ABSTRACT

PKCγ Expression in Adolescent and Adult Rats: Evidence for a Cerebellar Mechanism Underlying Age-Dependent Motor Impairments Produced by Acute Ethanol

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Adolescents are less sensitive to ethanol-induced motor impairments compared to adults; however, a definitive mechanism underlying this difference has not been identified. Compared to wild-type littermates, PKCγ knock-out mice exhibit reduced motor sensitivity to ethanol; it is plausible that adolescent rats also have reduced PKCγ expression in brain regions responsible for motor function, specifically the cerebellum and cortex. Reduced PKCγ expression in these regions may govern the age-dependent motor impairments produced by ethanol. The current study analyzed membrane-bound PKCγ expression in adolescent and adult rats 40 minutes after an acute ethanol or saline injection. Western blot analysis indicates adolescent rats have reduced PKCγ expression in the cerebellum and cortex compared to adults. It is concluded that PKCγ expression may be part of a larger mechanism regulating the age-dependent motor impairments produced by acute ethanol administration.

PKCγ Expression in Adolescent and Adult Rats: Evidence for a Cerebellar Mechanism Underlying Age-Dependent Motor Impairments Produced by Acute Ethanol

by

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A Thesis

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LIST OF ABBREVIATIONS

ADHD	Attention Deficit Hyperactivity Disorder
AUD	Alcohol Use Disorder
BEC(s)	Blood Ethanol Concentration(s)
$GABA_A$	γ-amino butyric acid, type A
$GABA_AR$	γ-amino butyric acid, type A Receptor
PBS	Phosphate-Buffered Saline
PD	Postnatal Day
PFC	Prefrontal Cortex
PKC	Protein Kinase C
PKCγ	γ isoform of Protein Kinase C
PTSD	Post-Traumatic Stress Disorder
PVDF	Polyvinylidene difluroide
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

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Lastly, I want to express my appreciation to you, the reader, for taking interest in my research.

DEDICATION

To anyone in need of inspiration:

I can do all things through Christ who strengthens me. (Phil. 4:13 NKJV)

CHAPTER ONE

Introduction and Background

Introduction

Alcohol has been used to produce intoxication since times dating before Christ; and it seems as though excessive consumption has historically been cause for concern, as implied in this quote from Socrates, "Bad men live that they may eat and drink, whereas good men eat and drink that they may live."

Adolescence represents a unique period of altered sensitivity to many of ethanol's effects. Compared to adults, adolescents are less sensitive to motor impairments produced by alcohol, which typically serve as cues to moderate ethanol intake (Hefner & Holmes, 2007; Lisenbardt, Moore, Gross, Golfarb, Blackman, & Boehm, 2009; Little, Kuhn, Wilson, & Swartzwelder, 1996; Pian, Criado, Walker, & Ehlers, 2008; Ristuccia & Spear, 2008; Silveri & Spear, 2001; Spear & Varlinskaya, 2005 for review; Varlinskaya & Spear, 2002; White, Truesdale, Bae, Ahmad, Wilson, Best & Swartzwelder, 2002). This reduction in motor sensitivity to ethanol could contribute to the high proportion of binge and heavy alcohol consumption in adolescence and young adulthood relative to moderate drinking (Substance Abuse and Mental Health Services Administration, 2010).

Personality changes that accompany the adolescent time period might also predispose this age group to experiment with alcohol, as adolescence is characterized by increased sensation-seeking and risk-taking behaviors (Arnett, 1996; Spear, 2000; Steinberg, Albert, Cauffman, Banich, Graham, & Woolard, 2008). While these factors may serve to initiate alcohol use, the underlying reduction in sensitivity to ethanol-

induced motor impairments may contribute to the maintenance of excessive alcohol consumption. In fact, binge and heavy alcohol consumption increases throughout human adolescence and young adulthood, peaking at 21-25 years of age (Substance Abuse and Mental Health Services Administration 2010).

Adolescence is a period of continuing brain development (Giedd, 2008; Pfefferbaum, Mathalon, Sullivan, Rawles, Zipursky, & Lim, 1994; Paus, Collins, Evans, Leonard, Pike, & Zijdenbos, 2001; Yu, Wang, Fritschy, Witte, & Redecker, 2006), and since adolescents who consume ethanol are likely to do so in an excessive manner, it is especially important to illuminate the immediate and long-term consequences of heavy alcohol use during these formative years. However, adolescents with alcohol use disorders (AUDs), usually defined as meeting the Diagnostic and Statistical Manual of Mental Disorders criteria for alcohol abuse or dependence, typically engage in poly-substance use, and many present with comorbid psychiatric disorders (Clark, Pollock, Bukstein, Mezzich, Bromberger, & Donovan, 1997), making it difficult to determine the abnormalities produced specifically by alcohol use. For instance, in McQueeny, Schweinsburg, Schweinsburg, Jacobus, Bava, Frank, et al. (2009), the sample size for adolescents aged 16 to 19 that met criteria for binge alcohol consumption was only 14. This sample was limited by excluding participants with a history of psychiatric disorders and/or a history of alcohol or other drug abuse or dependence. Alternatively, De Bellis, Clark, Beers, Soloff, Boring, Hall, Kersh, et al. (2000) did not employ such exclusion criteria, but instead reported cormorbid conditions like other substance dependence or abuse, and disorders of: depression, bipolar, generalized anxiety, conduct, attention deficit/hyperactivity, and oppositional defiance.

Adolescents cannot be recruited to the experimental laboratory and given alcohol, regardless of their history with alcohol or other drugs. Despite the potential scientific benefits of doing so, ethical issues deny us the opportunity because all of the experimental evidence indicates that alcohol use during adolescence is detrimental. For instance, alcohol exposure during the adolescent developmental period may produce irreversible abnormalities in neural function and brain structure (Pascual, Blanco, Cauli, Minarro, & Guerri, 2007) which can persist to alter ethanol preference and consumption in adulthood (Diaz-Granados & Graham, 2007; Pascual, Boix, Felipo, & Guerri, 2009). In addition, the legal issues regarding the provision of alcohol to minors as well as the consumption of alcohol as a minor further prohibits the conduction of such experiments. For these moral, ethical, and legal reasons, we need a reasonable approximation of adolescence in a non-human model.

To this end, many scientists have relied on rodent models to bridge this gap because they undergo rapid development, with adolescence in the rat spanning postnatal day (PD) 28 to 42. These are the days surrounding the time of sexual maturation, during which physiological and behavioral markers of adolescence are present (Spear, 2007; Spear & Brake, 1983). However, adolescence is an ill-defined period of transition, and the exact dates encompassing this developmental period in the rat are disputable, as changes have been reported to last until PD 55 in male rats (Odell, 1990).

While rats and humans are quite distinct in terms of morphology, many parallels can be drawn between the behavior and physiology of both species, especially as it relates to adolescence and alcohol use. For example, adolescent rodents exhibit more risk-taking behaviors than adult rodents (Macri, Adriani, Chiarotti, & Laviola, 2002).

Parallel ethanol consumption behaviors are also found in rodent models: adolescent rats and mice self-administer greater quantities of ethanol than adults (Doremus, Brunell, Rajendran, & Spear, 2005; Walker, Walker, & Ehlers, 2008), consuming 2-3 times more ethanol relative to body weight (Lancaster, Brown, Coker, Elliott, & Wren, 1996). Importantly, adolescent rats also demonstrate reduced sensitivity to ethanol-induced motor impairments compared to adults (Hefner & Holmes, 2007; Pian et al., 2008; Ristuccia & Spear, 2008; White et al., 2002), which is the primary focus of investigation in the present set of studies. Therefore, the rat appears to be an acceptable approximation to aid in determining potential factors that underlie the reduced motor sensitivity to ethanol during adolescence.

Our research objective in the present set of studies is to find a mechanism that underlies the age-dependent disturbances in motor coordination produced by ethanol. Protein kinase Cγ (PKCγ) knock-out mice exhibit reduced sensitivity to ethanol-induced motor impairments compared to their wild-type littermates (Bowers, Elliott, & Wehner, 2001; Bowers, Owen, Collins, Abeliovich, Tonegawa, & Wehner, 1999; Harris, McQuilkin, Paylor, Abeliovich, Tonegawa, & Wehner, 1995). This reduced motor sensitivity is similar to the reduced sensitivity of adolescents when compared to adults. However, the similarities between PKCγ knockouts and adolescents extend beyond ethanol responsiveness: both exhibit increased risk-taking behavior (Crone, Bullens, van der Plas, Kijkuit, & Zelazo, 2008; Macri et al., 2002; Spear, 2000) and voluntarily consume more ethanol per unit body weight than wild-types and adults, respectively (Doremus et al., 2005; Lancaster et al., 1996; Walker et al., 2008). Therefore, reduced

PKCγ expression may be a viable mechanism underlying the adolescent's reduced motor sensitivity to ethanol compared to their adult counterparts.

Additionally, PKCγ is distributed only within the neurons of the central nervous system, making it a prime contender for aiding in the modulation of the neurobiological effects of alcohol use. Specific to the brain regions of interest, PKCγ is abundantly expressed in the dendrites of cerebellar Purkinje neurons (Hashimoto et al., 1988; Saito, Kikkawa, Nishizuka, & Tanaka, 1988) that constitute the sole output of the cerebellum (Cesa & Strata, 2009). Since the mid-1960s, the cerebellum has been known to modulate activity in the motor cortex (Li & Tew, 1966) and its theorized purpose was to learn motor skills to be retrieved by a simple signal from the cortex (Brindley, 1964). The cerebellum also plays a role in timing (Arshavsky, Gelfand, & Orlovsky, 1983; Braitenberg, 1967) and synchrony (Heck, Thach, & Keating, 2007), both of which are important for motor coordination and precision. Therefore, our hypothesis is that adolescent rats, which are also less sensitive to motor impairments produced by ethanol when compared to adult rats, will also have reduced PKCγ expression in the cerebellum, a brain region concerned with movement. It is plausible that age-dependent differences in cerebellar PKCγ expression may account for differential motor impairments produced by ethanol, especially since PKCγ expression is abundant in Purkinje neurons (Hashimoto et al., 1988; Saito, et al., 1988), which serve as the sole efferent projection from the cerebellum (Cesa & Strata, 2009).

Another region highly involved in motor behavior is the cortex, specifically the motor and premotor cortices, responsible for initiating movement (Brown & Sherrington, 1911; Levy, York, McCaffrey, & Tanzer, 1984; for historical reference, see Fritsch &

Hitzig, 1870). Within the motor and premotor corticies of the rhesus macaque, the majority of PKCγ immunoreactivity occurs in the pyramidal cells of layers II, III, and VI, and within the neuropil of layers I and II (Tominaga, Saito, Tsujino, & Tanaka, 1993). The presence of PKCγ within the motor and premotor corticies indicates that alterations in PKCγ expression levels may facilitate the understanding of age-dependent motor impairments produced by ethanol.

Pyramidal cells of the hippocampus also have abundant PKCγ expression (Saito, Kikkawa, Nishizuka, & Tanaka, 1988), which has been implicated in modulating long-term potentiation, a synaptic model of memory (Bliss & Collingridge, 1993). In the hippocampus, PKCγ and its modulation of long-term potentiation aid in spatial memory. Indeed, PKCγ knock-out mice have altered hippocampal long-term potentiation (Abeliovich, Chen, Goda, Silva, Stevens, & Tonegawa, 1993), and exhibit mild learning deficits on spatial tasks (Abeliovich, Paylor, Chen, Kim, Wehner, & Tonegawa, 1993).

Ethanol impairs spatial memory (Acheson, Ross, & Swartzwelder, 2001; Berry & Matthews, 2004; Matthews, Simson, & Best, 1995); however, the relative sensitivities of adolescents and adults to ethanol-induced spatial memory insults are currently in debate. For more than a decade, adolescents (Markwiese, Acheson, Levin, Wilson, & Swartzwelder, 1998) and young adults (Acheson, Stein, & Swartzwelder, 1998) were thought to have a heightened sensitivity to the hippocampal-dependent memory impairments produced by ethanol compared to adults. However, others have found no age-related differences in spatial memory impairment using an appetitive sand-digging task (Rajendran & Spear, 2004), and the Morris water maze (Chin, Berry, & Matthews, 2009; Chin, Diaz-Granados, & Matthews, 2010a), along with other hippocampal-

dependent learning tasks, like contextual fear conditioning (Land & Spear, 2004). Our lab is among the producers of the data supporting the latter notion (Chin et al., 2009; Chin et al., 2010a); therefore, we expect to find similar levels of PKCγ expression in the hippocampus of adolescent and adult rats.

PKCγ knock-out mice show reduced hypnotic sensitivity to ethanol compared to wild-type littermates (Harris, McQuilkin, Paylor, Abeliovich, Tonegawa, & Wehner, 1995), which is similar to the comparison between adolescent and adult rats, with adolescents exhibiting less sensitivity to ethanol's motor impairing effects (Hefner & Holmes, 2007; Lisenbardt et al., 2009; Little et al., 1996; Pian et al., 2008; Ristuccia & Spear, 2008; Silveri & Spear, 2001; Varlinskaya & Spear, 2002; White et al., 2002). PKCγ phosphorylates the $GABA_AR$ (Kellenberger et al., 1992; Krishek et al., 1994; Qi et al., 2007; Song & Messing, 2005; Wafford, et al., 1991) , which is one of the molecular targets that confers some effects of ethanol (Aguayo, Peoples, Yeh, & Yevenes, 2002; Weiss, 1992). In the presence of ethanol, PKCγ phosphorylation enhances $GABA_AR$ activity (Kumar, Khisti, & Morrow, 2005). Accordingly, reduced PKCγ expression in brain regions associated with motor control, specifically the cerebellum and cortex, may be a viable mechanism underlying adolescent's reduced sensitivity to ethanol-induced motor impairments compared to adults.

