

EFFECTS OF REPEATED ETHANOL WITHDRAWAL AND
STRESS/WITHDRAWAL PARADIGMS IN ADOLESCENT RATS

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ABSTRACT

Tiffany A. Wills: Effects of Repeated Ethanol Withdrawals and Stress/Withdrawal
Paradigms in Adolescent Rats
(Under the Direction of Dr. George R. Breese)

Adolescence is a period of development that is marked with increased vulnerability to the use and abuse of alcohol. Many studies have illustrated that adolescents respond to ethanol in ways that are distinct from adults. The adult literature has established the importance of understating the negative affect (i.e anxiety) produced from alcohol withdrawal and how the cyclic nature of ethanol exposure can modulate its development. Initial studies showed that adolescent and adult rats seem to display similar withdrawal-related behaviors (anxiety and seizure thresholds) following repeated withdrawals once corrections were made for differences in ethanol intake. This anxiety-like behavior was shown to be sensitized by repeated ethanol withdrawals in adolescent rats, as was previously demonstrated in adult rats. However, this anxiety-like behavior in adolescent rats was much longer lasting than in adult rats. Additional work was conducted to determine the role of stress in the development of this anxiety-like behavior. Stress was shown to substitute for these early withdrawals and sensitize anxiety-like behavior in adolescent rats. In contrast to the effects of repeated withdrawals, the anxiety-like behavior of this stress/withdrawal paradigm was not long lasting. The reduced effect of stress in adolescents was also produced when assessing acute stress. A common mechanism between for both stress and ethanol actions may be related to corticotrophin releasing factor (CRF). Dose-response studies illustrated that CRF could substitute for

early stress/withdrawal episodes and produce anxiety-like behavior. The dose required to produce this effect was higher in adolescents than adults, which suggested a reduced sensitivity to CRF. The reduced sensitivity to stress and CRF in adolescents may be due to higher basal CRF levels found in adolescent rats. Finally, it was illustrated that repeated withdrawals decreased CRF immunoreactivity within the central nucleus of the amygdala only in adolescent rats. This work illustrates that adolescents are equally and sometimes more vulnerable to effects of repeated ethanol withdrawal. Further, there is a clear interaction of stress and CRF in this process. Therefore, this work would encourage the development and use of treatments that focus on the modulation of this system in adolescents.

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LIST OF ABBREVIATIONS

5-HT	serotonin
5-HT _{1A}	serotonin type 1A receptor
ACTH	adrenocorticotrophic hormone
ANOVA	analysis of variance
ASR	acoustic startle response
AUD	alcohol use disorder
BEC	blood ethanol concentration
BNST	bed nucleus of the stria terminalis
Bus	bupirone
CD	control diet
CeA	central nucleus of the amygdala
CON	continuous ethanol exposure; 15 days of ethanol diet
CP	CP-154,526
CRF	corticotrophin releasing factor
CRF ₁ R	corticotrophin releasing factor type 1 receptor
CRF ₂ R	corticotrophin releasing factor type 2 receptor
CRF-BP	corticotrophin releasing factor binding protein
CY1	single cycle; 5-days of ethanol diet
CY3	three cycles; three 5-day cycles of ethanol diet
D ₁	dopamine type 1 receptor

D ₂	dopamine type 2 receptor
D ₄	dopamine type 4 receptor
db	decibel
dIBNST	dorsal lateral portion of the bed nucleus of the stria terminalis
DSM IV	diagnostic and statistical manual of mental disorders fourth version
ED	ethanol diet
EEG	electroencephalography
Flum	flumazenil
GABA	gamma-Aminobutyric acid
H	Hour
HPA	hypothalamic-pituitary-adrenal axis
icv	intracerebroventricular
ip	intraperitoneal
IPSC	inhibitory postsynaptic current
IR	immunoreactivity
LTP	long-term potentiation
min	minute
mIPSC	mini inhibitory postsynaptic currents
mRNA	messenger ribosomal nucleic acid
NAc	nucleus accumbens
NMDA	N-methyl-D-aspartic acid
P	postnatal day
P rat	alcohol preferring rat

PCP	phencyclidine
pERK	phosphorylated extracellular signal-regulated kinase 1/2
PFC	prefrontal cortex
PPI	pre-pulse inhibition
PVN	paraventricular nucleus
SEM	standard error of the mean
SI	social interaction
sIPSC	spontaneous inhibitory postsynaptic currents
Str	stress
Veh	vehicle

CHAPTER I: GENERAL INTRODUCTION

Alcoholism is defined as a maladaptive pattern of alcohol consumption that results in impairment or distress in the user. Patients are diagnosed with alcohol dependence based on the presentation of 3 or more of the following criteria outlined by the DSM-IV (1994): tolerance, withdrawal, drinking longer than intended, inability to cut down/stop drinking, large amount of time spent to acquire alcohol, stopping or reducing important activities, and continued use in spite of physical or psychological problems caused by alcohol. Alcohol dependence and/or abuse have 7-8% prevalence in the population within a 12 month period and 18% prevalence throughout the lifetime (Grant & Harford, 1995). The annual economic cost associated with alcohol use disorders is estimated at about 184.6 billion (Harwood, 1998). It is also the second most common psychiatric disorder in the United States (Kessler et al., 1994). Therefore, understanding the mechanisms that underlie alcoholism is of critical importance.

Much of the research on this disease has focused on understanding the mechanisms of alcohol use in adults, a time when alcoholism is commonly diagnosed. However, it is known that problematic patterns of alcohol use are apparent long before a clinical diagnosis. The information presented here will demonstrate that most use of alcohol is initiated during adolescence and use during this time can have different consequences than alcohol use in adults. Further, alcohol use during this early period has effects that persist into adulthood. However, there are still gaps in our understanding of the effects of alcohol in adolescents. The adult literature has identified some fundamental

issues in alcoholism that will be identified herein and these issues will provide the framework for the work that remains in adolescents. This summary emphasizes the importance of understanding the effects of alcohol use during adolescence for future treatment and prevention of alcoholism.

Adolescence

Adolescence is a time of transition between childhood and adulthood and is, therefore, not confined to strict age limits. Instead, adolescence is characterized by hormonal, behavioral, and neurological changes which seem to be common in a number of species. This time frame includes but is not restricted to the onset of puberty and development of sexual maturity. In humans, the age range for adolescence has typically been defined as 9 to 18 years old (Buchanan et al., 1992) but others feel that people in their early 20s could also be included in this group (Baumrind, 1987). Similar age ranges have also been established in rodents from P28-42 (Spear, 2000) although again this range can vary.

One of the factors included in this period is puberty. In rodents, mature diurnal gonadotropin cycling can be seen at P28 while sexual maturity is typically not complete until P60 in males and P38 in females (Ojeda & Urbanski, 1994). These data illustrate that gender is an additional source of variability with males generally maturing more slowly than females. Another factor also frequently associated with adolescence is a rapid increase in growth (growth spurt), which can be seen in both humans and rodents (Frisch, 1984; Kennedy & Mirta, 1963).

Various other changes are also taking place in the brain during adolescence. There is an overall peak in grey matter volume during adolescence that declines into

adulthood (Giedd et al., 1999; Giedd, 2004). This effect is caused, in part, by the overproduction of neuronal axons and dendrites in early adolescence followed by the pruning of synapses later in adolescence (Andersen et al., 2000; Andersen & Teicher, 2004; Giedd et al., 1999). Some regions that undergo the most reorganization are the prefrontal cortex (PFC; Sowell et al., 1999, 2001; Van Eden et al., 1990), hippocampus, amygdala (Zehr et al., 2006), nucleus accumbens (NAc; Tarazi et al., 1998; Teicher et al., 1995), and hypothalamus (Choi & Kellogg, 1992; Choi et al., 1997). In the PFC, there are large decreases in glutamatergic synapses (Huttenlocher, 1984; Zecevic et al., 1989), which likely contribute to the decline in grey matter. More specifically, there is a loss of up to 1/3 of NMDA receptor binding from P28-60 (Insel et al., 1990). While glutamate synapses are being reduced, dopamine innervations to the PFC are increased in both nonhuman primates (Rosenberg & Lewis, 1994; 1995) and rats (Kalsbeek et al., 1988; Leslie et al., 1991). Additionally, cholinergic inputs into the PFC also increase during adolescence (Gould et al., 1991). The hippocampus also undergoes significant pruning of glutamatergic synapses, which is illustrated by a decrease of 1/4 of the NMDA receptors within the hippocampal pyramidal region between P28-60 (Insel et al., 1990). These data illustrate that there are large degrees of regional specific reorganization that occur during adolescence.

In addition to the regional changes in glutamate described above, there are also more global changes in this neurotransmitter system. Glutamatergic function throughout the brain declines during adolescence and results in a general loss of excitatory tone. This effect was described above in the PFC and hippocampus but can also be found in the NAc (Frantz & Van Hartesveldt, 1999). This decrease in excitatory tone (especially loss

of NMDA receptors) is most likely responsible for changes in long term potentiation (LTP) induction found during this period. Schramm et al. (2002) illustrated that LTP was more frequently induced in the NAc of adolescent mice compared to their adult counterparts. These differences in synaptic plasticity (through induction of LTP) could lead to enhanced learning and memory processes during this period. While this enhancement of learning and memory are beneficial in most instances, this enhancement could also potentially effect negative/pathological learning processes (like addiction).

GABA function is also modulated during adolescence, mainly through activation of GABA_A receptors. It has been shown that there is a steady increase in GABA_A expression into adulthood (P60; Behringer et al., 1996; Xia & Haddad, 1992). However, in certain brain regions it was illustrated that during early adolescence (P20-28) there is a plateau in the rate of GABA_A receptor expression (measured by zolpidem binding) followed by a rapid increase during later adolescence (P36-60; Moy et al., 1998). These data suggest that expression of GABA_A receptors may be distinct during different stages of adolescence.

GABA_A receptors can be composed of a wide-range of subunit combinations, which can change their pharmacological and electrophysiological properties. During adolescence, these subunit combinations can vary with age and affect the functional response of the GABA_A receptor. The synaptic GABA_A receptor is composed of two α , two β , and one γ subunits, while the extrasynaptic GABA_A receptor contains a δ subunit instead of γ . Both synaptic and extrasynaptic receptors modulate GABA transmission in different ways and would lead to different behavioral phenotypes. Electrophysiological studies in hippocampal slices showed that tonic current mediated through extrasynaptic

GABA_A receptors was larger in the dentate gyrus of the hippocampus from adult rats compared to their adolescent counterparts (Fleming et al., 2007). This effect is most likely due to the developmental increase in expression of the δ subunit within this region (Laurie et al., 1992). Further, various splice variants of the α subunit are also known to change in expression throughout development. Alpha 1 subunits peak during adolescence and then slowly decline into adulthood (P90), while α_3 subunits decline later (9 months) and the α_2 subunit remains stable within the frontal cortex (Yu et al., 2006). Alpha 5 subunits are high postnatally but then decline through adolescence into adulthood in the frontal cortex (Yu et al., 2006).

Further alterations can also be found in the transition from adolescence to adulthood when dopamine is considered. In the frontal cortex, hippocampus, and entorhinal cortex, there is an increase in D₁, D₂, and D₄ receptors that peaks in mid-adolescence and then remains stable throughout adulthood (Tarazi & Baldessaeini, 2000). In the striatum and NAc, however, there is a reduction in 1/3 of dopamine receptors during adolescence (Tarazi & Baldessaeini, 2000; Teicher et al., 1995). Dopamine transporters in striatum, on the other hand, steadily increase into adulthood (Tarazi et al., 1998). These neurological changes, along with many others not discussed here, are responsible for the behavioral phenotypes that differ between adolescents and adults.

A number of these behavioral phenotypes have been evaluated during adolescence and adulthood. One of the behaviors increased during adolescence is peer related interactions. In fact in humans, adolescents spend about 33% of their waking time communicating with peers (only 8% talking to adults) in a given week during the school year (Csikszentmihalyi et al., 1977). While it is well known that human adolescents

display this increase in peer-related interaction, the same is also true in rodents. Adolescent rats have increased social interaction (Primus & Kellogg, 1989) and play behavior (Spear, 2000) compared to their adult counterparts. This transition from family focused interactions to peer focused interactions is likely a way for adolescents to develop the independence that they will need in adulthood (Spear, 2000).

Another behavior that is increased during this period is risk taking. Data illustrated that 80% of adolescents had one or more of the following problems in the last month: disobeying parents, school misconduct, drug use, or antisocial behavior (theft or fighting; Maggs et al., 1995). Additionally, a review on antisocial behavior determined that increases in these behaviors during adolescence are so common that they seem to be the norm (Moffitt, 1993). Clinical data also showed that a moderate amount of drug taking (a type of risk taking behavior) is not necessarily associated with negative outcomes. In fact, these moderate drug users were found to be more socially competent, than abstainers or frequent drug users (Shedler & Block, 1990). However, increases in risk taking behaviors also do have detrimental consequences. For example, 85% of deaths during adolescence can be attributed to homicides, suicides, or accidents (Irwin, 1989). The following sections will evaluate both the short term and long term consequences of one of these risky behaviors (alcohol use) during adolescence.

Adolescence and Alcohol

Adolescence is known to be a time of increased risk taking behaviors, which includes increased use of alcohol. Recent statistics showed that 73% of high school students had used alcohol by the time they graduated (Johnston et al., 2007). Of considerable concern is the fact that the strongest predictor of alcohol dependence in

adulthood is the use of alcohol before the age of 14 (Grant, 1998). Not only is experimentation prevalent but 30% of 12th graders admitted to have engaged in heavy drinking (5+ drinks in one occasion) in the last month. It is estimated that 6% of adolescents drink in patterns that qualify for alcohol abuse or dependence (Rohde et al., 1996). Furthermore, the dependency course (time between initial use and dependence) is more rapid in adolescents than adults (Clark et al., 1998). These and other clinical data make it evident that understanding the effects of alcohol use and abuse during this period is critical.

Acute Effects of Alcohol during Adolescence

Basic research in rats has demonstrated clear differences between adolescent and adult rats in their responses to acute ethanol administration. For example, adolescents have lower sensitivity to the sedative (Little et al., 1996; Silveri & Spear, 1998), hypothermic (Silveri & Spear, 2000), and motor impairing (White et al., 2002) effects of acute ethanol injections compared to adults. These differences do not seem to be due to differences in ethanol metabolism between adolescent and adult rats. Little et al. (1996) illustrated that a more rapid recovery of the righting reflex (an index of sedation) was also accompanied by a higher blood ethanol concentration at the time of recovery in adolescents compared to adult rats. Additionally, Silveri and Spear (2000) found that rats at P16 had slower metabolism but that by P26 (within the adolescent age range) ethanol metabolism was not different from adults.

A mechanism that might account for this general insensitivity to ethanol's sedative properties during adolescence is a change in the transmission of GABA during development. Activation of the GABA_A receptor is a main contributor to the sedative

properties of ethanol. Previously, it was illustrated that changes in expression and subunit composition of this receptor change during this period of development. Silveri & Spear (2002) conducted experiments to determine whether pretreatment with muscimol (GABA_A receptor agonist) or (+) MK-801 (NMDA antagonist) could increase the sedative properties of ethanol in adolescent and adult rats. They found that (+)MK-801 increased the sedative properties of ethanol but there was no age-related difference. However, treatment with muscimol enhanced the sedative effects of ethanol in adolescent but not adult rats (Silveri & Spear, 2002). Therefore, the data indicated that ethanol had a reduced ability to enhance GABA_A function in adolescent rats compared to adults and that this effect is most likely responsible for the difference in sedative properties of ethanol between ages. Additional support was provided with studies of GABA_A-mediated inhibitory post-synaptic currents (IPSCs) from hippocampal whole cell recordings in juvenile (2 wks), adolescent (4wks), and adult (16wks) rats. In these recordings, greater ethanol-induced enhancement of IPSCs was demonstrated in adults compared to both juvenile and adolescent rats (Li et al., 2003). Later work from this group went on to show that this developmental effect of ethanol was specific to spontaneous IPSCs (sIPSCs) frequency but did not effect miniature IPSCs (mIPSCs; Li et al., 2006). The authors conclude that this result indicates a presynaptic mechanism mediated either through interneurons or changes in subunit composition. Regardless of the specific mechanism, these data illustrate that the reduced sedative effects of ethanol in adolescents is most likely a result from a developmentally regulated increase in ethanol-induced GABA_A function.

The previous work described a reduced sensitivity to some of the effects of ethanol in adolescents; however, there are also effects where adolescents are more sensitive. These behaviors include both ethanol induced memory impairment and social facilitation (Markwiese et al., 1998; Varlinskaya & Spear 2002; 2006). Further, the effects of ethanol-induced memory impairment were not due to differences in learning, which were the same in the absence of ethanol administration (White & Swartzwelder, 2004). The mechanisms underling this response are most likely related to the effect of ethanol on the NMDA receptor. It is generally accepted that the processes of learning and memory involve LTP in the hippocampus, which is mediated through activation of the NMDA receptors. Ethanol is known to more potently inhibit NMDA receptors in the hippocampus of adolescents compared to adults (Swartzwelder et al., 1995b). This increased inhibition in adolescent rats would thereby decrease NMDA's ability to induce LTP. NMDA-mediated LTP disruption by ethanol in hippocampal slices was more inducible in adolescents than adults (Pyapali et al. 1999; Swartzwelder et al., 1995a). These data indicated that the increased ability of ethanol to induce memory impairment in adolescents is most likely due to the increased effect of ethanol on NMDA receptors at this age. Similar to the work with the developmental regulation of the GABA_A receptor, these differences in the effect of ethanol could be caused by differences in subunit composition or expression.

These studies of the acute effects of ethanol during adolescence have illustrated that adolescent rats do not respond to ethanol in the same way as adult rats on many indices. Additionally, this work showed that adolescents may be uniquely vulnerable to alcohol dependence. It was illustrated that adolescents are less sensitive to the sedative

and motor impairing effects of ethanol. With these reduced sensitivities, adolescents would be able to consume much larger quantities of alcohol before feeling the adverse effects of alcohol. Furthermore, adolescents would be able to drink more alcohol before they “pass out”, which increases the risk for alcohol poisoning and alcohol-related fatalities. The above studies also illustrated that adolescents are more sensitive to the memory impairing effect of ethanol. Therefore, less alcohol is required to impair memory in adolescents. This observation coupled with the fact that adolescents are most likely consuming more alcohol produces a very dangerous situation.

Chronic Effects of Alcohol during Adolescence

In addition to the work on the acute effects of ethanol, research has also been conducted on the effects of more chronic ethanol use during adolescence. A number of studies have been performed where adolescent or adult rats are given chronic ethanol treatments and then tested when all rats are adults. They then looked at the effects of this ethanol history on future ethanol responses. These studies found that the differences in sensitivity to acute ethanol displayed by adolescents (described under acute effects) can also be maintained into adulthood (Slawecki, 2002; White et al., 2000; 2002). For example, White et al. (2000) found that adult rats treated chronically as adolescents showed greater ethanol-induced working memory impairments relative to adults chronically treated as adults. Similar ethanol treatments during adolescence resulted in reduced sensitivity to motor impairments that persisted into adulthood (White et al., 2002). Further, alcohol preferring (P) rats exposed to ethanol during adolescence but not in adulthood affected later ethanol operant responding. That is, adolescent exposure in P rats tested as adults led to more rapid acquisition of ethanol responding, greater resistance

to extinction, and increased responding to ethanol upon re-exposure (Rodd-Henricks et al., 2002a/b). Finally, Crews et al. (2000) showed that a 4-day binge ethanol exposure caused brain damage in select regions that does not appear in adults. This evidence indicated that ethanol treatment during the adolescent period can affect future responses to ethanol as adults.

