

**Alcohol Exposure Leads to Differential Neuroadaptations in the ERK/MAPK Signaling
Pathway in Adolescents as Compared to Adults**

Marina Spanos

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in
partial fulfillment of the requirements for the degree of Doctor of Philosophy in the
Neurobiology Curriculum.

Chapel Hill

2010

Approved By:

Dr. Clyde Hodge

Dr. Todd Thiele

Dr. Darin Knapp

Dr. Joyce Besheer

Dr. Josephine Johns

ABSTRACT

Marina Spanos: Alcohol Exposure Leads to Differential Neuroadaptations in the ERK/MAPK Signaling Pathway in Adolescents as Compared to Adults

(Under the Direction of Dr. Clyde W. Hodge)

Evidence suggests that behavioral and neurobiological changes may leave adolescents vulnerable to experimenting with drugs of abuse and to drug-induced neuroadaptations. Neuroadaptations in cell signaling pathways mediate behavioral pathologies in drug addiction including alcohol addiction. The ERK/MAPK signaling pathway has received attention in addiction due to its fundamental role in neuroplasticity and other cellular processes. However, the influence of this signaling pathway as a potential mediator of adolescent vulnerability to alcohol has not been studied. Results of this dissertation show that adolescent C57BL/6J mice are more sensitive to acute alcohol-induced changes in ERK1/2 activity than their adult counterparts. Increased alcohol-induced p-ERK1/2 in the BLA during adolescence may mediate associative learning processes that could lead to a potential addiction during adulthood. Decreased alcohol-induced p-ERK1/2 in the hippocampus was associated with age-related differences in novel object recognition task (NOR). Increased sensitivity in the ERK/MAPK pathway during adolescence may allow for immediate consequences including those observed in the NOR test or more long-term changes that are brought about by the first exposure to alcohol. Chronic alcohol exposure shows that inhibition of p-ERK1/2 caused an age-dependent effect on alcohol intake (g/kg) in C57BL/6J mice. Systemic administration of the MEK/ERK inhibitor, increased alcohol

intake in adolescent mice. Since chronic alcohol self-administration increased p-ERK1/2 in a brain region known to regulate self-administration (CeA) these data are consistent with the hypothesis that p-ERK1/2 in the CeA is an important pharmacological effect of alcohol that maintains self-administration in adolescents. Adolescents exposed to chronic alcohol prior to an acute stressor had unaffected CORT responses however, adult CORT responses were blunted by alcohol self-administration. Stress-induced, circulating levels of glucocorticoids may increase the pleasurable effects of drinking and HPA axis activation is thought to contribute to initiation, maintenance and relapse to alcohol abuse. An inability of the HPA axis to adapt, to stress may make the adolescent brain more vulnerable to a future addiction. Overall, a lack of sensitivity to the negative effects of alcohol and an increase in the pleasurable effects of alcohol during adolescence may increase their vulnerability to future alcohol dependence.

To my parents Aris and Evie, sisters Stella and Alexia and husband Aaron this could never have happened without your support, help and unconditional love. To my little one I have not forgotten you.

ACKNOWLEDGEMENTS

This dissertation would not have been completed without the guidance, opportunities and encouragement afforded to me by my advisor Dr. Clyde W. Hodge. I must also thank the members of my committee Drs. Todd Thiele, Josephine Johns, Darin Knapp and Joyce Besheer who were invaluable with their suggestions, and critiques throughout this process. I would like to acknowledge Dr. Joyce Besheer who patiently spent a great deal of her time helping me become the scientist I am today. I would also like to thank all the members of the Hodge lab who have helped me through the years and encouraged me to keep on going. Finally, I would like to thank my family and friends (you know who you are) for making this process a very fulfilling experience and one that I will never forget.

TABLE OF CONTENTS

LIST OF TABLES.....	viii
LIST OF FIGURES.....	ix
LIST OF ABBREVIATIONS.....	xi
Chapter	
I. GENERAL INTRODUCTION	1
1.1. Alcoholism.....	1
1.2. Adolescent Critical Period.....	2
1.3. Neurobiological Changes During Adolescence.....	3
1.4. Increased Behaviors During Adolescence.....	6
1.5. Adolescence And Stress.....	7
1.6. Adolescence, The HPA Axis (Stress) And Alcohol.....	8
1.7. Differential Response To Alcohol.....	10
1.8. The ERK/MAPK Pathway.....	11
1.9. ERK1 and ERK2.....	12

1.10.	ERK 1/2 and Stress.....	13
1.11.	Effects Of Alcohol On The ERK/MAPK Pathway.....	15
1.12.	Summary And Overall Goals Of This Research.....	17
II.	ACUTE ALCOHOL ADMINISTRATION ALTERS EXTRACELLULAR-SIGNAL REGULATED KINASE ACTIVITY IN AN AGE AND BRAIN REGION DEPENDENT MANNER IN C57BL/6J MICE.....	23
2.1.	Introduction.....	23
2.2.	Methods.....	26
2.3.	Results.....	29
2.4.	Discussion.....	33
III.	NOVEL MOLECULAR MECHANISM OF INCREASED ADOLESCENT ALCOHOL DRINKING.....	48
3.1.	Introduction.....	48
3.2.	Methods.....	51
3.3.	Results.....	55
3.4.	Discussion.....	58
IV.	DIFFERENCES IN AGE RELATED BINGE-LIKE ALCOHOL CONSUMPTION: CONDITIONED TASTE AVERSION AS A POSSIBLE BEHAVIORAL MECHANISM.....	70
4.1.	Introduction.....	70
4.2.	Methods.....	73

4.3. Results.....	77
4.4. Discussion.....	78
V. AGE-DEPENDENT EFFECTS OF VOLUNTARY DRINKING ON STRESS AND THE ERK/MAPK PATHWAY.....	88
5.1. Introduction.....	88
5.2. Methods.....	90
5.3. Results.....	95
5.4. Discussion.....	98
VI. CONCLUSIONS AND FUTURE DIRECTIONS.....	105
VII. REFERENCES.....	114

LIST OF TABLES

Table 2.1. Blood alcohol concentration (BAC) 30 minutes after acute alcohol injection in adolescent and adult mice.....	38
Table 2.2. Basal levels of adolescent and adult p-ERK1/2 Immunoreactivity.....	39
Table 2.3. Effect of p-ERK1/2 immunoreactivity in adolescent and adult brain.....	40
Table 3.1. Basal levels of p-ERK1/2 IR in ethanol naïve adolescents and adult mice in specific mesocorticolimbic brain regions.....	63
Table 3.2. Effects of ethanol drinking on p-ERK1/2 IR in mesocorticolimbic brain regions from adolescent and adult mice.....	64

LIST OF FIGURES

Figure 1.1. Representation of the ERK1/2 signaling pathway.....	22
Figure 2.1. Novel Object Recognition Task.....	41
Figure 2.2 Effect of acute alcohol (3g/kg) on p-ERK1/2 IR in the amygdala of adolescents and adult mice.....	42
Figure 2.3. Effect of acute alcohol (1g/kg) on p-ERK1/2 IR in the dentate gyrus of the hippocampus in adolescent and adult mice.....	43
Figure 2.4. Effect of acute ethanol (3 g/kg) on p-ERK _{1/2} IR in the dentate gyrus of the hippocampus in adolescent and adult mice.....	44
Figure 2.5. Sample object exposure 1 and 2.....	45
Figure 2.6. Age-dependent effect of acute ethanol (1 g/kg) on adolescent and adult performance in the novel object recognition task.....	46
Figure 2.7. Measures of locomotor activity in ethanol (1 g/kg) treated adolescent and adult mice during the novel object task.....	47
Figure 3.1. Measures of voluntary ethanol drinking as a function of developmental stage.....	65
Figure 3.2. P-ERK1/2 response in the central nucleus of the amygdala and representative photomicrographs.....	66
Figure 3.3. P-ERK1/2 response in the dentate gyrus of the hippocampus and representative photomicrographs.....	67
Figure 3.4. Alcohol intake after SL327 injection in adolescent and adult mice.....	68

Figure 3.5. The effect of the MEK/ERK inhibitor SL327 on locomotor activity.....	69
Figure 4.1. Drinking in the Dark Procedure.....	83
Figure 4.2. Alcohol intake and blood alcohol concentration in adolescent and adult mice in the 4 hr binge procedure.....	84
Figure 4.3. Age of Onset of Binge Drinking.....	85
Figure 4.4. Adult (PND 93-107) alcohol intake and age of binge alcohol onset.....	86
Figure 4.5. Adolescent mice are less sensitive to alcohol-induced conditioned taste aversion than adult mice.....	87
Figure 5.1. Basal and stress-induced changes in plasma corticosterone in adolescent and adult mice.....	102
Figure 5.2. Alcohol self-administration and corticosterone response to stress in alcohol experienced and naïve mice.....	103
Figure 5.3. Effect of stress on p-ERK1/2 IR in the PVN and CeA of adolescent and adult mice with a history of alcohol drinking.....	104

LIST OF ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
BDNF	brain derived neurotrophic factor
BAL	blood alcohol levels
BLA	basolateral amygdala
CeA	central nucleus of the amygdala
CORT	corticosterone
CPP	conditioned place preference
CREB	cyclic AMP response element-binding protein
CRH	corticotrophin-releasing hormone
CTA	conditioned taste aversion
DA	dopamine
DG	dentate gyrus
DID	drinking in the dark
ERK1/2	extracellular signal-regulated kinases 1 and 2
ETOH	ethanol
GPCR	G-protein coupled receptor
GR	glucocorticoid receptor
HPA	hypothalamic-pituitary-adrenal axis
IHC	immunohistochemistry
IP	Intra-peritoneal
IR	immunoreactivity
LTP	long-term potentiation
MAPK	mitogen activated protein kinase
NESARC	national epidemiologic survey on alcohol and related conditions
NMDA	N-methyl-D-aspartic acid

NOR	novel object recognition
NuAcc	nucleus accumbens
PFC	prefrontal cortex
PND	post natal day
SHAC	scheduled high alcohol consumption
SOS	son of sevenless
SRE	serum response element

CHAPTER 1: GENERAL INTRODUCTION

ALCOHOLISM

Alcohol is the most widely abused psychoactive drug in the United States. More than 50% of Americans have a close family member who has been diagnosed with alcohol abuse problems (Dawson and Grant 1998). A study in 2002 by the National Epidemiologic Survey on Alcohol and Related Conditions (NESARC) found that approximately 18 million Americans either abuse alcohol or are alcohol dependent. The DSM-VI defines alcohol abuse as repeated use despite recurrent adverse consequences. Such consequences include social/legal problems as well as an inability to fulfill obligations at work and home. Alcohol dependence or alcoholism includes the symptoms of alcohol abuse as well as several other criteria including craving or “compulsion” to consume alcohol, an inability to stop drinking, tolerance and finally withdrawal. A combination of genetic and environmental influences increases an individual’s risk to develop such conditions. One of the strongest genetic factors that could predict alcohol abuse problems includes a family history of alcoholism (Bierut, Dinwiddie et al. 1998). Efforts to develop effective treatments for alcoholism have been improved with the recognition of alcoholism as a disease. To date, there are four approved medications for the treatment of alcohol dependence these include disulfiram, acamprosate, naltrexone (oral), and naltrexone (injectable). All approved medications have shown some efficacy however the results are often inconsistent. It is thought that more efficacious pharmacotherapies could be developed through increased knowledge of the mechanisms of alcohol dependence (Garbutt 2009).

Differences in rates of alcohol dependence exist between men and women as well as between various ethnicities. Alcohol dependence rates are higher in men than women and the prevalence of alcohol dependence is higher in Caucasians, Native Americans, and Hispanics compared with Asians and African Americans (Dawson 2004). Age-related differences in alcohol dependence are also quite prevalent. The rates of alcohol abuse are highest in 18-29 year olds (Grant et al., 2004). Adolescent drinking is a major problem in the United States and is important to address because it has been estimated that over three million teenagers are alcohol dependent and several million more have a serious drinking problem that they cannot manage on their own (Grant et al., 2004). It is also important to note that the earlier an individual begins drinking the more likely they are to develop alcohol dependence problems later in adulthood (Dawson et al., 2008).

Much of the research on alcohol dependence focuses on understanding cellular signaling pathways and mechanisms of alcoholism in adults. However, as can be observed by the epidemiological data above, adolescence is the developmental stage where drinking is initiated and more importantly drinking during this time period has long-term effects on future alcohol dependence. The information presented here will expand the current knowledge by addressing a key cellular signaling system that may be altered in adolescents and adults upon alcohol exposure. Studying this developmental stage is crucial to our understanding of the effects of alcohol during adolescence and how these effects then lead to dependence problems in the future and allow for the development of pharmacotherapies to prevent alcoholism.

ADOLESCENT CRITICAL PERIOD

Adolescence represents a critical stage in development that marks the transition from childhood to adulthood. Adolescence is characterized by numerous behavioral, hormonal and neurobiological changes and not strict age limits. In humans, the age range of

adolescence is between 9-18 years old however some researchers believe that individuals in their early to mid twenties could also be included (Baumrind 1987; Buchanan, Eccles et al. 1992; Petersen, Compas et al. 1993; Spear 2000). In rodents, this age range includes post natal days (PND) 28-45 although this range also can vary (Spear 2000). Puberty or attainment of sexual maturity is one of the developmental changes occurring during adolescence and involves activation of the hypothalamic-pituitary-adrenal (HPA) axis (Buchanan, Eccles et al. 1992). The HPA axis is one of the physiological systems involved with coping with environmental challenges (McCormick, Merrick et al. 2007). Behavioral and cognitive systems mature at different rates during adolescence (Paus, Zijdenbos et al. 1999). Limbic motivational systems mature in early adolescence and cortical executive control systems mature later in adulthood (Bourgeois, Goldman-Rakic et al. 1994; Paus, Zijdenbos et al. 1999; Casey, Giedd et al. 2000). The disjunction in maturation of behavioral and cognitive systems during this critical developmental period leaves the adolescent vulnerable to increased emotional drive and decreased decision making abilities which then often leads to an increased risk of emotional/ behavioral problems including substance abuse (Steinberg 2005; White 2009).

NEUROBIOLOGICAL CHANGES DURING ADOLESCENCE

Numerous neurobiological changes take place during adolescence. There is a large peak in grey matter volume during adolescence that then declines into adulthood (Giedd, Blumenthal et al. 1999; Giedd 2004). It is thought that this decline is due to an overproduction of axons and dendrites in adolescence and then subsequent pruning later in adolescence (Giedd, Blumenthal et al. 1999; Gogtay, Giedd et al. 2004; Toga, Thompson et al. 2006). There is a substantial loss of synapses in specific areas of the adolescent brain including the prefrontal cortex (PFC), hippocampus, Nucleus accumbens (NuAcc) and amygdala (Tarazi, Tomasini et al. 1998; Sowell, Thompson et al. 2001; Zehr, Todd et al.

2006). There is culling of close to 50% of synaptic connections in some cortical regions (Rakic, Bourgeois et al. 1994). The result of synaptic pruning is greater focal cortical activity, which can be viewed as more efficient information processing as the adolescent transitions to adulthood. Progressive myelination of axons from adolescence to adulthood results in considerable increase in white matter which is presumed to increase the speed of information processing within the brain (Sowell, Peterson et al. 2003; Wozniak and Lim 2006). Increases in both synaptic pruning and myelination indicate that the adolescent time period is characterized by refinements in brain processing that help to sustain integration of information and encourage higher-order cognitive processes (Goldman-Rakic 1988). Although information processing is near adult levels in adolescence, the continued increases in myelination and synaptic pruning indicate limitations in connectivity. The neurocircuitry in adolescence therefore is similar to that of adults but with immaturities that allow the adult more effective control of executive functions (Geier and Luna 2009; Luna 2009).

Overall, the prefrontal cortex takes much longer than the limbic system to develop during adolescence (Steinberg 2005). Among the alterations observed in the limbic and prefrontal cortical regions are specific alterations in dopamine (DA) production and utilization. DA synthesis and turnover is higher in early adolescence than in late adolescence and adulthood in the PFC (Tarazi, Tomasini et al. 1998). In fact, dopamine innervation of the PFC peaks during adolescence and stabilizes into adulthood (Kalsbeek, Voorn et al. 1988; Rosenberg and Lewis 1994; Andersen, Thompson et al. 2000; Tarazi and Baldessarini 2000). Conversely, in the nucleus accumbens and striatum dopamine levels peak and then decline into adulthood (Teicher, Andersen et al. 1995; Tarazi and Baldessarini 2000). Therefore, during adolescence there is a shift from mesolimbic

dopaminergic control (reward) to more mesocortical dopaminergic control (cognitive function)(White 2009).

Glutamate synapses are pruned during adolescence which accounts for some of the reduction in grey matter (Zecevic, Bourgeois et al. 1989). Synaptic pruning of glutamatergic excitatory neurotransmission produces a loss of one-third of NMDA receptors by post-natal day 60 (adulthood) and a loss of excitatory glutamate input to the limbic brain regions (Tarazi and Baldessarini 2000). The loss of glutamate synapses also produces changes in long-term potentiation (LTP) an effect allowing greater induction during adolescence and a reduction into adulthood (Izumi, Kirby-Sharkey et al. 1995; Liao and Malinow 1996; Schramm, Egli et al. 2002). LTP is a cellular mechanism of synaptic plasticity that underlies learning and memory. Age-related differences in induction of LTP could promote improved learning and memory during adolescence including negative learning processes such as those of addiction.

GABAergic neurotransmission is also altered during adolescence. GABAergic remodeling of synapses occurs from adolescence through adulthood (Behringer, Gault et al. 1996). GABA A receptor expression during early adolescence is stable and then rapidly increases from mid-adolescence to adulthood (Laurie, Wisden et al. 1992). Remodeling of GABAergic neurotransmission has been thought to improve signal discrimination and increase processing (Crews, He et al. 2007).

Neurobiological changes during this critical developmental period may underlie the known vulnerability for drug use during adolescence to lead to drug abuse problems in adulthood (Grant and Dawson 1998). A developing prefrontal cortex increases impulsivity and allows the adolescent to disregard negative consequences of their behavior (Steinberg 2005). In addition, a developing nucleus accumbens increases the tendency of adolescents

to seek out novel/exciting activities that involve little effort. It has also been suggested that the effect of an immature amygdala is that adolescents subjectively report greater feelings of social disinhibition when drinking alcohol compared to adults (Spear 2002; White 2009). Increases in adolescent social disinhibition when drinking are thought to promote a more rewarding social experience. The developing brain during the adolescent time point exhibits impulsivity without thought to negative consequences, and increased sensitivity to alcohol-induced social disinhibition these positive effects may promote the initial use of alcohol and also encourage future use (Spear 2002; Steinberg 2005; White 2009).

INCREASED BEHAVIORS DURING ADOLESCENCE

Neurobiological changes to the adolescent brain may have an effect on adolescent behaviors (Kelley, Schochet et al. 2004). Increases in risk taking, novelty seeking and peer-directed social interaction are hallmarks of adolescent behavior (Kelley, Schochet et al. 2004). Engaging in risk taking, novelty seeking and peer-directed social interaction produces feelings of positive arousal and independence and may help adolescents mediate the developmental transition from dependence to independence (Spear 2000). As a result, however, epidemiological data indicate that human adolescents are more involved in the expression of risky novel behaviors, including drug abuse, compared to adults (Laviola, Macri et al. 2003). It has also been suggested that high levels of novelty-seeking and risk taking during adolescence are good predictors of alcohol and drug use (Wills, Vaccaro et al. 1994). Increased peer-directed social interaction is also prevalent during adolescence. Adolescents spend one-third of their time with peers and less than 10% of their time communicating with adults. Drugs of abuse, specifically alcohol, help to facilitate peer interactions and are closely related to peer-social activity (Smith, Goldman et al. 1995; Beck and Treiman 1996).

ADOLESCENCE AND STRESS

Adolescents face a number of life transitions and as such adolescence is considered to be a stressful time of life (Allen and Matthews 1997). The sources of adolescent stress are many and include developmental challenges associated with puberty and the move towards independence (Allen and Matthews 1997; Spear 2000). Data suggest that the perception of stress may be increased in adolescents relative to adults (Seiffge-Krenke, Aunola et al. 2009). Specifically, data indicate that adolescents experience high levels of perceived stress during early adolescence up to age of 15, after which perceived stress begins to decrease into adulthood (Seiffge-Krenke, Aunola et al. 2009). Researchers have postulated that the presumed increase in anxiety and stress during adolescence contributes to the frequent initiation of alcohol or other drug use observed in adolescents (Wagner, 1993)(Spear 2000). Adolescents who begin using alcohol under these life stressors have been shown to adopt continued and more frequent alcohol use as a strategy of coping with their problems (Pohorecky 1991; Aseltine and Gore 2000). Stress is most convincingly associated with alcohol consumption in adolescence, with mixed findings evident in studies conducted in adults (Pohorecky 1991). It has also been observed that after peer substance use, the most powerful predictor of adolescent alcohol and drug use, was level of perceived stress (Wagner, Myers et al. 1999).

Studies in laboratory animals also indicate that adolescents are more negatively affected by stressful events than adults (Stone and Quartermain 1997; Wagner, Myers et al. 1999; Romeo, Lee et al. 2004). This difference in stress response is thought to be due to the developing stress response system (HPA axis) (Stone and Quartermain 1997). Chronic social stress brought on by a short daily encounter with a an unfamiliar adult male mouse for 5 days produced higher levels of plasma corticosterone in adolescent than adult male mice (Stone and Quartermain 1997). Adolescent rats show more immobility under stressful

situations, such as a forced swim test than adult mice (Walker, Trottier et al. 1995). Rats in early adolescence have a delayed rise and more prolonged corticosterone release to several different types of acute stressors compared with adults (Romeo, Lee et al. 2004; Prendergast and Little 2007). Specifically, adolescent and adult rats have a significant elevation in plasma corticosterone levels after the stressor, but a higher level was maintained in adolescent and not adult rats 45 minutes later (Romeo, Lee et al. 2004). In adult rats, it has been found that repeated restraint stress or chronic alcohol use decreases the HPA response (Wand and Dobs 1991). The reduced HPA response (blunting) helps protect the animal from high levels of circulating glucocorticoids while allowing the HPA axis to respond to a different type of stressor (Romeo, Bellani et al. 2006). The neuroadaptation or blunting of the HPA axis due to prolonged alcohol exposure has not been studied in adolescent animals and could be a critical difference in the response of adolescents and adults to both stress and drugs of abuse.

ADOLESCENCE, THE HPA AXIS (STRESS) AND ALCOHOL

The behavioral, hormonal and neurobiological changes that take place during this critical stage in development increase the susceptibility of the adolescent to experiment with drugs of abuse and to consequent drug-induced neuroadaptations (Grant and Dawson 1997; Crews, He et al. 2007; White 2009). Exposure to stressors and alcohol activate the HPA axis and set up a cascade of events which include hormone release from the hypothalamus (CRH), which binds to the corticotrophin-releasing hormone receptor (CRHR) in the pituitary gland and stimulates the release of the second-messenger cAMP in the target cells and production of adrenocorticotrophic hormone (ACTH) (Prendergast and Little 2007). The primary target of ACTH is the adrenal gland which releases the glucocorticoids (corticosterone in rodents, cortisol in humans) in the blood stream (Prendergast and Little 2007). Activity within the HPA axis is under close negative feedback regulation, with the

glucocorticoids released after stress and then acting on glucocorticoid receptors in brain regions such as the hippocampus, amygdala, and prefrontal cortex to terminate the function of the HPA axis (Prendergast and Little 2007).

Stress (HPA activation) has been observed to increase, decrease or not influence alcohol consumption (Fidler and LoLordo 1996; Lynch, Kushner et al. 1999; Le, Harding et al. 2005). Although alcohol is consumed in part due to its anxiolytic properties, it activates the HPA axis causing release of corticosterone (Spencer and McEwen 1990). Corticosterone has been found to be reinforcing as adult rodents will self-administer a corticosterone solution (Piazza, Deroche et al. 1993). Activation of the HPA axis (stress) has been found to be an important factor for initiation, maintenance and relapse to alcohol abuse (Brown, Vik et al. 1990; Brady and Sonne 1999; Overstreet, Knapp et al. 2004). Adolescents who are stressed seem to adopt more continued and frequent alcohol use as a way to cope with their problems (Pohorecky 1991). It has also been found that the perception of stress may be increased in adolescents relative to children and adults (Seiffge-Krenke, Aunola et al. 2009). Given such data, it would suggest that adolescents are more sensitive to stress-induced drinking.

Developmental differences have been observed in HPA axis (stress) responses to acute alcohol (Silveri and Spear 2004; Song, Wang et al. 2007). Stress seems to play a role in acquisition of alcohol conditioned place preference (CPP) in adolescent mice. Adolescent mice were found to be less sensitive to the acquisition of alcohol CPP than adult mice (2g/kg). However, they acquired alcohol CPP(2g/kg) after one week of chronic stress a finding indicating that adolescent mice were more sensitive than the adults to stress (Song, Wang et al. 2007). Furthermore, using acute doses of alcohol (1.5g/kg and 4.5g/kg) as an activator of the HPA axis significant age differences were observed in corticosterone levels at 40 minutes, in fact, plasma corticosterone levels peaked in adolescence (PND 36) and

decreased into adulthood (PND 56) (Silveri and Spear 2004). Developmental differences in response to more chronic alcohol exposures and the effect of such exposures on HPA axis responses have not been examined to date. This gap in knowledge should be addressed given the increased sensitivity of adolescents to stress and drugs of abuse.

DIFFERENTIAL RESPONSE TO ALCOHOL

As previously discussed adolescence is a time of considerable neural restructuring of the brain (Steinberg 2005). Remodeling of the adolescent brain may underlie differential responses to alcohol in adolescents and adults that are often observed (White 2009). Studies have indicated that adolescent rodents are more sensitive to some of the acute effects of alcohol. These alcohol-induced behaviors include: hippocampal-dependent spatial memory, disruptions of hippocampal NMDA receptor mediated neurotransmission and long-term potentiation, locomotor stimulation, ataxia, anxiety, conditioned place preference, sensitization and social interaction (Markwiese, Acheson et al. 1998; Varlinskaya and Spear 2002; Philpot, Badanich et al. 2003; Yttri, Burk et al. 2004; Hefner and Holmes 2007). In contrast, other studies in rodents have shown that adolescents are less sensitive than adults to alcohol induced motor-impairments, intoxication, analgesia and sedation than adult rats (Hollstedt, Olsson et al. 1980; Spear 2000; White, Roberts et al. 2002; White, Truesdale et al. 2002).

A lower sensitivity to the effects of alcohol would be consistent with the observation that young people are capable of drinking large amounts of alcohol without intoxication. These differences in sensitivity to alcohol are important because it has been shown that a decreased sensitivity to alcohol during adolescence is a powerful predictor of future alcohol abuse (Schuckit 1994). Identifying neuroadaptations in cell signaling systems that underlie differential sensitivity from adolescence to adulthood is important to understanding how

exposure to alcohol early in life predisposes people to subsequent development of alcohol addiction.

THE ERK/MAPK PATHWAY

A possible point of convergence for many neurotransmitter systems (glutamate, dopamine) that may change during adolescence is the mitogen activated protein kinase pathway (MAPK) pathway (Vanhoutte, Barnier et al. 1999; Girault, Valjent et al. 2007). The MAPK pathway regulates biological processes that range from cellular proliferation to apoptosis (Qi and Elion 2005). Adaptive processes such as learning, memory and synaptic plasticity are modulated by ERK signaling cascades (Sweatt 2004). Drug-induced plasticity is also modulated by the ERK signaling pathway (Girault, Valjent et al. 2007; Pandey, Zhang et al. 2008). ERK1/2 activity regulates biological processes and gene transcription so that signals to the cell are able to influence long-term changes in central nervous system structure and function (Pearson, Robinson et al. 2001; Pandey, Zhang et al. 2008; Prakash, Zhang et al. 2008).

The MAPK pathway is activated by numerous extracellular signals and then activates downstream transcription factors that can alter gene expression (Vanhoutte, Barnier et al. 1999; Pandey, Zhang et al. 2008). Five families of MAPKs have been defined for mammals and they are all able to translocate from the cytoplasm to the nucleus to effect gene expression. These are the extracellular signal-regulated kinases (ERK1 and ERK2), Jun N-terminal kinases (JNK1, JNK2 and JNK3), p-38 kinases (p38 α , p38 β and p38 γ) ERK3, ERK4 and finally the most recently discovered ERK5 (Johnson and Lapadat 2002; Qi and Elion 2005).

MAPKs are regulated by phosphorylation cascades. Each pathway is activated by a three-tiered kinase cascade beginning with a MAP kinase kinase kinase (MAPKKK),

followed by a MAP kinase kinase (MAPKK) and finally the MAPK (Pearson, Robinson et al. 2001). The MAPKKK are often linked to the cell membrane by small GTPases or lipids. The MAPKKK have large regulatory domains and are activated by upstream regulators such as Rho or Ras (Robinson and Cobb 1997). The MAPKK differ in that they contain a smaller regulatory domain and they are activated by dual phosphorylation of serine and threonine residues in the catalytic domain (Robinson and Cobb 1997). There is great specificity between the MAPKK and the MAPK but the MAPKKK activate numerous MAPKK (Qi and Elion 2005). Cells receive numerous stimuli that influence the survival, interaction with other cells, and metabolic rate of the intracellular domain. The different MAPKKKs can then interact with specific MAPKK-MAPK groups to illicit a specialized response (Johnson and Lapadat 2002) (Figure 1.1)

ERK1 AND ERK2

ERK1 and ERK2 are widely expressed and are specifically involved with cellular growth, proliferation, cell survival and learning and memory in the central nervous system. ERK1 and ERK2 have 83% amino acid identity and are expressed in all tissues (Pearson, Robinson et al. 2001). Numerous extracellular stimuli activate this particular pathway including growth factors, cytokines and G-protein coupled receptors (Robinson and Cobb 1997; Vanhoutte, Barnier et al. 1999; Roux and Blenis 2004). ERK1/2 pathway consists of the MAPKKK (A-Raf, B-Raf and Raf-1), the MAPKK (MEK1 and MEK2) and finally the MAPK (ERK1 and ERK2).

Typically for an activation cascade, cell surface receptors such as G-protein coupled receptors (GPCRs) transmit activating signals to the Raf/MEK/ERK cascade through different isoforms of the Ras protein. Extracellular signals activate Ras by recruiting to the membrane Son of Sevenless (SOS), which is a Ras activating guanine exchange factor.

SOS stimulates the exchange of Ras from GDP to GTP activating it and allowing the downstream activation of Raf isoforms. Activated Raf now binds to and activates MEK1 and MEK2 via a serine/threonine dual phosphorylation sequence. The activation of the MEK1/2 kinase in turn phosphorylates ERK1/2 within a conserved Thr-Glu-Tyr motif in the activation loop (Roux and Blenis 2004). These in turn activate numerous transcription factors including p-ELK and phosphorylated cAMP response element-binding protein p-CREB (Choe, Chung et al. 2002). Activation of the ERK pathway via both a cAMP/PKA-dependent mechanism leading to activation of CREB and/or the serum response element (SRE) binding protein Elk-1, has been shown to occur in the hippocampus during acquisition of learning and memory and LTP (Cammarota, Bevilaqua et al. 2000; Davis, Vanhoutte et al. 2000). Cyclic AMP response element-binding protein (CREB) and Elk-1 are major transcriptional regulators in striatal projection neurons and are phosphorylated by only one member of MAPK family: extracellular signal-regulated kinase 1/2 (ERK1/2) (Sgambato, Pages et al. 1998; Vanhoutte, Barnier et al. 1999). By regulating the transcription of genes, the ERK1/2 pathway can convert cellular actions into long-term changes in neural functions, such as those observed in addiction (Figure 1.1).

ERK 1/2 and STRESS

All MAPKs have been shown to rapidly activate in response to physiological stress such as electroconvulsive shock and ischemic insult (Alessandrini, Namura et al. 1999; Oh, Ahn et al. 1999). In vitro, research suggests that corticosterone can induce a rapid activation of ERK1/2 in PC 12 cells (Qiu, Wang et al. 2001). In vivo, acute swim stress was found to increase ERK1/2 phosphorylation, in the hippocampus, neocortex, prefrontal cortex and striatum (Shen, Tsimberg et al. 2004). A single exposure to 30 minutes of restraint stress also elevated p-ERK1/2 in the hippocampus, medial prefrontal cortex and cingulate cortex (Meller, Shen et al. 2003). Region-specific effects of MAPK activation have not only been

noted in acute but chronic stress as well. Evidence suggests that 11 days (30min/day) of restraint stress reduced the levels of p-ERK1/2, in the hippocampus and prefrontal cortex (Meller, Shen et al. 2003). More recent work indicated that chronic restraint stress significantly reduced p-ERK1/2 in the rat brain (Imbe, Murakami et al. 2004). Rats that underwent chronic swim stress (14 d) were found to have decreased p-ERK1/2 levels in the hippocampus and prefrontal cortex (Qi, Lin et al. 2008). In contrast, rats undergoing chronic (21 days) of footshock stress were found to have an increase in p-ERK1/2 in the mPFC (Trentani, Kuipers et al. 2002). These discrepancies may be due to differences in brain regions examined, and type of stressor as well as duration of stress. What can be concluded from the studies is that stress affects p-ERK1/2 in numerous brain regions that are also involved with addiction.

Many of the behavioral consequences of stress are mediated by the activation of the glucocorticoid receptor by stress-induced glucocorticoid hormones including corticotropin-releasing hormone (CRH). CRH exerts its actions via CRH receptors of which there are two types (CRH1 and CRH2). In cultured cells, the MAPK pathway is activated by CRH and cAMP by a calcium dependent and independent mechanism and involves MEK and ERK activity (Kovalovsky, Refojo et al. 2002). Using a mutant mouse expressing a brain-specific mutation in the glucocorticoid receptor gene (GR) it was discovered that activation of glucocorticoid receptors (30 min of acute restraint stress) greatly increased the expression and activity of ERK1/2 60 minutes after stress and an increase in the phosphorylated forms of these proteins 120 minutes after stress (Revest, Di Blasi et al. 2005). Intracerebroventricular CRH injections in the mouse brain were found to increase p-ERK1/2 levels in the hippocampus and the basolateral amygdala. Conditional knockout mice were then utilized in order to distinguish between CRH1 or CRH2 involvement. CRH1 conditional knockouts inhibited the p-ERK1/2 increase and solidified the involvement of CRH1 in the

activation of MAPK pathways. (Refojo, Echenique et al. 2005). Interestingly, although adolescents have been shown to have higher levels of perceived stress than adults, activation of the MAPK pathway via stress has not been studied in an age-dependent manner and should be addressed.

