alterations in the behavioral response to alcohol [54].

The serotonin signaling systems have also been shown to respond to the presence of alcohol and mediate some of the effects of alcohol exposure. In rodent models, acute and chronic alcohol have both been shown to alter activity of serotonin neurons in the dorsal raphe nucleus [55], a region which projects to many brain areas critical to alcohol reward and consumption, including the prefrontal cortex, nucleus accumbens and amygdala [56]. Genetic variations in the serotonin transporter gene SERT have been associated with increased risk for alcoholism in human populations [57]. In preclinical models, selective serotonin reuptake
inhibitors (SSRIs) have been shown to reduce alcohol intake in rodents [58] and non-human primates [59], although human trials have had difficulty replicating this reduction even in populations of alcoholics with comorbid depression [60].

**Endocannabinoids**

The endocannabinoid signaling systems serve as retrograde regulators of neuronal activity. Activation of pre-synaptic CB1 or CB2 receptors by the postsynaptic release of the endogenous ligands anandamide (AEA) or 2-arachidonoylglycerol (2-AG) reduces firing of the presynaptic cell. Activity at CB1 receptors has been shown to be involved in the rewarding effects of natural reinforcers such as food as well as the euphoria accompanying marijuana use [61]. Endocannabinoid signaling is also involved in alcohol reward; inhibition of the CB1 receptor blunts alcohol intake and reduces alcohol preference, whereas activation of CB1 has been generally shown to enhance alcohol consumption [62]. In terms of the effects of alcohol on endocannabinoid signaling, acute alcohol has been shown to promote the release of endocannabinoids and potentiate CB1 signaling [63], whereas chronic alcohol appears to decrease CB1 receptor expression [64] as well as increase synthesis of AEA and 2-AG [65]. In human populations, decreases in CB1 receptor availability has been demonstrated in alcoholics [66] and variations in the CNR1 gene have been associated with increased risk for alcoholism [67].

**Glutamate**

Glutamate is the primary excitatory neurotransmitter in the mammalian CNS and plays a critical role in driving neurotransmission throughout the brain.
Glutamate activates multiple receptors with specific synaptic functions. Ionotropic N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainite receptors are expressed at the postsynaptic membrane; glutamate stimulation of these receptors results in direct opening of ion channels, allowing ingress of cations and facilitating depolarization of the postsynaptic neuron [68]. Metabotropic glutamate receptors indirectly open ion channels through the action of the βγ subunit of the coupled G protein [69], and can also exert a modulatory effect on synaptic transmission via the activation of second messenger signaling systems, leading to a diverse array of alterations in cellular activity. Group I metabotropic receptors (mGluR1 and mGluR5) are localized to the postsynaptic membrane; both receptors are Gαq coupled and therefore stimulate increases in cyclic adenosine monophosphate (cAMP) and associated activity within the cell [70]. Common downstream effects of cAMP stimulation include the activation of phospholipase C (PLC) and the subsequent synthesis of inositol triphosphate (IP3) and diacylglycerol (DAG), which in turn can alter genetic material, protein expression, and protein trafficking to the cell surface [71]. In contrast, Group II metabotropic glutamate receptors (mGluR2 and mGluR3) are located at the presynaptic membrane. These inhibitory Gι coupled proteins serve as autoreceptors, regulating the release of glutamate from the presynaptic membrane [72]. Stimulation of mGluR2/3 receptors results in decreased glutamate release and thus reduces the likelihood of a postsynaptic depolarization. The role of Group III metabotropic glutamate receptors in the brain (mGluR4, mGluR7 and mGluR8) is less well
characterized, but these receptors are also G_i coupled and may serve to regulate glutamate release in a manner similar to the Group II receptors [73].

Alcohol has been shown to directly impact the mammalian glutamate systems. Generally speaking, acute alcohol appears to increase extracellular glutamate in the rodent brain [74-77]. Chronic alcohol exposure also appears to upregulate the release of glutamate in limbic brain regions; Griffin and colleagues [78] found that mice exposed to chronic ethanol vapor exhibited twice the enhanced glutamate release in the nucleus accumbens following voluntary alcohol consumption as compared with water vapor controls. This enhancement of glutamate release was observed up to a week following alcohol drinking. Evidence from human populations also suggests that alcohol consumption is associated with alterations in glutamate signaling and function. Increased glutamate activity has been linked to alcohol craving in detoxified alcoholic patients [79] and alcohol withdrawal [80], and postmortem studies in the brains of AUD patients have indicated changes in metabotropic [81] and ionotropic [82] glutamate receptor expression and binding activity. In non-alcoholic participants, low dose alcohol has been shown to suppress excitability of the prefrontal cortex [83]. These findings illustrate that the interactions between alcohol and the glutamate systems vary depending upon population, alcohol dose and brain region. Lastly, one of the few FDA-approved treatments for AUD is acamprosate, a drug that has been shown to alter glutamatergic synaptic transmission (although the precise mechanism of action remains unclear) [84].
Acute alcohol has been shown to interact with the metabotropic glutamate receptors. Alcohol inhibits Group I metabotropic glutamate receptors through a protein kinase C (PKC) dependent mechanism [85], and may also inhibit the activity of Group 2 metabotropic glutamate receptors [86]. Exposure to alcohol also alters the expression of metabotropic glutamate receptors; acute alcohol has been shown to upregulate mGluR5 [87] and chronic alcohol has been found to reduce mGluR2/3 expression [86]. Evidence indicates that glutamate receptors can functionally regulate alcohol-associated behaviors. The metabotropic glutamate receptors have been shown to regulate a variety of behavioral responses to alcohol, including subjective effects [88], locomotor sedation [89], reinforcement [90], intake [91], and relapse-like behavior [92]. In general, inhibition of Group I metabotropic glutamate receptors has been found to reduce alcohol intake, whereas Group II/III metabotropic glutamate receptor agonists reduce alcohol intake and block alcohol discrimination, suggesting opposing roles for these receptor groups in the regulation of alcohol-associated behaviors [93]. mGluR1 antagonists specifically inhibit the alcohol-induced potentiation of dopamine release in the nucleus accumbens [94], which may be a mechanism by which mGluR1 activity modulates alcohol reward and alcohol drinking. The specific mechanism by which alcohol interacts with metabotropic glutamate receptors remains unclear, but the lack of evidence for direct binding of alcohol to metabotropic glutamate receptors suggests that alcohol exerts these effects upstream of mGluR activity.

The ionotropic glutamate receptors have also been shown to be involved in the brain response to alcohol. In mice, in vivo recording from hippocampal slices has
shown that acute alcohol inhibits NMDA-mediated excitatory synaptic transmission [95]. Chronic alcohol has been found to increase the expression of NMDA receptors over time [96], leading to a “hyperexcited” state that may be associated with seizures during alcohol withdrawal [97]. Chronic alcohol has also been found to alter the subunit composition of NMDA receptors [98], which has been independently associated with alterations in the sensitivity of the NMDA receptor to alcohol [99]. Findings from the postmortem brain tissue of human alcoholics have been mixed, with some reports indicating an increase in NMDA receptor expression and glutamate binding sensitivity [100] whereas others report decreases in both measures [101]. A functional role for NMDA receptor activity in alcohol drinking has also been established, as inhibition of NMDAR has been shown to reduce alcohol intake in rodent models of alcohol drinking [102].

The AMPA receptors have also been shown to respond to and regulate alcohol drinking. The specific mechanism by which alcohol interacts with AMPA receptors has not been conclusively established, although evidence suggests that alcohol may interfere with AMPA receptor desensitization [103]. Acute alcohol appears to suppress the activity of AMPA receptors [104, 105], but chronic alcohol exposure leads to an upregulation in the expression and activity of AMPARs, particularly in brain regions known to be involved in alcohol reward such as the prefrontal cortex [106], nucleus accumbens [107], dorsal striatum [108], amygdala [109-111] and ventral tegmental area [112]. Systemic enhancement of AMPAR activity increases self-administration of alcohol in P-rats [111], as does intra-amygdala AMPA activation [113], whereas AMPAR antagonists have been shown to
reduce alcohol intake in mice [110]. Primate models of chronic alcohol self-administration have also revealed alterations in AMPA receptor mRNA [114]. Human research has also indicated that alcoholism may be associated with changes in AMPA receptor expression, with divergent effects across different brain regions [115].

Taken together, these findings indicate that glutamate signaling is a critical component of alcohol consumption and a potential contributor to AUD. It has been suggested that the transition from alcohol use to alcohol dependence and abuse is characterized by an imbalance in inhibitory and excitatory signaling in the mesocorticolimbic regions of the brain, resulting in a hyper-excitatory state that enhances motivation to consume alcohol. A proposed consequence of this enhanced glutamatergic activity in the alcoholic brain is the activation of glutamatergic synaptic plasticity in addiction-associated brain regions.

**Synaptic Plasticity in Alcohol Addiction**

Synaptic plasticity refers to the phenomenon by which repeated stimulation of neurons can lead to long-lasting changes in the strength of synaptic connections. Synapses can be strengthened or weakened through plastic mechanisms depending upon the type of input received by the postsynaptic neuron. Generally, high-frequency stimulation has been shown to increase synaptic strength between the presynaptic and postsynaptic neuron. This process, better known as long-term potentiation (LTP), has been thought to underlie the ability of the brain to learn and form memories and has been most thoroughly characterized in the glutamatergic
neurons of the hippocampus. Electrophysiology has identified a decrease in the ratio of NMDA/AMPA receptors (generally reflecting an increase in AMPA receptors accompanied by no change in NMDA receptors) in the postsynaptic neuron as a key identifying feature of LTP. Evidence for LTP has also been observed in additional brain regions, including the prefrontal cortex [116], amygdala [117], nucleus accumbens [118] and ventral tegmental area [119]. Although these key changes are driven by alterations in glutamatergic signaling, many other neurotransmitters also contribute to LTP in the brain. Specifically within the cortex, both monoamines [120] and GABAergic receptors [121] are sensitive to plastic adaptation and functionally regulate the alterations in cellular activity seen after LTP.

Long-term depression (LTD) is a related phenomenon that typically translates low-frequency stimulation into a reduction of synaptic strength. At the postsynaptic membrane, LTD is accompanied by reductions in the AMPA/NMDA receptor ratio as AMPA receptors are removed from the synapse [122]. In addition to alterations in glutamate receptor expression, LTD has also been shown to require activity in the endocannabinoid systems, which act as retrograde facilitators of LTD from the postsynaptic membrane to the presynaptic terminal [123], and alterations in the inhibitory GABAergic signaling systems [124].

Molecular Mechanisms of Glutamatergic Synaptic Plasticity

Although glutamatergic synaptic plasticity could be broadly described as an increase in AMPA receptors at the postsynaptic membrane, there are a number of key molecular mediators that translate an initial high-frequency stimulus train into long-lasting changes at the dendrite. Endogenously, the initial stimulus to the
postsynaptic membrane takes the form of glutamate release, which activates NMDA receptors already present at the postsynaptic membrane. The opening of ionotropic NMDA receptors triggers an influx of cations, most notably Ca\(^{2+}\). Ca\(^{2+}\) is a critical regulator of a vast array of cellular functions, including metabolism, receptor trafficking, gene expression and cell death [125]. One of the distinct effects of Ca\(^{2+}\) influx is the activation of calmodulin, a Ca\(^{2+}\)-binding protein that facilitates many of the intracellular effects of Ca\(^{2+}\) [126]. Once calmodulin binds Ca\(^{2+}\) and thereby becomes active, the critical enzyme Ca\(^{2+}\)/calmodulin-dependent kinase II (CaMKII) can be activated by the complex.

CaMKII is a heterodimer composed of 6 α and 6 β subunits which exhibits the ability to engage in autophosphorylation, phosphorylating its own subunits perpetually upon activation until the enzyme is inactivated [127]. The ability to maintain initial phosphorylation over extended periods of time has led many investigators to conclude that CaMKII may be a physical representation of memory in the brain [128]. One of the important downstream effects of CaMKII activation is the phosphorylation of the GluA1 subunit of the AMPA receptor at serine 831 on the C-terminus, which increases the conductance of the receptor and is associated with trafficking of cytoplasmic AMPARs to the cell surface [129]. Enhanced phosphorylation of GluA1 is therefore a key component of the increased AMPA expression and activity observed at postsynaptic neurons exhibiting LTP. Several lines of evidence from in vivo and in vitro studies have established that CaMKII activation is both necessary and sufficient for LTP induction. Mutant mice with autophosphorylation-deficient CaMKII do not display LTP under normal stimulation.
procedures and exhibit memory deficits in learning tasks [130]. Conversely, the introduction of autophosphorylated CaMKII into the hippocampus triggers LTP [131].

GluA1 phosphorylation is dynamically regulated by phosphatases that dephosphorylate the subunit to reduce conductance and remove AMPARs from the synaptic membrane. A key phosphatase involved in this process is calcineurin, or protein phosphatase 3 (PPP3). Like CaMKII, PPP3 is a calcium-sensitive enzyme that is activated upon Ca$^2+$ influx to the postsynaptic neuron [132]. Upon activation, PPP3 dephosphorylates GluA1 at Ser845, a site phosphorylated by cAMP-dependent protein kinase (PKA) and associated with increased single-channel conductance, open probability, and insertion into the synaptic membrane (much like Ser831, the CaMKII phosphorylation site). Whereas phosphorylation of these sites by CaMKII/PKA is associated with LTP, dephosphorylation by PPP3 is associated with long-term depression (LTD), or a decrease in synaptic strength following an initial stimulus. Cell culture experiments with a PP2B peptide inhibitor demonstrated that PPP3 inhibition facilitates LTP [133], whereas overexpression of a constitutively active PPP3 inhibits LTP [134].

**Alcohol and Synaptic Plasticity**

In addition to the body of work identifying NMDA and AMPA receptors as targets of alcohol's action in the brain as previously discussed, direct evidence also implicates lasting changes in LTP and subcellular targets known to be involved in synaptic plasticity in the etiology of alcoholism. CaMKII appears to be alcohol-sensitive, mediate some behavioral responses to alcohol and be involved in the motivation to consume alcohol. Low-dose alcohol drinking has been shown to
increase α-CaMKII in the amygdala of male mice, and both CaMKII inhibitors and AMPAR antagonists microinjected into the amygdala reduced operant self-administration of alcohol in mice [110]. In a genetic model of CaMKII autophosphorylation-deficient mice, alcohol drinking and locomotor stimulation in response to alcohol were both reduced, and DA and 5-HT release in response to alcohol were altered in the nucleus accumbens and prefrontal cortex, respectively [135]. The same model was found to reduce conditioned place preference for alcohol in subsequent experiments [136]. Finally, SNPs in the human CAMK2A gene have been associated with alcoholism in humans [135].

Although PPP3 has an important role in synaptic plasticity and interacts with GluA1 in a way that opposes the activity of CaMKII, very few experiments have investigated the effects of alcohol on PPP3 and vice versa. Observations from alcoholics who undergo liver transplantation for alcohol-induced liver disease have noted that rates of relapse to alcoholism following transplantation are low, and some have attributed these reductions in drinking to the anti-rejection medication cyclosporine A, a PPP3 inhibitor [137]. Two studies have assessed the effects of PPP3 inhibitors on alcohol drinking in mice, finding that PPP3 inhibition decreases alcohol drinking [138, 139]. The extent to which PPP3 is alcohol-sensitive, the brain regions responsible for the effects of systemic PPP3 inhibition on alcohol intake, and the specific neural mechanisms that mediate these effects remain to be investigated.

A complexity in the relationship between alcohol and synaptic plasticity is the need to specify which synaptic inputs and neural circuits are being strengthened. In the prefrontal cortex, alcohol exposure appears to affect excitatory efferents with
input to downstream limbic regions involved in drug reward. Chronic alcohol exposure and repeated withdrawal has been shown to enhance the excitability of glutamate projection neurons in the PFC as well as increase the AMPA/NMDA ratio [106]. Recent work by Ma and colleagues [140] suggests that alcohol consumption potentiates synaptic strengthening of glutamatergic inputs from the PFC to the dorsal striatum via an AMPA-associated process, highlighting the importance of cortical regulation of limbic brain regions in the etiology of excessive alcohol consumption. In the amygdala, where some drug-associated efferents from the PFC terminate, chronic alcohol potentiates synaptic plasticity as indexed by an increase in the expression and activity of AMPA receptors [141].

**Vulnerability to Alcoholism**

The identification of factors that impart vulnerability to the development of an AUD is an important goal of alcoholism research. Risk factors for alcoholism have the potential to aid in the determination of AUD liability in individual patients, point towards avenues for pharmacotherapeutic intervention, and suggest strategies for the prevention of AUDs in the general population. To date, strong evidence supports a role for genetic, environmental, and developmental components of the composite risk for developing alcoholism over the lifespan.

Results from twin studies suggest the heritability of AUD is approximately 50% [142], and research on the descendants of alcoholics suggest that a genetic risk for alcoholism persists in the offspring of alcoholics even if they are not raised in an alcoholic household [143]. Several specific genetic polymorphisms have been linked to incidence of AUD, including the aldehyde dehydrogenase 2 gene [144] and
the α-2 subunit of the gamma-Aminobutyric acid (GABA) receptor [45]. However, the contribution of such polymorphisms to overall alcoholism liability appears to be small (2% or less of the variance), indicating that genetic and environmental factors both contribute to the development of the disease.

Environmental factors also contribute to the development of AUDs. Stress is well-characterized as a component of alcohol seeking and drinking behavior [145], and has been shown to increase alcohol intake in both alcoholics [146] and non-alcoholics [147] as well as precipitate relapse in abstinent alcoholics [148]. Socioeconomic status [149] and culture [150] have also been shown to influence the development of AUDs.

Finally, key developmental stages have been linked to the incidence of alcoholism over the lifespan. Prenatal exposure to alcohol can result in Fetal Alcoholism Spectrum Disorder (FASD), a condition characterized by a mosaic of physical and behavioral abnormalities including increased risk for alcohol addiction in adulthood [151]. Significantly, the vast majority of alcoholics initiate alcohol drinking during adolescence [152], and exposure to alcohol during this developmental stage is associated with much higher rates of lifetime AUD than exposure to alcohol during early adulthood [153]. These data make adolescence of particular interest in service of the overall aim of understanding and preventing the development of alcoholism.

**Adolescence**
Adolescence is a developmental period that occurs as an organism matures from a juvenile into an adult. In humans, adolescence can be broadly defined as occupying the years between pubertal development and complete physiological maturity (including adult brain development), usually between the early teens and early twenties [154]. Adolescence is conserved across mammalian species such that adolescent-typical physiological development and behavioral characteristics can be observed in rodents, primates and humans [155]. Evolutionarily, adolescent behaviors serve a drive to leave the familial environment and explore new territory, seek social peers and sexual partners, and establish independence as an adult [156]. As such, key behavioral hallmarks of adolescence across species include novelty-seeking, impulsivity, risk tolerance, and heightened sensitivity to reward [157]. These traits position adolescence as a developmental stage ripe with both opportunity and danger. Adolescence is the typical age of onset for several psychiatric illnesses, including anxiety, depression and psychotic disorders [158, 159]. Adolescents engage in risky behaviors such as dangerous driving, unprotected sex and drug use at higher rates than adults [160]. Rates of accidental death are highest for this age group, particularly for males [161]. These characteristics set adolescence apart as a distinct developmental period, as different from childhood and adulthood as those developmental periods are from one another.

Taken together, these features provide insight into some of the specific challenges of the adolescent epoch in humans. With increased novelty-seeking, impulsivity, reward sensitivity and risk tolerance, the adolescent is poised to experience many new challenges and temptations. Although these characteristics
serve an evolutionary purpose to facilitate the transition away from the family unit and into an independent new environment, they also put adolescents at a disadvantage in their motivated behavioral decision making as compared with adults [162].

**Animal Models of Adolescence**

The conservation of adolescence across species facilitates the use of animal models to replicate key behavioral features of human adolescence for use in developmental neurobiology [163]. Behavioral procedures using rodent models have revealed strong similarity with the adolescent phenotype observed in humans. The increased propensity for novelty-seeking has been assessed in rodents with the novel objection recognition (NOR) task, in which the subject is presented with a novel object in the test environment and the amount of time spent investigating/interacting with the novel object is measured. Adolescent rats have consistently been shown to spend more time with a novel object than adults [164, 165] and prefer interactions with the novel object to a greater extent than adult rats [166]. Adolescent mice have also been shown to spend more time exploring a novel environment than adults and to prefer the novel setting to a familiar one [167], which mirrors the novelty-seeking observed in human adolescents.

Impulsivity can be assessed in rodents using an intolerance-to-delay (ID) task [168]. In this task, making a quick response results in a smaller reward (i.e. 1 food pellet), but delaying the response leads to a larger reward (i.e. 3-5 food pellets). Subjects that can withhold responding in favor of the larger reward in the future are interpreted to be less impulsive, and adolescent mice and rats have been found to
favor the immediate smaller reward over a future large reward (i.e. enhanced impulsivity) [169-171]. This behavior is reminiscent of human studies which have also showed diminished behavioral inhibition in human adolescents [172], as well as strong motivational salience of immediate rewards versus long-term payouts [173].

Risk-taking behavior is a complex phenomenon and can be assessed through the interpretation of several different behavioral tasks. The degree to which a rodent explores the open arms of an elevated plus maze has been interpreted as an index of risk-tolerance [174], and adolescent mice have been shown to more readily explore the open arm than adult mice [175]. This procedure may represent an animal correlate to the dangerous behaviors exhibited by adolescent humans that result in accidental injury or death [160]. Studies of risky decision-making in rats, wherein subjects have the choice between a certain but small reward and a larger but uncertain reward, have also demonstrated an increased tolerance to risk in adolescent versus adult rats [176]. These findings are reminiscent of human data indicating that adolescents tend to favor larger rewards over smaller [177].

Finally, increased sensitivity to reward is a feature of adolescence that can be directly assessed in rodents through self-administration of highly palatable foods and solutions. Adolescent mice display increased intake of sweetened solutions and foods, and show greater preference for these substances than their adult counterparts. Additionally, for adolescent rodents the presence of a peer is rewarding: adolescents spend more time with peers than adults [178, 179] and show stronger preference for social interaction than their adult counterparts [see 180 for review]. These same behaviors are a hallmark of adolescence in human
populations [181]. A related behavioral phenomenon of relevance to adolescent reward salience is aversion learning. Unlike the clear enhancement of reward sensitivity seen in adolescent rodents and humans, aversive stimuli appear to be less potent in adolescents than in adults. The conditioned taste aversion (CTA) task is a common method to assess aversion learning in rodents; a novel sweetened solution (conditioned stimulus) can be paired with an aversive injection of lithium chloride (unconditioned stimulus), and subsequent intake of the solution can be measured (conditioned response). Decreased intake following injection is interpreted as a measure of the aversion induced by the injection. Adolescent mice and rats have been shown to be less sensitive to CTA than their adult counterparts [182]. Taken together, the increased sensitivity to reward and decreased sensitivity to aversion exhibited by adolescents appears to drive increased behaviors directed towards obtaining rewards.

Across a variety of behavioral domains, the characteristic features of adolescence in humans can be readily observed and quantified in rodent models. Both mice and rats have been found to display a discrete adolescent period between the juvenile and adult stage accompanied by increases in impulsivity, novelty-seeking, risk taking and reward sensitivity. Although there are differences in the precise timing of the pubertal period and full maturation of the brain between mice and rats [163], both species clearly display the characteristic features of adolescent behavior. These features make rodent models ideal to address questions about the underlying neurobiology of adolescent brain development that cannot be ethically assessed in humans.
Neurobiology of Adolescent Brain Development

The behavioral characteristics that define adolescence are reflective of changes in brain anatomy and function that occur during this developmental stage.

Structural Maturation of the Adolescent Brain

Although the brain undergoes a legion of specific structural changes as it matures from childhood into adulthood, one of the defining features of the developing adolescent cortex is the phenomenon of neural pruning. From birth to childhood, the number of total neurons in the human cortex (as indexed by gray matter) increases in a linear fashion, reaching peak density between two and three years of age [183]. Beginning between ages six-ten and progressing well into the late twenties, notable decreases in cortical gray matter are observed in parallel with increases in white matter (reflective of myelinated processes) [184]. Similar decreases have been observed in rodent and primate models [185]. These changes have been shown to represent a process of synaptic refining, wherein some synaptic connections are pruned back whereas others are strengthened [186]. This process is dependent upon experience, prioritizing myelination of synapses with heavier traffic and pruning of neurons with inefficient pre- and post-synaptic connectivity [187]. Indeed, functional connectivity assessments in humans have revealed that increasing coordination between multiple brain regions is a defining feature of adolescent development [188, 189].

The process of structural maturation proceeds in an posterior-to-anterior fashion, with regions in the hindbrain and midbrain reaching structural maturity first whereas regions in the cortex lag behind [190]. The PFC is one of the last regions to
achieve structural maturity, continuing to develop into the second decade of life in some human MRI studies [191]. Importantly, limbic regions such as the amygdala and nucleus accumbens complete neural pruning well before the PFC has fully developed [192]. As the PFC exerts regulatory control over these downstream brain regions, a current theory of adolescent neurobehavioral development suggests that the immaturity of the PFC inhibits normal regulatory function over limbic brain areas, leading to adolescent-typical behaviors such as increased reward salience and risk-taking [157]. In support of this hypothesis, recent neuroimaging data suggest that amygdala-prefrontal cortex connectivity undergoes a developmental “switch” during adolescence, shifting in valence from positive functional connectivity to negative functional connectivity during late childhood [193]. As this change was accompanied by a reduction in amygdala reactivity, it likely reflects frontal regulation of limbic regions becoming available as the cortex matures.

Alterations in Neurotransmitter Signaling During Adolescent Brain Development

Virtually all major neurotransmitters have been shown to exhibit fluctuations in extracellular concentration and/or receptor density during adolescent brain development. Although the specific effects of these developmental alterations in neurotransmitter systems have not been concretely established, these processes suggest potential mechanisms that may underlie adolescent-typical behavior. Additionally, the same neurotransmitter systems can exhibit different developmental trajectories in different brain regions, complicating interpretation of these data.

Extensive research in animal models has indicated that GABA neurotransmission undergoes significant changes during adolescent brain
development. Between birth and adolescence, whole-brain GABA concentrations double in rodent models [194]. GABA\textsubscript{A} receptor density increases linearly during adolescence to reach adult levels in rats [195], whereas in non-human primates GABA\textsubscript{A} expression peaks during adolescence and then declines to achieve adult levels [196]. Importantly, age-related differences in the expression of GABA\textsubscript{A} subunits have also been reported and may be responsible for differences in GABAergic neurotransmission between adolescents and adults [197]. By contrast, work examining the effects of adolescence on GABA in the human brain has been limited. One MRS study has indicated that GABA levels in the anterior cingulate cortex of the frontal lobe were lower in adolescents versus adults [198]. Positron emission tomography (PET) has indicated that GABA\textsubscript{A} receptors reach adult density in subcortical regions during early adolescence, but do not achieve adult levels in the frontal cortex until the late teens [199]. These findings are generally consistent with overall trends reported in the rodent and primate literature to date. Lower levels of GABA during adolescence versus adulthood, particularly in the frontal cortex, have been hypothesized to represent a reduction in inhibitory control of limbic brain regions, and may therefore underlie some of the impulsive and risky behaviors associated with adolescence [198].

Of the monoamines, most research in adolescents has focused on dopamine fluctuations during brain development, particularly in brain regions known to be involved in reward, learning and executive function. The expression of dopamine receptors D1 and D2 peaks during early adolescence in the striatum and nucleus accumbens, whereas D1 and D2 expression in the mPFC peaks during late
adolescence, with both areas experiencing a subsequent decline to adult expression [200]. Dopamine levels in the midbrain have repeatedly been found to peak during adolescence [201], a finding which may underlie the increased reward sensitivity exhibited by adolescent mammals. Importantly, during the transition to adulthood tonic dopamine increases in the PFC [202] whereas dopamine levels in the striatum decrease [203]. These alterations represent a shift from juvenile mesolimbic dopaminergic regulation of behavior (promoting emotional/reward-related behaviors) towards mature mesocortical dopaminergic prominence (top-down regulation of limbic regions) [204]. Dopaminergic regulation of neuronal excitation via modulation of GABAergic interneurons appears to be particularly sensitive to developmental state, with D2 receptor antagonists mildly inhibiting excitability of cortical slices during the prepubertal period but becoming strongly excitatory on slices from young adult rats [205]. Dopamine signaling may also play an important role in plastic alterations in adolescent synaptogenesis, as activation of D2 receptors alters spine density in adolescent but not adult mice [206]. Due to the strong association between dopamine and reward learning, many of these alterations in dopamine signaling have been hypothesized to play a role in the demonstrated increase in reward salience exhibited by adolescents. Indeed, the adolescent nucleus accumbens has been shown to exhibit markedly different dopamine responses to reward across multiple brain regions (see [207] for review.)

The endocannabinoid systems undergo dramatic reorganization during the adolescent developmental period, particularly in the prefrontal cortex. In the human PFC, mRNA for the cannabinoid receptor 1 (CB1) peaks during childhood and
remains elevated throughout early adolescence before decreasing to adult levels [208]. Similar decreases have been observed in rat mRNA and CB1 protein [209, 210]. Concurrently, alterations in the endogenous ligands for the CB1 receptor (anandamide and 2-arachidonoylglycerol) are exhibited by adolescent rodents [211, 212]. Activation of the CB1 receptor suppresses neuronal firing via retrograde neurotransmission; thus, increased cortical CB1 expression and activity has been hypothesized to contribute to the hypofunction of the adolescent PFC [210]. Recent work has established a functional role for CB1 activity in several adolescent-typical behaviors. In rats, enhancement of CB1 activity in adults through a gain-of-function mutation increased risk taking, peer interaction, and reward sensitivity for natural and drug reinforcers [213]. Additionally, polymorphisms in the human CNR1 gene (which encodes the CB1 receptor) have been associated with impulsivity in adolescents [214].

Current understanding of synaptic plasticity strongly suggests a role for this process in the maturation of the adolescent brain [215], implicating changes in the glutamate signaling systems as important contributors to adolescent brain development and behavior. In rodents, glutamate activity steadily increases from birth, with a peak during adolescence and a subsequent decline into adult levels [216]. Expression of the NMDA receptor increases during the pubertal period and begins to decrease to adult levels at the start of the adolescence [217]. There are also dramatic ontogenetic shifts in the relative expression of the constitutive NMDAR subunits; GluN2B expression predominates at birth but gradually decreases across adolescence to reach adult levels, whereas NR2A levels are low at birth and reach
adult levels during early adolescence [218]. Cortical AMPA receptors are similarly regulated by age, with peak expression achieved in early adolescence followed by a decline to adult levels [219]. The AMPA receptor may also play a functional role in adolescent development of executive function. Overexpression of calcyon, an AMPA accessory protein that facilitates internalization of the AMPA receptor, produced learning and memory deficits only during the adolescent critical period [220].