Clinical studies also investigated the effects of chronic adolescent alcohol use on brain damage and learning/memory deficits. In these experiments, patients were either adolescents or young adults with history of alcohol use disorder (AUD) or those without. De Bellis and colleagues (2000) illustrated with MRI that subjects with a history of AUD had decreased hippocampal volume, which was also correlated with the onset and duration of alcohol use. Later work also showed that white matter was decreased in the corpus collosum of adolescents with AUD (Tapert et al., 2003). Further, subjects with AUD as adolescents showed a decrease in delayed memory function by 10% three weeks into abstinence (Brown et al., 2000).

These studies have illustrated two key points. One point is that chronic ethanol use during adolescence can effect the way that adults respond to ethanol. Importantly, this exposure causes adults to display sensitivities to ethanol like those seen in adolescents. The section above (acute effects of ethanol) describes why these changes in the sensitivity to ethanol can have negative consequences. These data reveal that adolescent alcohol use can cause adult rats to be more vulnerable to ethanol dependence. The second point illustrated by these data is that alcohol use during adolescence can cause damage to the brain and lead to cognitive deficits. While this damage may impact adult vulnerability to alcohol dependence, it could also lead to problems in many other

aspects of life. Overall these studies have shown that ethanol use during adolescence can produce long-lasting changes in the brain that affect adult behavior.

Stress-Induced Effects during Adolescence

In addition to the differential response to ethanol seen in adolescents, their responses to stress are also different from adults. Given the long standing appreciation for the importance of stress in alcoholism (Sinha, 2001), consideration of this issue in adolescents is key. It was demonstrated that adolescents are more vulnerable to acute stress (greater immobility in forced swim and intermittent foot shock tests; Spear, 2000; Walker et al., 1995). Also, administration of chronic stress during this time can produce a blunted ACTH response to future stress (Goliszek et al., 1996). Furthermore, in mice it has been illustrated that responses to stress (decreased weight gain and increased anxiety) were more enduring when stress was experienced during adolescence (Stone et al., 1997). Stress may also have complex interactions with alcohol consumption, for example, it was demonstrated that adolescents had significantly increased ethanol consumption compared to adults in response to foot shock stress (Siegmund et al., 2005). Clinical data also illustrated that the second largest predictor for ethanol consumption during adolescence is perceived stress (Wagner, 1993). These observations indicate that adolescents have increased vulnerability to stress, which may affect their responses to ethanol.

Alcoholism in Adults

The previous section characterized the differences between adolescents and adults in their response to ethanol and illustrated the increased vulnerability to the effects of ethanol during adolescence. However, many gaps still remain in our understanding of how alcohol use during this period can affect the progression of alcoholism. The

following discussion will introduce some key hypotheses and mechanisms of relevance. These data focus on research conducted in adults and will provide a framework for work that remains to be conducted in adolescent populations.

Tension Reduction Hypothesis

Alcoholism is known to be a chronic relapsing disorder. The progress from alcohol use to dependence can be modulated by systems which regulate the positive reinforcing effects of ethanol and those controlling the negative reinforcing effects. The reliance on these different types of reinforcement is thought to change throughout the process of ethanol dependence. Koob and Le Moal (1997) presented a model to explain these changes, as a process of hedonic homeostatic dysregulation. They outlined three stages of the addiction process: preoccupation/anticipation, binge/intoxication, and withdrawal/negative affect. During transition to ethanol dependence, there is a shift in the motivational drive for ethanol use. The beginning stages of ethanol use are driven primarily by the positive reinforcing effects of ethanol (pleasurable effects from ethanol). However, as addiction progresses, it is thought that the negative reinforcing properties of the ethanol (withdrawal/negative affect) are the motivational force behind the compulsive ethanol intake.

One of the early hypotheses on alcohol addiction was the tension reduction hypothesis (Cappell & Herman, 1972). This hypothesis stated that alcoholics were motivated to drink in order to reduce the negative symptoms associated with ethanol withdrawal. Early work focused on the outwardly negative physical symptoms of withdrawal (tremors, seizure, nausea, and delirium) and proposed that alcoholics maintained drinking to prevent these symptoms (Cappell & Herman, 1972). However,

later research indicated that this was not always the case. It was shown that less than 25% of patients continued to drink in order to alleviate physical symptoms of withdrawal (Hershon, 1977). Additionally, the physical symptoms of withdrawal typically last for only 12-72 hrs (Mello and Mendelson, 1972) but craving is reported in alcoholics for months (Roelofs, 1985). These data seemed to discount the original tenet of this hypothesis, which stated that alcoholics drank to alleviate the symptoms of withdrawal.

Later work, however, illustrated that other symptoms were more persistent, such as increased negative affect (depression and anxiety) that also accompanies ethanol withdrawal (De Solo et al., 1985; Roelofs, 1985). It was illustrated that 80% of alcoholics cite that relapse in drinking was due to depressed mood or anxiety (Hershon, 1977). Therefore, a modified version of the tension reduction hypothesis is that alcoholics maintain drinking or relapse to alleviate the negative affect associated with ethanol withdrawal. This hypothesis also incorporates the influence of stress (tension), which can promote these negative mood states or be a source of ethanol intake on its own. Early studies illustrated that acute stress increased alcohol consumption in social drinkers (Higgins and Marlatt, 1975). Furthermore, stress is known to induce alcohol craving and promote relapse (Sinha, 2001). Later sections will also illustrate the interactions between stress and ethanol withdrawal and suggest possible mechanisms.

Kindling Hypothesis

Another hypothesis has been put forth to emphasize the importance of ethanol intake patterns, specifically cyclic ethanol intake and subsequent withdrawal. The kindling hypothesis helps to explain a progressive worsening that occurs with chronic ethanol use. Kindling has been defined as the process in which the threshold to induce a

seizure is decreased following repeated stimulations (Goddard, 1967; Goddard et al., 1969). Ballinger and Post (1978) observed a positive association between the number of previous detoxifications from ethanol and the severity of seizures during withdrawal in the clinical population. They proposed that this enhancement of seizure susceptibility from repeated ethanol withdrawal could be caused by a “kindling”-like process. Later work (Brown et al., 1988) provided clinical support for this kindling hypothesis of ethanol withdrawal seizures. Rodent models also illustrated that increased seizure susceptibility could be produced from repeated ethanol withdrawals (Becker & Hale, 1993; Kokka et al., 1993; McCown & Breese, 1990).

Later work in animals examined whether repeated ethanol withdrawals could also sensitize (“kindle”) anxiety-like behavior, a form of negative affect discussed above. These studies showed that if rats were given repeated ethanol withdrawals, then they exhibited a decrease in social interaction (a validated index of increased anxiety-like behavior). In contrast, rats that received no ethanol or ethanol in a continuous fashion (only a single withdrawal episode) did not display this anxiety-like behavior (Overstreet et al., 2002). These data indicate that the kindling process identified in humans could also be modeled in rats with symptoms other than seizures.

Later experiments illustrated that this anxiety-like behavior does not have a long duration. Duration is defined as the amount of time that anxiety-like behavior can be detected following the final withdrawal. In adults, anxiety-like behavior following the final withdrawal in the repeated withdrawal paradigm was detected at 24 but not 48 hours (Overstreet et al., 2002). However, this paradigm did produce underlying changes in the brain that could be re-elicited with future challenges. In these studies, rats were given

repeated ethanol withdrawals and then allowed to recovery for a period of time. During this recovery, rats were exposed to a non-anxiogenic regimen of chronic ethanol exposure (which normally causes no effect). In rats that had a history of repeated ethanol withdrawals, this future ethanol exposure re-elicited their previous anxiety-like behavior (Overstreet et al., 2002). These data illustrated that adaptation caused by repeated withdrawals can effect future responses to ethanol.

Further work also illustrated the importance of stress in this kindling hypothesis. In these experiments, rats were exposed to two episodes of stress in place of the first two ethanol/withdrawal cycles, followed by one ethanol cycle (referred to as the stress/withdrawal paradigm). One ethanol cycle alone has no effect on social interaction normally but when rats are also exposed to stress the reduction in social interaction was elicited. Further, it was illustrated that three exposures to stress alone had no effect on social interaction (Breese et al., 2004). The adaptations produced from this paradigm were also shown to have long-term consequences in that future ethanol exposure 16 days following the stress/withdrawal paradigm could re-elicite anxiety (Breese et al., 2004).

This work illustrated that repeated ethanol withdrawals or stress could sensitize an emotional phenotype in rodents (i.e. anxiety) during withdrawal. Stress interactions with this kindling-like process have been incorporated into an expanded version of the above hypothesis referred to as the “The Kindling/Stress Hypothesis” (Breese et al., 2005a). The work referenced here documents the importance of understanding the cyclic nature of ethanol intake, ethanol withdrawal, and stress. This hypothesis illustrates that with increasing intake/withdrawal cycles, there is a progressive worsening of negative affect (anxiety) that can interact with stress. The tension reduction hypothesis would predict

that this increase in negative affect would lead to increased relapse. Therefore, the combination of these two hypotheses provided definable features that work to explain the progression of ethanol dependence. A large proportion of current research has been focused on identifying those systems responsible for this dependence, especially those contributing to ethanol withdrawal, stress, and negative affect. One of the systems that play an integral part in this process is the corticotrophin releasing factor (CRF) system.

Adaptations through CRF

CRF is a 41-residue polypeptide that can be found in the paraventricular nucleus (PVN) as well as in many extrahypothalamic sites (Sarnyai et al., 2001). These extrahypothalamic sites include the limbic regions (e.g. amygdala and bed nucleus of the stria terminalis, BNST) and brainstem (e.g. locus coeruleus and nucleus of solitary tract). CRF has binding affinity for CRF binding protein (CRF-BP), CRF type 1 receptors (CRF₁R), and CRF type 2 receptors (CRF₂R). The CRF₁R and CRF₂R are G-coupled receptors which are positively coupled to adenylate cyclase (primarily through G α_s). CRF₁R are found primarily in the amygdala, pituitary, hippocampus, cortex, and cerebellum. CRF₂R, on the other hand, are located mainly in lateral septum, hypothalamus, and amygdala (Sarnyai et al., 2001).

The behavioral effects of CRF at these extrahypothalamic sites are contingent on the environment situation (stressful or non-stressful). When CRF is given in a familiar environment (non-stressful), it resulted in behavioral activation including increased locomotor activity, rearing, and grooming (Jones et al., 1998). In contrast, CRF given in a novel environment (stressful) produced behavioral suppression including decreased activity in open field and elevated plus maze, food intake, and sexual behavior (Dunn &

Berridge, 1990). Reductions in explorations in open field and elevated plus maze are an index of increased anxiety-like behavior. Antagonist studies have also highlighted the behavioral effects of CRF. Administration of a non-specific CRF antagonist reversed stress-induced feeding (Krahn et al., 1998) and fighting (Tazi et al., 1987a), increased exploration in novel environments (Takahashi et al., 1989), blocked enhancement of acoustic startle (Swerdlow et al., 1989), and blocked anxiety-like responding in elevated plus maze (Heinrichs et al., 1994). Later work with receptor specific agonists, antagonists, and genetic knockouts (KO) showed behavioral effects of CRF through these different receptor subtypes were not the same. These studies illustrated that CRF₁R activation seems to be more involved in stress or CRF-induced anxiety and activity suppression (Contarino et al., 1999; 2000; Liebsch et al., 1999; Skutella et al., 1998; Tazi et al., 1987b). Alternatively, CRF₂R activation was shown to reduce food intake and had anti-anxiety effects, although data on these anti-anxiety effects are mixed (Bale et al., 2000; Reyes et al., 2001).

In addition to the well known effects of ethanol on the HPA axis, there are also many effects of ethanol that can be attributed to the modulation of CRF at extrahypothalamic sites. Low doses of ethanol are known to be anxiolytic and this effect may be regulated at least in part through suppression of CRF (Valdez & Koob, 2004). Chronic exposure to ethanol leads to a doubling in the amount of CRF-immunoreactivity (IR) in the amygdala, as found in microdialysis samples (Merlo Pich et al., 1995). Additionally, patients with alcohol dependence also show an increase in CRF in their cerebral spinal fluid (Bruijnzeel & Gold, 2005).

During ethanol withdrawal, CRF levels in the amygdala were shown with microdialysis to peak 11-12 hours after chronic ethanol exposure (Merlo-Pich et al., 1995). In immunohistochemical studies, it was shown that there was a decrease in CRF immunoreactivity in the amygdala, hippocampus, and frontal cortex 5-6 into withdrawal (Zorrilla et al., 2001). This decrease in CRF immunoreactivity was thought to be caused by increased CRF release in these regions which resulted in CRF depletion in the cell bodies. Additionally, it was determined that CRF₁R is particularly involved in the regulation of ethanol withdrawal-induced anxiety (Baldwin et al., 1991; Overstreet et al., 2004a; Timpl et al., 1998). Other work illustrated that microinjection of a CRF antagonist into the CeA, but not the nucleus accumbens shell or lateral BNST, blocked withdrawal-induced increases in self-administration in dependent rats (Funk et al., 2006). Therefore, these experiments define the contribution of CRF in the amygdala (CeA) in ethanol withdrawal-induced increases in self-administration and anxiety most likely through the CRF₁R.

One of the main features of ethanol dependence is that it is a chronically relapsing condition. It was described earlier that some of the factors that contribute to relapse are negative affect (anxiety-like behavior) and interaction of stress with these mood states. Therefore, studies were also performed to determine the role of adaptations in CRF in protracted ethanol withdrawal that relate to relapse. Administration of a CRF antagonist blocked the stress-induced anxiety in protracted withdrawal (6wks) from chronic ethanol (Valdez et al., 2003). Additionally, other work showed that a CRF₁R antagonist blocked foot-shock stress mediated reinstatement of ethanol self-administration (Lê et al., 2000). Experiments showed that 6 weeks into protracted withdrawal there was a recovery of

CRF levels except in the amygdala were levels of CRF were still increased (Zorrilla et al., 2001). Further work using EEG recordings illustrated that responsiveness to CRF was increased in the cortex and the amygdala during protracted withdrawal from ethanol (Slawecki et al., 1999).

Experiments have also been performed to evaluate the role of CRF in the sensitization process from repeated ethanol withdrawals described above (under Kindling Hypothesis). These studies showed that CRF (icv) substituted for early stress/withdrawal cycles and produced anxiety-like behavior after a final withdrawal (Overstreet et al., 2004a). Additionally, a CRF₁ receptor antagonist (CP-154,526) given systemically prior to the first two withdrawals or prior to stress blocked this anxiety-like behavior (Breese et al., 2004; 2005b). In contrast, CRF₂R antagonist did not have this effect (Overstreet et al., 2004a).

Collectively this work demonstrates a clear role for CRF in the processes inherent in ethanol dependence. Chronic ethanol use was shown to produce changes in the CRF system which are responsible for the production of anxiety-like behavior during withdrawal. The hypotheses mentioned above (Tension Reduction and Kindling) illustrated that negative affect (including anxiety) was responsible for relapse and craving for ethanol during abstinence. Data presented in this section showed that modulation of CRF could prevent stress-induced reinstatement and the sensitization of anxiety behavior. Therefore, understanding the development of changes in CRF from ethanol will be critical for treating and possibly preventing the development of this disease.

Dissertation

The focus of this dissertation will be to evaluate cyclic ethanol exposure, interactions of stress, and adaptations through CRF that lead to the development of alcohol dependence in a vulnerable population, adolescents. In adults, related work has established the importance of understating the negative affect (i.e anxiety) produced from alcohol withdrawal and how the cyclic nature of ethanol exposure can modulate its development. Further, it has been identified that the duration of these anxiety-like responses and the interactions with stress are vital. Finally, it was illustrated that adaptations in the CRF system are critical for the development of these behaviors and are likely targets for treatment.

The following work will investigate these key concepts in adolescents. The clinical data presented at the beginning of this section would indicate that alcohol use during adolescence is a large predictor for future alcoholism. These data would predict that adolescent rats should be able to undergo the adaptations thought to be important to the development of addiction. In the current work, it would be expected that adolescent rats are either more or equally sensitive to the production of withdrawal related behaviors (especially anxiety) from repeated ethanol withdrawal. Furthermore, stress should also be able to interact with these ethanol withdrawals to produce anxiety-like behavior and it is likely that this response may be more robust in adolescents. Furthermore, it is likely that these adaptations from repeated withdrawals and stress that produce this increased anxiety are mediated through the modulation of the CRF system. If adolescents undergo these adaptations that lead to the production of negative affect (i.e. anxiety), then it is likely that these behaviors will promote continued ethanol use in adulthood. Thus, the overall hypothesis tested is that adolescents will be vulnerable to the negative affect

inducing effects of stress and cycled chronic ethanol and this vulnerability will depend on CRF systems.

CHAPTER II: GENERAL METHODS

Animals

Male Sprague Dawley rats (Charles-River, Raleigh, NC) were obtained at 7 weeks of age (P49) for the adult groups and 3 weeks of age (P21) for the adolescent groups. Animals were group housed for 1 day to adapt to the local conditions (light/dark cycle of 12:12, with lights on between 07:00 and 19:00 hour). Rats were then individually housed (unless noted otherwise) for the remainder of the experiments with food and fluids monitored as described below. The experiments described here were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Liquid Diets

Following a day of adaptation, standard food chow was removed from the cages and all rats were placed on nutritionally complete liquid diets that has been used previously in this laboratory (e.g., Frye et al., 1983; Knapp et al., 1998; Moy et al., 1997). During this time, all rats had constant access to water. The diet used was lactalbumin/dextrose-based with vitamins, minerals, and other nutrients from Dyets (Bethlehem, PA). The number of calories from dextrose was equated with calories from the ethanol so that both control diet (CD) and ethanol diet (ED) were calorically balanced. Rats were given CD in a volume equivalent to that consumed by the ED group on the previous day to minimize differences in weight gain. Additionally, rats were weighed weekly to monitor weight gain throughout the experiment. ED and CD volumes were measured daily at the end of the dark cycle (08:00 hour) to determine g/kg intake

/day. Most behavioral measures were performed on the 20th day of diet administration during which time all rats maintained on ED were placed on CD. Most behavioral tests were then performed 5 hours into withdrawal when blood ethanol levels have fallen to 0 (Breese et al., 2004; Overstreet et al., 2002).

Social Interaction

The social interaction (SI) test was first described by File and Hyde (1978) and has been used regularly in our laboratory to assess anxiety-like behavior. In this test, a pair of rats are placed into an arena and the amount of time they are engaged in social behaviors is recorded. Studies have shown that anxiogenic stimuli (noisy environment and cat odor; File, 1994; Zangrossi and File, 1992), anxiogenic drugs (yohimbine, amphetamine, and PCP; Bhattacharya et al., 1997; File and Hyde, 1979; Sams-Dodd, 1995), and drug withdrawal (alcohol and nicotine; File et al., 1989; Irvine et al. 2001; Kampov-Polevoy et al., 2000) all produced a reduction in SI. Alternatively, a number of anxiolytic agents (summarized in File & Seth, 2003) have been shown to increase SI.

In the classic administration of the test, the pairs of rats were matched by both treatment and body weight. Additionally, SI scores of the pair were treated as a single unit. Several modifications have been made in our laboratory to this original SI test. Our studies showed that the social behavior of one member of a testing pair is independent of the other rat's behavior (Breese et al., 2004; Overstreet et al., 2002, 2003, 2004b). This allowed for the individual data collection of each animal of the pair rather than the average performance of the pair (Overstreet et al., 2003). Additionally, it was possible to test rodent pairs that did not receive the same treatment. Therefore, the sole criterion for pair selection in our studies was body weight.