EFFECTS OF ALCOHOL ON THE ERK/MAPK PATHWAY

Both acute and chronic alcohol affects the ERK/MAPK signaling pathway *in vitro* and *in vivo*. *In vitro* models, including both primary and transformed cells, have demonstrated both activation and inhibition of ERK1/2 by alcohol. In PC-12 cells treated chronically with alcohol ERK activity increased via a PKC dependent mechanism (Roivainen, Hundle et al. 1995). In contrast chronic alcohol treatment of neuroepithelial cells decreased carbachol stimulation of ERK1/2 in a dose-dependent manner (Ma, Li et al. 2003). Acute alcohol exposure models have also produced conflicting results. Acute alcohol exposure in cerebellar granule cells decreased stimulation of ERK1/2 by brain derived neurotrophic factor (BDNF) (Ohrman, Stancik et al. 2006). In contrast, no effect of acute alcohol exposure was observed in PMA stimulated ERK1/2 in cells pretreated with 150mM alcohol (Hallak, Seiler et al. 2001). Evidence suggests that the direction and magnitude of the effect is dependent on the exposure to alcohol in primary culture. It has been shown in fetal cortical neurons that acute alcohol inhibits and chronic exposure increases the phosphorylation of ERK (Kalluri and Ticku 2002). This however, does not account for all of the different effects observed between laboratories and cell types.

The effects of *In vivo* exposure to alcohol on the ERK/MAPK pathway has produced more consistent data. A study looking into the effects of acute alcohol on the ERK pathway in the rodent brain showed that at a dose of 3.5 g/kg of alcohol decreased p-ERK1/2 in the cerebral cortex and hippocampus in rat pups at PND5, PND21 and as adults (Chandler and

Sutton 2005). A study that extended the above findings used several acute doses ranging from 1.5 – 3.5 g/kg alcohol in mice from PND7 to adulthood showed a dose- and time-dependent decrease in p-ERK1/2 in the cerebral cortex (Kalluri and Ticku 2002). These studies indicate that acute alcohol rapidly alters p-ERK1/2 in specific brain regions related to drug addiction; however the adolescent developmental stage has not been examined to date.

The ERK pathway also plays an important role in neuroadaptations associated with chronic alcohol exposure. Rats chronically exposed to alcohol showed no effect on protein and mRNA levels of p-ERK1/2 in the various structures of amygdala. However, alcohol withdrawal (24 hrs after 15 days of alcohol exposure) produced significant reductions in the protein and mRNA levels of p-ERK1/2 in the central, and medial but not in the basolateral amygdala (Pandey, Roy et al. 2004). These results suggest the possibility that the decreased expression and function of p-ERK1/2 in the central and medial amygdala may be associated with the process of alcohol dependence, and this may be promoting the continued consumption of alcohol (Pandey, Roy et al. 2004). Rats exposed to continuous or intermittent alcohol in vapor chambers (BAL of approximately 200%) showed that chronic alcohol vapor decreased p-ERK1/2 immunoreactivity (IR) in the amygdala, cortex, cerebellum, and dorsal striatum. Conversely, withdrawal from alcohol vapor increased p-ERK1/2 levels that peaked at 13-h post withdrawal and remained elevated at 24-h (Sanna, Simpson et al. 2002).

Accumulating evidence indicates that the ERK/MAPK pathway is associated with some of the behavioral effects of alcohol, including alcohol self-administration and cue-induced reinstatement of alcohol seeking (Schroeder, Spanos et al. 2008; Faccidomo, Besheer et al. 2009). Mice trained to self-administer alcohol showed potentiated alcohol-reinforced responding after injection of the ERK/MEK inhibitor SL327 (Faccidomo, Besheer

et al. 2009). Rats trained to self administer alcohol were tested for cue-induced reinstatement of alcohol seeking after 30 days of abstinence. Re-exposure to the alcohol associated context and discrete cues activated ERK_{1/2} in the basolateral amygdala (BLA) (Radwanska, Wrobel et al. 2008). A more recent study in P-rats (a genetic model of alcoholism) showed that reinstatement of alcohol-seeking behavior, produced by response-contingent presentation of alcohol-associated cues, is associated with an increase in ERK_{1/2} phosphorylation in both the basolateral nucleus of the amygdala and the nucleus accumbens shell. This increase was subsequently blocked using, the metabotropic glutamate receptor subtype 5 antagonist MPEP (Schroeder, Spanos et al. 2008). These observations suggest that the ERK/MAPK pathway may be specifically involved in some of the behavioral effects of alcohol induced by discrete cues in specific brain regions and that the metabotropic glutamate receptor may be involved.

Research on alcohol's effects on the developing adolescent brain is still in its earliest stages, despite the fact that this is the time during which many people begin drinking. Few researchers have looked for developmental differences in cell signaling systems that may underlie the differential behavioral responses to alcohol. To date no studies have evaluated the effects of alcohol (both acute and chronic) on the ERK/MAPK pathway during adolescence. There are also no studies addressing the effects of alcohol and stress on the ERK signaling pathway in an age dependent manner. As this is a key signaling pathway that is altered by alcohol this gap in our knowledge must be addressed. Just how alcohol use impacts the ERK/MAPK pathway during adolescence or whether these changes in the pathway influence future alcohol use is unknown.

SUMMARY AND OVERALL GOALS OF THIS RESEARCH

Recent evidence suggests that behavioral and neurobiological changes may leave adolescents vulnerable to experimenting with drugs of abuse and to drug-induced

neuroadaptations (Spear 2002). Neuroadaptations in cell signaling and gene transcription pathways mediate behavioral pathologies in drug addiction including alcohol addiction. The ERK/MAPK signaling pathway is one such molecular mechanism that has received growing attention in addiction due to its fundamental role in neuroplasticity and other cellular processes. However, the role of this key molecular signaling pathway as a potential mediator of adolescent vulnerability to alcohol has not been studied. The principal goal of this dissertation was to test facets of the overall hypothesis that: **alcohol exposure leads to differential neuroadaptations in the MAPK signaling pathway in adolescents as compared to adults.**

The differences observed in alcohol sensitivity of adolescent rodents compared to adults are of significant interest because it has been shown that a decreased response to acute alcohol challenge during adolescence is a potent predictor of future alcoholism (Schuckit 1994). There are no known neurobiological mechanisms for these developmental differences in behavioral effects to alcohol in rodents. It is therefore important to identify neuroadaptations in cell signaling pathways that underlie the differential responses to alcohol observed in adolescents and adults. Research on alcohol's acute effects on the developing adolescent, especially as it pertains to signaling systems, including ERK, are still in its early stages despite the fact that this is the time during which many people begin drinking. To date there has been no systematic examination of alcohol's effects on adolescent cellular signaling systems, including ERK. Accordingly, the purpose experiments in chapter 2 was to *characterize developmental and brain regional differences in the ERK/MAPK signaling pathway associated with acute alcohol administration in C57Bl6/J mice. Experiments in this study will also address the functional significance of age-related changes in ERK activity using behavioral assays that complement changes in brain ERK/MAPK signaling.*

Early alcohol use may have long-lasting consequences. Adolescents who begin drinking before age 15 are three times more likely to develop alcohol dependence at some point in their lives compared with those who have their first drink at age 20 or older (Grant and Dawson 1997). Developmental differences in cell signaling systems, including ERK that may underlie changes associated with alcohol drinking must be studied. Studying age-dependent neuroadaptations in the ERK/MAPK pathway associated with chronic alcohol self-administration may provide insight to changes in the brain that could potentially lead to alcohol dependence problems in adulthood. A functional role for ERK1/2 in voluntary drinking can be studied in an age-dependent manner by injecting a MEK/ERK inhibitor, SL327. To this point there has been no examination of chronic alcohol's effects on adolescent cellular signaling systems, including ERK. Accordingly, experiments in chapter 3 will *characterize developmental and brain regional differences in the ERK/MAPK signaling pathway associated with chronic alcohol self-administration in C57Bl6/J mice. Differences in p-ERK1/2 can then be assessed in an age-related manner. Using the ERK/MEK inhibitor SL32L during chronic self-administration will enable the functional significance of brain areas associated with age-related effects of intake to be revealed.*

Human adolescents drink less often than adults however they drink more per occasion. We often define as drinking large amounts of alcohol in a short amount of time a binge. According to the 2002 National Household Study on Drug Abuse, nearly 1 in 5 adolescents between the ages of 12 and 20 engaged in binge alcohol drinking (5 or more drinks on 1 occasion) during the previous month. Binge alcohol use peaks at 21 years of age and gradually declines throughout life. Importantly, high levels of alcohol abuse during adolescence are associated with future alcohol abuse (Bates and Labouvie 1997). To better understand and prevent this serious public health problem, it is important to study the effect of binge alcohol use on adolescents. Experiments in chapter 4 will investigate binge-alcohol

drinking in adolescents and adult C57BL/6J mice. Using a binge-drinking alcohol intake model, drinking in the dark, alcohol intake can then be studied in adolescent and adult mice. The age of onset of binge-like drinking and its effects on adult (future) alcohol self-administration will be examined.

In humans, stressful life events and maladaptive responses to stress increase alcohol drinking (Pohorecky 1991). Studies have found that the perception of stress may be increased in adolescents relative to children and adults (Spear 2000). Adolescents who begin using alcohol under these life stressors have been shown to adopt continued and more frequent alcohol use as a strategy of coping with their problems (Pohorecky 1991). Consequently, understanding the cellular and biochemical mechanisms that transduce stress signals may inform us of the factors that lead to alcoholism as well as produce new avenues for therapeutic intervention. Given that stress modulates alcohol intake and p-ERK1/2 has been directly linked to acute, chronic effects of alcohol and stress it is imperative to determine the effects alcohol drinking on the stress response by observing its link to the MAPK pathway in an age-dependent manner. To date there has been no examination of chronic alcohol self-administration's effects on adolescent and adult cellular signaling systems, including ERK after activation of the HPA axis. *Accordingly, experiments in Chapter 5 will investigate the stress response (corticosterone) in adolescents and adults and how this response is linked to alcohol intake and the ERK/MAPK pathway.*

Overall, these three integrated but separate studies will extend the current knowledge of differences between adolescent and adult mice by addressing an important cellular signaling pathway (ERK) and its involvement in alcohol exposure. These studies provide new insight into the cellular signaling cascades that may underlie behavioral differences in adolescent and adult mice and may also provide insight into neurobiological

mechanisms by which adolescent alcohol use increases the probability of alcohol dependence in adulthood.

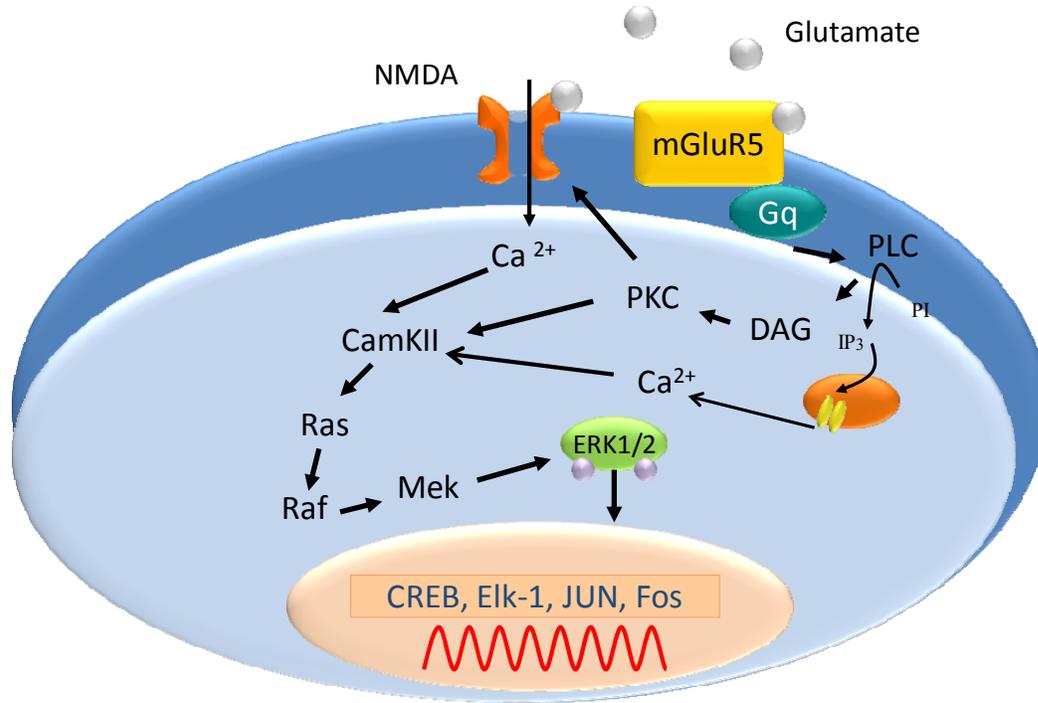


Figure 1.1. Representation of the ERK1/2 signaling pathway. In this depiction, glutamate activates G-protein coupled receptors (mGluR5) at the cell membrane to increase intracellular calcium via ionotropic receptors or intracellular release via IP₃. This in turn activates CaMKII alpha which then initiates the MAPK three-tiered signaling cascade. The phosphorylation of ERK by MEK then causes the protein to translocate into the nucleus where it affects gene transcription.

**CHAPTER II: ACUTE ALCOHOL ADMINISTRATION ALTERS EXTRACELLULAR-
SIGNAL REGULATED KINASE ACTIVITY IN AN AGE AND BRAIN REGION
DEPENDENT MANNER IN C57BL/6J MICE**

INTRODUCTION

Adolescence is a critical developmental period during which young mammals undergo behavioral and neurobiological changes that lead to the transition into adulthood (Spear 2000). During this developmental period individuals exhibit certain characteristic behaviors including increases in novelty seeking and risk-taking which may lead to experimentation with drugs of abuse (Wills, Vaccaro et al. 1994; Laviola, Macri et al. 2003; Kelley, Schochet et al. 2004). Accordingly, adolescence is the period during which alcohol use in humans is often initiated (Johnston, O'Malley et al. 2009). The 2000 Monitoring the Future Survey of the National Institute of Drug Abuse found that 73% of high school seniors reported using alcohol in that past year and 32% reported drinking to intoxication in the past month. Moreover, individuals who begin drinking during adolescence are four times more likely to become alcohol dependent as adults (Grant and Dawson 1998).

Human studies have suggested that a potent predictor of future alcoholism is a decreased response to acute alcohol challenge during adolescence (Schuckit 1994; Schuckit and Smith 2000). It is therefore important to address the effects of acute alcohol during this ontogenetic stage and rodent studies have indicated that adolescents and adults are differentially sensitive to the effects of acute alcohol. Some studies have shown that adolescent rodents are less sensitive to alcohol-induced acute withdrawal, intoxication,

motor-impairments, and sedation as compared to adults (Silveri and Spear 1998; Varlinskaya and Spear 2002; White, Truesdale et al. 2002; Doremus, Brunell et al. 2005; Doremus-Fitzwater and Spear 2007; Hefner and Holmes 2007). Other studies have shown that adolescent rodents are more sensitive to alcohol-induced locomotor activation, hypothermia, anxiolysis, memory impairment, and social interaction than adult rodents (Markwiese, Acheson et al. 1998; Varlinskaya and Spear 2002; Philpot, Badanich et al. 2003; Yttri, Burk et al. 2004; Hefner and Holmes 2007). The neurobiological mechanisms that underlie the variety of developmental differences in behavioral and physiological responses in acute alcohol exposure have not been characterized.

The goal of this study was to identify potential adaptations in neural cell signaling associated with differential responses to acute alcohol observed between adolescents and adults. The extracellular signal-regulated kinase (ERK) signaling pathway is an ideal candidate for study based on its regulation of key neurotransmitter systems, including dopamine and glutamate, which continue to mature during adolescence (Insel, Miller et al. 1990; Tarazi and Baldessarini 2000) and modulate acute response to ethanol (Gessa, Muntoni et al. 1985; Di Chiara and Imperato 1986; Roberto, Schweitzer et al. 2004). Although recent evidence indicates that two weeks of intermittent ethanol (3 g/kg) injection decreases expression of dopamine D2 receptors and NMDA NR2B phosphorylation in prefrontal cortex of adolescent but not adult rats (Pascual, Blanco et al. 2007), it is not known if ethanol alters ERK signaling.

The two closely related isoforms of ERK (ERK1 and ERK2, or ERK1/2) are widely expressed in the central nervous system and are involved in cell growth, proliferation, and survival, and have been shown to be modulated by drugs of abuse (Valjent, Corvol et al. 2000; Sanna, Simpson et al. 2002; Qi and Elion 2005; Pandey, Zhang et al. 2008). Specifically, ERK1/2 activation (phosphorylation) is modulated by alcohol in a dose,

treatment (acute vs. chronic), and brain region-dependent manner. Acute alcohol administration (1.5 – 3.5 g/kg) produced dose- and time-dependent decreases in ERK1/2 phosphorylation (p- ERK1/2) in the cerebral cortex of mice without effecting total ERK (Kalluri and Ticku 2002). Further evidence showed that an acute alcohol injection of 3.5 g/kg decreased p-ERK1/2 in the cerebral cortex and hippocampus in rat pups at PND5, PND21 and as adults (Chandler and Sutton 2005). ERK1/2 activation increased in the Edinger-Westphal nucleus of mice after a 3g/kg acute alcohol injection (Bachtell, Tsivkovskaia et al. 2002). Although these studies indicate that acute alcohol rapidly alters p-ERK1/2 in specific brain regions, the extent to which acute alcohol-induced effects vary as a function of ontogeny remains to be addressed.

ERK1/2 signaling is also critical for neural and behavioral plasticity (Sweatt 2004), which may be of special importance to adolescent response to ethanol (Monti, Miranda et al. 2005). In fact, activation of the ERK/MAPK cascade in the hippocampus is critical for memory consolidation (Blum, Moore et al. 1999; Schafe, Atkins et al. 2000; Kelly, Laroche et al. 2003; Runyan, Moore et al. 2004), and blocking hippocampal ERK activation with the MEK inhibitor SL327 impairs memory consolidation in several tasks (Blum, Moore et al. 1999; Walz, Roesler et al. 2000; Runyan, Moore et al. 2004; Zhang, Zhao et al. 2004). In particular, intracerebroventricular MEK inhibition disrupts object memory consolidation (Bozon, Kelly et al. 2003; Kelly, Laroche et al. 2003). As a sedative-hypnotic compound, ethanol has long been known to produce dose-dependent effects on memory (Ryback 1970; Ryback 1971) and recent evidence indicates that adolescent rodents are more sensitive to ethanol-induced disruption of memory acquisition than adults (Markwiese, Acheson et al. 1998). To date there has been no systematic examination of the effects of acute alcohol on adolescent cellular signaling systems, including ERK, that are important to learning and memory and addiction.

To address this gap in knowledge, the present study sought to characterize ontogenetic and brain regional differences in the ERK/MAPK signaling pathway associated with acute alcohol administration in C57BL/6J mice. Adolescent (40 days) and adult mice (84 days) were administered a range of acute alcohol doses (0, 0.5, 1, 3g/kg IP) and were sacrificed 30 minutes later for immunohistochemical evaluation. P-ERK1/2 immunoreactivity (IR) was then examined in brain regions specifically associated with addiction, such as the medial prefrontal cortex, nucleus accumbens, amygdala and hippocampus. In addition, ontogenetic effects of acute alcohol (1 g/kg) were examined on the novel object recognition test (Bevins and Besheer 2006) followed by evaluation of p-ERK1/2 IR in the hippocampus.

METHODS

Animals. Male C57BL/6J mice (Jackson Laboratories, Bar Harbor, Maine) were group housed in standard Plexiglas cages. Food and water were available *ad libitum* in the home cage. The mice were 21 days (juvenile) and 70 days (adult) upon arrival to the facility. The colony room was maintained at 27°C on a 12-hour light/dark cycle (lights on 20:00), and experiments were performed during the dark cycle. The mice were handled and weighed for three weeks before experiments began to acclimate to the reverse light cycle, resume normal sleep/wake cycles (Wexler and Moore-Ede 1986) and allow the mice to reach the specific ages required for adolescent (PND 42) and adult mice (PND 84). Animals were under continuous care and monitoring by the Division of Laboratory Animal Medicine (DLAM) at UNC-Chapel Hill. All procedures were carried out in accordance with the NIH *Guide to Care and Use of laboratory Animals* (National Research Council, 1996) and institutional guidelines.

Acute Alcohol Treatment. Adolescent mice (PND 42) and adult mice (PND 84) were habituated to intraperitoneal injection (saline, 0.1ml/10g), for three days prior to the start of the experiment. Alcohol (95%w/v) was diluted in physiological saline (0.9%) to a concentration of 20% (v/v) and administered in various volumes to obtain the appropriate dose. On the day of the experiment, mice received alcohol (0, 0.5, 1 or 3 g/kg, IP).

Immunohistochemistry. Thirty minutes after an alcohol or saline injection mice were deeply anesthetized with 60 mg/kg pentobarbital. The mice were then transcardially perfused with 0.1M phosphate buffered saline (PBS) followed by 4% paraformaldehyde. The brains were extracted from the skulls washed in PBS and sliced on a vibratome into 40 μ m coronal sections. These free-floating sections were then stored in cryoprotectant at -20 $^{\circ}$ C until immunohistochemical processing. The free-floating sections were rinsed in PBS, and then placed in 1% hydrogen peroxide in PBS for 5 minutes to block endogenous peroxidase. Next sections underwent antigen retrieval using citra buffer at 70 $^{\circ}$ C for 30 min (Antigen Retrieval Citra, BioGenex). Sections were then blocked in 0.1% triton-X in PBS with 5% goat serum for 1 hour and then were incubated at +4 $^{\circ}$ C overnight in primary polyclonal antibody to p-ERK1/2 (1:200 Cell Signaling Technology, Danvers, MA). The sections were incubated in Dako EnVision Kit (Dako, Carpinteria, CA) secondary antibody for one hour at room temperature and immunoreactivity was detected using nickel enhanced diaminobenzene (Dako EnVision Kit) as a chromagen. The sections were counterstained using toluidine blue, mounted onto slides, and coverslipped.

Immunoreactivity was visualized using an Olympus CX41 light microscope (Olympus America, Center Valley, PA). Images were acquired using a digital camera (Regita model, QImaging, Burnaby, BC) interfaced to a desktop computer (Dell, Round Rock, TX) image analysis software (Bioquant Nova Advanced Image Analysis; R&M Biometric, Nashville, TN). The microscope, camera, and software were background corrected and normalized to

preset light levels to ensure fidelity of data acquisition.

Data Analysis. Data for p-ERK1/2 IR data were acquired from a minimum of 4 sections/brain region/animal and averaged to obtain a single value per subject. Coordinates of brain regions analyzed were as follows: nucleus accumbens and proximal brain regions (+ 0.86 to 1.34 mm anterior to bregma), amygdala and proximal brain regions (-1.7 to -1.34 mm posterior to bregma), (Pixel density and cell count measurements were calculated from a circumscribed field (e.g., brain region) and divided by the area of the region and expressed as pixels/mm². Immunoreactivity (IR) data were collected by a researcher blind to treatment conditions. Due to variability that occurs across individual immunohistochemistry experiments independent parallel controls were run for each dose of ethanol (0.5g/kg, 1g/kg and 3g/kg), therefore, data were expressed and analyzed as a percent change from control.

Blood Alcohol Determination. In order to ensure the differences we were observing were not due to confounds of a differential dose, blood alcohol levels were examined. Approximately 20 µl of heart blood was collected at the time of perfusion. From the 20 µl centrifuged sample 5 µl of plasma was used to determine alcohol concentration using an AM1 Alcohol Analyzer (Analox Instruments, Lunenburg, MA).

Novel Object Recognition Task. Novel object recognition (NOR) was measured in eight covered sound attenuating, lighted Plexiglas chambers (30 cm², Med Associates, Georgia, VT). Two sets of 16 pulse-modulated infrared photobeams were located on opposite walls to record ambulatory movements in the X-Y (horizontal) plane. All software settings were the same for adult and adolescent mice. The activity chambers were computer-interfaced (Med Associates) for data sampling at 50-millisecond resolution. Adolescent mice (PND 42) and adult mice (PND 84) were habituated to IP saline injections (saline, 0.1ml/10g), cart transport and the chamber (30 minutes) for three days prior to the novel object recognition (NOR) task (Bevins and Besheer 2006).

On the test day, mice were transported via cart from the colony room to a procedure room. Mice were placed in the empty locomotor chambers for a 10 minute habituation period. Three test sessions, each 10 minutes long, were separated by 5 minute intervals. During session 1, two identical objects (either two filter tips or two eraser tops) were placed in the back right and left corners of the chamber for the sample object exposure (familiar objects). Ethanol (1g/kg) or saline was injected (IP) in adolescent and adult mice immediately before the first session (sample object exposure 1). In session 2, mice were re-exposed to the same two sample objects (familiar objects). In session 3, the novel object test, one familiar object was replaced with a novel object (whichever object had previously not been used). Objects and side of novel object presentation was counterbalanced across subjects.

Data analysis and acquisition. Proximity to novel and sample objects was evaluated post hoc using Med-Associates Zone Analysis software. Sample and test objects (micro filter and eraser top) were placed in the center of square zones (6.5 X 6.5 cm or 4 X 4 photo beams) located in the two back corners of the test environment (Figure 2.1). Object exploration was defined as the time spent in the zone of interest (breaking beams around each object) and was then converted to percent time spent with the novel object.

RESULTS

Blood alcohol concentration (BAC)

No differences were detected in BAC between C57BL/6J adolescent and adult mice following acute alcohol treatment (0.5g/kg, 1g/kg or 3g/kg) (Table 2.1). Blood alcohol concentrations ranging from 35-49 mg% were detected at 30 minutes after injection of 0.5 g/kg in adolescents and adults and did not differ (Students-t test $p = 0.4257$). Blood alcohol concentrations ranging from 62-98 mg% were detected after injection of 1g/kg in

adolescents and adults and did not differ (Students-t test $p = 0.9031$). BACs ranging from 265-304 mg% were detected after injection of 3g/kg in adolescents and adults and did not differ (Students-t test $p = 0.7309$).

Basal p-ERK in Adults and Adolescents in Mouse Brain

Basal levels of p-ERK1/2 IR were analyzed in brain regions that have been shown to regulate acute and chronic effects of ethanol, including acute response, reinforcement, drug discrimination and memory (Hodge, Samson et al. 1992; Hodge and Cox 1998; Markwiese, Acheson et al. 1998; Schroeder, Olive et al. 2003; White and Swartzwelder 2004; Wilkie, Besheer et al. 2007; Besheer, Schroeder et al. 2008; Schroeder, Spanos et al. 2008). Adolescent mice showed significantly higher levels of p-ERK1/2 IR in the central ($t(60) = 2.97$, $p=0.004$) and basolateral amygdala ($t(58)=2.2$, $p=0.03$) and prefrontal cortex ($t(62)=2.3$, $p=0.02$) as compared to adults (Table 2.2). No statistically significant differences were observed in nucleus accumbens, hippocampus, or entorhinal cortex. Qualitative observation also indicated the highest densities of p-ERK1/2 IR were observed in the medial prefrontal cortex and central amygdala with lower levels of immunostaining seen in the dentate gyrus, the nucleus accumbens (core and shell) and the basolateral amygdala (Table 2.2).

Effect of Acute Alcohol on p-ERK1/2 Immunoreactivity

Nucleus Accumbens. Acute alcohol did not alter p-ERK1/2 IR in the Nucleus accumbens core or shell in adolescent or adult mice at any dose (Table 2.3).

Basolateral Amygdala. Acute alcohol (0.5g/kg, 1g/kg) did not alter p-ERK1/2 IR in the basolateral amygdala (BLA) (Table 2.3). However, a 2-way ANOVA indicated a significant main effect of age ($F(1, 43) = 5.8$, $p=0.02$) and alcohol ($F(1, 43) = 14.2$, $p=0.0005$) after an acute 3g/kg injection of alcohol. In addition there was a significant interaction between

alcohol and age ($F(1, 43) = 5.8, p = 0.02$). Post hoc (Holm-Sidak) multiple comparisons showed that alcohol produced a significant increase in p-ERK1/2 in adolescents ($p = 0.02$) and not adult mice (Figure 2.2A, left). Representative photomicrographs illustrating the cytological pattern of p-ERK1/2 IR in the basolateral amygdala (Figure 2.2B, left).

Central Amygdala. Acute alcohol (0.5g/kg, 1g/kg) did not alter p-ERK1/2 IR in the central amygdala (CeA) (Table 2.3). However, a 3g/kg dose of alcohol increased p-ERK1/2 IR as indicated by a significant main effect of alcohol ($F(1, 43) = 75.02, p < 0.0001$) due to a significant increase in p-ERK1/2 IR in both adolescents and adults (Figure 2.2A, right). Effects of age or age x alcohol interactions were not significant. Representative photomicrographs illustrating the cytological pattern of p-ERK1/2 IR in the central amygdala (Figure 2.2B, right).

Medial prefrontal cortex. Acute alcohol (0.5g/kg, 1g/kg) did not alter p-ERK1/2 IR in the medial prefrontal cortex (mPFC) (Table 2.3). Acute alcohol (3g/kg) treatment increased p-ERK1/2 IR in the medial prefrontal cortex in both adolescents and adults as indicated by a significant main effect of ethanol ($F(1, 33) = 11, p < 0.01$). No main effects of age or alcohol x age interactions were observed.

Dentate Gyrus. Acute alcohol (0.5g/kg) did not alter p-ERK1/2 IR in the dentate gyrus in adolescent and adult mice. The 1g/kg dose of alcohol produced age-dependent effects, with significant decreases in p-ERK1/2 IR observed in the adolescent mice. Two-way ANOVA analysis showed main effects of alcohol ($F(1, 41) = 10.135, p = 0.003$) and age ($F(1, 41) = 6.506, p = 0.015$) and age x alcohol interactions ($F(1, 41) = 7.163, p = 0.011$). Post hoc (Holm-Sidak) comparisons showed that alcohol (1 g/kg) produced a significant decrease in p-ERK1/2 IR in adolescent ($p < 0.001$) but not adult mice (Figure 2.3A).

Representative photomicrographs illustrating the cytological pattern of p-ERK1/2 IR in the dentate gyrus after ethanol (1 g/kg) injection are shown in Figure 2.3B.

Acute alcohol (3g/kg) treatment decreased p-ERK1/2 IR in the dentate gyrus as indicated by a significant main effect of alcohol ($F(1,33) = 11, p < 0.01$). Post hoc comparisons showed that the main effect of alcohol was due to a significant reduction in both age groups (Holm-Sidak, $p < 0.05$) (Figure 2.4A). Effects of age and age x alcohol interactions were not significant. Representative photomicrographs illustrating the cytological pattern of p-ERK1/2 IR in the dentate gyrus of adolescents and adults after ethanol (3 g/kg) injection are shown in Figure 2.4B.

NOVEL OBJECT RECOGNITION TASK

Session 1 and 2: Sample object exposure. Adolescent and adult mice showed similar object interaction time (sec) with the filter and the eraser-top during the sample object exposure sessions (Student's t-test, $p > 0.05$) (Figure 2.5).

Novel Object Recognition (NOR) task. A two-way ANOVA of novel object interaction time following acute ethanol (1 g/kg) found a significant main effect of age ($F(1,41)=7.1, p=0.011$) and alcohol ($F(1,41)=24.7, p<0.001$). In addition there was a significant interaction between alcohol and age ($F(1,41)=5.5, p=0.02$) a finding that indicated that the effect of alcohol depends on age. Post hoc (Holm-Sidak) comparisons showed that alcohol produced a significant decrease in % time with the novel object in adolescent ($p=0.001$) but not in adult mice (Figure 2.6).

No differences were observed between age and treatment conditions on several other measures of general motor activity including total distance traveled, stereotypic counts and zone entries were examined (Figure 2.7 A-C). Lack of differences in these measures

ensures that the age-related differences in % time spent with the novel object were not due to differences in locomotor activity (activation or inhibition).

DISCUSSION

A growing body of evidence suggests that behavioral and neurobiological changes may leave adolescents vulnerable to experimenting with drugs of abuse and to drug-induced neuroadaptations (Spear 2002). Neuroadaptations in cell signaling and gene transcription pathways mediate behavioral pathologies in drug and alcohol addiction (Koob and Le Moal 2005). The present study addressed a key cellular mechanism, the ERK/MAPK pathway, that may contribute to behavioral differences to acute alcohol exposure noted in adolescent and adult rodents. Results indicate that acute alcohol exposure produces a unique set of neural changes in p-ERK1/2 IR (activation) between adults and adolescents. We found that upon first exposure to alcohol, age dependent increases in p-ERK 1/2 IR were produced in the basolateral amygdala (3g/kg) and decreases in the dentate gyrus (1g/kg). The adolescent age group was found to be more sensitive to alcohol-induced changes in p-ERK1/2 IR in these brain regions than their adult counterparts.

Our study found alcohol-induced increases in p-ERK1/2 IR in the CeA, in both adolescents and adults which is consistent with recent research that has shown increases in ERK activity after acute alcohol exposure (Bachtell, Tsivkovskaia et al. 2002; Kalluri and Ticku 2002; Chandler and Sutton 2005; Pandey, Zhang et al. 2008). Specifically, a recent study in adult rats examined the effects of acute alcohol using immunogold-labeling techniques and found that p-ERK1/2 as well as some downstream targets of p-ERK1/2 (Elk-1, CREB and Arc) were increased in the central amygdala (Pandey, Zhang et al. 2008). The present study is the first to report changes in p-ERK1/2 IR in adolescent mice. Increased p-

ERK1/2 in the CeA is related to decreased anxiety in adult rats therefore p-ERK1/2 may not be responsible for the behavioral differences in alcohol-induced anxiety between adolescents and adult rats reported in the literature (e.g., (Varlinskaya and Spear 2002; Wills, Knapp et al. 2009)). However p-ERK1/2 activity in the CeA is also important for incubation of craving and drug seeking (Lu, Hope et al. 2005) which may impact adolescent vulnerability to addiction.

Developmental differences in ERK1/2 phosphorylation were noted in the BLA after a 3g/kg acute alcohol injection. The BLA is known to mediate associative learning processes/cue learning such as Pavlovian fear conditioning (LeDoux 2000). Evidence has implicated the lateral and basal nuclei of the amygdala (BLA) in the plastic changes underlying acquisition and retention of Pavlovian fear conditioning (Fendt and Fanselow 1999; Maren 1999; LeDoux 2000). ERK/MAPK is transiently activated in the amygdala after fear conditioning and pharmacological blockade of ERK/MAPK activation in the amygdala impairs fear memory consolidation (Schafe, Atkins et al. 2000). Differences in activation of ERK1/2 in the BLA may suggest that adolescents attend to an acute alcohol cue, or to environmental stimuli paired with alcohol, differently than their adult counterparts. After an acute dose of alcohol the BLA of the developing adolescent responds to this salient “cue” by increasing ERK1/2 phosphorylation which may lead to an increased vulnerability to addiction. The functional significance of the 3g/kg dose of acute alcohol was not examined further as mice at this dose are sedated and behaviors associated with the BLA (such as fear conditioning) could not be studied.