Developmental increases in critical glutamate signaling molecules such as CaMKIIα have also been observed, with expression increasing during the immediate postnatal period and levels stabilizing only in adulthood [221]. Metabotropic glutamate receptors have received limited investigation in the context of adolescent brain development, but one previous report indicates that Type I mGluRs may have different impacts on LTD in the hippocampus during the early postnatal, adolescent and adult development epochs [222]. In primates, the density of excitatory dendritic spines in the PFC increases during postnatal development and stabilizes until adolescence, after which spine density decreases to adult levels [185]. In the human cortex, neural pruning appears to selectively target excitatory projection neurons, the loss of which accounts for 40% of the reduction in synapses observed during the adolescent development period [187, 223]. These varied alterations in glutamate receptor expression and function indicate that adolescence is accompanied by substantial shifts in excitatory neurotransmission, a process likely guided by synaptic plasticity in key brain regions such as a prefrontal cortex.
Alcohol Use During Adolescence

The initiation of drug use, including alcohol, during adolescence is a defining feature of neurobehavioral development in humans. This peak of interest in drugs of abuse has been replicated in animal models; human, non-human primate and rodent adolescents have been shown to self administer more alcohol (and most other drugs of abuse) than adults when intake is adjusted for differences in body weight. Adolescent exposure to alcohol also appears to be uniquely dangerous, with initiation of alcohol use during the early adolescent period associated with an approximately 35% increase in lifetime risk for the development of an AUD [225]. Unfortunately, alcohol use is ubiquitous amongst adolescents in the United States, with more than half of 12th-grade students reporting the consumption of intoxicating doses of alcohol within the past two weeks [226]. Although the specific neurobiological mechanisms that may be responsible for both increased alcohol intake during adolescence and long-term consequences of adolescent alcohol exposure remain unclear, insight from animal studies has indicated several potential explanations for these phenotypes.

Behavioral Mechanisms

A wealth of literature indicates that adolescents display altered sensitivity to many of the acute/intoxicating effects of alcohol. In rodent models, adolescents exhibit blunted sensitivity to the sedating/hypnotic effects of alcohol [227], locomotor deficits following an intoxicating dose of alcohol [228], conditioned taste aversion to alcohol [229, 230], social impairment from high-dose alcohol [231], and anxiety exhibited during withdrawal from high-dose alcohol [232]. However, adolescent
rodents display enhanced sensitivity to the social facilitation induced by low-
moderate dose alcohol [233] and the reinforcing properties of alcohol [234]. Taken
together, these findings suggest a pattern of enhanced sensitivity to the rewarding
effects of alcohol and reduced sensitivity to the aversive effects of alcohol [235].
Adolescent rats are also less sensitive to the interoceptive properties of alcohol,
requiring higher doses than adults for alcohol to serve as a discriminative stimulus
[236]. Many of these same age differences in the behavioral response to alcohol
have been observed in human adolescents, most notably diminished sensitivity to
the locomotor impairing effects of alcohol [237] and heightened sensitivity to the
stimulating [238] and social facilitating effects of alcohol [239]. This mosaic of
increased and decreased sensitivity results in adolescents who experience the
rewarding properties of alcohol more strongly, the aversive consequences less
harshly, and have difficulty assessing their level of intoxication relative to adults. The
biological underpinnings of this behavioral phenotype have not yet been definitively
identified.

Age-Related Changes in Dopamine

Due to the central role dopamine signaling plays in reward learning, and more
specifically alcohol reward, and the significant changes the dopamine systems
undergo during adolescence, the potential for dopamine to mediate age-related
changes in alcohol behavior has received considerable investigation. During
adolescence, experimenter-administered alcohol induces greater dopamine release
in the nucleus accumbens of adolescent rats as compared to adult rats [240].
Adolescent alcohol exposure has also been shown to lead to long-lasting increases
in basal dopamine [241] and alcohol-evoked dopamine release in the adult nucleus accumbens [242]. Similarly, adolescent alcohol has been shown to reduce D1 receptor modulation of neuronal activity in the adult mPFC [243]. Evidence also suggests a role for dopamine signaling in regulating the adolescent response to drugs of abuse, including alcohol. Overexpression of the dopamine D1 receptor in projection neurons of the PFC created adolescent-typical behaviors in adult rats, including heightened place preferences for low dose alcohol [244]. D1 receptor inhibition in the PFC also reduces the motivational salience of cocaine cues [245]. In human participants, the gene for the dopamine D2 receptor (DRD2) has been associated with elevated risk for alcoholism in adolescent populations [246].

Together, these findings indicate a role for dopaminergic signaling in some of the age-related differences in behavioral and brain response to drugs of abuse, including alcohol.

**Role of Other Neurotransmitter Systems in Adolescent Alcohol Use**

A complete review of the interactions between neurotransmission in the developing brain and the impact of alcohol on the adolescent is beyond the scope of the present work. However, the potential for age X dose interactions during adolescence is high, due to the nature of alcohol (a small molecule capable of interacting with a wealth of signaling targets) and the nature of the adolescent brain (an organ in a state of dramatic flux, impacting virtually all neurotransmitter systems to varying degrees.) Thus, good evidence also implicates GABA [197], serotonin [247] and endocannabinoid [248] signaling in the adolescent response to alcohol. Importantly, one of the most prominent unanswered questions with respect to
adolescence and alcohol remains the mechanism by which adolescent exposure to alcohol can impart such high risk for problem drinking later in life. The current state of the field would seem to indicate that most brain signaling systems contribute to this elevated risk in some way. However, a unifying theme for some of the acute responses to alcohol described above may be the degree to which they impact long-term changes in brain structure and function.

**Synaptic Plasticity: A Link between Adolescence and Alcoholism**

Synaptic plasticity is a critical component of adolescent brain development, playing a role in synaptic strengthening and synaptic pruning. Mechanisms of LTP have also been shown to be sensitive to disruption by alcohol, and to mediate some of the consequences of alcohol exposure. However, the degree to which molecular changes in the receptors and subcellular proteins that mediate synaptic plasticity are involved in the adolescent response to alcohol, and more importantly the enduring changes in the adolescent brain imposed by alcohol, has received relatively limited investigation to date.

Several experiments using rodent models have indicated that alcohol selectively alters glutamatergic signaling in adolescents. Repeated administration of alcohol downregulates the expression of the GluN2B subunit of the NMDA receptor in the adolescent but not adult PFC [240]. Santerre and colleagues [219] demonstrated that low-dose alcohol (1 g/kg) increased GluA2 phosphorylation in the adult hippocampus but reduced pGluA2 in the adolescent hippocampus, suggesting that alcohol may exert opposing effects on AMPA receptors in adolescent and adult
rats. The same group has also produced evidence for age differences in the expression and alcohol sensitivity of PKC, a regulator of AMPA receptor phosphorylation and mediator of synaptic plasticity [249]. In contrast, in the nucleus accumbens adolescent rats appear to be relatively insensitive to alcohol-induced changes in the expression of mGluR1, mGluR5, GluN2B, PKC and CaMKII [250] exhibited by adults. Another subcellular mediator of synaptic plasticity is extracellular signal-regulated kinase 1/2 (ERK1/2), which is a critical component of a signaling cascade known to be involved in plasticity and alcohol reward [251]. Adolescents have been shown to display greater expression of ERK1/2, and ERK1/2 is more sensitive to alcohol-induced phosphorylation changes in the adolescent hippocampus and amygdala versus adult [252]. Evidence also suggests that adolescent rats may be more sensitive than adults to alcohol-induced suppression of LTP in the hippocampus [253].

Evidence from human studies also indicates a role for alterations in glutamatergic signaling during adolescence in increased risk for problem drinking and AUDs across the lifespan. Elevated hippocampal glutamate levels have been associated with risky alcohol drinking in non-dependent adolescents [254], and adolescent-typical changes in glutamate/glutamine ratios have been shown to be altered in individuals with a family history of alcoholism [255]. Conversely, adolescent drinking to the point of blackout has also been associated with reductions in glutamate levels in the frontal cortex [256]. Genome-wide association studies (GWAS) have also linked alcoholism to Glun2a polymorphisms [257], which have
independently been associated with risky drinking during adolescence and a family history of alcoholism [258].

Findings from both preclinical animal models and assessments of adolescent drinkers point to mechanisms of glutamatergic plasticity being responsive to alcohol and involved in adolescent alcohol-related behavior. These observations lead to the hypothesis that **age-related changes in synaptic plasticity during adolescent brain development would be sensitive to alcohol and functionally involved in the increased risk for escalated alcohol consumption following adolescent alcohol exposure.**

**Rationale**

The experiments in this dissertation were aimed at three complementary goals:

1) To characterize the developmental trajectory of the adolescent brain both at baseline and under the influence of alcohol drinking

2) To determine the relative contributions of two key synaptic plasticity enzymes, CaMKII and PPP3R1, to alcohol drinking in adolescent and adult mice. These experiments focused on two key brain regions: the prefrontal cortex, for its role in the regulation of adolescent behavior, and the amygdala, for its role in alcohol reward and reinforcement. Initially, an unbiased proteomics screen of the adolescent and adult PFC revealed substantial developmental differences in this brain region, with major changes in the expression of proteins known to regulate synaptic plasticity. A subsequent proteomics experiment comparing the effects of alcohol or water drinking independently in adolescent and adult mice demonstrated that several of the developmentally-regulated proteins were alcohol-sensitive, illustrating the
qualitatively different responses of the adolescent and adult PFC to alcohol. The protein phosphatase calcineurin (PPP3R1) was chosen for functional evaluation in adolescent and adult alcohol drinking behavior because it was both subject to developmental regulation and alcohol-sensitive. Systemic calcineurin inhibition reduced alcohol drinking more potently in adolescent versus adult mice. Next, a model of adolescent alcohol exposure and subsequent operant self-administration in adulthood was developed in order to assess the role of PPP3R1 signaling in long-term vulnerability to alcoholism following adolescent drinking. The role of the PFC in mediating these effects was assessed using site-directed inhibition of PPP3R1 in adult mice that had been exposed to either alcohol or water during adolescence. To examine the downstream brain regions that are regulated by the PFC and involved in alcohol reinforcement, the amygdala, dorsal striatum and nucleus accumbens were assessed for alterations in plasticity-associated proteins. Age differences emerged in the amygdala, with CaMKII phosphorylation reduced in the adolescent but not adult amygdala accompanied by increased pGluA1 in the adult but not adolescent amygdala. Finally, systemic potentiation of CaMKII-pGluA1 activity was used to assess the effects of global alterations of synaptic plasticity on adolescent and adult drinking behavior.
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CHAPTER TWO: COMPARISON OF THE ADOLESCENT AND ADULT PREFRONTAL CORTEX PROTEOME

Introduction

Adolescence is a critical developmental period during which organisms make the transition from childhood to adulthood. This time period is characterized by significant changes in brain architecture [1], pubertal development and sexual maturity, and several unique behavioral characteristics, including increases in risk-taking, sociability, novelty-seeking, reward sensitivity and impulsivity [2]. Both the physical and behavioral manifestations of adolescence are conserved across mammalian species, facilitating the use of rodent models in the study of adolescent development [3]. Adolescence is also a potentially vulnerable time, associated with increased rates of alcohol and drug use, risky sexual practices, and reckless driving [4]. Additionally, adolescence in humans and rodents is an epoch of heightened stress, characterized by increases in anxiety-like behavior as well as enhanced sensitivity of the hypothalamic-pituitary-adrenal axis (HPA axis) response to stressors [5]. Importantly, adolescence is the typical time of onset of many neurological and psychiatric conditions, including epilepsy, neurodegenerative disorders and neuromuscular dysfunction [6] as well as anxiety, impulse-control, substance use, schizophrenia and mood disorders [7]. In spite of widespread

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recognition of the adaptive and maladaptive changes associated with adolescence, the specific neuronal mechanisms that usher the brain into adult maturity (and potentially mediate both behavior and dysfunction) remain unclear.

During adolescence, the brain undergoes substantial structural and functional alteration. Of considerable significance is the decline of cortical gray matter, which usually begins in late childhood/early adolescence [8] and may be driven by both synaptic pruning [9] and enhanced myelination of existing axons [10]. Notably, loss of gray matter density follows an anterior-to-posterior trajectory, with maturation occurring first in sensorimotor areas and last in higher-order regions such as the prefrontal cortex (PFC) [11]. The PFC is functionally involved in the executive control of behavior and decision-making processes [12], and the relative immaturity of the adolescent PFC is associated with lack of inhibitory control over behavior exhibited by adolescents [13, 14]. At the same time, limbic brain areas associated with emotional arousal and reward, such as the amygdala and nucleus accumbens, reach maturity earlier than cortical regions and receive fewer neural projections from the immature PFC [15], resulting in an imbalance in top-down control of limbic regions and greater reward seeking and impulsive behavior in adolescents [16]. The PFC is therefore both a site of significant neuronal development during adolescence and a potential contributor to adolescent behavioral phenotypes.

Previous reports have begun to characterize the development of the PFC proteome from birth to adulthood [17-20], but several important questions have not been addressed by the existing literature. The specific alterations of protein expression and network function during adolescence remain unclear, due to the
combination of pre-adolescent and adolescent data for comparison with adults in prior studies. Further, the majority of findings in the developing PFC to date have focused on the synaptic fraction of proteins. Although this strategy brings important insight into the development of synaptic connections and signaling during brain development, proteins that are expressed outside of the membrane fraction may play an important role in the maturation of the adolescent cortex. Additionally, some studies have failed to distinguish between the subregions of the PFC collected for analysis. The prelimbic and infralimbic PFC have different projections and different functional roles in behavior and therefore may be subject to different developmental processes during the adolescent period [21]. Finally, previous reports have focused exclusively on the expression of proteins in the PFC, creating uncertainty as to whether the observed protein expression differences in the PFC are unique to that region or part of a general developmental trend across multiple brain regions.

To investigate the subcellular machinery involved in adolescent brain development and behavior, we used a high-throughput unbiased proteomics analysis to characterize age differences in protein expression between adolescent and adult male C57BL/6J mice. Mice have a defined period of adolescence (approximately 2 weeks) in which they display “adolescent typical” behavior, such as impulsivity and novelty seeking [22], and were therefore a useful model for these studies. We chose to focus on the transition from early- to mid-adolescence in order to capture a snapshot of the adolescent brain midway through maturation to adulthood, and therefore collected tissue on post-natal day (PND) 36 [23]. We focused on the prelimbic medial prefrontal cortex (mPFC) as this region is critically
involved in executive control of behavior and sends projections to the amygdala and nucleus accumbens [24]. We also used a bioinformatics approach to identify protein networks that may play a role in adolescent brain development, particularly neurochemical signaling and structural alterations. By identifying proteins that are differentially expressed in the adult and adolescent mouse mPFC, as well as additional brain regions of interest, these experiments give insight into cellular correlates of adolescent-typical behaviors and dysfunctions.

**Materials and Methods**

**Subjects**

All procedures were performed in accordance with the NIH Guide to the Care and Use of Laboratory Animals [25] and approved by the Internal Review Board as compliant with all institutional guidelines at the University of North Carolina, Chapel Hill (approved protocol number: 13-217).

**Proteomic analysis**

Adolescent (postnatal day 21 [PND21]) and adult (PND65 ± 3) male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were pair-housed upon arrival in standard laboratory cages with corn-cob bedding and a small PVC tube for environmental enrichment. Food and water were available *ad libitum* for the duration of the experiment. Subjects were minimally handled throughout the experiment to minimize stress.
**Immunoblotting**

An additional group of adolescent (PND 21) and adult (PND 65 ± 3) male C57BL/6J mice (Jackson) were housed and handled under identical conditions to the proteomics cohort in order to confirm expression changes observed in the proteomics experiments using Western blots.

**Proteomic analysis**

Adolescent and adult mouse mPFC proteomes were analyzed utilizing 2-Dimentional in-gel electrophoresis (2D-DIGE) for protein expression profiling, DeCyder software for selection of significantly altered spots, and matrix-assisted laser desorption/ionization-Time of Flight (MALDI-TOF) mass spectrometry (MS) for protein identification (Fig 1 D) [26].

**Tissue collection**

Fifteen days after arrival (Fig 1A), 12 adolescent (P36) and 12 adult (P80 ± 3) mice were weighed and deeply anesthetized with sodium pentobarbital (150 mg/kg, IP). Once anesthetized, mice were transcardially perfused with ice-cold phosphate-buffered saline (PBS, 0.1M, pH 7.4) for 2 min at rate of 3 ml/min in order to remove blood from the brain tissue. The brain was then quickly isolated and flash-frozen in isopentane (2-methylbutane; Sigma-Aldrich, St. Louis, MO) at -20 – -30°C for 1 min, weighed, and stored at -80°C. To isolate the mPFC, tissue was sliced coronally on a cryostat (Leica CM3050S, Leica Biosystems, Buffalo Grove, IL), with a 0.5 mm tissue slice being taken between +1.5-2.0 mm (± 0.2) anterior to Bregma. The mPFC was dissected out bilaterally using a 1.0 mm tissue punch (Fig 1C). The isolated
tissue included the prelimbic and dorsal infralimbic cortices, as well as the posterior anterior cingulate cortex. Tissue was pooled from n=3 mice per age group resulting in a final group size of N=4 adolescent and N=4 adult mPFC samples for analysis on 4 replicate 2D gels. Protein samples were kept at -80°C and shipped to Applied Biomics (Hayward, CA) for 2-D DIGE analysis.

Tissue preparation

Tissue samples were sonicated on ice in 2D lysis buffer (2 M thiourea, 7 M urea, 4% CHAPS, 30 mM Tris-HCl, pH 8.8) and shaken for 30 min at room temperature. Samples were then spun at 14000 rpm at 4°C for 30 min, and the resulting supernatant was collected. Protein concentrations were determined using the Bio-Rad protein assay method (Hercules, CA), and samples were diluted to 5 mg/ml in 2-D lysis buffer.

CyDye labeling

Adult and adolescent tissue samples (30 μg) were combined within age group with 1.0 μl of the appropriate diluted CyDye (Cy2, Cy3, or Cy5; 1:5 concentration, diluted with dimethylformamide (DMF) from a 1 nmol/μl stock), vortexed, and kept on ice for 30 min in the dark. Afterwards, 1.0 μl of 10 mM Lysine was added to each sample, vortexed, and incubated on ice for 15 min in the dark. The CyDye-labeled samples (Cy2, Cy3, Cy5) for each age group were then combined and mixed with a 2X 2-D sample buffer (8 M urea, 4% CHAPS, 20 mg/ml dithiothreitol, 2% pharmalytes, trace amount of bromophenol blue) and 100 μl of Destreak Solution and Rehydration Buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mg/ml DTT, 1%
pharmalytes, trace amount of bormophenol blue) to a final volume of 350 μl for the
18 cm IPG strip. Labeled samples were mixed well and spun before loading into the
strip holder.

2D-DIGE

Following loading of samples into the 18 cm IPG strip holder, the strip was
placed facing down and 1.5 ml mineral oil was added to the top of the strip. Samples
were then run using isoelectric focusing (IEF) under dark conditions at 20°C using
an established protocol (GE Healthcare, Pittsburgh, PA). Following IEF, the IPG
strips were incubated in fresh equilibration buffer 1 (50 mM Tris-HCl, pH 8.8,
containing 6 M urea, 30% glycerol, 2% SDS, trace amount of bromophenol blue, 10
mM DTT) for 15 min with gentle shaking. Strips were then washed in fresh
equilibration buffer 2 (50 mM Tris-HCl, pH 8.8, containing 6 M urea, 30% glycerol,
2% SDS, trace amount of bromophenol blue, and 45 mg/ml lodacetamide) for 10 min
with gentle shaking. IPG strips were then washed and transferred to a 12% SDS gel
(prepared using low fluorescent glass plates) and sealed with 0.5% w/v agarose
solution in SDS-gel running buffer. Gels were run at 15°C.

Image scan and data analysis

Immediately following the SDS-PAGE portion of the 2D-DIGE experiment,
image scans were conducted using a Typhoon TRIO imager (GE Healthcare).
Scanned images were analyzed by ImageQuantTL (IQTL) software (GE Healthcare)
and subjected to both in-gel and cross-gel analyses using the DeCyder software
package (v. 6.5, GE Healthcare), which provided a ratio change of protein expression from the in-gel analyses.

**Spot picking and trypsin digestion**

Protein spots that met our *a priori* spot-picking criteria (differential expression in the same direction in all four gels, an overall significant difference in expression \(p<0.05\), and a 1.2-fold change or greater increase or decrease in expression) were isolated by the Ettan Spot Picker (GE Healthcare) (Fig 1D). Gel spots were washed and digested in-gel with a modified porcine trypsin protease (Trypsin Gold, Promega, Madison, WI). Digested peptides were desalted (Zip-tip C18 column, Millipore, Billerica, MA) and eluted with 0.5 μl of matrix solution (α-cyano-4-hydroxycinnamic acid, 5 mg/ml in 50% acetonitrile, 0.1% trifluoroacetic acid, 25 mM ammonium bicarbonate) and spotted on the matrix-assisted laser desorption/ionization (MALDI) plate.

**Mass spectrometry**

Both MALDI-TOF (time-of-flight) mass spectrometry (MS) and TOF/TOF (tandem MS/MS) analyses were performed on a 5800 mass spectrometer (AB Sciex, Redwood City, CA). Mass spectra from the MALDI-TOF analysis were acquired in reflectron positive ion mode (average of 2000 laser shots/spectrum), whereas the TOF/TOF tandem MS fragmentation spectra were acquired for each sample (average of 2000 laser shots/fragmentation spectrum) on each of the 10 most abundant ions present in the sample (with the exclusion of trypsin autolytic peptides and other background ions)
**Database search**

Resulting peptide masses and fragmentation spectra were submitted to GPS Explorer (v. 3.5) with the MASCOT search engine (Matrix Science, Boston, MA) in order to explore the database of the National Center for Biotechnology Information non-redundant (NCBI_nr). Searches were not constrained by protein molecular weight or isoelectric point; additionally, the search allowed for variable carboxamidomethylation of cysteine and oxidation of methionine residues, and one missed cleavage was allowed in the search parameters. Both ion score (statistical likelihood that a peptide sequence experimentally observed and identified in the MASCOT database are matched based on random chance \[-\log_{10} P\]) and protein score (sum of the highest ion scores for each sequence) were calculated; increased protein score indicates increased confidence in the identification of the protein. Candidate proteins with a protein score confidence interval greater than 95% were considered significant. For samples with multiple candidate proteins exceeding the identification criteria, an identity was assigned based on the highest protein score. Full identification data, including all candidate proteins for each spot, are available on the open access proteomics data repository ProteomeXchange (www.proteomexchange.org).

**Bioinformatics**

Protein identifiers, fold-change, and \(p\)-values from the proteomic analysis were uploaded to QIAGEN’s Ingenuity Pathway Analysis (IPA; QIAGEN Redwood City, CA) system for dataset enrichment. A Core Analysis was performed on the dataset, using the following parameters: reference set- Ingenuity Knowledge Base
(genes only), relationships- direct and indirect, networks- interaction, data sources-all, confidence- experimentally observed, species- mouse, tissues and cells lines-nervous system/CNS cell lines. Proteins were assessed via Global Functional Analysis (GFA) and Global Canonical Pathways (GCP) to identify functional protein networks and canonical signaling systems that were impacted by developmental state. Statistical significance of the predicted functions and pathways was determined using the right-tailed Fisher’s Exact Test, where significance indicates that an identified set of proteins is overrepresented in a set of proteins with known function and is interpreted to indicate altered function in the experimental set.

Protein interaction networks were derived via Ingenuity’s interconnectivity algorithm. p values, representing the probability of finding proteins identified in the proteomics analysis (Focus Molecules) in a set of n genes randomly selected from the Global Molecular Network, were calculated using Fisher’s Exact Test and displayed as p-scores [p-score = -log₁₀ (p-value); i.e. p-score indicates the exponent of the significance of the protein network identification]. Ingenuity Pathway Designer (QIAGEN) was used to visualize the statistically significant protein interaction networks revealed by GFA.

**Immunoblotting: mPFC confirmations and additional brain regions**

**Tissue collection**

Brain tissue was collected from adolescent (P36) and adult (P80 ± 3) [n=12/age] as described above. In addition to the mPFC as described, the dorsal striatum (dSTR), nucleus accumbens (NAc), primary motor cortex (MC), amygdala
(AMY) and ventral tegmental area (VTA) were isolated and dissected to analyze protein changes in additional brain areas with relevance to adolescent behavior. The coordinates for each region (relative to Bregma) were: +1.0-1.5 mm (± 0.2) for dSTR, NAc and M1, -0.9-1.4 mm (± 0.2) for AMY and -3.3-3.8 mm (± 0.2) for VTA.

Following dissection of regions of interest, tissue punches were homogenized by pulse sonication (4 s) in 10 mM Tris (pH 7.4 at 23°C) with 1% w/v SDS and 1:100 Halt EDTA-free Protease and Phosphatase Inhibitor Cocktail (Pierce, ThermoFisher Scientific, Rockford, IL). Brain tissue was stored at -80°C. The Pierce BCA kit (ThermoFisher Scientific) was used to determine protein concentrations (µg/µL) of each tissue sample.

Immunoblots

Protein samples from each brain region, at 5 µg per sample, were run on a TGX 4-15% 18-well gel (BioRad) with 1x tris-glycine-SDS running buffer (Tris 25 mM, Glycine 192 mM, 0.1%SDS) with Protein Plus Dual Color (Bio-Rad) and See Blue ladders (ThermoFisher Scientific) and dry-transferred onto a PDVF membrane using the Invitrogen iBlot protocol (ThermoFisher Scientific). Membranes were blocked for 2 hours at room temperature in 3% bovine serum albumin (BSA, for STXBP1 and DPYSL2; Sigma-Aldrich), 1% BSA (actin) or 5% w/v non-fat dry milk (for DNM1 and CFL1; ThermoFisher Scientific). Membranes were incubated with the following primary antibodies overnight at 4°C with gentle rocking: rabbit polyclonal anti-STXBP1-1 [1:1000 in 3% BSA; Cell Signaling Technology, Inc., Danvers, MA], rabbit polyclonal anti-CFL1 [1:5000 in 5% non-fat dry milk, Cell Signaling Technology, Inc. [27]], mouse monoclonal anti-DNM1 [1:1000 in 5% non-fat dry milk, Cell Signaling Technology, Inc. [27]], mouse monoclonal anti-DNM1 [1:1000 in 5% non-fat dry milk, Cell Signaling Technology, Inc. [27]],
Cell Signaling Technology, Inc.). Blots were incubated with rabbit polyclonal anti-DPYSL2 [1:10,000 in 3% BSA; AbCam, Cambridge, MA [28]] and mouse monoclonal anti-actin [1:5000; Millipore] for 1 hour at room temperature. Membranes were then extensively washed and incubated with an HRP-labeled goat-anti rabbit or goat-anti mouse secondary antibody (1:20,000 in the same blocking buffer as the primary antibody; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Protein expression was assessed via an enhanced chemiluminescence protocol (Pierce ECL, ThermoFisher Scientific), with exposure to autoradiography film (Bio Express, Kaysville, UT). Protein bands were quantified by optical density analysis (NIH/Scion Image) and normalized to actin which was used as a loading control.

Antibody selectivity for the target protein was established by the vendor (example blots are available on the manufacturers’ websites.) Prior to immunoblot analysis of experimental tissue, blots with additional adolescent and adult mouse brain homogenate were probed with each antibody to validate the vendor’s findings. All antibodies chosen for the confirmation experiments showed a single band at the correct molecular weight marker for the indicated protein. Actin was chosen as a loading control for these experiments because it was not found to show differential expression between adolescent and adult mice in the mPFC in the proteomics screen. To confirm that this housekeeping protein was appropriate, adolescent and adult actin optical density was compared in all brain regions tested during analysis. No age differences in actin optical density emerged in any brain region.
Data Analysis

Western blot data were analyzed using GraphPad Prism software (GraphPad Software, Inc, La Jolla, CA, v5). To determine the difference between adolescent and adult mice in the expression of proteins of interest, data were transformed to percent change in optical density from the adult control for each gel. As the immunoblotting represented a confirmation of the proteomics data, significant differences were analyzed by a one-tailed unpaired t-test, with significance set a priori at p ≤ 0.05. Subjects with a percent change less than or greater than 2 standard deviations away from the group mean were considered outliers and were removed from the analysis (one adult mouse was removed from the STXBP1 and DNM1 blots, respectively).

Results

Proteomic analysis

To identify proteins with developmentally altered expression in the mPFC, brains from adolescent (PND 36) and adult (PND 80) mice were collected (Fig 1A). At the time of brain tissue collection, adolescent body weight was significantly lower than adult mice \([t(22) = 8.10, p < 0.0001]\) but brain weight was equivalent among the two age groups \((p > 0.05; \text{Fig 1B})\). The mPFC was dissected from each brain (Fig 1C), homogenized and labeled with red (adult) and green (adolescent) Cy dyes, run on 2D-DIGE, analyzed for expression differences between ages, and finally identified using tandem MALDI TOF/TOF mass spectrometry (Fig 1D).
The automated proteomic analysis spot picker detected 87 spots with differential expression in the adolescent and adult mPFC (Fig 1E). Manual curation to fulfill the criteria set (≥20% difference in all 4 gels) resulted in 58 differentially expressed spots, while an additional 4 spots were significantly altered at ≥15% across all 4 gels. All 62 spots were identified using MALDI-TOF and tandem TOF/TOF mass spectrometry (Table 1). mPFC data were analyzed as adolescent / adult expression, with positive fold change representing decreased protein expression in adolescents compared to adults and negative fold change representing increased protein expression in adolescents relative to adults. The majority of the identified proteins fell within the functional categories of cell-to-cell signaling, cell growth and motility, and cell metabolism.
Figure 1. Proteomic analysis of the adolescent and adult medial prefrontal cortex (mPFC).

(A) Timeline of experimental procedure. (B) Adult body weight was significantly greater than adolescent body weight at tissue collection (left), but brain weight did not differ between the two ages (right). (C) Photomicrograph (left) and schematic (right) of mouse brain section showing location of mPFC tissue punch for two-dimensional differential in-gel electrophoresis (2D-DIGE) and immunoblot studies. (D) Schematic describing proteomics workflow. Adult tissue is combined with Cy3 (red) and adolescent with Cy2 (green) dye and run in 2D-DIGE, with protein separating in the y plane via molecular weight and the x plane via isoelectrical focusing (IEF). DeCyder software identifies protein spots with significantly different fluorescent signals. Selected spots are subjected to trypsin digestion and identified via tandem MALDI TOF/TOF mass spectrometry. (E) Representative 2D-DIGE gel run in the proteomics analysis of mPFC. Adult samples were combined with red Cydye (left top); adolescent samples were combined with green CyDye (left bottom). Overlay of adult and adolescent samples (right). IEF is indicated on the x axis with pH values and molecular weight is indicated on the y axis in kDa. Circles indicate location of differentially expressed spots on the gel, with numeric markers prior to identification. Focus proteins are indicated in bold, with red representing increased expression in adults and green representing increased expression in adolescents. (**** indicates p≤0.0001)
Table 1. 62 Differentially Expressed Proteins in adult versus adolescent mPFC
Identified in Proteomics Analysis.