Adolescence

Adolescence is a period of ongoing development, marked by physiological and neurobiological changes, as well as changes in personality and emotionality, which typically occurs between the ages of 8 to 20 in humans (Dahl, 2004). Conceptually, adolescence is a transitional period between childhood and adulthood, beginning with

sexual maturation and ending upon achievement of adult roles and responsibilities (Dahl & Spear, 2004). Adolescence is typically a tumultuous psychological transition as well. Personality changes include an increase in risk-taking (Spear, 2000), sensation-seeking (Steinberg et al., 2008), and reckless behaviors (Arnett, 1996). Furthermore, physiological maturation is driven by hormonal changes, and the negative emotional states that plague many adolescents have been shown to correlate with the levels of certain hormones (Archibald, Graber, & Brooks-Gunn, 2006). For instance, the health paradox of the adolescent manifests as exceptional physical health, with an increased rate of psychopathological disorders, like depression and conduct disorder (Dahl, 2004; Loeber & Keenan, 1994). This difficulty in controlling behavior and emotion contributes to an adolescent-specific doubling of morbidity and mortality rate, via increased rates of accidents, depression, suicide, substance abuse, and other reckless behaviors (Dahl, 2004). Despite the increased risk of disability and death, sensation-seeking and risk-taking behaviors during adolescence have been hypothesized to serve an adaptive function, permitting the adolescent to become better prepared for adulthood via increased exploration and new learning opportunities. Indeed, sensation-seeking during adolescence may be beneficial, as many species show an increase in risk-taking behavior during this period (Crone et al., 2008; Macri et al., 2002).

The most obvious change during adolescence is the increase in body size and development of secondary sex characteristics, both of which involve an increase and redistribution of muscle and adipose tissues. The process of physiological maturation begins early in the adolescent years, between 6-8 years of age, with an increase of androgen secretion from the adrenal, continuing throughout puberty. Sexual maturation

is initiated by gonadotropin-releasing hormone that stimulates the gonadotroph cells of the hypothalamus, which induces secretion of follicule-stimulating hormone and leutinizing hormone. The levels of these gonadotropins increase steadily from childhood to adulthood , to stimulate gonadal development in a gender-specific manner (Burr, Sizonenko, Kaplan, & Grumback, 1970; Neely, Hintz, Wilson, Lee, Gautier, Argente, et al. 1995; Sizonenko, Burr, Kaplan, & Grumback, 1970; Winter & Faiman, 1972).

Brain development, while not as obvious as the physical alterations, is pervasive during adolescence. However, brain development during this period is linear, indicating that adolescence should be considered a continued period of brain development (Durston et al., 2001; Giedd et al., 1999). Within the cerebrum, volume peaks between ages 11 and 14 (Lenroot, Gogtay, Greenstein, Wells, Wallace, Clasen et al., 2007), although the human brain reaches 90% of its adult weight by age 6 (Casey, Galvan, & Hare, 2005). Despite the minimal increase in volume after childhood, many other neurobiological changes are continuing to occur. Specifically, the ratio of white mater to gray matter increases globally and regionally throughout childhood and adolescence (Giedd, 2008; Paus et al., 2001 for review). Global corticial white matter volume shows a linear increase from age 4 to 22 (Pfefferbaum et al., 1994). In contrast, the decrease in cortical gray matter volume resembles an inverted U-shaped curve that is region-specific (Giedd, Blumenthal, Jeffries, Castellanos, Liu, & Zijdenbos et al., 1999). These regressive maturational changes in gray matter volume during adolescence correlate with behavioral performance: in general, regions involving primary sensory and motor functions are the first to mature, followed by higher-order association areas (Casey, Getz, & Galvan, 2008;

Durston, Hulshoff Pol, Casey, Giedd, Buitelaar, & van Engeland, 2001; Gogtay, Giedd, Lusk, Hayashi, Greenstein, Vaituzis et al., 2004).

Neurobiological changes are also occurring in the hippocampus: Hippocampal volume increases linearly from ages 4 to 18 at a rate of between 0.07 and 0.10 mL per year (Giedd, Castellanos, Rajapakse, Vaituzis, & Rapoport, 1997), although there may be a reduction in hippocampal volume around age 13 (Utsunomiya, Takano, Okazaki, & Mitsudome, 1999). Myelination of white matter fasciculi associated with the hippocampus, the fimbria and alveus, reaches its maximum during late childhood and early adolescence (Utsunomiya et al., 1999). Additionally, the subicular and presubicular regions, which serve as a relay between the hippocampus and cortex, continues to myelinate through adolescence and adulthood (Benes, Turtle, Khan, & Farol, 1994).

The cerebellum, responsible for learning motor skills (Brindley, 1964), timing (Arashavsky et al., 1983; Braitenberg, 1967) and synchrony (Heck, 2007) of motor functions, and modulating the activity in the motor cortex (Li & Tew, 1966), develops during adolescence and follows an inverted U shaped pattern (Tiemeier, Lenroot, Greenstein, Tran, Pierson, & Giedd, 2010). Longitudinal MRI analysis of cerebellar volumes from ages 5 to 24 indicates peak volume is attained between approximately 12 and 15 years of age. Peak volume is reached later in the cerebellum than in the cerebrum, indicating these two brain regions have different developmental trajectories (Tiemeier, et al., 2010). Interestingly, the cerebellum may show comparatively enhanced vulnerabilities to environmental insults compared to other brain regions since Purkinje cell neurogenesis occurs postnatally (Welsh, Yuen, Placantonakis, Vu, Haiss, O'Hearn, 2002) and the cerebellum is among the last brain regions to reach peak volume (Lenroot

et al., 2005; Tiemeier, et al., 2010). For instance, premature birth before 33 weeks gestation is associated with decreased cerebellum volume, full-scale IQ, and self-reported well being (Parker, Mitchell, Kalpakidu, Walshe, Jung, Nosarti, et al., 2008).

On the cellular level, volumetric changes and alterations in white to gray matter ratios are likely due to an overproduction of axons and synapses in late childhood or early adolescence, followed by pruning of excess neurons (Sowell, Thompson, Leonard, Welcome, Kan, & Toga, 2004). These changes likely result from the reorganization and refinement of connections between neurons and glial cells (Fields & Stevens-Graham, 2002) that partially produces a decrease in gray matter volume. Neural pruning is highly dependent on experience (Sowell et al., 2004), which makes the adolescent brain exceptionally malleable, capable of adapting to a wide array of environmental demands. In contrast, myelination of axons results in increases in white matter and functions to accelerate the processing of information within the brain (Sowell, Delis, Stiles, & Jernigan, 2001).

At the level of the receptor, many changes are occurring throughout adolescence in terms of function, structure, and receptor subunit distribution. For instance, during periadolescence, receptors are overproduced for a variety of neurotransmitter systems, including dopamine, serotonin, acetylcholine, and GABA, followed by pruning and elimination (Andersen, Thompson, Krenzel, & Teicher, 2002; Lidow, Goldman-Rakic, & Rakic, 1991; Lidow & Rakic, 1992). Ethanol potentiates $GABA_AR$-mediated chloride ion current (Aguayo, 1990; Reynolds, Prasad, & MacDonald, 1992), and is responsible for producing some of ethanol's effects (reviewed in Aguayo, Peoples, Yeh, & Yevenes, 2002; and Weiss, 1992). Animal models indicate that $GABA_AR$ subunits exhibit

different peptide and mRNA expression patterns based on age and brain region (Fritschy & Mohler, 1995; Fritschy, Paysan, Enna, & Mohler, 1994; Laurie, Wisden, & Seeburg, 1992; Wisden, Laurie, Monyer, & Seeburg, 1992). Specific to the adolescent developmental period, $GABA_A$ receptor subunit α1, α2, α3, and γ2 peptide expression in rat cortex decreases from high levels at PD 30 to moderate levels at PD 60 (Yu et al., 2006), which corresponds to the adolescent developmental period in rats (Spear, 2000). In the hippocampus, $GABA_A$ α1 peptide expression increases during adolescence, reaching adult expression levels at PD 90 (Yu et al., 2006). In postmortem human brain tissue, changes in expression patterns of $GABA_A$ γ2 and α1 peptides mirror the changes found in rat tissue. Specifically, between 12 and 46-75 years, $GABA_A$ γ2 expression decreases in the CA4 region of the hippocampus, while $GABA_A$ α1 expression increases in the CA1 and CA4 during the same time period (Kanaumi, Takashima, Iwasaki, Mitsudome, & Hirose, 2006). Within the human cerebellum, the number of $GABA_A$ receptors increases threefold from the age of 9 months to adulthood (Brooks-Kayal & Pritchett, 1993).

It is apparent that adolescence is a unique and important developmental time period requiring specific investigation: The physiology and neurobiology of the adolescent is rife with developmental alterations that may contribute to differential motor responsiveness after acute ethanol exposure. Ethanol metabolism does not differ between adolescents and adults (Silveri & Spear, 2000), making it especially important to consider the age-dependent alterations at the sites of action of ethanol, including the $GABA_AR$. Ethanol confers some of its effects through allosteric modulation of the $GABA_AR$ (Aguayo, Peoples, Yeh, & Yevenes, 2002; Weiss, 1992), which can be modulated by

PKCγ phosphorylation of the receptor (Kumar et al., 2005; Song & Messing, 2005). $GABA_AR$ subunit distribution changes throughout development (Bovolin, Santi, Memo, Costa, & Grayson, 1992; Brooks-Kayal & Pritchett, 1993; Gambarana, Pittman, & Siegal, 1990; Gutierrez, Khan, Miralles, Mehta, Ruano, Araujo, Vitorica, & De Blas, 1997; Henschel, Gipson, & Bordey, 2008; Laurie et al., 1992) and PKCγ expression is age-dependent in certain brain regions (Van Skike et al., 2010), which suggests the age-dependent effects of ethanol may be of neurobiological origin. The developmental alterations of $GABA_AR$ subunit composition and PKCγ expression in brain regions controlling motor behavior is especially important given the specific focus on the age-dependent differences in motor responsiveness to acute ethanol exposure. Although humans rarely consume only one alcoholic unit during their lifetime, a single isolated exposure can lay the behavioral foundation for future use, especially if this exposure occurs early, as evidenced by the prevalence of alcohol use and addiction that peaks during young adulthood, ages 18-25 (Substance Abuse and Mental Health Services Administration, 2009; 2010).

Ethanol

Ethanol is a widely used substance; in fact, about half of the adult population, 18 years and older, are currently regular consumers of alcohol, with an additional 14 percent engaging in infrequent consumption (Pleis, Lucas, & Ward, 2009). The prevalence and pattern of underage drinking is of immense concern: 16 percent of adolescents between the ages of 12 and 17 had their first alcoholic drink before age 13, with a lifetime use rate of 39 percent in this age group (Fryar, Merino, Hirsch, & Porter, 2009). Alcohol use begins as early as age 12, with 3.4 percent of 12- and 13- year-olds reporting past month

alcohol use. Among 14- and 15-year-olds this percentage rises to 13.1, doubles among 16- and 17-year-olds, reaching 48.7 percent with young adults aged 18 to 20, and peaks at 69.5 percent in adults 21 to 25 years of age (Substance Abuse and Mental Health Services Administration 2010). It is of pressing concern that over half of the past month alcohol consumption is accounted for by binge or heavy drinking in each of these age groups. In this report, binge drinking was defined as 5 or more drinks per one occasion during the past month; whereas heavy drinking was defined as binge drinking on 5 or more days during the past month. In fact, binge and heavy alcohol consumption accounts for over half of all alcohol use until ages 30 to 34; the 35- to 39-year-old age group was the first to report less binge and heavy alcohol use than current (non-binge and non-heavy) use (Substance Abuse and Mental Health Services Administration 2010). However, these distinctions were made irrespective of gender, which likely translates into an underrepresentation of binge drinking among females. The National Institute of Alcoholism and Alcohol Abuse suggests that binge drinking among females should be reduced to 4 or more drinks (National Institute of Alcoholism and Alcohol Abuse, 2004). Indeed, certain aspects of the pharmacokinetic properties of alcohol are gender-dependent. For instance, non-alcoholic women have an increased bioavailability of ingested alcohol due to reduced alcohol dehydrogenase activity in the gastrointestinal tract compared to men (Frezza, di Padova, Pozzato, Terpin, Baraona, & Lieber, 1990). Thus, upon first-pass metabolism in non-alcoholic women, less ethanol is metabolized, allowing more to enter the bloodstream. Additionally, women have less total body water compared to men, even after correcting for height and weight (Watson, Watson, & Batt,

1980), which allows the fully miscible ethanol to be more concentrated in the total body water of women.

With consumption patterns favoring binge and heavy alcohol use until the late 30s, there is no shortage of adults with alcoholism, alcohol use disorders, and other problematic drinking behaviors. In fact, prolonged ethanol consumption is associated with considerable neurological deficits of function and morphology. In general, adults with alcoholism have significant volumetric deficits in cortical and subcortical brain structures, including white and gray matter atrophy, which can occur independently of malnutrition (Crews & Nixon, 2009; Kubota, Nakazaki, Hirai, Saeki, Yamaura, & Kusaka, 2001; Pfefferbaum, Sullivan, Mathalon, Shear, Rosenbloom, & Lim, 1995). The frontal lobes are potentially the most insulted region by chronic ethanol consumption. Indeed, deficits of the frontal lobe, which regulates impulsivity, judgment, planning, and motivation, among many other things, may underlie the troublesome consumption patterns present in alcoholism (Crews & Boettiger, 2009).

Additionally, the hippocampus is especially sensitive to ethanol-induced insults (Chin et al., 2010b, for review), such that ethanol can be used to produce reversible functional disruptions that mimic hippocampal lesions (reviewed in Matthews & Silvers, 2004). For instance, alcohol consumption results in reduced hippocampal neurogenesis (Taffe, Kotzebue, Crean, Crawford, Edwards, & Mandyam, 2010) and volume loss (Beresford, Arciniegas, Alfers, Clapp, Martin, & Du, et al., 2006; Wilhelm, Frieling, Hillemacher, Degner, Kornhuber, & Bleich, 2008). Bilateral deficits in hippocampal volume produced by alcohol can also be found in adolescents (De Bellis, Clark, Beers, Soloff, Boring, Hall, Kersh, & Keshavan, 2000), indicating that insults to the

hippocampus can be produced with a relatively short duration of exposure. Indeed, cognitive disruptions produced by ethanol during adolescence can be long-lasting (Slawecki, Betancourt, Cole, & Ehlers, 2001), and can be observed with prenatal (Barbaccia, Scaccianoce, Del Bianco, Campolongo, Trezza, Tattoli, et al., 2007; Matthews & Simson, 1998) or early postnatal (Goodlett & Johnson, 1997) ethanol exposure.