An increase in p-ERK1/2 IR in the mPFC at a 3g/kg dose of alcohol was observed in both adolescents and adult mice. By contrast, other evidence shows a decrease in p-ERK1/2 using a dose of 3.5g/kg in the adult mouse cerebral cortex (Chandler and Sutton 2005). This discrepancy may be due to differences in methodology. Specifically, we

examined the medial prefrontal cortex, a subregion of the cortex known to regulate alcohol self-administration (Hodge, Chappelle et al. 1996). The majority of past studies were limited to one age group and a single brain region (cerebral cortex) using Western blot methods to assess changes in p-ERK1/2. By using immunohistochemical methods we have been able to evaluate more anatomically restricted changes in p-ERK1/2 in specific subnuclei of the brain known to be important for addiction. Since both adolescent and adults showed an increase in p-ERK1/2 IR following acute ethanol treatment, developmental differences in acute-alcohol high-dose behaviors, such as sedation, are probably not related to the function of mPFC and therefore the increased p-ERK1/2 in both adolescents and adult may not mitigate these behavioral effects. The mPFC may not be an underlying mechanism of adolescent vulnerability to future addiction however; other cellular signaling systems should be addressed.

Our finding that acute alcohol exposure (1g/kg and 3g/kg) decreased p-ERK1/2 IR in the dentate gyrus of the hippocampus is also consistent with previous studies (Chandler and Sutton 2005). For example, a 3.5 g/kg dose of ethanol decreased p-ERK1/2 levels in the adult rat hippocampus at sixty minutes post injection (Chandler and Sutton 2005). Acute alcohol exposure has also been found to decrease p-ERK1/2 levels *in vitro*. These studies include SH-SY5Y cells, cultured cortical neurons, and NG108-15 cells (Seiler, Ross et al. 2001; Kalluri and Ticku 2003; Constantinescu, Wu et al. 2004). Developmental differences in ERK1/2 phosphorylation were noted in the DG after a 1g/kg acute alcohol injection which may be related to differences that have been observed between these two age groups in alcohol-induced memory deficits (White and Swartzwelder 2004).

Using the hippocampal-dependent novel object recognition (NOR) task we examined the potential role of the differential p-ERK1/2 response in the dentate gyrus between adolescents and adults. Object recognition memory refers to the ability to judge a previously

encountered item as familiar and depends on the integrity of the hippocampus (Squire, Wixted et al. 2007). Tasks that assess object recognition memory have become increasingly useful tools for basic research investigating the neural basis of memory (Winters, Saksida et al. 2008). This data suggests that in a hippocampal-dependent NOR task, adolescents were more sensitive to alcohol-induced disruptions in memory than their adult counterparts. This effect was expressed as the adolescent alcohol treated mice not showing novel object preference. The difference in hippocampal-dependent object recognition memory in adolescent mice may be associated with the difference observed in p-ERK1/2 activity (decrease) after a 1g/kg alcohol exposure.

These data agree with the majority of literature on alcohol's effects on memory in adolescents and adults (rodent and human). Evidence suggests that adolescents are more sensitive to alcohol's memory-impairing effects than adults (Acheson, Stein et al. 1998; Markwiese, Acheson et al. 1998; Brown, Tapert et al. 2000; White, Truesdale et al. 2002; Tapert, Schweinsburg et al. 2004). Accumulating data also indicate that p-ERK1/2 activity is required for hippocampal-dependent memory tasks such as the novel object recognition task (Atkins et al 1998, Blum et al 1999, Bozon 2003, Sachafe 2000, Kelly 2003, Runyan 2004). The potential functional regulation of alcohol induced memory impairment in adolescent mice via decreased p-ERK1/2 in the hippocampus should be directly tested. Further studies microinjecting the ERK inhibitor SL327 in the hippocampus prior to the NOR task and observing its effects on memory could solidify the role of p-ERK1//2 in adolescent and adult learning and memory. Overall, these results suggest that adolescent mice are more sensitive to ethanol-induced memory impairment and that this effect may be mediated by a rapid reduction in ERK1/2 activity.

In summary, results of this study show that adolescent C57BL/6J mice are more sensitive to acute alcohol-induced changes in ERK1/2 activity than their adult counterparts.

The differences in ERK1/2 signaling observed between the two age groups may have behavioral implications (functional significance). Increased alcohol-induced ERK activation in the BLA during adolescence may mediate associative learning processes that could lead to a potential addiction during adulthood. Decreased alcohol-induced ERK activity in the hippocampus was associated with age-related differences in novel object recognition task. Increased sensitivity to changes in cell signaling systems such as the ERK/MAPK pathway during adolescence may allow for both immediate consequences including those observed in the novel object recognition test or more long-term changes that are brought about by the first exposure to alcohol. The long-term changes possibly induced by the altered ERK signaling system during adolescence could promote a central nervous system vulnerable to a future alcohol addiction.

Age	Alcohol Dose		
	0.5g/kg	1.0g/kg	3.0g/kg
Adolescent	43.2 ± 1.31	80.5 ± 3.19	288.6 ± 3.86
Adult	41.7 ± 1.24	81.1 ± 3.49	285.85 ± 6.88

Table 2.1. Blood alcohol concentration (BAC) 30 minutes after acute alcohol injection in adolescent and adult mice. No age-dependent differences were observed in blood alcohol content did not differ between adolescent and adult mice at any dose of alcohol tested. Data represent mean ± SEM BAC (mg/dL) analyzed with an Analox system.

Age	Brain regions	Basal p-ERK1/2 levels (IR)
Adolescent	Amygdala	
	Central	6043.72 ± 592.62*
	Basolateral	2797.44 ± 343.64*
	N. Accumbens	
	Core	3869.37 ± 314.80
	Shell	2491.55 ± 219.80
	Prefrontal Cortex	
	Medial	7764 ± 313.96*
	Hippocampus	
	DG	11.87 ± 0.42
Entorhinal Cortex	2103.75 ± 192.51	
Adult	Amygdala	
	Central	3838.67 ± 435.49
	Basolateral	1869.70 ± 241.14
	N. Accumbens	
	Core	4046 ± 439.95
	Shell	2018.16 ± 287.25
	Prefrontal Cortex	
	Medial	6794.64 ± 280.1
	Hippocampus	
	DG	11.37 ± 0.33
Entorhinal Cortex	2347.84 ± 213.90	

* significantly different from adult (p< 0.05)

Table 2.2. Basal levels of adolescent and adult p-ERK1/2 immunoreactivity. Basal p-ERK1/2 IR values (raw) ± SEM derived from adolescent (n=32) and adult (n=30) mice in specified brain regions. * - indicates significantly different from adult, p<0.05, t-test.

Age	0.5g/kg		1.0g/kg		3.0g/kg	
	Saline	Ethanol	Saline	Ethanol	Saline	Ethanol
Adolescent Amygdala						
Central	100 ± 17.46	110.65 ± 15.52	99.02 ± 14.92	106.6 ± 17.70	100 ± 9.43	460.34 ± 36.55*
Basolateral	100 ± 12.16	73.55 ± 8.52	100 ± 21.44	83.92 ± 13.87	100 ± 11.66	443 ± 27.54*
N. Accumbens						
Core	100 ± 12.2	110.33 ± 14.85	100 ± 13.40	106.61 ± 12.35	105.43 ± 11.95	100 ± 15.50
Shell	100 ± 18.55	75.29 ± 13.89	100 ± 17.14	91.69 ± 16.78	100 ± 10.11	100 ± 12.94
Prefrontal Cortex						
Medial	104.20 ± 7.35	101.47 ± 8.79	100 ± 6.31	104.26 ± 7.80	100 ± 3.86	176.60 ± 2.62*
Hippocampus						
DG	101.82 ± 5.18	93.6 ± 1.7	100 ± 8.29	56.29 ± 6.11*	101.01 ± 4.21	35.2 ± 4.64*
Adult						
Amygdala						
Central	100 ± 17.77	127.75 ± 16.18	100 ± 16.10	128.32 ± 12.82	106.67 ± 18.49	513.81 ± 77.18*
Basolateral	100 ± 23.06	53.01 ± 24.42	100 ± 27.03	127.08 ± 27.79	94.59 ± 9.22	121.38 ± 12.10
N. Accumbens						
Core	100 ± 11.46	115.07 ± 18.62	99.99 ± 14.60	128.66 ± 18.66	100 ± 10.21	92.95 ± 15.55
Shell	100 ± 30.28	87.53 ± 16.10	72.93 ± 22.96	93.64 ± 18.04	120.21 ± 22.24	97.37 ± 10.41
Prefrontal Cortex						
Medial	98.73 ± 8.91	96.92 ± 590.5	103.26 ± 7.61	105.84 ± 13.26	100 ± 5.48	139 ± 10.33*
Hippocampus						
DG	100 ± 6.33	103.54 ± 5.60	100 ± 5.82	89.39 ± 10.04	99.99 ± 3.28	38.33 ± 5.05*

* sig diff from saline p<0.05

Table 2.3. Effect of acute alcohol on p-ERK1/2 immunoreactivity in adolescent and adult brain. Data are shown as percent change in mean ± SEM p-ERK1/2 IR as compared to parallel saline controls at each dosage of ethanol tested. * - indicates significantly different from corresponding saline control, p<0.05.

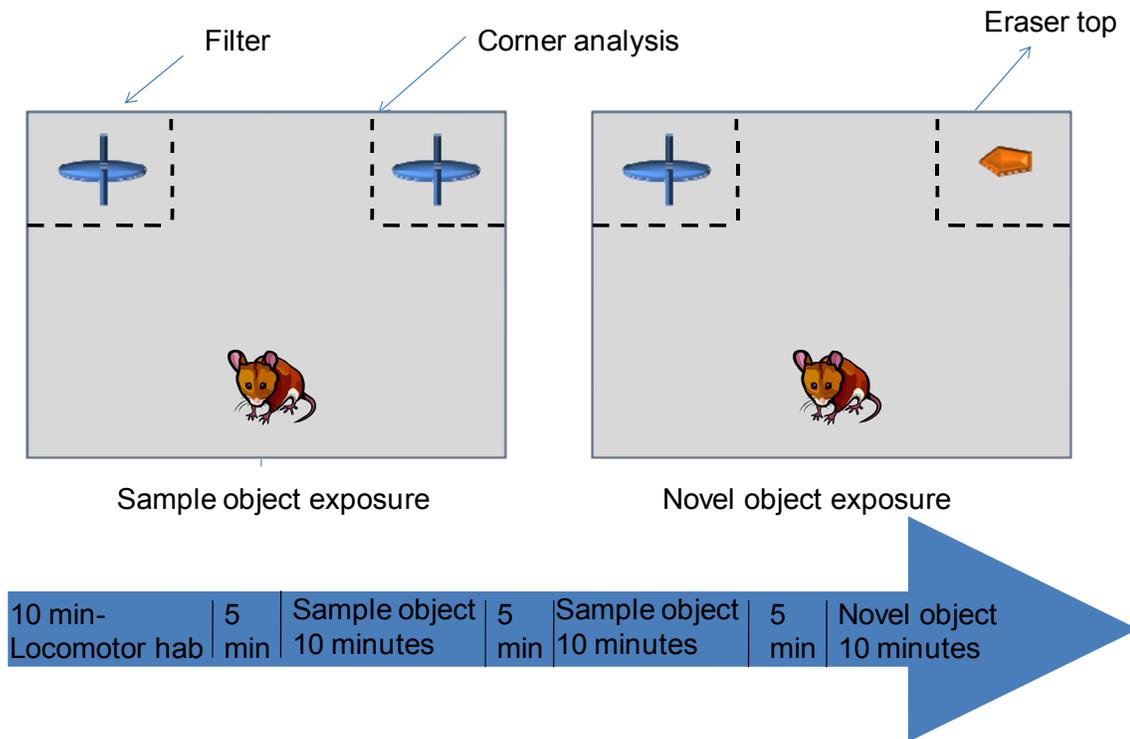


Figure 2.1. Novel Object Recognition Task. Schematic representation of the novel object recognition task as conducted in locomotor chambers. During sample object exposure (left panel), mice were free to explore two of the same object (i.e., filters). During novel object testing (right panel), mice were exposed to one familiar object (i.e., filter) and one novel object (i.e., eraser top). Dashed lines represent the 6.5 cm² zone around the in which object recognition was scored. The blue arrow shows the temporal sequence of procedural events.

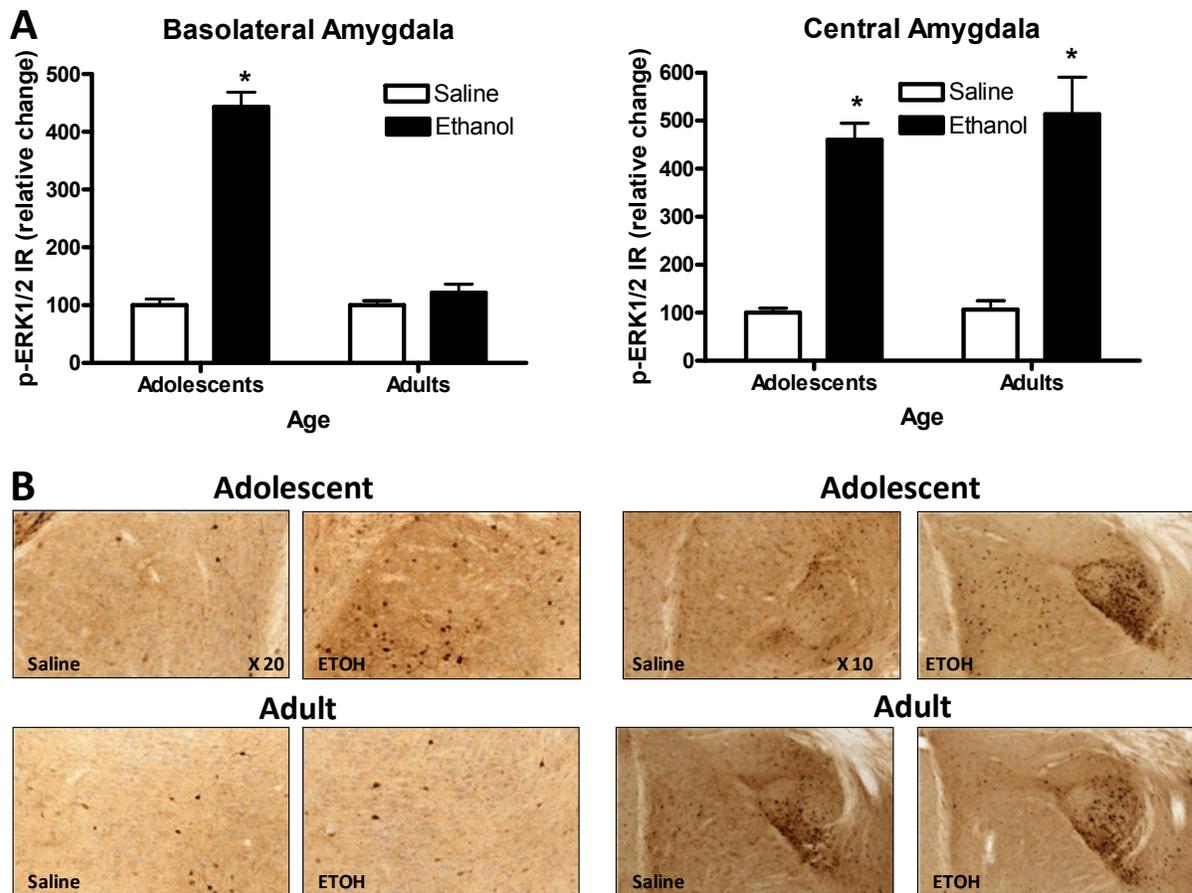


Figure 2.2. Effect of acute alcohol (3g/kg) on p-ERK1/2 IR in the amygdala of adolescents and adult mice. (A) Acute alcohol (3g/kg) produced a four-fold increase in p-ERK1/2 IR in the BLA of adolescent as compared to adult mice (A, left). The same dose of ethanol produced a four- to five-fold increase in both adolescent and adult mice in the CeA (A, right). (B) Representative images of p-ERK1/2 IR in the BLA and CeA from saline and EtOH injected mice. Data represent mean \pm SEM p-ERK1/2 IR and are plotted as percent change relative to saline control. * - indicates significantly different from saline control, $p < 0.05$.

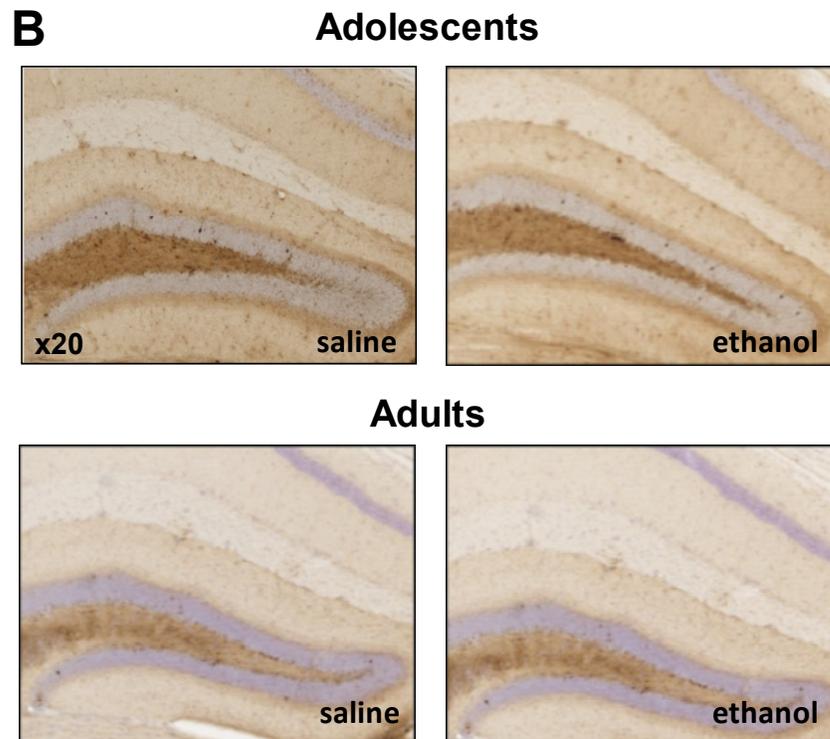
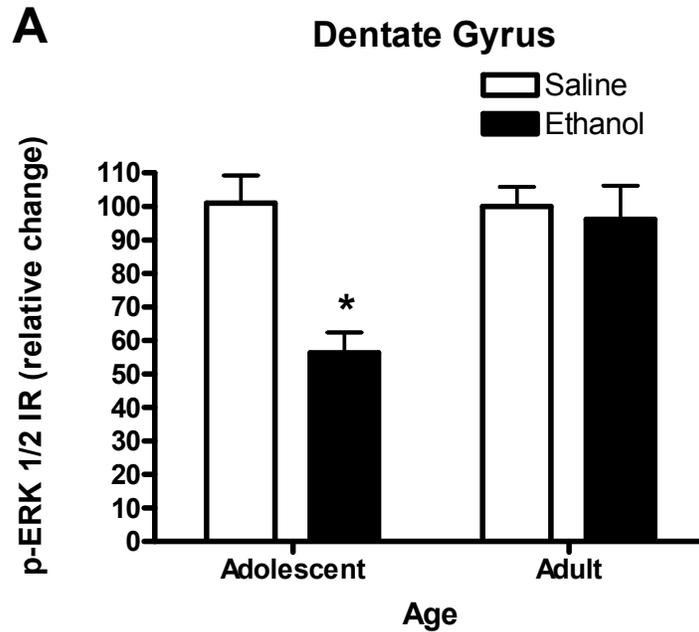


Figure 2.3. Effect of acute alcohol (1g/kg) on p-ERK1/2 IR in the dentate gyrus of the hippocampus in adolescent and adult mice. (A) Acute injection of ethanol (1g/kg) decreased p-ERK1/2 IR in the dentate gyrus of adolescent mice compared with adults. Data represent mean \pm SEM p-ERK1/2 IR plotted as a relative change (%) compared to parallel saline controls. * - indicates significantly different from saline within age group, ($p < 0.05$). **(B)** Representative images of p-ERK1/2 IR in the dentate gyrus from saline and ethanol injected mice.

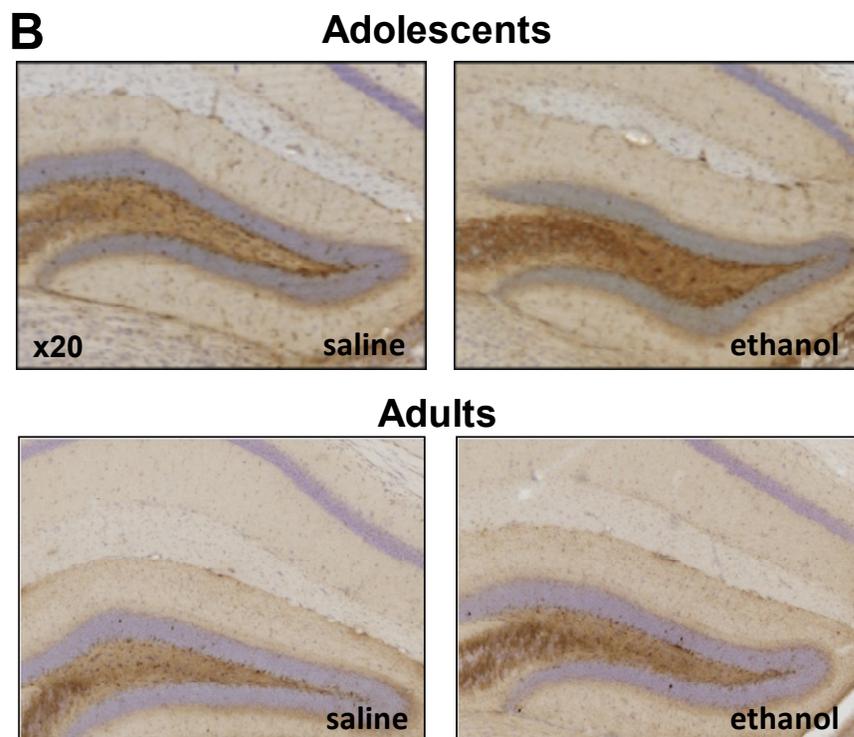
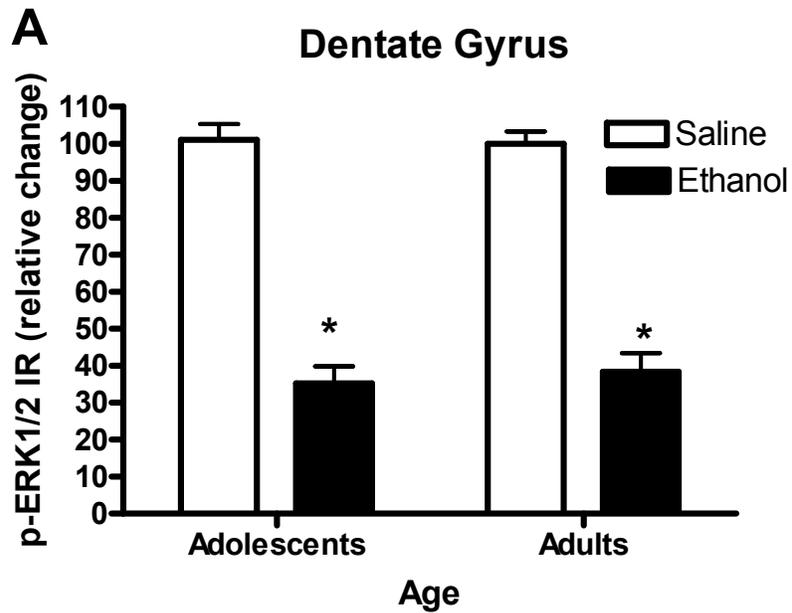


Figure 2.4. Effect of acute ethanol (3 g/kg) on p-ERK_{1/2} IR in the dentate gyrus of the hippocampus in adolescent and adult mice. (A) An acute injection (3g/kg) of ethanol significantly decreased p-ERK_{1/2} IR in the dentate gyrus of both adolescent and adult mice. Data represent mean \pm SEM p-ERK_{1/2} IR plotted as a relative change (%) compared to parallel saline controls. * - indicates significantly different from saline within age group, ($p < 0.05$). ($p < 0.01$) **(B)** Representative images of p-ERK_{1/2} IR in the DG from saline and 3g/kg EtOH injected mice.

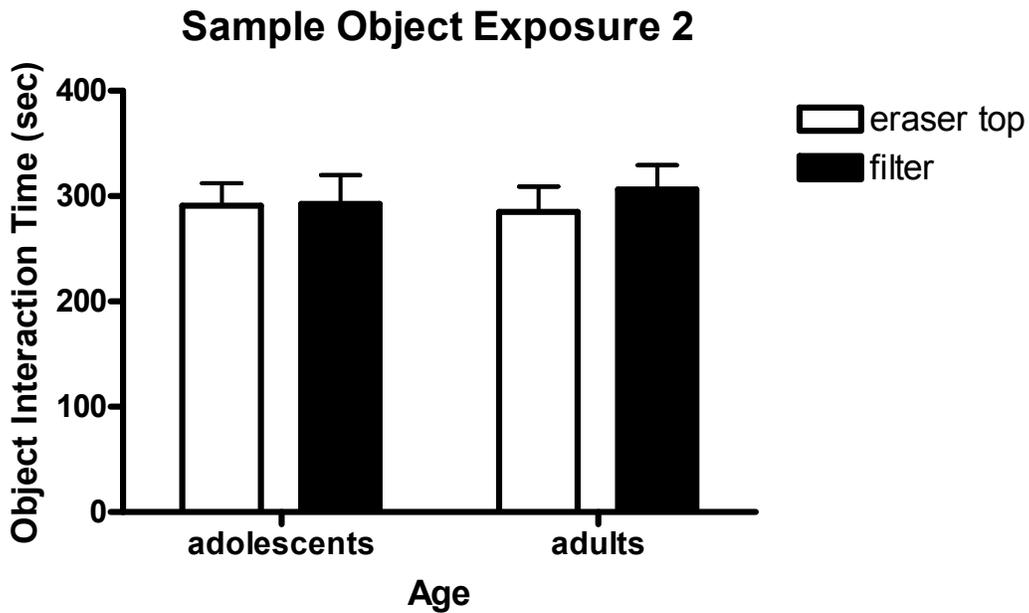
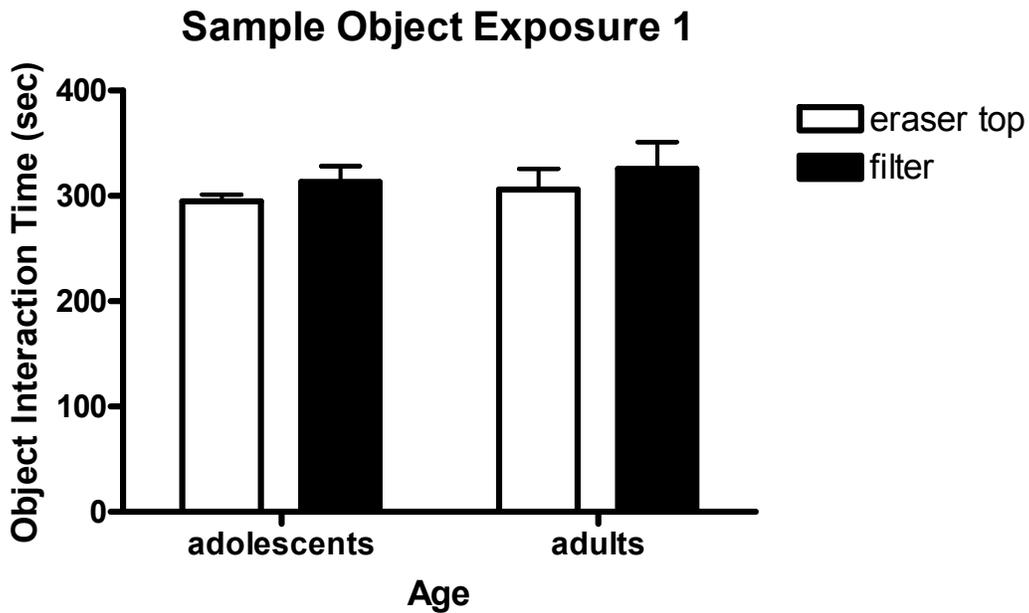


Figure 2.5. Sample object exposure 1 and 2. Object interaction time for adolescent and adult mice during sample object exposure phase (1 and 2). During sample object exposure adolescent and adult mice interacted with the filter and eraser top equally. Both adolescents and adults spent approximately 250-300 sec with each object. Session 1 students-t test ($p=0.295$), ($p=0.547$) respectively and session 2 students-t test ($p=0.966$), ($p=0.5311$) respectively. Data represent mean \pm SEM seconds ($n=6$ per condition).

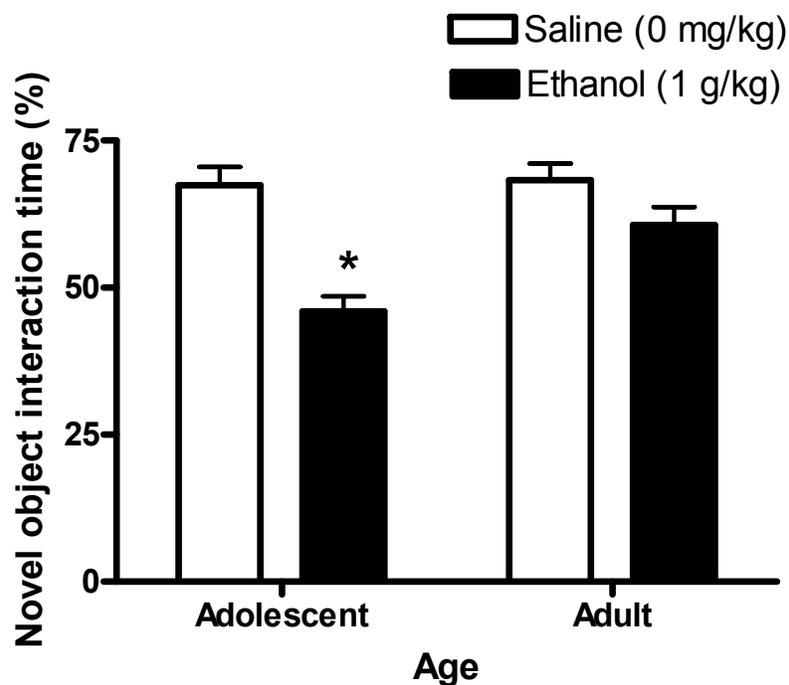


Figure 2.6. Age-dependent effect of acute ethanol (1 g/kg) on adolescent and adult performance in the novel object recognition task. Adolescent mice injected with acute ethanol (1 g/kg) interacted with the novel object significantly less than vehicle treated controls. No statistically significant effect of ethanol was observed in adult mice. Data represent mean \pm SEM percentage of time interacting with the novel object (relative to total time). * - indicates significantly different from saline within age group, ($p < 0.05$).

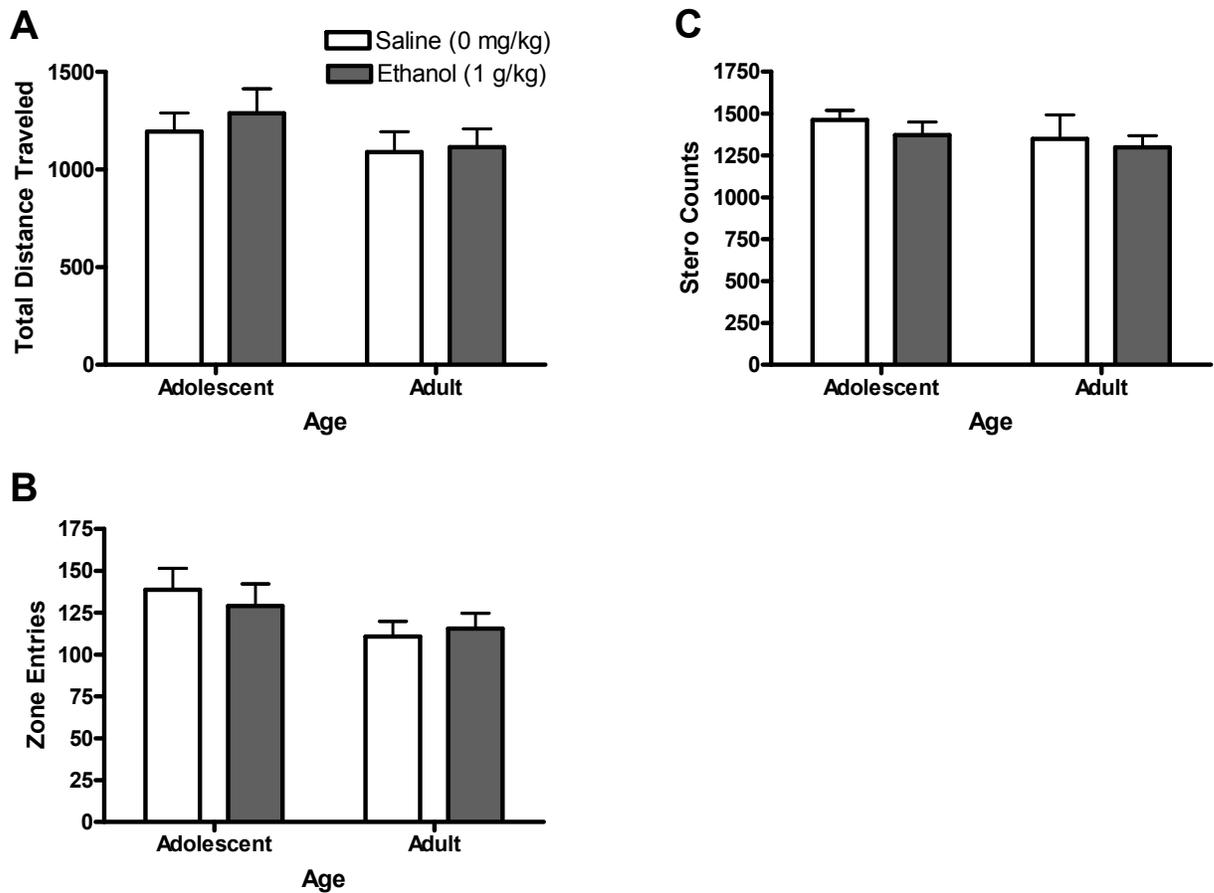


Figure 2.7. Measures of locomotor activity in ethanol (1 g/kg) treated adolescent and adult mice during the novel object task. (A) No differences were observed in total distance traveled in centimeters during the novel object test. **(B)** Number of entries in the novel object zone were not different among ages or ethanol treatment conditions. **(C)** There were also no differences in stereotypy (repeated discrete movements) counts during the test. Data represent mean \pm SEM (n=12 per condition).

CHAPTER III: NOVEL MOLECULAR MECHANISM OF INCREASED ADOLESCENT ALCOHOL DRINKING

INTRODUCTION

Adolescence is a precarious developmental period that defines the transition from childhood to adulthood. Adolescents exhibit new behavioral repertoires that reflect the appropriate acquisition of adult cognitive and emotional function (Anthony 1982) but also engage in a variety of health-risk behaviors that account for leading causes of morbidity and mortality among the adolescent age group (Merrick, Kandel et al. 2004). Epidemiological data indicate that human adolescents are more involved in the expression of risky novel behaviors, including drug abuse, compared to adults (Laviola, Macri et al. 2003) and that heavy alcohol use during adolescence has long-term, negative health consequences in early adulthood including obesity, hypertension, and unsafe driving practices (Oesterle, Hill et al. 2004). Adolescence is the period during which alcohol use in humans is often initiated and is the most widely abused drug in this age group (Johnston, O'Malley et al. 2009). Studies have indicated that chronic alcohol use results in brain dysmorphology in areas involved with addiction and adolescents are particularly vulnerable to the effects of alcohol as their brains are still developing (Crews, He et al. 2007). The most alarming data suggest that alcohol use during adolescence before age 14 increases the likelihood of adult alcohol dependency by four-fold when compared to initiation of alcohol drinking after age 20 (Grant and Dawson 1997).