In this modified version of the SI test, rats were placed into an unfamiliar 60 X 60 cm square open field with a 15 X 15 cm square grid floor under low lighting conditions (30 lx). Two rats, naive to the testing environment were monitored for 5 minutes. During this period, an observer blind to the treatment condition measured the time (in seconds) that each rat was engaged in social behavior (conspecific grooming, sniffing, following, crawling over/under) with its partner. Locomotor activity was also simultaneously measured during the test by the number of times a rat crossed the lines of the grid floor. Ethanol withdrawal in this modified test has been repeatedly shown to reduce SI and sometimes locomotor activity (Breese et al., 2004; Overstreet et al., 2002; 2003; 2004a,b). However, it is important to note that reductions in SI and locomotor activity seemed to be independent of each other, as they can be independently manipulated with changes in ethanol treatment conditions or drug treatments (Breese et al., 2004; 2005b; Knapp et al., 2005; Overstreet et al., 2002). Further, the validity of these results was also confirmed with another test used to evaluate anxiety-like behavior, the elevated plus maze (Overstreet et al., 2002; 2004b).

Blood Ethanol Concentrations

A separate group of rats were used for blood ethanol concentration (BEC) analysis and were not included in the behavioral tests. This step was taken to prevent any potential effects that multiple blood sampling might have on behavioral measures. For BEC analysis, blood was removed from the tip of the tail during the last hour of the dark cycle (06:00) on the days indicated in the following chapters.

Blood samples were then analyzed with gas chromatographic methods. Tail blood (6 µl) or standards (6 µl; 0 to 200 mg%) were combined with 375 µl of distilled water and

0.5 g NaCl in 12 X 75mm borosilicate glass culture tubes. These tubes were capped and then heated to 55°C for 10 minutes. After this time 1.5 ml of head-space gas was removed from the tube and injected directly into an SRI 8610C gas chromatograph (SRI Instruments, Inc., Torrance, CA), as previously described (Breese et al., 2004; Navarro et al., 2003; Overstreet et al., 2002).

CHAPTER III: EFFECTS OF REPEATED ETHANOL WITHDRAWALS

Introduction

Adolescence is a period of development that is marked with increased vulnerability to the use and abuse of alcohol. Many studies, presented in earlier sections, have illustrated that adolescents respond to ethanol in ways that are distinct from adults. Further, the work in adult rats highlighted the importance of understanding the behavioral consequences of alcohol withdrawal. This work showed that these behaviors can be critical in the maintenance of alcohol intake and relapse. Therefore, in evaluating the differential responses of adults versus adolescents on alcohol-related measures, it is also important to assess the effects of alcohol withdrawal (a measure of physical dependence).

There are a number of symptoms that are associated with ethanol withdrawal including seizures, anxiety, and activity suppression among others that have been evaluated in adolescent rodents. With regard to alcohol withdrawal seizures, Acheson et al. (1999) demonstrated that seizure induction following 5-days of intragastric ethanol infusions was more pronounced in adult mice than adolescents. In more recent studies, 2-weeks of exposure to ethanol vapor chamber produced no change in anxiety (light/dark box), a decrease in acoustic startle response (ASR), and an increase in pre-pulse inhibition (PPI) during withdrawal. There were no differences in these responses between adolescent and adult rats (Slawecki et al., 2006). Further evaluation of withdrawal from this ethanol treatment showed that high frequency power in a parietal cortical EEG was

selectively increased in adolescents, while hypoactivity in the light/dark box was produced only in adults (Slawecki et al., 2006). Other investigators (Doremus et al., 2003) assessed anxiety-like behavior during acute withdrawal from an intraperitoneal injection of ethanol. Using the elevated plus maze, they showed that adolescent rats were unable to display an anxiety-like behavior that is reliably produced in adult rats. Finally, Varlinskaya and Spear (2004) also found that adult rats, but not adolescents, exhibited increased anxiety-like behavior 18 hours following acute intraperitoneal (ip) ethanol administration in the social interaction test. Collectively, these studies suggest that adolescents may be less sensitive than adults to some of the behavioral consequences of ethanol withdrawal. However, they also indicate that differences in these behavioral responses can be dependent on the behavioral test used and type of ethanol exposure.

While these previous studies have provided important information about the age-related differences during ethanol withdrawal, more work is still needed on more chronic ethanol exposures. Further, adult data suggest that the pattern of ethanol intake can have significant effects of withdrawal-related behaviors. In adult humans and rodents, it has been demonstrated that repeated ethanol withdrawals (cycles of ethanol intake and withdrawal) increased the susceptibility for seizures (Ballenger & Post, 1978; Becker & Hale, 1993; Kokka et al., 1993; McCown & Breese, 1990). Further investigations in adult rodents demonstrated that other symptoms of withdrawal (i.e., anxiety) could also undergo this kindling-like process identified with seizures (Breese et al., 2004; Overstreet et al., 2002). It has been well characterized that teenagers typically consume alcohol in a “binge manner”, where high levels of intake are followed by periods of abstinence (Hiller-Strumhofel & Swartzwelder, 2004/2005). This pattern suggests that they likely

experience repeated withdrawals. Therefore, the repeated withdrawal model provided a novel way to address the effects of ethanol withdrawal in adolescent rats and offered a potentially valuable comparison with previous studies that showed little indication of acute withdrawal anxiety during this developmental period.

Therefore, the current studies were designed to evaluate whether repeated withdrawals from ethanol would produce withdrawal symptoms (anxiety-like behavior and seizure susceptibility) in adolescent rats. Additionally, it was investigated whether relative ethanol intake or blood ethanol concentrations (BEC) across ages might affect differences in susceptibility between adolescent and adult rats. These investigations were carried out by testing both ages in social interaction, audiogenic seizure induction, and bicuculline seizure induction following repeated withdrawals from ethanol diets (ED) known to be effective in adult rats.

Materials & Methods

Animals

For standard animal information and housing conditions, refer to General Methods (Chapter II).

Ethanol & Control Diets

For standard liquid diet administration procedures, refer to General Methods (Chapter II). Adult rats were then habituated for 3 days on control diet (CD) and then placed into 1 of 3 treatment groups. Generally, one-third of the rats received 19 days of CD and the other two-thirds received cycled administration (three 5-day cycles of ED separated by two 2-day CD exposures) of either 4.5% or 7% (w/v) ED. Adolescent CD and 4.5%ED groups were treated the same as adult rats, however, a slight modification was made for adolescent 7%ED group. This group was given 4.5%ED for the first cycle of treatment and then exposed to 7%ED for the remaining cycles. This modification was used because of reduced weight gain that occurred when adolescent rats received 7%ED during the first cycle (T.A. Wills, D.J. Knapp, D.H. Overstreet, and G.R. Breese, unpublished observation). Further, since adolescence is known to be period of rapid growth and liquid diets can modestly retard weight gain (Mason et al., 1992; Sampson et al., 1996), it was important to make sure liquid diet did not adversely effect adolescent rats. Therefore, a subgroup of adolescent rats were given standard chow food and compared to adolescent rats that received CD for the duration of the experiment in social interaction test. All behavioral measures were performed on the 20th day of diet administration during which time all rats maintained on ED were placed on CD. Behavioral tests were then performed 5 hours into withdrawal when blood ethanol levels

had fallen to 0 (Breese et al., 2004; Overstreet et al., 2002).

Social Interaction

For standard social interaction (SI) test procedures, refer to General Methods (Chapter II).

Audiogenic Seizure Test

Rats were tested for induction of audiogenic seizures 6 hours into withdrawal (following SI test). Rats were placed individually into a plastic container (30 gallon: 19.5 X 21.75 X 27.5 in), which contained an electric bell (100 db) and view window. Once the rat was placed into the container, the electric bell was turned on for 2 minutes. While the bell was tuned on, an observer blind to the experimental treatment condition scored the degree of seizure (seizure score 1 to 5) and latency to induce seizure. The seizure score was given based on the following criteria: 1 = no change in behavior, 2 = running with no convulsive movement, 3 = running/jumping and masticatory movements with mild facial clonus, 4 = running/jumping with startle followed by complete clonus of the forelimbs, and 5 = running/jumping followed by complete tonic extension of the hindlimbs and then generalized clonus to all limbs. The latency to induce a seizure was measured as the seconds between start of the bell and full seizure episode. Rats that experienced seizures were immediately injected with a lethal dose of pentobarbital. Rats that did not display audiogenic seizures were then tested in response to bicuculline. The results below will illustrate that only adolescent rats given 7%ED demonstrated an induction of audiogenic seizures. As these animals were therefore selected out of the bicuculline test, the n size was reduced from 10 to 4. This lowered sample size limited appropriate statistical

comparisons involving this group; thus, we tested an additional group of 7%ED treated adolescent rats that received the bicuculline test alone.

Bicuculline Threshold

Immediately following the audiogenic test, rats were infused with 0.05 mg/ml of bicuculline (GABA_A antagonist; MP Biomedicals, Solon, OH) into the lateral tail vein. The drug was injected with a syringe pump at a rate of 1.6 ml/min. The time required for the rat to exhibit a twitch of the head/neck was recorded. From this time, the minimum amount of drug required to produce the first evidence of seizure activity can be calculated.

Blood Ethanol Concentrations (BECs)

BECs were taken from groups of adolescent and adult rats that were cycled on either 4.5%ED or 7%ED in the manner described above. Blood was removed from the tip of the tail during the last hour of the dark cycle (06:00) on the first, fifth, sixth, tenth, and 11th day of ED. Additionally, on the last day of ED (15th day) blood was collected at the time of ethanol removal (hour 0) and then 2, 4, and 6 hours later. Other details on BEC analysis are described in General Methods (Chapter II).

Statistics

Analyses of SI and locomotor activity were conducted with one-way ANOVAs for each age group because of large differences in baseline SI (seen in CD groups). These baseline differences prevented comparison of data in a two-way ANOVA for these behavioral tests. Therefore, decrease from baseline scores were used to make comparisons between adolescent and adult rats. When two group comparisons were made, t-tests were utilized. Two-way ANOVAs were possible for audiogenic and

bicuculline tests. Daily ethanol intakes and BECs were analyzed with repeated measures ANOVAs for adolescent and adult rats while ethanol intakes averaged over cycles were analyzed with one-way ANOVAs. Differences between groups were determined with Fisher's post hoc tests.

Results

Social Interaction in Adolescent and Adult Rats

In adult rats, there was a significant difference in SI among groups [$F(2,41) = 4.69$, $p < 0.05$; Figure 3.1A]. Rats which experienced repeated withdrawals from ED (4.5%ED or 7%ED) spent less time engaged in SI compared to rats that received CD. There were no significant differences in SI between ED groups (4.5%ED and 7%ED). In adolescent rats, there was a significant difference in SI between groups where rats in both the 4.5%ED and 7%ED groups had reduced SI compared to rats in the CD group [$F(2,50) = 45.28$, $p < 0.0001$; Figure 3.1B]. There was also a significant difference between the ED treated groups, where rats that received 7%ED had significantly reduced SI compared to those receiving 4.5%ED. Regarding adolescent rats that received chow or CD, there was a significant difference between groups [$t(17) = 5.81$, $p < 0.0001$; data not shown] in SI where CD rats had higher SI scores than chow fed rats.

In CD groups, SI in adolescent rats was double that seen in adult rats. Therefore, reductions in SI from ethanol-treated animals were converted to decreases from baseline so the differences between age groups could be better determined. The decreases from baseline were calculated as a percent decrease in SI in the ethanol treatment groups compared to their age-matched controls (CD groups). Overall, there was a significant difference among the ethanol treatment groups and ages [$F(3,62) = 3.52$, $p < 0.05$; Figure 3.1C] in the decrease of SI from baseline. Adolescent rats treated with 7%ED showed a greater decrease from baseline compared to all other groups (4.5%ED adolescent, 4.5% and 7%ED adult). Additionally, no differences were found between adolescent rats given 4.5% ED and adult rats given either 4.5%ED or 7%ED.

Locomotor Activity in Adolescent and Adult Rats

There was a significant difference in locomotor activity in adult groups [$F(2,41) = 10.75$, $p < 0.0005$; Figure 3.2A), where adult rats treated with 7%ED had reduced line crosses compared to 4.5%ED and CD-treated rats. There was no significant difference in line crosses among the 4.5% ED and CD groups. In adolescent rats, there was a significant difference in line crosses among groups [$F(2,50) = 114.36$, $p < 0.0001$; Figure 3.2B] where the ED groups (4.5%ED and 7%ED) had decreased line crosses compared to the CD group and the 7%ED group had reduced line crosses compared to the 4.5%ED group. Regarding adolescent rats that received chow or CD, there was no significant difference between groups [$t(17) = 1.44$, NS; data not shown] in locomotor activity.

In CD groups, adolescent rats were found to have higher baseline activity than adult rats. Therefore, decreases in line crosses from ethanol-treated animals were converted to decreases from baseline, so that the differences between age groups could be better determined. There was a significant difference among ethanol treatment groups and ages [$F(3,62) = 22.41$, $p < 0.0001$; Figure 3.2C] in the decrease of line crosses from baseline. 7%ED-treated adolescent rats showed the largest decrease of line crosses compared to all other groups. Additionally, adult rats receiving 7%ED had a larger decrease from baseline than adolescent rats given 4.5%ED. No differences were found between adult and adolescent rats given 4.5% ED.

Audiogenic Seizures in Adolescent and Adult Rats

Audiogenic seizures were measured by the amount of time to induce a seizure (latency) and degree of seizure (seizure score). There was a main effect of both diet treatment [$F(2,75) = 17.38$, $p < 0.0001$] and age [$F(1,75) = 5.32$, $p < 0.05$], as well as an

interaction between diet treatment and age for latency [$F(2,75) = 8.63$, $p < 0.0005$; data not shown]. Additionally, seizure scores (Figure 3.3) also showed a main effect of diet treatment [$F(2,75) = 20.16$, $p < 0.0001$] and age [$F(1,75) = 5.84$, $p < 0.05$] along with an interaction between the two [$F(2,75) = 7.48$, $p < 0.005$]. For both latency and seizure scores, only 7%ED-treated adolescent rats were significantly different from all other groups. This group showed significantly reduced latency to induce audiogenic seizure and higher seizure scores. There were no differences between any other ethanol-treated group and their respective control groups.

Bicuculline Thresholds in Adolescent and Adult Rats

Bicuculline thresholds were determined in both ages by calculating the amount of bicuculline (mg/kg) required during an infusion to initiate a seizure. As 7%ED-treated adolescent rats exhibited audiogenic seizures, these animals were excluded from the subsequent bicuculline test. Thus, there was an additional group of 7%ED-treated adolescent rats that received the bicuculline test alone. There were no differences in the amount of bicuculline required for infusion between these groups, so all animals were collapsed into the 7%ED-treated adolescent group for further analysis. For bicuculline thresholds, there was a main effect of diet treatment [$F(2,76) = 7.66$, $p < 0.001$] but no main effect of age [$F(1,76) = 3.18$, NS] or an interaction between the two [$F(2,76) = 1.1$, NS, Figure 3.4]. Both 4.5%ED- and 7%ED-treated adolescent rats showed reduced bicuculline thresholds compared to rats given CD. However, only adult rats treated with 7%ED showed this reduction compared to their age-matched controls. There were no differences between ethanol-treated groups at either age. Additionally, there were no differences in ethanol-treated groups between adolescent and adult rats.

Daily Ethanol Intake

To determine how ethanol treatment could have influenced behavioral responses in withdrawal, the pattern of daily ethanol intake was assessed. In adult rats, daily ethanol intake was significantly different between 4.5%ED and 7%ED groups with a repeated measures ANOVA [$F(1,420) = 205.53, p < 0.0001$; Figure 3.5A]. Rats treated with 7%ED had higher g/kg ethanol consumption on these days compared to the 4.5%ED-treated rats.

In adolescent rats, both 4.5%ED and 7%ED groups received 4.5%ED for the first 5 days of ethanol treatment and there were no differences in ethanol intake between groups during these days. There were significant differences in ethanol intake between groups in the second and third cycles (days 6 to 15) [$F(1,378) = 26.08, p < 0.0001$; Figure 3.5B] measured with a repeated measures ANOVA.

Additionally, to determine differences between adolescent and adult rats ethanol intake was evaluated by cycles (average of daily intake for days 1 to 5 = cycle 1, days 6 to 10 = cycle 2, and days 11 to 15 = cycle 3). During cycle 1, there were significant differences among 4.5%ED- and 7%ED-treated adult and adolescent rats [$F(3,57) = 16.27, p < 0.0001$; Figure 3.6]. Adolescent rats treated with 4.5%ED and 7%ED drank more than adult rats which received the same ethanol treatments. There were no differences between 4.5%- and 7%ED-treated adolescent rats as all animals received 4.5%ED during this first cycle. However, both these adolescent groups consumed more ethanol than 7%ED-treated adults.

Averages of ethanol intake during the second cycle also illustrated significant differences among 4.5%ED- and 7%ED-treated adult and adolescent rats [$F(3,57) =$

30.49, $p < 0.0001$; Figure 3.6]. Again, adolescent rats treated with 4.5%ED and 7%ED drank more than adult rats which received the same ethanol treatments. Additionally, rats of both ages treated with 7%ED showed higher consumption than rats of the same age that were given 4.5%ED. Interestingly, when 4.5%ED-treated adolescent rats were compared to 7%ED-treated adult rats, they did not differ in their amount of consumption.

In the third cycle, group differences were also demonstrated among 4.5%ED-and 7%ED-treated adult and adolescent rats [$F(3,57) = 61.79$, $p < 0.0001$; Figure 3.6]. Adolescent rats treated with 4.5%ED and 7%ED drank more than adult rats which received the same ethanol treatments. Rats of both ages treated with 7%ED showed higher consumption than rats of the same age that were given 4.5%ED. However, during this last cycle, 7%ED adult rats and 4.5%ED adolescent rats were shown to be different with adult 7%ED-treated rats consuming more ethanol than 4.5%ED adolescent rats. This difference was caused by a slight increase in intake between cycle 2 and 3 for 7%ED adult rats and a corresponding decrease in ethanol intake for 4.5%ED adolescent rats.

Blood Ethanol Concentrations

In adolescent rats, there were significant differences between ED groups (4.5% and 7%ED) in BEC across the days examined [$F(1,352) = 9.36$, $p < 0.005$; Table 1]. BECs in adult rats were also significantly different between ED groups (4.5% and 7%ED) [$F(1,126) = 15.75$, $p < 0.001$; Table 1]. In both adolescent and adult rats, it was determined that BECs had returned to 0 by 6 hours into withdrawal on the final test day (day 15, Table 1). As illustrated in Table 1, 7%ED-treated adult and adolescent rats had higher BECs than their age-matched counterparts receiving 4.5% ED. Additionally, BECs in adolescent rats treated with 4.5%ED were similar to adult rats treated with

7%ED. These data, therefore, compliment ethanol intake data displaying comparable ethanol intakes between these 2 groups.

Body Weights

In adult rats, there was a significant difference between groups in body weight [$F(2,41) = 9.33$, $p < 0.001$, Table 2] where the 7%ED group have reduced body weight compared to CD and 4.5%ED groups. There was no difference between body weights of 4.5%ED and CD groups.

In adolescent rats, there was a significant difference between groups in body weight [$F(2,50) = 24.81$, $p < 0.0001$; Table 2] where the 7%ED group had reduced body weight compared to CD and 4.5%ED groups. There was no difference between body weights of 4.5%ED and CD groups. In a separate study, chow-fed adolescent rats had higher body weights (194 ± 3 g) than CD-exposed rats [$t(17) = 18.0$, $p < 0.0001$; data not shown].