Unlike the hippocampus, prolonged heavy alcohol use may be required to produce significant cerebellar changes, as some studies have reported cerebellar deficits only in alcohol-using patients with clinical manifestations of Wernicke's encephalopathy (Baker, Harding, Halliday, Krill, & Harper, 1999) or long-term (20-30 years) moderate daily ethanol consumption (Karhune, Erkinjuntti, & Laippala, 1994). Other cerebellar deficits produced by chronic ethanol consumption include a global reduction in Purkinje cell volume (Andersen, 2004) and region-specific volumetric deficits (Sullivan, Rohlfing, & Pfefferbaum, 2010). Within the cerebellar vermis of individuals with alcoholism, there is a rather drastic reduction of Purkinje cells, and shrinkage of the molecular and granular cell layers (Phillips, Harper, & Kril, 1987). However, disruptions in fine motor coordination that are consistent with symptoms of cerebellar dysfunction can be seen during acute intoxication on a handwriting task (Phillips, Ogeil, & Müller, 2009). This suggests that even though prolonged ethanol consumption might be required to produce noticeable cellular or volumetric deficits within the cerebellum, there are also cerebellar disruptions that occur during individual periods of acute intoxication.

The vast majority of the previously cited research was conducted in an adult population; however, physiology and neurobiology of the adolescent is different from the

adult, so alcohol produces some unique effects on the developing adolescent brain and must be considered separately. At the cellular level, adolescent and adults show similar volumetric and neuronal deficits produced by chronic ethanol exposure. For example, MRI studies of adolescents with AUDs reveal bilateral deficits in hippocampal volume compared to healthy matched controls (De Bellis et al., 2000); however, adolescent-onsent AUDs are typically comorbid with other disorders, including poly-substance abuse and/or dependence, mood and conduct disorders, ADHD, and PTSD (Clark et al., 1997). Although adolescents with AUDs, free from other psychiatric cormorbidities, have reduced left hippocampal volumes compared to controls (Nagel, Schweinsburg, Phan, & Tapert, 2005). Additionally, adolescents with AUDs without cormorbid mood or attention disorders have gender-specific prefrontal cortex (PFC) volume reductions: Adolescent females with AUDs had significantly smaller PFC volumes than control females, while adolescent males with AUDs had significantly enlarged PFC volumes compared to same-gender controls (Medina, McQueeny, Nagel, Hanson, Schweinsburg, & Tapert, 2008). However, studies of adolescents with AUDs that are free from comorbid disorders are comparatively rare. Other legal and ethical considerations, such as supplying alcohol to minors, permitting alcohol use in an underage participant, and withholding treatment from an adolescent with an AUD, prohibit laboratory-controlled investigations during single episodes of intoxication in an adolescent population. For these reasons, animal models of alcohol use have been used as a reasonable facsimile for studying alcohol use during adolescence. Additionally, animal models allow a unique opportunity to investigate structural, cellular, and molecular alterations that are specific to alcohol use in any age group of interest.

Although the complexity of the human brain and behavior can never be fully approximated with non-human animals, there are still numerous similarities between human adolescents and adolescent rodents. Behaviorally, adolescent rats and mice show increased risk-taking behavior, coupled with low levels of harm avoidance (Crone et al., 2008; Macri et al., 2002), which approximates the human adolescent-specific increase in risk-taking (Spear, 2000; Steinberg et al., 2008). The rat brain, like that of the human, continues to develop throughout adolescence, especially in terms of $GABA_AR$ subunit distribution and composition (Yu et al., 2006). With regard to ethanol, adolescent rats are quite similar to their human counterparts. For example, compared to adult rats, adolescents are less sensitive to ethanol-induced motor impairment (Hefner & Holmes, 2007; Lisenbardt, 2009; Little et al., 1996; Pian et al., 2008; Ristuccia & Spear, 2008; Silveri & Spear, 2001; Spear & Varlinskaya, 2005 for review; Van Skike, Botta, Chin, Tokunaga, McDaniel, Venard, et al., 2010; Varlinskaya & Spear, 2002; White et al., 2002). The adolescent's reduced sensitivity to ethanol-induced motor impairments, coupled with the propensity for risk-taking, may predispose adolescent rats to more readily consume ethanol: adolescent rats will self-administer ethanol in greater quantities than adults (Doremus et al., 2005; Walker et al., 2008), consuming up to 2 to 3 times more ethanol relative to body weight (Lancaster et al., 1996).

There is universal agreement on the relative insensitivity of adolescents to the motor-impairing effects of ethanol compared to adults. However, the mechanism responsible for producing these age-dependent effects has proven to be rather elusive. For example, metabolic differences do not account for these effects; in fact, the ethanol elimination rate of adolescent and adult rats is not significantly different (Silveri & Spear,

2000). Blood ethanol concentration (BEC) levels and their resulting motor effect between the two ages are almost counterintuitive. Specifically, 20 minutes after an intraperitoneal administration of 2.0 g/kg 10% w/v ethanol, the BEC level of the adolescent rat is nearly double that of the adult; despite this, the adolescent rat performs significantly better on the aerial righting reflex task at this time point than the adult (Van Skike et al., 2010). Clearly, ethanol metabolism and differing BEC levels do not contribute to the reduced ethanol-induced motor sensitivity of the adolescent. $GABA_A$ receptors are one of the molecular targets through which ethanol produces some of its intoxicating effects (Aguayo, Peoples, Yeh, & Yevenes, 2002; Weiss, 1992), including motor impairment via tonic inhibition of cerebellar granule cells (Hanchar, Dodson, Olsen, Otis, & Wallner, 2005). Therefore, differential alteration of $GABA_A$R-related function in the presence of ethanol is likely to produce viable insight into age-dependent motor impairments produced by ethanol.

$GABA_A$ Receptors and Acute Ethanol

$GABA_A$ receptors ($GABA_A$Rs) are ionotropic ligand-gated chloride ion channels responsible for mediating the majority of fast synaptic inhibition in the adult central nervous system (Mehta & Ticku, 1999). γ-aminobutyric acid (GABA) is the endogenous ligand, which binds at the interface of the α and β subunits to open the intrinsic anion channel (Mehta & Ticku, 1999; Zezula, Slany, & Sieghart, 1996). Human $GABA_A$Rs are heteromeric pentamers formed from 19 subunits, including α_1 to α_6, β_1 to β_3, γ_1 to γ_3, δ, ε, π, θ, and ρ_1 to ρ_3 (Simon, Wakimoto, Fujita, Lalande, & Barnard, 2004). However, the number of functional subunit combinations is limited by the region-specific distribution of receptor subunits (Hedblom & Kirkness, 1997; Pirker et al., 2000; Whiting et al.,

1997); additionally, fully functional receptors are formed preferentially from variations of α, β, and γ subunits (Fritschy, Benke, Mertens, Oertel, Bachi, & Mohler, 1992), with the most abundant combination of α1β2γ2 (Song & Messing, 2005), reported to comprise 60% of all $GABA_A$Rs (Mohler, 2006).

$GABA_A$R subunit distribution changes throughout development, which further highlights the need to investigate the GABAergic mechanisms of ethanol exposure during adolescence, since receptor subunit composition plays a prominent role in determining the pharmacological properties of the receptor (Helms, Rogers, & Grant, 2009; Sieghart, 1995; Thompson, Whiting, & Wafford, 1996). Levels of α_1, α_6, β_2, γ_2, and δ mRNA increase during development in the rat; while α_5, β_1, β_3, γ_1, and γ_3 peak early in ontogeny followed by a decline during subsequent development (Bovolin et al., 1992; Gambarana et al., 1990; Laurie et al., 1992). Additionally, postmortem analysis of human $GABA_A$ α_1 mRNA levels suggests that human cortex has relatively stable α_1 mRNA levels from 36 weeks to 50 weeks of development and nearly doubles during development into adulthood. In the cerebellum $GABA_A$ α_1 mRNA levels increase relatively linearly throughout development and into adulthood (Brooks-Kayal & Pritchett, 1993). However, developmental changes in receptor subunit distribution are brain region-specific. Much research has been done in rodents, which can help to illuminate the pattern of region-specific receptor subunit distributions throughout the lifespan. For instance, $GABA_A$ α_6 subunits, which are only present in the cerebellum, increase with age; while cerebellar expression levels of α_1, γ_2, β_2, β_3, and δ decrease throughout the lifespan (Gutierrez et al., 1997).

Not only does subunit distribution and receptor composition change throughout development, the number of $GABA_A$Rs also increases. Interestingly, functional $GABA_A$Rs are present in human embryos as early as 7 weeks after conception followed by a rapid increase between 8 and 11 weeks of prenatal development (Hebebrand et al., 1988). Furthermore, the human cerebellum shows a threefold increase in $GABA_A$Rs from birth to adulthood (Brooks-Kayal & Pritchett, 1993). These alterations in $GABA_A$R composition and quantity during development further emphasize the importance of investigating adolescence as a unique time period, since the adolescent brain differs from the adult in terms of $GABA_A$R quantity, subunit composition, and subunit distribution (Henschel et al., 2008).

The $GABA_A$R is a macromolecular complex that serves as the mechanism of action for many different substrates because it possesses five distinct binding sites for GABA, benzodiazepines, barbiturates, picrotoxin, and neurosteroids (Nestler, Hyman, & Malenka, 2009; Olsen & DeLorey, 1999). Although the $GABA_A$R does not possess a specific binding site for ethanol, it is an accepted mechanism of action for some of ethanol's acute and chronic effects. Indeed, many behavioral effects of ethanol are enhanced by $GABA_A$R agonists and diminished by $GABA_A$R antagonists or inverse agonists (Harris, 1990; Liljequist & Engel, 1982; Lister & Linnoila, 1991). The mechanism underlying the potentiation of GABAergic activity in response to acute ethanol administration is rather complex: It is dependent on the dose and brain region of interest (Grobin, Matthews, Devaud, & Morrow, 1998; Kumar, Porcu, Werner, Matthews, Diaz-Granados, Helfand, et al., 2009; VanDoren, Matthews, Janis, Grobin, Devaud, & Morrow, 2000) and often involves both pre- and post- synaptic mechanisms

(Carta, Ariwodola, Weiner, & Valenzuela, 2003; Carta, Mameli, & Valenzuela, 2004; Sanna, Talani, Busonero, Pisu, Purdy, Serra, & Biggio, 2004; Van Skike et al., 2010; Weiner, Ariwodola, Bates, Bryant, Silberman, & Daunais et al., 2005).

For example, ethanol can directly facilitate presynaptic GABA release and/or increase interneuronal excitability as indicated by *in vitro* electrophysiological recordings with slices from the hippocampus (Carta et al., 2003; Sanna et al., 2004; Weiner et al., 2005) and cerebellum (Carta et al, 2004; Van Skike et al., 2010). Postsynaptic effects of ethanol include a biphasic mIPSC amplitude increase in CA1 pyramidal neurons. During the initial ethanol application mIPSC amplitude is increased and is partially reversed following 10 minutes of continuous ethanol exposure, but after 30 minutes of continuous exposure there is another increase in mIPSC amplitude and decay is prolonged (Sanna et al., 2004). However, many of ethanol's actions at the $GABA_AR$ are indirect and brain-region specific. For instance, the aforementioned biphasic effect is produced indirectly through an increase in the local biosynthesis of neuroactive steroids, specifically 3α-hydroxy-5α-pregnan-20-one, or allopregnanolone (Sanna et al., 2004).

Neurosteroids are not the sole mechanism responsible for the indirect actions of ethanol at the $GABA_AR$: protein kinases can modulate the ethanol-induced potentiation of GABAergic activity (Weiner, Zhang, & Carlen, 1994) and also play a role in determining sensitivity to ethanol (Bowers et al., 2001; Bowers et al., 1999; Harris et al., 1995; Hodge, Mehmert, Kelley, McMahon, Haywood, Olive, Wang, Sanchez-Perez, & Messing, 1999; Van Skike et al., 2010). For instance, the ε-isoform of protein kinase C (PKC) may be inversely associated with sensitivity to ethanol-induced motor impairments, as PKCε knock-out mice show heightened sensitivity to motor impairments

following ethanol consumption (Hodge et al., 1999). Additionally, PKCγ may be positively associated with motor sensitivity to ethanol, as PKCγ knock-out mice show reduced sensitivity to the motor-impairing effects of ethanol (Bowers et al., 1999; Bowers et al., 2001; Harris et al., 1995). The age-dependent motor impairments produced by ethanol are widely accepted in the field; as such, differential PKCγ expression within the cerebellum and cortex, the regions that are responsible, respectively, for coordinating and initiating movement, may underlie age-dependent motor impairments following ethanol consumption. Specifically, on the basis of the PKCγ knock-out mice data, PKCγ expression is likely to be reduced in adolescents, who are less sensitive to ethanol's motor impairing effects.

Protein Kinase Cγ

Protein kinase C is a phospholipid-dependent kinase that transduces signals involving lipid second messengers (Nishizuka, 1995). Nine PKC isoforms transcribed from unique genes have been identified, and can be classified into three subgroups: conventional, novel, and atypical based on their sensitivities to calcium ions, phosphatidylserine and diacylglycerol (Mellor & Parker, 1998). Protein kinase Cγ (PKCγ), along with the α and β isoforms of PKC, belongs to the conventional PKC family, which is activated by calcium ions and diacylglycerol in the presence of phosphatidylserine (Nishizuka, 1995; reviewed in Song & Messing, 2005).

PKCγ has a unique distribution because it is only found within neurons of the central nervous system (Hashimoto et al., 1988). There is abundant expression of PKCγ in Purkinje neurons of the cerebellum and pyramidal cells of the hippocampus (Hashimoto et al., 1988; Saito et al., 1988), implicating PKCγ as a modulator of certain

forms of synaptic plasticity, such as long-term potentiation and long-term depression (Bliss & Collingridge, 1993; Ito, 1989). Compared to the other isoforms in its class, PKCγ also has a unique intracellular localization; specifically, it is located within the cytoplasm of the soma and dendrites (Kose, Ito, Saito, & Tanaka, 1990; Kose, Saito, Ito, Kikkawa, Nishizuka, & Tanaka, 1988), and upon activation, translocates to the cell membrane (Kumar et al., 2006).

Specifically related to ethanol's effects, PKC phosphorylates the β and γ_2 subunits of the $GABA_AR$ to modulate receptor sensitivity to ethanol (Kellenberger, Malherbe, & Siegel, 1992; Krishek, Xie, Blackstone, Huganir, Moss, & Smart, 1994; Qi, Song, Wallace, Wang, Newton, McMahon, et al., 2007; Song & Messing, 2005; Wafford, Burnett, Leidenheimer, Burt, Wang, Kofuji, et al., 1991), with PKCγ enhancing the effect of ethanol on $GABA_ARs$ (Kumar et al., 2005; Song & Messing, 2005) .