Age-dependent differences in chronic alcohol intake have been examined in both rats and mice with conflicting results. Studies have found that adolescent rats voluntarily

consume more alcohol than their adult counterparts under continuous voluntary access conditions (Brunell and Spear 2005; Doremus, Brunell et al. 2005). However, in contrast, at the onset of drinking adolescent rats were found to consume less alcohol and showed a lower preference than adults when given a choice between water and 5% and 20% alcohol solutions (Siegmund, Vengeliene et al. 2005). A more recent study in adolescent mice given a free choice between water and 10% alcohol (v/v) self-administered a similar amount as adults per kilogram of body weight (Hefner and Holmes 2007). While there are human and nonhuman data suggesting that drinking is initiated during adolescence and that alcohol drinking can promote adverse neural and cognitive changes, the cellular mechanisms underlying this require further elucidation.

The development of alcohol addiction (chronic alcohol use) is thought to entail the creation of long term adaptive changes in neurotransmitter and cellular signaling systems leading to altered responses at the cellular and neural circuit levels (Koob 2000). The mitogen-activated protein kinase (MAPK) signaling pathway is a three-tiered phosphorylation cascade that links events at the plasma membrane to activation of nuclear transcription proteins (including CREB) to influence more long-term changes in the cell such as those that occur with addiction (Pearson, Robinson et al. 2001; Prakash, Zhang et al. 2008). Extracellular regulated kinase (ERK) 1 and 2 (ERK1/2) are members of the MAPK pathway and regulate adaptive processes such as synaptic plasticity (English and Sweatt 1996; Martin, Michael et al. 1997; Bozon, Kelly et al. 2003; Sweatt 2004) and maladaptive processes such as drug-induced plasticity (Girault, Valjent et al. 2007). Given its role in mediating long-term neuroplasticity, the ERK/MAPK cell signaling system is an interesting candidate for study in adolescent vulnerability to alcohol abuse and alcoholism.

The ERK/MAPK signaling cascade is responsive to a variety of drugs of abuse (Valjent, Pages et al. 2004; Rubino, Forlani et al. 2005). Acute ethanol has been shown to

decrease p-ERK1/2 levels in cultured neurons (Kalluri and Ticku 2003), mouse and rat cortex (Kalluri and Ticku 2002; Chandler and Sutton 2005) and to increase p-ERK1/2 expression in the Edinger-Westphal nucleus of mice (Bachtell, Tsivkovskaia et al. 2002). The acute anxiolytic effects of ethanol (1 g/kg) are also associated with increased p-ERK1/2 IR in the central and medial amygdala of rats 1-h after administration (Pandey, Zhang et al. 2008). Chronic ethanol exposure has been shown to increase p-ERK1/2 expression in cultured cortical neurons (Kalluri and Ticku 2003) but long-term exposure to ethanol vapor produces decreases in p-ERK1/2 protein expression in a number of brain regions in rats (Sanna, Simpson et al. 2002). Ethanol withdrawal also increases ERK activity in several brain regions including hippocampus, amygdala, dorsal striatum, nucleus accumbens, and frontal cortex (Sanna, Simpson et al. 2002). To date, there have been no studies examining potential developmental differences in the effect of alcohol self-administration on ERK1/2 activity.

Recent evidence also indicates that ERK1/2 regulates alcohol self-administration and relapse-like behavior. Pharmacological inhibition of ERK1/2, but not JNK, activity specifically increases operant ethanol self-administration by C57BL/6J mice, a finding that suggests that ethanol-induced increases may underlie the reinforcing function of the drug (Faccidomo, Besheer et al. 2009). A recent study in P-rats (a genetic model of alcoholism) showed that cue induced reinstatement of alcohol-seeking behavior is associated with an increase in ERK1/2 phosphorylation in both the basolateral nucleus of the amygdala and the nucleus accumbens shell, a finding that suggests that ERK1/2 activity may mediate relapse-like behavior in individuals with genetic risk of developing alcoholism (Schroeder, Spanos et al. 2008). These studies support the possibility that the ERK/MAPK pathway may regulate alcohol self-administration.

To this point there has been no examination of chronic alcohol's effects on adolescent cellular signaling systems, including ERK1/2. The purpose of the present study was to characterize developmental and brain regional differences in the ERK/MAPK signaling pathway associated with chronic voluntary alcohol drinking in C57Bl6/J mice. Adolescent (27-41 days) and adult mice (70-84 days) were allowed to voluntarily consume 10% alcohol or water in the home cage. On the last day of drinking (day 14) mice were sacrificed for immunohistochemical evaluation. P-ERK1/2 immunoreactivity (IR) was examined in brain regions specifically associated with alcohol self-administration such as the nucleus accumbens core and shell (NuAcc-core, shell), central (CeA) and basolateral amygdala (BLA), medial prefrontal cortex (mPFC) and dentate gyrus (DG). To test potential functional regulation of alcohol drinking by ERK1/2 signaling, the effect of the MEK/ERK inhibitor SL327 on alcohol drinking was tested in adolescent (27-41 days) and adult mice (70-84 days).

METHODS

Animals. Male C57BL/6J mice (Jackson Laboratories, Bar Harbor, Maine) were singly housed in standard Plexiglas cages. Food and water was available *ad libitum* in the home cage. The mice were 21 days and 63 days upon arrival to the facility. The mice were handled and weighed before experiments began to acclimate to the reverse light cycle, resume normal sleep/wake cycles (Wexler and Moore-Ede 1986) and allow the mice to reach adolescence post natal day (PND 27) and adulthood (PND 70) before experiments were initiated. The colony room was maintained at 27°C on a 12-hour light/dark cycle (lights on 20:00), and experiments were performed during the dark cycle. Animals were under continuous care and monitoring by the Division of Laboratory Animal Medicine (DLAM) at UNC-Chapel Hill. All procedures were carried out in accordance with the NIH *Guide to Care*

and Use of laboratory Animals (National Research Council, 1996) and institutional guidelines.

EXPERIMENT 1: EFFECT OF ALCOHOL DRINKING ON P-ERK1/2 IMMUNOREACTIVITY (IR) IN ADOLESCENT AND ADULT MICE

Two-Bottle Self Administration. Mice self-administered alcohol 10%v/v using a two-bottle choice procedure. The alcohol drinking groups were given access to one bottle containing alcohol (10%v/v) for three days. The second bottle containing water was introduced three days later to bring the total drinking time to 14 days. Fluid intake levels and weights were taken each day to determine a daily alcohol intake measurement. The position (left or right) of each solution was alternated daily to control for side preferences. Parallel control mice (water drinking groups) were given access to two bottles as well, both containing water, and weighed daily.

Immunohistochemistry. On the last day of drinking (day14) mice were deeply anesthetized with 60 mg/kg pentobarbital. The mice were then transcardially perfused with 0.1M phosphate buffered saline (PBS) followed by 4% paraformaldehyde. The brains were extracted from the skulls washed in PBS and sliced on a vibratome into 40 μ m coronal sections. These free-floating sections were then stored in cryoprotectant at -20 $^{\circ}$ C until immunohistochemical processing. The free-floating sections were rinsed in PBS, and then placed in 1% hydrogen peroxide in PBS to block endogenous peroxidase. Next sections underwent antigen retrieval using citra buffer at 70 $^{\circ}$ C for 30 min (Antigen Retrieval Citra, BioGenex). Sections were then blocked in 0.1% triton-X in PBS with 5% goat serum for 1 hour and then were incubated at +4 $^{\circ}$ C overnight in primary polyclonal antibody to p-ERK_{1/2} (1:200 Cell Signaling Technology, Danvers, MA). The sections were incubated in Dako EnVision Kit (Dako, Carpinteria, CA) secondary antibody for one hour at room temperature

and immunoreactivity was detected using nickel enhanced diaminobenzene (Dako EnVision Kit) as a chromagen. The sections were counterstained using toluidine blue, mounted onto slides, and cover slipped.

Immunoreactivity was visualized using an Olympus CX41 light microscope (Olympus America, Center Valley, PA). Images were acquired using a digital camera (Regita model, QImaging, Burnaby, BC) interfaced to a desktop computer (Dell, Round Rock, TX). Image analysis software (Bioquant Nova Advanced Image Analysis; R&M Biometric, Nashville, TN). The microscope, camera, and software were background corrected and normalized to preset light levels to ensure fidelity of data acquisition.

Data Analysis. Data for p-ERK_{1/2} IR were acquired from a minimum of 4 sections/brain region/animal and averaged to obtain a single value per subject. Coordinates of brain regions analyzed were as follows: nucleus accumbens and proximal brain regions (+ 0.86 to 1.34 mm anterior to bregma), amygdala and proximal brain regions (-1.7 to -1.34 mm posterior to bregma). Pixel density and cell count measurements were calculated from a circumscribed field (e.g., brain region) and divided by the area of the region and expressed as pixels/mm². Raw data values were analyzed when comparing basal expression of for p-ERK1/2 IR between adolescent and adult mice. Basal expression in each brain region was compared statistically between age groups with Students t-test. Effects of alcohol drinking were analyzed statistically via two-way ANOVA (age x drinking history) followed by Holm-Sidak post hoc multiple comparisons where appropriate. Since differences in basal expression were detected, effects of ethanol were analyzed as a percentage change from parallel water control mice of the same age. P-ERK1/2 IR data were collected by a researcher blind to treatment conditions.

Blood Alcohol Determination. Blood alcohol measurements were taken at the time of perfusion in order to ensure the differences we were observing in p-ERK1/2 (adolescent

and adult mice) were not due to confounds of a differential dose. Approximately 20 µl of heart blood was collected at the time of perfusion. From the 20 µl centrifuged sample 5 µl of plasma was used to determine alcohol concentration using an AM1 Alcohol Analyzer (Analox Instruments, Lunenburg, MA).

EXPERIMENT 2: ALCOHOL SELF-ADMINISTRATION AND MEK/ERK INHIBITION

MEK/ERK inhibitor drinking study. A separate cohort of adolescent (27-41 days) and adult (70-84 days) mice self-administered alcohol 10%v/v using a two-bottle choice procedure. Fluid intake levels and weights were taken each day from 11:00-12:00 to determine a daily alcohol intake measurement. On drinking days 9, 10 and 11 mice were given habituation intraperitoneal injections (11:00-12:00) of the vehicle (0.1% CMC). On day 12 of drinking adolescent (PND 40) and adult (PND 83) mice were injected with the ERK/MEK inhibitor SL327 (0 or 30mg/kg) at (11:00-12:00) and fluid levels were monitored every two hours from 12:00-14:00 and again from 14:00-16:00 in order to obtain an alcohol intake measurement. Control mice (water drinking groups) were also injected with the vehicle and SL327 inhibitor and their fluid levels were also monitored every two hours from 12:00-14:00 and 14:00-16:00. Ethanol intake (g/kg), water intake (mls), and relative ethanol intake (preference) were analyzed by two-way ANOVA (age x drug) followed by Holm-Sidak multiple comparisons where appropriate.

Locomotor behavior. The open field activity (horizontal distance traveled, cm) of adolescent and adult mice (group 2) was measured in eight covered, sound attenuating Plexiglas chambers (27.9 cm², ENV-510, Med Associates, Georgia, VT). Two sets of 16 pulse-modulated infrared photobeams were located on opposite walls to record ambulatory movements in the X-Y (horizontal) plane. All software settings were the same for adult and

adolescent mice. The activity chambers were computer-interfaced (Med Associates) for data sampling at 100-millisecond resolution.

On day 14 of drinking, all mice were given an intraperitoneal (IP) injection of the ERK/MEK inhibitor SL327 or vehicle (0.1% CMC) and placed in the locomotor chambers for a 120 minute session. Locomotor tests were conducted in alcohol and water drinking mice both adolescent and adult (from group 2) to evaluate the locomotor effect of 30 mg/kg of SL327.

Drugs. The 10% alcohol solution (v/v) was prepared by diluting 95% alcohol (Pharmaco Products Inc., Brookfield, CT, USA) with tap water. The MEK1/2 inhibitor, SL327, (α -[amino][(4-aminophenyl) methylene]-2-(trifluoromethyl) benzenecetonitrile (Tocris bioscience, Ellisville, MO, USA) was diluted in carboxy methylcellulose (0.1%) to a concentration of 3.0 mg/ml and injected in a volume of 0.01 ml/g body weight.

RESULTS

Alcohol intake in Adolescent and Adult Mice

During the 14 day drinking period, adolescent mice consumed an average (mean \pm SEM) of 12.1 \pm 0.93 g/kg of alcohol (10% v/v) per day and adult mice consumed an average (mean \pm SEM) of 11.8 \pm 0.32 g/kg of alcohol (10% v/v) per day (Figure 3.1A). Statistical analysis indicated that alcohol intake did not differ between adolescent (PND 27-41) and adult (PND 70-84) C57BL/6J mice (Students-t, p=0.7594). Blood alcohol levels taken from adolescent mice at time of perfusion on day 14 showed a range of 22 – 130 mg/dL with a group mean \pm SEM of 77.8 \pm 8.8 mg/dL. Blood alcohol levels taken from adult mice at time of perfusion on day 14 showed a range of 13 – 201 mg/dL with a group mean \pm SEM of 76.7 \pm 20.1 mg/dL. Average blood alcohol concentrations did not differ between adolescent and adult mice (Students-t, p=0.96) at the time of perfusion (Figure

3.1B). There was also no difference in relative ethanol intake (ethanol preference) with each age group preferring the ethanol solution at approximately 75% as compared to water (Figure 3.1C). Total fluid consumption showed an age-appropriate statistical difference with adults consuming significantly more total fluid than adolescents ($t(20)=4.262$, $p=0004$) (Figure 3.1D).

Basal p-ERK1/2 IR in mesocorticolimbic brain regions of alcohol naïve adolescent and adult C57BL/6J mice

P-ERK1/2 IR was analyzed in various mesocorticolimbic brain regions from water drinking mice to characterize potential ontogenetic differences (Table 3.1). Qualitative observation indicated the highest densities of p-ERK1/2 IR were observed in the medial prefrontal cortex and central amygdala with lower levels of immunostaining seen in the dentate gyrus, the nucleus accumbens (core and shell) and the basolateral amygdala. Statistical analysis showed that adolescent mice have higher basal expression of p-ERK1/2 IR in the central amygdala ($t(21)=4.2, p=0.0004$), basolateral amygdala ($t(19)=2.3, p=0.03$), nucleus accumbens shell ($t(21)=2.2, p=0.04$), and medial prefrontal cortex ($t(20)=2.7, p=0.014$) (Table 3.1). No differences were observed in nucleus accumbens core or dentate gyrus.

Effect of Alcohol Drinking (10%v/v) on pERK Immunoreactivity (% change)

Central Amygdala. A 2-way ANOVA indicated a significant main effect of age ($F(1, 39) = 20.81$, $p < 0.001$) and 2 weeks of alcohol drinking ($F(1, 39) = 17.59$, $p < 0.001$) on p-ERK1/2 IR in the central amygdala. In addition there was a significant interaction between alcohol and age ($F(1, 39) = 20.71$, $p < 0.001$) indicating that effects of alcohol drinking depended on age. Post hoc (Holm-Sidak) comparisons showed that alcohol produced a significant increase in p-ERK1/2 IR in adolescents ($p < 0.001$) and not adult mice (Figure

3.2A). Representative photomicrographs illustrating the cytological pattern of p-ERK1/2 IR immunoreactivity in the central amygdala are shown in Figure 3.2B.

Basolateral Amygdala. Alcohol drinking (2-bottle self-administration) did not alter p-ERK1/2 IR in a basolateral amygdala in adolescent and adult mice (Table 3.2)

Nucleus Accumbens (core and shell). Alcohol drinking (2-bottle self-administration) did not alter p-ERK1/2 IR in the nucleus accumbens core or shell in adolescent and adult mice (Table 3.2).

Medial prefrontal cortex. Alcohol drinking (2-bottle self-administration) did not alter p-ERK1/2 IR in the medial prefrontal cortex (mPFC) (Table 3.2).

Dentate Gyrus. A 2-way ANOVA indicated a significant main effect of age ($F(1, 47) = 5.593, p = 0.024$) and alcohol ($F(1, 47) = 8.506, p = 0.006$) after 2 weeks of alcohol self-administration on p-ERK1/2 IR in the dentate gyrus. In addition there was a significant interaction between alcohol and age ($F(1, 47) = 5.493, p = 0.024$) Post hoc (Holm-Sidak) comparisons showed that alcohol produced a significant increase in p-ERK1/2 IR in adolescents ($p = 0.002$) and not adult mice (Figure 3.3A). Representative photomicrographs illustrating the cytological pattern of p-ERK IR in the dentate gyrus are shown in Figure 3.3B.

Effect of the MEK/ERK inhibitor (SL327) on alcohol self-administration

A 2-way ANOVA indicated a significant main effect of age ($F(1, 31) = 8.65, p = 0.048$) and SL327 (30mg/kg) inhibitor ($F(1, 31) = 20.69, p = 0.0034$) on alcohol self-administration during the first 2-hr (12pm-2pm) of alcohol drinking. In addition there was a significant interaction between age and SL327 injection ($F(1, 31) = 8.66, p = 0.0488$). Post hoc (Holm-Sidak) comparisons showed that SL327 produced a significant increase in p-ERK1/2 in adolescents ($p < 0.01$) and not adult mice (Figure 3.4). Ethanol intake data during the second 2-hr period (2pm-4pm) did not differ (data not shown). Water intake was not changed by

SL327 treatment in adolescent or adult mice. In addition, adolescent and adult water-only drinking control mice were injected with SL327 (0 or 30 mg/kg) and water intake (mls) was unaffected.

Effect of MEK/ERK inhibitor, SL327, on locomotor activity

The dose of SL327 (30 mg/kg) that increased ethanol intake in adolescent mice had no effect on spontaneous locomotor activity or habituation to the open-field environment (Figure 3.5).

DISCUSSION

The main goal of this study was to examine the potential involvement of the ERK/MAPK cell signaling system in voluntary alcohol drinking by adolescent as compared to adult mice. In the current study, adolescent and adult C57BL/6J mice consumed similar doses (g/kg) alcohol (10% v/v) in a 24-hr access 2-bottle choice self-administration procedure. Adolescent mice also showed similar preference for alcohol. These data are in agreement with other results showing that voluntary alcohol intake is not different between adolescent and adult mice under 24-hr access conditions (Hefner and Holmes 2007). Results of the present study are in contrast with evidence showing that adolescent rats either consume less (Siegmund, Vengeliene et al. 2005) or more alcohol (Brunell and Spear 2005; Doremus, Brunell et al. 2005) than their adult counterparts. However, various environmental and procedural variables have been shown to exert influences on alcohol intake (Doremus, Brunell et al. 2005) in those studies. Since the alcohol drinking procedures used in the present study did not reveal ontogenetic differences in alcohol intake, and mice from each age group showed similar BACs at the time of sacrifice, it was feasible to

examine effects of voluntary drinking on ERK/MAPK activation (phosphorylation) between adolescent and adult mice and to test effects of ERK1/2 inhibition on alcohol intake.

An initial finding of this study is that basal ERK1/2 activity (phosphorylation) differs between adolescent and adult mice in several mesocorticolimbic brain regions. Adolescent water exposed mice showed higher p-ERK1/2 IR in the central and basolateral amygdala, nucleus accumbens shell, and medial prefrontal cortex than adults. Although ERK1/2 activity has been shown to regulate alcohol self-administration in adult C57BL/6J mice (Faccidomo, Besheer et al. 2009), and ERK-associated receptor systems in these brain regions control alcohol self-administration in rats (Hodge, Samson et al. 1992; Hodge, Chappelle et al. 1996; Schroeder, Olive et al. 2003), it appears that basal differences in p-ERK1/2 IR in these brain regions did not influence the development of adolescent alcohol drinking under 24-hr access conditions. Basal differences in ERK1/2 may indicate that ERK1/2 activity in these brain regions is unrelated to alcohol drinking. It will be important to extend these results in future studies by examining ERK1/2 gene expression or protein levels using complementary methods.

Although basal differences in p-ERK1/2 IR did not appear to influence alcohol drinking, it is plausible that alcohol-induced adaptations in ERK1/2 activity may regulate drinking in an age-specific manner. To address this question, adolescent and adult brains were examined for p-ERK1/2 IR after two weeks of voluntary alcohol drinking. Adolescent mice voluntarily consumed alcohol during the two week period ranging from PND 28 – 42 whereas adult mice consumed alcohol during PND 77 – 91. Mice exposed to water only were run in parallel for each age group and data were expressed as a relative change from water controls.

Results showed that alcohol drinking was associated with a significant (40%) age-specific decrease in p-ERK1/2 IR in adolescent dentate gyrus, with no change observed in adults. Other evidence shows that chronic ethanol vapor (12 days continuous or

intermittent) exposure reduces p-ERK1/2 expression in adult rat hippocampus (Sanna, Simpson et al. 2002). This differential effect in adult rats, as compared to the present study, may reflect different routes of ethanol administration (oral vs. vapor), exposure method (voluntary vs. forced), anatomical variation (dentate gyrus vs. total hippocampus), species differences, or differences in immunohistochemistry vs. Western techniques. Alternatively, the present methods resulted in lower BAC (70 mg/dL) as compared to the vapor method (200 mg/dL), which may reflect differential sensitivity to the dose-dependent effects of ethanol on p-ERK1/2 levels in the hippocampus. Overall, since ERK1/2 activation in the hippocampus is critical for memory consolidation (Blum, Moore et al. 1999; Schafe, Atkins et al. 2000; Kelly, Laroche et al. 2003; Runyan, Moore et al. 2004), and adolescent rodents are more sensitive to ethanol-induced reductions in ERK1/2 in the dentate gyrus (Chapter 2), impairment of performance on a hippocampal-dependent memory test (Chapter 2), and memory acquisition (Markwiese, Acheson et al. 1998) as compared to adults, these data suggest that adaptations in ERK1/2 signaling in the dentate gyrus is one potential adaptation that may underlie adolescent vulnerability to cognitive impairing effects of alcohol as reported in humans (Brown, Tapert et al. 2000).

An age-specific increase in p-ERK1/2 after chronic alcohol consumption was noted in adolescents in the central nucleus of the amygdala (CeA) compared with adults. This result suggests that adolescent mice are more sensitive to effects of voluntary alcohol drinking on p-ERK1/2 IR in the CeA. Our results are consistent with a study showing that chronic exposure to an alcohol liquid diet for 15 days had no effect on protein levels of p-ERK_{1/2} in the amygdala of adult rats (Pandey, Zhang et al. 2008), highlighting the differential response of chronic alcohol on the adolescent amygdala. However, exposure to higher levels of ethanol via alcohol vapor chambers (BAL of approximately 200%) significantly decreased p-ERK_{1/2} in the amygdala (Sanna, Simpson et al. 2002). As noted above, however, alcohol exposure paradigms and duration of alcohol exposure was different and cannot be directly

compared. However, since alcohol-induced increases in p-ERK1/2 may underlie its reinforcing effects (Faccidomo, Besheer et al. 2009), increased p-ERK1/2 IR in the adolescent amygdala after alcohol drinking may reflect differential regulation of self-administration in this age group.

Thus, another goal of the study was to determine if the ERK/MAPK pathway is functionally involved in modulating alcohol intake in male adolescent and adult C57BL/6J mice. To accomplish this goal, we tested effects of the MEK/ERK inhibitor SL327 (0 or 30 mg/kg) on alcohol drinking by adolescent and adult mice using a 4-hr limited access procedure. If alcohol induced increases in p-ERK1/2 in CeA are an important pharmacological effect of the drug that maintains self-administration in adolescents, then inhibiting pERK1/2 should increase drinking. Alternatively, if alcohol-induced reductions in ERK1/2 activity in the dentate gyrus are important for the maintenance of alcohol drinking, then one might predict that ERK inhibition should reduce intake. Results of this study showed that an intraperitoneal injection (IP) of SL327 increased alcohol intake (g/kg) in adolescent but not adult C57BL/6J mice during the first 2-hr of access. No effects were seen on alcohol drinking during the second 2-hrs of alcohol drinking. Since adolescent mice show increased ERK1/2 phosphorylation in the CeA after voluntary alcohol drinking, it is possible that the inhibitor influenced alcohol drinking via actions in this brain region. This result is consistent with a variety of evidence showing that the CeA regulates alcohol self-administration via receptor systems that are associated with ERK/MAPK activity including, GABA-A, opiates, and NPY (Heyser, Roberts et al. 1999; Hyytia and Kiianmaa 2001; Schroeder, Olive et al. 2003) and other evidence that CeA function is important for alcohol drinking (McBride 2002). Injection of the MEK/ERK inhibitor in water-drinking control mice did not increase intake (mls) which indicates that inhibition of ERK specifically affects alcohol and not general intake. Open field studies indicated that there was no effect of the MEK/ERK inhibitor SL327 on locomotor activity in adolescents and adult mice. Our data

agrees with recently published data associating alcohol self-administration with the ERK signaling cascade (Faccidomo, Besheer et al. 2009). C57BL/6J mice trained to self-administer alcohol/sucrose showed increased alcohol-reinforced responding after injection of the ERK/MEK inhibitor SL327, but not the JNK-inhibitor (Faccidomo, Besheer et al. 2009). This result indicates that activation of ERK1/2 is involved the reinforcing properties of alcohol due to its selective potentiation of alcohol-reinforced responding (Faccidomo, Besheer et al. 2009). Results of the present study suggest that adolescent mice are more sensitive to both the effects of alcohol drinking on the ERK/MAPK system and to its functional regulation of drinking behavior. Future studies to determine whether the SL327 inhibitor effect is specific to alcohol or if it influences intake of other caloric compounds such as sucrose should be addressed.

In conclusion, findings of the present investigation show that inhibition of ERK1/2 phosphorylation caused an age-dependent effect on alcohol intake (g/kg) in C57BL/6J mice. Systemic administration of the MEK/ERK inhibitor SL 327, increased alcohol, but did not affect water intake in adolescent mice. Since chronic alcohol self-administration increased p-ERK1/2 in a brain region known to regulate self administration (CeA), these data are consistent with the hypothesis that ERK1/2 phosphorylation in the CeA is an important pharmacological effect of alcohol that maintains self-administration in adolescents. The neuroadaptations in the CeA induced by alcohol self-administration on the ERK signaling cascade during adolescence may enhance susceptibility to a future alcohol addiction.

Age	Brain regions	Basal p-ERK1/2 levels (IR)
Adolescent	Amygdala	
	Central	6407.97 ± 543.86*
	Basolateral	3789.617 ± 499.93*
	N. Accumbens	
	Core	3449.11 ± 143.14
	Shell	3158.85 ± 276.18*
	Prefrontal Cortex	
	Medial	7727 ± 484.25*
	Hippocampus	
	DG	12.25 ± 0.62
Adult	Amygdala	
	Central	3412.35 ± 170.16
	Basolateral	2654.15 ± 110.17
	N. Accumbens	
	Core	3314.35 ± 519.97
	Shell	2558.13 ± 108.33
	Prefrontal Cortex	
	Medial	6660.17 ± 482.74
	Hippocampus	
	DG	12.1 ± 0.56

* significantly different from adult (p < 0.05)

Table 3.1. Basal levels of p-ERK1/2 IR in ethanol naïve adolescents and adult mice in specific mesocorticolimbic brain regions. Data represent mean ± SEM pixels/mm² from n=11-12 mice per age / brain region.

Age	Brain regions	Drinking History	
		Water	Ethanol
Adolescent	Amygdala		
	Central	100 ± 6.05	181.59 ± 8.71*
	Basolateral	97.04 ± 11.22	79.72 ± 2.98
	N. Accumbens		
	Core	100 ± 5.14	86.64 ± 9.72
	Shell	94.41 ± 15.56	87.44 ± 17.19
	Prefrontal Cortex		
	Medial	100 ± 8.24	90.92 ± 10.482
	Hippocampus		
	DG	99.99 ± 10.76	59.11 ± 6.41*
Adult	Amygdala		
	Central	100 ± 13.07	96.56 ± 10.61
	Basolateral	100 ± 7.58	90.84 ± 12.41
	N. Accumbens		
	Core	100 ± 13.84	80.98 ± 9.53
	Shell	100 ± 18.82	63.16 ± 11.15
	Prefrontal Cortex		
	Medial	100 ± 17.98	97.52 ± 7.63
	Hippocampus		
	DG	102.52 ± 7.13	93.70 ± 4.86

* sig diff from adult p<0.05

Table 3.2. Effects of ethanol drinking on p-ERK1/2 IR in mesocorticolimbic brain regions from adolescent and adult mice. Data represent relative change (percentage) in mean±SEM pixels/mm² from n=8 adolescent and n=10 adult mice per group.

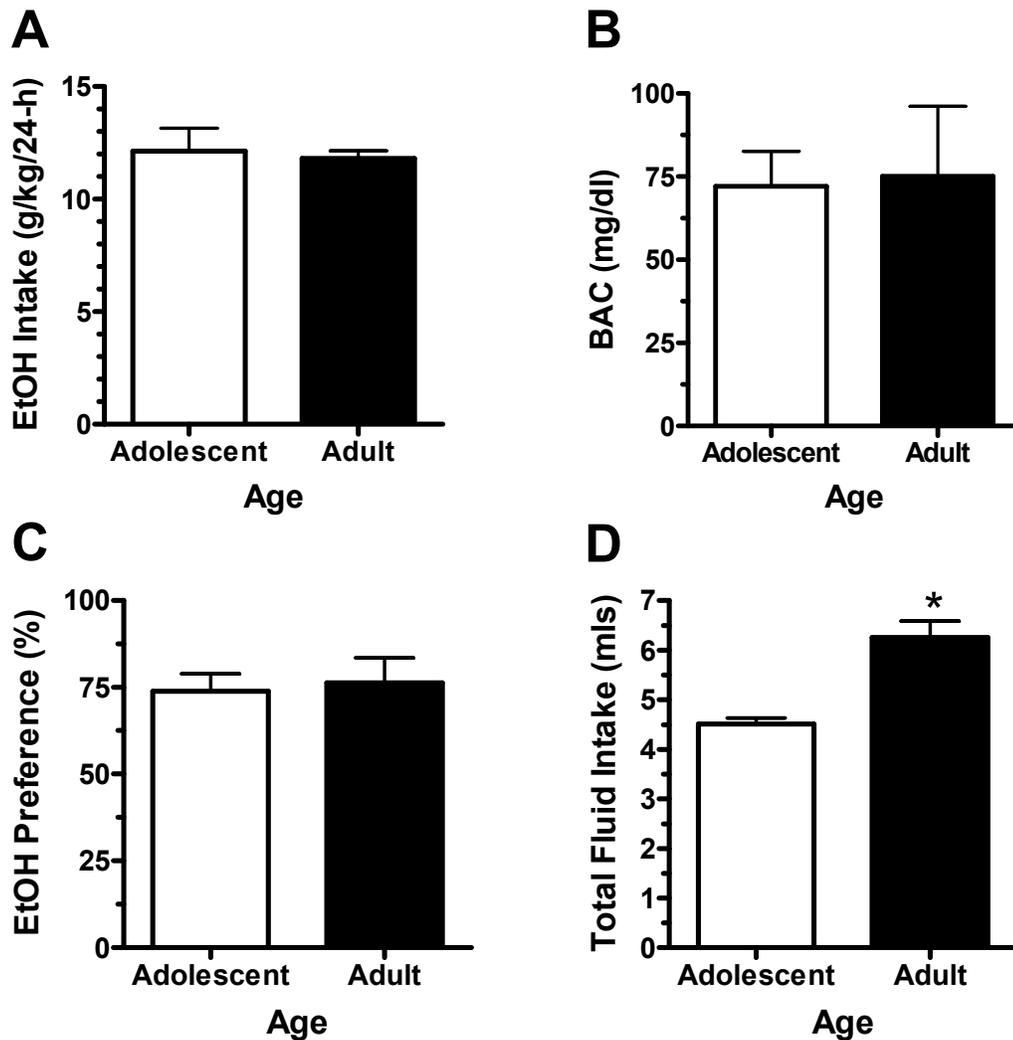


Figure 3.1. Measures of voluntary ethanol drinking as a function of developmental stage. (A) Dosage of ethanol (EtOH) intake (g/kg/24-h) plotted as a function of age. (B). Blood alcohol concentration (BAC) in mg/dL plotted as a function of age. (C) Mean EtOH preference (EtOH intake / total fluid intake in %) plotted as a function of age. (D) Mean total fluid intake (EtOH mls + water mls) in each age group. Although adult mice consumed more total fluid, adolescent and adult mice did not differ in measures that correct for differential body mass (EtOH intake, BAC, or preference). Data represent (mean \pm SEM) values from groups of adolescent (n=13) and adult mice (n=14). * - indicates significantly different from adolescent, p=0.0004 (Students t-test).