<table>
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<tr>
<th>Protein Name</th>
<th>Gene ID</th>
<th>Spot #</th>
<th>Peptide Count</th>
<th>Protein Score</th>
<th>Relative Change</th>
<th>p Value</th>
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<td>FABP7</td>
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<td>7</td>
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<td>Neurocalcin-δ</td>
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<td>75</td>
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<td>328</td>
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<td>16</td>
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<tr>
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<td>178</td>
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<tr>
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<td>Phosphoglycerate mutase 1</td>
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<td>17</td>
<td>817</td>
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84
<table>
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<td>Dihydropyrimidinase-like 4</td>
<td>DPYSL4</td>
<td>22</td>
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<td>1030</td>
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<td>Calreticulin</td>
<td>CALR</td>
<td>15</td>
<td>19</td>
<td>740</td>
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<td>Dihydropyrimidinase-like 3</td>
<td>DPYSL3*</td>
<td>19</td>
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<td>Protein phosphatase 3, regulatory subunit B, alpha</td>
<td>PPP3R1*</td>
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<td>Protein kinase C Y</td>
<td>PRKCG*</td>
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<td>Fascin actin-bundling protein 1</td>
<td>FSCN1</td>
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<td>17</td>
<td>674</td>
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<td>Drebrin</td>
<td>DBN1</td>
<td>72</td>
<td>23</td>
<td>858</td>
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<td>0.0018</td>
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<td>Collapsin Response Mediator Protein 1</td>
<td>CRMP1</td>
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<td>20</td>
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<td>&lt; 0.0001</td>
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<td>CB1 cannabinoid receptor-interacting protein 1</td>
<td>CNRP1</td>
<td>53</td>
<td>9</td>
<td>594</td>
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<td>3-oxoacid CoA transferase 1</td>
<td>OXCT1*</td>
<td>25</td>
<td>7</td>
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<td>3-oxoacid CoA transferase 1</td>
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<td>Protein Name</td>
<td>Gene Symbol</td>
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<td>p-Value</td>
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<tr>
<td>Fatty acid-binding protein</td>
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<td>11</td>
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<td>Voltage-dependent anion-selective channel protein 3</td>
<td>VDAC3</td>
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<td>11</td>
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<td>DNM1*</td>
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<td>Synapsin II</td>
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<td>13</td>
<td>328</td>
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<td>Guanine nucleotide binding protein (G protein), beta polypeptide 4</td>
<td>GNB4</td>
<td>37</td>
<td>15</td>
<td>231</td>
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<td>HYDIN, axonemal central pair apparatus protein</td>
<td>HYDIN</td>
<td>82</td>
<td>21</td>
<td>41</td>
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<td>Clathrin, light chain B</td>
<td>CLTB</td>
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<td>13</td>
<td>424</td>
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<td>Calbindin 2</td>
<td>CALB2</td>
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<td>14</td>
<td>388</td>
<td>-1.22</td>
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<td>3-hydroxybutyrate dehydrogenase, type 1</td>
<td>BDH1</td>
<td>49</td>
<td>12</td>
<td>489</td>
<td>-1.22</td>
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<tr>
<td>Fatty acid binding protein 3</td>
<td>FABP3</td>
<td>71</td>
<td>8</td>
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<td>GNB2L1</td>
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<td>972</td>
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<td>Dihydropyrimidinase-like 2</td>
<td>DPYS2L2*</td>
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<tr>
<td>Enolase 2 (gamma, neuronal)</td>
<td>ENO2</td>
<td>30</td>
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<td>0.0019</td>
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<td>Creatine kinase, brain</td>
<td>CKB</td>
<td>32</td>
<td>1.18</td>
<td>&lt; 0.0001</td>
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<tr>
<td>Ubiquinol-cytochrome c reductase core protein II</td>
<td>UQCRC2</td>
<td>69</td>
<td>1.19</td>
<td>0.017</td>
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<td>NADH dehydrogenase [ubiquinone] flavoprotein 1, mitochondrial</td>
<td>NDUFV1</td>
<td>77</td>
<td>1.20</td>
<td>&lt; 0.0001</td>
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<td>EF-hand domain-containing protein D2</td>
<td>EFHD2</td>
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<td>Bridging integrator 1</td>
<td>BIN1</td>
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<td>V-type proton ATPase subunit B, brain isoform</td>
<td>VATB2</td>
<td>81</td>
<td>1.23</td>
<td>0.0022</td>
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<td>Pyruvate kinase isozymes M1/M2</td>
<td>KPYM</td>
<td>80</td>
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<td>&lt; 0.0001</td>
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<tr>
<td>Voltage-dependent anion-selective channel protein 1</td>
<td>VDAC1*</td>
<td>47</td>
<td>1.25</td>
<td>0.0015</td>
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<tr>
<td>Syntaxin-binding protein 1</td>
<td>STXB1*</td>
<td>79</td>
<td>1.26</td>
<td>0.0002</td>
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<tr>
<td>Guanine nucleotide-binding protein G(o) subunit α</td>
<td>GNAO1</td>
<td>35</td>
<td>1.27</td>
<td>&lt; 0.0001</td>
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<tr>
<td>Glutathione S-transferase mu 5</td>
<td>GSTM5</td>
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<td>1.28</td>
<td>&lt; 0.0001</td>
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<tr>
<td>N-ethylmaleimide-sensitive factor</td>
<td>NSF</td>
<td>78</td>
<td>21</td>
<td>280</td>
<td>1.29</td>
<td>0.0078</td>
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<tr>
<td>Voltage-dependent anion-selective channel protein 1</td>
<td>VDAC1*</td>
<td>67</td>
<td>13</td>
<td>611</td>
<td>1.29</td>
<td>0.00029</td>
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<td>Dynamin-1</td>
<td>DNM1*</td>
<td>4</td>
<td>22</td>
<td>338</td>
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<td>Septin-3</td>
<td>SEPT3</td>
<td>34</td>
<td>6</td>
<td>103</td>
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<td>&lt; 0.0001</td>
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<td>Mitochondrial inner membrane protein</td>
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<td>28</td>
<td>625</td>
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<td>0.00026</td>
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<td>Carbonic anhydrase 2</td>
<td>CAH2</td>
<td>46</td>
<td>13</td>
<td>615</td>
<td>1.34</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Dihydropyrimidinase-like 2</td>
<td>DPYSL2*</td>
<td>13</td>
<td>25</td>
<td>719</td>
<td>1.35</td>
<td>0.00012</td>
</tr>
<tr>
<td>Cysteine and glycine-rich protein 1</td>
<td>CSRP1</td>
<td>51</td>
<td>8</td>
<td>334</td>
<td>1.36</td>
<td>0.017</td>
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<tr>
<td>Dihydropyrimidinase-like 2</td>
<td>DPYSL2*</td>
<td>12</td>
<td>26</td>
<td>800</td>
<td>1.38</td>
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<td>Syntaxin-binding protein 1</td>
<td>STXB1*</td>
<td>20</td>
<td>28</td>
<td>788</td>
<td>1.39</td>
<td>&lt; 0.0001</td>
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<tr>
<td>Complexin-2</td>
<td>CPLX2</td>
<td>57</td>
<td>6</td>
<td>163</td>
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<tr>
<td>Septin-2</td>
<td>SEPT2</td>
<td>33</td>
<td>10</td>
<td>352</td>
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<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>GAPDH*</td>
<td>48</td>
<td>12</td>
<td>280</td>
<td>4.33</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

**Table 1.** 58 spots showed ≤20% difference in expression in all 4 2D-DIGE gels

(p<0.05; standard font), with an additional 4 spots with ≤15% difference in
expression in all 4 2d-DIGE gels ($p<0.05$; italics). Each spot was identified via MALDI TOF/TOF mass spectrometry with a confidence of 1.0. Spot change was expressed as fold change ratio of adolescent from adult, with negative numbers reflecting reduced expression in adults relative to adolescents and positive numbers indicating greater expression in adults. Asterisks denote spots that appear more than once in the proteomics report.

**Ingenuity pathway analyses**

All proteins identified in the proteomics analysis were uploaded to Ingenuity Pathway Analysis for Global Functional Analysis (GFA) and Global Canonical Pathway (GCP) determination (Table 2). GFA revealed participation of identified proteins in cellular functions including cell-to-cell signaling and interaction, cellular morphology and cellular development. Additionally, identified proteins were shown to be involved in neurological disease, including schizophrenia and movement disorders. GCP analysis indicated the involvement of identified proteins in several known signaling cascades, including semaphorining signaling and axonal guidance signaling, androgen signaling, and glycolysis I and gluconeogenesis I signaling.

**Table 2. Role of identified proteins in biofunctions and disorders, and canonical signaling networks.**

<table>
<thead>
<tr>
<th>Biofunctions and disorders</th>
<th>Function/Disorder</th>
<th>$p$-Value</th>
<th>Higher in adults</th>
<th>Higher in adolescents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-to-cell Signaling and Interaction</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Process</td>
<td>p-Value</td>
<td>Genes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>---------</td>
<td>-------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synaptic transmission of synapse</td>
<td>0.000033</td>
<td>STXBP1, VDAC1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synaptic transmission of nervous tissue</td>
<td>0.000054</td>
<td>NSF, STXBP1, VDAC1</td>
<td></td>
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<td>Synaptic transmission</td>
<td>0.0000124</td>
<td>NSF, STXBP1, VDAC1</td>
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<tr>
<td>Long-term potentiation of synapse</td>
<td>0.00000198</td>
<td>CPLX2, VDAC1</td>
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<td></td>
</tr>
<tr>
<td>Long-term potentiation</td>
<td>0.00000453</td>
<td>CPLX2, VDAC1</td>
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</tr>
<tr>
<td>Synaptic depression</td>
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**Cellular Morphology**

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<tr>
<td>Size of neurons</td>
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</tr>
<tr>
<td>Morphology of neurites</td>
<td>0.0187</td>
</tr>
<tr>
<td>Morphology of neurons</td>
<td>0.00716</td>
</tr>
<tr>
<td>Length of neurites</td>
<td>0.00173</td>
</tr>
<tr>
<td>Length of neurons</td>
<td>0.000214</td>
</tr>
<tr>
<td>Formation of filopodia</td>
<td>0.000124</td>
</tr>
<tr>
<td>Extension of plasma membrane projections</td>
<td>0.0213</td>
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<tr>
<td>Outgrowth of neurites</td>
<td>0.011</td>
</tr>
<tr>
<td>Branching of neurons</td>
<td>0.000225</td>
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<tr>
<td>Morphogenesis of neurites</td>
<td>0.00276</td>
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Higher in adolescents

Higher in adults
<table>
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<tr>
<th>cellular Development</th>
<th>p-Value</th>
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</thead>
<tbody>
<tr>
<td>Abnormal morphology of cerebral cortex</td>
<td>0.00591</td>
<td>CKB, CPLX2</td>
</tr>
<tr>
<td>Abnormal morphology of brain</td>
<td>0.0138</td>
<td>CKB, CPLX2</td>
</tr>
<tr>
<td>Abnormal morphology of nervous system</td>
<td>0.0332</td>
<td>CKB, CPLX2, DNM1</td>
</tr>
<tr>
<td>Morphology of nervous system</td>
<td>0.00607</td>
<td>CKB, CPLX2, DNM1</td>
</tr>
<tr>
<td>Proliferation of cells</td>
<td>0.00297</td>
<td>CSRP1, DPYSL2, GNAO1, PKM</td>
</tr>
<tr>
<td>Proliferation of neuronal cells</td>
<td>0.00476</td>
<td>CSRP1, DPYSL2, GNAO1</td>
</tr>
<tr>
<td>Differentiation of cells</td>
<td>0.0014</td>
<td>CSRP1, DPYSL2</td>
</tr>
<tr>
<td>Transport of synaptic vesicles</td>
<td>0.00000475</td>
<td>BIN1, CPLX2, DNM1, DPYSL2</td>
</tr>
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<td>Endocytosis of synaptic vesicles</td>
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<td>BIN1, DNM1</td>
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<tr>
<td>Growth of neurites</td>
<td>0.0056</td>
<td>CSRP1, DPYSL2, GNAO1</td>
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<tr>
<td>Neurological Disease</td>
<td>p-Value</td>
<td>Higher in adults</td>
</tr>
<tr>
<td>--------------------------------------</td>
<td>-----------------</td>
<td>------------------</td>
</tr>
<tr>
<td>Neuromuscular disease</td>
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<td>CA2,CKB,ENO2</td>
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<td>Schizophrenia</td>
<td>0.00000118</td>
<td>ATP6V1B2, CSRP1,VDAC1</td>
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<td>Disorder of basal ganglia</td>
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<td>CA2,CKB,ENO2</td>
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<td>Movement Disorders</td>
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<td>CA2,CKB,ENO2</td>
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<tr>
<td>Huntington's Disease</td>
<td>0.000119</td>
<td>CA2,CKB,ENO2</td>
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</table>

### Canonical pathways and upstream regulators

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<th>p Value</th>
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<th>Higher in adolescents</th>
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<tbody>
<tr>
<td>Semaphorin Signaling in Neurons</td>
<td>2.31E-08</td>
<td>DPYSL2</td>
<td>CRMP1,CFL1,DPYSL3,DPYSL4,DPYSL5</td>
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<tr>
<td>Glycolysis I</td>
<td>7.01E-07</td>
<td>PKM,ENO2,GAPDH</td>
<td>PGAM1</td>
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<td>Huntington's Disease Signaling</td>
<td>8.93E-07</td>
<td>DNM1,NSF,CPLX2</td>
<td>CLTA,CLTB,GNB2L1, GNB4,PRKCG</td>
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<tr>
<td>Androgen Signaling</td>
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<td>GNAO1</td>
<td>CALR,GNB4,GNB2L1, PRKCG</td>
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<tr>
<td>Gluconeogenesis I</td>
<td>4.84E-05</td>
<td>ENO2,GAPDH</td>
<td>PGAM1</td>
</tr>
<tr>
<td>Axonal Guidance Signaling</td>
<td>7.26E-05</td>
<td>DPYSL2, GNAO1</td>
<td>GNB4, CFL1, PPP3R1, GNB2L1, DPYSL5, PRKCG</td>
</tr>
<tr>
<td>---------------------------</td>
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</tbody>
</table>

**Regulators**

| MAPT | 1.15E-16 | CKB, CPLX2, DPYSL2, ENO2, GAPDH, GNAO1, PKM, STXBP1, VDAC1, ATP5D, CFL1, CLTA, CLTB, PGAM1, SNCB |
| APP  | 3.88E-15 | CKB, CPLX2, DNM1, DPYSL2, ENO2, GAPDH, GNAO1, PKM, STXBP1, VDAC1, ATP5D, CFL1, CLTA, CLTB, DBN1, FABP3, GAP43, PGAM1, SNCB, SYN2 |
| PSEN1| 7.74E-14 | CKB, CPLX2, DPYSL2, ENO2, GAPDH, GNAO1, PKM, STXBP1, VDAC1, ATP5D, CFL1, CLTA, CLTB, PGAM1, SNCB |
| MKNK1| 2.84E-08 | CPLX2, GNAO1, STXBP1, CRMP1, DPYSL3, GAP43, SYN2 |
| BDNF | 6.22E-08 | CPLX2, GNAO1, STXBP1, CALB2, CRMP1, DPYSL3, FSCN1, GAP43, SYN2 |

**Table 2.** Proteomics results were analyzed via Ingenuity Pathway Analysis for known interactions with other proteins, signaling systems and networks in the Ingenuity Knowledge Base. Proteins that displayed higher expression during adulthood are shown on the left, and proteins that displayed higher expression during adolescence are shown on the right, as seen in Table 1. *p* values were derived from Ingenuity Pathway Analysis by right-tailed Fisher exact test and indicate relative overrepresentation of proteins in a given function compared with what is expected by chance.
Network analysis and Western blot confirmation

Ingenuity Pathway Analysis was performed to identify functional protein networks likely to be impacted by the developmentally regulated proteins identified in the proteomics analysis. Three significant networks were identified: Network 1, cellular assembly and organization, cellular function and maintenance, and cellular movement (p-score 29); Network 2, behavior, cell signaling and interaction, nervous system development and function (p-score 31); Network 3, neurological disease, skeletal and muscular disorders, and psychological disorders (p-score 22). Complete Western blot gel images for all proteins tested are shown in supporting information figures (S1 – S24 Figs).

Network 1

Network 1 (Cellular Assembly & Organization) includes 18 focus molecules identified in the proteomics analysis of adolescent and adult mPFC as well as 17 significant interaction proteins (Fig 2). Noteworthy predicted regulators of this network include interferon gamma (INFG) and reticulon 4 (RTN4).

Of the focus molecules, dihydropyrimidinase-like 2 (DPYSL2) was a locus of significant interconnectedness in Network 1 and was thus selected for further analysis. DPYSL2 appeared at Spot 12 in the proteomics analysis (Fig 1E), where its standardized log abundance was 0.041 in adults and -0.098 in adolescents, representing a 38% reduction in adolescents versus adults (Fig 3, A-C). Spot 12 was identified as DPYSL2 with a protein score of 800 (Table 1), and no other candidate proteins met the pre-hoc criteria for identification at Spot 12. Western blot analysis
confirmed the decrease observed in the proteomics analysis, with adolescent expression of DPYSL2 27% in mPFC lower than adult \[t(26) = 2.27, p <0.05; \text{Fig 3, D & E}\].

Figure 2. Adolescent development impacts a functional protein network involved in cellular assembly and organization, cellular function and maintenance, and cellular movement.
Visualization of a protein interaction network identified by Ingenuity Pathway Analysis as being altered by adolescent brain development (p-score= 29). Proteins shown in red were up-regulated in the adult mPFC, proteins in green were up-regulated in the adolescent mPFC, and proteins in yellow indicate statistically significant interaction proteins identified by IPA network analysis. Solid lines indicate a direct interaction, and dashed lines indicate an indirect interaction mediated by additional, non-significant proteins. Asterisks denote proteins that were identified multiple times in the proteomic analysis that have been consolidated into a single point in the functional network. Molecules for confirmation are indicated in bold, e.g. syntaxin binding protein 1 (STXBP1), dynamin-1 (DNM1) and dihydropyrimidinase-like-2 (DPYSL2).

In addition to the results of the proteomic analysis and Western blots in the mPFC, other brain regions were of interest based on their association with adolescent-typical behaviors. The dorsal striatum (dSTR), nucleus accumbens (NAc), primary motor cortex (MC), amygdala (AMY) and ventral tegmental area (VTA) were also analyzed for expression of the selected confirmation proteins. DPYSL2 expression was significantly reduced in adolescent mice as compared with adult mice in all brain regions examined excepting the VTA (p >0.05; Fig 3F). Age differences were most pronounced in the MC [22% decrease; \( t(23) = 9.00, p <0.0001 \)] and AMY [26% decrease; \( t(19) = 4.45, p <0.0001 \)], with less pronounced differences in the dSTR [18% decrease; \( t(22) = 3.59, p <0.001 \)] and NAc [9% decrease; \( t(23) = 2.42, p <0.05 \)].
Figure 3. Adult and adolescent expression of dihydropyrimidinase-like-2 (DPYSL2). (A) Representative 3D plot of DPYSL2 expression in adult (left) and adolescent (right) mice for Spot #20. (B) Standardized abundance (log)
of DPYSL2 demonstrating higher expression in adults versus adolescents. 

(C) Representative gel image of a Western blot for DPYSL2 expression to confirm 2D-DIGE changes. Both resulting bands were quantified. (D) Quantification of Western blot results, confirming reduced expression of DPYSL2 (normalized to actin) in adolescents as compared to adults. (E) Top, representative gel images for each brain region; bottom, quantification of Western blots for each brain region. Adults show increased expression of DPYSL2 in dStr, NAc, MC and Amy. No significant age differences were observed in the VTA ($p>0.05$). Data were expressed as percent change from mean adult within the same blot and graphed as mean ± SEM. (* indicates $p\leq0.05$, *** indicates $p\leq0.001$).

Dynamin-1 (DNM1) was also significantly interconnected in Network 1 and was subsequently analyzed. In the proteomics analysis DNM1 was identified twice, at Spot #4 and Spot #6 (Fig 1E). At Spot 4, DNM1 standardized log abundance was -0.003 for adults and -0.056 for adolescents, representing a 31% decrease in adolescent mice compared to adults (Fig 4, A-C). Spot 4 was identified as DNM1 with a peptide score of 338 (Table 1), and no other candidate proteins met the pre-hoc criteria for identification at Spot 12. At Spot 6, DNM1 standardized log abundance was -0.058 for adults and 0.400 for adolescents, representing a 26% increase in adolescent mice compared to adults (Fig 4, D-F). At Spot 6, the candidate proteins DNM1 and DNM2 both exceeded the pre-hoc criteria of 95% C.I., and DNM1 was selected as the identity for Spot 6 based on the higher peptide score for DNM1 (625) versus DNM2 (83). Western blot analysis indicated that DNM1
expression was decreased by 22% in adolescent mPFC as compared to adults \([t(25) = 5.84, p < 0.0001; \text{Fig 4, G & H}]\). DNM1 expression was also significantly lower in adolescents compared with adults in dSTR [12% decrease; \(t(22) = 4.48; p < 0.0001\)], MC [13% decrease; \(t(23) = 5.48, p < 0.0001\)], AMY (14% decrease; \(t(18) = 2.73, p < 0.01\)) and VTA (9% decrease; \(t(21) = 2.68, p < 0.01\), Fig 4 I]. The NAc displayed no age differences in DNM1 expression \((p > 0.05)\).
Figure 4. Adult and adolescent expression of dynamin-1 (DNM1).

(A) Representative 3D plot of DNM1 expression in adult (left) and adolescent (right) mice for Spot #4. (B) Standardized abundance (log) of DNM1 (Spot 4) demonstrating higher expression in adults versus adolescents (C) Representative 3D plot of DNM1 expression in adult (left) and adolescent (right)
mice for Spot #6. **(D)** Standardized abundance (log) of DNM1 (Spot 6) demonstrating higher expression in adults versus adolescents. **(E)** Representative gel image of a Western blot for DNM1 expression to confirm 2D-DIGE changes. **(F)** Quantification of Western blot results, confirming reduced expression of DNM1 (normalized to actin) in adolescents as compared to adults. **(G)** Top, representative gel images for each brain region; bottom, quantification of Western blots for each brain region. DNM1 expression was higher in adults in dStr, M1, Amy, and VTA. There was no significant change in DNM1 expression in NAc (p>0.05). Data were expressed as percent change from mean adult within the same blot and graphed as mean ± SEM. (* indicates p≤0.05, ** indicates p≤0.01, *** indicates p≤0.001, **** indicates p≤0.0001).

**Network 2**

Network 2 (Behavior/Signaling) includes 19 focus molecules identified in the proteomics analysis as well as 16 proteins statistically predicted to interact with the focus proteins in a functional network (Fig 5). Of the focus molecules, 9 exhibited higher expression in adult mPFC whereas 10 exhibited greater expression in adolescents Notable predicted regulators of this network include brain-derived neurotrophic factor (BDNF) and huntingtin (HTT).
Figure 5. Adolescent development impacts a functional protein network involved in behavior, cell-to-cell signaling and interaction, and nervous system development and function.

Visualization of a protein interaction network identified by Ingenuity Pathway Analysis as being altered by adolescent brain development (p score= 31).

Proteins shown in red were up-regulated in the adult mPFC, proteins in green were up-regulated in the adolescent mPFC, and proteins in yellow indicate statistically significant interaction proteins identified by IPA network analysis.
Solid lines indicate a direct interaction, and dashed lines indicate an indirect interaction mediated by additional, non-significant proteins. Asterisks denote proteins that were identified multiple times in the proteomic analysis that have been consolidated into a single point in the functional network. Focus molecule syntaxin binding protein 1 (STXBP1) is indicated in bold.

Of the focus molecules identified in the proteomics analysis in Network 2, syntaxin-binding protein 1 (STXBP1) was a significant hub of interconnectedness within the network and was therefore selected for further analysis. In the proteomics analysis of the mPFC STXBP1 was identified twice, at spot #20 and spot #79 (Fig 1E). At Spot 20, STXBP1 standardized log abundance was 0.039 for adults and -0.100 for adolescents, representing a 39% decrease in adolescent mice compared to adults (Fig 6, A-C). Spot 20 was identified as STXBP1 with a peptide score of 788, and no additional candidate proteins met the pre-hoc criteria for identification at that spot.
Figure 6. Adult and adolescent expression of syntaxin binding protein 1 (STXBP1).

(A) Representative 3D plot of STXBP expression in adult (left) and adolescent (right) mice for Spot #20. (B) Standardized abundance (log) of STXBP1 (Spot 20) demonstrating higher expression in adults versus adolescents. (C) Representative
3D plot of STXBP1 expression in adult (left) and adolescent (right) mice for Spot#79. (D) Standardized abundance (log) of STXBP1 (Spot 79) demonstrating higher expression in adults versus adolescents. (E) Representative gel image of a Western blot for STXBP1 expression to confirm 2D-DIGE changes. (F) Quantification of Western blot results, confirming reduced expression of STXBP1 (normalized to actin) in adolescents as compared to adults. (G) STXBP1 expression was greater in adults in dStr, NAc, M1, Amy, and VTA. Top, representative gel images for each brain region; bottom, quantification of Western blots for each brain region. (** indicates $p \leq 0.001$, **** indicates $p \leq 0.0001$.)

At Spot 79, the candidate proteins STXBP1 and TPX2 both exceeded the pre-hoc criteria of 95% C.I., and STXBP1 was selected as the identity of Spot 79 based on the higher peptide score of STXBP1 (716) versus TPX2 (65). STXBP1 standardized log abundance was 0.036 for adults and -0.058 for adolescents, representing a 26% decrease in adolescent mice compared to adults (Fig 6, D-F). Western blot analysis confirmed the decreases observed in the proteomics analysis with adolescent expression of STXBP1 18% lower than adult expression in mPFC [$t(25) = 3.89, p < 0.001$; Fig 6, G & H]. STXBP1 expression was consistently reduced in adolescent mice as compared to adult mice in all brain regions examined (Fig 6I). The age difference was most pronounced in MC, where adolescent STXBP1 expression was decreased by 39% compared to adults [$t(23) = 7.85, p < 0.0001$]. Age differences in the dSTR [11% decrease; $t(23) = 3.98, p < 0.001$], NAc [17% decrease; $t(23) = 3.35, p < 0.01$], AMY [11% decrease; $t(18) = 2.48, p < 0.05$] and VTA [15% decrease; $t(21) = 3.91, p < 0.001$] were more modest.
Network 3

Network 3 (Disease) includes 15 focus molecules identified in the proteomics analysis that were differentially expressed in adolescent as compared to adult mPFC as well as 20 significant interaction proteins (Fig 7). Of the focus molecules, 8 exhibited greater expression in adults whereas 7 had greater expression in adolescents. Significant predicted regulators of this network included amyloid-β precursor protein (APP), microtubule-associated protein tau (MAPT) and presenilin-1 (PSEN1).

The focus molecule cofilin-1 (CFL1) was a significant component of the network connectivity and was selected for further analysis. CFL1 appeared at Spot 54 in the proteomics analysis of the mPFC (Fig 1E), where its standardized log abundance was -0.378 in adults and -0.180 in adolescents, representing a 58% increase in adolescents versus adults (Fig 8, A-C). Both the candidate proteins CFL1 and CFL2 exceeded the pre-hoc criteria for identification, and CFL1 was chosen as the identity for Spot 54 based on the higher peptide score of CFL1 (158) versus CFL2 (149). Western blot analysis confirmed the increase observed in the proteomics analysis, with adolescent expression of CFL1 21% higher than adult \[t(26) =1.84, p <0.05; \text{Fig 8, D \\
& E}\] in the mPFC. CFL1 expression was also significantly higher in adolescents versus adults in the VTA [23% increase; \(t(21) =1.85, p <0.05; \text{Fig 8F}\)]. No significant age differences in CFL1 emerged in the dSTR, NAc, MC or AMY \((p >0.05)\).
Figure 7. Adolescent development impacts a functional protein network involved with neurological disease, skeletal and muscular disorders, and psychological disorders.

Visualization of a protein interaction network identified by Ingenuity Pathway Analysis as being altered by adolescent brain development ($p$-score= 22). Proteins shown in red were up-regulated in the adult mPFC, proteins in green were up-regulated in the adolescent mPFC, and proteins in yellow indicate statistically significant interaction proteins identified by IPA network analysis. Solid lines indicate a direct interaction, and dashed lines indicate an indirect interaction mediated by additional, non-significant proteins. Asterisks denote proteins that were identified
multiple times in the proteomic analysis that have been consolidated into a single point in the functional network. Focus molecule cofilin-1 (CFL1) is indicated in bold.

Discussion

Although significant structural changes in the prefrontal cortex during adolescence have been well established, the molecular changes that mediate these developmental alterations remain to be more fully characterized. The present study utilized a high-throughput unbiased proteomics approach to identify specific proteins and protein networks that show differential expression in adolescent compared to adult mPFC. 2D-DIGE followed by MALDI TOF/TOF identified 62 individual proteins with significant age-dependent differences in expression. Ingenuity Pathway Analysis identified 3 networks in which these target proteins were overexpressed. Further results confirmed key changes from previous investigations [20] while focusing the analysis on adolescent-specific protein changes, and extended these findings in additional brain regions (dSTR, MC, NAc, AMY and VTA). Together, the results indicate that, compared to adults, the adolescent mPFC has developmentally linked alterations in protein networks that regulate cellular organization/structure, neuronal signaling, anxiety-related behavior and neurological disease. These findings strengthen existing hypotheses about the progression of postnatal cortical development and point to several novel potential functional regulators of adolescent-typical behavior and vulnerability.
Figure 8. Adult and adolescent expression of cofilin-1 (CFL1). (A)

(A) Representative 3D plot of CFL1 expression in adult (left) and adolescent (right) mice for Spot #54. (B) Standardized abundance (log) of CFL1
demonstrating higher expression in adults versus adolescents. (C)

Representative gel image of a Western blot for CFL1 expression to confirm 2D-DIGE changes. (D) Quantification of Western blot results, confirming reduced expression of CFL1 (normalized to actin) in adolescents as compared to adults. (E) Top, representative gel images for each brain region; bottom, quantification of Western blots for each brain region. Adolescents show higher expression of CFL1 in VTA. There were no significant changes in CFL1 expression in dSTR, NAc, MC or Amy ($p>0.05$). (* indicates $p \leq 0.05$, *** indicates $p \leq 0.001$).

**Structural development**

The present results provide several lines of evidence that suggest that the adolescent mPFC is characterized by widespread alterations in protein expression related to the regulation of cellular assembly & structure, cellular organization and structural plasticity. Many of the proteins identified in the proteomic analysis have roles in cellular morphology and synaptic plasticity. Ingenuity Global Canonical Pathway analysis also suggested that canonical signaling pathways known to regulate cellular growth and development in the adult mPFC were likely to differ from the adolescent condition, based on the interactions between proteins identified in the proteomics screen (Table 2). Both semaphorin signaling and axonal guidance signaling were identified as canonical signaling pathways that were impacted by the developmental state of the mPFC (Table 2). The majority of identified proteins in these canonical pathways were up-regulated in the adolescent cortex. Semaphorins are a family of receptors and secretory proteins that have a well-established role in guiding axonal outgrowth during embryonic development [29] and are also involved
in neuronal maturation, synaptic plasticity and cell death in the adult cortex [30, 31]. Although the role of sempahorin signaling in adolescent brain maturation and synaptic pruning has not been investigated to date, this signaling system is a plausible mediator of morphological changes in adolescent cortex and merits examination in future studies.