Discussion

Adolescent rats demonstrated reduced SI following repeated withdrawals from both 4.5% and 7% ED. Adult rats also demonstrated this behavioral phenotype which is consistent with previous studies (Overstreet et al., 2002). As prior research showed that the SI test is a validated means to measure anxiety-like behavior (File & Seth, 2003), it would appear that repeated withdrawals from these EDs produced an anxiety-like phenotype in adolescent rats, as it is known to do in adult rats. Although the retardation of weight gain sometimes seen with liquid diets could arguably be stressful and impact negatively on SI, the fact that the CD-treated adolescent rat's SI scores were not lower than those of the chow-fed adolescent rats provides evidence against this hypothesis. Furthermore, the fact that the chow-fed rats and the CD-treated rats had no difference in locomotor activity suggests that neither group was unduly stressed. The presence of this anxiety-like phenotype after repeated ethanol withdrawals in adolescent rats was a novel finding, particularly in light of evidence from acute withdrawal tests that showed anxiety-like behavior in adult but not in adolescent rats (Doremus et al., 2003; Varlinskaya & Spear, 2004). Differences between these studies and the current investigation could be due to the duration and cycling of ethanol exposure. In adult animals, it has been shown that repeated withdrawals from 4.5%ED is critical to the expression of anxiety-like behavior (Breese et al., 2004; Overstreet et al., 2002). One 5-day cycle or continuous 15 days of 4.5%ED did not elicit an anxiety-like phenotype in these adult rats. Therefore, the difference between expression of anxiety in this study compared to acute administrations (Doremus et al., 2003) could have been due to the cyclical nature of the ethanol administration. Another potentially relevant difference between these studies was the age

of behavioral assessment for anxiety following ethanol withdrawal and the test used. The chronic ethanol administration that was used in the present procedure delays measurement of SI related anxiety until around P44, whereas in the study by Doremus et al. (2003), adolescent rats were tested at P33–35 in the elevated plus maze. Therefore, it might be the case that younger adolescent rats are able to undergo adaptations in the brain that contribute to anxiety but may not be able to express this phenotype until later stages of adolescence.

Adolescent rats were also shown to display higher baseline levels of activity and SI than adult rats. This result was consistent with previous reports of elevated exploration and social behavior in adolescent rats (Adriani et al., 1998; Primus & Kellogg, 1989; Vanderschuren et al., 1997). Comparisons between adolescent and adult rats in SI (after correction for baseline differences) demonstrated that adolescent rats treated with the higher concentration of ethanol (7%ED) exhibited a greater reduction in SI than in adult rats. One interpretation of these data is that adolescent rats may have greater sensitivity to the effects of this ethanol administration than adult rats.

The greater sensitivity of adolescent rats is also indicated in the other behavioral measurements (activity, audiogenic seizures, and bicuculline threshold). These data showed that lower doses of ED (4.5%) decreased activity in adolescent but not in adult rats. When corrections for baseline differences were made, it was found that adolescent rats treated with the higher concentration of ethanol (7%ED) exhibited a greater reduction in locomotor activity than adults receiving the same treatment. Further analysis of audiogenic seizures illustrated that the adolescent 7%ED group was the only group in which seizures were induced. For bicuculline thresholds, both ED concentrations were

able to decrease thresholds in adolescent rats where only the high concentration was able to do so in adult rats. These data were in contrast with results found by Acheson et al. (1999), who demonstrated that seizure induction following 5-days of intragastric ethanol infusions in mice was more pronounced in adults than adolescents. However, this study differed in a number of ways to those presented here: mice versus rats, single cycle versus repeated cycles, high ethanol doses versus moderate ethanol dose, pentylenetetrazol-induced seizures versus bicuculline and audiogenic induced seizures, and testing 15 versus 5 hours into withdrawal. Therefore, different results in these studies could have been caused by any one or more of these variables. Together, these data presented here suggested an overall greater sensitivity in adolescent rats compared to adult rats following repeated withdrawals in the behavioral measures that were tested.

Although the SI, locomotor, and seizure data appear to support the hypothesis that adolescent rats were more sensitive to the repeated ethanol withdrawal experiences, alternative interpretations should be considered based on other data collected from these animals. These behavioral differences could also be explained by differences in BEC and intake between ages. Analysis of ethanol intake between ages consistently showed that adolescent rats consumed higher g/kg of ethanol than their respective ED groups in adult rats. Other investigators have reported greater ethanol intake in adolescent rats compared to adult rats using ethanol administration paradigms (Bell et al., 2006; Doremus et al., 2005; Rodd-Henricks et al., 2002a,b; Vetter et al., 2007).

The results presented here emphasize the importance of monitoring ethanol intake between adolescent and adult rats to fully address potential age differences in behavioral responding to chronic ethanol exposure and withdrawal. While significant efforts were

made in the current study to control blood ethanol levels and intake across age, the multiple variables involved (differential intake, blood levels, physiological/metabolic differences) make this task formidable. When comparing the behavioral effects of treatments herein across 4.5%ED-treated adolescent rats and 7%ED-treated adult rats, it could be argued that relatively comparable behavioral effects were present. There were no differences between these groups in SI (after corrections were made for differences in baseline), audiogenic seizure measures (latency and seizure score), or bicuculline threshold. Furthermore, these groups were the two most closely matched in ethanol intake and BECs. Therefore, it could be concluded that when corrections were made for differences in ethanol intake and BECs between adolescent and adult rats, behavioral responses following repeated withdrawals from ethanol were similar. Additionally, it should be noted that high BECs obtained in adolescent rats given 7%ED, which appear to peak near hour 0 on day 15, might function as an acute stress. This additional stress exposure in the adolescent group should also be considered when evaluating the present results. Interpretations of behavioral differences between adolescent and adult rats with the use of liquid diet might be bolstered by further reducing dietary ethanol concentration in adolescent rats so that intakes and/or BECs are more tightly comparable across age. Regardless of the outcomes of such studies, one interpretation that seemed less challenging was that, like adult rats, adolescent rats showed relevant withdrawal responses on all of these measures in our model, and that these responses deserve further study.

With additional refinement of the liquid diet regimen, further study of other relevant variables known to impact this anxiety-like behavior in adult rats could be

examined. For example, anxiety-like behavior from repeated withdrawal is known to be sensitized in adult rats in that only repeated cycles of ethanol, but not a single or continuous exposure, produced anxiety-like behavior. This behavioral /physiological process, however, has not been evaluated in adolescent rats. Additionally, a corticotropin-releasing factor (CRF) type 1 receptor antagonist, benzodiazepine receptor antagonist, or a 5-HT_{1A} receptor agonist given during the early withdrawals blocked the induction of anxiety-like behavior (Knapp et al., 2004; Overstreet et al., 2003) in adult rats. Findings from these latter studies indicated the importance of GABA, 5-HT, and CRF in the adaptations that occur. It has also been illustrated that stress substitutes for early withdrawals in the production of anxiety-like behavior (Breese et al., 2004) in adult rats. This knowledge on the effects of repeated withdrawals in adult rats will be used in appropriately refined models to guide future research analyzing the effects of repeated withdrawals in adolescent rats.

In summary, the results of these experiments suggested initially that adolescents might be more sensitive to the consequences of repeated withdrawal from chronic ethanol exposure. This impression was based on greater decreases in SI behavior, locomotor deficits, and increased seizure sensitivity. However, further examination of the different blood ethanol levels and ethanol intake across age suggest that caution should be employed in interpreting such age-dependent effects. Adolescent rats, like adult rats, showed relevant withdrawal responses on all of these measures in our model. Given the relatively limited data of this type available for this age group, these responses should be further explored and studies should be expanded to assess additional relevant variables such as persistence, drug effects, and interactions with stress.

Figures & Tables

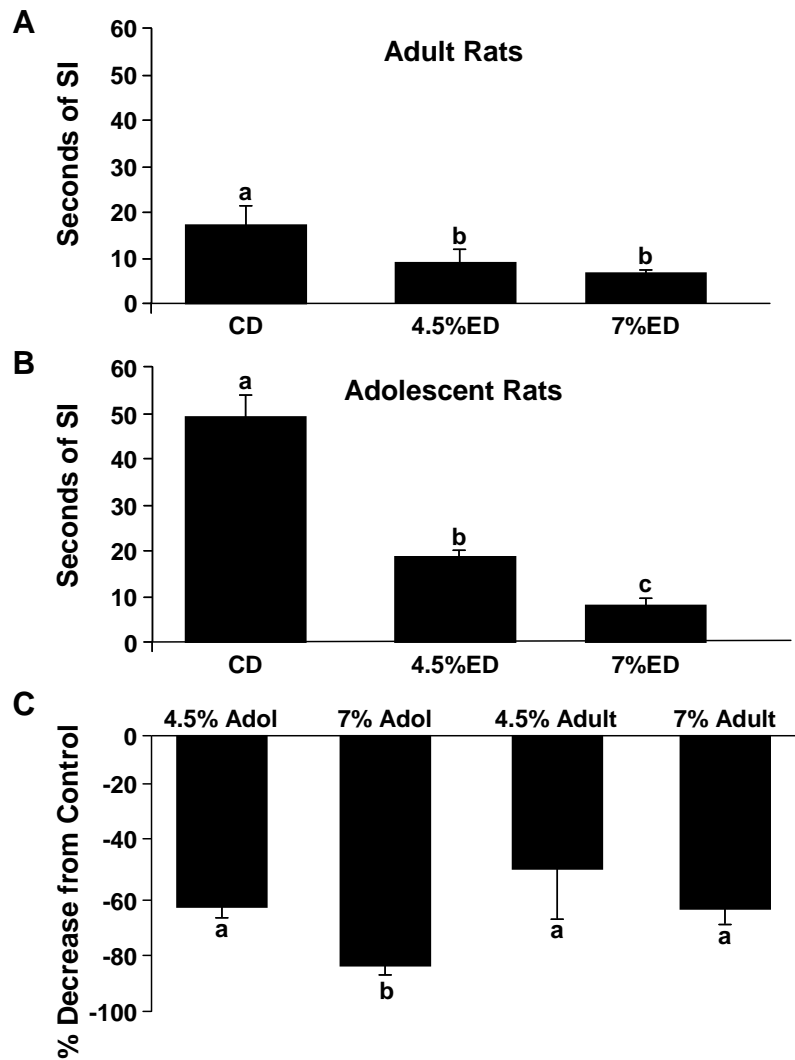


Figure 3.1 Effects of repeated ethanol exposure on social interaction in adult and adolescent rats (Panel A & B). Male adult and adolescent rats were given CD, 4.5%ED, or 7%ED. ED groups were exposed to three 5-day cycles of ED interspersed with two 2-day withdrawal periods, during which rats receive CD. Rats were tested 5 hours after removal of ethanol during the final withdrawal. Data represent mean \pm SEM for 8-10 rats/group. Panel C represents these data as a decrease from baseline to correct for differences in baseline (CD group) social interaction between ages. Groups with different letters are significantly different from each other ($p < 0.05$).

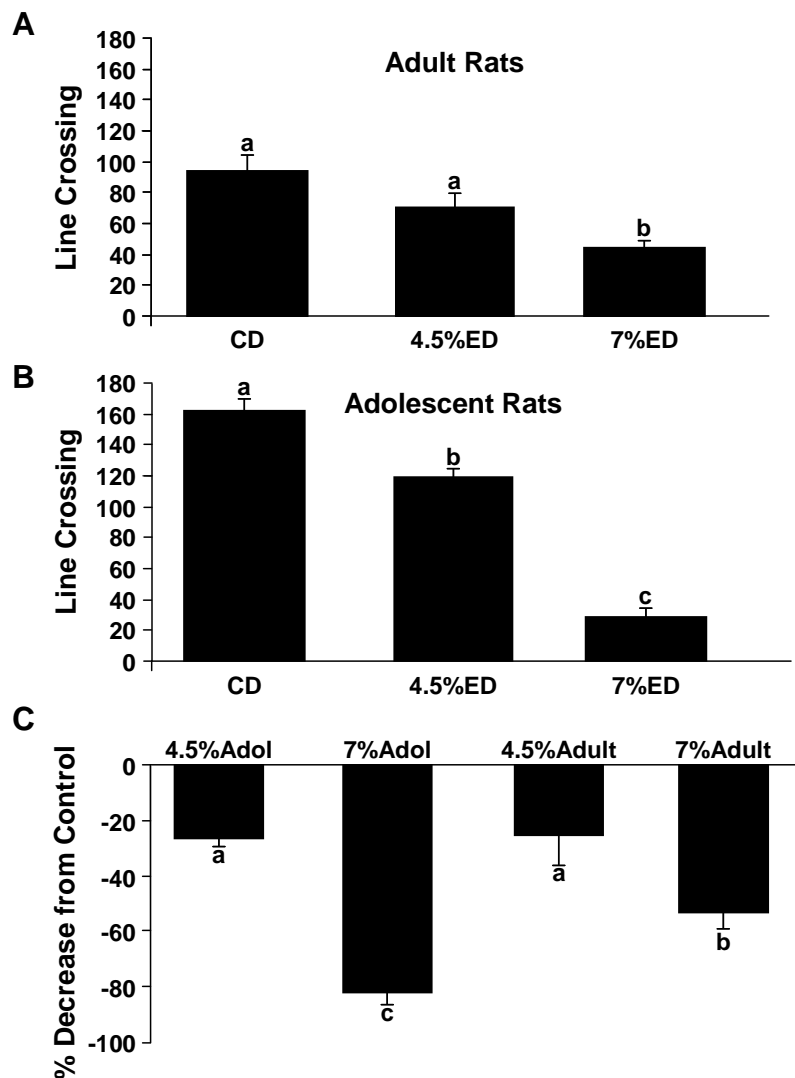


Figure 3.2 Effects of repeated ethanol exposure on locomotor activity in adult and adolescent rats (Panel A & B). Male adult and adolescent rats were given CD, 4.5%ED, or 7%ED. ED groups were exposed to three 5-day cycles of ED interspersed with two 2-day withdrawal periods, during which rats receive CD. Rats were tested 5 hours after removal of ethanol during the final withdrawal. Locomotor activity was measured concurrently with social interaction. Data represent mean \pm SEM for 8-10 rats/group. Panel C represents these data as a decrease from baseline to correct for differences in baseline (CD group) activity between ages. Groups with different letters are significantly different from each other ($p < 0.05$).

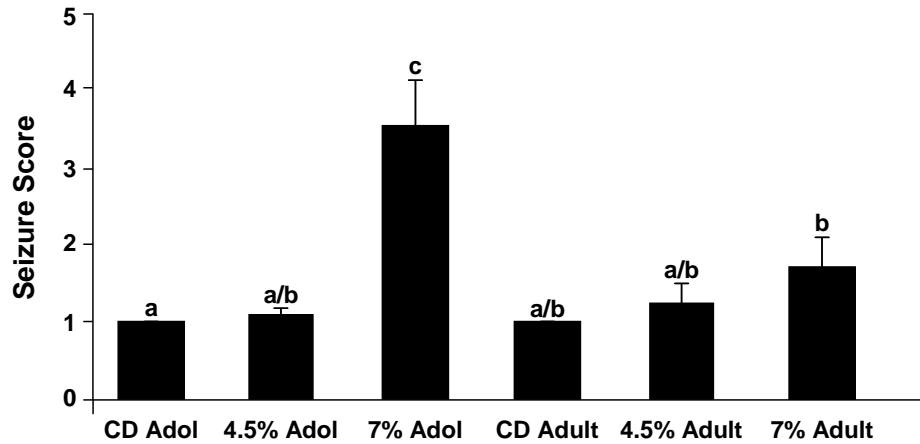


Figure 3.3 Effects of repeated ethanol exposure on audiogenic-induced seizures in adult and adolescent rats. Male adult and adolescent rats were given CD, 4.5%ED, or 7%ED. ED groups were exposed to three 5-day cycles of ED interspersed with two 2-day withdrawal periods, during which rats receive CD. Rats were tested 6 hours into final withdrawal following social interaction. See Materials and Methods for seizure scoring. Data represent mean \pm SEM for 8-10 rats/group. Groups with different letters are significantly different from each other ($p < 0.05$).

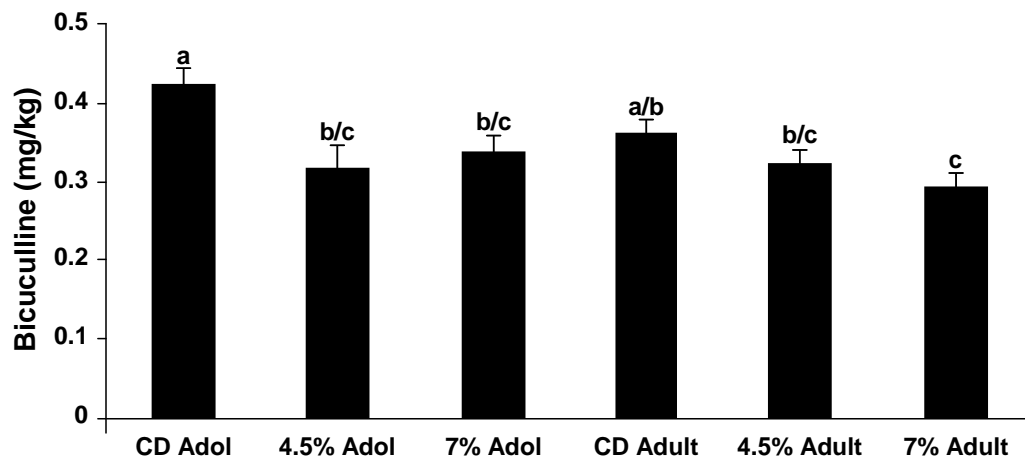


Figure 3.4 Effects of repeated ethanol exposure on bicuculline-induced seizures in adult and adolescent rats. Male adult and adolescent rats were given CD, 4.5%ED, or 7%ED. ED groups were exposed to three 5-day cycles of ED interspersed with two 2-day withdrawal periods, during which rats receive CD. Rats were tested 6 hours into final withdrawal in those rats in which audiogenic seizures were not detected. The amount of bicuculline infused until the first sign of head/neck twitch was recorded. Data represent mean \pm SEM for 8-10 rats/group. Groups with different letters are significantly different from each other ($p < 0.05$).

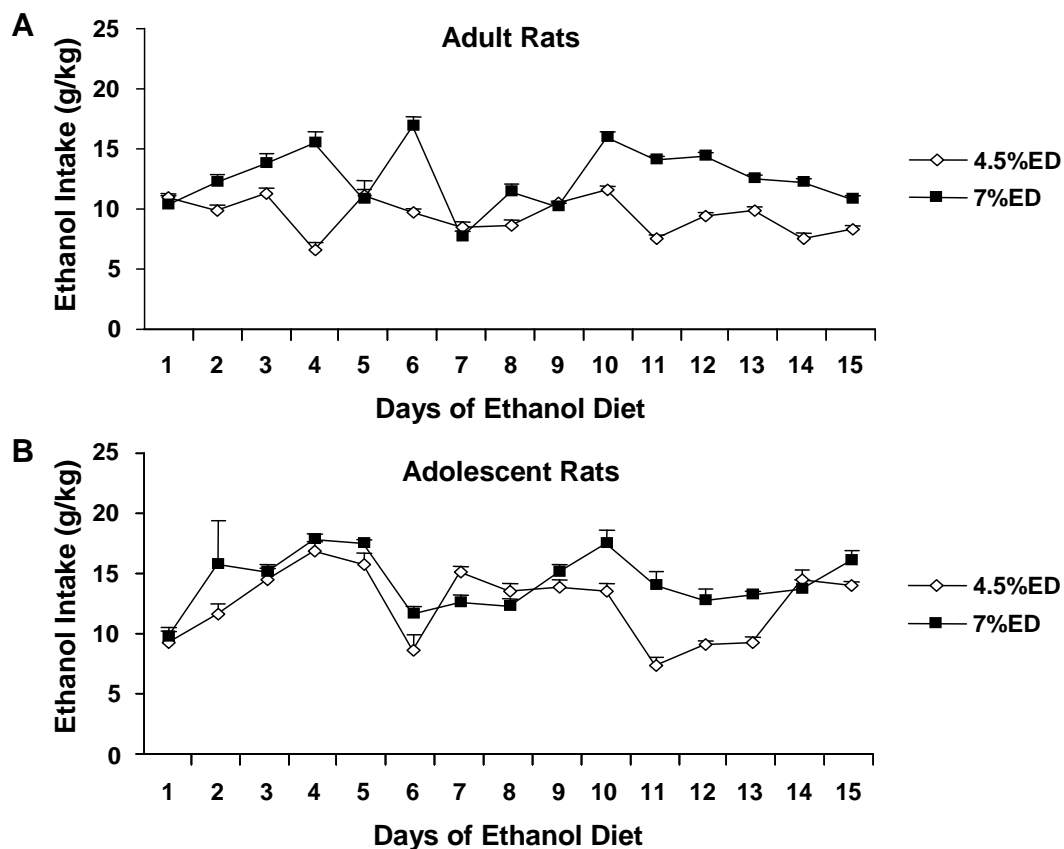


Figure 3.5 Daily ethanol intake in adult (Panel A) and adolescent rats (Panel B) exposed to 4.5%ED and 7%ED. ED groups were exposed to three 5-day cycles of ED interspersed with two 2-day withdrawal periods, during which rats received CD. Data represent mean \pm SEM for 8-10 rats/group.