Additionally, PKCγ expression following ethanol administration is time-dependent in adult rats (Kumar, Lane, & Morrow, 2006). Specifically, 2.0 g/kg ethanol has no effect on PKCγ expression in the P2 fraction 10 minutes after administration, but reduces PKCγ expression in the P2 by nearly 40 percent 60 minutes after injection. We chose to analyze membrane-bound PKCγ expression 40 minutes after a 2.0 g/kg administration of ethanol or saline. Not only does this time point provide a unique glimpse of ethanol's effect on PKCγ expression in the P2 fraction compared to previously published work (Kumar et al., 2006) between two different ages, it is also a time when BEC levels between adolescent and adult rats are similar, but slight differences in motor impairments are still manifested (Van Skike et al., 2010). Therefore, altered PKCγ expression levels at this time point could directly target an important mechanism

involved in producing age-dependent motor impairments when systemic ethanol concentrations are equivalent.

Indeed, some of the most enlightening work regarding the importance of PKCγ in determining ethanol sensitivity involves the use of knock-out mice.

PKCγ knock-out mice show reduced potentiation of $GABA_AR$ mediated ion flux in response to ethanol (Harris et al., 1995). While the behavioral effect of PKCγ deletion is a reduction in hypnotic and anxiolytic sensitivity to ethanol compared to wild-type littermates (Bowers et al., 1999; Bowers et al., 2001; Harris et al., 1995). Furthermore, PKCγ knock-outs have an enhanced preference for ethanol compared to wild-type littermates (Bowers & Wehner, 2001). These effects, along with the increased impulsivity of PKCγ knock-outs (Bowers & Wehner, 2001), are strikingly similar to the behaviors of adolescent rodents: reduced ethanol-induced ataxia and hypnosis (Hefner & Holmes, 2007; Lisenbardt et al., 2009; Little et al., 1996; Pian et al., 2008; Ristuccia & Spear, 2008; Silveri & Spear, 2001; Varlinskaya & Spear, 2002; White et al., 2002), enhanced ethanol preference (Doremus et al., 2005; Lancaster et al., 1996; Walker et al., 2008), and increased impulsivity (Macri et al., 2002).

It is important to consider the impact of PKCγ deletion in these mutant mice. Fortunately, PKCγ is not expressed at birth but becomes detectable within a week; therefore, it is not likely to affect early neural development of the knockout mouse (Hashimoto et al., 1988). However, PKCγ does play a role in hippocampal long-term potentiation, which is impaired in the mutant mouse, but synaptic transmission in the hippocampus remains intact (Abeliovich, Chen, Goda, Silva, Stevens, & Tonegawa, 1993a). Perhaps because of the impaired long-term potentiation in the hippocampus,

mutant mice display modest learning and memory impairments compared to wild-type littermates (Abeliovich et al., 1993b). Within the cerebellum, PKCγ is localized to the dendrites of Purkinje neurons (Hashimoto et al., 1988), and its deletion results in slight ataxia (Abeliovich, Paylor, Chen, Kim, Wehner, & Tonegawa, 1993b).

Although there are modest alterations resulting from PKCγ deletion, there is a striking similarity between ethanol responsiveness in adolescent rats and PKCγ knockout mice. As such, the current work investigates if the expression of PKCγ expression varies in accordance with the age-dependent effects of ethanol on motor behavior. It is hypothesized that compared to adults, adolescents will have reduced PKCγ peptide expression in brain regions responsible for motor production and precision, specifically the cortex and cerebellum. Since our research question targets reduced PKCγ expression as the mechanism underlying some of the age-dependent effects of ethanol, we hypothesize adolescents and adults will have equivalent PKCγ expression in the hippocampus based on our data showing similar spatial memory impairments between adolescents and adults in response to an ethanol challenge (Chin et al., 2009; Chin et al., 2010a).

Primary Investigative Goal

Age-dependent motor impairments produced by ethanol have been well documented, indicating adolescents exhibit reduced sensitivity to the motor-impairing effects of ethanol (Hefner & Holmes, 2007; Lisenbardt et al., 2009; Little et al., 1996; Pian et al., 2008; Ristuccia & Spear, 2008; Silveri & Spear, 2001; Varlinskaya & Spear, 2002; White et al., 2002). Ethanol-induced motor impairments serve as cues to moderate ethanol intake (Spear & Varlinskaya, 2005), which may contribute to amplified alcohol

use during this developmental time period. Indeed, recent national survey data reveal that adolescents are consuming alcohol at alarming rates (Substance Abuse and Mental Health Services Administration, 2010); yet the neural and molecular mechanisms underlying the adolescent's comparative reduction in alcohol sensitivity that potentially underlies the high drinking rates of adolescents has yet to be determined.

Accordingly, we investigated the expression of membrane-bound PKCγ as a potential mechanism underlying ethanol's ataxic effects since adolescent rats and PKCγ knock-out mice are similarly affected by ethanol, namely exhibiting a reduction in motor impairments following ethanol administration (Harris et al., 1995; Bowers et al., 1999; Bowers et al., 2001). We expect adolescent rats to have reduced PKCγ expression compared to adults in the cortex and cerebellum because these regions are responsible for motor production and precision, both of which could be involved in ethanol-induced motor impairments. Additionally, based on our data indicating adolescent and adult rats show similar spatial memory impairments after an acute ethanol challenge (Chin et al., 2009; Chin et al., 2010a), we hypothesize that adolescent and adult rats will have similar levels of PKCγ expression in the hippocampus.

CHAPTER TWO

Methods and Materials

Animals and Tissue Collection

Male Sprague-Dawley rats purchased from Harlan Laboratories (Indianapolis, IN) were pair-housed in an IACUC approved animal colony with ad libidum access to food and water. Animals were allotted a 2 day acclimation period before beginning any experimental procedures. Twelve adolescent (PD 40) and twelve adult (PD 120) male Sprague-Dawley rats received a single intraperitoneal injection of 2.0 g/kg 10% (w/v) ethanol (n = 6 per age) or equivalent volume of saline (n = 6 per age). Tissue was harvested via rapid decapitation 40 minutes post-administration, a time point when BECs are equivalent but differential motor impairments between adolescent and adult animals are observed (Van Skike et al., 2010). In addition, this time point following ethanol administration provides unique information concerning ethanol's effect on PKCγ expression compared to previously published work (Kumar, Lane, & Morrow, 2006). Whole cortex, cerebellum, and hippocampus were rapidly dissected over ice and stored at -80ºC until assayed.

Tissue Preparation

P2 fractions of individual brain regions were prepared by homogenizing the tissue sample in 0.32M sucrose in phosphate-buffered saline (PBS), followed by a 10 minute centrifugation at 1000 × *g*. The resulting supernatant was centrifuged again at 12,000 × *g* for 20 minutes. This pellet, the P2 fraction, was resuspended in PBS and stored at -80ºC.

Western Blot Procedure

A Bradford assay was conducted to determine the protein concentration of each P2 fraction. Equivalent amounts of protein (20 μg) were loaded into Tris-Glycine gels (8 to 16%), counterbalanced across conditions. Proteins were separated by SDS-PAGE, electroblotted to polyvinylidene difluoride (PVDF) membranes (Invitrogen, Carlsbad, CA), and targeted with PKCγ (Santa Cruz Biotechnology, Santa Cruz, CA) primary antibody that was diluted with blocking buffer (50 mL PBS, 25 μL Tween-20, 0.5 g milk powder) to a concentration of 1:2,000. A horseradish peroxidase-conjugated secondary antibody targeted against a rabbit host (Santa Cruz Biotechnology) was applied, with antibody concentration diluted to 1:2,000. Peptide labeling was detected by chemiluminescent substrates (Thermo/Scientific Pierce, Rockford, IL), and exposed to x-ray film under non-saturating conditions. Densitometric measurements of the resulting proteins bands were made with NIH Image J software. All blots were re-probed with β-actin (1:2,000) and an horseradish peroxidase conjugated secondary antibody targeted against a goat host (1:7,500) to verify equivalent protein loading and transfer. All data was normalized to actin expression.

Data Analysis

Densitometric measurements normalized to actin expression were analyzed with a two-way repeated ANOVA [Age (adolescent, adult) X Drug (saline, ethanol)].

More specifically, a generalized randomized block design with 2 treatment levels (GRB-2) was used to assign subjects to the experimental conditions (Kirk, 1995). The organismic variable of age restricted complete randomization of subjects; however,

subjects within each age group were randomly assigned to the treatment conditions of saline or ethanol administration (Kirk, 1995).

CHAPTER THREE

Results

PKCγ expression in adolescent rats is significantly reduced in cerebellum (F (7,14) = 5.08, $p < 0.05$, Figure 1) and cortex (F (7,14) = 16.91, $p < 0.01$, Figure 2) compared to adult peptide expression. However, the differences between PKCγ expression levels are modest, with approximately 7% reduction in adolescent cerebellum and 10% reduction in adolescent cortex from adult expression levels.

PKCγ expression in the hippocampus was not significantly affected by age (F (7,14) = 0.34, $p = 0.90$, Figure 3). Interestingly, acute ethanol administration did not have a significant effect on PKCγ expression in any brain region and did not significantly interact with age to alter PKCγ expression.

Since there was no effect of ethanol on PKCγ expression level in any brain region, all data presented has been collapsed across treatment level, leaving only the main effect of age to be plotted on the graph.

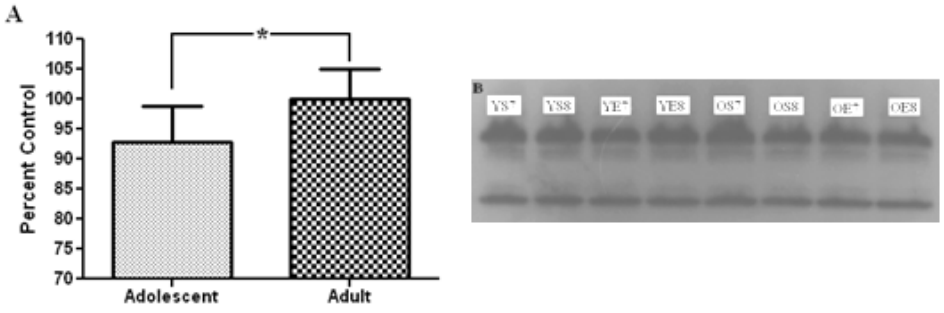

Figure 1. Adolescent rats have less PKCγ expression in the cerebellum. (A) Optical density of cerebellar PKCγ expression normalized to β-actin with adult set as control condition. (B) Representative PKCγ expression (top) and β-actin (bottom) in cerebellum. YS = adolescent saline, YE = adolescent ethanol, OS = adult saline, OE = adult ethanol; numeral indicates animal number. Error bars indicate S.E.M.

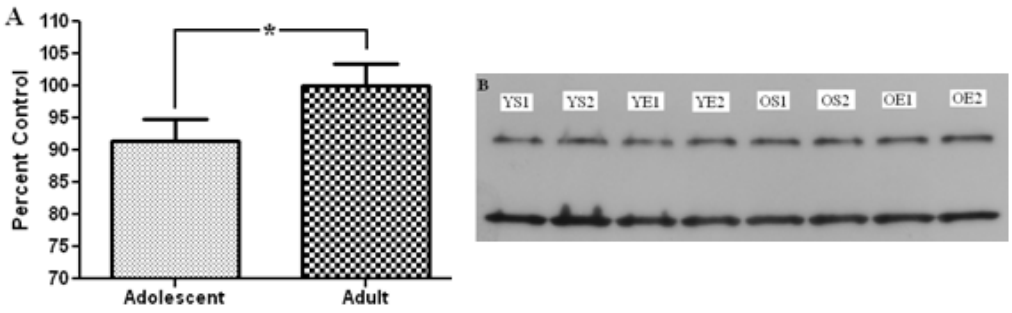

Figure 2. Adolescent rats have less PKCγ expression in the cortex. (A) Optical density of PKCγ expression in the cortex normalized to β-actin with adult set as the control condition. (B) Representative PKCγ expression (top) and β-actin (bottom) in cortex. YS = adolescent saline, YE = adolescent ethanol, OS = adult saline, OE = adult ethanol; numeral indicates animal number. Error bars indicate S.E.M.

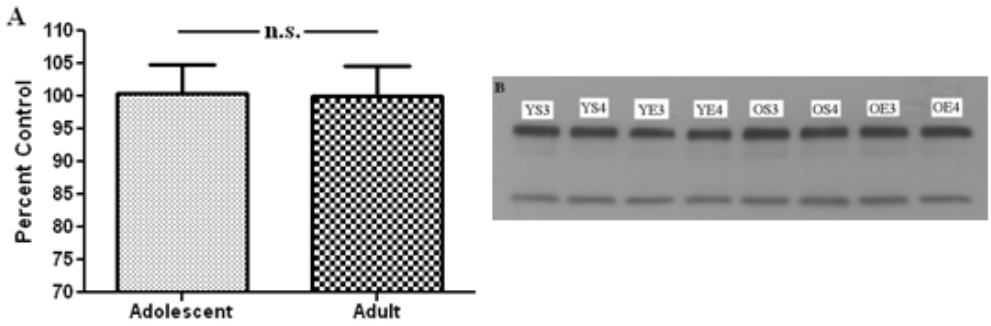

Figure 3. Adolescent and adult rats have equivalent PKCγ expression levels in the hippocampus. (A) Optical density of hippocampal PKCγ expression normalized to β-actin with adult set as the control condition. (B) Representative PKCγ expression (top) and β-actin (bottom) in cortex. YS = adolescent saline, YE = adolescent ethanol, OS = adult saline, OE = adult ethanol; numeral indicates animal number. Error bars indicate S.E.M.

CHAPTER FOUR

Discussion

Adolescents are less sensitive to ethanol-induced motor impairments (Hefner & Holmes, 2007; Lisenbardt et al., 2009; Little et al., 1996; Pian et al., 2008; Ristuccia & Spear, 2008; Silveri & Spear, 2001; Varlinskaya & Spear, 2002; White et al., 2002). This reduced motor sensitivity may contribute to the excessive consumption patterns that are typical of adolescents and young adults (Substance Abuse and Mental Health Services Administration, 2010) by increasing the threshold required to produce biofeedback cues that serve to moderate intake (Spear & Varlinskaya, 2005). Nearly one-third of adolescents and young adults aged 12-17 years consume alcohol (Substance Abuse and Mental Health Services Administration, 2009; 2010); subsequently, the prevalence of alcohol use and addiction is highest slightly thereafter, between 18-25 years of age (Substance Abuse and Mental Health Services Administration, 2009; 2010). The rate of alcohol use during adolescence and young adulthood are concerning, not only because of the potential neurobiological and general health consequences, but also because the reduced motor sensitivity of the adolescent and young adult might enable excessive consumption, which could lead to tolerance and to the formation of detrimental alcohol consumption behaviors. Therefore, it is especially pertinent to identify the neurobiological mechanism that contributes to the reduced ethanol-induced motor sensitivity of the adolescent.