Ingenuity Pathway Analysis identified a functional protein network involved in cellular assembly and organization that was significantly impacted by age (Fig 2). Several proteins identified in this network have been shown to play a role in synaptic development, including PP3R1 [32], CALR [33] and NSF [34], as well as the focus protein DPYSL2. DPYSL2 (or CRMP2) is a member of the CRMP family, which binds tubulin heterodimers to facilitate microtubule assembly [35]. These proteins function in growth cone formation, contributing to neuronal outgrowth [36] and may also play a role in cell death [37] and thus neural pruning. The expression of CRMP1 and DPYSL3, 4, and 5 was higher in the adolescent mPFC, consistent with increased neuronal outgrowth and synaptic formation during this developmental period (Table1). However, DPYSL2 expression was found to be consistently higher in adults in the mPFC, dSTR, NAc, M1, and AMY (Fig 4). The reason why this CRMP subtype, but not the others, is up-regulated in adulthood is unknown, but could be due to a developmental shift from dominant expression of CRMP 1, DPYSL3, 4, or 5 in the adolescent brain to DPYSL2 in the adult brain. DPYSL2 was identified three times in the proteomic screen, at spots #12, 13 and 14. Spots 13 and 14 appeared at the same molecular weight range but exhibited different isoelectrical focusing, which may suggest a posttranslational modification affecting one of the two
spots. Spot #12 exhibited shifts in both isoelectrical focusing and molecular weight, which could indicate a possibility of protein contamination, significant posttranslational modification, or both. Immunoblots confirmed the increase in total DPYSL2 in the adolescent mPFC, but additional assessment with antibodies targeting posttranslational modifications of DPYSL2 would provide clarity as to the variable spots detected in the proteomics screen.

**Signaling and behavior**

In addition to their roles as mediators of cellular assembly and development, many of the proteins identified in the proteomics analysis are known to be involved in cell-to-cell signaling and neurotransmission. Indeed, Ingenuity Pathway Analysis identified a significant protein interaction network associated with cell signaling and behavior that was impacted by adolescent brain maturation (Fig 4). The immaturity of the prefrontal cortex has been suggested to underlie many adolescent-typical behaviors, such as impulsivity, reward sensitivity and risk taking. Subcortical areas involved in emotional processing (such as the nucleus accumbens, ventral tegmental area and amygdala) reach adulthood before regulatory control of these regions from the PFC is fully mature [16], leading to enhanced response to reward and impairments in inhibitory control under emotionally salient conditions [38].

At the cellular level, previous studies have demonstrated that the adolescent mPFC has altered responses to neurotransmitters and cell signaling molecules, including dopamine [39, 40], glutamate [41] and GABA [42]. Moreover, several studies have provided evidence for the regulation of adolescent-typical behaviors by diverse signaling systems including the cannabinoid systems [43], glutamate [44],
dopamine [45] and GABA [46]. Within the network identified by IPA, several proteins with established roles in adolescent-related behaviors were observed, including BDNF [47, 48], GAP43 [49] and mTOR [50]. Regulation of both PFC function and behavior by these varied signaling systems may reflect large-scale structural changes occurring in the adolescent forebrain during development, consistent with the alterations in structural proteins we observed.

The focus proteins STXBP1 and DNM1 have both been shown to be involved in neurotransmitter signaling, and both displayed different expression patterns in the adolescent and adult mPFC. STXBP1 contributes to the regulation of exocytosis in cells, assisting in vesicle fusion and neurotransmitter release [51]. The proteomics screen revealed two individual spots that were each identified as STXBP1 (spot #20 and #79), both of which displayed decreased expression in adolescents versus adults (Fig 6). While it is possible that these multiple spots could represent contamination with other proteins, each spot was identified as STXBP1 with high confidence; spot #20 was identified as STXBP1 with 28 peptides and a peptide score of 788, and spot #79 was identified as STXBP1 with 26 peptides and a peptide score of 716. Based on these identifications, another explanation for the multiple identifications of STXBP1 in the proteomics screen is post-translational modifications. The presence of phosphate groups, methylation and other post-translational modifications can shift the isoelectrical focusing of a spot without altering the observed molecular weight or peptide identification. Immunoblotting confirmed that total STXBP1 expression is reduced in the adolescent mPFC relative to adults. This alteration in STXBP1 expression in the adolescent mPFC was also
observed in each additional brain region examined (dStr, NAc, M1, Amy and VTA), which may indicate that this developmental change in STXBP1 levels is part of a brain-wide process, perhaps in response to the widespread changes in neurotransmitter signaling reported throughout the brain during adolescence [2]. The increased STXBP1 observed in the mature brain may contribute to greater regulation of synaptic transmission in adults, and ultimately greater refinement in the executive functioning of the PFC.

DNM1 participates in vesicle budding in both clathrin-mediated endocytosis and activity-dependent bulk endocytosis [52, 53], and mutations in dynamin cause developmental abnormalities in *drosophila* [54]. Proteomic analysis of DNM1 revealed two individual spots that were each identified as DNM1 (spot #4 and #6) As was the case for STXBP1, each spot was identified as DNM1 with high confidence; spot #4 was identified as DNM1 with 22 peptides and a peptide score of 338, and spot #6 was identified as DNM1 with 34 peptides and a peptide score of 625. Unlike STXBP1, however, in this instance one DNM1 spot displayed increased expression in the adult mPFC (spot #4) while the other showed increased expression in the adolescent mPFC (spot #6). To resolve this apparent contradiction, immunoblotting for total DNM1 protein showed that DNM1 expression was lower in the adolescent versus the adult mPFC, consistent with spot #4. The increased expression in DNM1 at spot #6 may indicate that a posttranslational modification of DNM1 has higher expression in the adolescent mPFC, perhaps in compensation for reduced protein levels. Future studies should examine the phosphorylation state and activity of DNM1 in the adolescent and adult cortex to test this hypothesis. Similar to STXBP1,
Western blot analysis of total DNM1 expression in other brain regions showed higher DNM1 expression in adults across most brain regions, with the exception of the NAc where levels did not significantly differ between the two ages. Much like STXBP1, the generally elevated expression of DNM1 in the adult brain may represent more mature control of receptor expression at the cell surface and therefore refinement of synaptic signaling.

Whereas DPYSL2, STXBP1 and DNM1 showed a general decrease in the adolescent brain relative to the adult brain, the expression of the focus protein cofilin-1 (CFL1) was found to have higher expression in the adolescent prefrontal cortex in the proteomics analysis. Furthermore, the age-dependent alteration in CFL1 expression was relatively selective for the mPFC; of the additional brain regions tested, only the VTA showed a similar age difference (Fig 8). Spot 54 was identified as CFL1, but the 3D image rendered by DeCyder revealed a shoulder onto another spot, and the candidate protein CFL2 was also a significant identity for Spot 54. Ultimately CFL1 was chosen as the protein identity for Spot 54 based on the higher protein score of CFL1 versus CFL2, and immunoblotting experiments confirmed that the expression of CFL1 is increased in the adolescent mPFC versus the adult. However, the potential for CFL2 in addition to CFL1 being altered during adolescent brain development remains a strong possibility.

Both cofilin-1 and -2 are actin depolymerizing proteins [55] that contribute to spine growth and shrinkage [56, 57], cell migration [58] and AMPAR and NMDAR trafficking during both long-term potentiation (LTP) and long-term depression (LTD) [59, 60]. These observations make cofilin a particularly interesting protein in the
context of adolescent development of the cortex and the developmental disorders associated with adolescent cortical disruption, such as schizophrenia.

Schizophrenia is a psychotic disorder characterized by significant impairments in cognition, hallucinations and delusions, and social withdrawal and mood disturbances [61] that is usually diagnosed during adolescence [62]. A significant neurobiological component of schizophrenia appears to be alterations in cortical gray matter; schizophrenia patients present with both faster reductions in cortical gray matter during adolescence [63] and greater total volume reductions in the prefrontal cortex [64], which may predate symptom onset [65]. Decreased dendritic spines [66] and synaptic markers [67] have also been reported in the schizophrenic cortex. Taken together, these findings suggest a “hyper-adolescent” state in the schizophrenic cortex, such that adolescent-typical neural pruning occurs in excess [68], eliminating necessary synaptic connections in the PFC and reducing ability to regulate brain function and behavior.

Several recent reports have linked actin dynamics and coflin to schizophrenia [69, 70]. Additionally, Ingenuity Global Functional Analysis revealed significant enrichment of the identified protein dataset for proteins previously shown to be linked to schizophrenia, including GAP43 [71], VDAC1 [72] and SNCB [73], all of which have been shown to play a role in synaptic communication. These results underscore the association between normal adolescent brain development and pathology. However, neither the protein identifications nor the bioinformatics utilized in the present studies were able to assess a functional role for the observed protein alterations either in normal development or in disease. A major goal for future work
will be the evaluation of protein targets like cofilin that may regulate adolescent brain
development and be mechanistically involved in the etiology of developmental
disorders such as schizophrenia.

**Limitations and future directions**

The most significant limitation of the present work was the inability to assess
the functional relevance of protein changes observed in these experiments. Although
the identified proteins and subsequent bioinformatics analyses provide insight into
the biochemical processes that may underlie the development occurring in the
adolescent mPFC, these results are correlational and do not directly address the
causative relationship, if any, between protein expression changes and
structural/functional maturation. However, the high-throughput nature of the
proteomics analysis enabled the identification of several novel targets and signaling
pathways, providing significant heuristic value for subsequent investigations. The
Ingenuity Pathway Analysis provided useful insight into additional protein targets
whose activity, but not expression, may be altered during adolescent brain
development, but the suggested impact of development on predicted interaction
proteins was not directly assessed in these experiments. Future work should confirm
changes in the activity or phosphorylation state of protein predicted by IPA to be
altered in the adolescent mPFC.

The proteomic screen was conducted using 2D-DIGE and MALDI TOF/TOF
MS, a high-throughput and cost-effective means of obtaining a “snapshot” of the
mPFC proteome in adolescent and adult samples. However, gel-based proteomics
applications have important limitations, the most significant of which are the inability
to detect low-abundance proteins and the difficulty of resolving membrane-bound proteins [74]. Several previous studies have examined the synaptic fraction of the mPFC proteome in developing rodents [17-20], thus the present findings add to a preexisting literature by shedding light on the whole-cell protein alterations occurring during adolescent brain maturation. Still, the methods used in these experiments leave open the strong possibility that additional proteins not detected here may be altered during cortical development.

Additionally, the immunoblots were developed with radiography, which presents limited resolution compared with digital imaging systems. Subsequent experiments to expand the present findings could strengthen the conclusions reported herein by combining MS-based proteomics with digital imaging of immunoblots to confirm the protein expression changes observed. In the immunoblot experiments, actin was selected as a housekeeping protein due to its previous use as a loading control in development proteomics screens [18] and the lack of evidence for age differences in actin expression in the present proteomics assessment. Across all brain regions and proteins tested, the optical density of actin was not different between adolescents and adults. Nevertheless, the actin values were not compared to total protein in the immunoblot experiments, leaving open the possibility that loading errors could have occluded a genuine age difference, rendering actin inappropriate as a housekeeping protein. However, four previous proteomics screen have failed to identify actin as developmentally altered during adolescent brain development [17-20]. Combined with the consistent lack of age differences in
actin optical density observed in each brain region tested, the possibility of occult age differences in actin expression remains but seems unlikely.

During tissue collection, mice were perfused using 1.0M PBS to remove blood that could potentially contaminate brain tissue and alter the protein differences reported in the proteomics screen. However, this treatment entailed the use of an injected anesthetic, and injection stress could have altered the expression of identified proteins, as could the perfusion process itself. To avoid identifying age differences in proteins based on stress/perfusion alone, both adolescent and adult samples were treated identically during anesthesia and perfusion. Still, protein abundance in both ages could have been altered by these procedures.

**Conclusion**

The present findings supplement an existing body of work that suggests that adolescence is characterized by enhancement of neural pruning, synaptic plasticity, and morphological changes as the brain matures into adulthood. Importantly, many of the proteins identified in the current experiments have previously been observed to be altered at the protein level in mouse cortex [20] and at the gene expression level in the postmortem human adolescent cortex [75]. The results of the proteomics analysis provide novel insight into a wider array of molecular alterations that may underlie the large-scale alterations in cortical gray matter and connectivity observed during adolescence. Further, functional protein networks involved in cellular assembly and signaling point toward pathways for future research into the mechanistic regulation of postnatal brain development. Future experiments to evaluate the role of the identified proteins and signaling systems in adolescent brain
maturation, particularly in the context of psychiatric diseases such as schizophrenia, will shed further light on this crucial developmental period.
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CHAPTER THREE- THE DRUNKEN ADOLESCENT PREFRONTAL CORTEX PROTEOME: CALCINEURIN AS A MOLECULAR MECHANISM OF ENHANCED ALCOHOL INTAKE AFTER ADOLESCENT ALCOHOL EXPOSURE

Introduction

Alcoholism is a debilitating neuropsychiatric condition characterized by excessive alcohol consumption, physical dependence upon alcohol, and continued use of alcohol in the face of negative consequences of drinking. Incidence of alcoholism in the United States is approximately 6-10%, and alcohol-related disease and injury is the fifth leading cause of death in the US [1]. Current treatment modalities for alcoholism include cognitive-behavioral therapy, support groups such as Alcoholics Anonymous, and the medications Antabuse, acamprosate and naltrexone [2]. Unfortunately, despite these interventions relapse rates amongst alcoholics remain high (as much as 90% over four years) [3]. There is a pressing need for new treatments to help reduce alcohol drinking as well as interventions to prevent risky drinking from transitioning to an Alcohol Use Disorder (AUD). The study of at-risk populations advances both goals as it can inform prevention efforts as well as identify new molecular mechanisms of alcohol activity to target for medication development. One such at-risk population is adolescents, or children/young adults between ages 13-22.
Alcohol use during adolescence is associated with significantly increased risk for AUD during adulthood [4], suggesting that unique features of the adolescent brain may play a role in the lasting effects of alcohol exposure.

Adolescence is a distinct developmental period occurring during the transition from childhood to adulthood. This epoch is defined by unique behavioral and physiological characteristics that are conserved across mammalian species. Behaviorally, adolescents have been shown to exhibit increased impulsivity, risk-taking and reward-related behaviors while also displaying cognitive deficits (particularly when under emotional duress) [5]. The behavioral phenotype of adolescence arises as a result of the differing developmental trajectories of sub-regions of the brain. In both rodents and humans, adolescent brain development is driven by increases in white matter (indicative of myelination of maturing synapses) and decreases in gray matter (indicative of pruning of extraneous synaptic connections to increase synaptic efficacy) [6]. This development proceeds in an anterior-to-posterior fashion, with limbic brain regions such as the nucleus accumbens and amygdala maturing faster than forebrain regions such as the medial prefrontal cortex (mPFC) which participate in executive control of emotional behavior [7]. This mosaic of mature brain regions associated with reward, aversion and emotion and relatively immature forebrain regions that regulate these downstream regions may be responsible for the adolescent-typical behaviors exhibited by humans and rodents.

Adolescent experimentation with drugs of abuse, including alcohol, is extremely common; recent estimates in the United States indicate that 42% of high
school students routinely consume alcohol [8]. This widespread level of adolescent alcohol consumption is alarming, given that alcohol intake prior to age 21 is associated with an increase in lifetime risk for AUD ranging from 10-30% (with younger age of drinking onset associated with greater lifetime risk) [9]. Adolescents also consume more alcohol than adults in both humans and rodent models when intake is adjusted for body weight [10]. In accordance with the general behavioral manifestations of adolescent brain development, adolescents have been shown to have altered sensitivity to many of the acute effects of alcohol. Adolescents appear to be more sensitive to the rewarding and social facilitation effects of alcohol consumption but relatively insensitive to the aversive properties of alcohol [11]. Evidence also suggests that the cognitive impairment imparted by alcohol drinking may be more severe in adolescent drinkers [12]. However, the neurobiological mechanisms that mediate both altered sensitivity to alcohol during adolescence and long-term increased risk for AUDs following adolescent alcohol drinking remain unclear.

To investigate the role of adolescent brain development on sensitivity to alcohol, an unbiased high-throughput proteomics screen of the effects of alcohol on the adolescent and adult mPFC, respectively, was conducted following two weeks of intermittent 24-hour two-bottle choice drinking. The mPFC was chosen due to its clear role in regulating both alcohol drinking behavior [13] and adolescent-typical behavior [14], and previous work suggesting substantial differences in the adolescent and adult mPFC at the level of protein expression [15]. Next, to assess the functional relevance of the identified proteins in adolescent alcohol drinking, the
candidate protein calcineurin (PPP3) was selected for further manipulation. Systemic inhibition of calcineurin was achieved with the inhibitor FK506 and the effects of this drug on adolescent and adult alcohol and sucrose consumption were examined. Finally, to determine the role of calcineurin in the long-term risk for increased alcohol consumption following adolescent alcohol exposure, a model was developed using adolescent intermittent 24-hour TBC drinking followed by operant self-administration of alcohol in adulthood. Adolescent but not adult alcohol exposure produced long-lasting increases in operant responding for alcohol, which was manipulated via systemic calcineurin inhibition with FK506. To determine whether calcineurin activity in the mPFC was responsible for the effects of FK506 on operant responding for alcohol, cannulae aimed at the mPFC were implanted and drug was infused directly into the mPFC. The findings suggest that differences between the adolescent and adult response of the mPFC to alcohol, and particularly calcineurin signaling in the mPFC, may be responsible for age differences in the effects of alcohol intake and mediate some of the risk for enhanced alcohol consumption following adolescent alcohol exposure.

**Materials and Methods**

**Subjects**

Adolescent (postnatal day 21 [PND21]) and adult (PND65 ± 3) male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were single-housed upon arrival in standard laboratory cages with corn-cob bedding and a small nestlet for
environmental enrichment. The colony was maintained on a 12-hour reverse light/dark cycle (lights on at 1900). All testing occurred during the dark cycle. Food and water were available ad libitum except where noted. All procedures were performed in accordance with the NIH Guide to the Care and Use of Laboratory Animals [16] and approved by the Internal Review Board as compliant with all institutional guidelines at the University of North Carolina, Chapel Hill.

**Proteomic analysis**

*Intermittent Alcohol Drinking*  

All mice (n=12/age/dose) were given seven days to acclimate to vivarium conditions prior to testing. Beginning at 10:00AM on PND 28 (adolescent)/PND 72 (adult), the home-cage water bottle was removed and replaced with two drinking tubes; mice in the alcohol groups received one tube with 20% alcohol (v/v) and one tap water tube, whereas mice in the water groups received two water tubes. The following day, all tubes were removed and replaced with two water tubes for all subjects. This intermittent home-cage access procedure was repeated at 24-hour intervals for two weeks, from PND 28-42 (adolescent) and PND 72-86 (adult). Intake was recorded at 2, 4 and 24-hour intervals throughout the drinking period.

*Tissue collection*  

Beginning at 8:00AM on the last drinking day (PND 42/86), a time when blood alcohol levels were naturally low, all mice were weighed and deeply anesthetized with sodium pentobarbital (150 mg/kg, IP). Once anesthetized, mice were transcardially perfused with ice-cold phosphate-buffered saline (PBS, 0.1M, pH 7.4)
for 2 min at rate of 3 ml/min. Brains were flash-frozen in isopentane (2-methylbutane; Sigma-Aldrich, St. Louis, MO) at -20 – -30°C for 1 min and stored at -80°C. Tissue was sectioned coronally on a cryostat (Leica CM3050S, Leica Biosystems, Buffalo Grove, IL) and the mPFC was isolated with a 0.5 mm tissue slice taken +1.5-2.0 mm (± 0.2) anterior to Bregma. The mPFC was bilaterally dissected using a 1.0 mm sterile tissue punch. Tissue was pooled from n=3 mice per age, per dose resulting in a final group size of N=4 in each group for analysis on 4 replicate 2D gels. Protein samples were kept at -80°C and shipped to Applied Biomics (Hayward, CA) for 2-D DIGE analysis.

**Proteomics**

Tissue samples were prepared for proteomics analysis as described in Chapter Two. Briefly, tissue was homogenized in lysis buffer, spun for supernatant, and protein concentration was determined using the Bio-Rad protein assay (Hercules, CA). All samples were diluted to 5 mg/ml in 2-D lysis buffer. Samples in each dose group were combined with the appropriate CyDye (water, Cy3 [green]; alcohol, Cy5 [red]) and prepared for electrophoresis.

All 2D gels were run separately between the two ages, with alcohol and water compared within each age group only. Samples were run using isoelectric focusing (IEF) under dark conditions at 20°C using an established protocol (GE Healthcare, Pittsburgh, PA). Following IEF, the IPG strips were incubated in fresh equilibration buffer, washed and transferred to a 12% SDS gel for separation based on molecular weight (polyacramide gel electrophersis [SDS-PAGE]). Each of the four replicate gels within each age groups was scanned using a Typhoon TRIO imager.
Scanned images were analyzed by ImageQuantTL (IQTL) software (GE Healthcare) and subjected to both in-gel and cross-gel analyses using the DeCyder software package (v. 6.5, GE Healthcare), which provided a ratio change of protein expression from the in-gel analyses.

Protein spots that met our a priori spot-picking criteria (differential expression in the same direction in all four gels, an overall significant difference in expression \([p<0.05]\), and a 1.15-fold change or greater increase or decrease in expression) were isolated by the Ettan Spot Picker (GE Healthcare). Gel spots were washed and digested in-gel with a modified porcine trypsin protease (Trypsin Gold, Promega, Madison, WI) and spotted on the matrix-assisted laser desorption/ionization (MALDI) plate. Both MALDI-TOF (time-of-flight) mass spectrometry (MS) and TOF/TOF (tandem MS/MS) analyses were performed on a 5800 mass spectrometer (AB Sciex, Redwood City, CA).

Peptide masses and fragmentation spectra were submitted to the MASCOT search engine (Matrix Science, Boston, MA) via GPS Explorer (v. 3.5) in order to search the database of the National Center for Biotechnology Information non-redundant (NCBI) for protein identifications. Ion score (chance that an experimentally observed peptide sequence and MASCOT database identity are matched based on random chance \([-\log_{10}P]\)) and protein score (sum of the highest ion scores for each sequence) were calculated; putative identifications with a protein score confidence interval greater than 95% were considered significant. Samples with multiple candidate proteins exceeding the identification criteria were assigned the putative identity with the highest protein score.
Phosphorylated Protein Expression Profiling

Following the total protein expression profiling, 2D-DIGE gels were fixed overnight and stained with ProQ Diamond (Fisher) to identify phosphoproteins in the gel. Gels were rinsed and imaged with the Typhoon TRIO Imager (GE Healthcare), then each image was compared with the total protein gel obtained during the protein expression experiment. Comparisons in the phospho-protein experiment were made within a single representative gel, with the Ettan Spot Picker identifying spots with significantly different phosphorylation staining between the alcohol and water samples, respectively within each age. Selected spots were then identified using tandem MS/MS as described above. Differences in phosphorylation between alcohol and water drinking samples were quantified using an internal control spot, astrocytic phosphoprotein PEA-15 (spot 14), that did not differ between the two drinking conditions in either adolescent or adult gels. p-values were not calculated for the phosphoprotein experiments since each experiment was conducted within a single gel, rather than across four replicates as with the total protein experiment.

Bioinformatics

Protein identifiers, fold-change, and p-values from the proteomic analyses were uploaded to QIAGEN’s Ingenuity Pathway Analysis (IPA; QIAGEN Redwood City, CA) system for dataset enrichment as described in Chapter Two. Proteins were assessed via Global Functional Analysis (GFA) and Global Canonical Pathways (GCP) to identify functional protein networks and canonical signaling systems that were impacted by alcohol exposure. Statistical significance was determined using
the right-tailed Fisher’s Exact Test to assess potential overrepresentation of target proteins biofunctions, disorders and canonical signaling pathways. Protein interaction networks were derived via Ingenuity’s interconnectivity algorithm. \( p \) values, representing the probability of finding proteins identified in the proteomics analysis (Focus Molecules) in a set of \( n \) genes randomly selected from the Global Molecular Network, were calculated using Fisher’s Exact Test and displayed as \( p \)-scores \[ p\text{-score} = -\log_{10}(p\text{-value}) \]; i.e. \( p \)-score indicates the exponent of the significance of the protein network identification. Ingenuity Pathway Designer (QIAGEN) was used to visualize the statistically significant protein interaction networks revealed by GFA.

**Pharmacology**

*Acute Alcohol Drinking in Adolescence and Adulthood*

Adolescent (\( n=12 \)) and adult (\( n=12 \)) mice arrived at PND 21/65 and were housed as previously described. Beginning on PND 28/72, the home cage water bottle was removed at 10:00AM and replaced with one tube containing 20% alcohol (v/v) and one tube containing tap water for all subjects. The following day, all tubes were removed and replaced with two tubes containing tap water for all subjects. This intermittent access procedure was repeated for the duration of the experiment. During the first week of drinking, mice received habituation intraperitoneal (i.p.) injections of 20% \( \beta \)-cyclodextrin (BCD; Sigma) and intake was recorded at 2, 4 and 24-hour intervals. Beginning on PND 36 (adolescent) and PND79 (adult), all subjects received the calcineurin inhibitor FK506 (0, 0.56, 1, or 3 mg/kg) 30 minutes prior to
the alcohol drinking session in a Latin square design. Drug testing continued on PND 38/81, 40/83 and 42/85.

To determine if effects of FK506 were associated with nonspecific changes in motor function, on PND 44/83 mice were given the lowest effective dose (1 g/kg FK506 i.p.) 30 minutes prior to a 2-hour open-field locomotor activity test.

To determine whether the effects of FK506 were selective for alcohol or generalized to other reinforcing solutions, a separate cohort of adolescent (n=6) and adult (n=6) was treated identically as above except drinking solutions consisted of 1% sucrose and tap water. Beginning on PND 36/79, mice received either the lowest effective dose of FK506 (1 mg/kg i.p.) or vehicle in a randomized order. Injections were repeated on PND 38/81 in a within-subjects design.

Model of Adult Operant Self-Administration Following Adolescent or Adult Alcohol Drinking

Adolescent (n=16) and adult (n=16) mice arrived at PND21/655 and were housed as previously described. Beginning on PND 28/72, the home cage water bottle was removed at 10:00AM and replaced with one tube containing 20% alcohol (v/v) and one tube containing tap water (alcohol exposure group; n=8/age) or two tubes containing tap water (water exposure group; n=8/age). The following day, all tubes were removed and replaced with two tubes containing tap water for all subjects. Intake was recorded at 2, 4 and 24-hour intervals. This intermittent access procedure was repeated for two weeks, with the final drinking day occurring on PND 42/85. Beginning on PND 43/86, the home-cage water bottle was returned to each
cage and mice were left undisturbed for a period of six weeks, apart from weekly handling and weighing.

Beginning on PND 89 (adolescent exposure groups)/PND 134 (adult exposure groups), mice were presented with one tube containing the reinforcing solution of 9% alcohol (v/v)/2% sucrose (w/v) and one tube containing tap water for 24 hours in the home cage to familiarize the subjects with the solution. The following day, all bottles were removed and mice were water deprived for 23 hours to facilitate operant responding for the reinforcing solution. At the end of the deprivation period, beginning at 5:00PM mice began a 16-hour overnight operant self-administration session on a fixed-ratio 1 (FR-1) schedule with 0% alcohol/2% sucrose as the reinforcing solution. For the next three days, overnight operant sessions were conducted on an escalating fixed ratio schedule; beginning with FR-1, after each mouse achieved 25 reinforcements the schedule was increased to FR-2 (i.e. two lever presses yield one reinforcement), and after each subject achieved 25 reinforcements on an FR-2 schedule the schedule was increased to FR-4 (i.e. four lever presses yield one reinforcement). Once the FR-4 schedule was achieved, mice remained on this schedule for the duration of the overnight training sessions.

After the fourth overnight session, all subjects had achieved stable responding on the FR-4 schedule. Beginning on Monday of the following week, operant sessions were shortened to one hour occurring between 2:00PM-4:00PM daily Monday-Friday and all subjects responded on an FR-4 schedule. For the first week of operant testing, the concentration of alcohol in the reinforcing solution was increased daily from 0% to 3%-6%-9%-15% (while the sucrose concentration was
maintained at 2% throughout) to test the effect of age of alcohol exposure on operant responding across a range of doses. On the second week of operant testing, the alcohol concentration was reduced to 9% alcohol/2% sucrose and maintained at this concentration for the remainder of all operant experiments. To establish baseline differences in the effect of alcohol or water exposure on subsequent operant responding for sweetened alcohol in adulthood, mice continued operant sessions for 12 weeks, from PND 103-187 (adolescent exposure groups)/147-231 (adult exposure groups).

Effect of Systemic Calcineurin Inhibition on Operant Responding for Alcohol

After baseline operant responding had been established, beginning on PND 190 (adolescent exposure)/PND 233 (adult exposure) mice were given habituation injections of 20% BCD 30 minutes prior to operant sessions. A total of 8 habituation injections (2 injections/week) were administered until operant responding no longer decreased following injection. Beginning on PND 218 (adolescent exposure)/261 (adult exposure), subjects were injected with 0, 0.56 or 1 mg/kg FK506 i.p. in a Latin square design 30 minutes prior to operant testing. Mice were injected over the course of two weeks, with at least two days between each injection.

Effect of Intra-mPFC Microinjection of FK506 on Operant Responding for Alcohol

Following systemic pharmacology, mice in the adult exposure group were approaching PND 300 and were therefore transitioning into a geriatric state of development. Therefore, the remainder of the experiments were conducted in the adolescent alcohol or water exposure groups only.
Two weeks after the cessation of systemic pharmacology experiments, beginning on PND 246 mice were anesthetized with 3% isoflurane (Vet One, Boise, ID) placed within a stereotaxic frame (Kopf Instruments, Tujunga, CA) and implanted with unilateral 26-gauge guide cannulae (Plastics One, Roanoke, VA) aimed at the right or left mPFC (approximate coordinates: AP +1.7mm; ML ±0.4mm; DV −1.2mm, from skull surface.) Cannulae were secured to the skull with dental cement (Durelon, Butler Schein, Dublin, OH) and 33-gauge obturators (Plastics One) were inserted. All subjects were given one week for recovery, during which each mouse was weighed daily, monitored for health problems, and administered ibuprofen (15 mg/kg i.g.) for analgesia.

The following week, all subjects resumed operant responding for 9% alcohol/2% sucrose on an FR-4 schedule until operant responding returned to baseline for each subject. Once operant responding had stabilized, mice were given two sham injections and one habituation injection of 20% BCD to familiarize them with the injection procedure. After responding re-stabilized, mice were injected with either 20% BCD or FK506 (1 µg) immediately prior to the operant session.

**Materials**

*Home-Cage Drinking*

All drinking solutions (alcohol, sucrose and tap water) were presented to mice within tubes constructed from 10mL serological pipettes fitted with double ball-bearing sippers. Customized ventilation mouse cage lids fitted with screws were used to fasten the tube securely to the lid. Each drinking experiment included two
empty cages with tubes of each fluid type to control for leakage. The amount leaked each day was averaged across all drinking days at the end of the testing period and subtracted from all mouse intake values (within solution).