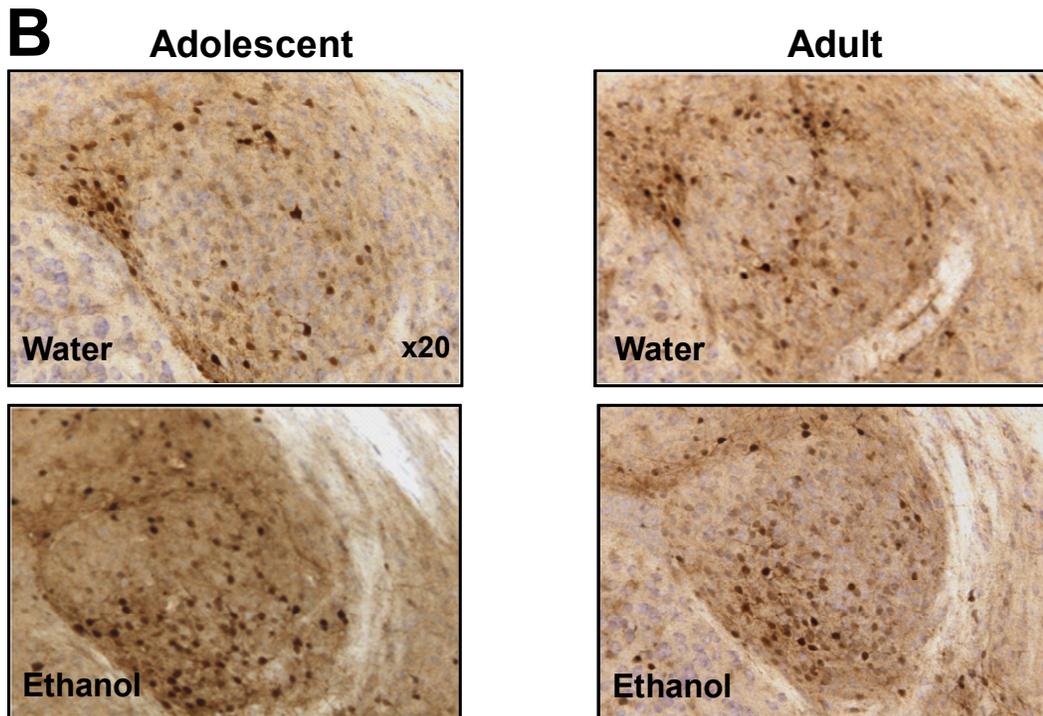
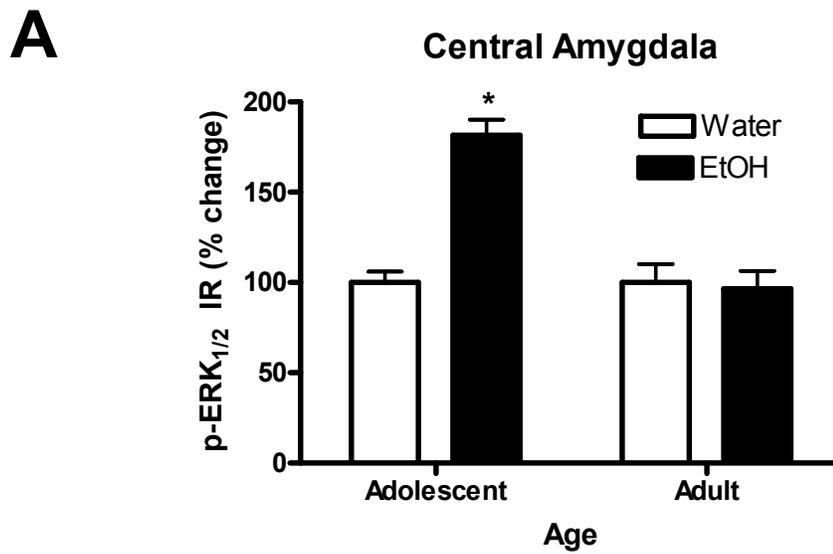


Figure 3.2. P-ERK1/2 response in the central nucleus of the amygdala and representative photomicrographs. Alcohol drinking (2-bottle self-administration) increased p-ERK1/2 in adolescents (* $p < 0.05$). Representative photomicrographs illustrating the cytological pattern of pERK immunoreactivity in the central amygdala.

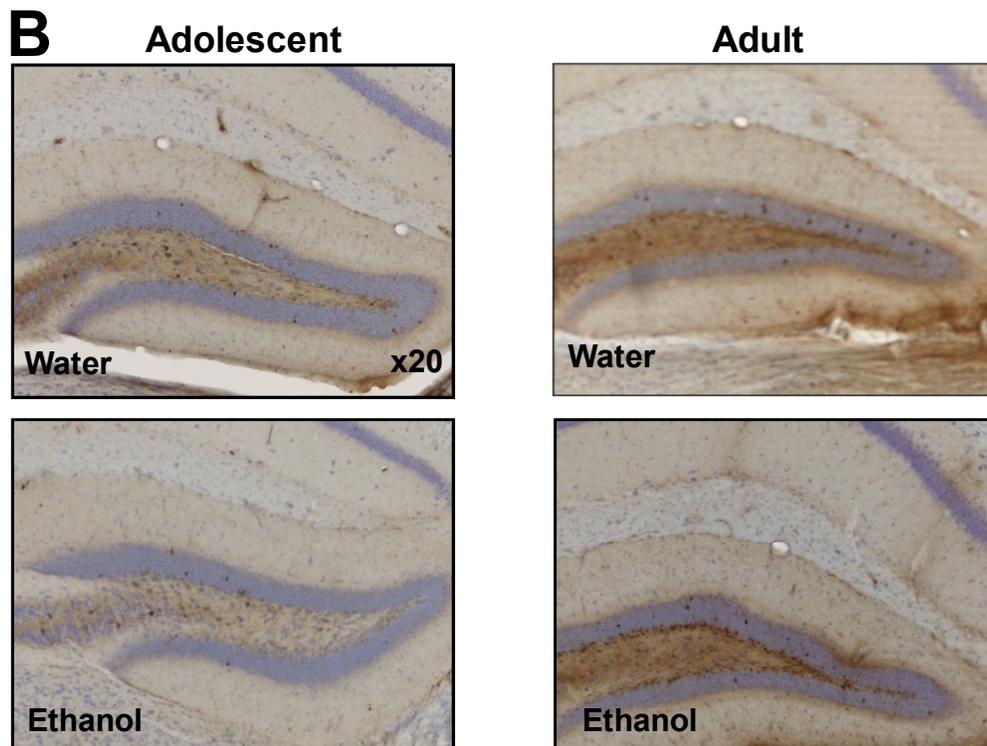
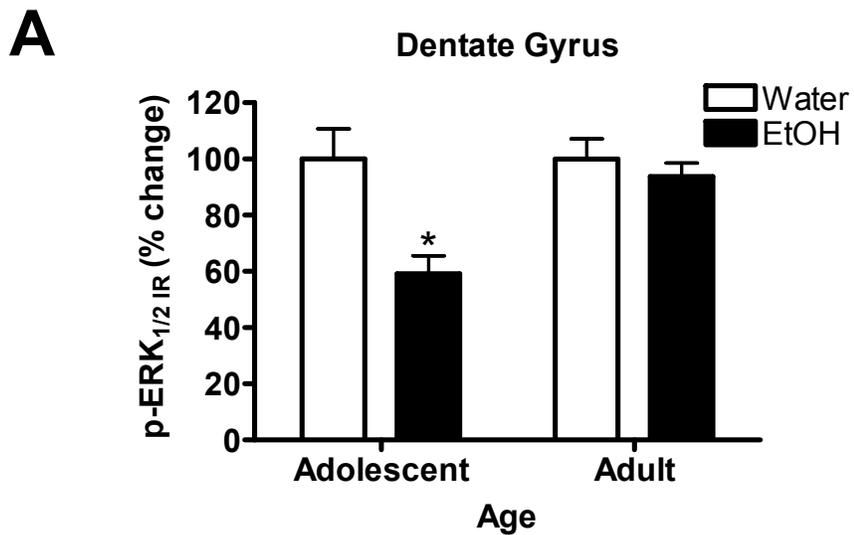


Figure 3.3. P-ERK1/2 response in the dentate gyrus of the hippocampus and representative photomicrographs. Alcohol drinking (2-bottle self-administration) decreased p-ERK1/2 IR in adolescent mice (* $p < 0.05$). Representative photomicrographs illustrating the cytological pattern of p-ERK1/2 immunoreactivity in the dentate gyrus

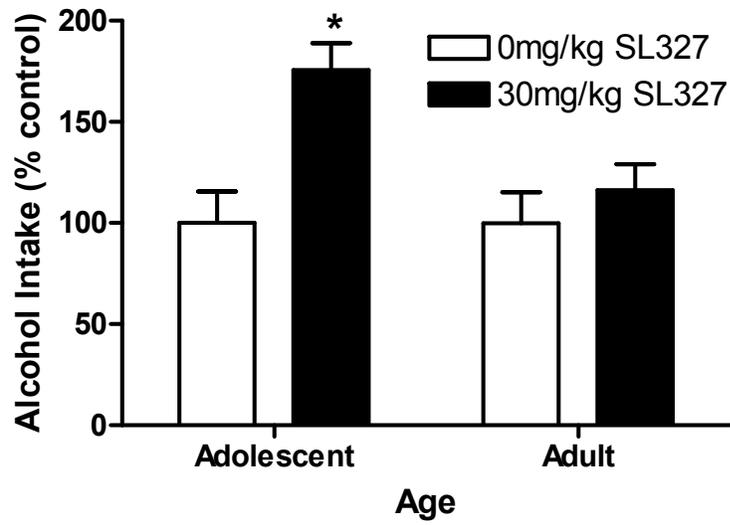


Figure 3.4. Alcohol intake after SL327 injection in adolescent and adult mice. 30mg/kg dose of the ERK/MEK inhibitor increased alcohol self-administration (g/kg) in adolescent but not adult mice ($p < 0.01$).

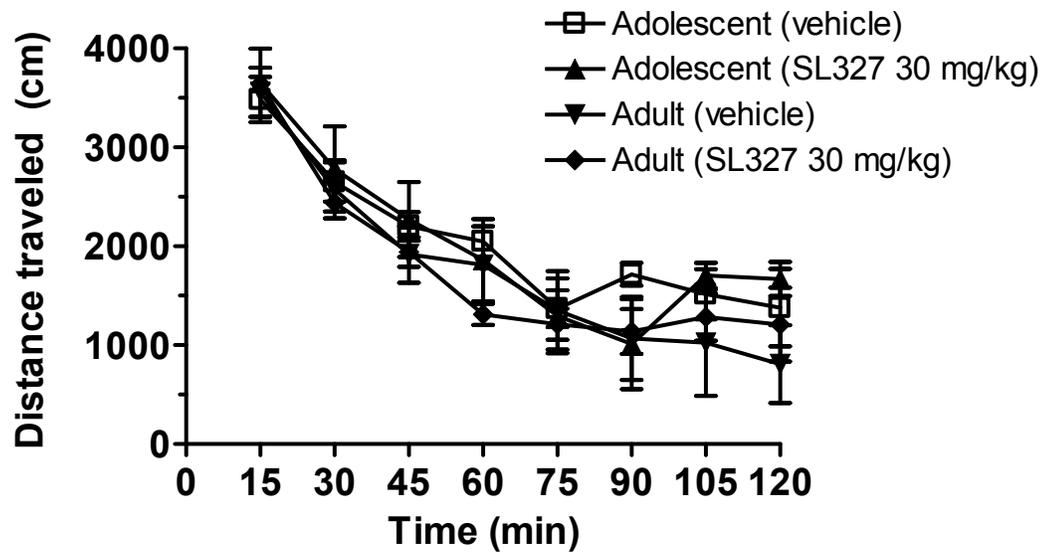


Figure 3.5. The effect of the MEK/ERK inhibitor SL327 on locomotor activity. SL327 30 mg/kg had no effect on spontaneous locomotor activity, or habituation to the novel environment, measured for 2-hr following injection in adolescent and adult C57BL/6J mice.

CHAPTER IV: DIFFERENCES IN AGE RELATED BINGE-LIKE ALCOHOL CONSUMPTION: CONDITIONED TASTE AVERSION AS A POSSIBLE BEHAVIORAL MECHANISM

INTRODUCTION

This chapter focuses on the pattern of alcohol consumption that is of the greatest public health concern for adolescents: episodic heavy drinking, or “binge drinking.” Initiation of alcohol consumption often begins during adolescence and it is characterized by heavy episodic “binge” drinking (Harford, Grant et al. 2005). The National Institute on Alcohol Abuse and Alcoholism (NIAAA) advisory council in 2004 defined binge drinking as a pattern of consumption of alcohol that rapidly brings blood alcohol concentrations to 0.08 mg% or above (5 or more drinks/2hrs). In 2006, a national survey of 8th, 10th, and 12th graders indicated that 11%, 22% and 29%, respectively, engaged in binge drinking (Johnston, 2007). Further, evidence suggests that over 50% of alcohol drinking adolescents engaged in binge-like drinking (Zeigler, Wang et al. 2005). These statistics are of great concern because several epidemiological studies have suggested that initiation of alcohol drinking at an early age is associated with an increased risk of developing an alcohol use disorder later in life (Grant and Dawson 1998; DeWit, Adlaf et al. 2000; Grant, Stinson et al. 2001). Nonetheless, few studies using animal models have investigated the relationship between age of onset of “binge” drinking and the relationship to voluntary alcohol drinking in adulthood.

Rodent models of alcohol consumption provide a valuable approach for examining adolescent alcohol intake and subsequent adult drinking. To date, there has been conflicting data suggesting both an increase and no difference in adult intake after adolescent exposure due to differences in methodology. Adolescent C57BL/6J mice exposed to alcohol consumed more alcohol in adulthood than mice that were not exposed to alcohol during adolescence (Ho, Chin et al. 1989). In contrast, another study reported that exposure to alcohol in C57BL/6J mice during adolescence or adulthood for a three week period did not affect 10% alcohol intake when measured in a 2-bottle choice paradigm during adulthood (Hefner and Holmes 2007). In a recent study, male and female BALB/cByJ and BALB/cJ adolescent mice were exposed to alcohol either gradually, via forced consumption, or via 2 bottle choice, and then switched to a 2-bottle choice paradigm. Under these conditions, all three alcohol exposure paradigms increased adult preference in BALB/cByJ mice. However, in the BALB/cJ mice, only the gradual alcohol access groups increased alcohol preference in adulthood, suggesting that genetic background and method of exposure are important factors for consideration (Blizard, Vandenberg et al. 2004). Studies using rats have not found an increase in adult intake when exposed to alcohol during adolescence (Slawecki and Betancourt 2002; Siegmund, Vengeliene et al. 2005). These studies suggest that methodological as well as species and genetic factors influence alcohol intake in adulthood.

High intake models of alcohol drinking have been developed and are more translational as they mimic human adolescent binge drinking behavior. In the scheduled high alcohol consumption (SHAC) procedure mice consume between 1.5-2g/kg alcohol within a 30min time period over multiple alcohol presentations and achieve blood alcohol levels above 100mg/dl. The SHAC procedure indicated that adolescent C57BL/6J mice drank significantly more alcohol than their adult conspecifics (Strong, Yoneyama et al. 2009). When subsequently tested in a 24 hr alcohol intake paradigm, the adolescent male mice with binge experience also consumed more alcohol than naïve control mice (Strong,

Yoneyama et al. 2009). Another study examined alcohol intake in adolescent and adult C57BL/6J and DBA/2J mice using another limited access alcohol paradigm known as drinking in the dark (DID), where mice drink to intoxication (Rhodes, Best et al. 2005). Adolescent binge alcohol exposure was found to increase subsequent alcohol intake in C57BL/6J mice were re-exposed to the same paradigm during adulthood (Moore, Mariani et al.). To date, binge drinking studies in rodents have come to a similar conclusion that adolescent drinking leads to an increase in drinking in adulthood.

Given the clear importance of adolescent alcohol exposure to future alcohol intake, it is important to address the reasons underlying adolescent binge drinking. Human adolescents drink more per occasion (binge); however, they are less sensitive to the negative effects of alcohol including sedation and acute withdrawal, which may serve to curb alcohol intake (Silveri and Spear 1998; Draski, Bice et al. 2001; Varlinskaya and Spear 2002). One potential contributor to high alcohol intake (binge) may be insensitivity to the adverse effects of alcohol that serve as cues to terminate drinking. Conditioned taste aversion (CTA) is a procedure that can be used to assess these aversive motivational effects of alcohol (Chester, Lumeng et al. 2003). During CTA, an association is established between a neutral environmental stimulus, referred to as the conditioned stimulus (CS), and a drug unconditioned stimulus (US) (Lucas and McMillen 2002). A negative correlation has been established between alcohol-induced CTA and alcohol intake (Elkins, 1991). For example, inbred strains that consume large amounts of alcohol, such as the C57BL/6J strain, are relatively insensitive to alcohol CTA, whereas DBA/2J mice, a strain that consumes very little alcohol, show strong alcohol CTA (Risinger and Cunningham 1995; Broadbent, Linder et al. 1996). Another study using 15 inbred mouse strains has also found a negative correlation of CTA to alcohol intake (Broadbent, Muccino et al. 2002). Rat models of low alcohol intake have also been found to be more sensitive to an alcohol CTA (Quintanilla, Callejas et al. 2001; Brunetti, Carai et al. 2002). Although, the negative

relationship between alcohol intake and CTA has been well established in rodents, only one study to date has examined the adolescent developmental period, despite the fact that it is a time of increased alcohol intake (Vetter-O'Hagen, Varlinskaya et al. 2009).

To date, there has been no examination of age of onset of drinking using a “binge” intake paradigm (drinking in the dark, DID) in adolescent and adult C57BL/6J mice and the subsequent relationship to voluntary alcohol drinking in adulthood. In the present study, adolescent (PND 25-41), late adolescent (PND 42-58), early adult (PND 59-75) and adult mice (PND 76-92) were allowed to self-administer 20% (v/v) alcohol in the home cage using the DID procedure for 16 days. The mice were then switched to a 2-bottle alcohol self-administration procedure (10% v/v) and adult intake was assessed between weeks 12-14 of age. Adolescent and adult mice were also used to determine whether adolescent C57BL/6J mice are less sensitive to the aversive properties of alcohol, which may in part explain their increased consumption of alcohol, by examining age-related differences in sensitivity to alcohol-induced conditioned taste aversion (CTA). Based on previous studies examining age differences in binge-like alcohol intake and effects of adolescent alcohol consumption on alcohol intake in adulthood using high intake models, it was predicted that adolescent mice would consume considerably more alcohol than adult mice and that mice which began the alcohol binge paradigm (DID) in adulthood would consume significantly less alcohol than adult mice first exposed to the binge paradigm during adolescence (PND 25).

METHODS

Animals. Male C57BL/6J mice (Jackson Laboratories, Bar Harbor, Maine) were singly housed in standard Plexiglas cages with corn-cob bedding. Food and water was available *ad libitum* in the home cage. The mice were 21 days and 63 days of age upon arrival to the facility. The mice were handled and weighed for at a minimum of 5 days before experiments began to acclimate to the reverse light cycle, resume normal sleep/wake

cycles (Wexler and Moore-Ede 1986) and allow the mice to reach the specific ages required (late adolescence and adulthood). The colony room was maintained at $20 \pm 1^\circ\text{C}$ on a 12-hr light/dark cycle (lights on 20:00 hrs), and experiments were performed during the dark cycle. Animals were under continuous care and monitoring by the Division of Laboratory Animal Medicine (DLAM) at UNC-Chapel Hill. All procedures were carried out in accordance with the NIH *Guide to Care and Use of Laboratory Animals* (National Research Council, 1996) and were approved by the UNC-Chapel Hill Institutional Animal Care and Use Committee.

EXPERIMENT 1: BINGE-LIKE DRINKING IN ADOLESCENT AND ADULT MICE

Adolescent (PND 25-41 days) and adult (PND 76-92) C57BL/6J mice were allowed to self-administer alcohol (20% v/v) in a limited access procedure (DID). The procedure is similar to that of (Rhodes, Best et al. 2005). In this method, a water bottle was replaced with an alcohol bottle (20% v/v) for three days, at a time point 3 hrs into the dark cycle for a total of two hrs per day (11:00-13:00 hrs). On the fourth day, the bottle of water was again replaced with an alcohol bottle (20%v/v); however, mice were given access to the bottle for 4 hrs (11:00-15:00). This process was repeated four times to bring the total drinking time to 16 days (Figure 4.1). Fluid intake levels (before and after drinking) and weights (2 hrs before drinking) were taken each day to determine an alcohol intake measurement.

Blood Alcohol Determination. Blood alcohol measurements were taken at day 16 after the last 4 hr binge session. Approximately 20 μl of tail blood was collected and centrifuged. Plasma was collected and used to determine alcohol concentration using an AM1 Alcohol Analyzer (Analox Instruments, Lunenburg, MA).

EXPERIMENT 2: AGE OF ONSET OF BINGE-LIKE DRINKING

Adolescent (PND 25-41), late adolescent (PND 42-58), early adult (PND 59-75) and adult (PND 76-92) C57BL/6J mice self-administered alcohol 20% v/v in a limited access procedure (DID) for 16 days. On the final day of binge drinking, adolescent (PND 41), late adolescent (PND 58), early adult (PND 75) and adult mice (PND 92) were immediately switched to an alcohol 2-bottle self-administration paradigm. The mice were given 23 hr access to two bottles in the home cage, one bottle containing alcohol (10% v/v) and the second bottle containing water. The position (left or right) of each solution was alternated daily to control for side preferences. Fluid intake levels and weights were taken each day to determine a daily alcohol intake (g/kg) measurement. Two-bottle choice drinking was assessed in all groups between PND 93-107.

EXPERIMENT 3: ALCOHOL-INDUCED CONDITIONED TASTE AVERSION (CTA)

CTA procedures were similar to previous published methods (Palmer, Sharpe et al. 2004). Adolescent (PND 21) and adult (PND 63) were isolate housed upon arrival to the animal colony and allowed to habituate for 2 days prior to handling. On day 3 mice were weighed and water bottles were removed at 14:00 hrs. On days 4-7, mice were weighed and water access was provided between 12:00-14:00 hrs, initial and final volumes were recorded to habituate them to restricted water access. On day 8, mice were weighed and presented with a 10 ml calibrated sipper tube containing a 0.2M NaCl solution (CS) in place of water for 1 hr (12:00-13:00 hrs). This initial 1 hr exposure was used to acclimate the mice to a novel tastant before conditioning trials began. Moreover, NaCl was used as a tastant because it promotes a faster conditioned response than sweet solutions (Palmer, Sharpe et al. 2004). Water was then provided for 30 min four hrs after the removal of the 0.2M NaCl bottle (17:00 hrs). On days 9, 11,13,15,17 mice were weighed and 2 hrs access to water

was provided (12:00-14:00 hrs). On days 10,12,14,16 (Trials 1-4), conditioning trials were performed. Mice were weighed and provided 1 hr access to 0.2M NaCl, recording initial and final volume. Immediately after the recording of the final volume, mice were administered an intraperitoneal injection of 0, 3 or 4 g/kg of alcohol. Each mouse received the same dose of alcohol after each conditioning session. Water was provided for 30 min, 4 hrs after the removal of the 0.2M NaCl bottle at 17:00 hrs. On day 18 (Trial 5), mice were weighed and provided to 1 hr access to 0.2M NaCl solution, and final volume was recorded. No alcohol injection was administered after this trial.

Data Analysis. *Experiment 1 and 2.* Drinking results are expressed as mean \pm S.E.M. Blood alcohol levels are expressed as mg/dl. Significance was determined by either an analysis of variance (ANOVA) followed by post hoc Tukeys test or the Student's t test as appropriate. Analyses were performed using the software GraphPad Prism version 4. *Experiment 3,* The development of CTA was indexed as the difference in 0.2M NaCl consumption from conditioning trial 1 to trial 5 (trial 5 – trial 1). Thus, negative values indicate reduced NaCl intake on trial 5, which is consistent with a taste aversion. Data were analyzed with a two-way ANOVA (ethanol dose x age). Follow up comparisons were evaluated with post-hoc Tukey's tests as appropriate.

Drugs. The 10 and 20% alcohol drinking solutions (v/v) were prepared by diluting 95% alcohol (Pharmaco Products Inc., Brookfield, CT, USA) with tap water. Injected alcohol (95% w/v) was prepared fresh daily and diluted in physiological saline (0.9%) to a concentration of 20% (v/v), with volume adjusted for dose (the 0 g/kg dose was administered at an equivalent volume to the 4 g/kg dose).

RESULTS

Experiment 1: Alcohol intake in Adolescent and Adult Mice in the Binge Procedure. Under binge-like exposure conditions, adolescent mice consumed considerably more alcohol than adult mice (Figure 4.2A). During the 16 day drinking period, adolescent mice (PND 25-41) consumed an average (\pm SEM) of 9.2 (\pm 0.3) g/kg of alcohol (20% v/v) during the 4 hr binge, whereas adult mice (PND 70-84) consumed an average of 7.5 (\pm 0.1) g/kg of alcohol during this same period (Figure 4.2A), a difference which was statistically significant (t (22) =5.08, p <0.0001). During the same 16 day drinking period, adolescent mice (PND 25-41) consumed an average (\pm SEM) of 6.1 (\pm 0.2) g/kg of alcohol (20% v/v) during the 2 hr binge days, whereas adult mice (PND 70-84) consumed an average of 5.2 (\pm 0.2) g/kg of alcohol during this same period, a difference which was also statistically significant (t (22) =3.1, p =0.0046). Blood alcohol levels taken on day 16 immediately after the 4 hr binge were statistically different (t (20) =2.9, p =0.007), with adolescent mice having a significantly higher BAL (180.6 \pm 18.1 mg/dL; range: 92 – 265 mg/dL) than adult mice (119.6 \pm 9.5 mg/dL; range: 70 – 183 mg/dL) (Figure 4.2B).

Experiment 2: Age of Alcohol Binge Onset and Adult Alcohol Self-Administration. As shown in figure 4.3, using a binge procedure, adolescent (PND 25-41) mice consumed significantly more alcohol (g/kg) than, late adolescent (PND 42-58), early adult (PND 59-75) and adult (PND 76-92) C57BL/6J mice. During the 16 day binge drinking period, adolescent, late adolescent, early adult, and adult mice consumed an average of 9.4 (\pm 0.2), 8.5 (\pm 0.3), 7.5 (\pm 0.21), and 7.1 (\pm 0.2) g/kg of alcohol, respectively, during the 4 hr binge. A one-way ANOVA indicated that there was a main effect of age F (3,95)=17.35, p <0.0001. Post-hoc tests indicated that late adolescent, early adult, and adult mice drank significantly less alcohol (g/kg) than the adolescent group (p <0.05) (4.3). The adolescent and late adolescent groups were different to all other groups; however, early adult vs. adult

were not statistically different. On the final day of the 16 day binge, adolescent (day 41), late adolescent (day 58), early adult (day 75) and adult mice (day 92) were immediately switched to an alcohol 2-bottle self-administration paradigm and alcohol intake (g/kg) was assessed when all mice were adults (PND 93-107). A one-way ANOVA indicated that there was a main effect of age ($F(3, 51) = 3.6, p = 0.02$). Post-hoc tests indicated that mice that began the alcohol binge paradigm (DID) in adulthood (starting at PND 59 or 76) consumed significantly less alcohol than adult mice first exposed to the binge paradigm during adolescence (PND 25). The late adolescent group did not differ in intake g/kg at (PND 93-107) from the adolescent, early adult or adult group (Figure 4.4).

Experiment 3. Alcohol – Induced Conditioned Taste Aversion (CTA).

The difference in NaCl intake (conditioning trial 5 – trial 1) was evaluated as an index of the effects of ethanol on CTA in adolescent and adult mice. Two-way ANOVA identified a statistically significant main effect of ethanol ($F(2, 23) = 50.18, p < 0.0001$) and age ($F(1, 23) = 16.48, p < 0.0005$) on CTA. In addition, the effects of ethanol depended on age as indicated by a significant age x ethanol interaction ($F(2, 23) = 3.6, p < 0.04$). Post hoc multiple comparison procedures showed that all doses of ethanol differed from vehicle within each age group (Tukey, $p < 0.05$). Importantly, adults showed a significant CTA following ethanol (3 g/kg) injection as compared to adolescents, who did not (Tukey, $p < 0.05$; Figure 4.5)

DISCUSSION

The main goal of this study was to examine effects of age or onset of binge-like drinking on subsequent alcohol drinking during adulthood in inbred alcohol-preferring C57Bl/6J mice. During an initial assessment, adolescent C57Bl/6J mice consumed significantly more alcohol and achieved a significantly higher BAC than adult mice during a 4-hr binge procedure. In a follow up experiment, adolescent mice (PND 25) consumed a

significantly higher dose of alcohol (g/kg) during a 4-hr binge event than late adolescent (PND 42), early adult (PND 59) or adult (PND 76) mice on binge exposure days using the DID procedure. These data agree with studies that have examined high alcohol intake models of drinking, where mice drink to blood alcohol concentrations above 100 mg/dl (Strong, Yoneyama et al. 2009; Moore, Mariani et al. 2010). Under continuous voluntary access conditions adolescent rats have also been found to voluntarily consume more alcohol than their adult counterparts (Brunell and Spear 2005; Doremus, Brunell et al. 2005). In contrast, other studies have not observed an adolescent increase in alcohol drinking compared to adult drinking. For example, adolescent rats were found to consume less alcohol than adults under a voluntary two-bottle choice procedure (Siegmund, Vengeliene et al. 2005). In a more recent study of mice given a free choice between water and 10% alcohol (v/v), adolescent mice self-administered a similar amount as adults per kilogram of body weight (Hefner and Holmes 2007). The majority of studies that have not found an increase in drinking in adulthood contain an assortment of alcohol exposure paradigms; however, high alcohol intake models should be the focus of rodent adolescent drinking studies as they mimic human adolescent drinking and have increased translational value.

The age-of onset binge drinking experiment found that mice that experienced binge-like alcohol drinking during adolescence (25-41 days) drank significantly more alcohol (g/kg) in adulthood than those who began drinking in late adolescence (42-58 days), early adulthood (59-75 days), or adulthood (76-92 days). These data are in line with other published reports that have found that exposure to alcohol during adolescence increased adult intake (Ho, Chin et al. 1989; Blizard, Vandenbergh et al. 2004; Moore, Mariani et al. 2010). In contrast, a study examining forced exposure to alcohol vapor during adolescence did not find a later effect on adult operant alcohol self-administration (Slawecki and Betancourt 2002). Another study using male Wistar rats also failed to show an increase in adult drinking when exposed to alcohol during adolescence (Siegmund, Vengeliene et al.

2005). Differences in adolescent exposure to alcohol, drinking versus inhalation, or the deprivation phase between adolescent exposure and adult alcohol self-administration may account for the observed discrepancies. A potential limitation of the design of the current study is that the increased adolescent alcohol intake may also be related to the fact that adolescent mice have a longer history of drinking. The alternative would be to allow an abstinent period that would equate time of alcohol exposure however; this method is not as translational and should not be compared to human epidemiological data. A number of studies have indicated that differential sensitivities to alcohol exhibited by adolescents may be maintained in adulthood (Slawecki and Betancourt 2002; Wills, Knapp et al. 2009).

These observations of long-lasting effects in rodents are strikingly similar to epidemiological evidence suggesting that alcohol exposure during adolescence is associated with an increased risk of developing an alcohol use disorder later in life (Grant and Dawson 1998; DeWit, Adlaf et al. 2000; Grant, Stinson et al. 2001). Using pharmacological methods, opioid, dopaminergic, and glutaminergic mechanisms underlying high alcohol intake (DID) can be examined in adolescent mice and the effects on drinking assessed. For example, studies have examined the role of endogenous opioid signaling in DID drinking in adult mice and found that they play a necessary role in high alcohol intake (Kamdar, Miller et al. 2007); however, the role of the opioid system in adolescent high alcohol intake has not been assessed. Further studies using the adolescent DID alcohol exposure procedure could provide greater insight into the variety of neurobiological changes that occur during adolescence to promote increased alcohol intake in adulthood, and whether pharmacological manipulations used to curb intake in adults show promise or are even effective at reducing high alcohol intake in adolescents.

Results of the present study also showed that adolescent mice are less sensitive to an alcohol-induced CTA than adult mice, which could be an underlying behavioral mechanism for increased alcohol consumption during adolescence. These data are

supported by a recent study that found that age differences in alcohol intake in male rats during adolescence are associated with the relative insensitivity of the male adolescent to alcohol's aversive properties (Vetter-O'Hagen, Varlinskaya et al. 2009). These data add to a number of studies indicating that adolescent rodents are less sensitive to some of the negative behavioral effects of alcohol than their adult counterparts including alcohol-induced motor impairment, sedation, anxiolysis and social inhibition (Silveri and Spear 1998; Spear 2000; Varlinskaya and Spear 2002). Thus, increased alcohol intake during adolescence relative to adulthood may be associated with decreased sensitivity of adolescents to some of the aversive effects of alcohol. A limitation of the current experiment is that the decreased sensitivity to alcohol-induced CTA in adolescents versus adults could be related to greater alcohol-induced memory impairment observed during adolescence (Acheson, Stein et al. 1998; Markwiese, Acheson et al. 1998; Tapert, Schweinsburg et al. 2004). Studies have shown that activation of ERK is critical for memory consolidation in CTA (Swank 2000; Languille, Davis et al. 2009), and adolescent rodents are more sensitive to ethanol-induced reductions in ERK1/2 (Chapter 2), impairment of performance in the novel object test (Chapter 2), and memory acquisition (Markwiese, Acheson et al. 1998) as compared to adults. A control experiment using lithium chloride (LiCl) as the aversive unconditioned stimulus could help to decipher if greater impairment to the alcohol-induced CTA is due to the memory impairing effects of alcohol or insensitivity to the alcohol in adolescent mice.

In summary, results of this study showed that adolescent (PND 25-41) C57BL/6J mice consumed significantly more alcohol (g/kg) than early adults (PND 59-75) and adult (PND 76-92) mice in the DID procedure. We also show that mice that began the alcohol binge procedure (DID) during adolescence drank significantly more alcohol (g/kg) as adults than those who began drinking in early adulthood and adulthood. Finally, we show that adolescent mice are less sensitive to alcohol-induced conditioned taste aversion than adult

mice. The adolescent insensitivity to the aversive effects of alcohol could be a potential contributor to high alcohol intake (binging) during adolescence. These data suggest that the blunted sensitivity to alcohol-induced CTA during adolescence may be a factor that contributes to increased risk of alcohol abuse in adulthood.

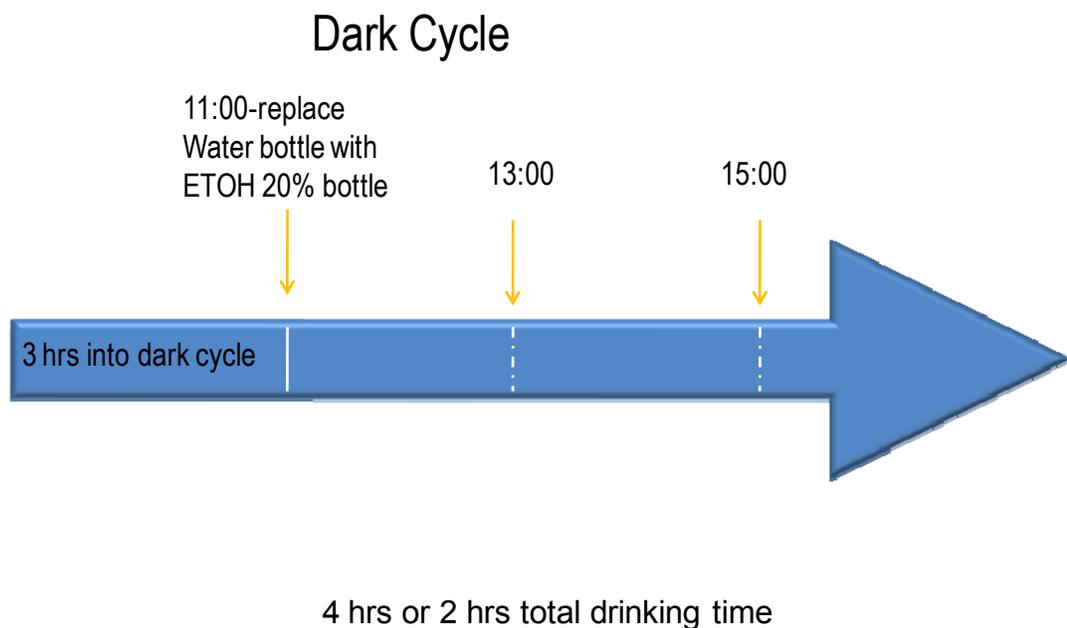


Figure 4.1. Drinking in the Dark Procedure. The DID procedure is repeated 4 times to bring the total number of drinking days to 16. Days 1-3 have 2 hours access to the 20% alcohol. On day 4 mice have access to the alcohol from 11:00-15:00.