PKCγ knockout mice are less sensitive to ethanol-induced motor impairments compared to their wild-type littermates (Harris et al., 1995). Additionally, PKCγ

knockouts show other behavioral traits that approximate human adolescence, like increased risk taking (Bowers & Wehner, 2001; Macri et al., 2002) and enhanced preference for ethanol compared to wild-type littermates (Bowers & Wehner, 2001), further implicating reduced PKCγ expression in adolescent rats as a neurobiological mechanism that would underlie the age-dependent sensitivities to ethanol. Accordingly, we hypothesized that adolescent rats may have reduced PKCγ expression in areas involved in movement precision and production, specifically the cerebellum and cortex, that could help explain the reduced sensitivity to ethanol-induced motor impairments seen in adolescent rats (Hefner & Holmes, 2007; Lisenbardt et al., 2009; Little et al., 1996; Pian et al., 2008; Ristuccia & Spear, 2008; Silveri & Spear, 2001; Varlinskaya & Spear, 2002; White et al., 2002).

Contrasting with the age-dependent motor impairments, it is currently unclear if ethanol produces age-dependent effects on hippocampal-dependent spatial memory (Chin et al., 2009; Chin et al., 2010a; Rajendran & Spear, 2004; Markwiese et al., 1998). Although there is data in opposition (Markweise et al., 1998), data from our lab indicates adolescents and adults have equivalent hippocampal-dependent spatial memory impairments (Chin et al., 2009; Chin et al., 2010a). Because of these cognitive similarities between adolescent and adult rats, we expected PKCγ expression in the hippocampus would be comparable between the two age groups.

We investigated membrane-bound PKCγ expression 40 minutes after a 2.0 g/kg intraperitoneal dose of ethanol or saline to directly target a $GABA_AR$-mediated mechanism that potentially contributes to age-dependent motor impairments (Kumar et al., 2005). At this time point, systemic ethanol concentrations of the adolescent and adult

rat are equivalent; however, slight differences in motor impairments are still observed (Van Skike et al., 2010). Therefore, differences in PKCγ binding expression when BECs are equivalent are more likely to pinpoint a molecular mechanism that differentially affects the $GABA_AR$ to produce some of the age-dependent motor impairments produced by ethanol.

Consistent with our hypotheses, PKCγ expression levels were reduced in brain regions controlling motor functioning. Within the cerebellum, adolescent PKCγ expression was reduced approximately 7% from expression levels of the adult rat. Additionally, cortical PKCγ expression of the adolescent was reduced approximately 10% from adult expression levels. Reduced expression in these brain regions may result in attenuated phosphorylation of the $GABA_AR$, and therefore less enhancement of chloride ion flux in the presence of ethanol, which may translate into mitigated motor impairments in adolescent rats.

In accordance with our hypotheses, adolescents and adults had similar levels of PKCγ expression in the hippocampus. While confirming the null hypothesis does not allow for the generation of strong conclusions, when taken with the cognitive-behavioral data (Chin et al., 2009; Chin et al., 2010a, Chin et al., 2011; Rajendran & Spear, 2004), it provides additional evidence that the adolescent hippocampus may not show enhanced vulnerabilities to ethanol-induced insults when compared with the adult hippocampus. For over a decade adolescents were thought to be more sensitive to ethanol-induced spatial memory impairments than adults (Markwiese et al., 1998); however, recent data demonstrates this may not be the case (discussed in Chin et al., 2010b and Chin et al., 2011). In brief, it has been shown that adolescents learn the MWM task more slowly

than adults, especially during initial acquisition (Chin et al., 2010a). Furthermore, more recent data refutes that initial claim, instead suggesting that adolescents and adults show similar ethanol-induced spatial memory impairments, both in an appetitive sand-digging task (Rajendran & Spear, 2004), and in the MWM (Chin et al., 2009; Chin et al., 2010a, Chin et al., 2011). Therefore, the current cognitive-behavioral (Chin et al., 2009; Chin et al., 2010a; Rajendran & Spear, 2004) and molecular data (Van Skike et al., 2010) are in support of equivalent spatial memory impairments produced by ethanol in both adolescent and adult rats.

The modest, but significant, reductions of PKCγ expression in the cerebellum and cortex of the adolescent rat compared to the adult could be due to several different factors. For instance, within the cerebellum, PKCγ is localized to Purkinje cells (Hashimoto et al., 1988; Metzger & Kapfhammer, 2000), which serve as the sole output of the cerebellum (Cesa & Strata, 2009). Once again, our method of harvesting whole cerebellum may have attenuated our result. This could be resolved in the future by preparing organotypic cerebellar slice cultures, followed by western blot analysis, as described in Schrenk, Kapfhammer, and Metzger (2001).

Within the cortex, it is plausible that alterations in PKCγ expression important for producing age-dependent motor responses to ethanol may be localized to cortical areas specialized in motor production, like the motor and premotor cortex. Alterations in these areas would be especially important in the investigation of an underlying difference in motor sensitivity to ethanol; however, we harvested and analyzed whole cortex, which could have washed out potentially large changes in specific cortical regions. Our method of tissue collection was chosen as an omnibus investigation because of the novelty of our

experimental investigation. To the best of our knowledge, this is the first experiment to consider age-dependent expression of PKCγ, especially in its relation to age-dependent motor sensitivities to ethanol administration. Future investigations could use microdissection to isolate the motor cortex and accessory areas responsible for motor production. Additionally, cortical PKCγ expression in the P2 fraction after ethanol administration is time-dependent in young adult rats: the expression level is stable 10 minutes after ethanol administration, but is significantly reduced at 60 minutes (Kumar et al., 2006), so the entire time-course of PKCγ expression following ethanol administration could be analyzed in the future using both membrane-bound and cytosolic PKCγ expression.

PKCγ is a cytosolic protein that translocates to the cell membrane upon activation (Kumar et al., 2006). Because of this and the novelty of our investigation, we focused our analysis specifically on the P2 fraction, which contains only membrane-bound proteins. If PKCγ expression were to be a plausible mechanism underlying motor sensitivity to ethanol, it would likely manifest itself in membrane-bound protein expression. Our analysis revealed ethanol administration had no effect on PKCγ expression in any brain region of interest. This result is not completely inconsistent with previous data indicating cortical PKCγ expression is significantly reduced in the P2 fraction 60 minutes, but not 10 minutes, after ethanol administration in young adult rats (Kumar et al., 2006). This indicates membrane-bound PKCγ expression is stable up to 40 minutes after ethanol administration, with decreased protein translocation occurring sometime between 40 and 60 minutes post-ethanol (Kumar et al., 2006). This effect may span across several different life stages of the rat; for instance, previously published

research investigated young adult rats (approximately PD 70-84, Kumar et al., 2006), while the current study used adolescent (PD 40) and adult (PD 120) rats. Future research should confirm this, for if decreased protein translocation occurs at the same time point following ethanol administration independently of age, our result of decreased membrane-bound PKCγ expression in adolescent rats is likely due to a global reduction in PKCγ availability. However, at this point, we cannot determine whether our result is due to an inherent reduction of PKCγ within the cytosol or if adolescents have reduced PKCγ translocation. As such, cytosolic PKCγ expression could be an avenue for future research to help determine the nature and origin of the reduced PKCγ expression in the P2 fraction of adolescent cerebellum and cortex.

Additionally, PKCγ expression at other time points following ethanol administration should be considered, especially at time points where there are larger manifestations of differential motor impairments between adolescents and adults. The analysis in this study was conducted at a time point where BECs are equivalent, yet slight differences in motor impairments are still observable. In contrast, 20 minutes following ethanol administration, adolescents display less motor impairment than adults, despite having a BEC level nearly twice as great as the adult (Van Skike et al., 2010). Systemic ethanol concentrations do not account for the age-dependent motor impairments at either of these time points; for instance, in the current analysis at 40 minutes, BECs are equivalent, while adolescents at 20 minutes have twice the BEC level of the adult and exhibit less deficits in motor functioning. Analysis at the 20 minute time point, which corresponds to a greater behavioral difference, may yield a bigger effect of differential PKCγ expression.

PKCγ phosphorylates the $GABA_AR$ (Kellenberger et al., 1992; Krishek et al., 1994; Qi et al., 2007; Song & Messing, 2005; Wafford, et al., 1991), and in the presence of ethanol, will enhance ethanol's effects at the receptor (Kumar et al., 2005; Song & Messing, 2005) by increasing ethanol-induced potentiation of GABAergic activity (Weiner et al., 1994). Intuitively, PKCγ deletion results in diminished sensitivity to the motor-impairing effects of ethanol (Bowers et al., 1999; Bowers et al., 2001; Harris et al., 1995) and reduced potentiation of $GABA_AR$ mediated ion flux in response to ethanol (Harris et al., 1995).

We have demonstrated that adolescent rats have significantly reduced PKCγ expression compared to adults in the cortex and cerebellum, brain regions responsible for motor production and precision (Arshavsky et al., 1983; Braitenberg, 1967 Heck, Thach, & Keating, 2007 Brindley, 1964; Brown & Sherrington, 1911; Fritsch & Hitzig, 1870; Levy et al., 1984; Li & Tew, 1966). Therefore, we propose that reduced PKCγ expression may be part of a larger $GABA_AR$-mediated mechanism that helps to explain the age-dependent motor impairments produced by ethanol, especially within the cerebellum. For instance, 1.5 g/kg ethanol decreases the *in vivo* spontaneous firing of Purkinje neurons in adult rats by about 20%, but results in a 5% excitation in adolescent rats (Van Skike et al., 2010). This electrophysiological effect correlates with the age-dependent motor impairments: adults exhibit a greater degree of ethanol-induced motor impairments and *in vivo* electrophysiological depression of spontaneous cerebellar Purkinje firing. Purkinje neurons are the sole output of the cerebellum (Cesa & Strata, 2009); therefore, ethanol-induced alterations of their firing rate may translate into deficits in motor coordination since the cerebellum plays a role in timing (Arshavsky et al., 1983;

Braitenberg, 1967) and synchrony (Heck et al., 2007). Additionally, PKCγ is abundantly expressed in the dendrites of Purkinje neurons (Hashimoto et al., 1988; Saito et al., 1988). Adolescent rats have slightly reduced PKCγ expression in the cerebellum compared to adults, which may attenuate the extent of ethanol-induced potentiation of GABAergic activity within cerebellar Purkinje neurons, thereby mitigating some of the motor impairments produced by ethanol in adolescent rats. Alternatively, adults have comparatively higher membrane-bound cerebellar PKCγ expression, which would likely enhance the $GABA_AR$-mediated activity, producing a greater motor impairment in adult rats compared to adolescent rats.

This mechanism, partially responsible for reduced motor sensitivity to ethanol in adolescents and young adults, likely plays a role in enabling excessive alcohol consumption during adolescence and young adulthood (Substance Abuse and Mental Health Services Administration, 2010). For instance, PKCγ knockout mice voluntarily consume more ethanol than their wild-type littermates (Bowers & Wehner, 2001), perhaps because they are more resistant to motor impairments that serve as feedback cues to regulate ethanol intake (Spear & Varlinskaya, 2005). Consistent with this idea, adolescent rats, which have reduced cortical and cerebellar PKCγ expression and are less sensitive to ethanol's motor impairments, also voluntarily consume more ethanol compared to adult rats (Doremus et al., 2005; Lancaster et al., 1996; Walker et al., 2008). Indeed, this pattern of excessive drinking in adolescents and young adults is readily found in human populations as well (Substance Abuse and Mental Health Services Administration, 2010). Because adolescents and young adults exhibit reduced motor sensitivity to ethanol, which may be partially mediated by PKCγ and the associated

alterations of ethanol-induced potentiation of $GABA_AR$-mediated ion flux, they must consume greater quantities of ethanol to produce the feedback cues that signal to attenuate consumption. Specifically, adolescent PKCγ expression is reduced by approximately 10% in the cortex and around 7% in the cerebellum compared to adult PKCγ expression levels. This reduction, especially in the cerebellum where PKCγ is concentrated in Purkinje neurons (Hashimoto et al., 1988; Saito et al., 1988) that are the sole output of the cerebellum (Cesa & Strata, 2009), may result in attenuated potentiation of $GABA_AR$ mediated ion flux by ethanol, thereby reducing the manifestation of motor impairments in the adolescent. Additionally, the *in vivo* spontaneous firing rate of the adolescent Purkinje neuron is resilient to ethanol-induced depression, while the spontaneous firing rate of the adult Purkinje neuron is reduced approximately 20% following ethanol administration (Van Skike et al., 2010). Together, this mechanism contributes to the adolescent's reduced sensitivity to the motor impairing effects. With motor impairments serving as feedback cues to attenuate ethanol consumption (Spear & Varlinskaya, 2005), our findings demonstrate evidence toward a neurobiological mechanism that predisposes adolescents and young adults to consume alcohol in an excessive manner.

REFERENCES

Abeliovich, A., Chen, C., Goda, Y., Silva, A. J., Stevens, C. F., & Tonegawa, S. (1993a). Modified hippocampal long-term potentiation in PKCγ-mutant mice. *Cell, 75*(7), 1253-1262.

Abeliovich, A., Paylor, R., Chen, C., Kim, J. J., Wehner, J. M., & Tonegawa, S. (1993b). PKCγ mutant mice exhibit mild deficits in spatial and contextual leanring. *Cell, 75*(7), 1263-1271.

Acheson, S. K., Ross, E. L., & Swartzwelder, H. S. (2001). Age-independent and dose-response effects of ethanol on spatial memory in rats. *Alcohol, 23*, 167-175.

Acheson, S. K., Stein, R. M., & Swartzwelder, H. S. (1998). Impairment of semantic and figural memory by acute ethanol: age-dependent effects. *Alcoholism: Clinical and Experimental Research, 22 (7)*, 1437-1442.

Aguayo, L. G. (1990). Ethanol potentiates the GABAA-activated Cl- current in mouse hippocampal and cortical neurons. *European Journal of Pharmacology, 187 (1)*, 127-130.

Aguayo, L. G., Peoples, R. W., Yeh, H. H., & Yevenes, G. E. (2002). GABA(A) receptors as molecular sites of ethanol action. Direct or indirect actions? *Current Topics in Medicinal Chemistry, 2 (8)*, 869-885.

Andersen, B. B. (2004). Reduction of Purkinje cell volume in cerebellum of alcoholics. *Brain Research, 1007*, 10-18.