**Operant Self-Administration**

Self-administration sessions were conducted in 16 operant conditioning chambers (Med Associates, St. Albans, VT). Each operant chamber was housed within a sound-attenuating cubicle with a fan for ventilation and ambient noise suppression. Both lateral walls of each chamber contained a stainless steel lever with a cue light directly above and a drinking trough immediately adjacent. Responding on only one of the levers (the “active” lever) resulted in the delivery of a 0.14 mL 9% alcohol (v/v)/2% sucrose (w/v) reinforcement via a connected syringe and pump, as well as the illumination of the cue light, whereas responding on the second lever (the “inactive” lever) had no contingency. Responses during reinforcement delivery (800ms) were measured but did not contribute towards the response requirement (time-out period). Each chamber was connected to an interface and computer that recorded lever presses (active and inactive), number of reinforcements delivered and head entries into the drinking trough for each subject (MED-PC for Windows v.4.1).

**Locomotor Activity**

Open field activity was measured in Plexiglas activity monitor chambers (27.9 cm²; ENV-510, Med Associates, Georgia, VT). Two sets of 16 pulse-modulated infrared photobeams were located on opposite walls and recorded X–Y ambulatory
movements. Distance traveled (in meters) throughout the session was quantified by assessing the mouse’s position in the open field every 100 milliseconds. Data from each chamber were collected by a computer.

Drugs

Home cage alcohol drinking solutions were prepared by diluting 95% ethanol (Pharmco Products Inc., Brookfield, CT) with tap water (v/v). Home cage sucrose drinking solutions were prepared by dissolving sucrose in tap water (w/v). Operant reinforcing solutions were prepared by diluting 95% ethanol with tap water (v/v) and dissolving sucrose in the resulting solution (w/v). Sodium pentobarbital was freshly dissolved in 0.9% saline immediately prior to tissue collection. The calcineurin inhibitor FK506 was added to 20% β-cyclodextrin (BCD), sonicated and spun at room temperature to create a fine suspension. Doses were determined based on Beresford et al. 2012 [17]. Isoflurane was administered via inhalation through a nosecone attached to the stereotax and delivered at a flow rate of 0.05-0.08 L/min throughout the surgical procedures, beginning with 3% isoflurane/97% oxygen for anesthesia induction, 2% for drilling and cannula placement, and 1.5% for cementing and suturing.

Data Analysis

All statistical analyses and graphical representation of behavioral data were performed using GraphPad Prism (version 6.0; GraphPad, La Jolla, CA).

In the home-cage drinking experiments, adolescent and adult alcohol and water drinking, respectively, and alcohol preference ratio were compared via two-
way ANOVA (age X time). Blood alcohol levels were compared via two-tailed t-test (adolescent vs. adult). The effects of the calcineurin inhibitor FK506 on home-cage alcohol drinking were assessed via two-way repeated measures ANOVA (Dose X Time) separately between adolescents and adults, with Dunnett’s multiple comparisons test as the post-hoc analysis. The effects of FK506 on home-cage sucrose self-administration were assessed via two-tailed t-tests separately between adolescents and adults. The effects of FK506 on open-field locomotor activity were analyzed via two-way repeated measures ANOVA (dose X time) separately between adolescents and adults. The effects of FK506 on total distance traveled were analyzed via two-tailed t-test separately between adolescents and adults. One adolescent mouse was excluded from the alcohol drinking analysis due to failure to consume alcohol (average 24hr alcohol consumption < 3 g/kg).

In the operant experiments, operant responding for alcohol was averaged for each subject per week and analyzed via two-way repeated measures ANOVA (water vs. alcohol exposure, separately between adolescent and adult exposure). Adolescent and adult alcohol exposure mice were also directly compared across an increasing alcohol concentration curve via two-way repeated measures ANOVA (exposure age X dose). The effects of FK506 on operant responding for alcohol were assessed via two-way repeated measures ANOVA (exposure condition [alcohol vs. water] X FK506 dose) separately between adolescent exposure and adult exposure groups, with Sidak’s multiple comparisons test as the post-hoc analysis. The effects of mPFC microinjection of FK506 on operant responding for
alcohol were analyzed via two-tailed t-test separately between alcohol and water exposure groups.

Results

Proteomics

Two-Bottle Choice Alcohol Drinking during Adolescence and Adulthood

Figure 9A depicts a timeline of the alcohol exposure procedure. Adolescent and adult mice consumed roughly equivalent amounts of alcohol (Figure 9B) and water (data not shown) over the two weeks of intermittent two-bottle choice drinking. A main effect of Day emerged ($F[6, 132] = 19.03, p < 0.0001$), with both adolescents and adults increasing their intake of alcohol over time. A trend for an Age X Day interaction emerged ($p = 0.10$), likely driven by the increase in alcohol intake exhibited by adolescent mice on the last two days of alcohol drinking. Average intake on the last drinking day was 31 g/kg/24hr for adolescents and 25 g/kg/24hr for adults. Analysis of alcohol preference revealed an Age X Day interaction, $F(6, 132) = 2.549, p < 0.05$. 
### Table A

<table>
<thead>
<tr>
<th>Days</th>
<th>Acclimation</th>
<th>Exposure</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
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<td>Acclimation</td>
<td>Exposure</td>
<td>Day 21</td>
</tr>
<tr>
<td>P21, 65</td>
<td>P28, 72</td>
<td>P42, 85</td>
<td></td>
</tr>
</tbody>
</table>

**Exposure**: 20% EtOH or H2O

### Graph B

**20% Alcohol Intake (g/kg/24 Hour)**

- **Adolescent**
- **Adult**

### Graph C

**Alcohol Preference % Total Fluid Intake**

### Graph D

**Blood Alcohol Concentration (mg/dL)**

- **Adolescent**
- **Adult**

### Graph E

**Blood Alcohol Concentration (mg/dL)**

- **Adolescent**
- **Adult**
Figure 9. Two-bottle choice home cage drinking in adolescent and adult male C57BL/6J mice. (A) Timeline of experimental procedure. (B) Adolescent and adult mice consumed similar amounts of 20% unsweetened alcohol in the 24-hour, every-other-day two bottle choice procedure. (C) Adults displayed greater preference for alcohol on the first two days of drinking, after which both age groups exhibited similarly high preference for alcohol. (D) Blood alcohol levels obtained during tissue collection were equally low in both adolescent and adult mice. (E) Blood alcohol levels obtained from a separate cohort of mice treated identically to the proteomics animals were similarly high in both adolescent and adult subjects. (* indicates $p <0.05$)

Sidak’s multiple comparisons test indicated that on the first two days of alcohol drinking, adult mice displayed increased preference for alcohol relative to adults (Day 1, $p <0.05$; Day 3, $p < 0.0001$; Figure 9C). Adolescent preference subsequently rose to match adult preferences on Day 5 and remained high for the duration of testing; adolescent preference on the last drinking day was 77% and adult preference was 83%. Blood alcohol samples obtained during tissue collection following the last drinking session revealed that both adolescent and adult BACs were minimal at the time of brain extraction (adolescent average BAC = 7.2 mg/dL, adult average BAC = 10.7 mg/dL; Figure 9D), and no age differences in BAC emerged ($p >0.05$). Blood samples from a separate cohort of mice run identically to the proteomics subjects were collected two hours into the drinking session on the last day of drinking. These samples confirmed that the two-bottle choice drinking procedure resulted in intoxicating levels of alcohol consumption, but no age
differences in BAC emerged from this cohort (adolescent average BAC = 149 mg/DL, adult average BAC = 152 mg/dL, \(p > 0.05\); Figure 9E). \textit{Proteomics Analysis}

To identify proteins within the adolescent and adult mPFC, respectively, that were sensitive to alcohol drinking in terms of their expression, following two weeks of home-cage two-bottle choice alcohol or water drinking brains were collected and mPFC tissue was dissected for 2D-DIGE and subsequent mass spectrometry. The automated Ettan Spot Picker identified 43 spots with differential expression between the adolescent water and adolescent alcohol gels, of which 24 met the \textit{a priori} inclusion criteria (Figure 10A). All 24 spots were identified using tandem MS/MS (Table 3).

The automated Spot Picker identified 44 spots with differential expression between the adult water and adult alcohol gels, of which 16 met the \textit{a priori} inclusion criteria (Figure 11A), and all 16 spots were identified using tandem MS/MS (Table 4).

Data in both age groups were analyzed as alcohol / water expression, with positive fold change representing increased protein expression following alcohol drinking and negative fold change representing decreased protein expression following alcohol drinking.
Figure 10. Total protein and phosphoprotein 2D-DIGE gels of the adolescent mouse mPFC.

(A) Adolescent total protein 2D-DIGE gel. Green CyDye labels water samples, red CyDye labels alcohol samples, and the predominantly yellow image shows the merge of the alcohol and water digital images. Significantly different spots are indicated in white on the merged image. Proteins are separated by IEF on the X axis and molecular weight on the Y axis. (B) Adolescent phosphoprotein staining for water (left panel) and alcohol (right panel) drinking. In each composite image, red staining indicates phosphoproteins and green indicates total protein imaged from the total protein experiment (i.e. Figure 10A).

Table 3. 24 proteins with differential expression following two weeks of intermittent alcohol drinking in the adolescent mPFC.

<table>
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<tr>
<th>Protein Name</th>
<th>Protein ID</th>
<th>Spot #</th>
<th>Peptide Count</th>
<th>Protein Score</th>
<th>Relative Change</th>
<th>p-value</th>
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</thead>
<tbody>
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<td>CB1 cannabinoid receptor-interacting protein 1</td>
<td>CRIP1A</td>
<td>14</td>
<td>9</td>
<td>199</td>
<td>-2.16</td>
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<td>Calreticulin</td>
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<td>ACTB</td>
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<td>PEX19</td>
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<td>4</td>
<td>75</td>
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<td>0.0018</td>
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<td>23</td>
<td>5</td>
<td>199</td>
<td>1.32</td>
<td>0.0024</td>
</tr>
<tr>
<td>Dynactin subunit 3</td>
<td>DCTN3</td>
<td>11</td>
<td>5</td>
<td>199</td>
<td>1.35</td>
<td>0.0025</td>
</tr>
<tr>
<td>Cofilin-1</td>
<td>CFL1</td>
<td>13</td>
<td>5</td>
<td>199</td>
<td>1.38</td>
<td>0.072</td>
</tr>
<tr>
<td>Gamma-enolase</td>
<td>ENO2</td>
<td>2</td>
<td>13</td>
<td>173</td>
<td>1.4</td>
<td>0.087</td>
</tr>
<tr>
<td>Cytochrome c oxidase subunit 6B1</td>
<td>COX6B</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>&lt;</td>
</tr>
<tr>
<td>Complexin-2</td>
<td>CPLX2</td>
<td>18</td>
<td>8</td>
<td>199</td>
<td>1.47</td>
<td>0.026</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>GAPDH</td>
<td>9</td>
<td>11</td>
<td>199</td>
<td>2.37</td>
<td>0.045</td>
</tr>
</tbody>
</table>

**Table 3.** 24 spots showed ≥15% difference in expression in all 4 adolescent 2D-DIGE gels (p<0.05). Each spot was identified via MALDI TOF/TOF mass spectrometry with a confidence of 1.0. Spot change was expressed as fold change of alcohol from water, with negative numbers reflecting reduced expression in alcohol relative to water and positive numbers indicating greater expression in alcohol relative to water. Table is presented in order of lowest expression in the alcohol group to highest expression in the alcohol group.
ADULT

A

B

H₂O Phosphoproteins

EtOH Phosphoproteins
Figure 11. Total protein and phosphoprotein 2D-DIGE gels of the adult mouse mPFC.

(A) Adult total protein 2D-DIGE gel. Green CyDye labels water samples, red CyDye labels alcohol samples, and the predominantly yellow image shows the merge of the alcohol and water digital images. Significantly different spots are indicated in white on the merged image. Proteins are separated by IEF on the X axis and molecular weight on the Y axis. (B) Adult phosphoprotein staining for water (left panel) and alcohol (right panel) drinking. In each composite image, red staining indicates phosphoproteins and green indicates total protein imaged from the total protein experiment (i.e. Figure 11A).

Table 4. 16 proteins with differential expression following two weeks of alcohol drinking in the adult mPFC.

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Protein ID</th>
<th>Spot #</th>
<th>Peptide Count</th>
<th>Protein Score</th>
<th>Relative Change</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bis(5'-nucleosyl)-tetraphosphatase</td>
<td>NUDT2</td>
<td>10</td>
<td>3</td>
<td>85</td>
<td>-1.7</td>
<td>0.0038</td>
</tr>
<tr>
<td>Clathrin light chain A</td>
<td>CLTA</td>
<td>3</td>
<td>9</td>
<td>283</td>
<td>-1.52</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Myosin light polypeptide 6</td>
<td>MYL6</td>
<td>11</td>
<td>9</td>
<td>234</td>
<td>-1.52</td>
<td>0.00077</td>
</tr>
<tr>
<td>Myosin light polypeptide 6</td>
<td>MYL6</td>
<td>13</td>
<td>6</td>
<td>158</td>
<td>-1.46</td>
<td>0.00062</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>CALM1</td>
<td>5</td>
<td>3</td>
<td>145</td>
<td>-1.39</td>
<td>0.0033</td>
</tr>
<tr>
<td>Clathrin light chain A</td>
<td>CLTA</td>
<td>2</td>
<td>10</td>
<td>331</td>
<td>-1.35</td>
<td>0.07</td>
</tr>
<tr>
<td>Myosin light polypeptide 6</td>
<td>MYL6</td>
<td>12</td>
<td>8</td>
<td>175</td>
<td>-1.35</td>
<td>0.011</td>
</tr>
<tr>
<td>Cofilin-2</td>
<td>CFL2</td>
<td>8</td>
<td>8</td>
<td>337</td>
<td>-1.32</td>
<td>0.0046</td>
</tr>
<tr>
<td>Calreticulin</td>
<td>CALR</td>
<td>1</td>
<td>20</td>
<td>738</td>
<td>-1.28</td>
<td>0.02</td>
</tr>
<tr>
<td>Cytochrome c oxidase subunit 6B1</td>
<td>COX6B1</td>
<td>16</td>
<td>6</td>
<td>330</td>
<td>1.2</td>
<td>0.0036</td>
</tr>
<tr>
<td>Protein Phosphorylation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4. 16 spots showed ≥15% difference in expression in all 4 adult 2D-DIGE gels ($p<0.05$). Each spot was identified via MALDI TOF/TOF mass spectrometry with a confidence of 1.0. Spot change was expressed as fold change of alcohol from water, with negative numbers reflecting reduced expression in alcohol relative to water and positive numbers indicating greater expression in alcohol relative to water. Table is presented in order of lowest expression in the alcohol group to highest expression in the alcohol group.

<table>
<thead>
<tr>
<th>Protein Phosphorylation</th>
</tr>
</thead>
</table>

Protein Phosphorylation

To determine the effects of alcohol drinking on protein phosphorylation in the adolescent and adult mPFC, the same gels used in the total protein expression assays were stained for phosphorylation and imaged. The automated Ettan Spot Picker identified 13 spots in the adolescent gels (Figure 10B) and 34 spots in the adult gels (Figure 11B) that exhibited differential phosphorylation between the alcohol and water conditions. All 47 spots were identified using tandem MS/MS (Table 5 [Adolescent] and Table 6 [Adult]).
Table 5. 14 spots with differential phosphorylation following two weeks of alcohol drinking in the adolescent mPFC.

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Protein ID</th>
<th>Spot #</th>
<th>Peptide Count</th>
<th>Protein Score</th>
<th>Relative Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myc box-dependent-interacting protein 1</td>
<td>BIN1</td>
<td>B</td>
<td>5</td>
<td>80</td>
<td>-2.24</td>
</tr>
<tr>
<td>Calcineurin subunit alpha</td>
<td>PPP3A</td>
<td>C</td>
<td>20</td>
<td>409</td>
<td>-0.55</td>
</tr>
<tr>
<td>Tubulin alpha-1B chain</td>
<td>TBA1B</td>
<td>4</td>
<td>22</td>
<td>623</td>
<td>0.76</td>
</tr>
<tr>
<td>Phosphatidylethanolamine binding protein 1</td>
<td>PEBP1</td>
<td>10</td>
<td>8</td>
<td>67</td>
<td>1.18</td>
</tr>
<tr>
<td>Stathmin</td>
<td>STMN1</td>
<td>11</td>
<td>7</td>
<td>86</td>
<td>1.22</td>
</tr>
<tr>
<td>Neurabin-2</td>
<td>NEB2</td>
<td>1</td>
<td>17</td>
<td>136</td>
<td>1.28</td>
</tr>
<tr>
<td>Heterogeneous nuclear ribonucleoprotein K</td>
<td>HNRPK</td>
<td>2</td>
<td>20</td>
<td>457</td>
<td>1.42</td>
</tr>
<tr>
<td>Guanine nucleotide-binding protein G(o) subunit alpha</td>
<td>GNAO</td>
<td>5</td>
<td>17</td>
<td>772</td>
<td>1.54</td>
</tr>
<tr>
<td>Alpha-soluble NSF attachment protein</td>
<td>SNAA</td>
<td>8</td>
<td>25</td>
<td>590</td>
<td>1.63</td>
</tr>
<tr>
<td>NADH dehydrogenase 1 alpha subcomplex subunit 8</td>
<td>NDUA8</td>
<td>13</td>
<td>12</td>
<td>409</td>
<td>2.27</td>
</tr>
<tr>
<td>Spermatogenesis-associated protein 22</td>
<td>SPT22</td>
<td>12</td>
<td>5</td>
<td>42</td>
<td>2.43</td>
</tr>
<tr>
<td>Drebrin</td>
<td>DREB</td>
<td>50</td>
<td>14</td>
<td>323</td>
<td>2.67</td>
</tr>
<tr>
<td>Aspartate aminotransferase, mitochondrial</td>
<td>AATM</td>
<td>7</td>
<td>23</td>
<td>588</td>
<td>4.49</td>
</tr>
<tr>
<td>Actin, cytoplasmic 2</td>
<td>ACTG</td>
<td>9</td>
<td>13</td>
<td>71</td>
<td>5.17</td>
</tr>
<tr>
<td>Astrocytic phosphoprotein PEA-15</td>
<td>PEA15</td>
<td>14</td>
<td>9</td>
<td>458</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Table 5. 14 spots showed ≥15% difference in expression in one adolescent 2D-DIGE gel (p<0.05). Each spot was identified via MALDI TOF/TOF mass spectrometry with a confidence of 1.0. Spot change was expressed as fold change of alcohol from water, with negative numbers reflecting reduced
expression in alcohol relative to water and positive numbers indicating greater
expression in alcohol relative to water. Table is presented in order of lowest
expression in the alcohol group to highest expression in the alcohol group.

*Italics* indicate the protein PEA15, which was used as an internal standard for
relative change determination based on lack of change between alcohol and
water conditions (fold change 1.00).

Table 6. 34 spots with differential phosphorylation following two weeks of
alcohol drinking in the adult mPFC.

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Protein ID</th>
<th>Spot #</th>
<th>Peptide Count</th>
<th>Protein Score</th>
<th>Relative Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>14-3-3 protein zeta/delta</td>
<td>1433Z</td>
<td>J</td>
<td>19</td>
<td>792</td>
<td>-2.41</td>
</tr>
<tr>
<td>Calcium/calmodulin-dependent protein kinase type II subunit alpha</td>
<td>KCC2A</td>
<td>G</td>
<td>14</td>
<td>116</td>
<td>-1.91</td>
</tr>
<tr>
<td>Calcium/calmodulin-dependent protein kinase type II subunit alpha</td>
<td>KCC2A</td>
<td>H</td>
<td>8</td>
<td>82</td>
<td>-1.27</td>
</tr>
<tr>
<td>Calcium/calmodulin-dependent protein kinase type II subunit alpha</td>
<td>KCC2A</td>
<td>I</td>
<td>21</td>
<td>687</td>
<td>-0.57</td>
</tr>
<tr>
<td>Actin, cytoplasmic 2</td>
<td>ACTG</td>
<td>33</td>
<td>16</td>
<td>336</td>
<td>-0.41</td>
</tr>
<tr>
<td>14-3-3 protein gamma</td>
<td>1433G</td>
<td>K</td>
<td>6</td>
<td>62</td>
<td>-0.39</td>
</tr>
<tr>
<td>Enolase-phosphatase E1</td>
<td>ENOPH</td>
<td>41</td>
<td>9</td>
<td>263</td>
<td>-0.36</td>
</tr>
<tr>
<td>Dihydropyrimidinase-related protein 2</td>
<td>DPYL2</td>
<td>27</td>
<td>32</td>
<td>829</td>
<td>-0.06</td>
</tr>
<tr>
<td>Myc box-dependent-interacting protein 1</td>
<td>BIN1</td>
<td>25</td>
<td>28</td>
<td>467</td>
<td>0.04</td>
</tr>
<tr>
<td>Calcineurin subunit alpha</td>
<td>PP2BA</td>
<td>28</td>
<td>22</td>
<td>236</td>
<td>0.15</td>
</tr>
<tr>
<td>Tubulin alpha-1A chain</td>
<td>TBA1A</td>
<td>29</td>
<td>16</td>
<td>330</td>
<td>0.23</td>
</tr>
<tr>
<td>UMP-CMP kinase</td>
<td>KCY</td>
<td>46</td>
<td>13</td>
<td>382</td>
<td>0.25</td>
</tr>
<tr>
<td>Sushi repeat-containing protein SRPX2</td>
<td>SRPX2</td>
<td>42</td>
<td>15</td>
<td>36</td>
<td>0.26</td>
</tr>
<tr>
<td>Creatine kinase B-type</td>
<td>KCRB</td>
<td>32</td>
<td>14</td>
<td>158</td>
<td>0.26</td>
</tr>
<tr>
<td>Serine racemase</td>
<td>SRR</td>
<td>36</td>
<td>11</td>
<td>281</td>
<td>0.30</td>
</tr>
<tr>
<td>Dynactin subunit 3</td>
<td>DCTN3</td>
<td>47</td>
<td>7</td>
<td>223</td>
<td>0.34</td>
</tr>
<tr>
<td>Transcriptional activator protein Pur-alpha</td>
<td>PURA</td>
<td>35</td>
<td>8</td>
<td>336</td>
<td>0.37</td>
</tr>
<tr>
<td>Protein Name</td>
<td>Gene Symbol</td>
<td>Spot Value</td>
<td>Fold Change</td>
<td>Z-Score</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
<td>------------</td>
<td>-------------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td>Amphiphysin</td>
<td>AMPH</td>
<td>22</td>
<td>585</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>Actin, cytoplasmic 2</td>
<td>ACTG</td>
<td>34</td>
<td>529</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>Pleckstrin homology-like domain family A member 1</td>
<td>PHLA1</td>
<td>45</td>
<td>39</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>Serine/threonine-protein phosphatase PP1-alpha catalytic subunit</td>
<td>PP1A</td>
<td>37</td>
<td>513</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2</td>
<td>GBB2</td>
<td>38</td>
<td>551</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td>Malate dehydrogenase, cytoplasmic</td>
<td>MDHC</td>
<td>39</td>
<td>242</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td>Actin-related protein 3</td>
<td>ARP3</td>
<td>31</td>
<td>650</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td>Neurofilament medium polypeptide</td>
<td>NFM</td>
<td>21</td>
<td>772</td>
<td>0.79</td>
<td></td>
</tr>
<tr>
<td>S-phase kinase-associated protein 1</td>
<td>SKP1</td>
<td>48</td>
<td>379</td>
<td>0.82</td>
<td></td>
</tr>
<tr>
<td>Actin, cytoplasmic 1</td>
<td>ACTB</td>
<td>43</td>
<td>179</td>
<td>0.83</td>
<td></td>
</tr>
<tr>
<td>Gamma-enolase</td>
<td>ENOG</td>
<td>30</td>
<td>349</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td>Microtubule-associated protein RP/EB family member 1</td>
<td>MARE1</td>
<td>40</td>
<td>347</td>
<td>0.90</td>
<td></td>
</tr>
<tr>
<td>78 kDa glucose-regulated protein</td>
<td>GRP78</td>
<td>26</td>
<td>951</td>
<td>1.06</td>
<td></td>
</tr>
<tr>
<td>Heat shock protein HSP 90-beta</td>
<td>HS90B</td>
<td>23</td>
<td>732</td>
<td>1.64</td>
<td></td>
</tr>
<tr>
<td>Sepiapterin reductase</td>
<td>SPRE</td>
<td>44</td>
<td>181</td>
<td>2.11</td>
<td></td>
</tr>
<tr>
<td>Heat shock protein HSP 90-beta</td>
<td>HS90B</td>
<td>24</td>
<td>726</td>
<td>2.53</td>
<td></td>
</tr>
<tr>
<td>ATP synthase subunit delta</td>
<td>ATPD</td>
<td>49</td>
<td>167</td>
<td>3.60</td>
<td></td>
</tr>
<tr>
<td>Astrocytic phosphoprotein PEA-15</td>
<td>PEA15</td>
<td>14</td>
<td>458</td>
<td>1.00</td>
<td></td>
</tr>
</tbody>
</table>

**Table 6.** 34 spots showed ≥15% difference in expression in one adult 2D-DIGE gel (p<0.05). Each spot was identified via MALDI TOF/TOF mass spectrometry with a confidence of 1.0. Spot change was expressed as fold change of alcohol from water, with negative numbers reflecting reduced expression in alcohol relative to water and positive numbers indicating greater expression in alcohol relative to water. Table is presented in order of lowest expression in the alcohol group to highest expression in the alcohol group. *Italics* indicate the protein PEA15, which was used as an internal standard for relative expression.
change determination based on lack of change between alcohol and water conditions (fold change 1.00).

Data in both groups were expressed as a fold change of alcohol / water phosphorylation, with fold changes > 1.0 indicating increased phosphorylation following alcohol drinking and ratios < 1.0 indicating decreased phosphorylation following alcohol drinking (Tables 2 and 3).

**Bioinformatics**

*Ingenuity Global Functional Analysis and Global Canonical Pathway Analysis.*

All proteins identified in the total protein expression analysis in both age groups were separately uploaded to Ingenuity Pathway Analysis for Global Functional Analysis (GFA) and Global Canonical Pathway (GCP) assessment. Among adolescent mice, GFA indicated that two weeks of intermittent alcohol drinking was associated with changes in several major cellular processes, including cellular signaling, cellular assembly, cellular function, nervous system development, neurological disease and behavior (Table 7). Five canonical pathways, including actin cytoskeletal signaling, were predicted to be impacted by the drinking procedure in adolescents, with key upstream regulators IGF2BP1, MECP2, FMR1, ADORA2A and PPARG.

In adult mice, two weeks of intermittent alcohol drinking was associated with changes in cellular signaling, function and assembly only (Table 8). Four canonical pathways, including Ephrin B signaling, were predicted to be
impacted by the drinking procedure in adolescents, with the key upstream regulator PPARG.

Table 7. Role of proteins identified in the adolescent mPFC in biofunctions, disorders and canonical signaling pathways.

<table>
<thead>
<tr>
<th>Function/Disorder</th>
<th>p-value</th>
<th>Higher in water</th>
<th>Higher in alcohol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell-to-Cell Signaling and Interaction</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neurotransmission</td>
<td>0.00112</td>
<td>PPP3R1, SNCB</td>
<td>CPLX2</td>
</tr>
<tr>
<td>Synaptic transmission</td>
<td>0.0123</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Excitatory postsynaptic potential of neurons</td>
<td>0.00748</td>
<td>CPLX2</td>
<td></td>
</tr>
<tr>
<td>Excitatory postsynaptic potential of cerebral cortex</td>
<td>0.003</td>
<td>CPLX2</td>
<td></td>
</tr>
<tr>
<td>Miniature excitatory postsynaptic currents</td>
<td>0.003</td>
<td>CPLX2</td>
<td></td>
</tr>
<tr>
<td>Long-term potentiation of mossy fibers</td>
<td>0.00897</td>
<td>CPLX2</td>
<td></td>
</tr>
<tr>
<td>Long-term depression</td>
<td>0.0119</td>
<td>PPP3R1, CFL1</td>
<td></td>
</tr>
<tr>
<td>Long-term depression of hippocampus CA1</td>
<td></td>
<td></td>
<td>CFL1</td>
</tr>
<tr>
<td>Delay in initiation of calcium clearance</td>
<td>0.0045</td>
<td>CFL1</td>
<td></td>
</tr>
<tr>
<td><strong>Cellular Assembly/Organization &amp; Development</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transport of synaptic vesicles</td>
<td>0.000972</td>
<td>SNCB</td>
<td>CPLX2</td>
</tr>
<tr>
<td>Exocytosis of synaptic vesicles</td>
<td>0.0149</td>
<td>CPLX2</td>
<td></td>
</tr>
<tr>
<td>Size of excitatory synapses</td>
<td>0.0045</td>
<td>SNCB</td>
<td>CPLX2</td>
</tr>
<tr>
<td>Priming of vesicles</td>
<td>0.00208</td>
<td></td>
<td>CPLX2</td>
</tr>
<tr>
<td>Quantity of nerve endings</td>
<td>0.00282</td>
<td>SNCB</td>
<td></td>
</tr>
<tr>
<td><strong>Behavior</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Learning</td>
<td>0.00501</td>
<td>PPP3R1</td>
<td>CFL1</td>
</tr>
<tr>
<td>Aversion learning</td>
<td>0.0015</td>
<td>CFL1</td>
<td></td>
</tr>
<tr>
<td>Reward learning</td>
<td>0.0015</td>
<td>CFL1</td>
<td></td>
</tr>
<tr>
<td>Object localization task learning</td>
<td>0.0015</td>
<td>CFL1</td>
<td></td>
</tr>
</tbody>
</table>
Working memory

**Cellular Function & Molecular Transport**
- Calcium-triggered exocytosis
- Exocytosis by neurons

**Nervous System Development**
- Development of Schwann cells
- Neurogenesis of dentate gyrus
- Abnormal morphology of striatum

**Neurological Disease**
- Huntington’s Disease
- Progressive high-frequency hearing impairment

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### Canonical Pathways and Upstream Regulators

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**Table 7.** Adolescent proteomics results were analyzed via Ingenuity Pathway Analysis for known interactions with other proteins, signaling systems and networks in the Ingenuity Knowledge Base. Proteins with decreased expression after alcohol drinking are shown on the right, and proteins with
increased expression after alcohol drinking are shown on the left. *p*-values were derived from Ingenuity Pathway Analysis by right-tailed Fisher exact test and indicate relative overrepresentation of proteins in a given function compared with what is expected by chance.

### Table 8. Role of proteins identified in the adult mPFC in biofunctions, disorders and canonical signaling pathways.

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<td><strong>Canonical Pathways and Upstream Regulators</strong></td>
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Table 8. Adult proteomics results were analyzed via Ingenuity Pathway Analysis for known interactions with other proteins, signaling systems and networks in the Ingenuity Knowledge Base. Proteins with decreased expression after alcohol drinking are shown on the right, and proteins with increased expression after alcohol drinking are shown on the left. p-values were derived from Ingenuity Pathway Analysis by right-tailed Fisher exact test and indicate relative overrepresentation of proteins in a given function compared with what is expected by chance.

Ingenuity Pathway Analysis

Ingenuity Pathway Analysis was performed to identify protein networks statistically predicted to be impacted by the effects of alcohol in the adolescent and adult mPFC, respectively. In the adolescent mPFC, four statistically significant protein interaction networks were predicted to have altered function following alcohol drinking. The most significant interaction network was associated with behavior, cell-to-cell signaling and interaction, and nervous system development/function and contained the identified proteins PPP3R1, ACTB, GAPDH, CYB5A and CFL1 (Figure 12).