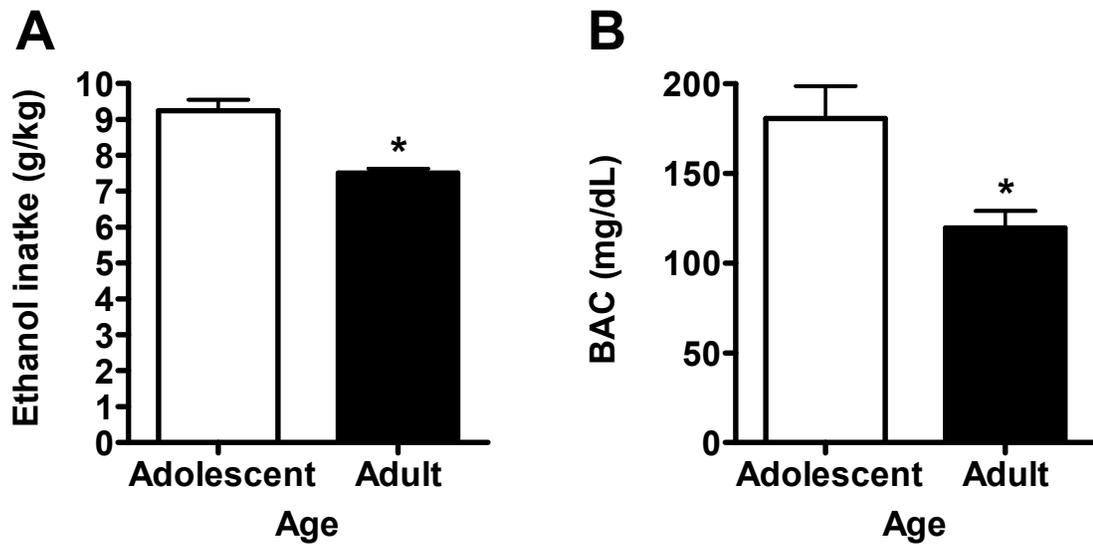


Figure 4.2. Alcohol intake and blood alcohol concentration in adolescent and adult mice in the 4 hr binge procedure. Alcohol intake in adolescent (PND 27-41) and adult (PND 70-84) C57BL/6J mice was statistically different ($p < 0.0001$) during the 4 hr binge. Blood alcohol concentrations were also statistically different between adolescent and adult mice at the time of perfusion (Students-t, $p = 0.0072$).

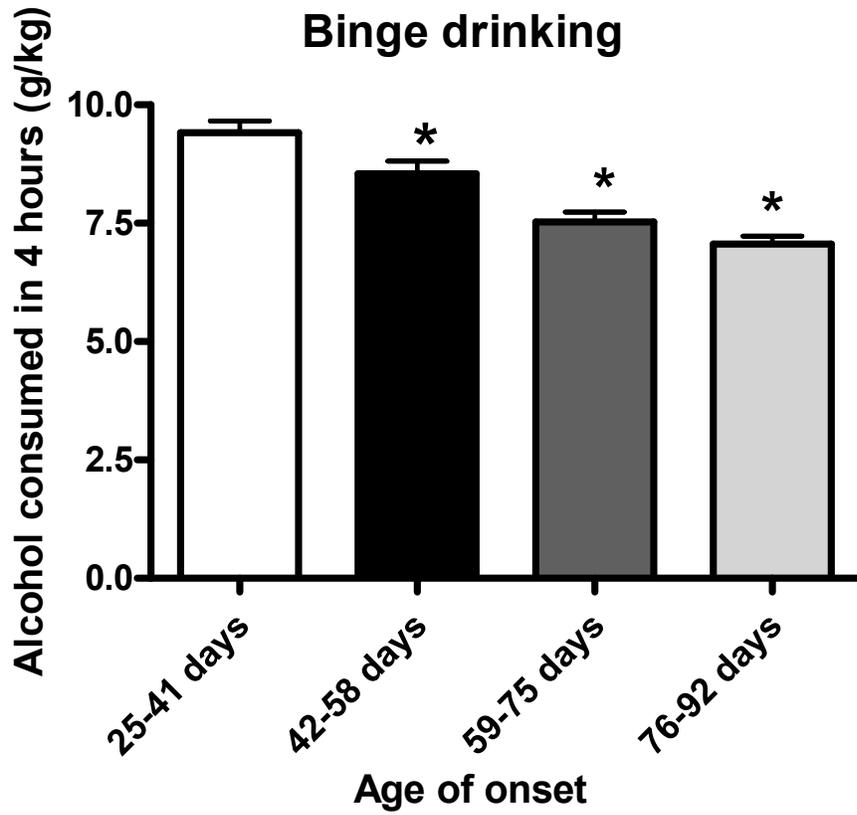


Figure 4.3. Age of Onset of Binge Drinking. Adolescent (PND 25-41) mice consumed significantly more alcohol (g/kg) than late adolescent (PND-42-58), early adult (PND 59-75) and adult (76-92) mice on binge exposure days while undergoing the DID procedure for 16 days.

2-Bottle Alcohol Self-administration during PND 93-107

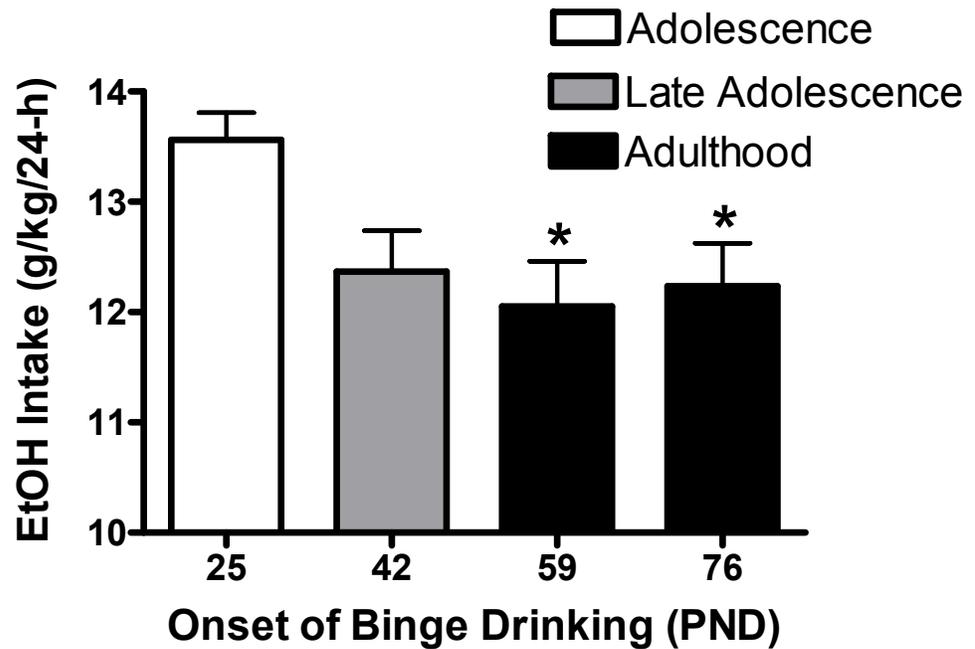


Figure 4.4. Adult (PND 93-107) alcohol intake and age of binge alcohol onset. Ethanol intake (g/kg) during PND (93-107) plotted as a function of age of onset of binge drinking. Mice that began drinking in adulthood drank significantly less alcohol than mice who starting binge drinking during the adolescent period (PND 25). Data represent mean \pm SEM from $n = 12 - 14$ per group. * - indicates significantly different from PND 25, Dunnett's t-test, $p < 0.05$.

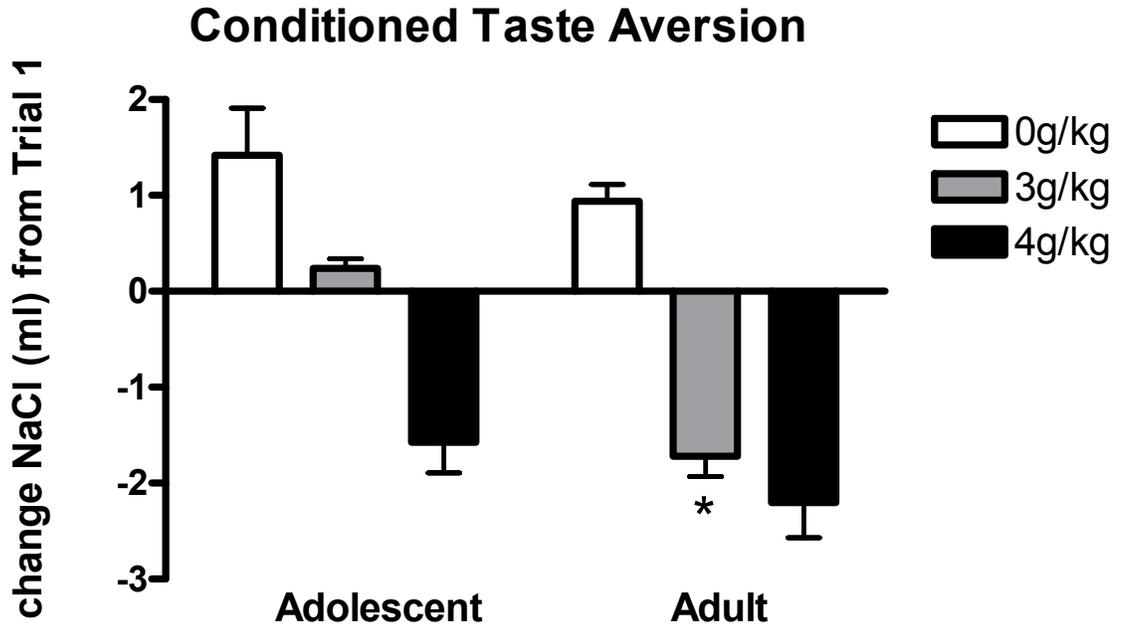


Figure 4.5. Adolescent mice are less sensitive to alcohol-induced conditioned taste aversion than adult mice. Data are plotted as the change in NaCl intake from trial 1 to trial 5 as a function of age. Values are mean \pm SEM from $n = 5$ per condition. * - indicates significantly different from adolescent mice at the corresponding dosage of ethanol, Dunnett's t-test, $p < 0.05$.

CHAPTER V: AGE-DEPENDENT EFFECTS OF VOLUNTARY DRINKING ON STRESS AND THE ERK/MAPK PATHWAY

INTRODUCTION

Adolescents are faced by a number of life transitions including developmental challenges associated with puberty and the transition toward independence, and as such this critical developmental period is widely considered to be a stressful time of life (Allen and Matthews 1997; Spear 2000). Recent research has indicated that the perception of stress may be increased in adolescents relative to adults. The presumed increase in stress during adolescence may contribute to the frequent initiation of alcohol or other drug use observed in adolescents (Wagner, Myers et al. 1999; Spear 2000; Seiffge-Krenke, Aunola et al. 2009). Adolescents who begin using alcohol under these life stressors have been shown to adopt continued and more frequent alcohol use as a strategy of coping with their problems (Pohorecky 1991; Aseltine and Gore 2000). It has also been observed that after peer substance use, the most powerful predictor of adolescent alcohol and drug use, was level of perceived stress (Wagner, Myers et al. 1999). Although stress is widely known to regulate the etiology and progression of alcoholism in adolescents and adults (Brown, Vik et al. 1990; Pohorecky 1991; Zweben, Clark et al. 1994; Brady and Sonne 1999; Aseltine and Gore 2000; Dai, Thavundayil et al. 2002; Breese, Overstreet et al. 2005; Siegmund, Vengeliene et al. 2005; Tambour, Brown et al. 2008), the molecular neurobiological mechanisms that mediate age-dependent differences in stress response and effects of alcohol remain to be characterized.

Studies in laboratory animals also indicate that adolescents are more negatively affected by stressful events than adults (Stone and Quartermain 1997; Wagner, Myers et al. 1999; Romeo, Lee et al. 2004). This difference in stress response is thought to be due to the developing stress response system (Hypothalamic pituitary adrenal or HPA axis) (Stone and Quartermain 1997). Rats in early adolescence have a delayed rise and more prolonged corticosterone (CORT) release to several different types of acute stressors compared with adults (Romeo, Lee et al. 2004; Prendergast and Little 2007). HPA axis function has been found to be an important factor for initiation, maintenance and relapse to alcohol abuse in adult rodents (Brown, Vik et al. 1990; Zweben, Clark et al. 1994; Brady and Sonne 1999; Overstreet, Knapp et al. 2004). Thus, increased stress responses noted in adolescent animals may cause an increase in alcohol consumption. Developmental differences in the HPA axis activation after chronic alcohol exposure have not been examined to date. This gap in knowledge should be addressed given the increased sensitivity of adolescents, to stress and drugs of abuse. Underlying cellular mechanisms involved with regulation of the stress response system in adolescents have also not been characterized.

The ERK signaling cascade has been implicated in both acute and chronic effects of drugs of abuse, including alcohol (Sanna, Simpson et al. 2002; Chandler and Sutton 2005; Lu, Hope et al. 2005). Previous studies (see chapters 2 and 3) have shown developmental differences in p-ERK1/2 activation following acute and chronic alcohol exposure. The ERK1/2 signaling cascade has been shown to be rapidly activated in response to physiological stress (Alessandrini, Namura et al. 1999; Oh, Ahn et al. 1999) and therefore may play a role in developmental differences in HPA axis activation following chronic alcohol exposure. Both chronic and acute stress alters ERK1/2 activation. Acute swim stress was found to increase p-ERK1/2 in the hippocampus, neocortex and prefrontal cortex (Shen, Tsimberg et al. 2004). By contrast, chronic restraint stress or chronic swim stress reduced

the levels of p-ERK1/2 in the hippocampus and prefrontal cortex (Meller, Shen et al. 2003; Qi and Elion 2005). Moreover, acute or repeated restraint stress increases p-ERK1/2 levels in the paraventricular nucleus of the hypothalamus (Sasaguri, Kikuchi et al. 2005; Kwon, Seo et al. 2006; Uchida, Nishida et al. 2008) and amygdala (Shen, Tsimberg et al. 2004). These studies demonstrate that activation of the HPA axis by stress alters p-ERK1/2 levels in hypothalamic and extra-hypothalamic brain regions that are also involved with alcohol abuse and alcoholism (Hodge, Chappelle et al. 1996; Hodge, Slawecki et al. 1996; Kelley, Nannini et al. 2001; Schroeder, Olive et al. 2003; Stevenson, Schroeder et al. 2009).

Adolescents are more sensitive to the effects of stress and these differences in sensitivity could promote future alcohol abuse problems. Developmental differences to chronic alcohol exposure and the effect of such exposure on HPA axis responses and cellular signaling systems, including ERK1/2, have not been examined to date. To address this gap in knowledge, the present study was designed to elucidate the developmental differences in HPA axis response and ERK1/2 activation (phosphorylation) after chronic alcohol exposure. Adolescent (27-41 days) and adult mice (70-84 days) were allowed to self-administer 10% alcohol or water in the home cage. On the last day of drinking (day 14) mice were acutely stressed (swim stress) and were either sacrificed for immunohistochemical evaluation (p-ERK1/2) or for RIA assessment of plasma CORT after stress. P-ERK1/2 immunoreactivity (IR) was examined in the paraventricular nucleus of the hypothalamus (PVN) and the central amygdala which are both areas of the brain known to mediate stress responses.

METHODS

Animals. Male C57BL/6J mice (Jackson Laboratories, Bar Harbor, Maine) were singly housed in standard Plexiglas cages with corn-cob bedding. Food and water was

available *ad libitum* in the home cage. The mice were 21 days and 70 days of age upon arrival to the facility. The mice were handled and weighed for approximately one week before experiments began to acclimate to the reverse light cycle, resume normal sleep/wake cycles (Wexler and Moore-Ede 1986) and allow the mice to reach adolescence post natal day (PND) 27 and adulthood (PND77) before experiments were initiated. The colony room was maintained at $20 \pm 1^\circ\text{C}$ on a 12-hr light/dark cycle (lights on 20:00), and experiments were performed during the dark cycle. Animals were under continuous care and monitoring by the Division of Laboratory Animal Medicine (DLAM) at UNC-Chapel Hill. All procedures were carried out in accordance with the NIH *Guide to Care and Use of Laboratory Animals* (National Research Council, 1996) and were approved by the UNC-Chapel Hill Institutional Animal Care and Use Committee.

EXPERIMENT 5.1: BASAL AND STRESS INDUCED CORTICOSTERONE LEVELS IN ADOLESCENT AND ADULT MICE

Acute swim stress. Adolescent (PND 41) and adult (PND 91) mice were subjected to a forced swim stress in a round glass beaker filled with water at 20 degrees Celsius. Control mice were not stressed and remained in the colony room. During the swim stress procedure, mice were forced to swim for 7 minutes and were then decapitated 30 minutes later. Trunk blood was collected for a plasma corticosterone assay.

Radioimmunoassay-Corticosterone Response. Plasma corticosterone levels were measured using an ImmuChem double antibody ^{125}I Corticosterone RIA kit (MP Biomedicals, LLC, Orangeburg, NY). The procedure followed was as outlined in the kit manual; a standard curve was generated by using standards provided in the kit and then the plasma levels of corticosterone were calculated by interpolation of unknown values against the standard curve generated.

EXPERIMENT 5.2: ALCOHOL-SELF ADMINISTRATION STRESS AND CORTICOSTERONE RESPONSES

Two-Bottle Self Administration. Adolescent (PND 27-41) and adult (PND77-91) mice self-administered alcohol (10%v/v) vs. water using a 24-h access two-bottle choice procedure. The alcohol drinking groups were given access to one bottle containing alcohol (10%v/v) for three days. The second bottle containing water was introduced three days later to bring the total drinking time to 14 days. Fluid intake levels and weights were taken each day to determine a daily alcohol intake measurement. The position (left or right) of each solution was alternated daily to control for side preferences. Age-matched control mice (water-only groups) were run in parallel and given access to two bottles, both containing water, and weighed daily.

Acute stress procedure. On the last day of drinking (day 14) water and alcohol drinking adolescent (PND 27-41) and adult (PND 77-91) mice were subjected to forced swim stress in a round glass beaker filled with water at 20 degrees Celsius. Age-matched control mice from both water and alcohol drinking conditions were not exposed to swim stress and remained in the colony room. During the swim stress procedure, mice were forced to swim for 7 minutes and then were decapitated 30 minutes later. Trunk blood was collected for a plasma corticosterone assay and blood alcohol concentrations.

Blood Alcohol Determination. Blood alcohol measurements were taken at the time of sacrifice in order to ensure the differences we were observing in CORT (adolescent and adult mice) were not due to confounds of a differential alcohol exposure (dose). 5 μ l of plasma was used to determine alcohol concentration using an AM1 Alcohol Analyzer (Analox Instruments, Lunenburg, MA).

Radioimmunoassay-Corticosterone Response. Plasma corticosterone levels were measured using an ImmuChem double antibody 125I Corticosterone RIA kit (MP

Biomedicals, LLC, Orangeburg, NY). The procedure followed was as outlined in the kit manual; a standard curve was generated by using standards provided in the kit and then the plasma levels of corticosterone were calculated by interpolation of unknown values against the standard curve generated.

EXPERIMENT 5.3: ALCOHOL SELF-ADMINISTRATION STRESS AND P-ERK1/2 RESPONSE

Two-Bottle Self Administration. Adolescent (PND 27-41) and adult (77-91) mice self-administered alcohol 10%v/v using a two-bottle choice procedure. The alcohol drinking groups were given access to one bottle containing alcohol (10%v/v) for three days. The second bottle containing water was introduced three days later to bring the total drinking time to 14 days. Fluid intake levels and weights were taken each day to determine a daily alcohol intake measurement. The position (left or right) of each solution was alternated daily to control for side preferences. Control mice (water drinking groups) were given access to two bottles as well, both containing water, and weighed daily.

Acute stress procedure. On the last day of drinking (day 14) water and alcohol drinking adolescent (PND 27-41) and adult (77-91)mice) were subjected to a forced swim stress in a round glass beaker filled with water at 20 degrees Celsius. Water-drinking and alcohol-drinking control animals were not stressed and remained in the colony room. During the swim stress procedure, mice were forced to swim for 7 minutes and then perfused 30 minutes after the end of the swim test to collect brains for immunohistochemical analysis of p-ERK1/2.

Blood Alcohol Determination. Blood alcohol measurements were taken at the time of perfusion in order to ensure the differences we were observing in p-ERK1/2 (adolescent and adult mice) were not due to confounds of a differential dose. Approximately 20 μ l of

heart blood was collected at the time of perfusion. From the 20 μ l centrifuged sample 5 μ l of plasma was used to determine alcohol concentration using an AM1 Alcohol Analyzer (Analox Instruments, Lunenburg, MA).

Immunohistochemistry. Mice were deeply anesthetized with 60 mg/kg pentobarbital. The mice were then transcardially perfused with 0.1M phosphate buffered saline (PBS) followed by 4% paraformaldehyde. The brains were extracted from the skulls washed in PBS and sliced on a vibratome into 40 μ m coronal sections. These free-floating sections were then stored in cryoprotectant at -20 $^{\circ}$ C until immunohistochemical processing. The free-floating sections were rinsed in PBS, and then placed in 1% hydrogen peroxide in PBS to block endogenous peroxidase. Next sections underwent antigen retrieval using citra buffer at 70 $^{\circ}$ C for 30 min (Antigen Retrieval Citra, BioGenex). Sections were then blocked in 0.1% triton-X in PBS with 5% goat serum for 1 hour and then were incubated at +4 $^{\circ}$ C overnight in primary polyclonal antibody to p-ERK1/2 (1:200 Cell Signaling Technology, Danvers, MA). The sections were incubated in Dako EnVision Kit (Dako, Carpinteria, CA) secondary antibody for one hour at room temperature and immunoreactivity was detected using nickel enhanced diaminobenzene (Dako EnVision Kit) as a chromagen. The sections were counterstained using toluidine blue, mounted onto slides, and coverslipped.

Immunoreactivity was visualized using an Olympus CX41 light microscope (Olympus America, Center Valley, PA). Images were acquired using a digital camera (Regita model, QImaging, Burnaby, BC) interfaced to a desktop computer (Dell, Round Rock, TX). Image analysis software (Bioquant Nova Advanced Image Analysis; R&M Biometric, Nashville, TN). The microscope, camera, and software were background corrected and normalized to preset light levels to ensure fidelity of data acquisition.

Data Analysis. Data for p-ERK1/2 IR data were acquired from a minimum of 2 sections/brain region/animal and averaged to obtain a single value per subject. Coordinates

of the brain region analyzed were as follows: Pixel density measurements were calculated from a circumscribed field (e.g., brain region) and divided by the area of the region and expressed as pixels/mm². Immunoreactivity (IR) data were collected by a researcher blind to treatment conditions.

Data Analysis. Drinking results are expressed as mean \pm S.E.M. Blood alcohol levels are expressed as mg/dl. Significance was determined by a 2-way analysis of variance (ANOVA) followed by post hoc Holm-Sidak or other post hoc tests as appropriate. Analyses were performed using the software GraphPad Prism version 4 and SigmaStat version 3.

Drugs. The 10% alcohol drinking solutions (v/v) were prepared by diluting 95% alcohol (Pharmaco Products Inc., Brookfield, CT, USA) with tap water.

RESULTS

EXPERIMENT 5.1: Basal and Stress induced Corticosterone Levels in Adolescent and Adult Mice. As shown in Figure 5.1, adolescent mice had higher levels of plasma CORT than adult mice 30 minutes after an acute swim stress procedure. Basal levels of CORT were not different between the age groups. A 2-way ANOVA indicated a significant main effect of age ($F(1, 28) = 6.9, p < 0.014$) and stress ($F(1, 28) = 134.1, p < 0.001$). In addition there was a significant interaction between stress and age ($F(1, 28) = 4.9, p < 0.037$). Post hoc (Holm-Sidak) comparisons showed that stress produced a significant increase in corticosterone (ng/ml) in adolescents ($p < 0.05$) and adults ($p < 0.05$), but the adolescent response was significantly greater than the adult response ($p < 0.05$).

EXPERIMENT 5.2: Alcohol Self-Administration and Corticosterone Responses. During the 14 day drinking period, adolescent mice consumed an average (mean \pm SEM) of 10.9 \pm 1.4 g/kg of alcohol (10% v/v) per day. Adult mice consumed an average (mean \pm SEM) of

11.8 ±0.9 g/kg of alcohol (10% v/v) per day (Figure 5.2A). Alcohol intake in adolescent (PND 27-41) and adult (PND 70-84) C57BL/6J mice did not differ ($t=0.067$, 31, $p=0.95$). Blood alcohol levels taken from adolescent mice at time of perfusion on day 14 showed a range of 62 – 117 mg/dL with a group mean±SEM of 87.0± 6.2 mg/dL. Blood alcohol levels taken from adult mice at time of perfusion on day 14 showed a range of 58 – 112 mg/dL with a group mean±SEM of 81.6± 6.0 mg/dL (Figure 5.2B). Blood alcohol concentrations did not differ between adolescent and adult mice at the time of perfusion ($t=0.62$, 14, $p=0.55$).

Corticosterone response. As shown in figure 5.2, a history of 2-bottle alcohol self-administration, had no effect on CORT response in adolescent mice after acute swim stress, however, chronic alcohol-drinking blunted the acute CORT response in adult mice. Basal levels of CORT were not different. A 2-way ANOVA indicated a significant main effect of age ($F(1, 28) = 6.9$, $p < 0.014$) and alcohol ($F(1, 28) = 134.1$, $p < 0.001$), in addition there was a significant interaction between alcohol and age ($F(1, 28) = 4.9$, $p < 0.037$). Post hoc (Holm-Sidak) comparisons showed that a history of alcohol drinking was associated with a blunted stress-induced increase in corticosterone (ng/ml) in adults ($p < 0.05$) compared to adolescent mice (Figure 5.2C). There was also a significant difference between water-drinking and alcohol-drinking adult mice ($p < 0.05$).

EXPERIMENT 5.3: Alcohol Self-Administration and p-ERK1/2 Responses. Alcohol intake in adolescent and adult mice did not differ (g/kg). During the 14 day drinking period, adolescent mice consumed an average (mean±SEM) of 11.4 ±0.8 g/kg of alcohol (10% v/v) per day. Adult mice consumed an average (mean±SEM) of 10.1 ±0.51 g/kg of alcohol (10% v/v) per day. Alcohol intake in adolescent (PND 27-41) and adult (PND 70-84) C57BL/6J mice did not differ ($t=0.598$, 30, $p=0.55$). Blood alcohol levels taken from adolescent mice at time of perfusion on day 14 showed a range of 41 – 122 mg/dL with a group mean±SEM of

87.0± 9.3 mg/dL. Blood alcohol levels taken from adult mice at time of perfusion on day 14 showed a range of 51 – 102 mg/dL with a group mean±SEM of 78.9± 5.8 mg/dL. Blood alcohol concentrations did not differ between adolescent and adult mice at the time of perfusion ($t=0.74$, 14, $p=0.47$) (Data not shown).

P-ERK1/2 response in the PVN and CeA. P-ERK1/2 IR was evaluated in 8 groups of mice ($n=72$ total). Adolescent and adult alcohol and water exposed mice were exposed to swim stress and p-ERK1/2 response was evaluated as compared to age- and treatment-matched controls. Data from the 4 stress groups are expressed as percent change from 4 parallel no-stress groups.

Paraventricular nucleus of the hypothalamus. Two-way ANOVA indicated a significant main effect of alcohol drinking ($F(1, 29) = 21.46$, $p < 0.001$) on stress-induced changes in p-ERK1/2 IR in the PVN of adolescent and adult mice. However there was no effect of age and no interaction between alcohol and age indicating that the effect of alcohol occurred in both age groups (Figure 5.3A). Planned comparisons showed that alcohol blunted the stress response in both age groups (Bonferroni t-test, $p < 0.05$) (Figure 5.3A). Thus, a history of alcohol self-administration was associated with blunted stress-induced activation of p-ERK1/2 in the PVN response in both adolescent and adult mice.

Central Amygdala. Two-way ANOVA indicated a significant main effect of alcohol drinking ($F(1, 31) = 9.4$, $p = 0.049$) and age ($F(1, 31) = 21.53$, $p = 0.004$) on stress-induced changes in p-ERK1/2 IR in the CeA of adolescent and adult mice. However there was no interaction between alcohol and age (Figure 5.3B). Although there was a large trend in both age groups, planned comparisons showed that alcohol produced a statistically significant blunting of the stress response only in the adolescent mice (Bonferroni t-test, $p < 0.05$) (Figure 5.3B). Thus, adults show an overall reduction in stress-induced activation of p-ERK

in the CeA as compared to adolescent mice, and a history of alcohol self-administration was associated with a significant blunting of stress-induced activation of p-ERK1/2 in the CeA response in both adolescent and adult mice.

DISCUSSION

Epidemiological data indicate that adolescent drug use is a significant predictor of developing dependence during adulthood (Grant and Dawson 1998). Evidence also indicates that stress during adolescence may contribute to alcohol and drug use (Allen and Matthews 1997; Spear 2000; Seiffge-Krenke, Aunola et al. 2009). The purpose of this study was to investigate potential ontogenetic differences in stress-induced increases in CORT and how alcohol drinking may alter this response. In addition, this study examined ERK1/2 activation as a potential neural correlate of age-dependent differences in the stress response. Identifying molecular mechanisms of ontogenetic differences in stress response, and their modulation by alcohol use, has the potential to lead to new understanding of adolescent vulnerability to addiction.

Results of an initial experiment showed that adolescent C57BL/6J mice had significantly higher levels of plasma CORT than adult mice 30 minutes after exposure to an acute swim stress procedure. Basal plasma CORT levels were not different between adolescent and adult mice. These results agree with several studies in which adolescents have shown a greater and more sustained rise in plasma CORT as compared to adult mice (Stone and Quartermain 1997; Wagner, Myers et al. 1999; Romeo, Lee et al. 2004). Specifically, chronic social stress brought on by a short daily encounter with a an unfamiliar adult male mouse for 5 days produced higher levels of plasma CORT in adolescent than adult male mice (Stone and Quartermain 1997). Adolescent rats also show more immobility

under stressful situations, such as a forced swim test than adult mice (Walker, Trottier et al. 1995). Rats in adolescence have a delayed rise and more prolonged corticosterone release to several different types of acute stressors compared with adults (Romeo, Lee et al. 2004; Prendergast and Little 2007). Specifically, adolescent and adult rats have a significant elevation in plasma corticosterone levels after the stressor, but a higher level was maintained in adolescent and not adult rats 45 minutes later (Romeo, Lee et al. 2004). These differences in the stress response are thought to indicate that the HPA axis continues to develop substantially during adolescence and a more tightly regulated stress response system may emerge in adulthood (Romeo, Bellani et al. 2006).

When adolescent mice were exposed to chronic voluntary alcohol self-administration for 14 days, swim-stress induced changes in CORT were not different from water controls. However, adult CORT response to swim stress was blunted in mice with a history of alcohol self-administration as compared to parallel water controls. Thus, these data are consistent with the interpretation that the HPA axis was dysregulated by alcohol drinking in adult but not adolescent mice. The adaptation or blunting of the stress response agrees with studies to date suggesting that repeated restraint stress or chronic alcohol use causes a decrease in the HPA response in adult humans and rodents (Wand and Dobs 1991). It has been suggested that this adaptation may negatively affect the ability of the HPA axis and related physiological systems to maintain their normal function (Romeo, Bellani et al. 2006). These data also agree with studies looking at the progression of stress responses in adolescent and adult mice. Although alcohol is consumed in part due to its anxiolytic properties, it activates the HPA axis causing release of corticosterone (Spencer and McEwen 1990). In more chronic exposure paradigms such as alcohol self-administration, activation of the HPA axis occurs daily as the rodent drinks bouts of alcohol and as such may be considered a chronic stress state. Therefore our data agree with experiments that have shown that

adolescent rats exposed to daily restraint stress had high levels of plasma ACTH and CORT after the seventh episode, as rats of the same age undergoing a first restraint. In contrast, adult rats secreted less corticosterone to a 7th episode of restraint stress than a first episode (blunting) (Romeo, Bellani et al. 2006; McCormick, Merrick et al. 2007). Overall, experiments have shown that, adolescent rats do not “adapt” to chronic stress or alcohol self-administration as adult rats do.

This study investigated the ERK1/2 signaling system as a possible molecular mechanism that may underlie ontogenetic differences in adaptation to chronic alcohol-induced modulation of HPA activation. It was expected that changes in p-ERK1/2 IR would mimic the CORT response after alcohol self-administration; however, both adolescents and adults showed an alcohol drinking-induced blunting of the stress-induced activation of p-ERK1/2 IR response in the PVN hypothalamus. These data agree with studies suggesting that chronic stress reduces the levels of p-ERK1/2, in the brain (Meller, Shen et al. 2003; Imbe, Murakami et al. 2004; Qi and Elion 2005). Additional analysis indicated that p-ERK1/2 IR was increased in response to stress in the CeA only in adolescent mice, and post hoc comparisons showed that this response was blunted in alcohol experienced adolescent mice. It has been demonstrated that ERK1/2 activation in the CeA may have a delayed rise in comparison to other stress related brain regions (Yang, Huang et al. 2008). Thus, adolescent mice may respond to the stress induced ERK1/2 activation at a faster rate than adult mice and at the time point sampled adult ERK1/2 activation may not have been fully captured. This suggests that adolescents may be more sensitive to stress induced ERK1/2 activation in the amygdala. Future studies should investigate a more complete time course in order to assess this potential ontogenetic difference. Taken together, these data suggest that the effects of alcohol drinking on stress-induced ERK1/2 activation in these brain regions are not CORT induced as they do not mimic the CORT response. Further analysis

of extrahypothalamic areas of the brain, such as the hippocampus, which also feeds back to the HPA axis, should also be examined to determine if p-ERK1/2 responses are associated with CORT.

In summary, results of this study showed that adolescent mice had higher levels of corticosterone than adult mice 30 minutes after an acute swim stress procedure. We also show that adolescents exposed to chronic voluntary alcohol self-administration prior to an acute stressor had unaffected CORT responses however, adult CORT responses were blunted by alcohol self-administration. Finally, we show that the decrease in p-ERK1/2 in the PVN hypothalamus of adolescents and adults was not induced by the altered CORT response. Stress (HPA activation) has been observed to increase, decrease or not influence alcohol consumption (Fidler and LoLordo 1996; Lynch, Kushner et al. 1999; Le, Harding et al. 2005). However, high circulating levels of glucocorticoids may increase the pleasurable effects of drinking. Corticosterone has been found to be reinforcing, as adult rodents will self-administer a corticosterone solution (Piazza, Deroche et al. 1993) and HPA axis activation is thought to contribute to initiation, maintenance and relapse to alcohol abuse (Brown, Vik et al. 1990; Brady and Sonne 1999; Overstreet, Knapp et al. 2004). HPA activation is a factor in acquisition of alcohol conditioned place preference (CPP) in adolescent mice indicating its importance in alcohol-seeking. An inability of the HPA axis to adapt to stress may make the adolescent brain more vulnerable to a future addiction.