Andersen, S. L., Thompson, A. P., Krenzel, E., & Teicher, M. H. (2002). Pubtertal changes in gonadal hormones do not underlie adolescent dopamine receptor overproduction. *Psychoneuroendocrinology, 27* (6), 683-691.

Archibald, A. B., Graber, J. A., & Brooks-Gunn, J. (2006). Pubertal Processes and Physiological Growth in Adolescence. From *Blackwell Handbook of Adolescence.* doi: 10.1002/9780470756607.ch2

Arnett, J. J. (1996). Sensation seeking, aggressiveness, and adolescent reckless behavior. *Personality and Individual Differences, 20*(6), 693-702.

Arshavsky, Y., Gelfand, I., & Orlovsky, G. (1983). The cerebellum and control of rhythmical movements. *Trends in Neurosciences, 6,* 417-422.

Baker KG, Harding AJ, Halliday GM, Krill JJ, & Harper CG. (1999) Neuronal loss in functional zones of the cerebellum of chronic alcoholics with and without Wernicke's encephalopathy. *Neuroscience, 91*(2), 429-438.

Barbaccia, M. L., Scaccianoce, S., Del Bianco, P., Campolongo, P., Trezza, V., Tattoli, M., et al. (2007). Cognitive impairment and increased brain neurosteroids in adult rats perinatally exposed to low millimolar blood alcohol concentrations. *Psychoneuroendocrinology, 32*, 931–942.

Benes, F. M., Turtle, M., Khan, Y., & Farol, P. (1994). Myelination of a key relay zone in the hippocampal formation occurs in the human brain during childhood, adolescence, and adulthood. *Archives of General Psychiatry, 51*, 477-484.

Beresford, T. P., Arciniegas, D. B., Alfers, J., Clapp, L., Martin, B., & Du, Y., et al. (2006). Hippocampus volume loss due to chronic heavy drinking. *Alcoholism: Clinical and Experimental Research, 30 (11),* 1866-1870.

Berry, R. B. & Matthews, D. B. (2004). Acute ethanol administration selectively impairs spatial memory in C57BL/6J mice. *Alcohol, 32*, 9-18.

Blednov, Y. A., Walker, D., Alva, H., Creech, K., Findlay, G., & Harris, R. A. (2003). GABAA receptor alpha 1 and beta 2 subunit null mutant mice: behavioral responses to ethanol. *Journal of Pharmacology and Experimental Therapeutics, 305*(3), 854-863.

Bliss, T. V. P., & Collingridge, G. L. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. *Nature, 361*, 31-39.

Bovolin, P., Santi, M., Memo, M., Costa, E., & Grayson, D. R. (1992). Distinct developmental patterns of expression of rat alpha 1, alpha 5, gamma 2S, and gamma 2L receptor subunit mRNAs in vivo and in vitro. *J. Neurochem, 59*(1), 62-72.

Bowers, B. J. & Wehner, J. M. (2001). Ethanol consumption and behavioral impulsivity are increased in protein kinase Cgamma null mutant mice. *J. Neurosci, 21*(21), RC180.

Bowers, B. J., Elliott, K. J., & Wehner, J. M. (2001). Differential sensitivity to the anxiolytic effects of ethanol and flunitrazepam in PKCgamma null mutant mice. *Pharacol. Biochem. Behav,. 69*(1-2), 99-110.

Bowers, B. J., Owen, E. H., Collins, A. C., Abeliovich, A., Tonegawa, S., & Wehner, J. M. (1999). Decreased ethanol sensitivity and tolerance development in gamma-protein kinase C null mutant mice is dependent on genetic background. *Alcoholism: Clinical and Experimental Research, 23*(3), 387-397.

Braitenberg, V. (1967). Is the cerebellar cortex a biological clock in the millisecond range? *Progress in Brain Research, 25*, 334-346.

Brasser, S. M. & Spear, L. P. (2002). Physiological and behavioral effects of acute ethanol hangover in juvenile, adolescent, and adult rats. *Behav. Neurosci., 116*, 305-320.

Brindley, G. S. (1964). The use made by the cerebellum of the information that it receives from sense organs. *Int. Brain. Res. Org. Bulletin 3*, 80.

Brooks-Kayal, A. R. & Pritchett, D. B. (1993). Developmental changes in human gamma-aminobutyric acidA receptor subunit composition. *Ann. Neurol., 34*, 687-693.

Brown, G., & Sherrington, C. S. (1911). Observations on the localization in the motor cortex of the baboon ("papio anubis"). *Journal of Physiology, 43*, 209-218.

Brown, S. A., Tapert, S. F., Granholm, E., & Delis, D. C. (2000). Neurocognitive functioning of adolescents: effects of protracted alcohol use. *Alcoholism: Clinical and Experimental Research, 24*(2), 164-171.

Burr, I. M., Sizonenko, P. C., Kaplan, S. L., & Grumback, M. M. (1970). Hormonal changes in puberty: I. Correlation of serum luteinizing hormone and follicle stimulating hormone with stages of puberty, testicular size, and bone age in normal boys. *Pediatric Research, 4*, 23.

Carta, M., Ariwodola, O. J., Weiner, J. L., & Valenzuela, C. F. (2003). Alcohol potently inhibits the kainite receptor-dependent excitatory drive of hippocampal interneurons. *Proc. Natl. Acad. Sci. U. S. A., 100*(11), 6813-6818.

Carta, M., Mameli, M., & Valenzuela, C. F. (2004). Alcohol enhances GABAergic transmission to cerebellar granule cells via an increase in Golgi cell excitability. *J. Neurosci, 24*(15), 3746-3751.

Casey, B. J., Galvan, A., & Hare, T. A. (2005). Changes in cerebral functional organization during cognitive development. *Curr. Opin. Neurobiol., 15*, 239-244.

Casey, B. J., Getz, S., & Galvan, A. (2008). The adolescent brain. *Dev. Rev., 28*, 62-77.

Cesa, R., & Strata, P. (2009). Axonal competition in the synaptic wiring of the cerebellar cortex during development and in the mature cerebellum. *Neuroscience 162*, 624-632.

Chin, V. S., Berry, R. B., & Matthews, D. B. (2009). Adolescent rats are less sensitive than adult rats to the spatial memory impairing effects of acute ethanol. *Alcoholism: Clinical and Experimental Research, 33*, p. 216A.

Chin, V. S., Diaz-Granados, J. L., & Matthews, D. B. (2010a). Effects of low dose acute ethanol on spatial memory in adolescent and adult rats. Alcohol Clin Exp Res 3: 30A.

Chin, V.S., Van Skike, C. E., Berry, R. B., Diaz-Granados, J. L., & Matthews, D. B. (2010b). Effects of ethanol on hippocampal function during adolescence: a look at the past and thoughts on the future. *Alcohol ,44,* 3-14.

Chin, V. S., Van Skike, C. E., Berry, R., Kirk, R., Diaz-Granados., Matthews, D. B. (2011). Effect of acute ethanol and acute allopregnanolone on spatial memory in adolescent and adult rats. *Alcohol, In Press.*

Clark, D. B., Pollock, N., Bukstein O. G., Mezzich, A. C., Bromberger, J. T., & Donovan, J. E. (1997). Gender and comorbid psychopathology in adolescents with alcohol dependence. *J. Am. Acad. Child Adolesc. Psychiatry, 36*, 1195-1203.

Crews, F. T., & Boettiger, C. A. (2009). Impulsivity, frontal lobes and risk for addiction. *Pharmacol. Biochem. Behav., 93 (3)*, 237-247

Crews, F. T., & Nixon, K. (2009). Mechanisms of neurodegeneration and regeneration in alcoholism. *Alcohol and Alcoholism, 44* (2), 115-127. doi:10.1093/alcalc/agn079

Criswell, H. E. & Breese, G. R. (2005). A conceptualization of integrated actions of ethanol contributing to its GABAmimetic profile: a commentary. *Neuropsychopharmacology, 30*, 1407-1425.

Crone, E. A., Bullens, L., van der Plas, E. A., Kijkuit, E. J., & Zelazo, P.D. (2008). Developmental changes and individual differences in risk and perspective taking in adolescence. *Dev. Psychopathol., 20*(4), 1213-1229.

Dahl, R. E. & Spear, L. P. (2004). Adolescent brain development. *Annals of the New York Academy of Sciences, 1021*, 1-22.

Dahl, R. E. (2004). Adolescent brain development: a period of vulnerabilities and opportunities. *Ann. N. Y. Acad. Sci., 1021*, 1-22.

De Bellis, M. D., Clark, D. B., Beers, S. R., Soloff, P. H., Boring, A. M., Hall, J., Kersh, A., & Keshavan, M. S. (2000). Hippocampal volume in adolescent-onset alcohol use disorders. *American Journal of Psychiatry, 157*, 737-744.

De Bellis, M. D., Keshavan, M. S., Beers, S. R., Hall, J., Frustaci, K., Masalehdan, A., Noll, J., Boring, A. M. (2001). Sex differences in brain and maturation during childhood and adolescence. *Cereb. Cortex 11*(6), 552-557.

Diaz-Granados, J. L. & Graham, D. L. (2007). The effects of continuous and intermittent ethanol exposure in adolescence on the aversive properties of ethanol during adulthood. *Alcoholism: Clinical and Experimental Research, 31*, 2020-2027.

Doremus, T. L., Brunell, S. C., Rajendran, P., & Spear L. P. (2005). Factors influencing elevated ethanol consumption in adolescent relative to adult rats. *Alcohol Clin Exp Res, 29*, 1796-1808.

Durston, S., Hulshoff Pol, H. E., Casey, B. J., Giedd, J. N., Buitelaar, J. K., & van Engeland, H. (2001). Anatomical MRI of the developing human brain: what have we learned? *J. Am. Acad. Child Adolesc. Psychiatry 40*, 1012-1020.

Fields, R. D., & Stevens-Graham, B. (2002). New insights into neuron-glia communication. *Science, 298* (5593), 556-562.

Frezza, M., di Padova, C., Pozzato, G., Terpin, M., Baraona, E., & Lieber, C. S. (1990). High blood alcohol levels in women – the role of decreased gastric alcohol dehydrogenase activity and first-pass metabolism. *The New England Journal of Medicine, 322*, 95-99.

Fritsch, G., & Hitzig, E. (1870). Ueber die elektrische erregbarkeit des grosshirns. *Arch fuer Anatomie, Physiologie und Wissenschafiliche Medicin*, 300-332. [English translation in Von Bonin, 1960.]

Fritschy, J. M. & Mohler, H. (1995). GABAA-receptor heterogeneity in the adult rat brain: differential regional and cellular distribution of seven major subunits. *J. Comp. Neurol., 359*(1), 154-194.

Fritschy, J. M., Benke, D., Mertens, S., Oertel, W. H., Bachi, T., & Mohler, H. (1992). Five subtypes of type A gamma-aminobutyric acid receptors identified in neurons by double and triple immunofluorescence staining with subunit-specific antibodies. *Proc. Natl. Acad. Sci. U. S. A., 89* (15), 6726-6730.

Fritschy, J. M., Paysan, J., Enna, A., & Mohler, H. (1994). Switch in the expression of rat GABAA-receptor subtypes during postnatal development: an immunohistochemical study. *J. Neurosci., 14*, 5302-5324.

Fryar, C. D., Merino, M. C., Hirsch, R., & Porter, K. S. (2009). Smoking, alcohol use, and illicit drug use reported by adolescents aged 12-17 years: United States, 1999-2004. *National Health Statistics Reports,* 15, 1-26.

Gambarana C., Pittman, R., & Siegal, R. E. (1990). Developmental expression of the GABAa Receptor α1 subunit mRNA in the rat brain. *J Neurobiol, 21,* 1169-1179.

Giedd, J. N. (2008). The teen brain: insights from neuroimaging. *Journal of Adolescent Health, 42*(4), 335-343.

Giedd, J. N., Blumenthal, J., Jeffries, N. O., Castellanos, F. X., Liu, H., Zijdenbos et al. (1999). Brain development during childhood and adolescence: a longitudinal MRI study. *Nat. Neurosci.,* 2, 861-863.

Giedd, J. N., Castellanos, F. X., Rajapakse, J. C., Vaituzis, A. C., & Rapoport, J. L. (1997). Sexual dimorphism of the developing human brain. *Prog. Neuropsychopharmacol. Biol Psychiatry, 21*, 1185-1201.

Giedd, J. N., Vaituzis, A. C., Hamburger, S. D., Lange, N., Rajapakse, J. C., Kaysen, D., Vauss, Y. C., Rapoport, J. L. (1996). Quantitative MRI of the temporal lobe, amygdale, and hippocampus in normal human development: ages 4-18 years. *J. Comp. Neurol., 366*(2), 223-230.

Gogtay, N., Giedd, J. N., Lusk, L., Hayashi, K. M., Greensteain, D., Vaituzis, A. C., et al. (2004). Dynamic mapping of human cortical development during childhood through early adulthood. *Proc. Natl. Acad. Sci. U. S. A. 101*, 8174-8179.

Goldstein, J. M., Seidman, L. J., Horton, N. J., Makris, N., Kennedy, D. N., Caviness, V. S., Faraone, S. V., & Tsuang, M. T. (2001). Normal sexual dimorphism of the adult human brain assessed by in vivo magnetic resonance imaging. *Cereb. Cortex, 11*(6), 490-497.

Gonzales-Burgos, I., Alejandre-Gomez, M., & Cervantes, M. (2005). Spine-type densities of hippocampal CA1 neurons vary in proestrus and estrus rats. *Neurosci. Lett., 379*, 52-54.

Goodlett, C. R., & Johnson, T. B. (1997). Neonatal binge ethanol exposure using intubation: time and dose effects on place learning. *Neurotoxicology and Teratology, 19,* 435-446.

Grobin, A. C., Matthews, D. B., Devaud, L. L., & Morrow, A. L. (1998). The role of $GABA_A$ receptors in the acute and chronic effects of ethanol. *Psychopharmacology, 139,* 2-19.

Gutierrez, A., Khan, Z. U., Miralles, C. P., Mehta, A. K., Ruano, D., Araujo, F., Vitorica, J., & De Blas, A. L. (1997). GABAa receptor subunit expression changes in the rat cerebellum and cerebral cortex during aging. *Molecular Brain Research, 45*(1), 59-70.

Hanchar, H. J., Dodson, P. D., Olsen, R. W., Otis, T. S., & Wallner, M. (2005). Alcohol-induced motor impairment caused by increased extrasynaptic GABA(A) receptor activity. *Nature Neuroscience, 8 (3)*, 339-345.