In the adult mPFC, two statistically significant protein interaction networks were found to be impacted by alcohol drinking. The most significant network was associated with neurological disease, organismal injury/abnormality and cellular
compromise and contained the identified proteins CFL2, CLTA and CPLX2 (Figure 13).

Figure 12. Adolescent alcohol drinking impacts a functional protein network involved in behavior, cell-to-cell signaling/interaction and nervous system development/function. Visualization of a protein interaction network in the adolescent mPFC identified by Ingenuity Pathway Analysis as being altered by alcohol drinking (p-score= 9). Proteins shown in green exhibited decreased expression in the alcohol drinking tissue, proteins in green exhibited increased expression in the alcohol drinking tissue, and proteins in yellow indicate statistically significant interaction proteins identified by IPA network analysis. Solid lines indicate a direct interaction, and dashed
lines indicate an indirect interaction mediated by additional, non-significant proteins.

Figure 13. Adult alcohol drinking impacts a functional protein network involved in neurological disease, organismal injury/abnormality and cellular compromise. Visualization of a protein interaction network in the adult mPFC identified as being altered by alcohol drinking (p-score = 8). Proteins shown in green exhibited decreased expression in the alcohol drinking tissue, proteins in green exhibited increased expression in the alcohol drinking tissue, and proteins in yellow indicate statistically significant interaction proteins identified by IPA network analysis. Solid lines indicate a direct interaction, and dashed lines indicate an indirect interaction mediated by additional, non-significant proteins.
Identification of Calcineurin in the Adolescent and Adult mPFC

The protein calcineurin (PPP3R1) was identified as having alcohol-sensitive total protein expression in the adolescent mPFC. Calcineurin appeared in two spots on the adolescent gels, Spot #21 (Figure 14A) and Spot #22 (Figure 14B). At Spot #21, the expression of total calcineurin protein was decreased by 51% following alcohol drinking (Figure 14A). At Spot #22, the expression of total calcineurin protein was decreased by 24% following alcohol drinking (Figure 14B). Changes in calcineurin expression were not detected in the adult alcohol/water gels. In the phosphorylated protein assay, calcineurin was detected in both the adolescent and adult alcohol / water comparisons, respectively. In the adult gel, calcineurin phosphorylation was increased by 15% following alcohol drinking (Figure 14C, left gray bar). In the adolescent gel, calcineurin phosphorylation was decreased by 55% following alcohol drinking (Figure 14C, right blue bar). Based on the age differences in alcohol sensitivity to both protein expression and protein phosphorylation, calcineurin was selected as a focus protein for further pharmacological assessment in adolescent and adult mice.
Figure 14. Alcohol drinking reduces calcineurin expression and phosphorylation in the adolescent but not adult mPFC. (A) At Spot #21 in the adolescent mPFC gel, calcineurin expression was reduced by 51% in alcohol drinking mice compared with water. (B) At Spot #22 in the adolescent mPFC gel, calcineurin expression was reduced by 24% in alcohol drinking mice compared with water. (C) In the adult mPFC, calcineurin phosphorylation was increased by 15% in alcohol drinking mice compared with water (left gray bar). In the adolescent mPFC, calcineurin phosphorylation was decreased by 55% in alcohol drinking mice relative to water drinking mice (right blue bar). (** indicates $p < 0.01$)
Role of Calcineurin Signaling in Alcohol Drinking

Effects of FK506 on Adolescent and Adult Two-Bottle Choice Drinking

To determine the contributions of calcineurin signaling to adolescent and adult alcohol drinking, the selective calcineurin inhibitor FK506 was administered 30 minutes prior to a 24-hour intermittent alcohol drinking procedure in adolescent and adult mice. Figure 7A depicts a timeline of the experimental procedure. Overall, the 24-hour intake data displayed a trend for increased adolescent alcohol consumption relative to adults (adolescent average intake = 19 g/kg, adult average intake = 15 g/kg, \( p = 0.09 \); data not shown.) Analysis of adolescent alcohol intake data revealed main effects of Dose (\( F[3, 40] = 6.912, p < 0.001 \)) and Time (\( F[1, 40] = 43.60, p < 0.0001 \)), as well as a Dose X Time interaction (\( F[3, 40] = 4.322, p < 0.01 \)). Dunnett’s multiple comparisons test indicated that at the four-hour time point, both the 1 and 3 mg/kg doses of FK506 reduced alcohol drinking in adolescent mice (Figure 7B).

Adult alcohol intake data also indicated main effects of Dose (\( F[3, 44] = 4.838, p < 0.01 \)) and Time (\( F[1, 44] = 64.59, p < 0.0001 \)), as well as a Dose X Time interaction (\( F[3, 44] = 6.112, p < 0.01 \)). Dunnett’s multiple comparison test indicated that at the four-hour time point, only the 3 mg/kg dose of FK506 was effective at reducing alcohol intake in adult mice (Figure 7C). In both adolescent and adult mice, no effects of FK506 remained by the 24-hour time point (\( p > 0.05 \); data not shown.)
Figure 15. Adolescent mice exhibit increased sensitivity to the reduction in alcohol drinking induced by pretreatment with a calcineurin inhibitor. (A) Timeline of experimental procedures. (B) Both 1 and 3 mg/kg FK506 reduced alcohol drinking in adolescent mice at the four-hour time point. (C) Only the 3 mg/kg dose of FK506 reduced alcohol drinking in adult mice. (D) FK506 had no effect on sucrose drinking in adolescents. (E) FK506 had no effect on sucrose drinking in adults. (F) FK506 did not alter open-field locomotor activity in adolescents in terms of distance over time (main image) or total distance traveled (insert). (G) FK506 did not alter open-field locomotor activity in adults in terms of distance over time (main image) or total distance traveled (insert).

To determine whether the effects of FK506 were selective for alcohol or generalized to other reinforcing solutions, a separate group of adolescent and adult (n =6) male mice consumed 1% sucrose and tap water in a two-bottle choice procedure in the home cage and were tested with the lowest effective dose of FK506 (1 mg/kg). Neither adolescent (Figure 15D) nor adult (Figure 15E) mice displayed any changes in sucrose intake following FK506 pretreatment (p > 0.05).

In the open-field test, a main effect of time emerged in adolescent mice, F(23, 230) = 37.93, p < 0.0001, with mice in both treatment groups tending to decrease locomotor activity over the course of the two-hour test. No effect of FK506 emerged in adolescent mice, either in the distance over time (Figure 15F, main panel) or total distance traveled (Figure 15F, insert) data (p > 0.05). Adult mice displayed a similar
lack of effect of FK506 (p > 0.05) on locomotor activity in distance over time (Figure 15G, main panel) and total distance traveled (Figure 15G, insert).

**Effects of Adolescent and Adult Alcohol Drinking on Subsequent Operant Responding for Alcohol**

To establish a model for the lasting effects of adolescent alcohol exposure on adult behavior, adolescent and adult mice were exposed to seven days of 24-hour two-bottle choice home cage alcohol or water drinking. Following a six and a half-week abstinence period, during which adolescent mice matured to adulthood, all subjects were trained to self-administer 9% alcohol/2% sucrose in operant chambers. Figure 16A depicts a timeline for the entirety of the operant behavior experiments. During the initial two-bottle choice drinking, no age differences emerged in 24-hour alcohol intake (adolescent average intake = 17 g/kg, adult average intake = 14 g/kg on the last drinking day, p > 0.05; Figure 16B) or alcohol preference (adolescent average preference = 46%, adult average preference = 56%, p > 0.05; Figure 16C). Blood alcohol concentration also did not differ between the two ages when assayed two hours into the drinking session on the last day of drinking (adolescent average BAC = 63 mg/dL, adult average BAC = 77 mg/dL, p > 0.05; Figure 16D).
Following the six and a half weeks of aging, mice were first tested on an increasing alcohol concentration curve during one-hour test sessions in the operant chambers. A main effect of concentration emerged, $F(3, 39) = 99.83, p < 0.0001$, indicating that mice achieved a higher dose of alcohol with increasing alcohol concentration. A main effect of exposure age was also evident, $F(1, 13) = 5.9610.05$, indicating that across all concentrations tested, mice exposed to alcohol during adolescence achieved a higher dose of alcohol than mice exposed to alcohol during adulthood (Figure 16E).

**Figure 16. Adolescent but not adult alcohol drinking increases operant responding for sweetened alcohol in adulthood.** (A) Timeline of the entirety of experiments in the operant self-administration context. (B) Adolescent and adult mice consumed similar amounts of alcohol in a two-bottle choice home-cage drinking procedure. (C) Preference for alcohol in the home-cage drinking did not differ between adolescent and adult mice. (D) Blood alcohol levels collected two hours into the last day of home cage alcohol drinking did not differ between adolescent and adult mice. After adolescent mice had reached adulthood (P90), both adolescent and adult mice were tested for subsequent operant responding for alcohol. (E) Upon initial testing across an alcohol concentration curve, mice exposed to alcohol during adolescence exhibited increased operant self-administration of alcohol compared with mice exposed to alcohol during adulthood. (F) Over a 12-week baseline testing period, mice with exposure to alcohol during adolescence exhibited increased operant responding for alcohol as compared with mice
exposed to water during adolescence. (G) No differences emerged between adults with a history of either alcohol or water drinking. (** indicates $p < 0.01$)

Over the next 12 weeks of testing, operant responding for 9% alcohol/2% sucrose was compared within each treatment age to determine the specific effects of the timing of alcohol exposure on subsequent operant responding for alcohol. In mice exposed during adolescence, a main effect of Time emerged, $F(11, 154) = 8.811, p < 0.0001$, with mice exposed to both alcohol and water increasing their operant responding for alcohol over time. A significant Exposure Condition X Time interaction also emerged, $F(11, 154) = 2.637, p < 0.01$. Although no individual day reached significance via Sidak’s multiple comparisons test, the trend evidenced by the data indicate that after the first six weeks of responding, the adolescent alcohol exposure group exhibited increased responding for alcohol compared to the adolescent water exposure group (Figure 16F). The same Exposure Condition X Time interaction emerged in the number of reinforcements earned ($F[11, 154] = 2.532, p < 0.01$) and dose of alcohol achieved ($F[11, 154] = 2.781, p < 0.01$; data not shown.)

In mice exposed during adulthood, only a main effect of Time emerged, with mice exposed to both alcohol and water during adulthood increasing their operant responding for alcohol over time ($F[11, 154] = 5.818, p < 0.0001$; Figure 16G). No significant main effects or interactions by Exposure Condition emerged for active lever responses, reinforcements earned, or dose of alcohol achieved, $p > 0.05$. 

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Effects of Systemic Calcineurin Inhibition on Operant Responding for Alcohol

After baseline responding had been established, the calcineurin inhibitor FK506 was administered systemically via i.p injection 30 minutes prior to a 1-hour operant self-administration session. This experiment assessed the effects of FK506 on the reinforcing properties of alcohol in mice with or without a history of adolescent alcohol drinking. In mice exposed to alcohol or water during adolescence, a Dose X Exposure Condition interaction emerged, \( F(2, 24) = 5.902, p < 0.01 \); Figure 17A.

Sidak’s multiple comparisons test revealed that mice exposed to alcohol drinking during adolescence exhibited reductions in operant responding for alcohol after pretreatment with both the 0.56 (\( p < 0.05 \)) and 1 mg/kg (\( p < 0.01 \)) doses of FK506 (Figure 17A). Mice exposed to water drinking were insensitive to both doses, \( p > 0.05 \). The same interaction was observed in the dose of alcohol achieved, \( F(2, 24) = 6.236, p < 0.01 \), with Sidak’s test indicating that both the 0.56 (\( p < 0.05 \)) and 1 mg/kg (\( p < 0.01 \)) doses of FK506 reducing g/kg alcohol intake in the operant setting (Figure 17B). No differences in percent responding on the active lever were observed in either the water or alcohol exposure groups (\( p > 0.05 \), Figure 17C.)

In mice exposed to either alcohol or water during adulthood, no significant effects of FK506 on operant responding for alcohol emerged in either exposure condition. Active lever responses (Figure 17D), dose of alcohol achieved (Figure 17E), and percent responding on the active lever (Figure 17F) were all unaffected by FK506 pretreatment (\( p > 0.05 \)).
Figure 17. The calcineurin inhibitor FK506 selectively reduces operant self-administration of sweetened alcohol only in mice with a prior history of adolescent alcohol exposure. (A) FK506 reduced operant responding for alcohol in mice with a history of adolescent alcohol (dark blue) but not water (light blue) drinking. (B) FK506 reduced the dose of alcohol obtained by mice with a history of adolescent alcohol (dark blue) but not water (light blue) drinking. (C) FK506 did not alter the proportion of responding on the active versus inactive lever in mice with a history of adolescent alcohol (dark blue) or water (light blue) drinking. (D) FK506 did not alter operant responding for alcohol in mice with exposure to either alcohol (dark grey) or water (light grey) in adulthood. (E) FK506 did not alter alcohol dose achieved of mice with either a history of adult alcohol (dark grey) or water (light grey) drinking. (F) FK506 did not alter the proportion of responses on the active versus inactive lever in adult mice with a prior history of alcohol (dark grey) or water (light grey) drinking.

Effect of Intra-mPFC Calcineurin Inhibition on Operant Responding for Alcohol

In contrast to the systemic experiments, this site-directed experiment was designed to determine the effects of calcineurin inhibition specifically within the mPFC on the reinforcing properties of alcohol in mice with or without a history of adolescent alcohol exposure. Owing to the advanced age of the mice in the adult exposure condition and the insensitivity to the systemic effects of FK506 in these groups, FK506 microinjection experiments were conducted in the adolescent alcohol and water exposure groups only. Among mice exposed to alcohol during
adolescence, 1µg of FK506 microinjected into the mPFC significantly increased operant responding for alcohol ($t[3] = 4.70, p < 0.05$; Figure 18A) and dose of alcohol achieved during the operant session ($t[3] = 4.08, p < 0.05$; Figure 18B). FK506 microinjection did not affect percent responding on the active lever, $p > 0.05$ (Figure 18C).

Among mice exposed to water during adolescence, 1µg of FK506 microinjected into the mPFC did not alter operant responding for alcohol (Figure 18D), dose of alcohol achieved during the operant session (Figure 18E), or percent responding on the active lever (Figure 18F; $p > 0.05$ for all comparisons).

**Discussion**

The use of alcohol by adolescents is both widespread and associated with increased risk for alcoholism during adulthood, but the neurobiological mechanisms behind these related phenomena remain unclear. The present experiments were designed to identify differences between the adolescent and adult mPFC proteome response to voluntary alcohol drinking, and assess the functional relevance of alcohol-sensitive proteins in the risk for increased alcohol self-administration in adulthood after alcohol drinking during adolescence. Here we provide evidence of qualitatively distinct responses of the adolescent and adult mPFC, respectively, to voluntary alcohol drinking.
Figure 18. Intra-mPFC calcineurin inhibition increases operant responding for alcohol selectively in mice with a history of adolescent alcohol, but not water, drinking. (A) FK506 (1µg) increased operant responding for alcohol in mice with prior history of alcohol drinking during adolescence. (B) FK506
increased the dose of alcohol consumed in mice with a prior history of alcohol drinking during adolescence. (C) FK506 did not alter percent responding on the active lever in mice with a prior history of alcohol drinking during adolescence. (D) FK506 did not alter operant responding for alcohol in mice with a prior history of water drinking during adolescence. (E) FK506 did not alter dose of alcohol consumed in mice with a prior history of water drinking during adolescence. (F) FK506 did not alter percent responding on the active lever in mice with a prior history of alcohol drinking during adolescence. (* indicates $p < 0.05$, ** indicates $p < 0.01$)

In terms of the degree of protein expression impacted by alcohol and extent of functional protein networks altered by drinking, the adolescent mPFC appears to be more sensitive to the effects of alcohol than the adult mPFC. The protein calcineurin (PPP3) was chosen for functional assessment based on baseline age differences in expression and differential sensitivity to alcohol between adolescent and adult mice. When tested during the adolescent versus adult development periods, respectively, calcineurin inhibition more potently reduced alcohol intake in adolescent mice compared with adult mice. Next, we provide evidence that seven days of alcohol drinking in adolescence increases operant self-administration during adulthood, a model of findings reported in the human literature. In mice with a history of adolescent, but not adult, alcohol drinking, systemic calcineurin inhibition reduces operant alcohol self-administration. Lastly, inhibition of calcineurin in the mPFC increased operant alcohol self-administration in mice with a history of alcohol, but
not water, drinking during adolescence. Taken together, these findings suggest that acute age differences in the protein-level response of the mPFC to alcohol may have lasting effects that underlie the longitudinal vulnerability to AUDs observed after adolescent alcohol drinking.

**Proteomics**

In the adolescent mPFC, 24 proteins were identified as alcohol-sensitive. Of these 24, two-thirds (16) were downregulated by alcohol drinking whereas the remainder (8) were upregulated by alcohol drinking (Table 1). Of the identified proteins, six have established roles in neurotransmitter signaling: CRIP1A [18], CALR [19], TOLLIP [20], DCTN3 [21], NSFL1C [22] and PTGES3 [23]. An additional four proteins have explicit roles in neurotransmission related to synaptic plasticity: SNCB [24], PPP3R1 [25], CFL1 and CFL2 [26] and CPLX2 [27]. Three proteins have been shown to be involved in neuronal structure: ACTB [28], CLTA [29] and BRICK1 [30]. The remaining proteins are mostly related to cellular metabolism.

In the adult mPFC, 16 proteins were identified as alcohol-sensitive. Of these, there was a roughly even split between proteins upregulated and downregulated by alcohol drinking (Table 2). Of the identified proteins, five were also identified in the adolescent mPFC: CLTA, CPLX2, COX6B1, DCTN3, NUDT2. Of the remaining seven unique proteins identified in the adult mPFC, two had discrete roles in neurotransmitter signaling (ACP1 [31] and CALR [32]) while an additional two have specific functions in synaptic plasticity: CALM [33] and NRGN [34]. One structural protein was unique to the adult mPFC (MYL6 [35]). The histone protein H4 was also identified, which plays a key role in gene regulation [36].
The design of the proteomics experiments directly compared mPFC tissue from alcohol and water drinking mice separately within each age group. As such, quantitative comparisons between adolescents and adults as to the amount of expression change at individual spots are not appropriate. However, qualitative comparisons between the two age groups reveal important differences in their respective responses to alcohol. In the protein expression experiment, about 75% of the identified proteins were unique to the adolescent mPFC, with only five proteins exhibiting alcohol sensitivity in both the adolescent and adult tissue. This result speaks to the markedly different impact that alcohol has on the mPFC between the two developmental stages. In the adolescent mPFC, a total of 24 proteins were identified as alcohol-sensitive, with three proteins appearing twice in the list for a total of 21 unique spots. These duplicates likely represent differing post-translational modifications of the identified proteins; as can be seen in Figure 2A, all three duplicates (actin, spots #3 and 5; calcineurin, spots #21 and 22; β-synuclein, spots#16 and 17) are in close proximity to one another and exhibit small shifts in IEF or molecular weight. In the adult mPFC, a total of 16 alcohol-sensitive proteins were identified, with two proteins appearing twice in the list (calmodulin, spots#5 and 6; clathrin light chain A, spots#2 and 3) and one protein appearing three times (myosin light polypeptide 6, spots#11, 12 and 13; Figure 3A) for a total of 12 unique spots. Accounting for duplicates, nearly twice as many proteins in the adolescent mPFC were alcohol-sensitive compared to the adult mPFC.

In the protein phosphorylation experiment, the opposite pattern of age differences was observed. In the adolescent mPFC, alcohol drinking altered the
phosphorylation of 14 unique proteins, all but two of which displayed increased phosphorylation in the alcohol drinking tissue. In the adult mPFC, alcohol drinking altered the phosphorylation of 34 proteins, including two proteins that appeared twice (actin, spots #33 and 34; Heat shock protein HSP 90-beta, spots #23 and 24) and one protein that appeared three times (calcium/calmodulin-dependent protein kinase II-α, spots G, H and I) for a total of 30 unique phosphoproteins. Similar to the adolescent phosphoproteins, approximately 75% of the identified phosphoproteins in the adult samples displayed increased phosphorylation in the alcohol-drinking tissue.

Taken together, these findings may indicate one potential explanation for the increased sensitivity of the adolescent mPFC to the effects of alcohol. The mature adult mPFC appears to respond to alcohol drinking with widespread increases in protein phosphorylation and relatively limited changes in protein expression, which may represent a homeostatic mechanism that favors changes in activity (i.e. protein phosphorylation) over changes in protein quantity (i.e. protein expression changes) after alcohol exposure. The adolescent mPFC responds to alcohol with far fewer phosphorylation changes, and perhaps as a consequence goes on to display many more proteins with alcohol-induced expression changes than the adult mPFC.

**Bioinformatics**

Ingenuity Pathway Analysis (IPA) identified an array of biofunctions/disorders and canonical signaling pathways in the adolescent and adult mPFC, respectively that were disrupted by alcohol drinking. In both age groups, alcohol drinking appeared to significantly impact a variety of neuronal functions related to neurotransmitter signaling. In the adolescent and adult mPFC, vesicular transport
and exocytosis appear to be altered by alcohol exposure. Acute alcohol has been shown to alter extracellular concentrations of glutamate [13], GABA and dopamine [37] in the PFC in adolescent and adult rodents, which accords with the protein-level findings observed in the present study. IPA identified more disruption in neurotransmission-related biofunctions and canonical signaling pathways in the adolescent mPFC after alcohol drinking than the adult mPFC, in accordance with the more substantial effects of alcohol on protein expression seen in the adolescent mPFC. The increased number of alcohol-sensitive functions related to exocytosis in the adolescent experiment may relate to age differences in the effects of alcohol on neurotransmission in the PFC. Recent findings suggest that the adolescent PFC is more sensitive to alcohol-induced alterations in spontaneous glutamatergic neurotransmission than the adult mPFC as indexed by alcohol-evoked changes in glutamate transients [38]. Adolescent alcohol exposure has also been shown to cause lasting changes in GABAergic transmission in the PFC [39]. These findings, combined with the present data indicating substantial impact of adolescent drinking on neurotransmission-associated protein in the mPFC, may indicate one potential mediator of age differences in sensitivity to the acute and lasting effects of alcohol exposure. IPA indicated that adolescent, but not adult, alcohol drinking altered biofunctions related to behavior, and particularly learning. Previous research has shown that adolescents are more sensitive to the cognitive impairment induced by alcohol [40], and it has been suggested that these age differences are mediated by the relative immaturity of the adolescent PFC [12]. The present findings provide support for this hypothesis at the protein level, and suggest that calcineurin and
cofilin-1 may be in part responsible for the increased sensitivity of the adolescent to alcohol-induced cognitive impairment. Further assessment is necessary to confirm a functional relationship between these protein changes and memory impairment following alcohol drinking in adolescents.

IPA also identified two unique functional protein interaction networks in the adolescent and adult mPFC, respectively, that were predicted to be impacted by alcohol drinking. In the adolescent mPFC, a network related to behavior, cell-to-cell signaling/interaction and nervous system development/function was statistically predicted to have altered activity following alcohol drinking (Figure 4). The identified network contained several proteins that have previously been shown to regulate both the cellular and behavioral response of the rodent brain to alcohol, including subunits of the GABA\textsubscript{A} and NMDA receptors [41], the adenosine 2A receptor [42], calcium/calmodulin-activated kinase II [43], mitogen-activated protein kinase [44] and fragile X mental retardation 1 [45]. Importantly, many of these same proteins have important roles in both neuronal development and learning/plasticity in the brain. The network therefore points towards a noxious interaction of increases in the expression/activity of these signaling systems during adolescence [15] and the sensitivity of these systems to alcohol.

In adult tissue, voluntary alcohol drinking was associated with a functional protein interaction network involved in neurological disease, organismal injury/abnormality and cellular compromise. One of the predicted interaction proteins, brain-derived neurotropic factor (BDNF), has repeatedly been shown to play a role in the cellular and behavioral response to alcohol [46]. Alcohol is a
physiological stressor that activates innate immune signaling in the rodent brain [47] and can induce brain damage in humans with chronic use [48]. Two of the interaction proteins identified by IPA, amyloid precursor protein (APP) and presenilin-1 (PSEN1), have well-established roles in other neurodegenerative disorders such as Alzheimer’s disease [49, 50], but to date the role of these proteins in alcohol-induced neurodegeneration has not be investigated. The adult interaction network therefore speaks to the impact of limited alcohol drinking on systems that underlie neurodegeneration, underscore the impact of alcohol drinking across the lifespan, and suggest some potential new avenues for research.

Comparatively, the adolescent interaction network was comprised of five proteins identified in the proteomics screen and 23 proteins that were predicted to be functionally impacted by alcohol drinking. The adult network was comprised of three proteins identified in the proteomics screen and an additional seven proteins that were predicted to be functionally impacted by alcohol drinking. The adolescent network was thus more complex than the adult network and implicated alcohol impacting the activity of many more proteins downstream of the proteins that exhibited expression changes following alcohol drinking. These differences in functional interaction networks are a logical consequence of the greater number of alcohol-sensitive proteins identified in the adolescent mPFC, and illustrate the impact of relatively widespread protein expression changes in the developing brain and how short-term alcohol drinking may impact neural development.

A limitation of the 2D-DIGE approach to proteomics is the difficulty in solubilizing membrane-bound proteins, particularly neurotransmitter receptors. The
functional interaction networks identified by IPA give indications of receptors that were not detected in the proteomics screen that may nevertheless be affected by adolescent alcohol drinking in terms of their expression or activity. In particular, the adolescent interaction network identified the GABA<sub>A</sub> receptor, the adenosine 2A receptor, and the NMDA as being impacted by intermittent alcohol drinking. As all three of these proteins have well-established roles in alcohol drinking [51, 52], they represent useful targets for subsequent expression analysis using immoblotting or immunohistochemistry and functional assessment via pharmacological activation or inhibition of these receptors in adolescent and adult mice.

**Calcineurin**

Of the proteins identified in the adolescent and adult alcohol proteomics experiments, calcineurin (PPP3R1) was selected for evaluation of a functional role in adolescent and adult alcohol drinking behavior. Calcineurin represented an attractive target for such assessment because it has previously been reported to exhibit increased expression in the adolescent relative to the adult mPFC [15] and was shown to be downregulated by alcohol drinking in the adolescent but not adult mPFC in the present study.

Calcineurin is a protein phosphatase that plays an integral role in synaptic plasticity [25]. The only calcium-sensitive protein phosphatase identified to date, calcineurin dephosphorylates the GluA1 subunit of the glutamate AMPA receptor at Ser<sup>854</sup>, the phosphorylation site of protein kinase A (PKA) [53]. Dephosphorylation at this residue is associated with reductions in AMPA-mediated glutamatergic signaling and internalization of the receptor [54]. Accordingly, calcineurin overexpression has
been shown to block long-term potentiation (LTP) and long-term depression (LTD) of glutamatergic synapses [55], whereas forebrain-specific calcineurin inhibition has been shown to enhance LTP and improve memory function in rodents [56].

To date, the role of calcineurin in alcohol drinking has received limited investigation. Observations from human alcoholics who undergo liver transplantation have suggested that the anti-rejection medication cyclosporine A (CSA), a calcineurin inhibitor, may be associated with reductions in alcohol drinking in this population. Two previous studies in rodent models have indicated that CSA and FK506 (another calcineurin inhibitor) reduce alcohol drinking in adults. However, age differences in sensitivity to calcineurin inhibition in the context of alcohol drinking have not be previously investigated.

In the present experiments, pretreatment with FK506 prior to an intermittent two-bottle choice home cage drinking procedure reduced alcohol drinking in adolescent and adult mice (Figure 7), consistent with previous reports. However, both the 1 mg/kg and 3 mg/kg dose of FK506 reduced alcohol drinking in adolescent mice, whereas only the high dose produced a reduction in adult subjects. This finding suggests increased efficacy of calcineurin inhibition in adolescent mice. The proteomics findings indicated that alcohol drinking produces decreases in calcineurin expression in the adolescent, but not adult, mPFC. Adolescent-specific alcohol-induced reductions in calcineurin during the initial week of alcohol drinking prior to drug testing may therefore have been responsible for the increased sensitivity of the adolescent mice to FK506. The 1 mg/kg dose did not significantly alter sucrose consumption in adolescent or adult mice, suggesting some degree of selectivity for
alcohol, and failed to alter open-field locomotor activity, indicating that the effects of FK506 were not due to nonspecific reductions in locomotor behavior.

**Modeling Long-term Risk for Increased Alcohol Consumption Following Adolescent Alcohol Exposure**

The finding that adolescents were more sensitive to calcineurin regulation of alcohol drinking than adults was significant, but did not speak to the long-term risk for alcoholism following adolescent alcohol exposure. Therefore, a model of adolescent and adult alcohol drinking and subsequent operant responding for alcohol when all subjects had reached adulthood was developed. Mice in both age groups drank alcohol and water or water alone, respectively, in a two-bottle choice home-cage drinking procedure identical to the procedure used in both the proteomics experiments and the initial FK506 pharmacology experiments. Following two weeks of every-other-day alcohol drinking, alcohol access was removed for 6 weeks, until adolescent subjects had reached adulthood (PND 90). Mice were next trained to operantly self-administer sweetened alcohol on an FR4 schedule.

The use of both age-matched water control mice and groups that had been exposed during adolescence or adulthood enabled assessment of differences induced by type of fluid exposure and timing of fluid exposure on subsequent operant responding for alcohol. When adolescent alcohol exposure and adult alcohol exposure mice were directly compared during the first week of operant testing, the adolescent exposure group consumed significantly more alcohol than the adult exposure group across doses ranging from 3-15% alcohol by volume. When comparisons were made between water and alcohol exposure within age, mice with
adolescent alcohol exposure exhibited modest but significant increases in operant self-administration compared with adolescent water exposure mice. This effect was not evident between mice that received alcohol during adulthood when compared with mice who received water during adulthood. Together, these findings indicate that adolescent exposure to alcohol uniquely leads to a lasting increase in the reinforcing effects of alcohol as measured by operant self-administration.

These results are particularly significant in light of the limited exposure to alcohol during adolescence in these experiments. Adolescent and adult mice drank alcohol in an intermittent (every-other-day) two-bottle choice procedure for the two-week period conservatively estimated to comprise adolescence in rodents. This resulted in a total of only seven days of alcohol drinking, following by an extended period of abstinence. Nevertheless, this drinking procedure was sufficient to both cause extensive alterations in protein expression and predicted changes in interaction network function in the adolescent mPFC, and produce age differences in alcohol drinking following adolescent alcohol consumption that persisted for months. The results therefore indicate that even limited access to alcohol during adolescence may have lasting consequences for the adult brain.

A significant limitation of the model is the use of the pause period between alcohol drinking and subsequent operant responding for alcohol. The current model was designed to isolate the effects of alcohol specifically during the adolescent period on subsequent operant responding. However, human adolescents experience no such pause in their consumption during late adolescence but rather regularly consume alcohol throughout the adolescent period and into adulthood. In this sense,
the model does not accurately reflect the drinking patterns typical amongst human adolescents. Continuing the drinking procedure throughout adolescence and into adulthood would most likely yield more significant changes in both brain protein expression and accompanying behaviors, and would therefore be predicted to produce more robust increases in operant self-administration during adulthood. For this initial assessment a more conservative approach was taken, but future studies to investigate the timing of the initiation of alcohol drinking will yield useful insights.