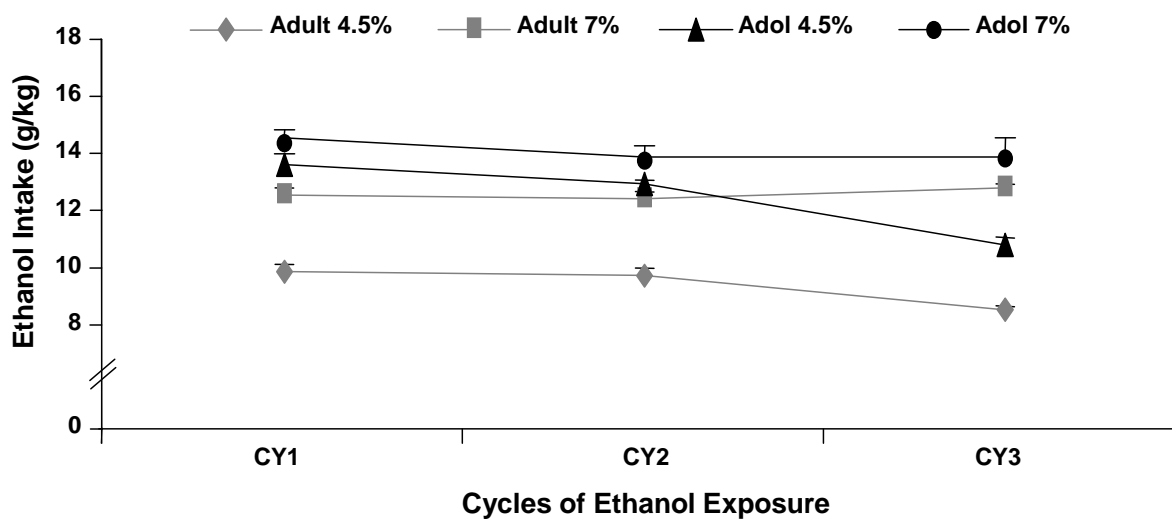


Figure 3.6 Ethanol intake averaged by cycles for adult and adolescent male rats. ED groups were exposed to three 5-day cycles of ED interspersed with two 2-day withdrawal periods, during which rats received CD. These data are an average of daily intake for each 5-day cycle. Data represent mean \pm SEM for 8-10 rats/group.

	Day1	Day5	Day6	Day10	Day11	Day15H0	Day15H2	Day15H4	Day15H6
Adult 4.5%ED	63 ± 11	88 ± 10	94 ± 14	107 ± 12	95 ± 12	40 ± 17	8 ± 8	0 ± 2	0 ± 0
Adult 7%ED	70 ± 11	149 ± 18	123 ± 18	187 ± 24	121 ± 17	187 ± 19	76 ± 15	3 ± 4	0 ± 0
Adolescent 4.5%ED	121 ± 9	160 ± 15	139 ± 15	183 ± 19	130 ± 11	160 ± 30	75 ± 18	21 ± 7	0 ± 1
Adolescent 7%ED	154 ± 12	158 ± 13	129 ± 16	246 ± 16	171 ± 10	284 ± 17	154 ± 15	30 ± 10	0 ± 1

Table 3.1 Blood Ethanol Concentrations in Adult and Adolescent Rats. Adolescent and adult rats given 4.5%ED and 7%ED, which were exposed to three 5-day cycles of ethanol diet interspersed with two 2-day withdrawals. Blood was collected from the tip of the tail during the last hour of darkness on day 1, 5, 6, 10, and 11 of ethanol diet. In addition, blood was collected when ethanol was removed on Day 15 (H0) and during withdrawal (2, 4, & 6 hours). Data represents mean mg% ± SEM for 23-25 rats/group.

	CD	4.5%ED	7%ED
Adult	311 ± 5	310 ± 7	283 ± 5 ^a
Adolescent	153 ± 4	165 ± 6	116 ± 6 ^a

Table 3.2 Body Weights in Adult and Adolescent Rats. Body weights for the three treatment groups (CD, 4.5%ED, and 7%ED) were collected the day before behavioral tests. Data represent mean in grams ± SEM for 8-10 rats/group. ^a Significantly different from CD and 4.5% groups for both adolescents and adults (p<0.05).

CHAPTER IV: SENSITIZATION OF ANXIETY-LIKE BEHAVIOR FOLLOWING REPEATED ETHANOL WITHDRAWALS

Introduction

Previous work (Chapter III) illustrated that adolescent rats have increased anxiety-like behavior and reduced seizure thresholds following exposure to repeated ethanol withdrawals (Wills et al., 2008). While behavioral comparisons indicated an increased vulnerability to these effects in adolescents, increased ethanol intake and differential blood ethanol concentrations (BECs) could also have accounted for age related differences. Nonetheless, these experiments showed that adolescent rats displayed at least as much withdrawal related behaviors as adult rats. This effect was distinct from previous investigations where adolescent rats had either reduced or absent withdrawal-related behaviors compared to adults (Acheson et al., 1999; Doremus et al., 2003; Varlinskaya & Spear, 2004). A number of experimental variables could have accounted for these differences, including route of ethanol administration, species used, age of behavioral testing, and test used to measure anxiety. One potentially critical factor is the use of cycled chronic ethanol exposure (Wills et al., 2008); a variable not employed in these other investigations. While it has been illustrated that adolescent rats can exhibit anxiety-like behavior following repeated withdrawals, it was not established whether repeated ethanol withdrawals actually sensitized anxiety-like behaviors, as has been shown in adult rats (Overstreet et al., 2002; Wills et al., 2008). In order to illustrate sensitization of anxiety-like behavior in adolescent rats it would need to be shown that

neither continuous nor a single cycle of ethanol produced anxiety, which was not demonstrated in previous work (Wills et al., 2008). Furthermore, adult studies have illustrated that pretreatment during early withdrawals with a benzodiazepine receptor antagonist, CRF₁ receptor antagonist, or 5-HT_{1A} receptor agonist was able to block the sensitization of anxiety-like behavior from repeated ethanol withdrawals (Breese et al., 2005b; Overstreet et al., 2003; 2004a).

The current studies were performed to evaluate whether repeated withdrawals sensitize anxiety-like behavior in adolescent rats. Additionally, it was evaluated whether there were age-related differences in the sensitivity to the effects of repeated withdrawals between adolescent and adult rats. Further experiments determined the duration (amount of time following the final withdrawal) of anxiety-like behavior in both adolescent and adult rats. In all of these studies, ethanol intake and blood ethanol concentrations were measured to make sure behavioral effects were not dependent on differences in BECs. Finally, it was also determined whether certain drug pretreatments (benzodiazepine receptor antagonist, CRF₁ receptor antagonist, or 5-HT_{1A} receptor agonist) would prevent the development of sensitized anxiety-like behavior in adolescents.

Materials & Methods

Animals

For standard animal information and housing conditions, refer to General Methods (Chapter II).

Ethanol and Control Diets

General information regarding administration of liquid diet can be found in the General Methods (Chapter II). Rats were then habituated for 3 days on control diet (CD) and then placed into one of three treatment groups. Generally, one-third of the rats received 19 days of CD and the other two-thirds received ethanol diet (3.5% or 2.5%ED). Ethanol diet was administered in a continuous (CON; 15 days of continuous ED; see Figure 4.1A), single cycle (CY1; 14 days of CD followed by one 5-day cycle of ED; see Figure 4.1B), or repeated cycles (CY3; three 5-day cycles of ED separated by two 2-day CD exposures; see Figure 4.1C). Behavioral measures were obtained at various times into the final withdrawal and will be discussed in the following sections.

Social Interaction Test

For standard social interaction (SI) test procedures, refer to General Methods (Chapter II).

Repeated Versus a Single Withdrawal

These experiments determined whether anxiety-like behavior was sensitized in adolescent rats. Adult rats previously showed reduced seconds of social interaction following repeated withdrawals but not after a single 5-day cycle or continuous exposure of 4.5%ED (Breese et al., 2004; Overstreet et al., 2002). In an initial experiment, it was illustrated that a single 5-day cycle of 4.5%ED reduced social interaction compared to

rats that received CD. This effect of anxiety-like behavior following a single cycle of 4.5%ED is most likely due to the increased ethanol intake demonstrated by adolescent rats. Therefore, additional experiments were needed with adolescent and adult rats consuming lower ethanol diet concentrations. These rats were placed into one of five groups: CD throughout the study (CD), 2.5% or 3.5%ED for three 5-day cycles interspersed with two 2-day withdrawal periods (rats received CD; 2.5%CY3 or 3.5%CY3), 4 days of CD followed by 15 continuous days of 2.5%ED (2.5%CON), or 14 days of CD followed by a single 5-day cycle of 2.5%ED (2.5%CY1). All behavioral measures were performed on the 20th day of diet administration when rats maintained on ED were placed on CD followed by behavioral testing starting 5 hours into withdrawal when blood ethanol levels have fallen to zero (Breese et al., 2004; Overstreet et al., 2002; Wills et al., 2008). On the test day, all adolescent rats were the same age.

Duration of Reduced Social Interaction Following Repeated Withdrawals

These experiments were conducted to determine the duration of anxiety-like behavior (measured by reduction in social interaction) following repeated withdrawal in both adolescent and adult rats. Previous work in adult rats illustrated that anxiety-like behavior returns to baseline (CD values) by 48 hours following the final withdrawal in rats exposed to repeated withdrawals from 7%ED (Overstreet et al., 2002). In these experiments adolescent rats were exposed to either repeated withdrawals from 2.5%ED (as described above) or CD. Following these treatments, separate groups of rats were given social interaction tests at either 5 hours, 1, 2, 3, 7, 14, or 18 days following the final withdrawal. At each time point a separate control group was tested to correct for any decreases in social interaction scores that might occur with age. Duration of anxiety-like

behavior was also evaluated in adult rats. Adults were exposed to either repeated withdrawals from 3.5%ED (this concentration provides equivalent g/kg intake and BEC compared to 2.5%ED treatment in adolescents) or CD. Separate groups of adult rats were given social interaction tests at either 5 hours, 1, or 2 days following the final withdrawal. All rats are maintained on CD from the final withdrawal until the time of testing.

Blood Ethanol Concentration

BECs were taken from groups of adolescent rats that received either continuous 2.5%ED, a single cycle of 2.5%ED, or repeated cycles of 2.5% or 3.5%ED, in the manner described above. BECs were also collected from adult rats that received repeated cycles of 2.5%ED or 3.5%ED. Blood was removed from the tip of the tail during the last hour of the dark cycle (0600) on days 1st, 5th, 6th, 10th and 11th days of ED. Additionally, on the last day of ethanol diet (15th day) blood was collected at the time of ethanol removal (hour 0) and then 2, and 4 hrs later. Additional information on BEC analysis can be found in the General Methods (Chapter II).

Drug Testing

The following experiments were conducted to determine the effects of specific neural systems in the sensitization that occurs in adolescent rats during repeated ethanol withdrawals. A CRF₁-receptor antagonist (CP-154,525; 10 mg/kg; Pfizer Inc., Groton, CT), a benzodiazepine receptor antagonist (flumazenil; 5 mg/kg; Roche, Basel, Switzerland), or a 5-HT_{1A}-receptor agonist (buspirone; 0.6 mg/kg; RBI-Sigma, St. Louis, MO) was administered 4 hours following the removal of ethanol diet during each of the initial two cycles (see Figure 4.1C). CP-154,526 and flumazenil were prepared as microfine suspensions in 0.5% carboxy-methylcellulose, while buspirone was dissolved

in 0.9% saline. All drugs were injected at a volume of 2 mls/kg. These drug doses were chosen based on previous studies in adults that illustrated the effectiveness of these drugs to block the sensitization of anxiety-like behavior following repeated withdrawals (Breese et al., 2005b; Overstreet et al, 2003; 2004a). Importantly, the drugs were not administered during the final (3rd) withdrawal, thus animals were tested for anxiety in a drug free state. This important feature of the design permits assessments of drug effects on the sensitization or maladaptation that develops over repeated withdrawals, rather than on the acute behavioral manifestation of withdrawal. Animals that were not injected with drugs (CD-Veh and ED-Veh) were injected with 0.5% carboxy-methylcellulose to control for effects of vehicle.

Statistics

Analyses of social interaction, locomotor activity, ethanol intake, and BECs were conducted with one-way ANOVAs. Comparisons of BECs between adolescent and adult rats were analyzed with repeated measures ANOVA. Differences between individual groups were determined with Fisher's post-hoc tests.

Results

Sensitization of Anxiety-like Behavior from Repeated Withdrawals in Adolescent Rats

Previous work demonstrated that repeated withdrawals from 4.5%ED reduced social interaction in adolescents (Wills et al., 2008). However, it was unclear whether a sensitization of anxiety-like behavior from repeated withdrawals occurred in adolescent rats as it was shown to do in adults. Analysis of social interaction in adolescent rats given lower ethanol concentrations revealed a main effect of diet treatment [$F(3,28) = 6.57$, $p < .005$; Figure 4.2A]. It was demonstrated that adolescent rats given repeated withdrawals from 2.5%ED (CY3) had lower social interaction compared to rats given CD, continuous 2.5%ED (CON), or a single cycle of 2.5%ED (CY1). There was no significant difference among groups in locomotor activity [$F(3,28) = 0.32$, N.S.; Figure 4.2B].

Ethanol Intake in Adolescents Rats

Ethanol intake was compared among adolescent rats that received either continuous ethanol (Con), repeated withdrawals (CY3), or a single cycle (CY1) of 2.5%ED. Ethanol intake was averaged across cycles (average of daily intake for days 1-5 = cycle 1, days 6-10 = cycle 2, and days 11-15 = cycle 3) for each group. During cycle 1, there was a significant effect of group on ethanol intake [$F(1,14) = 25.38$, $p < .0005$; Table 1]. Adolescents that received continuous ethanol diet displayed higher ethanol intake compared to those in the repeated withdrawal group. The single cycle group was left out of the comparisons between cycle 1 and 2 because these rats were receiving CD during this period. Additionally, during the second cycle there was a significant difference between ethanol treatment groups [$F(1,14) = 12.68$, $p < .005$], with continuously exposed rats still having higher ethanol intake than rats given repeated

withdrawals. During the third cycle, there was also a significant effect of ethanol treatment [$F(2,21) = 4.27, p < .05$]. During this cycle, the single ethanol cycle group had the highest intake and was significantly higher than the repeated withdrawal rats but not the continuously exposed rats. Continuously exposed rats and those given repeated withdrawals were not different during this cycle.

Blood Ethanol Concentrations in Adolescent Rats

BECs were analyzed among groups of adolescent rats given three cycles of 2.5%ED (CY3; repeated withdrawal paradigm), 15 days of continuous 2.5%ED (Con), or a single 5-day cycle of 2.5%ED (CY1). Repeated measures ANOVA revealed no significant effect of group (diet treatment) either during the first two weeks of treatment [$F(1,42) = 2.26, NS$; Table 2] or during the final week [$F(2,63) = .49, NS$; Table 2].

Anxiety-like Behavior from Lower Ethanol Diet Concentrations in Adult Rats

The next set of experiments determined whether adult rats given these lower ethanol concentrations also demonstrated anxiety-like behavior. In these studies, there was a main effect of ethanol treatment [$F(2,23) = 6.87, p < .005$; Figure 4.3A]. Adult rats given repeated withdrawals from either 2.5% or 3.5%ED showed a reduction in social interaction compared to rats given CD. Additionally, a main effect of treatment was found for locomotor activity [$F(2,23) = 4.08, p < .05$; Figure 4.3B] in these adult rats. Rats given repeated withdrawals from 3.5%ED had lower activity than rats given CD. There were no differences in locomotor activity between CD and 2.5%ED or 2.5%ED and 3.5%ED rats.

Comparison of Ethanol Intake between Adolescent and Adult Rats

Further experiments were aimed at directly comparing behavioral differences between adolescent and adult rats. It was therefore necessary to determine what ethanol concentration would be able to produce comparable ethanol intake between adolescent and adult rats. Previous work with ethanol diets illustrated that adolescent rats given the same ethanol diet concentration as adults will have higher g/kg intake (Wills et al., 2008). A simplistic way to compare adolescents and adults is to evaluate ethanol intake by cycles. During cycle 1 there were significant differences among 2.5% and 3.5%ED treated adults and adolescents [$F(2,25) = 76.10$, $p < .0001$; Table 3]. Adolescents treated with 2.5%ED drank more than adults who received the same ethanol treatment. Additionally, adults treated with 3.5%ED drank more than adults given 2.5%ED. Finally, adolescents treated with 2.5%ED consumed more ethanol than adults who received 3.5%ED.

Averages of ethanol intake during cycle 2 were also different among groups of 2.5% and 3.5% ED-treated adults and adolescents [$F(2,25) = 80.46$, $p < .0001$; Table 3]. Again, adolescents treated with 2.5%ED drank more than adults who received the same ethanol treatment. Additionally, adult rats treated with 3.5%ED showed higher consumption than those given 2.5%ED. Similar to cycle 1, adolescents treated with 2.5%ED consumed more ethanol than adults who received 3.5%ED.

In the third cycle, group differences were also demonstrated among 2.5% and 3.5% treated adults and adolescents [$F(2,25) = 68.41$, $p < .0001$; Table 3]. Adolescents treated with 2.5%ED drank more than adults who received the same ethanol treatment. Adult rats treated with 3.5%ED showed higher consumption than rats given 2.5%ED.

Importantly, during this last cycle adolescents given 2.5%ED and adults given 3.5%ED were not different in their ethanol intake.

Comparison of Blood Ethanol Concentrations between Adolescent and Adult Rats

BECs were analyzed between adolescent rats treated with 2.5%ED and adults given either 2.5% or 3.5%ED. Repeated measures ANOVA revealed a significant effect of group (Adolescent 2.5%ED, Adult 2.5%ED & 3.5%ED) across the days examined [$F(2,147) = 14.64$, $p < .0001$; Table 4]. Additionally, a separate repeated measures ANOVA between 2.5%ED treated adolescent and adult rats showed there was no significant effect of group [$F(1,98) = .63$, NS; Table 4]. However, a repeated measures ANOVA between 2.5%ED treated adolescent and 3.5%ED treated adult rats exposed a significant difference between these groups [$F(1,98) = 14.34$, $p < .005$; Table 4] with adult rats receiving 3.5%ED having slightly higher BECs. Blood ethanol concentrations (BECs) in both adolescents and adults returned to 0 mg% six hours into withdrawal on the final test day (day 15, data not shown).