Harris, R. A. (1990). Distinct actions of alcohols, barbiturates and benzodiazepines on GABA-activated chloride channels. *Alcohol, 7 (3)*, 273-275.

Harris, R. A., McQuilkin, S. J., Paylor, R., Abeliovich, A., Tonegawa, S., & Wehner, J. M. (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. *Proceedings of the National Academy of Sciences of the United States of America, 92*(9), 3658-3662.

Hashimoto, T., Ase, K., Sawamura, S., Kikkawa, U., Saito, N., Tanaka, C., & Nishizuka, Y. (1988). Postnatal development of a brain-specific subspecies of protein kinase C in rat. *J. Neurosci, 8*(5), 1678-1683.

Hebebrand, J., Hofmann, D., Reichelt, R., Schnarr, S., Kapp, M., Propping, P., & Fodisch, H. J. (1988). Early ontogeny of the central benzodiazepine receptor in human embryos and fetuses. *Life Sci, 43*(25), 2127-2136.

Heck, D. H., Thach, W. T., & Keating, J. G. (2007). On-beam synchrony in the cerebellum as the mechanism for the timing and coordination of movement. *Proceedings of the National Academy of Sciences of the United States of America, 104 (18)*, 7658-7663.

Hefner, K. & Holmes, A. (2007). An investigation of the behavioral actions of ethanol across adolescence in mice. *Psychopharmacology, 191*, 311-322.

Helms, C. M., Rogers, L. S., & Grant, K. A. (2009). Antagonism of the ethanol-like discriminative stimulus effects of ethanol, pentobarbital, and midazolam in cynomolgus monkeys reveals involvement of specific GABA(A) receptor subtypes. *Journal of Pharmacology and Experimental Therapeutics, 331 (1)*, 142-152.

Henschel, O., Gipson, K. E., & Bordey, A. (2008). GABAA receptors, anesthetics and anticonvulsants in brain development. *CNS and Neurological Disorders Drug Targets, 7*, 211-224.

Hodge, C. W., Mehmert, K. K., Kelley, S. P., McMahon, T., Haywood, A., Olive, M. F., Wang, D., Sanchez-Perez, A. M., & Messing, R. O. (1999). Supersensitivity to allosteric GABA(A) receptor modulators and alcohol in mice lacking PKCepsilon. *Nat Neurosci, 2*(11), 997-1002.

Ito, M. (1989). Long term depression. *Annual Review of Neuroscience, 12*, 85-102.

June, H. L. Sr., Foster, K. L., Eiler, W. J. 2nd, Goergen, J., Cook, J. B., Johnson, N., et al. (2007). Dopamine and benzodiazepine-dependent mechanisms regulate the EtOH-enhanced locomotor stimulation in the GABAA alpha1 subunit null mutant mice. *Neuropsychopharmacology, 32*(1), 137-152.

Kanaumi, T., Takashima, S., Iwasaki, H., Mitsudome, A., & Hirose, S. (2006). Developmental changes in the expression of GABAA receptor alpha1 and gamma2 subunits in human temporal lobe, hippocampus and basal ganglia: an implication for consideration on age-related epilepsy. *Epilepsy Res., 71*, 47-53.

Kang-Park, M., Kieffer, B. L., Roberts, A. J., Siggins, G. R., & Moore, S. D. (2006). Presynaptic delta opioid receptors regulate ethanol actions in central amygdala. *Journal of Pharmacology and Experimental Therapeutics, 320* (2), 917-925.

Karhune, PJ, Erkinjuntti T, & Laippala, P. (1994). Moderate alcohol consumption and loss of cerebellar Purkinje cells. *BMJ, 308*(6945), 1663-1667.

Kellenberger, S., Malherbe, P., & Sigel, E. (1992). Function of the α1β2γ2S γ-aminobutyric acid type A receptor is modulated by protein kinase C via multiple phosphorylation sites. *J. Biol. Chem., 267*, 25660-25663.

Kirk, R. E. (1995). Experimental design: Procedures for the behavioral sciences. Pacific Grove, CA: Brooks/Cole Publishing Company.

Kose, A., Saito, N., Ito, H., Kikkawa, U., Nishizuka, Y., & Tanaka, C. (1988). Electron microscopic localization of type I protein kinase C in rat purkinje cells. *Journal of Neuroscience, 8*, 4262-4268.

Kose, A., Ito, A., Saito, N., & Tanaka, C. (1990). Electron microscopic localization of γ- and βII-subspecies of protein kinase C in rat hippocampus. *Brain Research, 518*, 209-217.

Krishek, B. J., Xie, X., Blackstone, C., Huganir, R. L., Moss, S. J., & Smart, T. G. (1994). Regulation of $GABA_A$ receptor function by protein kinase C phosphorylation. *Neuron, 12*, 1081-1095.

Kubota, M., Nakazaki, S., Hirai, S., Saeki, N., Yamaura, A., & Kusaka, T. (2001). Alcohol consumption and frontal lobe shrinkage: study of 1432 non-alcoholic subjects. *J. Neurol. Neurosurg. Psychiatry, 71*, 104-106.

Kumar, S., Khisti, R. T., & Morrow, A. L. (2005). Regulation of native GABAA receptors by PKC and protein phosphatase activity. *Psychopharmacology, 183*(2), 241-247.

Kumar, S., Lane, B. M., & Morrow, A. L. (2006). Differential effects of systemic ethanol administration on protein kinase c epsilon, gamma, and beta isoform expression, membrane translocation, and target phosphorylation: reversal by chronic ethanol exposure. *J. Pharmacol. Exp. Ther., 319* (3), 1366-1375.

Kumar, S., Porcu, P., Werner, D. F., Matthews, D. B., Diaz-Granados, J. L., Helfand, R. S., et al. (2009). The role of GABA(A) receptors in the acute and chronic effects of ethanol: a decade of progress. *Psychopharmacology (Berl.), 205*, 529-564.

Lancaster, F. E., Brown, T. D., Coker, K. L., Elliott, J. A., & Wren, S. B. (1996). Sex differences in alcohol preference and drinking patterns emerge during the early postpubertal period. *Alcoholism: Clinical and Experimental Research, 20*(6), 1043-1049.

Land, C. & Spear, N. E. (2004). Fear conditioning is impaired in adult rats by ethanol doses that do not affect periadolescents. *International Journal of Developmental Neuroscience, 22 (5-6)*, 355-362.

Laurie, D. J., Wisden, W. T., & Seeburg, P. H. (1992). The distribution of 13 GABAa receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. *J Neurosci, 12,* 4151-4172.

Lenroot, R. K., Gogtay, N., Greenstein, D. K., Wells, E. M., Wallace, G. L., Clasen, L. S., et al (2007). Sexual dimorphism of brain developmental trajectories during childhood and adolescence. *Neuroimage, 36*(4), 1065-1073.

Leonard, C. M., Towler, S., Welcome, S., Halderman, L. K., Otto, R., Eckert, M. A., & Chiarello, C. (2008). Size matters: cerebral volume influences sex differences in neuroanatomy. *Cereb. Cortex, 18*(12), 2920-2931.

Levy, W. J., York, D. H., McCaffrey, M., & Tanzer, F. (1984). Motor evoked potentials from transcranial stimulation of the motor cortex in humans. *Neurosurgery, 15*(3), 287-302.

Lewohl, J. M., Crane, D. I., & Dodd, P. R. (1997). Expression of the alpha 1, alpha 2 and alpha 3 isoforms of the GABAA receptor in human alcoholic brain. *Brain Res, 751*(1), 102-112.

Li, C. & Tew, J. M. Jr. (1966). The effect of cerebellar stimulation on neuronal activity in the motor cortex. *Experimental Neurology, 14 (3)*, 317-327.

Lidow, M. S. & Rakic, P. (1992). Scheduling of monoaminergic neurotransmitter receptor expression in the primate neocortex during postnatal development. *Cerebral Cortex, 2*, 401-416.

Lidow, M. S., Goldman-Rakic, P. S., & Rakic, P. (1991). Synchronized overproduction of neurotransmitter receptors in diverse regions of the primate cerebral cortex. *Proceedings of the National Academy of Sciences of the United States of America, 88*, 10218-10221.

Liljequist, S. & Engel, J. (1982). Effects of GABAergic agonists and antagonists on various ethanol-induced behavioral changes. *Psychopharmacology (Berlin), 78 (1)*, 71-75.

Linsenbardt, D. N., Moore, E. M., Gross, C. D., Golfarb, K. J., Blackman, L. C., & Boehm, S. L. (2009). Sensitivity and tolerance to the Hypnotic and Ataxic Effects of Ethanol in Adolescent and Adult C57BL/6J and DBA/2J Mice. *Alcoholism: Clinical and Experimental Research, 33*(3), 464-476.

Lister, R. G., & Linnoila, M. (1991). Alcohol, the chloride ionophore and endogenous ligands for benzodiazepine receptors. *Neuropharmacology, 30*(12B), 1435-1440.

Little, P. J., Kuhn, C. M., Wilson, W. A., & Swartzwelder, H. S. (1996). Differential effects of ethanol in adolescent and adult rats. *Alcoholism: Clinical and Experimental Research, 20*(8), 1346-1351.

Loeber, R. & Keenan, K., (1994). The interaction between conduct disorder and its comorbid conditions: Effects of age and gender. *Clinical Psychology Review*, *14*, 497-523.

Macrì, S., Adriani, W., Chiarotti, F., & Laviola, G. (2002). Risk taking during exploration of a plus-maze is greater in adolescent than in juvenile or adult mice. *Animal Behavior, 64*, 541-546.

Markwiese, B. J., Acheson, S. K., Levin, E. D., Wilson, S. A., & Swartzwelder, H. S. (1998). Differential effects of ethanol on memory in adolescent and adult rats. *Alcoholism: Clinical and Experimental Research, 22*(2), 416-421.

Matthews, D. B. & Silvers, J. L. (2004). The use of acute ethanol administration as a tool to investigate multiple memory systems. *Neurobiol. Learn. Mem., 82*, 299-308.

Matthews, D. B. & Simson, P. E. (1998). Prenatal exposure to ethanol disrupts spatial memory: effect of the training-testing delay period. *Physiology and Behavior, 64*, 63-67.

Matthews, D. B., Simson, P. E., & Best, P. J. (1995). Acute ethanol impairs the use of spatial memory but not the use of stimulus/response memory in the rat. *Alcoholism: Clinical and Experimental Research, 19*, 902-909.

McQueeny, T., Schweinsburg, B. C., Schweinsburg, A. D., Jacobus, J., Bava, S., Frank, L. R., et al. (2009). Altered white matter integrity in adolescent binge drinkers. *Alcoholism: Clinical and Experimental Research, 33 (7)*, 1278-1285.

Medina, K. L., McQueeny, T., Nagel, B. G., Hanson, K. L., Schweinsburg, A. D., & Tapert, S. F. (2008). Prefrontal cortex volumes in adolescents with alcohol use disorders: unique gender effects. *Alcoholism: Clinical and Experimental Research, 32*(3), 386-394.

Mehta, A. K., & Ticku, M. K. (1999). An update on GABAA receptors. *Brain Research Reviews, 29*, 196-217.

Mellor, H., & Parker, P. J. (1998). The extended protein kinase C superfamily. *Biochemical Journal, 332*, 281-292.

Metzger, F. & Kapfhammer, J. P. (2000). Protein kinase C activity modulates dendritic differentiation of rat purkinje cells in cerebellar slice cultures. *European Journal of Neuroscience, 12*, 1193-2005.

Mitsuyama, H., Little, K. Y., Sieghart, W., Devaud, L. L., & Morrow, A. L. (1998). GABA(A) receptor alpha1, alpha4, and beta3 subunit mRNA and protein expression in the frontal cortex of human alcoholics. *Alcoholism: Clinical and Experimental Research, 22*(4), 815-822.

Mohler, H. (2006). GABAA receptor diversity and pharmacology. *Cell Tissue Res., 326*, 505-516.

Nagel, B. J., Schweinsburg, A. D., Phan, V., & Tapert, S. F. (2005). Reduced hippocampal volume among adolescents with alcohol use disorders without psychiatric comorbidity. *Psychiatry Research: Neuroimaging, 139*, 181-190.

National Institute of Alcohol Abuse and Alcoholism. (2004). NIAAA council approves definition of binge drinking. *NIAAA Newsletter, 3*, 3.

Neely, E. K., Hintz, R. L., Wilson, D. M., Lee, P. A., Gautier, T., Argente, J., et al. (1995). Normal ranges for immunochemiluminometric gonadotropin assays. *Journal of Pediatrics, 127 (1)*, 40-46.

Nestler, E. J., Hyman, S. E., & Malenka, R. C. (2009). Molecular neuropharmacology: A foundation for clinical neuroscience (2nd ed.). New York, NY: The McGraw-Hill Companies, Inc.

Nie, Z., Schweitzer, P., Roberts, A. J., Madamba, S. G., Moore, S. D., & Siggins, G. R. (2004). Ethanol augments GABAergic transmission in the central amygdala via CRF1 receptors. *Science, 303* (5663), 1512-1514.

Nishizuka, Y. (1995). Protein kinase C and lipid signaling for sustained cellular responses. FASEB J., *9*, 484-496.

Odell, W. D. Sexual maturation in the rat. In: Grumbach, M. M., Sizonenko, P. C. and Aubert, M. L. (Eds.) Control of the Onset of Puberty, Philadelphia, PA: Lippincott Williams & Wilkins, 1990, pp. 183-210.

Olsen, R.W., & DeLorey, T.M. (1999). GABA and glycine. In: Siegel,G.J., Agranoff, B.W., Albers, R.W., Fisher, S.K., Uhler, M.D. (Eds.), Basic Neurochemistry. Lipincott Williams and Wilkins, Philadelphia, 335–346.

Parker, J., Mitchell, A., Kalpakidou, A., Walshe, M., Jung, H-Y., Nosarti, C., et al. (2008). Cerebellar growth and behavioural & neuropsychological outcome in preterm adolescents. *Brain, 131* (5), 1344-1351.

Pascual, M., Blanco, A. M., Cauli, O., Minarro, J., and Guerri, C. (2007). Intermittent ethanol exposure induces inflammatory brain damage and causes long-term behavioural alterations in adolescent rats. *Eur. J. Neurosci,* 25, 541-550.

Pascual, M., Boix, J., Felipo, V., & Guerri, C. (2009). Repeated alcohol administration during adolescence causes changes in the mesolimbic dopaminergic and glutamatergic systems and promotes alcohol intake in the adult rat. *Journal of Neurochemistry, 108*(4), 920-931.