**Calcineurin Selectively Regulates Alcohol Self-Administration in Mice with a History of Adolescent Alcohol Drinking**

The model of adolescent and adult alcohol exposure was next used to assess the effects of calcineurin inhibition on operant self-administration of alcohol. When injected systemically, FK506 reduced operant responding for alcohol only in mice with a prior history of alcohol drinking (Figure 16). Mice that consumed water during adolescence as well as mice that consumed alcohol during adulthood were insensitive to both doses of FK506 tested. The reduction in operant responding exhibited by the adolescent alcohol exposure group was accompanying by a reduction in alcohol dose achieved during the operant session, but no changes in percent responding on the active lever, indicating that the effects of FK506 were not due to nonspecific learning deficits.

To establish the relevance of calcineurin signaling in the prefrontal cortex on operant self-administration, cannulae aimed at the mPFC were implanted into mice in the adolescent alcohol exposure and adolescent water exposure groups. When 1µg of FK506 was microinjected into the mPFC, it increased operant responding for
alcohol in mice with a history of adolescent alcohol drinking, but not in mice with a history of adolescent water drinking. This increase was accompanied by an increase in alcohol dose achieved during the operant session and no change in percent responding on the active lever, indicating that the effects of FK506 in the mPFC were not due to learning impairments.

The finding that systemic calcineurin inhibition reduces alcohol drinking and operant self-administration but intra-mPFC calcineurin inhibition increases alcohol self-administration speaks to the importance of assessing brain region-dependent effects of pharmacological manipulation of alcohol-related behaviors. In addition to the PFC, calcineurin expression is also dense within the nucleus accumbens, amygdala and ventral tegmental area, limbic regions associated with alcohol reward and reinforcement. As reductions in calcineurin expression and phosphorylation appear to be a drug effect of alcohol drinking in adolescents, systemic inhibition of calcineurin signaling may therefore be mimicking the effect of the alcohol, which would be expected to reduce the amount of alcohol necessary to achieve an intoxicating dose. This effect of systemic inhibition may be mediated not by the prefrontal cortex, but by the limbic reward-related brain regions detailed above. Adult animals are insensitive to this effect of alcohol on calcineurin expression as seen in the proteomics screen, which may explain why a higher dose was necessary to achieve reductions in alcohol drinking in the systemic alcohol drinking experiment. Such an explanation is consistent with behavioral reports from rodent models of adolescence and human studies which indicate that adolescents have increased
sensitivity to the rewarding effects of alcohol relative to adults. Calcineurin may be a molecular mechanism underlying these age-dependent behavioral manifestations.

Within the PFC, calcineurin inhibition would be expected to dephosphorylate the GluA1 subunit of the AMPA receptor, resulting in reduced glutamatergic signaling in this region. Importantly, the major glutamatergic projections of the mPFC terminate in the nucleus accumbens and amygdala, but synapse onto inhibitory interneurons in those regions. This network is believed to underlie the “executive control” exerted by the PFC over limbic regions, a feature that is relatively immature in the adolescent PFC relative to adults. In mice with a history of adolescent alcohol drinking, calcineurin inhibition in the PFC results in dysregulation of cortical output to limbic regions, which may increase responsivity to alcohol and related cues and motivate increased self-administration of alcohol. This effect of calcineurin inhibition appears to be blunted or absent in mice without a history of adolescent alcohol exposure, which is consistent with reports in humans and rodents of the persistence of an “adolescent phenotype” into adulthood following adolescent alcohol exposure. Overall, the dual findings that 1) alcohol drinking during adolescence downregulated calcineurin in the PFC, and 2) calcineurin inhibition in the PFC increased operant alcohol self-administration suggest that alcohol-induced downregulation of calcineurin in the PFC may increase the reinforcing effects of alcohol in this vulnerable population.
**Limitations and Future Directions**

Although the present findings provide an initial assessment, several questions remain as to the specific role of calcineurin in alcohol consumption across developmental stage. First, it would be informative to determine the longitudinal effects of alcohol exposure during adolescence on calcineurin expression during adulthood. Although the pharmacological data presented here indicate that adolescent alcohol exposure imparts lasting changes in calcineurin signaling, these effects could be due to adaptations in proteins or receptors downstream of calcineurin itself. It would also be valuable to assess the effects of this drinking procedure on GluA1, since it is a direct target of calcineurin activity and thought to mediate many of the effects of calcineurin. Additionally, since systemic and intramPFC calcineurin inhibition created divergent effects on operant self-administration of alcohol, future studies will assess the effects of alcohol on calcineurin expression in other brain regions, particularly those known to be involved in alcohol reward such as the nucleus accumbens, amygdala and ventral tegmental area. Microinjection of FK506 into these additional brain regions would also help clarify the systemic findings. Lastly, although the home-cage drinking experiments suggested that the effects of calcineurin inhibition were selective for alcohol and not sucrose drinking, the selectivity of FK506 was not assessed in the operant experiments. The effects of adolescent alcohol exposure on subsequent operant responding for sucrose would indicate whether calcineurin signaling selectively alters alcohol-related pathways in the brain, or functions through pathways involved in reward more generally.
Conclusion

Here we report that only seven days of voluntary alcohol drinking produced profound effects on the adolescent mPFC proteome. This limited adolescent alcohol exposure was further associated with long-term effects on the reinforcing properties of alcohol in adult mice. The results further provide initial evidence that calcineurin is selectively alcohol-sensitive in the adolescent mPFC and may mediate some of the long-term risk for increased alcohol self-administration observed following adolescent alcohol exposure. As personalized medicine gains prominence in the addiction field, these insights may be useful in developing treatments specifically for alcoholics with a history of heavy drinking during adolescence. One FDA-approved calcineurin inhibitor, cyclosporine A, has been suggested as a potential therapy for alcoholism, although significant side effects may pose a barrier to clinical evaluation. Additionally, this evidence for the unique vulnerability of the adolescent brain to the effects of alcohol may arm education and prevention efforts with more information about the harm of underage drinking.
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CHAPTER FOUR: CaMKIIα-GluA1 ACTIVITY UNDERLIES VULNERABILITY TO ADOLESCENT BINGE ALCOHOL DRINKING

Introduction

Adolescence is a distinct developmental period that in humans occurs from the early teens to the early twenties. This developmental stage is characterized by increased exploration and risk-taking, and is the time at which experimentation with drugs of abuse, including alcohol, is usually initiated [1]. Alarming data show that 25 – 37% of U.S. high school students engage in heavy episodic or binge drinking [2] at more than twice the rate of adults [3], and a recent survey revealed that approximately 22% of 12th grade students had engaged in binge drinking in the last two weeks [4]. Binge drinking during adolescence is particularly troubling in light of evidence that individuals who initiate alcohol use during early adolescence are at substantially higher risk for the development of alcohol use disorders (AUDs) than those who initiate alcohol use as young adults [5, 6]. These data suggest that adolescent alcohol users are uniquely vulnerable to the development of AUDs, and indicate that a significant portion of the population is subject to this increased risk. However, the precise neurobiological mechanisms that mediate this effect have yet to be determined [7].

2 This chapter previously appeared as an article in Alcoholism: Clinical and Experimental Research. The original citation is as follows: Agoglia AE, Holstein SE, Reid GT, Hodge CW (2015). CaMKIIα-GluA1 activity underlies vulnerability to adolescent binge alcohol drinking. Alcoholism: Clinical and Experimental Research, 39(9) 1680-90.
In humans and in rodent models, adolescence is characterized by increased neuronal plasticity as the brain matures from childhood to adulthood [8]. This suggests that adolescents may be more vulnerable to the effects of alcohol on plasticity-linked cellular functions. Indeed, evidence indicates that adolescent rodents are more sensitive to alcohol-induced disruption of long-term potentiation [9], which is a cellular mechanism that underlies behavioral plasticity. Similarly, we have shown that adolescent mice are more sensitive than adults to acute alcohol-induced changes in ERK MAP kinase activation in the amygdala [10] which is required for LTP in this region [11]. Further, alcohol exposure blunts LTP in the amygdala [12] where plasticity-linked proteins regulate alcohol-seeking behavior [13, 14]. Thus, increased understanding of alcohol-induced changes in signaling systems that regulate cellular plasticity may be of interest to identify mechanisms underlying adolescent vulnerability to development of AUDs.

Glutamate is the primary excitatory neurotransmitter in the mammalian brain and its receptors and cellular signaling pathways are required for adaptive plasticity. Calcium/calmodulin-dependent protein kinase II (CaMKII) is a 12-subunit protein expressed primarily in glutamatergic synapses, where it has a well-established role in synaptic plasticity, learning and memory [15]. CaMKII has been shown to be involved in alcohol consumption and reward. Mice expressing autophosphorylation-deficient CaMKIIα have been shown to drink less alcohol than wild type littermates [16] and display altered conditioned place preference for alcohol [17]. We have shown that voluntary alcohol drinking increases expression of the active (phosphorylated) form of CaMKIIαT286 (pCaMKIIαT286) in the mouse amygdala, and
that intra-amygdala inhibition of CaMKII activity reduces the positive reinforcing effects of alcohol [13]. CaMKII is activated upon phosphorylation, allowing it to phosphorylate several downstream targets including the GluA1$_{S831}$ subunit of the AMPA subtype of glutamate receptors [18]. Phosphorylation of GluA1$_{S831}$ (pGluA1$_{S831}$) is associated with increased stability of AMPARs in the synapse, which promotes synaptic plasticity and learning [19]. Our work also shows that AMPAR activity in the amygdala is required for the reinforcing effects of alcohol [13]. Together, these data suggest a critical role for CaMKIIa-GluA1 signaling in alcohol self-administration and reinforcement. However, age-dependent differences in sensitivity to alcohol-induced modifications of this system have not been explored.

To address this question, the present study was designed to test the hypothesis that adolescents are more sensitive to alcohol-induced changes in CaMKIIa and AMPAR GluA1protein phosphorylation and expression than adults. We chose to investigate protein changes in the amygdala and striatum based on our previous results as well as a wealth of literature indicating that these regions are functionally involved in alcohol self-administration and reinforcement [13, 20]. To evaluate the functional involvement of this system in adolescent and adult alcohol drinking, we used the drug tianeptine, a systemic upregulator of CaMKIIa-dependent GluA1 activation (e.g., phosphorylation). A binge-like model of alcohol access was utilized in these experiments in order to most closely mimic the drinking patterns exhibited by adolescents in the United States.
Materials and Methods

Animals

Male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were individually housed in standard Plexiglass cages with a small PVC pipe for environmental enrichment. Adolescent mice were postnatal day 21 (PND 21) and adults were (PND 63±2) upon arrival in our facility. Food and water were available ad libitum in home cages except where noted. The colony room was maintained on a 12-h light/dark cycle (lights on at 20:00 in the initial binge exposure experiment and lights on at 19:00 in the pharmacology experiments) at 21°C. All experimental manipulations and testing occurred during the dark cycle. All procedures were carried out in accordance with the NIH Guide to Care and Use of Laboratory Animals [21] and approved by the Internal Review Board of the University of North Carolina, Chapel Hill.

Experiment 1: Daily Binge Alcohol Exposure and Protein Changes in the Adolescent and Adult Mouse Brain

Binge Alcohol Exposure Procedure

Adolescent and adult mice (N=40) were allowed one week (PND 21-27) or (PND 63-69) to habituate to our colony and acclimate to the light/dark cycle (Fig 19A). During habituation, mice were handled and weighed daily. Mice were then brought to consume alcohol in a binge-like access procedure adapted from Rhodes et al. [22]. Beginning on PND 28 or PND 70, mice were weighed and home cage water bottles were removed at 11:00AM and replaced with a single drinking tube
containing either 20% (v/v) alcohol (alcohol treated animals) or water (water control animals) for four hours, n=10/treatment/age. The drinking tubes consisted of a 10mL serological pipette fitted with a ball-bearing sipper tube and fastened to the wire cage lid with a medium binder clip. This limited access procedure was repeated daily for two weeks, ending on PND 42 for adolescents or PND 84 for adults.

Tissue Collection

Immediately after the cessation of drinking, on the last day of testing, mice were rapidly decapitated and blood alcohol concentration (BAC) was assessed. Approximately 20µL of trunk blood was centrifuged to obtain 5µL of plasma for use in an AM1 Alcohol Analyzer (Analox Instruments, Lunenburg, MA). The brains were extracted from the skulls and flash-frozen in -30°C isopentane.

Western Blot Analysis

Brain regions of interest were dissected from coronal brain sections (1mm slices) using a 1mm sterile tissue punch (Stoelting, Wood Dale, IL) and homogenized in buffer (10ml: 1.1g sucrose, 50µL 1M HEPES buffer, 1:100 protease/phosphatase inhibitor cocktail, 1mL 10% SDS, 26.55mL ultra-pure water.) Protein concentration was measured using a calorimetric assay kit (Pierce Biotechnology, Rockford, IL). Protein (5µg) was diluted 4:1 with lithium dodecyl sulfate sample buffer (40–70% glycerol), 10:1 with sample reducing agent, vortexed, loaded onto a Nu-Page 4–15% Tris-glycine polyacrylamide gel (Invitrogen, Carlsbad, CA) for gel electrophoresis separation, and transferred to nitrocellulose membrane using an iBlot dry blotting system (Life Technologies, Grand Island, NY). Membranes
were blocked with 3% albumin bovine serum (Sigma-Aldrich, St. Louis, MO) before being incubated with primary antibodies [rabbit anti-pCaMKII\textsubscript{T286} (1:2 500; Abcam, Cambridge, MA), -pGluR1\textsubscript{S831} (1:1 000, EMD Millipore, Billerica, MA) -tGluR1 (1:1 000; Abcam) and mouse anti-tCaMKII\textalpha (1:10 000; Millipore), in blocking solution at 4°C overnight and β-actin (mouse monoclonal, 1:5 000; Sigma) in blocking solution 1 h at room temperature] and washed before incubation with secondary antibodies (HRP-conjugated goat anti-rabbit and goat anti-mouse, 1:10 000; Jackson ImmunoResearch, West Grove, PA). Membranes were then visualized using enhanced chemiluminescence substrate (Pierce), and bands were quantified using optical density measurements (NIH/Scion Image).

**Experiment 2: Pharmacological Manipulation of Intermittent Binge Drinking**

**Binge Alcohol or Sucrose Drinking Procedure**

Mice (N=24/experiment) were allowed a one-week (PND 21-27) or (PND 63-69) habituation period (Fig 4A). During habituation, mice were handled and weighed daily and given saline injections to habituate to the injection procedure. Beginning on PND 28 (adolescent) or PND 70 (adult), mice were weighed and the home cage water bottles were removed at 10:00AM and replaced with a drinking tube containing 20% (v/v) alcohol (alcohol experiment) or 0.5% sucrose (sucrose experiment). Mice had access to alcohol or sucrose for four hours every other day. Beginning on PND 36 or PND 80, mice were treated with 0, 3, 10 or 17 mg/kg tianeptine (i.p.) 30 minutes prior to alcohol or sucrose tube access according to a Latin square dosing regimen. Drug pretreatment and drinking continued on PND 38, 40, and 42 (adolescent) or PND 82, 84, and 86 (adult). Tianeptine dose range and pretreatment
time were determined from a study showing behavioral effects in alcohol-exposed rodents [23].

**Locomotor Testing**

On PND 46 or PND 90, mice were pretreated with either saline or the effective dose of tianeptine (10 mg/kg for adolescents, 17 mg/kg for adults) for 2 hours prior to a locomotor activity test (n=6/treatment/age). Open field activity was measured in Plexiglas activity monitor chambers (27.9 cm$^2$; ENV-510, Med Associates, Georgia, VT). Two sets of 16 pulse-modulated infrared photobeams were located on opposite walls and recorded X–Y ambulatory movements. Distance traveled (in meters) throughout the session was quantified by assessing the mouse’s position in the open field every 100 milliseconds. Data from each chamber were collected by a computer.

**Blood Alcohol Clearance**

On PND 47-48 or PND 91-92, adolescent (n=6) and adult (n=6) mice were pretreated with the effective dose of tianeptine (10 mg/kg for adolescents, 17 mg/kg for adults) or saline. Thirty minutes later, mice were injected with 4.0 g/kg alcohol (20% w/v) i.p. Beginning at 10 minutes post-alcohol injection, mice were confined in clear Plexiglas restraint tubes (Braintree Scientific, Braintree, MA), tail blood was collected using a heparinized microcapillary tube. Additional samples were collected at 30 minutes, 1 hour, 2 hours and 4 hours post-alcohol injection. Blood samples were analyzed as in Experiment 1.
Drugs

Alcohol solutions (v/v) were prepared by diluting 95% ethanol (Pharmco Products Inc., Brookfield, CT) with tap water (for drinking) or 0.9% saline (for injection). The GluA1 modulator tianeptine (Tocris Bioscience; Ellisville, MO) was freshly dissolved in saline before each day of testing.

Data Analysis

All analyses were performed using Prism v. 6.0 (GraphPad, La Jolla, CA). For drinking experiments, alcohol intake data were reported as grams of intake per kilogram of body weight and water intake data were reported as milliliters of fluid consumed per kilogram of body weight. Intake of both solutions was analyzed via two-way repeated measures ANOVA (Age x Day). BAC was analyzed via t-test to compare adolescent and adult values.

Western blot data were expressed as percent change from age-matched water drinking controls, and all protein levels were expressed as a ratio to β-Actin. Data were analyzed via independent t-tests comparing alcohol and water treated animals separately within each age group.

In the two tianeptine pretreatment experiments, alcohol and sucrose intake were analyzed via two-way repeated measures ANOVA (Age x Dose) respectively. Tukey’s LSD test was used for all post-hoc analyses. BEC was analyzed via two-way repeated measures ANOVA (Dose x Time) separately within each age. Open field locomotor data were collapsed into 20 minute time bins and activity was assessed via two-way repeated measures ANOVA (Dose x Time) separately within
each age. Locomotor data were further analyzed for potential age or drug-induced differences in anxiety-like behavior in an open-field test. Thigmotaxis was evaluated by comparing distance (cm) traveled in the center zone (inner 25% of the area) to distance traveled in the periphery (outer 75% of the area) as previously described [24]. α was set at 0.05 for all comparisons.

Results

Experiment 1

Binge-like Alcohol Consumption

Adolescent and adult mice consumed equivalent amounts of alcohol and water over the two-week daily access period (adolescents averaged 5.01 ±.22 g/kg and adults averaged 5.09 ±.13 g/kg, Figures 19B and C). Alcohol drinking did not alter body weight in adolescents or adults (Figure 19D). Blood alcohol levels immediately after drinking on the last experimental day exceeded the NIAAA criteria for a binge drinking session (80 mg/dL), with no significant differences between the two age groups (Figure 19E) [25].

CaMKIIα Changes in the Amygdala

Western blot analysis of adolescent amygdala revealed that a two-week history of alcohol drinking significantly decreased the phosphorylation of CaMKIIαT286 by approximately 30%, t(17)= 1.916, p<0.05 (Figure 20A). No differences in total CaMKIIα expression emerged in adolescents (Figure 20B). In contrast, neither pCaMKIIαT286 nor tCaMKIIα were affected by alcohol drinking in the adult amygdala (Figure 20C and D).
Figure 19. Adolescent and adult mice achieve binge levels of alcohol consumption in a daily limited access procedure. (A) Timeline of binge-like drinking procedure. Adolescent and adult alcohol (B) and water (C) intake did not differ over two weeks of daily drinking sessions. (D) Alcohol drinking did not alter body weight in either adolescents or adults. (E) Blood ethanol concentration did not differ between alcohol-drinking adolescent and adult
mice immediately following the drinking session on the last drinking day.
Dashed line indicates the NIAAA criteria for a binge drinking session (≥ 80 mg/dL).

CaMKIIα Changes in the Striatum

Analysis of the adolescent and adult nucleus accumbens via Western blot showed no effect of alcohol exposure on pCaMKIIα\textsubscript{T286} in either age (Table 9). Similarly, pCaMKIIα\textsubscript{T286} levels were unaltered by alcohol drinking in both ages in the dorsal striatum. Total expression of CaMKIIα was also unaffected by alcohol exposure in adolescents and adults in both regions.

GluA1 Changes in the Amygdala

To examine a downstream target of phosphorylated CaMKII activity, the GluA1 subunit of the AMPAR receptor was analyzed in the adolescent and adult amygdala. Western blot analysis showed that in the adolescent amygdala, a two-week history of alcohol drinking had a non-significant trend to decrease pGluA1\textsubscript{Ser831}, \( p = 0.11 \) (Figure 21A). In the adult amygdala, alcohol drinking significantly increased pGluA1\textsubscript{Ser831} by approximately 65%, \( t(17) = 1.262, \ p = 0.05 \) (Figure 21C). No effect of alcohol drinking on total GluA1 expression emerged in either age (Figure 21B and D).
### Adolescent

**A**

- **pCaMKIIα**
- **β-Actin**

*pCaMKIIα(T286)/β-Actin % Change from Water*

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*B*

- **tCaMKIIα**
- **β-Actin**

*tCaMKIIα/β-Actin % Change from Water*

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### Adult

**C**

- **pCaMKIIα**
- **β-Actin**

*pCaMKIIα(T286)/β-Actin % Change from Water*

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**D**

- **tCaMKIIα**
- **β-Actin**

*tCaMKIIα/β-Actin % Change from Water*

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**Figure 20.** CaMKII phosphorylation is altered by two weeks of daily binge-like alcohol drinking in the adolescent but not adult amygdala. (A) Phospho-CaMKIIα\[\text{T286}\] (pCaMKIIα\[\text{T286}\]) was decreased in the amygdala of adolescent mice exposed to alcohol in the daily binge drinking procedure (*p < 0.05). (B) Total CaMKIIα expression was not different between alcohol and water drinking adolescents. (C) pCaMKIIα\[\text{T286}\] did not differ in the amygdala of adult mice exposed to alcohol or water in the daily binge drinking procedure. (D) tCaMKIIα expression was also not altered by alcohol treatment in adults.

**Experiment 2**

*Effect of Tianeptine Pretreatment on Binge-like Alcohol Consumption*

Prior to drug treatment, baseline 4-hour every-other-day alcohol intake was significantly greater among adolescents (5.6 g/kg ±0.69) than adults (3.7 g/kg ±0.69) [Main effect of Age, \( F(1, 22) = 8.896, p < 0.01 \)]. Over the course of the entire experiment, adolescent body weight increased steadily from an average of 14g on PND 28 to an average of 21g on PND42. The rate of body weight gain did not change following tianeptine pretreatment and is consistent with the published literature for mouse body weight at these ages [26].
Table 9. Phospho- and total-CaMKIIα is unaffected by alcohol drinking in the adolescent and adult nucleus accumbens and dorsal striatum. Data are represented as the mean optical density/β-Actin for each age and treatment condition ±SEM.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>pCaMKIIα T286</th>
<th>tCaMKIIα</th>
<th>Water</th>
<th>Alcohol</th>
<th>Water</th>
<th>Alcohol</th>
</tr>
</thead>
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<tr>
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</table>
Figure 21. Differential effects of a two-week history of daily binge alcohol exposure on GluA1 phosphorylation in the adolescent and adult amygdala (A) Phospho-GluA1$_{S831}$ (pGluA1$_{S831}$) had a tendency to decrease in the amygdala of adolescent mice exposed to alcohol in the daily binge drinking procedure (#p < 0.1). (B) Total GluA1 expression was not different between alcohol and water drinking adolescents. (C) pGluA1$_{S831}$ was increased in the amygdala of adult mice exposed to alcohol in the daily binge drinking procedure (*p = 0.05). (D) Total GluA1 expression was not different between alcohol and water drinking adults.

Following 30 minute pretreatment with tianeptine, a Dose x Age interaction emerged, $F(3, 66) = 5.062$, $p < 0.001$. Tukey’s multiple comparisons test revealed that among adolescent mice, the 10mg/kg dose of tianeptine significantly increased alcohol consumption relative to vehicle ($p < 0.05$, Figure 22B). Adolescent alcohol intake increased from 5.4 g/kg to 6.6 g/kg (22%). In contrast, the 17mg/kg dose of tianeptine significantly decreased alcohol consumption relative to vehicle in adults ($p < 0.05$). Adult alcohol intake decreased from 4.2 g/kg to 3.2 g/kg (25%). Dose effects were only apparent during the last two hours and total four-hour intake period (data not shown.) A main effect of Age also emerged [$F(1, 22) = 45.37$, $p < 0.0001$] with adolescents consuming significantly more alcohol than adults.

Selectivity of Tianeptine Effects

To assess behavioral (alcohol) specificity, we evaluated effects of tianeptine pretreatment on binge-like consumption of sucrose, a non-drug solution that has rewarding properties. Analysis of 4-hour sucrose consumption following 30-minute
pretreatment with tianeptine revealed a main effect of Dose, $F(3, 63) = 3.579$, $p < 0.05$, indicating that tianeptine decreased sucrose consumption similarly in adolescent and adult mice. No Age x Dose interaction emerged ($p > 0.05$, Figure 22C).

To determine if tianeptine effects were associated with nonspecific locomotor effects, adolescent and adult mice were pretreated with either vehicle or the effective dose of tianeptine (10mg/kg for adolescents and 17mg/kg for adults) 2 hours prior to a 2 hour open-field locomotor activity test in order to correspond with the time interval during which tianeptine effects on alcohol drinking were observed. Analysis of cumulative locomotor activity in both adolescents and adults failed to detect any significant effect of tianeptine pretreatment ($p > 0.05$, Figure 22D and E).

To evaluate anxiety-like behavior, locomotor data were analyzed for time spent in the center versus perimeter of the chamber. A main effect of zone emerged, such that mice in both age groups spent more time in the perimeter of the chamber than in the center zone, $F(1, 10) = 504.0$, $p < 0.0001$. Under vehicle treatment conditions, both adolescents and adults spent approximately 80% of their time in the perimeter zone. No significant effect of tianeptine pretreatment or age emerged in either zone ($p > 0.05$, data not shown.)

To determine if tianeptine affected alcohol clearance, adolescent and adult mice were pretreated with tianeptine 30 minutes prior to a 4g/kg intraperitoneal injection of alcohol. Analysis of blood alcohol samples collected 10, 30, 60, 120, and 240 minutes after the alcohol injection failed to reveal any significant effects of tianeptine pretreatment on BACs in either age ($p > 0.05$, Figure 22F and G).
Figure 22. Tianeptine pretreatment alters alcohol, but not sucrose, intake in opposite directions in adolescent and adult male mice. (A) Timeline of tianeptine pretreatment binge drinking procedure. (B) Tianeptine dose-dependently increased alcohol intake in adolescent mice but decreased alcohol intake in adult mice (*p <0.05). (C) Tianeptine comparably decreased sucrose intake in adolescent and adult mice. The open-field locomotor behavior of adolescent (D) and adult (E) mice injected with the effective dose of tianeptine in each age did not differ from age-matched controls injected with vehicle. Adolescent (F) and adult (G) mice pretreated with tianeptine did not differ in blood alcohol concentration following an acute alcohol injection.

Discussion

Although human [5, 6] and rodent studies [27-29] indicate that adolescent alcohol exposure increases the risk for lifetime AUDs, the neurobiological mechanisms of this age-specific vulnerability remain to be determined. To address this gap in knowledge, we first evaluated effects of voluntary binge-like alcohol drinking on protein expression and activation (e.g., phosphorylation) of CaMKIIαT286 and the GluA1S831 subunit of AMPARs in adolescent and adult mice. To determine if CaMKIIα-GluA1 signaling mechanistically regulates age-dependent binge drinking, we next assessed the effects of systemic administration of the antidepressant Tianeptine, which potentiates AMPAR GluA1 activity via activation of CaMKIIα (Szegedi et al 2011), in adolescent and adult mice. Results indicate that voluntary binge-like alcohol drinking produces age-dependent differential effects on
pCaMKIIαT286 and pGluA1S831 in the amygdala. Accordingly, positive modulation of CaMKII-dependent AMPA signaling via systemic Tianeptine administration increased binge-like alcohol drinking specifically in adolescent mice and reduced drinking in adults. Together, these data indicate that binge drinking produces age-dependent effects on CaMKIIα and GluA1 signaling that regulate the amount of alcohol consumed during binge drinking episodes.

Effects of Binge-Drinking on CaMKIIα and GluA1 Expression and Phosphorylation

Adolescent and adult male C57BL/6J mice voluntarily consumed similar doses of alcohol during the two-week exposure period, each achieving blood alcohol levels consistent with binge exposure (e.g., > 80 mg/dL). Binge drinking produced no effects on overall health as indexed by body weight comparisons to parallel water-only controls. The lack of age differences in alcohol intake in Experiment 1 was advantageous because it ruled out alcohol dose-dependent differences in protein expression, allowing us to more clearly interpret age-dependent differences (i.e., without the confound of differential alcohol dose).

Adolescent. Binge-like alcohol drinking produced an age-dependent decrease in pCaMKIIαT286 in adolescent amygdala with no change in total protein expression. Since pCaMKIIαT286 is a primary molecular mechanism of synaptic plasticity that is required for associative learning and memory processes mediated by the amygdala (Lisman et al., 2002, Rodrigues et al., 2004), binge alcohol-induced downregulation of CaMKIIα activity in the adolescent amygdala may underlie age-dependent inhibition of memory processes as previously observed following alcohol exposure [10, 30, 31]. This observation is highly significant from a translational perspective.
since estimates indicate that over 50% of college-age frequent binge drinkers have experienced significant associative memory loss, including complete blackouts [32]. Moreover, emerging adults (age 18-24) who have experienced binge-induced blackouts have lower levels of glutamate in the anterior cingulate cortex as compared to light drinkers [33]. Thus, binge drinking may produce adolescent-specific cognitive deficits by disruption of basic molecular mechanisms of memory and plasticity in the amygdala.

At the molecular and cellular level, alcohol-induced inhibition of pCaMKIIα T286 expression in adolescents may disrupt numerous functions subserved by the kinase including regulation of membrane current, neurotransmitter synthesis and release, cytoskeletal organization, dendrite maturation, and gene expression [15, 34-36]. Further, a crucial function of CaMKIIα is to phosphorylate AMPARs at the GluA1 S831 site, which leads to potentiation of AMPA-mediated synaptic activity [37, 38], promotes AMPAR membrane insertion, and enhances their function [15, 34, 35]. Here, we observed a trend for decreased pGluA1 S831 following adolescent binge-like alcohol intake. Binge drinking may disrupt a variety of critical neural and behavioral functions in the adolescent brain via inhibition of CaMKIIα activity and downstream mechanisms. This suggests that binge-induced changes in CaMKIIα activity may be associated with subtle changes in AMPAR signaling.