Duration of Anxiety-like Behavior from Repeated Withdrawals

Further experiments determined the duration of anxiety-like behavior (measured at various times after the final withdrawal) in adolescent and adult rats following repeated withdrawals. In adult rats exposed to repeated withdrawals from 3.5%ED, there was a significant difference in social interaction between groups tested at different durations [$F(3,36) = 6.60$, $p < .005$; Figure 4.4]. Adult rats tested 5 hours into the final withdrawal showed reduced social interaction compared to CD-treated rats (as shown previously above). However, adult rats tested 24 hours and 48 hours into the final withdrawal were different from rats tested at 5 hours but not from CD-treated rats. These same

experiments were performed in adolescent rats exposed to repeated withdrawals from 2.5%ED. These data are presented as a percent of control to account for the decrease in social interaction that occurs with age. In adolescent rats, there was a significant difference in social interaction between groups tested at different durations [$F(7,80) = 9.02$, $p < .0001$; Figure 4.5]. Groups tested 5 hours, 1, 2, 3, and 7 days into the final withdrawal showed significantly reduced social interaction (% of control values) compared to control groups. However, groups tested 14 and 18 days into the final withdrawal were not different from controls.

Effects of Selective Drugs on the Sensitization of Anxiety-like Behavior Following Repeated Withdrawals in Adolescent Rats

A final set of experiments were performed to determine whether a CRF_1 antagonist (CP-154,526), a benzodiazepine receptor antagonist (flumazenil), or a 5-HT_{1A}-receptor agonist (buspirone) would block the sensitization of anxiety-like behavior following repeated withdrawal in adolescent rats, as they have been shown to do in adult rats (Knapp et al., 2004; Overstreet et al., 2003). In adolescent rats, there was a significant difference between drug treatments in social interaction [$F(4,97) = 2.58$, $p < .05$; Figure 4.6A]. Adolescent rats given buspirone, flumazenil, or CP-154,526 had social interaction scores not different from those of CD-treated rats. Additionally, adolescent rats injected with vehicle showed reduced social interaction compared to all drug treated groups. Locomotor activity was also evaluated in the social interaction test following these drug treatments and no significant differences among treatment groups were found [$F(4,97) = .828$, NS; Figure 4.6B].

Discussion

Adolescent rats exhibited sensitized anxiety-like behavior following repeated withdrawals, which has been previously demonstrated with 4.5% ethanol diet (ED) in adults (Overstreet et al., 2002). This effect was illustrated by a reduction in social interaction (a validated measure of anxiety-like behavior; File & Seth, 2003) in adolescent rats that experienced repeated withdrawals from 2.5%ED. Importantly, adolescents given either a single 5-day cycle or continuous 15 days of 2.5%ED (same ethanol exposure as repeated withdrawal groups) did not exhibit this reduction in social interaction. These data, therefore, indicate that the production of anxiety-like behavior (with this ED concentration) was dependent on the cycled nature of the ethanol exposure. Additionally, these studies illustrated that locomotor activity was not reduced by any of these ethanol treatments in adolescent rats. Previous research from our laboratory demonstrated that adolescent rats given repeated withdrawals from higher ED concentrations can exhibit anxiety-like behavior following repeated withdrawals (Wills et al., 2008). These experiments, however, are the first to demonstrate that a sensitization of this anxiety-like behavior can occur in adolescent rats. Previous acute ethanol withdrawal tests in adolescent rats showed reduced or no anxiety-like response at this age compared to adult rats (Doremus et al., 2003; Varlinskaya & Spear, 2004). Therefore, the presence of this anxiety-like phenotype after repeated ethanol withdrawals in adolescent rats establishes that the cycled nature of ethanol exposure and age of testing are critical to producing anxiety-like behavior during ethanol withdrawal.

Earlier work in adult rats illustrated that they too can undergo sensitization of anxiety-like behavior (e.g., Overstreet et al., 2002). These studies used somewhat higher ED concentration (4.5%ED), so it was unknown whether these lower ED concentrations (2.5% & 3.5%ED) would also produce anxiety-like behavior in adult rats. The results established that indeed repeated withdrawals from these lower ED concentrations produced anxiety-like behavior in adult rats. These data indicated that although adolescent rats demonstrate sensitized anxiety-like behavior, that this response was not unique from the behavioral response of adult rats at these lower ethanol diet concentrations. It is notable that although we report here that rats receive two formal extended withdrawals periods of 48 hours, it is reasonable to assume that the rats may also occasionally experience a type of brief withdrawal between meals across the circadian cycle (e.g., during sleep bouts). Despite the possibility of these mini-withdrawals, previous research shows that the formal withdrawals capture the essence of the sensitization process as continuous ethanol exposure or a single 5-day cycle (with both types of groups conceivably experiencing occasional mini-withdrawals) do not lead to substantial anxiety relative to non-ethanol exposed rats (e.g. Overstreet et al., 2002).

Analysis of ethanol intakes and blood ethanol concentrations (BECs) illustrated that differences in behavior among adolescent groups (CY1, Con, & CY3) were not related to differences in ethanol intake and BECs. Additionally, a comparison between the ethanol intake of adolescent and adult rats revealed adolescents consumed more g/kg of ethanol than adults given the same treatment. This increased ethanol intake in adolescent rats has been previously demonstrated in our laboratory with liquid diet (Wills et al., 2008), as well as in other laboratories with other ethanol administration paradigms

(Bell et al., 2006; Doremus et al., 2005; Rodd-Henricks et al., 2002a/b; Vetter et al., 2007; Walker et al., 2008). Furthermore, these data illustrated that adolescent rats given 2.5%ED had comparable ethanol intake to adults given 3.5%ED. BEC comparisons between these two groups show that even with comparable ethanol intake, adult rats treated with 3.5%ED had a slightly higher BEC than 2.5%ED treated adolescent rats. Since both of these groups showed the most comparable ethanol intake and BEC, they were used for comparisons in duration experiments. It is worth noting also that BECs in both adolescent and adult rats may represent the BEC on the falling end of the curve and therefore may be slightly lower than peak BEC on a given day.

In adolescent rats, it was demonstrated that anxiety-like behavior was present up to one week following repeated ethanol withdrawals. This effect is in stark contrast to the effect in adult rats where anxiety-like behavior returns to baseline after only 24 hours. These data illustrate that the anxiety-like behavior produced from repeated withdrawals is much longer lasting in adolescent versus adult rats. Therefore, it appears that adolescent rats may be more vulnerable to the effects of repeated withdrawals on this measure. The relative importance of this finding to adolescent versus adult risk for further alcohol abuse/relapse is unknown. However, previous research supports the idea that adolescent ethanol exposure can affect future responses to ethanol in adulthood. A body of evidence shows that the differential sensitivities to ethanol displayed by adolescents can also be maintained into adulthood (Slawecki, 2002; White et al., 2000; 2002). Additionally, Crews et al. (2000) showed that a 4-day binge ethanol exposure in adolescents caused brain damage in select regions that does not appear in adults. Further, in P rats it was illustrated that prior ethanol exposure during adolescence, but not in adulthood, affects

later ethanol responding. That is, adolescent exposure in P rats led to faster responses to ethanol, more resistance to extinction, and increased responding to ethanol upon re-exposure (Rodd-Henricks et al., 2002a/b). This evidence indicates ethanol treatment during the adolescent period can affect future responses to ethanol. These observations of long lasting effects in rodents may relate to clinical evidence demonstrating that alcohol use during adolescence is the largest predictor for future alcoholism (Grant, 1998).

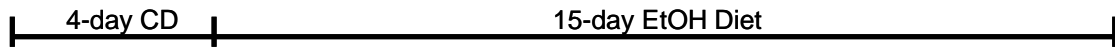
Finally, it was illustrated that this increased anxiety-like behavior produced from repeated withdrawals could be blocked by pretreatment with buspirone, flumazenil, or CP-154,526. Notably, these drugs blocked the induction of sensitized anxiety-like behavior since no drugs were administered during the final withdrawal when rats were tested for anxiety-like behavior. Previous work in adults has also shown that pretreatment with these drugs also blocked anxiety-like behavior (Breese et al., 2005b; Overstreet et al, 2003; 2004a). The effectiveness of these drugs in both age groups indicates that mechanisms that are responsible for this anxiety-like behavior are likely similar between adolescent and adult rats. These findings suggest that at least some future pharmacological treatment strategies in alcoholism might apply broadly across age.

In summary, these experiments illustrated that adolescent rats exhibit sensitized anxiety-like behavior that had been previously demonstrated in adult rats (Overstreet et al., 2002). Comparisons of adolescent and adult rats at these lower ethanol diet concentrations revealed that the sensitivity of this response seemed to be similar for both ages. However, the duration of this anxiety-like response was found to be much longer in adolescent rats that experienced repeated withdrawals compared to their adult

counterparts. On the other hand, it was illustrated that drugs known to be effective in preventing the sensitization of anxiety-like behavior in adult rats are also effective in adolescents. Future work will further explore the mechanisms that might be responsible for the extended duration of anxiety-like behavior in adolescent rats.

Figures & Tables

(A) Continuous Ethanol (CON) Paradigm:



(B) Single Cycle Ethanol (CY1) Paradigm:



(C) Repeated Withdrawal (CY3) & Drug Pretreatment Paradigm:

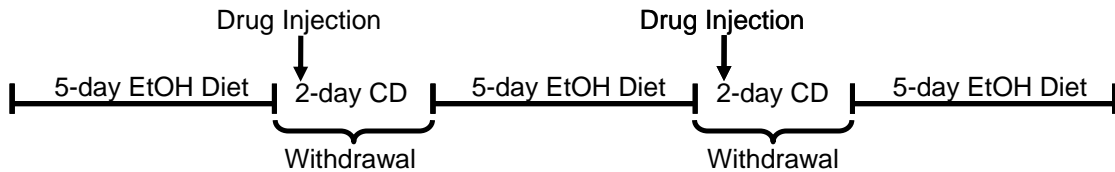


Figure 4.1 Procedure for diet administrations. (Panel A) Continuous ethanol paradigm (Con): rats were given 4 days of control diet (CD) followed by 15 days of ethanol diet (ED). (Panel B) Single cycle ethanol paradigm (CY1): rats were given 14 days of CD followed by one 5-day cycle of ED. (Panel C) Repeated withdrawal (CY3) and drug pretreatment paradigm: rats were given three 5-day cycles of ED interspersed with two 2-day withdrawal periods, during which time rats receive CD. Some rats were injected with CP-154,516, flumazenil, or buspirone four hours into the first two withdrawal periods.

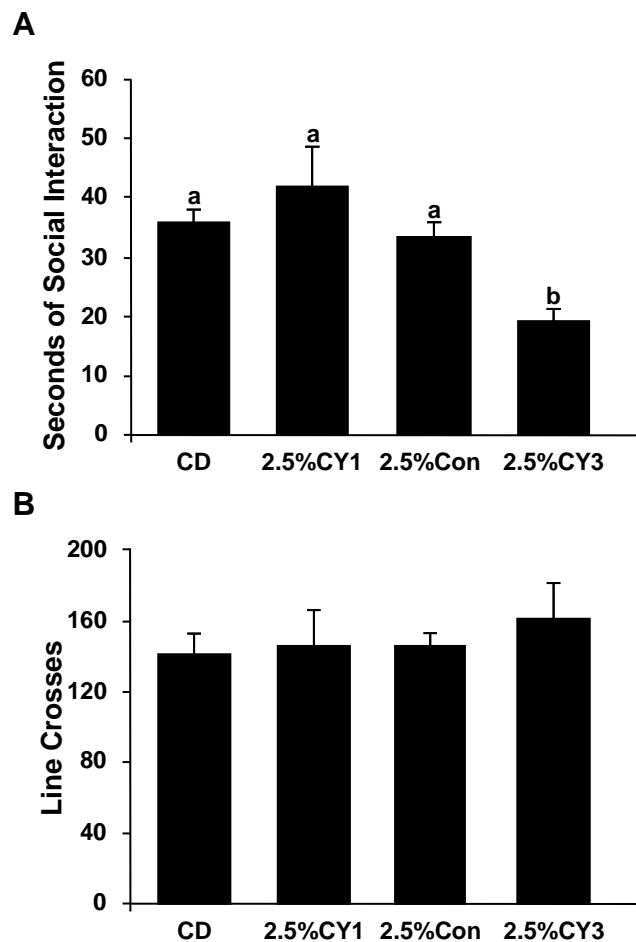


Figure 4.2 Social interaction and locomotor activity in adolescent rats (Panel A & B). Male adolescent rats were given either control diet (CD), continuous 15 days of 2.5% ethanol diet (ED) continuously (2.5%Con), one 5-day cycle of 2.5% ED (2.5%CY1), or repeated ethanol withdrawals from 2.5%ED (CY3). Repeated withdrawal groups were exposed to three 5-day cycles of ED interspersed with two 2-day withdrawal periods, during which rats receive CD. All adolescent rats were tested 5 hours after removal of ethanol during the final withdrawal. Data represent means \pm SEM for 8 rats/group. Groups with different letters are significantly different from each other ($p < 0.05$).

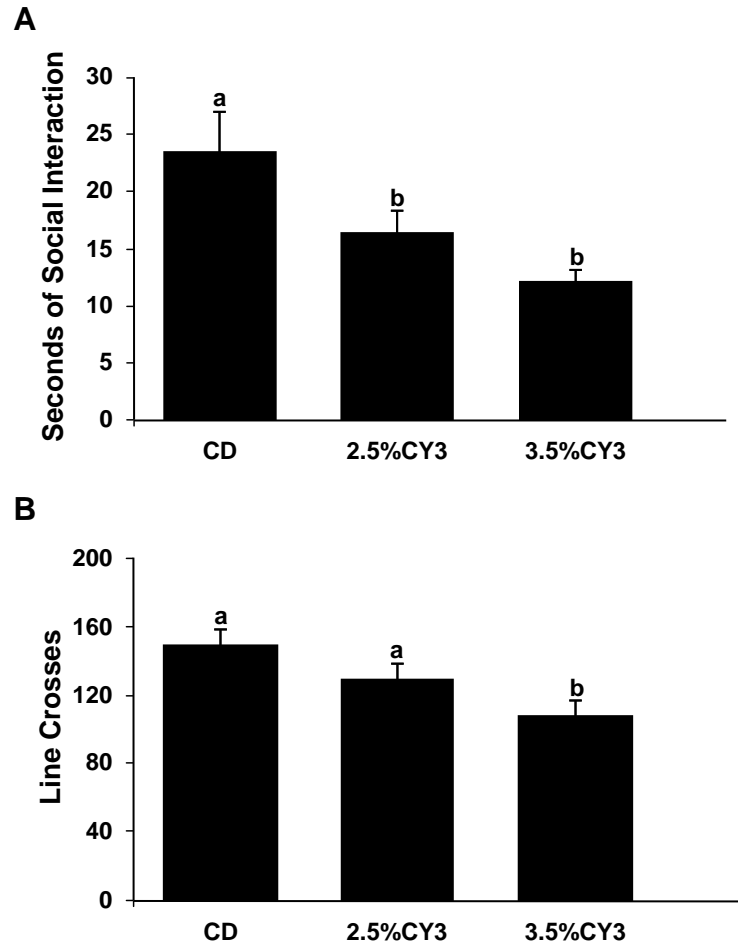


Figure 4.3 Social interaction and locomotor activity in adult rats given repeated ethanol withdrawals from lower ethanol diet concentrations (Panel A & B). Male adult rats were given control diet (CD), 2.5% ethanol diet (ED), or 3.5%ED. ED groups were exposed to three 5-day cycles of ED interspersed with two 2-day withdrawal periods (CY3), during which rats receive CD. Rats were tested 5 hours after removal of ethanol during the final withdrawal. Data represent means \pm SEM for 8 rats/group. Groups with different letters are significantly different from each other ($p < 0.05$).

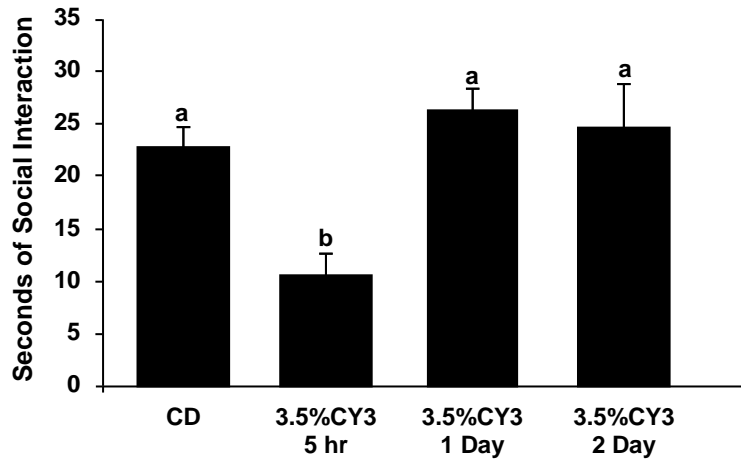


Figure 4.4 Duration of anxiety-like behavior in adult rats exposed to repeated ethanol withdrawals. Male adult rats were given either control diet (CD) or 3.5% ethanol diet (ED) for three 5-day cycles interspersed with two 2-day withdrawals (CY3). Rats were tested 5 hours, 1 day, or 2 days after the removal of ethanol during the final withdrawal. Data represent means \pm SEM for 8 rats/group. Groups with different letters are significantly different from each other ($p < 0.05$).

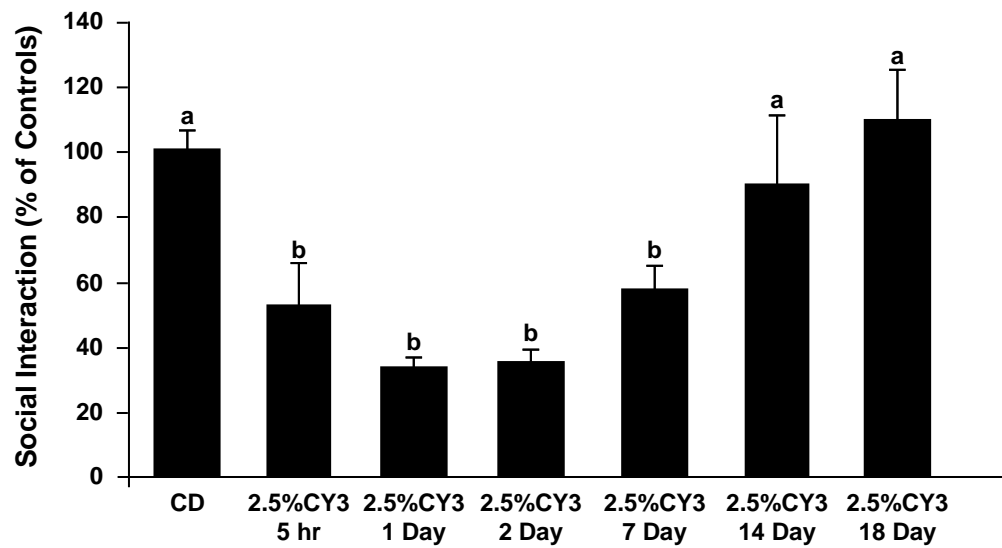


Figure 4.5 Duration of anxiety-like behavior in adolescent rats exposed to repeated ethanol withdrawals. Male adolescent rats were given either control diet (CD) or 2.5% ethanol diet (ED) for three 5-day cycles interspersed with two 2-day withdrawals (CY3). Rats were tested 5 hours, 1, 2, 7, 14, or 18 days after the removal of ethanol during the final withdrawal. Data represent mean \pm SEM for 7-10 rats/group. Groups with different letters are significantly different from each other ($p < 0.05$).