Paus, T., Collins, D. L., Evans, A. C., Leonard, G., Pike, B., & Zijdenbos, A. (2001). Maturation of white matter in the human brain: a review of magnetic resonance studies. *Brain Res Bull, 54*(3), 255-266.

Perrin, J. S., Herve, P. Y., Leonard, G., Perron, M., Pike, G. B., Pitiot, A., et al. (2008). Growth of white matter in the adolescent brain: role of testosterone and androgen receptor. *J. Neurosci, 28*(38), 9519-9524.

Pfefferbaum, A., Mathalon, D. H., Sullivan, E. V., Rawles, J. M., Zipursky, R. B., & Lim, K. O. (1994). Frontal lobe volume loss observed with magnetic resonance imaging in older chronic alcoholics. *Alcohol. Clin. Exp. Res., 21*, 521-529.

Pfefferbaum, A., Sullivan, E. V., Mathalon, D. H., Shear, P. K., Rosenbloom, M. J., & Lim, K. O. (1995). Longitudinal changes in magnetic resonance imaging brain volumes in abstinent and relapsed alcoholics. *Alcoholism: Clinical and Experimental Research, 19 (5)*, 1177-1191.

Phillips, J. G., Ogeil, R. P., & Müller, F. (2009). Alcohol consumption and handwriting: a kinematic analysis. *Human Movement Science, 28 (5)*, 619-632.

Phillips, S. C., Harper, C. G., & Kril, J. (1987). A quantitative histological study of the cerebellar vermis in alcoholic patients. *Brain, 110 (pt 2)*, 301-314.

Pian, J. P., Criado, J. R., Walker, B. M., & Ehlers, C. L. (2008). Differential effects of acute alcohol on EEG and sedative responses in adolescent and adult Wistar rats. *Brain Research, 1194*, 28-36.

Pirker, S., Schwarzer, C., Wieselthaler, A., Sieghart, W., & Sperk, G. (2000). GABA(A) receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. *Neuroscience, 101*(4), 815-850.

Pleis, J. R., Lucas, J. W., & Ward, B. W. (2009). Summary health statistics for U. S. adults: national health interview sulrvey, 2008. National Center for Health Statistics. *Vital and Health Statistics, 10*(242).

Qi, Z., Song, M., Wallace, M. J., Wang, D., Newton, P. M., McMahon, T., Chou, W., Zhang, C., Shokat, K. M., & Messing, R. O. (2007). Protein kinase Cε regulates γ-aminobutyrate type A receptor sensitivity to ethanol and benzodiazepines through phosphorylation of γ2 subunits. *J. of Biol. Chem., 282*(45), 33052-33063.

Rajendran, P. & Spear, L. P. (2004). The effects of ethanol on spatial and nonspatial memory in adolescent and adult rats studied using an appetitive paradigm. *Ann. N. Y. Acad. Sci., 1021*, 441-444.

Reynolds, J. N., Prasad, A., & MacDonald, J. F. (1992). Ethanol modulation of GABA receptor-activated Cl- currents in neurons of the chick, rat and mouse central nervous system. *European Journal of Pharmacology, 224 (2-3)*, 173-181.

Ristuccia, R. C. & Spear, L. P. (2004). Adolescent ethanol sensitivity: hypothermia and acute tolerance. *Ann. N. Y. Acad. Sci., 1021*, 445-447.

Ristuccia, R. C. & Spear, L. P. (2005). Sensitivity and tolerance to autonomic effects of ethanol in adolescent and adult rats during repeated vapor inhalation sessions. *Alcoholism: Clinical and Experimental Research, 29*, 1809-1820.

Ristuccia, R. C. & Spear, L. P. (2008). Autonomic responses to ethanol in adolescent and adult rats: a dose-response analysis. *Alcohol, 42*(8), 623-629.

Saito, N., Kikkawa, U., Nishizuka, Y., & Tanaka, C. (1988). Distribution of prontein kinase C-like immunoreactive neurons in rat brain. *Journal of Neuroscience, 8*, 369-382.

Saito, N. & Shirai, Y. (2002). Protein kinase Cγ (PKCγ): function of neuron specific isotype. *J. Biochem, 132,* 683-687.

Sanna, E., Talani, G., Busonero, F., Pisu, M. G., Purdy, R. H., Serra, M., Biggio, G. (2004). Brain steroidogenesis mediates ethanol modulation of GABAA receptor activity in rat hippocampus. *J. Neurosci 24*(29), 6521-6530.

Schmahmann, J. D., MacMore, J., & Vangel, M. (2009). Cerebellar stroke without motor deficit: clinical evidence for motor and non-motor domains within the human cerebellum. *Neuroscience, 162* (3), 852-861.

Schrenk, K., Kapfhammer, J. P., & Metzger, F. (2001). Altered dendritic development of cerebellar purkinje cells in slice cultures from protein kinase Cγ-deficient mice. *Neuroscience, 110 (4)*, 675-689.

Sieghart, W. (1995). Structure and pharmacology of gamma-aminobutyric acidA receptor subtypes. *Pharmacological Reviews, 47*, 181-234.

Silveri , M. M., & Spear, L. P. (2000). Ontogeny of ethanol elimination and ethanol-induced hypothermia. Alcohol 20(1): 45-53.

Silveri, M. M., & Spear, L. P. (2001). Acute, rapid, and chronic tolerance during ontogeny: observations when equating ethanol perturbation across age. Alcohol Clin Exp Res 25(9): 1301-1308.

Simon, J., Wakimoto, H., Fugita, N., Lalande, M., & Barnard, E. A. (2004). Analysis of the set of GABAA receptor genes in the human genome. *Journal of Biological Chemistry, 279*, 41422-41435.

Sizonenko, P. C., Burr, I. M., Kaplan, S. L., & Grumbach, M. M. (1970). Hormonal changes in puberty: II. Correlation of serum luteinizing hormone and follicle stimulating hormone with stages of puberty and bone age in normal girls. *Pediatric Research, 4*, 36.

Slawecki, C. J., Betancourt, M., Cole, M., & Ehlers, C. L. (2001). Periadolescent alcohol exposure has lasting effects on adult neurophysiological function in rats. *Brain Research. Developmental Brain Research, 128 (1)*, 63–72.

Song, M. & Messing, R. O. (2005). Protein kinase C regulation of $GABA_A$ receptors. *Cellular and Molecular Life Sciences, 62*, 119-127.

Sowell, E. R., Delis, D., Stiles, J., & Jernigan, T. L. (2001). Improved memory functioning and frontal lobe maturation between childhood and adolescence: a structural MRI study. *J. Int. Neurophychol. Soc., 7*(3), 312-322.

Sowell, E. R., Thompson, P. M., Leonard, C. M., Welcome, S. E., Kan, E., & Toga, A. W. (2004). Longitudinal mapping of cortical thickness and brain growth in normal children. *Journal of Neuroscience, 24*(38), 8223-8231.

Spear L. P. (2007). Assessment of adolescent neurotoxicity: rationale and methodological considerations. *Neurotoxicology and Teratology, 29*, 1-9.

Spear, L. P. & Brake, S. C. (1983). Periadolescence: Age-dependent behavior and psychopharmacological responsivity in rats. *Devel. Psychobiol., 16,* 83-109.

Spear, L. P. & Varlinskaya, E. I. (2005). Adolescence. Alcohol sensitivity, tolerance, and intake. *Recent Developments in Alcoholism, 17*, 143-159.

Spear, L. P. (2000). The adolescent brain and age-related behavioral manifestations. *Neurosci. Behav. Physiol., 24*, 417-463.

Steinberg, L., Albert, D., Cauffman, E., Banich, M., Graham, S., & Woolard, J. (2008) Age differences in sensation seeking and impulsivity as indexed by behavior and self-report: evidence for a dual systems model. *Developmental Psychology, 44*(6), 1764-1778.

Substance Abuse and Mental Health Services Administration. (2009). Results from the 2008 national survey on drug use and health: national findings. (Office of Applied Studies, NSDUH Series H-36, HHS Publication No. SMA 09-4434). Rockville, MD.

Substance Abuse and Mental Health Services Administration. (2010). Results from the 2009 national survey on drug use and health: volume I. Summary of national findings (Office of Applied Studies, NSDUH Series H-38A, HHS Publication No. SMA 10-4856 Findings). Rockville, MD.

Sullivan, E. V., Rohlfing, T., & Pfefferbaum, A. (2010). Pontocerebellar volume deficits in alcoholic men and women: no evidence for "telescoping." *Psychopharmacology, 208*, 279-290.

Swartzwelder, H. S., Richardson, R. C., Markwiese-Foerch, B., Wilson, W. A., Little, P. J. (1998). Developmental differences in the acquisition of tolerance to ethanol. *Alcohol, 15*(4), 311-314.

Taffe, M. A., Kotzebue, R. W., Crean, R. D., Crawford, E. F., Edwards, S., & Mandyam, C. D. (2010). Long-lasting reduction in hippocampal neurogenesis by alcohol consumption in adolescent nonhuman primates. *PNAS, 107*(24), 11104-11109.

Thompson, S. A., Whiting, P. J., & Wafford, K. A. (1996). Barbiturate interactions at the human GABAA receptor: dependence on receptor subunit composition. *British Journal of Pharmacology, 117 (3)*, 521-527.

Tiemeier, H., Lenroot, R. K., Greenstein, D. K., Tran, L., Pierson, R., & Giedd, J. N. (2010). Cerebellum development during childhood and adolescence: a longitudinal morphometric MRI study. *NeuroImage, 49*(1), 63-70.

Tominaga, S., Saito, N., Tsujino, T., & Tanaka, C. (1993). Immunocytochemical localization of α-, βI-, βII-, and γ-subspecies of protein kinase C in the motor and premotor cortices of the rhesus monkey. *Neuroscience Research, 16 (4)*, 275-286.

Utsunomiya, H., Takano, K., Okazaki, M., & Mitsudome, A. (1999). Development of the temporal lobe in infants and children: analysis by MR-based volumetry. *American Journal of Neuroradiology, 20 (4)*, 717-723.

Van Skike, C. E., Botta, P., Chin, V. S., Tokunaga, S., McDaniel, J. M., Venard, et al. (2010). Behavioral effects of ethanol in cerebellum are age dependent: potential system and molecular mechanisms. Alcoholism: Clinical and Experimental Research, *34*(12), 2070-2080.

VanDoren, M. J., Matthews, D. B., Janis, G. C., Grobin, A. C., Devaud, L. L., & Morrow, A. L. (2000). Neuroactive steroid 3alpha-hydroxy-5alpha-pregnan-20-one modulates electrophysiological and behavioral actions of ethanol. *J. Neurosci., 20*(5), 1982-1989.

Varlinskaya, E. I. & Spear, L. P. (2002). Acute effects of ethanol on social behavior of adolescent and adult rats: role of familiarity of the test situation. *Alcoholism: Clinical and Experimental Research, 26,* 1502-1511.

Wafford, K. A., Burnett, D. M., Leidenheimer, N. J., Burt, D. R., Wang, J. B., Kofuji, P., et al. (1991). Ethanol sensitivity of the GABAA receptor expressed in xenopus oocytes requires 8 amino acids contained in the gamma 2L subunit. *Neuron, 7*, 27-33.

Walker, B. M. & Ehlers, C. L. (2009). Age-related differences in the blood alcohol levels of wistar rats. *Pharmacology, Biochemistry, and Behavior, 91* (4), 560-565.

Walker, B. M., Walker, J. L., & Ehlers, C. L. (2008). Dissociable effects of ethanol consumption during the light and dark phase in adolescent and adult wistar rats. *Alcohol, 42*(2), 83-89.

Watson, P. E., Watson, I. D., & Batt, R. D. (1980). Total body water volumes for adult males and females estimated from simple anthropometric measurements. *The American Journal of Clinical Nutrition, 33*, 27-39.

Weiner, J. L., Ariwodola, O. J., Bates, W. H., Bryant, V., Silberman, Y., Daunais, J. B., et al. (2005). Presynaptic mechanisms underlying ethanol actions at GABAergic synapses in rat and monkey hippocampus. *Alcoholism: Clinical and Experimental Research, 29*, 187A.

Weiner, J. L., Zhang, L., & Carlen, P. L. (1994). Potentiation of GABAA-mediated synaptic current by ethanol in hippocampal CA1 neurons: possible role of protein kinase C. *Journal of Pharmacology and Experimental Therapeutics, 268*(3), 1388-1395.

Weiss, D. S. (1992). Actions of ethanol on gamma-aminobutyric acid-activated chloride channels. *Journal of the Florida Medical Association, 79 (7)*, 473-475.

Welsh, J.P., Yuen, G., Placantonakis, D.G., Vu, T.Q., Haiss, F., O'Hearn, E., et al. (2002). Why do Purkinje cells die so easily after global brain ischemia? Aldolase C, EAAT4, and the cerebellar contribution to posthypoxic myoclonus. *Advances in Neurology, 89,* 331–359.

White, A. M., Truesdale, M. C., Bae, J. G., Ahmad, S., Wilson, W. A., Best, P. J., & Swartzwelder, H. S. (2002). Differential effects of ethanol on motor coordination in adolescent and adult rats. *Pharmacology, Biochemistry, and Behavior, 73,* 673-677.

Whiting, P. J., McAllister, G., Vassilatis, D., Bonnert, T. P., Heavens, R. P., Smith, D. W., et al. (1997). Neuronally restricted RNA splicing regulates the expression of a novel GABAa receptor subunit conferring atypical functional properties. *J. Neurosci., 17*(13), 5027-5037.

Winter, J. S. D., & Faiman, C. (1972). Pituitary-gonadal relations in male children and adolescents. *Pediatric Research, 6*, 126-135.

Wisden, W., Laurie, D. J., Monyer, H., & Seeburg, P. H. (1992). The distribution of 13 GABAA receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. *J. Neuroci., 12*, 1040-1062.

Witelson,S. F., Beresh, H., & Kigar, D. L. (2006). Intelligence and brain size in 100 postmortem brains: sex, lateralization and age factors. *Brain, 129*, 386-398.

Yu, Z. Y., Wang, W., Fritschy, J. M., Witte, O. W., & Redecker, C. (2006). Changes in neocortical and hippocampal GABAA receptor subunit distribution during brain maturation and aging. *Brain Research, 1099*, 73-81.

Zezula, J., Slany, A., & Sieghart, W. (1996). Interaction of allosteric ligands with GABAA receptors containing one, two, or three different subunits. *Eur. J. Pharmacol, 301*, 207-214.