Adult. In contrast to the adolescent, a two-week history of daily binge alcohol drinking specifically increased pGluA1 S831 in adult amygdala with no change in pCaMKIIα T286 or total protein expression. These findings are consistent with previous evidence showing that pGluA1 S831 is increased by voluntary 24-h home-cage
drinking or low-dose operant alcohol self-administration in adult mice [13], and by chronic intermittent high-dose alcohol vapor in adult rats [39]. Phosphorylation of the GluA1 subunit of the AMPAR results in increased single-channel conductance [18, 40] and has been shown to be critical for the induction of LTP [19]. Recent work in our lab has demonstrated that CaMKII and GluA1 activity in the amygdala functionally regulate alcohol self-administration [13]. Thus, increased pGluA1S831 may be indicative of increased activity of AMPARs in the adult amygdala after alcohol exposure, and may contribute to long-term plastic adaptations to alcohol. Since CaMKIIα phosphorylation was not increased in adults, it is plausible binge-like alcohol drinking does not specifically alter CaMKII signaling in this age group. Thus, GluA1S831 phosphorylation may have been mediated by another alcohol-sensitive kinase, such as PKC [41-44]. These results add weight to a growing body of evidence suggesting that glutamate signaling in the amygdala is a target of alcohol self-administration.

Overall, these data show differential age-dependent effects of binge drinking on CaMKIIαT286 and GluA1S831 phosphorylation between adolescent and adult mice. Although the specific mechanism(s) for these effects are unknown, it is plausible that well-documented age-dependent maturation of the target systems is partly accountable. For instance, CaMKIIα expression increases linearly in rat forebrain by 10-fold from PND 5 through 25 [45]. Similarly, CaMKIIα mRNA increases 10-fold between PND 1 and 21 with an additional 5-fold increase by PND 90, which spans the adolescent and early adult periods examined in the present study. Additionally, the subcellular distribution of CaMKII shifts from primarily cytosolic to membrane
localization during development [46, 47]. Developmentally regulated protein concentration and localization in neural tissue may influence the effects of alcohol on CaMKIIα phosphorylation.

Tissue for these experiments was collected immediately following the last drinking session, when mice had achieved a binge-level of alcohol consumption. Therefore, the results should be considered in the context of alcohol-induced brain protein changes in the presence of alcohol. The present findings were also selective for the amygdala, with no changes in pCaMKIIαT286 or pGluA1S831 observed in the nucleus accumbens or the dorsal striatum. Therefore, glutamatergic signaling in the striatum may be less relevant than in the amygdala in terms of the regulation of binge-like alcohol self-administration. The amygdala is a complex region comprised of several subnuclei, including the central, basolateral and lateral subregions, and these nuclei may play different roles in the regulation of alcohol drinking behavior [48, 49]. The current experiment is limited by the use of whole amygdala tissue, making it difficult to establish the relevance of different sub-nuclei in these findings. Future studies utilizing immunohistochemistry would clarify the contributions of different subregions of the amygdala to the effects observed in the present study.

Effects of the pGluA1S831 Upregulator Tianeptine on Binge-Drinking

In Experiment 2, adolescent animals consumed more alcohol than adult animals both at baseline and during drug treatment under vehicle conditions. This result is in contrast to Experiment 1, where adolescent and adult alcohol intake was equivalent. Although adolescents have been reported to consume more alcohol than adults in some studies [50, 51], other reports have failed to find differences in
adolescent and adult intake [52, 53]. Procedural differences between experiments are likely to account for discrepancies in age differences across studies. Interestingly, the studies above that failed to find age differences in alcohol self-administration utilized daily or continuous access procedures (as in Experiment 1) whereas the studies that demonstrated increased alcohol consumption during adolescence made use of intermittent drinking protocols (as in Experiment 2). These findings suggest that intermittent drinking procedures may be advantageous for experiments in which age differences in alcohol consumption are under investigation, whereas daily drinking procedures may be useful when dose differences between ages represent a confounding variable.

Binge alcohol consumption increased pGluA1_{S831} in the adult amygdala. Mimicking this effect via systemic pretreatment with the AMPAR positive modulator Tianeptine dose-dependently decreased binge-like alcohol consumption in adult mice. Since Tianeptine increases pGluA1_{S831} in a CaMKII-dependent manner [54], it may have substituted for a pharmacological effect of alcohol in adult mice, leading to decreased alcohol consumption. This finding is consistent with previous studies which have shown that tianeptine pretreatment decreases alcohol intake [55] and attenuates alcohol withdrawal symptoms in adult rats [23]. Moreover, alcohol self-administration increases pGluA1_{S831} expression in adult mouse amygdala where AMPAR activity is required for the positive reinforcing effects of alcohol [13] and increased non-NMDA glutamatergic signaling in the amygdala is associated with alcohol conditioned reward [56]. Importantly, tianeptine pretreatment also decreased sucrose consumption in adult mice, suggesting that tianeptine may globally reduce
the consumption of palatable solutions. These findings add growing evidence that AMPAR activity is critical for the reinforcing effects of alcohol and other palatable solutions in adults.

In contrast to adults, binge-like alcohol drinking resulted in a trend for decreased pGluA1<sub>831</sub> in the adolescent amygdala. Opposing this alcohol-induced effect via systemic pretreatment with tianeptine increased binge-like alcohol consumption in this age group. Tianeptine may therefore have blocked a pharmacological effect of alcohol at AMPARs in adolescent mice, leading to a compensatory increase in alcohol drinking. The effects of tianeptine on adolescent binge drinking formed an inverted U-shaped dose-response curve, with an intermediate dose leading to increased alcohol consumption and a high dose returning to baseline. It is possible that testing a higher dose would reveal decreased alcohol consumption in adolescents, indicating a rightward shift of the dose-response curve in adolescents corresponding to the effect in adults. Significantly, tianeptine pretreatment produced comparable decreases in sucrose consumption in adolescent and adult mice, indicating that the increase in alcohol drinking seen after tianeptine pretreatment in adolescents is selective for alcohol and not generalized to other reinforcing solutions. The alterations in glutamate signaling reported here in the adolescent mouse may therefore be both selective for the adolescent brain and specific for alcohol. Together, these data provide evidence that CaMKIIα-GluA1 signaling differentially regulates binge-like drinking by adolescent and adult mice. Upregulation of AMPAR activity in the amygdala may promote increased drinking during adolescence but inhibit this behavioral pathology during adulthood. Future
studies that target AMPAR activity specifically in the amygdala would clarify the anatomical basis of this age-dependent differential response.

Tianeptine pretreatment failed to significantly alter open-field activity in both adolescents and adults, and blood alcohol clearance was similarly unaffected by tianeptine pretreatment in both ages, indicating a lack of effect of tianeptine on gross locomotor activity and alcohol metabolism. It is possible that tianeptine and alcohol may interact to alter locomotor behavior in adolescent and adult mice. While no obvious locomotor effects were observed during drinking, this possibility limits the interpretation of the locomotor results. Tianeptine may alter the metabolism of lower doses of alcohol but not the higher doses tested here, limiting the interpretation of the blood alcohol clearance experiments. Additionally, the injections administered in these experiments may have represented a significant stressor in the experimental design, potentially contributing to the age differences observed in alcohol drinking. However, zone analysis of adolescent and adult locomotor behavior under vehicle conditions revealed no evidence for anxiety-like behavior in either age, and tianeptine pretreatment did not alter time spent in the center or perimeter of the open field. Therefore, stress-related explanations for these findings are unlikely.

A potential limitation of these findings is the possibility for tianeptine activity at targets other than CaMKII/GluA1 signaling. Unlike most antidepressants, tianeptine has very low affinity for monoamine receptors or transporters [57] and the drug has not been found to interact directly with NMDA receptors [58]. However, activation of CaMKII also prompts the protein to bind with the NR2B subunit of the NMDA receptor [59]. Therefore, activation of CaMKII via tianeptine pretreatment could alter
NMDA receptor activity as well as GluA1\textsubscript{Ser831} phosphorylation. Additionally, a recent report suggests that tianeptine may have some affinity for the µ-opioid receptor [60], which could represent another mechanism for tianeptine’s effects on alcohol drinking. However, activation of µ-opioid receptors has been shown to be an upstream trigger of the signaling cascade that stimulates CaMKII activation [61], and therefore may be part of the pGluA\textsubscript{Ser831} potentiation seen after tianeptine treatment.

In conclusion, the findings from this study indicate that binge-like alcohol drinking impacts amygdala glutamate signaling systems of adolescent and adult animals in very different ways. Glutamate signaling in the amygdala, as indexed by CaMKII\textsubscript{α} and GluA1 phosphorylation, appears to be downregulated by alcohol in adolescence and upregulated in adulthood. Results from this study suggest that CaMKII\textsubscript{α}-GluA1 signaling in the adolescent amygdala is especially vulnerable to binge-induced insult. Understanding the age-dependent differences in the impact of binge alcohol drinking on glutamate signaling and how these systems, in turn, regulate intake is critical to understanding adolescent vulnerability to alcohol addiction.
REFERENCES


56. Zhu W, Bie B, Pan ZZ. Involvement of non-NMDA glutamate receptors in central amygdala in synaptic actions of ethanol and ethanol-induced reward behavior. The Journal of neuroscience : the official journal of the Society for


CHAPTER FIVE: GENERAL DISCUSSION

Adolescent alcohol drinking is both extremely prevalent and particularly dangerous, as it is associated with nearly a 50% increase in lifetime risk for developing an alcohol use disorder (AUD). Insights from research in alcoholic populations [2] and preclinical findings from rodent models [3] have suggested that the same process of synaptic plasticity that ushers the adolescent brain into maturity may also be responsible for the unique vulnerability of this age group to the lasting deleterious effects of alcohol use. However, the specific molecular mechanisms that mediate this developmental risk have not been completely elucidated. Thus, the overarching goals of this dissertation were to first identify novel targets of alcohol drinking in the adolescent brain and then to validate their involvement in behavioral processes that underlie vulnerability to alcohol addiction in adulthood.

In the present study, unbiased high-throughput proteomics and hypothesis-driven pharmacological assessments were combined to provide insight into developmental differences between the adolescent and adult brain that play a role in alcohol-related behaviors. First, a proteomics screen of the adolescent and adult medial prefrontal cortex (mPFC) in the absence of alcohol revealed substantial age-related alterations in protein expression and implicated functional protein networks involved in behavior and neurological disease as being affected by the immaturity
of the adolescent brain. The results of this study provide further evidence that adolescence is distinct from both childhood and adulthood and represents a period of significant protein-level change in the adolescent and adult mPFC following two weeks of intermittent home-cage alcohol drinking pointed to the increased vulnerability of the adolescent mPFC to the effects of alcohol drinking on protein expression. Alcohol drinking in adolescent mice was associated with profound alterations in proteins and interaction networks associated with learning and synaptic plasticity, in support of the hypothesis that the heightened plasticity during adolescent brain development confers some of the vulnerability to alcoholism observed following adolescent alcohol exposure. The protein calcineurin was chosen for functional assessment due to its identification in both the developmental and alcohol drinking proteomics experiments (downregulated by alcohol in adolescent mPFC) and its central role in regulating the plasticity of glutamatergic synapses. Initial behavioral pharmacology studies showed that adolescent mice were more sensitive than adult mice to reductions in alcohol drinking induced by the calcineurin inhibitor FK506, which is consistent with alcohol-induced downregulation of total calcineurin protein by alcohol drinking in this vulnerable age group. To assess the effects of calcineurin activity on the long-term risk for increased alcohol consumption following adolescent alcohol exposure, a model of adolescent alcohol drinking and subsequent operant responding for alcohol in adulthood was developed. Mice exposed to alcohol during adolescence exhibited increased operant self-administration compared with mice exposed to water during adolescence and mice exposed to alcohol during adulthood, indicating that
adolescent drinking increases the positive reinforcing effects of alcohol later in life. Mice with a history of adolescent alcohol drinking were also sensitive to FK506-induced decreases in operant responding for alcohol whereas mice with a history of adolescent water drinking and mice with a history of adult alcohol or water drinking were all insensitive to the effects of calcineurin inhibition. To establish the role of the mPFC in mediating some of the effects of calcineurin inhibition on operant responding for alcohol, FK506 was infused directly into the mPFC. Results showed that inhibition of calcineurin specifically in the mPFC increased the positive reinforcing effects of alcohol only in mice with a history of adolescent alcohol, but not water, consumption. As a whole, these findings indicate that calcineurin activity in the mPFC selectively regulates the reinforcing effects of alcohol during adulthood following adolescent alcohol exposure.

Lastly, the synaptic plasticity-associated protein calcium/calmodulin-dependent protein kinase II (CaMKII) was evaluated for sensitivity to binge-like alcohol drinking in adolescent and adult mice. CaMKIIα phosphorylation was decreased in adolescent but not adult mice selectively within the amygdala, whereas phosphorylation of the GluA1 subunit of the AMPA receptor was enhanced in adult mice but trended towards suppression in adolescent mice. To establish a functional role for CaMKII-GluA1 signaling in adolescent binge-like alcohol drinking, adolescent and adult mice were pretreated with the CaMKII-dependent pGluA1 enhancer tianeptine. Tianeptine increased binge drinking in adolescent mice but decreased binge drinking in adults, an effect that was selective for alcohol but not sucrose drinking. These findings provide a separate line of evidence that suggests
mechanisms of synaptic plasticity play a critical role in mediating immediate age differences between adolescent and adult alcohol drinking.

The experiments described above made use of three different procedures for modeling alcohol consumption in rodents: binge-like limited access single bottle alcohol drinking, 24-hour intermittent two-bottle choice alcohol drinking, and operant alcohol self-administration. Each of these procedures presents advantages and disadvantages in terms of their ability to replicate differing aspects of human alcohol use. Binge-like limited access drinking arguably provides the best model of adolescent-typical alcohol consumption available in mice. Binge drinking is extremely widespread amongst human adolescents who consume alcohol [4], and indeed has been reported to be the preferred method of alcohol consumption among this age group [5]. Every-other-day binge drinking has reliably modeled increased alcohol drinking in adolescent versus adult mice both in the present findings and in previous reports [6-9], further validating it as an accurate reflection of adolescent-typical drinking patterns observed in the human literature. The disadvantage of limited-access binge drinking is the limited access; although mice readily consume intoxicating quantities of alcohol during the binge drinking session, the total dose of alcohol achieved by each mouse is artificially limited by the experimenter. In the two-bottle choice 24-hour access model, mice receive one tube containing alcohol and one tube containing water for 24-hours every other day. This procedure is advantageous when higher alcohol consumption is desirable; in the present experiments, mice routinely achieved alcohol consumption in excess of 20 g/kg during 24-hour drinking sessions, whereas the limited access binge-drinking model
produced alcohol consumption of 4-6 g/kg over four hours. Both an advantage and disadvantage of the two-bottle choice 24-hour access procedure is the lack of age differences in alcohol drinking observed in this model in the present studies. Although this procedure does not recapitulate the age differences in alcohol consumption observed in humans, the similar alcohol consumption of adolescent and adult mice removes dose as a variable from the experiment, allowing brain and behavior changes to be attributed directly to developmental stage and not to merely increased or decreased intake between the two ages. Although this model is a less accurate reflection of the drinking patterns of a typical human adolescent, 24-hour drinking sessions are not unheard of in the human literature and the model does offer insight into what high-dose alcohol exposure may do to the adolescent brain, a topic that has gained additional relevance as extreme alcohol drinking continues to increase among adolescents in the US [10]. Operant self-administration of alcohol offers several advantages over home-cage drinking models. Operant behavior is far more quantitative than home cage drinking and affords the experimenter the ability to measure not just consumption, but motivation to consume alcohol (indexed via responding for alcohol), alcohol seeking (indexed via head entries into the reward trough) and a proxy measure of learning (indexed via the accuracy of responding on the active versus inactive lever) [11]. Operant behavior is also exquisitely sensitive to pharmacological manipulation as compared with home-cage drinking, making it a particularly attractive model for use in assessing the molecular mechanisms that regulate alcohol intake. The disadvantage of operant models is the limited amount of alcohol animals are able to self-administer; in the present experiments, mice
achieved doses of 1-2 g/kg over the course of a one-hour operant session, making this procedure even more limited than the limited-access binge-like alcohol drinking model.

The choice of which drinking model to use for a given experiment was predicated on the experimental question at hand. Thus, acute age differences in alcohol drinking were best assessed in a limited-access binge model in which those differences were most prominent, the effects of alcohol drinking on protein expression in the adolescent and adult brain benefitted from the highest dose of alcohol voluntarily consumed by the mice (i.e. 24-hour two-bottle choice drinking), and the pharmacological assessment of the longitudinal increased risk for alcohol consumption in adulthood following adolescent alcohol exposure called for the most sensitive method of quantitating intake possible, i.e. operant self-administration. However, it would be useful to conduct some of these same assessments in different models of alcohol drinking to clarify the effect of alcohol dose/access procedure on brain and behavioral actions of alcohol in the adolescent mouse. Of particular interest would be determining whether the protein-level changes observed in the high-dose 24-hour two-bottle choice experiments would also be observed in lower-dose drinking procedures that more accurately reflect adolescent alcohol drinking in humans, i.e. the binge-like limited-access procedure.

An important caveat about the interpretation of the present experiments in terms of alcoholism is that none of the three models used in these studies attempted to reflect alcohol dependence, withdrawal or relapse, three key characteristics of alcoholism in humans. The current results identified initial effects of alcoholism
during adolescence and long-term effects on adult alcohol consumption following adolescent exposure, but did not continue to the predicted outcome of alcohol dependence following adolescent exposure. Thus, important questions remain as to the mechanisms that translate escalated alcohol drinking following adolescent alcohol exposure into full-blown alcohol dependence. The model developed here could readily be adapted to address this question. In the adolescent exposure-adult operant model, a pause period was build into the procedure to isolate the effects of alcohol exposure during adolescence. To assess the effects of adolescent alcohol drinking on subsequent alcohol exposure, the pause period could be removed in favor of continuous access to alcohol throughout adolescence and into adulthood. Previous studies have demonstrated that intermittent two-bottle choice alcohol drinking produces physiological dependence upon alcohol (as indexed by tolerance and withdrawal) [12], making this procedure appropriate for assessments that more directly concern “alcoholism” in rodents. Alternatively, repeatedly cycles of intermittent binge alcohol exposure have similarly been shown to produce increased alcohol intake over time in a way that may reflect the early transition from use to dependence [13]. Such a procedure could be used to introduce age differences evident in binge-like alcohol drinking into the model to more accurately reflect the human condition. After dependence upon alcohol had been established, operant self-administration could be used as a model for relapse to alcohol seeking if mice were trained to self-administer alcohol, taken through extinction of operant responding, and then presented with an alcohol-associated cue to induce reinstatement of alcohol seeking. This procedure has been successfully used to
model relapse in previous studies [14, 15] and would be particularly appropriate to address this aspect of human alcoholism.

The experiments in this dissertation focused primarily on two brain regions: the prefrontal cortex (PFC) and the amygdala. The PFC was a major focus of this work because of its dual-role in the neurobiology of alcohol addiction and the neurobiology of adolescence. In both humans and rodents, the PFC has been shown to be critical for executive function; that is, learning and memory, decision making, and regulation of emotional reactivity and related behaviors [16]. The PFC exerts this regulatory control via projections to downstream brain regions involved in emotionally relevant stimuli and behavioral responses to them, most notably the nucleus accumbens, amygdala, ventral tegmental area, and the thalamus. Within the PFC, differing subregions have varying projections and associated functions. Generally, in humans the lateral PFC contains projections that extend to other cortical areas, the medial PFC (mPFC) contains dense connections to the thalamus, hippocampus and striatum, and the orbitofrontal cortex contains reciprocal connections with the amygdala, basal ganglia and other prefrontal subregions [17]. Rodents also have a PFC as defined by both structural projections similar those seen in primates and the functional impact of lesions to this same area on executive function [18]. The rodent PFC has similar subdivisions as the primate PFC, including a mPFC with similar projections to limbic brain areas that can be divided in the dorsal pre-limbic and ventral infra-limbic regions [19]. The topography of the rodent mPFC connections roughly matches the primate mPFC, with more dorsal regions (PL-mPFC) projecting to dorsal portions of the striatum and ventral regions (IL-
mPFC) projecting to ventral striatum, although fiber tracts in both subdivisions can project throughout the striatum [17]. (It should be noted that the present experiments did not differentiate the subregions of the mPFC.) Although the major projection neurons of the PFC are excitatory glutamatergic neurons, many of these projections terminate onto GABAergic interneurons within the striatum and amygdala [20]. In this way, excitatory output from the PFC inhibits the activity of limbic regions, underlying cortical regulation of emotional behavior.

Alcohol has direct effects on neurotransmission in the PFC. *In vitro* studies have demonstrated that acute alcohol reduces spontaneous spiking and upstates of glutamatergic neurons within the PFC [21]. *In vivo* animal studies have indicated that alcohol induces widespread alterations in neurotransmitter receptors within the PFC, including opioid [22], serotonin [23] and endocannabinoid receptors [24], as well as direct effects on glutamate concentration and receptors. Further, alcohol consumption-related behaviors can be manipulated with intra-PFC infusion of several neuromodulatory drugs [25, 26]. In studies with human participants, acute alcohol has been shown to inhibit several PFC-dependent behavioral tasks, including memory and decision making [17]. Acute alcohol also increases blood flow to the PFC at low doses but reduces blood flow at high doses [27]. Additionally, trans-magnetic cranial stimulation (TMS)-evoked PFC activity is reduced by acute alcohol [28]. In studies with alcoholic participants, reductions in PFC gray matter have been noted alongside reductions in white matter volume [29]. Together, these findings indicate that the PFC is both a site of the actions of acute alcohol as well as a regulator of some of the effects of acute alcohol on behavior.
As discussed in Chapter One, the PFC is one of the last brain regions to mature during adolescent development, and the delay in PFC maturation is thought to underlie some of the adolescent-typical behaviors exhibited during this epoch [30]. Thus, the effects of adolescence and alcohol on the brain would seem to converge on the PFC, making it an attractive target for housing cellular and molecular mechanisms of age differences in alcohol drinking and vulnerability to alcoholism following adolescent alcohol intake. Indeed, in Chapter Three significant qualitative differences in the response of the adolescent and adult mPFC to alcohol drinking were observed. Notably, many of these protein changes (including the focus protein calcineurin) were related to synaptic plasticity and learning and memory, both key features of cortical regulation of limbic brain regions and hallmarks of adolescent brain maturation. Previous evidence has indicated that alcoholics exhibit cognitive deficits as well as abnormalities in emotional processing which have been suggested to represent a persistence of the adolescence phenotype into adulthood induced by alcohol exposure [31]. The present findings accord with this view and suggest that the PFC may play a role in this phenomenon.

The amygdala, like much of the limbic system, develops earlier in adolescence than the PFC [32], but remains immature in terms of its functional connectivity to cortical brain regions throughout adolescence. Importantly, it has been demonstrated that PFC input from reward-associated brain regions such as the nucleus accumbens matures more rapidly than input from the aversion-associated amygdala, which may underlie the increased sensitivity to the rewarding effects of drugs of abuse but blunted sensitivity to the aversive properties observed in
adolescent rodents and humans [33]. Such a view of the relationship between the PFC and amygdala may be overly simplistic, since the amygdala (particularly the CeA and BLA) also mediates some of the reinforcing effects of drugs of abuse [34] and may indeed be a “salience detector” rather than an aversion response system (with regions in the extended amygdala such as the bed nucleus of the stria terminalis applying negative emotional valence to aversive stimuli) [35, 36]. What is clear is that across the course of adolescent brain development, functional connectivity between the PFC and amygdala shifts from a positive to a negative correlation; that is, in children increased activity of the PFC is associated with increased amygdala activity, but in adults increased PFC activity is associated with decreased amygdala activity [37]. Thus, even though the amygdala may be structurally mature during early adolescence, it remains immature in terms of its network-wide signaling until adulthood.

The present findings characterized the role of two synaptic plasticity-regulating enzymes in the effects of adolescent alcohol drinking. First, in the PFC calcineurin was found to be increased in adolescents relative to adults, to be sensitive to alcohol drinking in adolescents but not adults, and to regulate both acute alcohol drinking in adolescence and the long-term increases in alcohol drinking following adolescent alcohol exposure. Calcineurin is a negative regulator of synaptic plasticity; activation of this protein phosphatase prompts dephosphorylation of the GluA1 subunit of the AMPA receptor, reduced AMPA-mediated currents, and reductions in the magnitude of the postsynaptic response to glutamate. Thus, in the adolescent cortex alcohol drinking would be predicted to have a net effect of
enhanced AMAPA receptor signaling (via reductions in calcineurin expression and activity), and potentially increase glutamatergic output of the PFC onto downstream limbic brain regions.

In the amygdala, alcohol drinking reduced the phosphorylation of CaMKIIα in adolescent but not adult mice; accordingly, alcohol drinking was associated with increases in GluA1 phosphorylation in the adult but not adolescent amygdala. Taken together with the findings in the PFC, the results of these experiments suggest that increased excitatory output of the PFC onto inhibitory interneurons within the amygdala may reduce alcohol-associated activity in that region. As amygdala glutamate signaling has been shown to be involved in alcohol self-administration in adult rodents, this inhibition of a pharmacological effect of alcohol may drive adolescent mice to consume more alcohol in order to achieve an intoxicating dose. Adolescent rodents and humans have repeatedly been shown to be less sensitive to the acute effects of alcohol than their adult counterparts [38], and the present findings provide a hypothesis as to the molecular mechanisms behind this behavioral phenotype. Thus, systemic enhancement of CaMKII-GluA1 signaling may drive increased alcohol self-administration in adolescents by blocking a direct pharmacological effect of alcohol (i.e. reductions in CaMKII-GluA1 activity) in the amygdala, whereas mPFC-specific calcineurin inhibition increases alcohol self-administration via indirect blockade of this pharmacological effect via increased PFC inhibition of amygdala activity. This hypothetical model is illustrated in Figure 23.
Figure 23. Proposed model of effects of alcohol on synaptic plasticity in the adolescent brain. (A) At baseline, calcineurin (PPP3) activity in the PFC (left) and CaMKII activity in the amygdala (right) maintain normal balance of PFC glutamatergic output onto inhibitory GABAergic interneurons in the amygdala (center). (B) Under the influence of alcohol, both calcineurin and
CaMKII activity are blunted in the PFC and amygdala, respectively, leading to net reductions in GluA1 phosphorylation and related activity in the adolescent amygdala. These features may lead to a blunted response of the adolescent amygdala to the presence of alcohol, requiring adolescents to consume a higher dose in order to achieve subjective intoxication.

Although the present findings support this hypothesis, several additional experiments would more concretely establish a role for PFC regulation of amygdala responses to alcohol in adolescent alcohol drinking. First, additional assessments of the protein-level changes in the PFC and amygdala induced by adolescent alcohol drinking are necessary. In Chapter Two, CaMKII was shown to exhibit reduced phosphorylation in the adult but not adolescent PFC. Reductions in CaMKII activity would reduce glutamatergic activity in the PFC, and could therefore be a compensatory response to avoid the inordinate glutamatergic output to limbic regions that has been hypothesized to go awry in the adolescent brain. However, the effects of alcohol on calcineurin expression and activity in the amygdala have not been assessed in these experiments. Immunoblotting or immunohistochemistry would provide important insight into the response of calcineurin to alcohol in the adolescent amygdala.

Secondly, the changes in protein expression observed in the present work have been predicted to alter glutamatergic neurotransmission in the PFC and amygdala, respectively, but assessment of AMPA signaling in both regions using electrophysiology would directly test that hypothesis. Based on the protein-level and
pharmacological findings, adolescent alcohol exposure would be predicted to enhance AMPA currents in the PFC and reduce AMAPA currents in the amygdala.

Thirdly, the net effect of adolescent alcohol is herein proposed to blunt glutamate signaling in the amygdala, thereby necessitating more alcohol consumption to achieve intoxicating effects in adolescent versus adult mice. Reductions in the subjective effects of alcohol have been previously associated with increases in alcohol consumption [39], and glutamate activity specifically within the amygdala has been shown to underlie some of the discriminative-stimulus properties of alcohol [40]. Additionally, adolescent rats have been shown to display blunted sensitivity to the discriminative stimulus effects of alcohol [41]. Although all of these findings provide additional support for the proposed hypothesis, a direct assessment of the role of glutamate signaling in adolescent alcohol discrimination would be better evidence. Adolescent and adult mice could be trained to discriminate injected alcohol from saline, then administered tianeptine systemically as in Chapter Four to test for age differences in sensitivity to the effect of enhanced CaMKII-GluA1 signaling on the interoceptive effects of alcohol. Enhancement of amygdalar CaMKII-GluA1 signaling would be predicted to restore adult-typical enhancement of this activity following alcohol consumption and would therefore most likely confer sensitivity to the interoceptive effects of lower doses of alcohol in adolescent mice. Further, discrimination training of adult mice with a history of adolescent alcohol or water drinking would determine whether any evident age differences in glutamate regulation of the subjective effects of alcohol persist following adolescent alcohol exposure. Microinjection of tianeptine into the amygdala could then be assessed in
the discrimination procedure, and would be predicted to restore sensitivity to the interoceptive effects of lower dose alcohol in mice with a history of adolescent alcohol drinking.

Lastly, the proposed hypothesis involves signaling changes between the PFC and amygdala mediated by glutamatergic transmission. To conclusively demonstrate the relevance of this circuitry in adolescent alcohol drinking, an ideal experiment would be to utilize chemo- or optogenetic enhancement of PFC-amygdala projection neurons in adolescent and adult mice. Based on the present findings, excitation of PFC-amygdala glutamatergic projection neurons would be predicted to increase alcohol consumption in adolescents but not adults. Unfortunately, due to the limited period of adolescence in rodent models (approximately two-four weeks), time for viral transfection and surgical recovery are insurmountable obstacles. However, such an experiment would be more feasible in adult mice with a history of adolescent alcohol or water drinking, respectively, where enhanced PFC-amygdala excitation would be expected to increase alcohol consumption selectively in subjects with a history of adolescent alcohol drinking.

Age differences in adolescent behavior and brain function have been well-characterized in the literature, but the connections between these phenomena and the degree to which they relate to increased risk for alcoholism following adolescent exposure have been difficult to establish. As the results of the age comparison experiments show, the adolescent brain is in a unique developmental stage that is completely distinct from the adult state at the protein level in the PFC. Alcohol induces widespread alterations in protein networks within the adolescent PFC,
including the synaptic plasticity-associated proteins CaMKII and calcineurin, and seems to produce more lasting alterations in the adolescent cortex versus the adult cortex. Pharmacological manipulation of CaMKII and calcineurin point to a regulatory role for these proteins in mediating some of the acute age differences in adolescent and adult alcohol drinking as well as the long-term risk for increased alcohol drinking following adolescent alcohol exposure. These findings support the hypothesis that the increased activity and expression of plasticity-associated proteins in the adolescent brain may underlie adolescent vulnerability to addiction during adulthood. The results also suggest that such changes may persist into the adult organism, leading to calcineurin regulation of alcohol drinking selectively in individuals with a history of adolescent alcohol exposure. This finding points to age of drinking onset as a potential means of distinguishing different types of alcoholics for targeted prevention and treatment efforts. Problem drinking that begins during adolescence may impact distinct neurotransmitter systems from problem drinking that develops in midlife, and patients with adolescent alcohol exposure may respond differently to medications for alcoholism treatment. This type of analysis may help retrospective reviews of published clinical trials make sense of individual differences in drug efficacy as well as guide the analysis of future studies in humans. Finally, the current experiments provide further justification for the continued development of pharmacological and neurobehavioral treatments for alcoholism based on altering glutamatergic plasticity. Both the field at large and these results specifically strongly caution against underage drinking. However, they also offer hope that the lasting
effects of adolescent alcohol drinking may still be sensitive to therapeutic intervention in adulthood.
REFERENCES