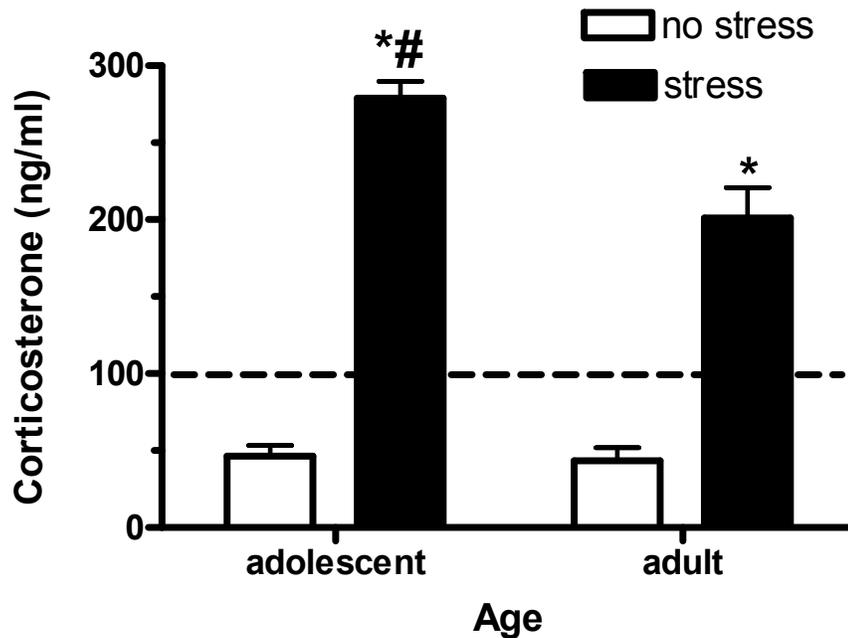


Figure 5.1. Basal and stress-induced changes in plasma corticosterone in adolescent and adult mice. Average \pm SEM plasma CORT (ng/ml) plotted as a function of stress and age. A 2-way ANOVA indicated that adolescent mice had higher levels of corticosterone than adult mice 30 minutes after an acute swim stress procedure. Multiple comparison procedures indicated that stress produced a significant increase in corticosterone (ng/ml) in adolescents ($p < 0.05$) and adult ($p < 0.05$) mice, but the CORT response was greater in adolescents than in adults ($p < 0.05$). Basal plasma CORT levels were not different between adolescent and adult mice. Dashed line indicates control response. * - significantly different from no stress within age; # - significantly difference from adult within stress condition, Holm-Sidak test, $p < 0.05$.

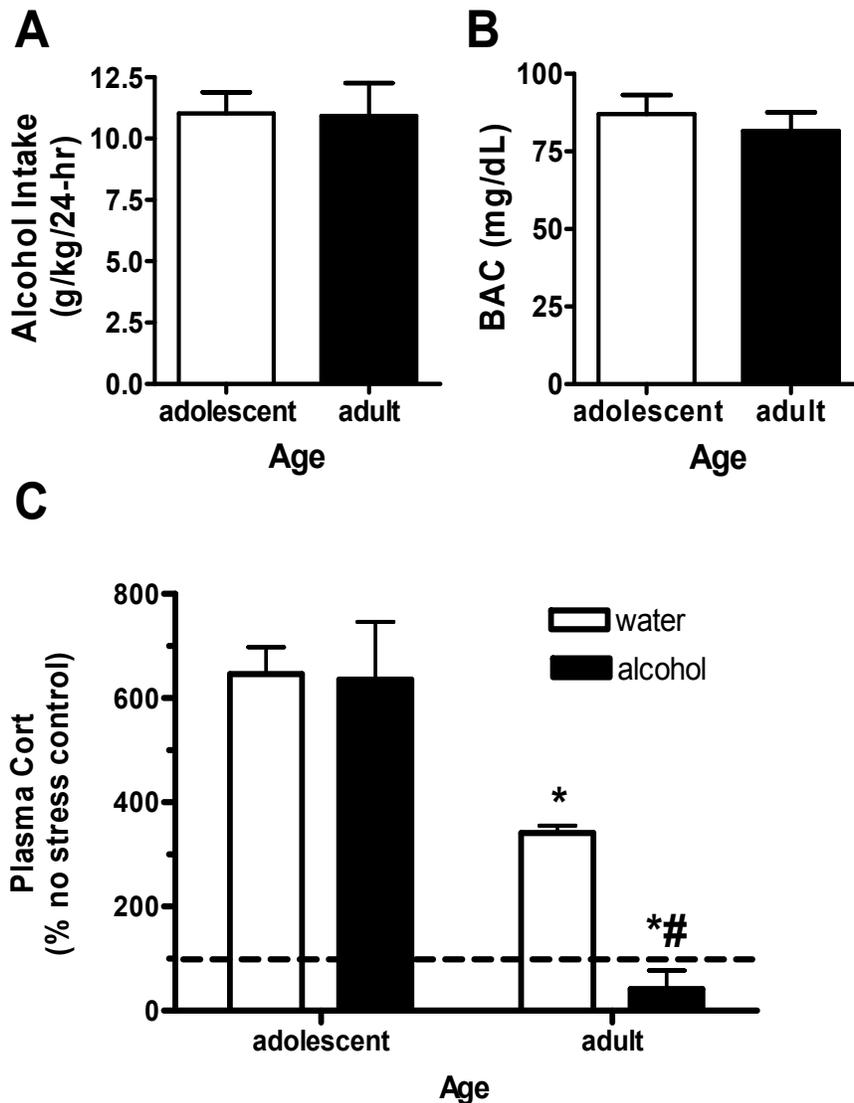


Figure 5.2. Alcohol self-administration and corticosterone response to stress in alcohol experienced and naïve mice. (A – B) Average alcohol intake (g/kg/24-hr) and BAC (mg/dL) sampled at time of sacrifice plotted as a function of age group. **(C)** Effect of swim stress on average plasma CORT (ng/ml) shown as a relative change from no-stress control and plotted after stress as a function of age for water and ethanol exposed mice. Two-way ANOVA indicated that a history of 2-bottle alcohol self-administration had no effect on stress-induced CORT response in adolescent mice after acute swim stress; however, chronic alcohol-drinking blunted the acute CORT response in adult mice. Post-hoc comparisons showed that alcohol produced a significant decrease in corticosterone (ng/ml) in adults compared to adolescent mice. Dashed line indicates control response. * - significantly different from no stress within age; # - significantly difference from adult within stress condition, Holm-Sidak test, $p < 0.05$.

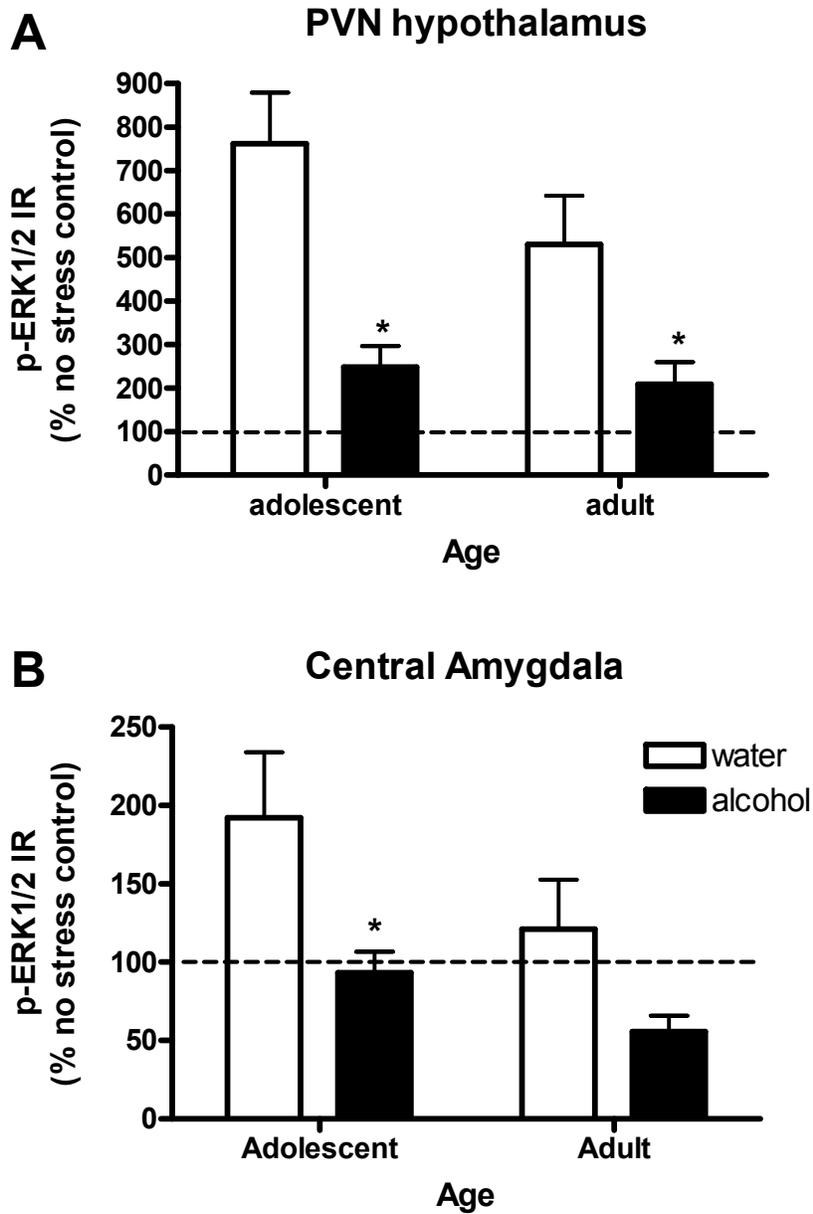


Figure 5.3. Effect of stress on p-ERK1/2 IR in the PVN and CeA of adolescent and adult mice with a history of alcohol drinking. Data are plotted as percent change from parallel no-stress controls in p-ERK1/2 IR as a function of age in the (A) paraventricular nucleus of the hypothalamus and (B) the central nucleus of the amygdala. Dashed line indicates control response. * - indicates significantly different from water control within age group, planned t-test ($p < 0.05$).

CHAPTER VI: CONCLUSIONS AND FUTURE DIRECTIONS

The work carried out for this dissertation extended the current knowledge of alcoholism by addressing a key cell signaling system, the ERK/MAPK pathway, and its potential role in adolescent vulnerability to addiction. Adolescent and adult alcohol-induced differences in behavior have been thoroughly examined; however, underlying cell signaling systems have not been addressed. These studies explored ontogenetic differences in effects of acute and chronic alcohol on activation of ERK/MAPK. Studies also explored how adolescent binge alcohol use leads to increases in abuse liability during adulthood. Finally, the interaction between HPA axis function and chronic alcohol consumption were investigated in relation to the ERK/MAPK signaling pathway. Thus, the present body of work attempts to identify differential developmentally dependent alcohol-induced responses to the ERK/MAPK pathway.

The goal of chapter 2 was to identify potential neuroadaptations in cell signaling that may underlie the differential behavioral responses to acute alcohol observed in adolescents and adults. The ERK/MAPK pathway is an ideal candidate for study since it has been associated with acute effects of alcohol (Kalluri and Ticku 2002; Chandler and Sutton 2005) and is modulated by other drugs of abuse and neurotransmitter that are altered by alcohol (Vanhoutte, Barnier et al. 1999; Valjent, Corvol et al. 2000; Girault, Valjent et al. 2007). Experiments examined the role of the ERK/MAPK signaling pathway in response to acute alcohol exposure in adolescents and adults. Differential developmental changes in p-ERK1/2 IR emerged upon first exposure to alcohol. The data show for the first time that adolescents are more sensitive to acute alcohol-induced p-ERK1/2 activation than their

adult counterparts in two brain regions: the basolateral amygdala and dentate gyrus. These brain regions are crucially involved with addiction neurocircuitry and have now been shown to respond differently to acute alcohol exposure in adolescents and adults. The difference in p-ERK1/2 activation is intriguing as nothing was known about intracellular signaling cascades that may underlie acute differential behavioral responses in adolescents and adults.

The BLA has been associated with drug relapse, and our lab has previously shown that ERK1/2 is activated in the BLA by response-contingent cue-induced reinstatement of alcohol-seeking behavior (Schroeder, Spanos et al. 2008). ERK1/2 and the BLA also play a more general role in learning and memory that may underlie changes in this system associated with drug relapse. For example, the BLA is known to mediate associative learning processes, such as cue learning in Pavlovian fear conditioning (LeDoux 2000). ERK/MAPK is activated in the amygdala after fear conditioning and pharmacological blockade of ERK/MAPK activation in the amygdala impairs fear memory consolidation (Schafe, Atkins et al. 2000). Moreover, enhanced activation of memory circuits by drugs of abuse is considered to be a major molecular mechanism that underlies the long-term persistent nature of addiction (Hyman, Malenka et al. 2006). In this way, enhanced alcohol-induced increases in p-ERK1/2 in the amygdala of adolescent mice may reflect increased vulnerability of the developing brain to addiction.

Age-dependent differential responses in the ERK/MAPK pathway were also observed in the hippocampus (DG) following a single administration of alcohol (1 g/kg). This finding is significant, in part, because p-ERK1/2 activity is required for hippocampal-dependent memory tasks (Blum, Moore et al. 1999; Schafe, Atkins et al. 2000; Bozon, Kelly et al. 2003; Kelly, Laroche et al. 2003; Runyan, Moore et al. 2004). Research has shown that adolescents are more sensitive to alcohol-induced memory deficits than adults

(Markwiese, Acheson et al. 1998; White and Swartzwelder 2004). Results from the current studies showed that administration of a 1g/kg dose of alcohol to adolescent mice disrupted performance on a hippocampal-dependent memory task to a greater extent than adult mice. Thus, ERK1/2 inhibition in the hippocampus may underlie adolescent vulnerability to alcohol-induced memory disruption. Future studies might obtain corresponding evidence by microinjecting an ERK inhibitor in the hippocampus prior to the NOR task and observing its effects on memory in adolescent and adult mice.

Both the BLA and DG play important roles in addiction and learning and memory circuits. Learning and memory and drug addiction are thought to be modulated by the same intracellular signaling cascades and depend on activation of similar transcription factors (Nestler 2002). It would be prudent to examine downstream transcription factors, such as p-CREB, in an age-dependent manner in order to determine differences in cell signaling proteins that may be relevant to both addiction and memory pathways. CREB is activated upon phosphorylation which occurs through a number of protein kinases including ERK and calcium /calmodulin kinase IV. Thus, CREB represents a site of convergence at which numerous signaling pathways, and the external stimuli that activate them, produce plastic changes by altering gene expression. CREB has been found to play a critical role in behavioral memory (Kida, Josselyn et al. 2002; Pittenger, Huang et al. 2002). Experiments have directly implicated CREB in the amygdala and hippocampus in mediating the effects of CREB in behavioral memory (Blendy and Maldonado 1998; Berke and Hyman 2000). Most importantly CREB has not been examined to date in adolescents in either acute or chronic effects of alcohol and should be addressed.

To date, there have been no studies examining potential developmental differences in the effect of alcohol self-administration on ERK1/2 activity. Experiments conducted in chapter 3 addressed this gap in knowledge by testing potential functional regulation of

alcohol drinking by ERK1/2 signaling. An age-specific decrease in p-ERK1/2 after chronic alcohol consumption was noted in adolescents in the dentate gyrus (DG) compared with adults (similar to acute alcohol injection). Alcohol (acute and chronic) has been observed to have differential behavioral effects on adolescent and adult learning and memory therefore, the alcohol-induced p-ERK1/2 neuroadaptations in the adolescent hippocampus (both acute and chronic) may be functionally important for future effects on learning and memory and addiction. These data suggest that adaptation in ERK1/2 signaling in the dentate gyrus is one potential mechanism that may underlie adolescent vulnerability to cognitive impairing effects of alcohol as reported in humans (Brown, Tapert et al. 2000). Any lasting impact of p-ERK1/2 on the hippocampus may in turn effect the development of an addiction as so many of the downstream molecular and cellular changes underlying both learning and memory and addiction are overlapping. Future studies should address adolescent and adult (acute and chronic) alcohol-induced p-ERK1/2 differences in the hippocampus and examine the functional role of these neuroadaptations on future learning and memory and addiction.

An age-specific increase in p-ERK1/2 after chronic alcohol consumption was also noted in adolescents in the central nucleus of the amygdala (CeA) compared with adults. To date, there are conflicting data in the literature concerning chronic alcohol use and p-ERK1/2 changes and therefore the results obtained from chronic alcohol exposure make it difficult to identify a functional role for ERK1/2 in chronic alcohol abuse. However, given that the CeA is critically involved in alcohol-drinking behavior of mice and rats (McBride 2002) another goal of the study was to determine, using the MEK/ERK inhibitor SL327, whether the ERK/MAPK pathway is functionally involved with modulating alcohol intake in adolescent and adult mice. Interestingly, an IP injection of the MEK/ERK inhibitor increased alcohol intake (g/kg) in adolescent but not adult C57BL/6J mice. Since an age-related increase in ERK1/2 activity was observed in the CeA of adolescents and not adult mice after 14 days of

2-bottle alcohol self-administration, it is probable that the inhibitor increased alcohol intake in adolescent mice via inhibition of ERK1/2 in the CeA. Future studies should examine upstream and downstream proteins in the ERK/MAPK signaling system that may also be involved in the differential increase in alcohol intake between adolescents and adults. The mGluR5 receptor is upstream of the ERK/MAPK signaling pathway and should be addressed as a potential mechanism in adolescent drinking as it has been shown to regulate alcohol self-administration in adults (Schroeder, Overstreet et al. 2005; Hodge, Miles et al. 2006; Besheer, Grondin et al. 2009). There are also a few interesting candidate proteins in the PKC family that may also be involved, including PKC ϵ and PKC γ (Hodge, Mehmert et al. 1999; Besheer, Lepoutre et al. 2006). It has been suggested that alcohol regulates activation of ERK1/2 by a PKC-dependent mechanism (Roivainen, Hundle et al. 1995). Studies have shown that PKC ϵ knock-out mice exhibit opposite alcohol-induced behaviors to adolescent mice including increased sensitivity to alcohol sedation and decreased alcohol consumption (Hodge, Mehmert et al. 1999). PKC γ knockout mice have a decreased initial sensitivity to alcohol-induced sedation, a trait they share with adolescent rodents (Harris, McQuilkin et al. 1995).

A third set of experiments focused on the pattern of alcohol consumption that is of the greatest public health concern for adolescents: episodic heavy drinking, or “binge drinking.” To this point, researchers have addressed the issue of high alcohol intake in a rodent model and agree that adolescent drinking leads to an increase in drinking in adulthood; however, no studies had examined age of onset of binge drinking and its impact on adult voluntary drinking. The age-of onset binge drinking experiment indicated that mice that began the alcohol binge procedure (DID) during adolescence drank significantly more alcohol (g/kg) in adulthood than those who began drinking in early adulthood and later adulthood. This conclusion is strikingly similar to epidemiological evidence suggesting that

alcohol exposure during adolescence is associated with an increased risk of developing an alcohol use disorder later in life and gives great translational value to the method. Pharmacological methods examining the role of opioid, dopaminergic, and glutamatergic mechanisms underlying high alcohol intake (DID) should be employed in adolescent mice and their effects on drinking assessed. Studies in adult animals have examined the role of endogenous opioid signaling in DID drinking and discovered that they play a necessary role in high alcohol intake (Kamdar, Miller et al. 2007); however, the role of the opioid system in adolescent high alcohol intake has not been assessed. Further studies using the adolescent DID alcohol exposure procedure could provide greater insight into the variety of neurobiological changes that occur during adolescence to promote increased alcohol intake in adulthood, and whether pharmacological manipulations used to curb intake in adults show promise or are even effective at reducing high alcohol intake in adolescents.

Adolescent and adult mice were also used to determine whether adolescent C57BL/6J mice are less sensitive to the aversive properties of alcohol, by examining age-related differences in sensitivity to alcohol-induced conditioned taste aversion (CTA). Adolescent mice were found to be less sensitive to an alcohol-induced CTA than adult mice, which could be an underlying behavioral mechanism for increased alcohol consumption during adolescence. A limitation of the current experiment is that the decreased sensitivity to alcohol-induced CTA in adolescents versus adults could be related to greater alcohol-induced memory impairment observed during adolescence (Acheson, Stein et al. 1998; Markwiese, Acheson et al. 1998; Tapert, Schweinsburg et al. 2004). Studies have shown that activation of ERK is critical for memory consolidation in CTA (Swank 2000; Languille, Davis et al. 2009), and adolescent rodents are more sensitive to ethanol-induced reductions in ERK1/2 (Chapter 2), impairment of performance in the novel object test (Chapter 2), and memory acquisition (Markwiese, Acheson et al. 1998) as compared to adults. A control experiment using lithium chloride (LiCl) as the aversive unconditioned stimulus could help to

decipher if greater impairment to the alcohol-induced CTA is due to the memory impairing effects of alcohol or insensitivity to the alcohol in adolescent mice. These data add to a number of studies indicating that adolescent rodents are less sensitive to a number of the negative behavioral effects of alcohol than their adult counterparts including alcohol-induced motor impairment, sedation, anxiolysis and social inhibition (Silveri and Spear 1998; Hefner and Holmes 2007). The adolescent insensitivity to the aversive effects of alcohol could be a potential contributor to high alcohol intake (binging) during adolescence. These data suggest that the blunted sensitivity to alcohol-induced CTA during adolescence may be a factor that contributes to increased risk of alcohol abuse in adulthood.

The last set of experiments addressed age-related differences in stress, drinking and the ERK/MAPK pathway. Research has suggested that the perception of stress may be increased in adolescents relative to adults and this presumed increase in stress during adolescence contributes to the frequent initiation of alcohol use observed in adolescents. Developmental differences to chronic alcohol exposure and the effect of such exposure on HPA axis responses and cellular signaling systems, including ERK, have not been examined to date. To address this gap in knowledge, the present study elucidated the developmental differences in HPA axis response and ERK1/2 activation after chronic alcohol exposure. When exposed to chronic voluntary alcohol self-administration for 14 days prior to an acute stressor adolescent CORT responses were unaffected; however, the adult CORT responses were blunted by alcohol self-administration. Dysregulation of the HPA axis was observed in the adult but not the adolescent mice.

It would be interesting to further investigate age-related differences in HPA axis function after chronic alcohol consumption by examining glucocorticoid receptor number and location and CRF in paraventricular nucleus of the hypothalamus. In response to stressors the HPA axis is driven by the release of CRF from the paraventricular nucleus (PVN) of the hypothalamus. This neuropeptide stimulates the anterior pituitary to release ACTH, which in

turn stimulates the adrenals to release corticosterone (Herman, Figueiredo et al. 2003). Feedback systems in the HPA axis appear to respond differently to an acute stressor after a history of alcohol consumption. Examining developmental differences in glucocorticoid receptor changes or CRF after a history of alcohol consumption may give more insight into the changes that occur in the HPA axis. A dexamethasone suppression test, which assesses the sensitivity of the hypothalamus and pituitary to negative feedback from circulating levels of cort, could also elucidate the developmental differences in feedback regulation after a history of alcohol consumption. Other HPA axis hormones such as ACTH and allopregnanolone should also be examined to give a better insight into the differences in the adolescent and adult HPA axis after chronic alcohol consumption as well as baseline age differences. Circulating allopregnanolone is increased in humans and rats after CRF and ACTH administration (Genazzani, Petraglia et al. 1998; Torres, Ruiz et al. 2001). Allopregnanolone may therefore be a part of a novel feedback mechanism within the HPA axis, as previously proposed (Torres and Ortega 2003). Studies have demonstrated that repeated alcohol exposure blunts the ACTH response to alcohol challenge but not footshock (Rivier and Lee 2001). This demonstrates the complexity of HPA regulation and that each step may be independently affected by alcohol exposure and thus should be studied independently. HPA function should also be examined in adulthood in order to establish any long-term effects of adolescent chronic alcohol exposure on the developing stress axis.

The ERK signaling system was investigated as a possible underlying cell signaling mechanism, which may lead to the age-related differences in adaptation to chronic alcohol-induced HPA activation. The p-ERK1/2 response would be expected to mimic the CORT response observed after alcohol self-administration; however both adolescents and adults showed a blunting of the stress-induced p-ERK1/2 IR response in the PVN hypothalamus indicating that there are no age-specific effects. However, other extrahypothalamic regions of the brain including the hippocampus and amygdala which are also part of the feedback

mechanism should be examined for possible age-related differences in CORT-activated p-ERK1/2 IR.

HPA axis activation is thought to contribute to initiation, maintenance and relapse to alcohol abuse (Brown, Vik et al. 1990; Brady and Sonne 1999; Overstreet, Knapp et al. 2004). It has been suggested that high circulating levels of glucocorticoids like those observed in the adolescent mice may increase the pleasurable effects of drinking. In fact, corticosterone has been found to be reinforcing, as adult rodents will self-administer a corticosterone solution (Piazza, Deroche et al. 1993). Activation of the HPA axis is a factor in acquisition of alcohol conditioned place preference (CPP) in adolescent mice indicating its importance in alcohol-seeking (Song, Wang et al. 2007). An inability of the HPA axis to adapt, to stress may therefore make the adolescent brain more vulnerable to a future addiction as it may enhance the pleasurable effects of alcohol while not allowing the adolescent brain to experience the dysregulation of the HPA axis observed in adult mice.

This dissertation illustrated the importance of examining underlying cell signaling systems during adolescence that may be altered by alcohol consumption. Age-related differences in the ERK/MAPK signaling pathway were observed after both acute and chronic alcohol consumption. Using the MEK/ERK inhibitor in the NOR task and in alcohol self-administration highlighted the importance of this pathway in adolescent learning and memory as well as addiction. Age-related differences in HPA axis adaptation to chronic alcohol may make the adolescent brain more vulnerable to a future addiction. Overall, a lack of sensitivity to the negative effects of alcohol and an increase in the more pleasurable effects of alcohol consumption during adolescence may make them more vulnerable to future alcohol dependence.

REFERENCES

- Acheson, S. K., R. M. Stein, et al. (1998). "Impairment of semantic and figural memory by acute ethanol: age-dependent effects." Alcohol Clin Exp Res **22**(7): 1437-42.
- Alessandrini, A., S. Namura, et al. (1999). "MEK1 protein kinase inhibition protects against damage resulting from focal cerebral ischemia." Proc Natl Acad Sci U S A **96**(22): 12866-9.
- Allen, M. T. and K. A. Matthews (1997). "Hemodynamic responses to laboratory stressors in children and adolescents: the influences of age, race, and gender." Psychophysiology **34**(3): 329-39.
- Andersen, S. L., A. T. Thompson, et al. (2000). "Dopamine receptor pruning in prefrontal cortex during the periadolescent period in rats." Synapse **37**(2): 167-9.
- Anthony, E. J. (1982). "Normal adolescent development from a cognitive viewpoint." J Am Acad Child Psychiatry **21**(4): 318-27.
- Aseltine, R. H., Jr. and S. L. Gore (2000). "The variable effects of stress on alcohol use from adolescence to early adulthood." Subst Use Misuse **35**(5): 643-68.
- Bachtell, R. K., N. O. Tsivkovskaia, et al. (2002). "Alcohol-induced c-Fos expression in the Edinger-Westphal nucleus: pharmacological and signal transduction mechanisms." J Pharmacol Exp Ther **302**(2): 516-24.
- Bates, M. E. and E. W. Labouvie (1997). "Adolescent risk factors and the prediction of persistent alcohol and drug use into adulthood." Alcohol Clin Exp Res **21**(5): 944-50.
- Baumrind, D. (1987). "A developmental perspective on adolescent risk taking in contemporary America." New Dir Child Dev(37): 93-125.
- Beck, K. H. and K. A. Treiman (1996). "The relationship of social context of drinking, perceived social norms, and parental influence to various drinking patterns of adolescents." Addict Behav **21**(5): 633-44.
- Behringer, K. A., L. M. Gault, et al. (1996). "Differential regulation of GABA A receptor subunit mRNAs in rat cerebellar granule neurons: importance of environmental cues." J Neurochem **66**(4): 1347-53.

- Berke, J. D. and S. E. Hyman (2000). "Addiction, dopamine, and the molecular mechanisms of memory." Neuron **25**(3): 515-32.
- Besheer, J., J. J. Grondin, et al. (2009). "Metabotropic Glutamate Receptor 5 Activity in the Nucleus Accumbens Is Required for the Maintenance of Ethanol Self-Administration in a Rat Genetic Model of High Alcohol Intake." Biol Psychiatry.
- Besheer, J., V. Lepoutre, et al. (2006). "GABAA receptor regulation of voluntary ethanol drinking requires PKCepsilon." Synapse **60**(6): 411-9.
- Besheer, J., J. P. Schroeder, et al. (2008). "Ethanol-induced alterations of c-Fos immunoreactivity in specific limbic brain regions following ethanol discrimination training." Brain Res **1232**: 124-31.
- Bevins, R. A. and J. Besheer (2006). "Object recognition in rats and mice: a one-trial non-matching-to-sample learning task to study 'recognition memory'." Nat Protoc **1**(3): 1306-11.
- Bierut, L. J., S. H. Dinwiddie, et al. (1998). "Familial transmission of substance dependence: alcohol, marijuana, cocaine, and habitual smoking: a report from the Collaborative Study on the Genetics of Alcoholism." Arch Gen Psychiatry **55**(11): 982-8.
- Blendy, J. A. and R. Maldonado (1998). "Genetic analysis of drug addiction: the role of cAMP response element binding protein." J Mol Med **76**(2): 104-10.
- Blizard, D. A., D. J. Vandenberg, et al. (2004). "Effects of periadolescent ethanol exposure on alcohol preference in two BALB substrains." Alcohol **34**(2-3): 177-85.
- Blum, S., A. N. Moore, et al. (1999). "A mitogen-activated protein kinase cascade in the CA1/CA2 subfield of the dorsal hippocampus is essential for long-term spatial memory." J Neurosci **19**(9): 3535-44.
- Bourgeois, J. P., P. S. Goldman-Rakic, et al. (1994). "Synaptogenesis in the prefrontal cortex of rhesus monkeys." Cereb Cortex **4**(1): 78-96.
- Bozon, B., A. Kelly, et al. (2003). "MAPK, CREB and zif268 are all required for the consolidation of recognition memory." Philos Trans R Soc Lond B Biol Sci **358**(1432): 805-14.
- Brady, K. T. and S. C. Sonne (1999). "The role of stress in alcohol use, alcoholism treatment, and relapse." Alcohol Res Health **23**(4): 263-71.

- Breese, G. R., D. H. Overstreet, et al. (2005). "Conceptual framework for the etiology of alcoholism: a "kindling"/stress hypothesis." Psychopharmacology (Berl) **178**(4): 367-80.
- Broadbent, J., H. V. Linder, et al. (1996). "Genetic differences in naloxone enhancement of ethanol-induced conditioned taste aversion." Psychopharmacology (Berl) **126**(2): 147-55.
- Broadbent, J., K. J. Muccino, et al. (2002). "Ethanol-induced conditioned taste aversion in 15 inbred mouse strains." Behav Neurosci **116**(1): 138-48.
- Brown, S. A., S. F. Tapert, et al. (2000). "Neurocognitive functioning of adolescents: effects of protracted alcohol use." Alcohol Clin Exp Res **24**(2): 164-71.
- Brown, S. A., P. W. Vik, et al. (1990). "Severity of psychosocial stress and outcome of alcoholism treatment." J Abnorm Psychol **99**(4): 344-8.
- Brunell, S. C. and L. P. Spear (2005). "Effect of stress on the voluntary intake of a sweetened ethanol solution in pair-housed adolescent and adult rats." Alcohol Clin Exp Res **29**(9): 1641-53.
- Brunetti, G., M. A. Carai, et al. (2002). "Differences in ethanol-induced conditioned taste aversion in Sardinian alcohol-preferring and Sardinian alcohol-nonpreferring rats." Alcohol **26**(3): 167-72.
- Buchanan, C. M., J. S. Eccles, et al. (1992). "Are adolescents the victims of raging hormones: evidence for activational effects of hormones on moods and behavior at adolescence." Psychol Bull **111**(1): 62-107.
- Cammarota, M., L. R. Bevilaqua, et al. (2000). "Learning-associated activation of nuclear MAPK, CREB and Elk-1, along with Fos production, in the rat hippocampus after a one-trial avoidance learning: abolition by NMDA receptor blockade." Brain Res Mol Brain Res **76**(1): 36-46.
- Casey, B. J., J. N. Giedd, et al. (2000). "Structural and functional brain development and its relation to cognitive development." Biol Psychol **54**(1-3): 241-57.
- Chandler, L. J. and G. Sutton (2005). "Acute ethanol inhibits extracellular signal-regulated kinase, protein kinase B, and adenosine 3':5'-cyclic monophosphate response element binding protein activity in an age- and brain region-specific manner." Alcohol Clin Exp Res **29**(4): 672-82.

- Chester, J. A., L. Lumeng, et al. (2003). "High- and low-alcohol-preferring mice show differences in conditioned taste aversion to alcohol." Alcohol Clin Exp Res **27**(1): 12-8.
- Choe, E. S., K. T. Chung, et al. (2002). "Amphetamine increases phosphorylation of extracellular signal-regulated kinase and transcription factors in the rat striatum via group I metabotropic glutamate receptors." Neuropsychopharmacology **27**(4): 565-75.
- Constantinescu, A., M. Wu, et al. (2004). "cAMP-dependent protein kinase type I regulates ethanol-induced cAMP response element-mediated gene expression via activation of CREB-binding protein and inhibition of MAPK." J Biol Chem **279**(41): 43321-9.
- Crews, F., J. He, et al. (2007). "Adolescent cortical development: a critical period of vulnerability for addiction." Pharmacol Biochem Behav **86**(2): 189-99.
- Dai, X., J. Thavundayil, et al. (2002). "Response of the hypothalamic-pituitary-adrenal axis to stress in the absence and presence of ethanol in subjects at high and low risk of alcoholism." Neuropsychopharmacology **27**(3): 442-52.
- Davis, S., P. Vanhoutte, et al. (2000). "The MAPK/ERK cascade targets both Elk-1 and cAMP response element-binding protein to control long-term potentiation-dependent gene expression in the dentate gyrus in vivo." J Neurosci **20**(12): 4563-72.
- DeWit, D. J., E. M. Adlaf, et al. (2000). "Age at first alcohol use: a risk factor for the development of alcohol disorders." Am J Psychiatry **157**(5): 745-50.
- Di Chiara, G. and A. Imperato (1986). "Preferential stimulation of dopamine release in the nucleus accumbens by opiates, alcohol, and barbiturates: studies with transcerebral dialysis in freely moving rats." Ann N Y Acad Sci **473**: 367-81.
- Doremus-Fitzwater, T. L. and L. P. Spear (2007). "Developmental differences in acute ethanol withdrawal in adolescent and adult rats." Alcohol Clin Exp Res **31**(9): 1516-27.
- Doremus, T. L., S. C. Brunell, et al. (2005). "Factors influencing elevated ethanol consumption in adolescent relative to adult rats." Alcohol Clin Exp Res **29**(10): 1796-808.
- Draski, L. J., P. J. Bice, et al. (2001). "Developmental alterations of ethanol sensitivity in selectively bred high and low alcohol sensitive rats." Pharmacol Biochem Behav **70**(2-3): 387-96.