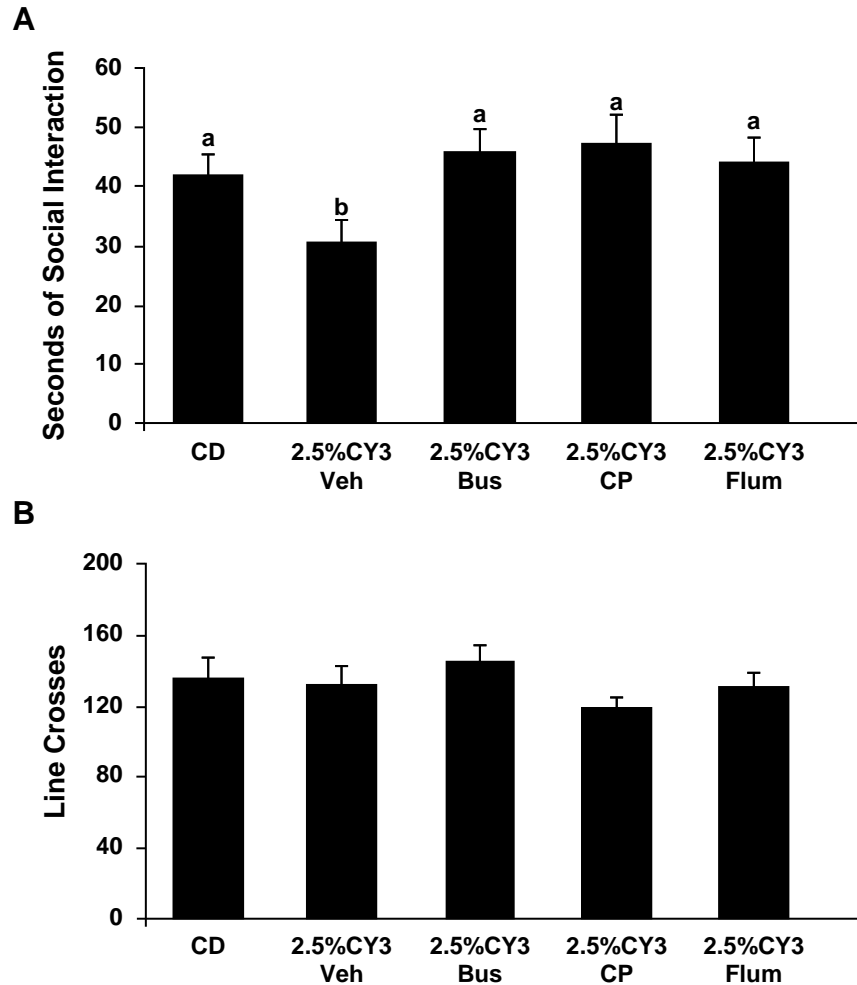


Figure 4.6 Effect of drug pretreatments during repeated withdrawals on social interaction and locomotor activity in adolescent rats (Panel A & B). The 5-HT_{1A} receptor agonist, buspirone, (Bus; 0.6 mg/kg), benzodiazepine antagonist, flumazenil, (Flum; 5 mg/kg), CRF₁ receptor antagonist, CP-154,526 (CP; 10 mg/kg), or vehicle was given during the first two early withdrawals of adolescent rats given repeated ethanol withdrawals from 2.5% ethanol diet (ED; CY3). Rats were tested 5 hours after the removal of ethanol during the final withdrawal. Data represent means ± SEM for 16-25 rats/group. Groups with different letters are significantly different from each other ($p < 0.05$).

	Cycle 1	Cycle 2	Cycle 3
2.5% CY1	-	-	^a 7.16 ± 0.1
2.5% Con	^a 11.34 ± 0.5	^a 8.62 ± 0.2	^b 6.83 ± 0.1
2.5% CY3	^b 8.49 ± 0.3	^b 7.70 ± 0.2	^b 6.66 ± 0.1

Table 4.1 Ethanol Intake (Averaged by Cycles) in Adolescent Rats. Adolescent rats were exposed to 2.5% ethanol diet for either: one 5-day cycle of ethanol diet (CY1), 15 continuous days of ethanol diet (Con), or three 5-day cycles of ethanol diet interspersed with two 2-day withdrawals (CY3). Data are an averaged daily intake for each 5-day cycle and represent mean g/kg ± SEM for 8 rats/group. Groups with different letters are significantly different from other groups within each cycle (p<0.05).

	Day1	Day5	Day6	Day10	Day11	Day15H0	Day15H2	Day15H4
2.5% CY1					36 ± 5	22 ± 6	1 ± 1	0
2.5% Con	51 ± 12	22 ± 9	30 ± 8	12 ± 4	30 ± 4	13 ± 4	0	0
2.5% CY3	49 ± 11	40 ± 11	52 ± 9	36 ± 9	37 ± 5	15 ± 4	1 ± 1	0

Table 4.2 Blood Ethanol Concentrations in Adolescent Rats. Adolescent rats were exposed to 2.5% ethanol diet for either one 5-day cycle of ethanol diet (CY1), 15 continuous days of ethanol diet (Con), or three 5-day cycles of ethanol diet interspersed with two 2-day withdrawals (CY3). Blood was collected from the tip of the tail during the last hour of darkness on day 1, 5, 6, 10, and 11 of ethanol diet. In addition, blood was collected when ethanol was removed on Day 15 (H0) and during withdrawal (2 & 4 hours; H2 & H4 respectively). Data represent mean mg% ± SEM for 8 rats/group.

	Cycle 1	Cycle 2	Cycle 3
<i>Adolescent 2.5% CY3</i>	^a 8.49 ± 0.3	^a 7.70 ± 0.2	^a 6.66 ± 0.1
<i>Adult 2.5% CY3</i>	^b 5.26 ± 0.1	^b 5.13 ± 0.1	^b 4.8 ± .01
<i>Adult 3.5% CY3</i>	^c 7.21 ± 0.2	^c 7.0 ± 0.2	^a 6.88 ± 0.2

Table 4.3 Comparison of Ethanol Intake (Averaged by Cycles) between Adolescent and Adult Rats. Ethanol intake averaged by cycles for male adult and adolescent rats. 2.5% and 3.5% ethanol diet (ED) groups were exposed to three 5-day cycles of ED interspersed with two 2-day withdrawal periods, during which rats received control diet (CD). These data are an average of daily intake for each 5-day cycle. Data represent means ± SEM for 8 rats/group. Groups with different letters are significantly different from other groups within each cycle (p<0.05).

	Day1	Day5	Day6	Day10	Day11	Day15H0	Day15H2	Day15H4
Adolescent 2.5% CY3	^a 49 ± 11	^a 40 ± 11	^{a/b} 52 ± 9	^a 36 ± 9	^a 37 ± 5	^a 15 ± 4	^a 1 ± 1	0
Adult 2.5% CY3	^b 21 ± 4	^a 19 ± 3	^a 35 ± 3	^a 33 ± 10	^a 42 ± 6	^b 35 ± 4	^a 9 ± 3	1 ± 1
Adult 3.5% CY3	^a 47 ± 6	^b 77 ± 12	^b 65 ± 5	^b 64 ± 5	^b 71 ± 9	^c 58 ± 6	^b 21 ± 6	1 ± 0

Table 4.4 Comparison of Blood Ethanol Concentrations between Adolescent and Adult Rats. Blood ethanol levels (BECs) in adult and adolescent rats were exposed to either 2.5% or 3.5% ethanol diet (ED) for three 5-day cycles interspersed with two 2-day withdrawals. Blood was collected from the tip of the tail during the last hour of darkness on day 1, 5, 6, 10, and 11 of ethanol diet. In addition, blood was collected when ethanol was removed on Day 15 (H0) and during withdrawal (2 & 4 hours; H2 & H4 respectively). Data represent means mg% ± SEM for 8 rats/group. Groups with different letters are significantly different from other groups within each day (p<0.05).

CHAPTER V: SUBSTITUTION OF STRESS AND CRF IN ADOLESCENT RATS

Introduction

There is a growing body of evidence from both basic and clinical research illustrating that stress can increase the risk of relapse in alcoholism (Lê et al., 2000; Sinha, 2001). Furthermore, it has been suggested that stress during adolescence is a risk factor for the development of future psychiatric disorders, including alcohol dependence (Arnsten & Shansky, 2004; Enoch, 2006). Clinical data also illustrated that the second largest predictor for ethanol consumption during adolescence is perceived stress (Wagner, 1993). Studies in adolescent rodents have generated mixed findings regarding the consequences of stress on ethanol consumption. Doremus et al. (2005) showed that isolate housing (a stress to adolescents) did not effect ethanol consumption more in adolescent rats than adults. In another experiment, daily footshock stress reduced concurrent ethanol consumption in a homecage setting in adolescent rats compared to adults (Brunell & Spear, 2005). Further, in a genetically alcohol preferring mouse strain (HAD1 mice) footstock stress during adolescence increased ethanol intake in adulthood more than if stress was given during adulthood (Chester et al., 2008). These data indicate that the interactions between stress, adolescence, and ethanol are likely complex and require additional investigation. Additionally, it is known that adaptations in stress-responsive corticotrophin releasing factor (CRF) system are associated with anxiety, ethanol use, withdrawal, and relapse (discussed in previous sections). It is therefore, necessary to evaluate the interactions of stress and CRF during adolescence.

Previous work in our laboratory with adult and adolescent rats illustrated that repeated withdrawals from ethanol can sensitize anxiety-like behavior which is not demonstrated after continuous ethanol exposure (Overstreet et al., 2002; Wills et al., 2009). Furthermore, in adult rats it has been shown that two weekly one hour restraint stress sessions substitute for early withdrawals (stress/withdrawal paradigm) to sensitize anxiety-like behavior (Breese et al., 2004). One hour of restraint stress was used because this amount of stress acutely produced anxiety-like behavior 30 minutes following the termination of stress (Breese et al., 2005b). This anxiety-like behavior produced by the stress/withdrawal paradigm seems to arise from extra-hypothalamic sites since two weekly injections of corticosterone were unable to mimic the anxiety-like behavior caused by repeated restraint stress (Breese et al., 2004). Other work in adult rats indicates that CRF acting through these extra-hypothalamic targets is at least partially responsible for the production of anxiety-like behavior from these paradigms. In adult rats, the development of anxiety-like behavior can be prevented in these paradigms by giving a CRF type 1 antagonist during early withdrawals or stress (Breese et al., 2004; Knapp et al., 2004). Additionally it was later illustrated that CRF administered intraventricularly could also substitute for either early withdrawals or stress to produce anxiety-like behavior (Overstreet et al., 2004a).

In addition to these effects on CRF there are also intracellular signaling cascades, like the Erk_{1/2}/MAPK pathway, that are thought to be important in the processes that underlie addiction (Russo et al., 2008; Zhai et al., 2008). This Erk_{1/2}/MAPK pathway has been shown to be modulated by acute ethanol, while the direction of this modulation is mixed. Several studies have indicated increases in the phosphorylation in Erk_{1/2} (pErk)

following acute ethanol (Sharko & Hodge, 2007), while others find a decrease in pErk (Chandler & Sutton, 2005; Hendrickson et al., 1998; Kalluri & Ticku, 2002; Tsuji et al., 2003). Sanna et al. (2002) also illustrated a reduction in pErk during ethanol exposure but found that pErk during ethanol withdrawal was increased in most brain areas. Furthermore, in this study it was illustrated that this increase in pErk was further enhanced in the amygdala during withdrawal from chronic intermittent ethanol exposure (similar to repeated ethanol withdrawals) versus continuous ethanol exposure (Sanna et al., 2002).

The present work set out to determine the role of stress and CRF in the production of anxiety-like behavior from ethanol withdrawal in adolescent rats. First, it was evaluated whether the stress/withdrawal paradigm produced anxiety-like behavior in adolescent rats, as it has been shown to do in adult rats. Further, it was evaluated how long this anxiety-like behavior following stress/withdrawal is present. Next, studies in adolescents were conducted to determine the acute effects of stress on anxiety-like behavior. Further, it was evaluated if housing condition affected this response, since there is some evidence that isolate housing (used in our experiments) during adolescence could function as a chronic stressor (Hall, 1998). Additional studies were performed to determine if CRF substituted for early withdrawals or stress to produce anxiety-like behavior in adolescent rats and if the effective doses were similar to those used in adult rats. Finally, experiments were performed to assess the levels of CRF and phosphorylated extracellular signal-regulated kinase 1/2 (pErk) in the central nucleus of the amygdala (CeA), paraventricular nucleus of the hypothalamus (PVN), and dorsal

lateral bed nucleus of the stria terminalis (dIBNST) following stress/withdrawal and repeated withdrawal paradigms in adolescent and adult rats.

Materials & Methods

Animals

For standard animal information and housing conditions, refer to General Methods (Chapter II). One group of adolescent rats were group housed 3/cage for the length of the experiment.

Ethanol and Control Diets

General information regarding administration of liquid diet can be found in the General Methods (Chapter II).

Social Interaction Test

For standard social interaction (SI) test procedures, refer to General Methods (Chapter II).

Stress/Withdrawal Paradigm

The Stress/Withdrawal Paradigm involves the substitution of stress for the first two early withdrawals. The stress used in these experiments is one hour of restraint stress in a plastic cone. Adolescents were placed in one of the following groups: 19 days of control diet (CD) (no stress; CD), 4 days of CD followed by 15 days of continuous 2.5%ethanol diet (ED) with stress (given on Day 6 and 11 of ED; 2.5%CON-Str; Figure 5.1A), 14 days of CD with stress (given on Day 6 & 13) followed by one 5-day cycle of 2.5%ED (2.5%CY1-Str; Figure 5.1B), or 19 days of CD with stress (given on Day 6, 13, 19; CD-Str; Figure 5.1C). Social interaction was performed five hours after the final withdrawal or stress.

Duration of Reduced Social Interaction Following Stress/Withdrawal Paradigm

These experiments were conducted to determine the duration of anxiety-like behavior (measured by reduction in social interaction) following the stress/withdrawal paradigm in adolescent rats. The stress/withdrawal paradigm used in these experiments involved giving a group two stress sessions (on Day 6 and 13) followed by 5 days of 2.5%ED. For comparison, one group of rats were maintained on CD through out the experiment. Following these treatments, separate groups of rats were given social interaction tests at either 5, 24, or 48 hours following the final withdrawal. All rats are maintained on CD from the final withdrawal until the time of testing.

Acute Stress Protocol

Adolescent rats were maintained on CD in either isolate or group housing conditions until P29. Rats were then placed in plastic cones and stressed using for 45, 90, or 120 minutes (isolate housed rats) and 60 or 90 minutes (group housed rats). Thirty minutes following the termination of stress, rats were tested in the social interaction test.

Surgery

Surgery was performed under 2.5% isoflurane anesthesia and then placed in a stereotaxic instrument (Kopf Instruments, Tujunga, CA). After exposing the dorsal surface of the skull, holes were drilled in the skull at the appropriate locations, and cannulae were inserted at the appropriate depth. Jeweler's screws were implanted into the skull, and dental acrylic was applied to secure the cannulae to the skull. All cannulae were made from 26-gauge stainless steel tubing. Once recovered from anesthesia, the rats were given acetaminophen (children's Q-Pap, cherry flavor, 6 mg/ml) in the drinking water for 48 hours.

CRF Administration

Adolescent rats were microinjected with 5.0, 7.5, or 10.0 µg/5µl of CRF into the lateral ventricle (AP = -0.6, ML = -1.2, DV = -1.5). Two injections of CRF or Veh (artificial cerebral spinal fluid) were administered one week apart starting one week after recovery from surgery. Rats in these experiments were given one of the following diet conditions in combination with CRF or Veh administration. Rats combined into the control group received either 19 days of CD or 14 days of CD followed by a single 5-day cycle of 2.5%ED and all rats received Veh microinjections. For each dose of CRF, rats were given either 19 days of CD or 14 days of CD followed by a single 5-day cycle of 2.5%ED.

Immunohistochemistry

For analysis of immunohistochemistry for CRF and pErk, adolescent and adult rats were divided into three treatment groups: repeated ethanol withdrawal groups which received three 5-day cycles of either 2.5%ED (adolescents; 2.5%CY3) or 3.5%ED (adults; 3.5%CY3) interspersed with two 2-day withdrawal periods (given CD); stress/withdrawal groups which received 14 days of CD with stress (given on Day 6 & 13) followed by one 5-day cycle of 2.5%ED (adolescents; 2.5%CY1-Str) or 3.5%ED (adults; 3.5%CY1-Str), or control groups which received 19 day of ethanol diet (CD). Different ethanol diet concentrations were used in adolescent and adult rats because of previous data showing that adolescent rats have higher g/kg ethanol intake than their adult counterparts (Wills et al., 2008; 2009). In previous experiments, it was shown that treating adolescent rats with 2.5%ED and adult rats with 3.5%ED produces similar ethanol intakes (Wills et al., 2009). All of these rats were tested in social interaction 5 hours into the final ethanol withdrawal or 20th day of CD.

Immediately following the social interaction test, rats were perfused with PBS and then 4% paraformaldehyde. After postfixing brains for at least 24 hours, free floating 40 micron coronal sections throughout the brain were collected with a vibrating microtome at 4°C. Immunohistochemical assays of CRF (rabbit anti-CRF human, rat; Peninsula laboratories; 1:5000) and pErk [Phospho-p44/42 MAP kinase (Thr202/Try204) Antibody; Cell Signaling; 1:500] were conducted by using a modification of a standard avidin-biotin/horseradish peroxidase method described previously (Knapp et al., 1998; 2001). Four representative sections of the central nucleus of amygdala (CeA), two representative sections of the paraventricular nucleus of the hypothalamus (PVN), and two representative sections of the dorsal lateral bed nucleus of the stria terminalis (dIBNST) for each rat were photographed digitally at 10X, captured with Bioquant Life Sciences (Ver. 8.0), and then analyzed using the Image J program. Cell counts were also performed at 20X magnification on the same four representative sections of the CeA and two representative sections of the PVN in adolescent and adult rats that received control diet.

Statistics

Analyses of social interaction, locomotor activity, and immunohistochemistry were conducted with one-way ANOVAs. Comparisons between two groups in immunohistochemical studies used t-tests. Differences between groups were determined with Fisher's post hoc tests.

Results

Stress/Withdrawal Paradigm in Adolescent Rats (Social Interaction & Locomotor Activity)

Previous work demonstrated that stress substituted for early withdrawals to produce anxiety-like behavior in adult rats; however, this same process had not been assessed in adolescent rats (Breese et al., 2004). Analysis of social interaction in adolescent rats given the stress/withdrawal paradigm (2.5%Con-Str & 2.5%CY1-Str; Figure 5.1A & B) revealed a main effect of stress/diet treatment [$F(3,27) = 8.26$, $p < .0005$; Figure 5.2A]. Adolescent rats given stress/withdrawal paradigm (2.5%Con-Str & 2.5%CY1-Str) had lower social interaction compared to rats given CD. Additionally, there was no significant difference between CD-Str and CD rats.

During the social interaction test, locomotor activity was also simultaneously measured. Analysis of locomotor activity in these groups also showed a significant main effect of stress/diet treatment [$F(3,27) = 3.11$, $p < .05$; Figure 5.2B]. Rats given stress with a single ethanol cycle (2.5%CY1-Str) showed significantly lower locomotor activity compared to CD and 2.5%Con-Str treated rats. There were no significant differences among other treatment groups.

Duration of Anxiety-like Behavior Following Stress/Withdrawal Paradigm

Further experiments determined the duration of anxiety-like behavior (measured at various times after the final withdrawal) in adolescent rats following stress/withdrawal paradigm (2.5%CY1-Str). Evaluation of social interaction showed a significant difference between groups tested at different durations [$F(3,36) = 3.96$, $p < .05$; Figure 5.3A]. Adolescent rats tested 5 hours into the withdrawal showed reduced social interaction

compared to CD-treated rats (as shown previously above). However, adolescent rats tested 24 hours and 48 hours into the final withdrawal were different from rats tested at 5 hours but not from CD-treated rats. Additionally, there were no significant differences in locomotor activity [$F(3,36) = .25$, NS; Figure 5.3B].