- English, J. D. and J. D. Sweatt (1996). "Activation of p42 mitogen-activated protein kinase in hippocampal long term potentiation." J Biol Chem **271**(40): 24329-32.
- Faccidomo, S., J. Besheer, et al. (2009). "Increased operant responding for ethanol in male C57BL/6J mice: specific regulation by the ERK1/2, but not JNK, MAP kinase pathway." Psychopharmacology (Berl) **204**(1): 135-47.
- Fendt, M. and M. S. Fanselow (1999). "The neuroanatomical and neurochemical basis of conditioned fear." Neurosci Biobehav Rev **23**(5): 743-60.
- Fidler, T. L. and V. M. LoLordo (1996). "Failure to find postshock increases in ethanol preference." Alcohol Clin Exp Res **20**(1): 110-21.
- Garbutt, J. C. (2009). "The state of pharmacotherapy for the treatment of alcohol dependence." J Subst Abuse Treat **36**(1): S15-23; quiz S24-5.
- Geier, C. and B. Luna (2009). "The maturation of incentive processing and cognitive control." Pharmacol Biochem Behav **93**(3): 212-21.
- Genazzani, A. R., F. Petraglia, et al. (1998). "Circulating levels of allopregnanolone in humans: gender, age, and endocrine influences." J Clin Endocrinol Metab **83**(6): 2099-103.
- Gessa, G. L., F. Muntoni, et al. (1985). "Low doses of ethanol activate dopaminergic neurons in the ventral tegmental area." Brain Res **348**(1): 201-3.
- Giedd, J. N. (2004). "Structural magnetic resonance imaging of the adolescent brain." Ann N Y Acad Sci **1021**: 77-85.
- Giedd, J. N., J. Blumenthal, et al. (1999). "Brain development during childhood and adolescence: a longitudinal MRI study." Nat Neurosci **2**(10): 861-3.
- Girault, J. A., E. Valjent, et al. (2007). "ERK2: a logical AND gate critical for drug-induced plasticity?" Curr Opin Pharmacol **7**(1): 77-85.
- Gogtay, N., J. N. Giedd, et al. (2004). "Dynamic mapping of human cortical development during childhood through early adulthood." Proc Natl Acad Sci U S A **101**(21): 8174-9.

- Goldman-Rakic, P. S. (1988). "Topography of cognition: parallel distributed networks in primate association cortex." Annu Rev Neurosci **11**: 137-56.
- Grant, B. F. and D. A. Dawson (1997). "Age at onset of alcohol use and its association with DSM-IV alcohol abuse and dependence: results from the National Longitudinal Alcohol Epidemiologic Survey." J Subst Abuse **9**: 103-10.
- Grant, B. F. and D. A. Dawson (1998). "Age of onset of drug use and its association with DSM-IV drug abuse and dependence: results from the National Longitudinal Alcohol Epidemiologic Survey." J Subst Abuse **10**(2): 163-73.
- Grant, B. F., F. S. Stinson, et al. (2001). "Age at onset of alcohol use and DSM-IV alcohol abuse and dependence: a 12-year follow-up." J Subst Abuse **13**(4): 493-504.
- Hallak, H., A. E. Seiler, et al. (2001). "Inhibition of insulin-like growth factor-I signaling by ethanol in neuronal cells." Alcohol Clin Exp Res **25**(7): 1058-64.
- Harford, T. C., B. F. Grant, et al. (2005). "Patterns of DSM-IV alcohol abuse and dependence criteria among adolescents and adults: results from the 2001 National Household Survey on Drug Abuse." Alcohol Clin Exp Res **29**(5): 810-28.
- Harris, R. A., S. J. McQuilkin, et al. (1995). "Mutant mice lacking the gamma isoform of protein kinase C show decreased behavioral actions of ethanol and altered function of gamma-aminobutyrate type A receptors." Proc Natl Acad Sci U S A **92**(9): 3658-62.
- Hefner, K. and A. Holmes (2007). "An investigation of the behavioral actions of ethanol across adolescence in mice." Psychopharmacology (Berl) **191**(2): 311-22.
- Herman, J. P., H. Figueiredo, et al. (2003). "Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness." Front Neuroendocrinol **24**(3): 151-80.
- Heyser, C. J., A. J. Roberts, et al. (1999). "Central administration of an opiate antagonist decreases oral ethanol self-administration in rats." Alcohol Clin Exp Res **23**(9): 1468-76.
- Ho, A., A. J. Chin, et al. (1989). "Early experience and the consumption of alcohol by adult C57BL/6J mice." Alcohol **6**(6): 511-5.

- Hodge, C. W., A. M. Chappelle, et al. (1996). "Dopamine receptors in the medial prefrontal cortex influence ethanol and sucrose-reinforced responding." Alcohol Clin Exp Res **20**(9): 1631-8.
- Hodge, C. W. and A. A. Cox (1998). "The discriminative stimulus effects of ethanol are mediated by NMDA and GABA(A) receptors in specific limbic brain regions." Psychopharmacology (Berl) **139**(1-2): 95-107.
- Hodge, C. W., K. K. Mehmert, et al. (1999). "Supersensitivity to allosteric GABA(A) receptor modulators and alcohol in mice lacking PKCepsilon." Nat Neurosci **2**(11): 997-1002.
- Hodge, C. W., M. F. Miles, et al. (2006). "The mGluR5 antagonist MPEP selectively inhibits the onset and maintenance of ethanol self-administration in C57BL/6J mice." Psychopharmacology (Berl) **183**(4): 429-38.
- Hodge, C. W., H. H. Samson, et al. (1992). "Microinjections of dopamine agonists in the nucleus accumbens increase ethanol-reinforced responding." Pharmacol Biochem Behav **43**(1): 249-54.
- Hodge, C. W., C. J. Slawecki, et al. (1996). "Norepinephrine and serotonin receptors in the paraventricular nucleus interactively modulate ethanol consumption." Alcohol Clin Exp Res **20**(9): 1669-74.
- Hollstedt, C., O. Olsson, et al. (1980). "Effects of ethanol on the developing rat. II. Coordination as measured by the tilting-plane test." Med Biol **58**(3): 164-8.
- Hyman, S. E., R. C. Malenka, et al. (2006). "Neural mechanisms of addiction: the role of reward-related learning and memory." Annu Rev Neurosci **29**: 565-98.
- Hyytia, P. and K. Kiianmaa (2001). "Suppression of ethanol responding by centrally administered CTOP and naltrindole in AA and Wistar rats." Alcohol Clin Exp Res **25**(1): 25-33.
- Imbe, H., S. Murakami, et al. (2004). "The effects of acute and chronic restraint stress on activation of ERK in the rostral ventromedial medulla and locus coeruleus." Pain **112**(3): 361-71.
- Insel, T. R., L. P. Miller, et al. (1990). "The ontogeny of excitatory amino acid receptors in rat forebrain--I. N-methyl-D-aspartate and quisqualate receptors." Neuroscience **35**(1): 31-43.

- Izumi, Y., C. O. Kirby-Sharkey, et al. (1995). "Age dependent sensitivity of the rat retina to the excitotoxic action of N-methyl-D-aspartate." Neurobiol Dis **2**(3): 139-44.
- Johnson, G. L. and R. Lapadat (2002). "Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases." Science **298**(5600): 1911-2.
- Johnston, L. D., P. M. O'Malley, et al. (2009). "Monitoring the Future national survey results on drug use, 1975–2008: Volume I, Secondary school students." (NIH Publication No. 09-7402). Bethesda, MD: National Institute on Drug Abuse.
- Kalluri, H. S. and M. K. Ticku (2002). "Ethanol-mediated inhibition of mitogen-activated protein kinase phosphorylation in mouse brain." Eur J Pharmacol **439**(1-3): 53-8.
- Kalluri, H. S. and M. K. Ticku (2003). "Regulation of ERK phosphorylation by ethanol in fetal cortical neurons." Neurochem Res **28**(5): 765-9.
- Kalsbeek, A., P. Voorn, et al. (1988). "Development of the dopaminergic innervation in the prefrontal cortex of the rat." J Comp Neurol **269**(1): 58-72.
- Kamdar, N. K., S. A. Miller, et al. (2007). "Acute effects of naltrexone and GBR 12909 on ethanol drinking-in-the-dark in C57BL/6J mice." Psychopharmacology (Berl) **192**(2): 207-17.
- Kelley, A. E., T. Schochet, et al. (2004). "Risk taking and novelty seeking in adolescence: introduction to part I." Ann N Y Acad Sci **1021**: 27-32.
- Kelley, S. P., M. A. Nannini, et al. (2001). "Neuropeptide-Y in the paraventricular nucleus increases ethanol self-administration." Peptides **22**(3): 515-22.
- Kelly, A., S. Laroche, et al. (2003). "Activation of mitogen-activated protein kinase/extracellular signal-regulated kinase in hippocampal circuitry is required for consolidation and reconsolidation of recognition memory." J Neurosci **23**(12): 5354-60.
- Kida, S., S. A. Josselyn, et al. (2002). "CREB required for the stability of new and reactivated fear memories." Nat Neurosci **5**(4): 348-55.
- Koob, G. F. (2000). "Animal models of craving for ethanol." Addiction **95 Suppl 2**: S73-81.

- Koob, G. F. and M. Le Moal (2005). "Plasticity of reward neurocircuitry and the 'dark side' of drug addiction." Nat Neurosci **8**(11): 1442-4.
- Kovalovsky, D., D. Refojo, et al. (2002). "Activation and induction of NUR77/NURR1 in corticotrophs by CRH/cAMP: involvement of calcium, protein kinase A, and MAPK pathways." Mol Endocrinol **16**(7): 1638-51.
- Kwon, M. S., Y. J. Seo, et al. (2006). "The effect of single or repeated restraint stress on several signal molecules in paraventricular nucleus, arcuate nucleus and locus coeruleus." Neuroscience **142**(4): 1281-92.
- Languille, S., S. Davis, et al. (2009). "Extracellular signal-regulated kinase activation is required for consolidation and reconsolidation of memory at an early stage of ontogenesis." Eur J Neurosci **30**(10): 1923-30.
- Laurie, D. J., W. Wisden, et al. (1992). "The distribution of thirteen GABAA receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development." J Neurosci **12**(11): 4151-72.
- Laviola, G., S. Macri, et al. (2003). "Risk-taking behavior in adolescent mice: psychobiological determinants and early epigenetic influence." Neurosci Biobehav Rev **27**(1-2): 19-31.
- Le, A. D., S. Harding, et al. (2005). "Role of alpha-2 adrenoceptors in stress-induced reinstatement of alcohol seeking and alcohol self-administration in rats." Psychopharmacology (Berl) **179**(2): 366-73.
- LeDoux, J. E. (2000). "Emotion circuits in the brain." Annu Rev Neurosci **23**: 155-84.
- Liao, D. and R. Malinow (1996). "Deficiency in induction but not expression of LTP in hippocampal slices from young rats." Learn Mem **3**(2-3): 138-49.
- Lu, L., B. T. Hope, et al. (2005). "Central amygdala ERK signaling pathway is critical to incubation of cocaine craving." Nat Neurosci **8**(2): 212-9.
- Lucas, L. A. and B. A. McMillen (2002). "Conditioned taste aversion and the Myers' high-ethanol-preferring rat." Alcohol Alcohol **37**(5): 427-31.
- Luna, B. (2009). "Developmental changes in cognitive control through adolescence." Adv Child Dev Behav **37**: 233-78.

- Lynch, W. J., M. G. Kushner, et al. (1999). "The effects of restraint stress on voluntary ethanol consumption in rats." Exp Clin Psychopharmacol **7**(4): 318-23.
- Ma, W., B. S. Li, et al. (2003). "Ethanol blocks both basic fibroblast growth factor- and carbachol-mediated neuroepithelial cell expansion with differential effects on carbachol-activated signaling pathways." Neuroscience **118**(1): 37-47.
- Maren, S. (1999). "Long-term potentiation in the amygdala: a mechanism for emotional learning and memory." Trends Neurosci **22**(12): 561-7.
- Markwiese, B. J., S. K. Acheson, et al. (1998). "Differential effects of ethanol on memory in adolescent and adult rats." Alcohol Clin Exp Res **22**(2): 416-21.
- Martin, K. C., D. Michael, et al. (1997). "MAP kinase translocates into the nucleus of the presynaptic cell and is required for long-term facilitation in Aplysia." Neuron **18**(6): 899-912.
- McBride, W. J. (2002). "Central nucleus of the amygdala and the effects of alcohol and alcohol-drinking behavior in rodents." Pharmacol Biochem Behav **71**(3): 509-15.
- McCormick, C. M., A. Merrick, et al. (2007). "Social instability in adolescence alters the central and peripheral hypothalamic-pituitary-adrenal responses to a repeated homotypic stressor in male and female rats." J Neuroendocrinol **19**(2): 116-26.
- Meller, E., C. Shen, et al. (2003). "Region-specific effects of acute and repeated restraint stress on the phosphorylation of mitogen-activated protein kinases." Brain Res **979**(1-2): 57-64.
- Merrick, J., I. Kandel, et al. (2004). "Adolescent injury risk behavior." Int J Adolesc Med Health **16**(3): 207-13.
- Monti, P. M., R. Miranda, Jr., et al. (2005). "Adolescence: booze, brains, and behavior." Alcohol Clin Exp Res **29**(2): 207-20.
- Moore, E. M., J. N. Mariani, et al. (2010). "Adolescent C57BL/6J (but not DBA/2J) Mice Consume Greater Amounts of Limited-Access Ethanol Compared to Adults and Display Continued Elevated Ethanol Intake into Adulthood." Alcohol Clin Exp Res.
- Nestler, E. J. (2002). "Common molecular and cellular substrates of addiction and memory." Neurobiol Learn Mem **78**(3): 637-47.

- Oesterle, S., K. G. Hill, et al. (2004). "Adolescent heavy episodic drinking trajectories and health in young adulthood." J Stud Alcohol **65**(2): 204-12.
- Oh, S. W., Y. M. Ahn, et al. (1999). "Differential activation of c-Jun N-terminal protein kinase and p38 in rat hippocampus and cerebellum after electroconvulsive shock." Neurosci Lett **271**(2): 101-4.
- Ohrtmann, J. D., E. K. Stancik, et al. (2006). "Ethanol inhibits brain-derived neurotrophic factor stimulation of extracellular signal-regulated/mitogen-activated protein kinase in cerebellar granule cells." Alcohol **39**(1): 29-37.
- Overstreet, D. H., D. J. Knapp, et al. (2004). "Similar anxiety-like responses in male and female rats exposed to repeated withdrawals from ethanol." Pharmacol Biochem Behav **78**(3): 459-64.
- Palmer, A. A., A. L. Sharpe, et al. (2004). "Corticotropin-releasing factor overexpression decreases ethanol drinking and increases sensitivity to the sedative effects of ethanol." Psychopharmacology (Berl) **176**(3-4): 386-97.
- Pandey, S. C., A. Roy, et al. (2004). "Partial deletion of the cAMP response element-binding protein gene promotes alcohol-drinking behaviors." J Neurosci **24**(21): 5022-30.
- Pandey, S. C., H. Zhang, et al. (2008). "Effector immediate-early gene arc in the amygdala plays a critical role in alcoholism." J Neurosci **28**(10): 2589-600.
- Pascual, M., A. M. Blanco, et al. (2007). "Intermittent ethanol exposure induces inflammatory brain damage and causes long-term behavioural alterations in adolescent rats." Eur J Neurosci **25**(2): 541-50.
- Paus, T., A. Zijdenbos, et al. (1999). "Structural maturation of neural pathways in children and adolescents: in vivo study." Science **283**(5409): 1908-11.
- Pearson, G., F. Robinson, et al. (2001). "Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions." Endocr Rev **22**(2): 153-83.
- Petersen, A. C., B. E. Compas, et al. (1993). "Depression in adolescence." Am Psychol **48**(2): 155-68.
- Philpot, R. M., K. A. Badanich, et al. (2003). "Place conditioning: age-related changes in the rewarding and aversive effects of alcohol." Alcohol Clin Exp Res **27**(4): 593-9.

- Piazza, P. V., V. Deroche, et al. (1993). "Corticosterone in the range of stress-induced levels possesses reinforcing properties: implications for sensation-seeking behaviors." Proc Natl Acad Sci U S A **90**(24): 11738-42.
- Pittenger, C., Y. Y. Huang, et al. (2002). "Reversible inhibition of CREB/ATF transcription factors in region CA1 of the dorsal hippocampus disrupts hippocampus-dependent spatial memory." Neuron **34**(3): 447-62.
- Pohorecky, L. A. (1991). "Stress and alcohol interaction: an update of human research." Alcohol Clin Exp Res **15**(3): 438-59.
- Prakash, A., H. Zhang, et al. (2008). "Innate differences in the expression of brain-derived neurotrophic factor in the regions within the extended amygdala between alcohol preferring and nonpreferring rats." Alcohol Clin Exp Res **32**(6): 909-20.
- Prendergast, M. A. and H. J. Little (2007). "Adolescence, glucocorticoids and alcohol." Pharmacol Biochem Behav **86**(2): 234-45.
- Qi, M. and E. A. Elion (2005). "MAP kinase pathways." J Cell Sci **118**(Pt 16): 3569-72.
- Qi, X., W. Lin, et al. (2008). "Fluoxetine increases the activity of the ERK-CREB signal system and alleviates the depressive-like behavior in rats exposed to chronic forced swim stress." Neurobiol Dis **31**(2): 278-85.
- Qiu, J., P. Wang, et al. (2001). "Rapid activation of ERK1/2 mitogen-activated protein kinase by corticosterone in PC12 cells." Biochem Biophys Res Commun **287**(4): 1017-24.
- Quintanilla, M. E., O. Callejas, et al. (2001). "Differences in sensitivity to the aversive effects of ethanol in low-alcohol drinking (UChA) and high-alcohol drinking (UChB) rats." Alcohol **23**(3): 177-82.
- Radwanska, K., E. Wrobel, et al. (2008). "Alcohol relapse induced by discrete cues activates components of AP-1 transcription factor and ERK pathway in the rat basolateral and central amygdala." Neuropsychopharmacology **33**(8): 1835-46.
- Rakic, P., J. P. Bourgeois, et al. (1994). "Synaptic development of the cerebral cortex: implications for learning, memory, and mental illness." Prog Brain Res **102**: 227-43.
- Refojo, D., C. Echenique, et al. (2005). "Corticotropin-releasing hormone activates ERK1/2 MAPK in specific brain areas." Proc Natl Acad Sci U S A **102**(17): 6183-8.

- Revest, J. M., F. Di Blasi, et al. (2005). "The MAPK pathway and Egr-1 mediate stress-related behavioral effects of glucocorticoids." Nat Neurosci **8**(5): 664-72.
- Rhodes, J. S., K. Best, et al. (2005). "Evaluation of a simple model of ethanol drinking to intoxication in C57BL/6J mice." Physiol Behav **84**(1): 53-63.
- Risinger, F. O. and C. L. Cunningham (1995). "Genetic differences in ethanol-induced conditioned taste aversion after ethanol preexposure." Alcohol **12**(6): 535-9.
- Rivier, C. and S. Lee (2001). "Effect of repeated exposure to alcohol on the response of the hypothalamic-pituitary-adrenal axis of the rat: II. Role of the length and regimen of alcohol treatment." Alcohol Clin Exp Res **25**(1): 106-11.
- Roberto, M., P. Schweitzer, et al. (2004). "Acute and chronic ethanol alter glutamatergic transmission in rat central amygdala: an in vitro and in vivo analysis." J Neurosci **24**(7): 1594-603.
- Robinson, M. J. and M. H. Cobb (1997). "Mitogen-activated protein kinase pathways." Curr Opin Cell Biol **9**(2): 180-6.
- Roivainen, R., B. Hundle, et al. (1995). "Ethanol enhances growth factor activation of mitogen-activated protein kinases by a protein kinase C-dependent mechanism." Proc Natl Acad Sci U S A **92**(6): 1891-5.
- Romeo, R. D., R. Bellani, et al. (2006). "Stress history and pubertal development interact to shape hypothalamic-pituitary-adrenal axis plasticity." Endocrinology **147**(4): 1664-74.
- Romeo, R. D., S. J. Lee, et al. (2004). "Testosterone cannot activate an adult-like stress response in prepubertal male rats." Neuroendocrinology **79**(3): 125-32.
- Rosenberg, D. R. and D. A. Lewis (1994). "Changes in the dopaminergic innervation of monkey prefrontal cortex during late postnatal development: a tyrosine hydroxylase immunohistochemical study." Biol Psychiatry **36**(4): 272-7.
- Roux, P. P. and J. Blenis (2004). "ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions." Microbiol Mol Biol Rev **68**(2): 320-44.
- Rubino, T., G. Forlani, et al. (2005). "Ras/ERK signalling in cannabinoid tolerance: from behaviour to cellular aspects." J Neurochem **93**(4): 984-91.

- Runyan, J. D., A. N. Moore, et al. (2004). "A role for prefrontal cortex in memory storage for trace fear conditioning." J Neurosci **24**(6): 1288-95.
- Ryback, R. S. (1970). "Alcohol amnesia." JAMA **212**(9): 1524.
- Ryback, R. S. (1971). "The continuum and specificity of the effects of alcohol on memory. A review." Q J Stud Alcohol **32**(4): 995-1016.
- Sanna, P. P., C. Simpson, et al. (2002). "ERK regulation in chronic ethanol exposure and withdrawal." Brain Res **948**(1-2): 186-91.
- Sasaguri, K., M. Kikuchi, et al. (2005). "Suppression of stress immobilization-induced phosphorylation of ERK 1/2 by biting in the rat hypothalamic paraventricular nucleus." Neurosci Lett **383**(1-2): 160-4.
- Schafe, G. E., C. M. Atkins, et al. (2000). "Activation of ERK/MAP kinase in the amygdala is required for memory consolidation of pavlovian fear conditioning." J Neurosci **20**(21): 8177-87.
- Schramm, N. L., R. E. Egli, et al. (2002). "LTP in the mouse nucleus accumbens is developmentally regulated." Synapse **45**(4): 213-9.
- Schroeder, J. P., F. Olive, et al. (2003). "Intra-amygdala infusion of the NPY Y1 receptor antagonist BIBP 3226 attenuates operant ethanol self-administration." Alcohol Clin Exp Res **27**(12): 1884-91.
- Schroeder, J. P., D. H. Overstreet, et al. (2005). "The mGluR5 antagonist MPEP decreases operant ethanol self-administration during maintenance and after repeated alcohol deprivations in alcohol-preferring (P) rats." Psychopharmacology (Berl) **179**(1): 262-70.
- Schroeder, J. P., M. Spanos, et al. (2008). "Cue-induced reinstatement of alcohol-seeking behavior is associated with increased ERK1/2 phosphorylation in specific limbic brain regions: blockade by the mGluR5 antagonist MPEP." Neuropharmacology **55**(4): 546-54.
- Schuckit, M. A. (1994). "Low level of response to alcohol as a predictor of future alcoholism." Am J Psychiatry **151**(2): 184-9.

- Schuckit, M. A. and T. L. Smith (2000). "The relationships of a family history of alcohol dependence, a low level of response to alcohol and six domains of life functioning to the development of alcohol use disorders." J Stud Alcohol **61**(6): 827-35.
- Seiffge-Krenke, I., K. Aunola, et al. (2009). "Changes in stress perception and coping during adolescence: the role of situational and personal factors." Child Dev **80**(1): 259-79.
- Seiler, A. E., B. N. Ross, et al. (2001). "Inhibition of insulin-like growth factor-1 receptor and IRS-2 signaling by ethanol in SH-SY5Y neuroblastoma cells." J Neurochem **76**(2): 573-81.
- Sgambato, V., C. Pages, et al. (1998). "Extracellular signal-regulated kinase (ERK) controls immediate early gene induction on corticostriatal stimulation." J Neurosci **18**(21): 8814-25.
- Shen, C. P., Y. Tsimberg, et al. (2004). "Activation of Erk and JNK MAPK pathways by acute swim stress in rat brain regions." BMC Neurosci **5**: 36.
- Siegmund, S., V. Vengeliene, et al. (2005). "Influence of age at drinking onset on long-term ethanol self-administration with deprivation and stress phases." Alcohol Clin Exp Res **29**(7): 1139-45.
- Silveri, M. M. and L. P. Spear (1998). "Decreased sensitivity to the hypnotic effects of ethanol early in ontogeny." Alcohol Clin Exp Res **22**(3): 670-6.
- Silveri, M. M. and L. P. Spear (2004). "Characterizing the ontogeny of ethanol-associated increases in corticosterone." Alcohol **32**(2): 145-55.
- Slawecki, C. J. and M. Betancourt (2002). "Effects of adolescent ethanol exposure on ethanol consumption in adult rats." Alcohol **26**(1): 23-30.
- Smith, G. T., M. S. Goldman, et al. (1995). "Expectancy for social facilitation from drinking: the divergent paths of high-expectancy and low-expectancy adolescents." J Abnorm Psychol **104**(1): 32-40.
- Song, M., X. Y. Wang, et al. (2007). "Role of stress in acquisition of alcohol-conditioned place preference in adolescent and adult mice." Alcohol Clin Exp Res **31**(12): 2001-5.
- Sowell, E. R., B. S. Peterson, et al. (2003). "Mapping cortical change across the human life span." Nat Neurosci **6**(3): 309-15.

- Sowell, E. R., P. M. Thompson, et al. (2001). "Mapping continued brain growth and gray matter density reduction in dorsal frontal cortex: Inverse relationships during postadolescent brain maturation." J Neurosci **21**(22): 8819-29.
- Spear, L. P. (2000). "The adolescent brain and age-related behavioral manifestations." Neurosci Biobehav Rev **24**(4): 417-63.
- Spear, L. P. (2002). "The adolescent brain and the college drinker: biological basis of propensity to use and misuse alcohol." J Stud Alcohol Suppl(14): 71-81.
- Spencer, R. L. and B. S. McEwen (1990). "Adaptation of the hypothalamic-pituitary-adrenal axis to chronic ethanol stress." Neuroendocrinology **52**(5): 481-9.
- Squire, L. R., J. T. Wixted, et al. (2007). "Recognition memory and the medial temporal lobe: a new perspective." Nat Rev Neurosci **8**(11): 872-83.
- Steinberg, L. (2005). "Cognitive and affective development in adolescence." Trends Cogn Sci **9**(2): 69-74.
- Stevenson, J. R., J. P. Schroeder, et al. (2009). "Abstinence following alcohol drinking produces depression-like behavior and reduced hippocampal neurogenesis in mice." Neuropsychopharmacology **34**(5): 1209-22.
- Stone, E. A. and D. Quartermain (1997). "Greater behavioral effects of stress in immature as compared to mature male mice." Physiol Behav **63**(1): 143-5.
- Strong, M. N., N. Yoneyama, et al. (2009). "'Binge' drinking experience in adolescent mice shows sex differences and elevated ethanol intake in adulthood." Horm Behav.
- Swank, M. W. (2000). "Phosphorylation of MAP kinase and CREB in mouse cortex and amygdala during taste aversion learning." Neuroreport **11**(8): 1625-30.
- Sweatt, J. D. (2004). "Mitogen-activated protein kinases in synaptic plasticity and memory." Curr Opin Neurobiol **14**(3): 311-7.
- Tambour, S., L. L. Brown, et al. (2008). "Gender and age at drinking onset affect voluntary alcohol consumption but neither the alcohol deprivation effect nor the response to stress in mice." Alcohol Clin Exp Res **32**(12): 2100-6.

- Tapert, S. F., A. D. Schweinsburg, et al. (2004). "Blood oxygen level dependent response and spatial working memory in adolescents with alcohol use disorders." Alcohol Clin Exp Res **28**(10): 1577-86.
- Tarazi, F. I. and R. J. Baldessarini (2000). "Comparative postnatal development of dopamine D(1), D(2) and D(4) receptors in rat forebrain." Int J Dev Neurosci **18**(1): 29-37.
- Tarazi, F. I., E. C. Tomasini, et al. (1998). "Postnatal development of dopamine and serotonin transporters in rat caudate-putamen and nucleus accumbens septi." Neurosci Lett **254**(1): 21-4.
- Teicher, M. H., S. L. Andersen, et al. (1995). "Evidence for dopamine receptor pruning between adolescence and adulthood in striatum but not nucleus accumbens." Brain Res Dev Brain Res **89**(2): 167-72.
- Toga, A. W., P. M. Thompson, et al. (2006). "Mapping brain maturation." Trends Neurosci **29**(3): 148-59.
- Torres, J. M. and E. Ortega (2003). "Alcohol intoxication increases allopregnanolone levels in female adolescent humans." Neuropsychopharmacology **28**(6): 1207-9.
- Torres, J. M., E. Ruiz, et al. (2001). "Effects of CRH and ACTH administration on plasma and brain neurosteroid levels." Neurochem Res **26**(5): 555-8.
- Trentani, A., S. D. Kuipers, et al. (2002). "Selective chronic stress-induced in vivo ERK1/2 hyperphosphorylation in medial prefrontocortical dendrites: implications for stress-related cortical pathology?" Eur J Neurosci **15**(10): 1681-91.
- Uchida, S., A. Nishida, et al. (2008). "Characterization of the vulnerability to repeated stress in Fischer 344 rats: possible involvement of microRNA-mediated down-regulation of the glucocorticoid receptor." Eur J Neurosci **27**(9): 2250-61.
- Valjent, E., J. C. Corvol, et al. (2000). "Involvement of the extracellular signal-regulated kinase cascade for cocaine-rewarding properties." J Neurosci **20**(23): 8701-9.
- Valjent, E., C. Pages, et al. (2004). "Addictive and non-addictive drugs induce distinct and specific patterns of ERK activation in mouse brain." Eur J Neurosci **19**(7): 1826-36.

- Vanhoutte, P., J. V. Barnier, et al. (1999). "Glutamate induces phosphorylation of Elk-1 and CREB, along with c-fos activation, via an extracellular signal-regulated kinase-dependent pathway in brain slices." Mol Cell Biol **19**(1): 136-46.
- Varlinskaya, E. I. and L. P. Spear (2002). "Acute effects of ethanol on social behavior of adolescent and adult rats: role of familiarity of the test situation." Alcohol Clin Exp Res **26**(10): 1502-11.
- Vetter-O'Hagen, C., E. Varlinskaya, et al. (2009). "Sex differences in ethanol intake and sensitivity to aversive effects during adolescence and adulthood." Alcohol Alcohol **44**(6): 547-54.
- Wagner, E. F., M. G. Myers, et al. (1999). "Stress-coping and temptation-coping as predictors of adolescent substance use." Addict Behav **24**(6): 769-79.
- Walker, C. D., G. Trottier, et al. (1995). "Dissociation between behavioral and hormonal responses to the forced swim stress in lactating rats." J Neuroendocrinol **7**(8): 615-22.
- Walz, R., R. Roesler, et al. (2000). "Time-dependent impairment of inhibitory avoidance retention in rats by posttraining infusion of a mitogen-activated protein kinase kinase inhibitor into cortical and limbic structures." Neurobiol Learn Mem **73**(1): 11-20.
- Wand, G. S. and A. S. Dobs (1991). "Alterations in the hypothalamic-pituitary-adrenal axis in actively drinking alcoholics." J Clin Endocrinol Metab **72**(6): 1290-5.
- Wexler, D. B. and M. C. Moore-Ede (1986). "Resynchronization of circadian sleep-wake and temperature cycles in the squirrel monkey following phase shifts of the environmental light-dark cycle." Aviat Space Environ Med **57**(12 Pt 1): 1144-9.
- White, A. M. (2009). "Understanding adolescent brain development and its implications for the clinician." Adolesc Med State Art Rev **20**(1): 73-90, viii-ix.
- White, A. M., D. C. Roberts, et al. (2002). "Context-specific tolerance to the ataxic effects of alcohol." Pharmacol Biochem Behav **72**(1-2): 107-10.
- White, A. M. and H. S. Swartzwelder (2004). "Hippocampal function during adolescence: a unique target of ethanol effects." Ann N Y Acad Sci **1021**: 206-20.
- White, A. M., M. C. Truesdale, et al. (2002). "Differential effects of ethanol on motor coordination in adolescent and adult rats." Pharmacol Biochem Behav **73**(3): 673-7.

- Wilkie, M. B., J. Besheer, et al. (2007). "Acute ethanol administration rapidly increases phosphorylation of conventional protein kinase C in specific mammalian brain regions in vivo." Alcohol Clin Exp Res **31**(7): 1259-67.
- Wills, T. A., D. J. Knapp, et al. (2009). "Sensitization, duration, and pharmacological blockade of anxiety-like behavior following repeated ethanol withdrawal in adolescent and adult rats." Alcohol Clin Exp Res **33**(3): 455-63.
- Wills, T. A., D. Vaccaro, et al. (1994). "Novelty seeking, risk taking, and related constructs as predictors of adolescent substance use: an application of Cloninger's theory." J Subst Abuse **6**(1): 1-20.
- Winters, B. D., L. M. Saksida, et al. (2008). "Object recognition memory: neurobiological mechanisms of encoding, consolidation and retrieval." Neurosci Biobehav Rev **32**(5): 1055-70.
- Wozniak, J. R. and K. O. Lim (2006). "Advances in white matter imaging: a review of in vivo magnetic resonance methodologies and their applicability to the study of development and aging." Neurosci Biobehav Rev **30**(6): 762-74.
- Yang, C. H., C. C. Huang, et al. (2008). "Differential roles of basolateral and central amygdala on the effects of uncontrollable stress on hippocampal synaptic plasticity." Hippocampus **18**(6): 548-63.
- Yttri, E. A., J. A. Burk, et al. (2004). "Intermittent ethanol exposure in adolescent rats: dose-dependent impairments in trace conditioning." Alcohol Clin Exp Res **28**(10): 1433-6.
- Zecevic, N., J. P. Bourgeois, et al. (1989). "Changes in synaptic density in motor cortex of rhesus monkey during fetal and postnatal life." Brain Res Dev Brain Res **50**(1): 11-32.
- Zehr, J. L., B. J. Todd, et al. (2006). "Dendritic pruning of the medial amygdala during pubertal development of the male Syrian hamster." J Neurobiol **66**(6): 578-90.
- Zeigler, D. W., C. C. Wang, et al. (2005). "The neurocognitive effects of alcohol on adolescents and college students." Prev Med **40**(1): 23-32.
- Zhang, H. T., Y. Zhao, et al. (2004). "Inhibition of the phosphodiesterase 4 (PDE4) enzyme reverses memory deficits produced by infusion of the MEK inhibitor U0126 into the CA1 subregion of the rat hippocampus." Neuropsychopharmacology **29**(8): 1432-9.

Zweben, J. E., H. W. Clark, et al. (1994). "Traumatic experiences and substance abuse: mapping the territory." J Psychoactive Drugs **26**(4): 327-44.