Social Interaction Following Acute Restraint Stress in Isolate and Group-Housed Adolescent Rats

Experiments in adult rats demonstrated that 60 minutes of restraint stress induces anxiety-like behavior (reduction in social interaction; Breese et al., 2005b); however, it is unknown how adolescents might respond to similar stress periods. Individually housed adolescent rats showed no significant differences in social interaction after various periods of stress [$F(3,28) = .81$, NS; Figure 5.4A]. There were also no significant differences in locomotor activity [$F(3,28) = 2.55$, NS; data not shown].

Since there is some evidence (Hall, 1998) that isolate housing may alter adolescent rats response to stress, social interaction was also performed in group housed adolescents following acute restraint stress. In these rats, there were also no significant differences in social interaction following stress [$F(2,21) = 1.00$, NS; Figure 5.4B]. Additionally, no significant differences were found in locomotor activity [$F(2,21) = 1.91$, NS; data not shown].

CRF/Withdrawal Paradigm in Adolescent Rats (Social Interaction & Locomotor Activity)

Previous work demonstrated that intraventricular CRF at 5 μ g substituted for early withdrawals to produce anxiety-like behavior in adult rats (Overstreet et al., 2004a). Additionally, we previously demonstrated that a CRF type 1 antagonist prevented the adaptations caused by repeated withdrawals in adolescent rats (Wills et al., 2009). These

data are presented as a percent of control because multiple studies needed to be combined to fully illustrate the effects of the various doses of CRF administered. Additionally, controls represent rats treated with CD and those given a single cycle of 2.5%ED. Analysis of social interaction in adolescent rats given the CRF/withdrawal paradigm revealed a main effect of CRF/diet treatment [$F(6,103) = 4.33$, $p < .001$; Figure 5.5A]. Adolescent rats given 7.5 μ g of CRF and ethanol diet (7.5CRF-ED) had lower social interaction compared to controls, 5 μ g dose with ED or CD, or 7.5 μ g dose with CD. Neither the 5 μ g or 10 μ g dose of CRF combined with ED was significantly different from controls. In rats that received CRF with CD, social interaction compared to controls was increased with 7.5 μ g, decreased with 10 μ g, and unchanged with 5 μ g of CRF.

There was also a main effect of CRF/diet treatment on locomotor activity [$F(6,103) = 2.30$, $p < .05$; Figure 5.5B]. There was an increase in locomotor activity in rats treated with 10 μ g of CRF-ED compared to all groups except 7.5 μ g-CD.

CRF Immunohistochemistry: Baseline Differences between Adolescent & Adult Rats

Experiments were conducted to evaluate differences in baseline levels (rats receiving CD) of CRF between adolescents and adults. In the central nucleus of the amygdala (CeA) there was a significant difference in CRF levels between adolescents and adults [$t(16) = 2.90$, $p < .05$; Figure 5.6A]. In this region, there was a greater total density of CRF-immunoreactivity in adolescent compared to adult rats. There were also differences in total density of CRF between age groups in the paraventricular nucleus of hypothalamus [PVN; $t(15) = 2.54$, $p < .05$; Figure 5.6B]. In the dorsal lateral portion of the bed nucleus of the stria terminalis (dIBNST), there were no significant differences between ages [$t(16) = .87$, NS; Figure 5.6C].

Cell counts were also conducted in adolescent and adult rats that received CD. In the CeA, there was a significant difference in the number of cell bodies with CRF-immunoreactivity between ages [$t(16) = 3.16$, $p < .01$; data not shown]. This was a result of more CRF-immunoreactive cells in the CeA of adolescent rats compared to adults. In the PVN, there was also a significant difference in the number of cell bodies with CRF-immunoreactivity between ages [$t(15) = 3.05$, $p < .01$; data not shown]. There were more CRF-immunoreactive cells in the PVN of adolescent rats compared to adults.

CRF Immunohistochemistry: Repeated Ethanol Withdrawal, Stress/Withdrawal, or CD in Adult Rats

Further experiments were performed to assess whether there were changes in CRF levels in the CeA, PVN, or dlBNST during withdrawal from repeated withdrawals and stress/withdrawal paradigms in adult rats. There were no significant differences between treatment groups in the CeA [$F(2,21) = .44$, NS; Figure 5.7A], PVN [$F(2,20) = .05$, NS; Figure 5.7B], or dlBNST [$F(2,21) = 2.78$, NS; Figure 5.7C].

CRF Immunohistochemistry: Repeated Ethanol Withdrawal, Stress/Withdrawal, or CD in Adolescent Rats

These same treatments were also evaluated in adolescent rats. There were significant differences between treatment groups in the CeA in adolescent rats [$F(2,27) = 7.82$, $p < .005$, Figure 5.8A]. Adolescent rats that experienced repeated ethanol withdrawals showed decreased total density of CRF-immunoreactivity in the CeA compared to stress/withdrawal and control rats. However, there were no differences between treatment groups in total density of CRF-immunoreactivity in the PVN [$F(2,26) = .34$, NS; Figure 5.8B] or DLBNST [$F(2,27) = .09$, NS; Figure 5.8C].

pErk Immunohistochemistry: Repeated Ethanol Withdrawal, Stress/Withdrawal, or CD in Adolescent Rats

Finally, experiments were performed to determine whether proteins downstream of CRF receptors (pErk) were changed as a consequence of repeated ethanol withdrawals in adolescent rats. Quantifications of pErk were performed within the CeA and in regions where CRF neurons in the CeA project (dlBNST and PVN). In the CeA, there was a significant difference between treatment groups in total density area of pErk [$F(2,25) = 4.25$, $p < .05$; Figure 5.9A]. This difference was caused by an increase in pErk immunoreactivity in stress/withdrawal treated adolescent rats compared to controls. There were no significant differences between repeated withdrawal treated rats and either stress/withdrawal or controls. There were no significant differences between treatment groups in the dlBNST [$F(2,25) = .31$, NS; Figure 5.9B] or PVN [$F(2,27) = .55$, NS; Figure 5.9C].

Discussion

This work has illustrated that in adolescent rats stress coupled with a single ethanol withdrawal can reduce social interaction. This reduction in social interaction is a validated measure of increased anxiety-like behavior (File & Seth, 2003). Importantly, it was demonstrated that the combination of both stress and ethanol withdrawal are critical, since neither stress nor a single ethanol withdrawal alone produced this anxiety-like behavior (Wills et al., 2009). Similar findings were previously found in adult rats, where stress substituted for early withdrawals to produce anxiety-like behavior (Breese et al., 2004). The changes in locomotor activity following the stress/withdrawal paradigm in adolescent rats were also similar to data in adults. In both age groups, a reduction was found in locomotor activity for rats given two stress episodes followed by a single 5-day cycle of ethanol diet (Breese et al., 2004). It is unlikely that this reduction in activity effected the social interaction of this group. In the duration experiment, this same stress/withdrawal treatment (2.5%CY1-Str at 5hrs) elicited a similar reduction in social interaction without a reduction in activity. These results reconfirm the idea that social interaction and locomotor activity are independently manipulatable and not necessarily contingent on one another (Overstreet et al., 2002).

Further, the anxiety-like behavior produced from the stress/withdrawal paradigm was present at 5 hours but had returned to baseline by 24 hours into withdrawal. This profile of recovery is similar to that seen with repeated ethanol withdrawals and stress/withdrawal paradigms in adult rats (Wills et al., 2009; unpublished data). However, this duration is very different from that found with repeated ethanol withdrawals in adolescent rats (Wills et al., 2009). In these rats, it was illustrated that anxiety-like behavior could be

detected up to one week following the final withdrawal. These experiments, therefore, determined that anxiety-like behavior from the stress/withdrawal paradigm has a similar duration to adult rats but was not as long lasting as anxiety-like behavior from repeated ethanol withdrawals in adolescent rats. The reason for differences in duration between these paradigms may be due to adolescent rat's resistance to the anxiogenic effects of acute restraint stress. In adult rats, it was previously shown that 60 minutes of restraint stress was sufficient to produce anxiety-like behavior (Breese et al., 2005b). However, the current experiments determined that up to 120 minutes of restraint stress was unable to reduce social interaction in adolescent rats.

There is some evidence that isolate housing during adolescence could function as a chronic stressor (Hall, 1998). It is possible that isolate housing, in these experiments might lead to an inability of rats to provide normal responses to stress. Therefore, to evaluate whether housing condition was responsible for this effect, we also tested adolescent rats that were group housed. These experiments showed no reduction in social interaction even after 90 minutes of restraint stress in group housed adolescent rats. These results illustrated that adolescent rats seemed to have a reduced sensitivity to stress. However, it is important to note that stress is not without effect since the stress/withdrawal paradigm did produce anxiety.

The next set of experiments established a role of CRF in the sensitization of anxiety-like behavior. Adolescent rats given 7.5µg of CRF in combination with ethanol withdrawal exhibited anxiety-like behavior (reduction in social interaction), which was not found with either of these treatments alone (CRF or ED). This dose of CRF (7.5µg) in adolescents was higher than doses that have previously been shown to be effective in

producing anxiety-like behavior in adult studies (5µg; Overstreet et al., 2004a). Therefore, it seems that not only are adolescent rats less sensitive to the acute effects of stress but also to the effects of CRF.

Additionally, immunohistochemical experiments indicated that adolescent rats seem to have higher basal immunoreactivity of CRF (increased density and cell bodies) in the CeA and the PVN compared to adult rats. Interestingly, this increase in CRF immunoreactivity in adolescent rats was region specific since CRF immunoreactivity were comparable between ages in the dlBNST. Therefore, it does not appear that there is a mere global increase in CRF in adolescent rats. These increased basal levels of CRF between adolescent and adults may be responsible the differences in sensitivity between these ages in the stress and CRF substitution experiments.

Analyses were also made of CRF levels in adolescent and adult rats that received repeated withdrawals, stress/withdrawals, or control diet. Results illustrated that CRF immunoreactivity was not significantly changed by any of the adult treatments in any of the regions that were evaluated (CeA, PVN, dlBNST). However, in adolescent rats there was a difference in CRF immunoreactivity in the CeA but not dlBNST or PVN. This difference was caused by a decrease in density of CRF in the CeA in adolescent rats that experienced repeated ethanol withdrawal. We hypothesize that this decrease in CRF levels in this treatment group relates to an increase in CRF release during withdrawal. An increase in CRF release in this group would presumably deplete the supply of CRF contained within the cell body and would lead to the decreased immuno-density that was found. Similar results have been found under more chronic ethanol conditions. Zorrilla et al. (2001) illustrated that during the first day of withdrawal there was a decrease in CRF

immunoreactivity in the amygdala. They also speculate that this reduction in immunoreactivity was an indication of increased CRF release during this period. This idea can be further supported by microdialysis studies which showed that CRF levels increased and peaked 11-12 hours into withdrawal from chronic ethanol exposure (Merlo Pich et al., 1995). This reduction in CRF levels in the CeA was also only found in adolescent rats that experienced repeated ethanol withdrawals, not following stress/withdrawal procedure, and not following either treatment in adult rats. Both of these treatments in adolescent and adults were shown to produce anxiety-like behavior, therefore, this change in CRF is most likely unrelated to this acute (5 hours into withdrawal) anxiety-like behavior. This change is most likely related to the extended anxiety-like response (up to 1 week into withdrawal) which is present only in adolescent rats given repeated ethanol withdrawals.

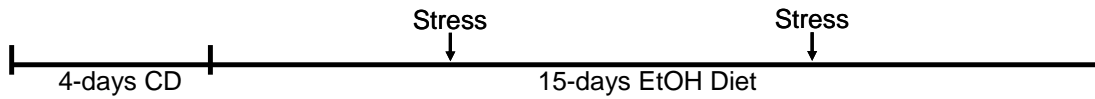
Finally, it was investigated if these adolescent treatments resulted in changes in levels of phosphorylated extracellular signal-regulated kinase 1/2 (pErk). Erk is downstream of CRF receptors and has been found to be phosphorylated by their activation (Arzt & Holsboer, 2006). Additionally, there is evidence of increased pErk during withdrawal from chronic intermittent ethanol in the amygdala of adult rats (Sanna et al. 2002). In our evaluation of pErk it was illustrated that the stress/withdrawal but not the repeated ethanol withdrawal paradigm increased pErk in the CeA. There was a trend for increased pErk in repeated ethanol withdrawal rats but it was not significant because of high variability. This variability was a function of rats either displaying moderate levels of pErk immunoreactivity or no pErk. The lack of a graded response indicates a possible threshold effect, where by sufficient activation needed to be met before pErk

expression was increased. It is unclear; however, what these changes might indicate since repeated ethanol withdrawals did not significantly increase this expression. Experiments were also performed to evaluate pErk in regions where CRF neurons in the CeA project (PVN and dlBNST). These regions showed no difference in pErk immunoreactivity from any of the adolescent treatments. Further investigations are needed to fully understand how these changes in pErk related to ethanol withdrawal related behaviors.

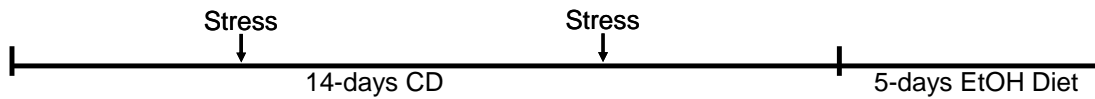
These experiments have illustrated that both stress and CRF substitute for early withdrawals and sensitize anxiety-like behavior. Further, it was shown adolescent rats have reduced sensitivity to the effects of acute stress and CRF. This reduced sensitivity in adolescent rats might be tied to the increased basal CRF found at this age. Finally, it was demonstrated that repeated ethanol withdrawals in adolescent rats caused a change in CRF with the CeA. Future studies will extend this work to evaluate the role of increased basal CRF in adolescents and how changes in CRF following repeated withdrawals might be tied to the extended anxiety-like behavior found in this group.

Figures & Tables

(A) Continuous Ethanol Stress (CON-Str):



(B) Single Cycle Ethanol Stress (CY1-Str):



(C) Control Stress (CD-Str):



Figure 5.1 Procedure for diet and stress administrations. (Panel A) Continuous ethanol stress paradigm (Con-Str): rats were given 4 days of control diet (CD) followed by 15 days of ethanol diet (ED). (Panel B) Single cycle ethanol stress paradigm (CY1-Str): rats were given 14 days of CD followed by one 5-day cycle of ED. (Panel C) Control stress paradigm (CD-Str): rats were given 19 days of CD. The stress used was one hour of restraint stress, which was administered at weekly intervals in the periods indicated by the diagrams above.

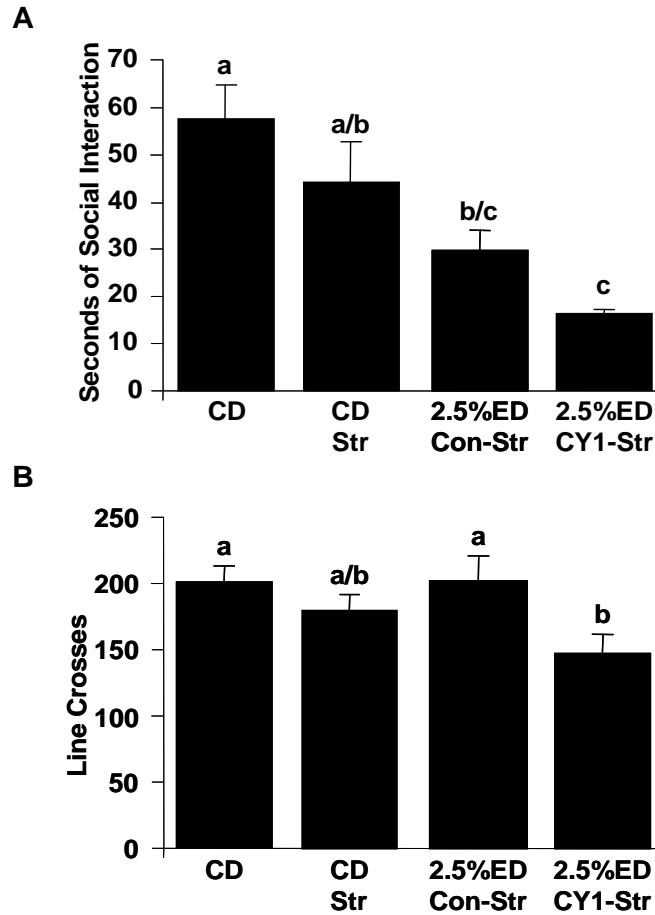


Figure 5.2 Social interaction and locomotor activity in adolescent rats (Panel A & B). Male adolescent rats were given either control diet (CD), CD with stress (CD-Str), 15 days of continuous 2.5% ethanol diet (ED) with stress (2.5%Con-Str), or one 5-day cycle of 2.5%ED with stress (2.5%CY1-Str). Stress was one hour of restraint stress given in weekly intervals. CD-Str received three stress episodes, while ED-Str groups received two. All adolescent rats were tested 5 hours into the final ethanol withdrawal or final stress (for CD-Str group) during the final withdrawal. Data represent means \pm SEM for 8 rats/group. Groups with different letters are significantly different from each other ($p < 0.05$).

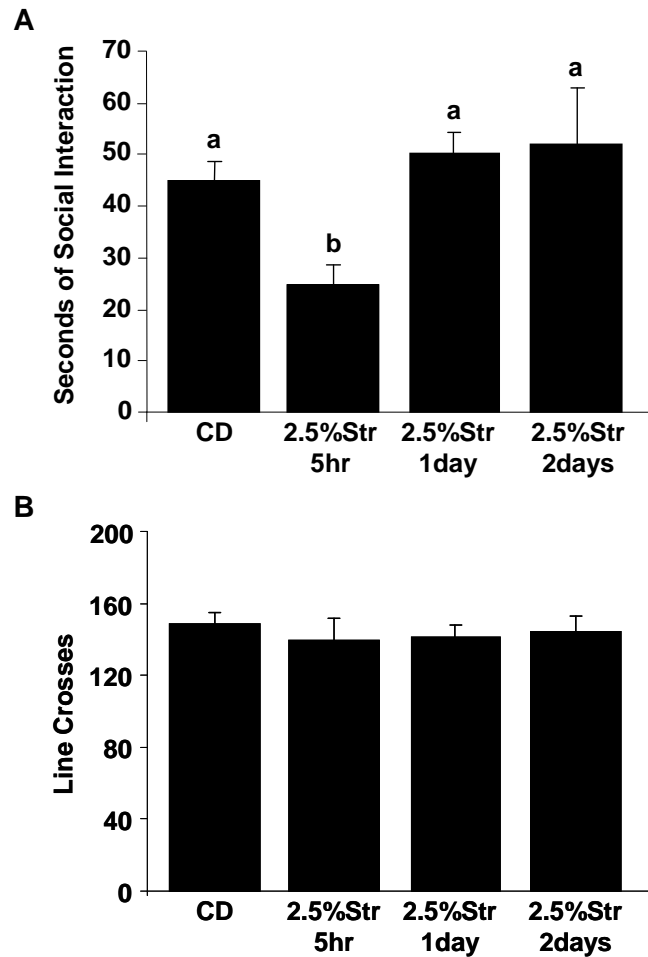


Figure 5.3 Duration of anxiety-like behavior in adolescent rats exposed to stress/withdrawal paradigm (Panel A & B). Male adolescent rats were given either control diet (CD) or 14-days of CD, in which two stress episodes were given one week apart, followed by a single 5-day cycle of 2.5% ethanol diet (ED; 2.5%Str). Stress was one hour of restraint stress. Rats were tested 5 hours, 1 day, or 2 days after the removal of ethanol during the final withdrawal. Data represent means \pm SEM for 8 rats/group. Groups with different letters are significantly different from each other ($p < 0.05$).

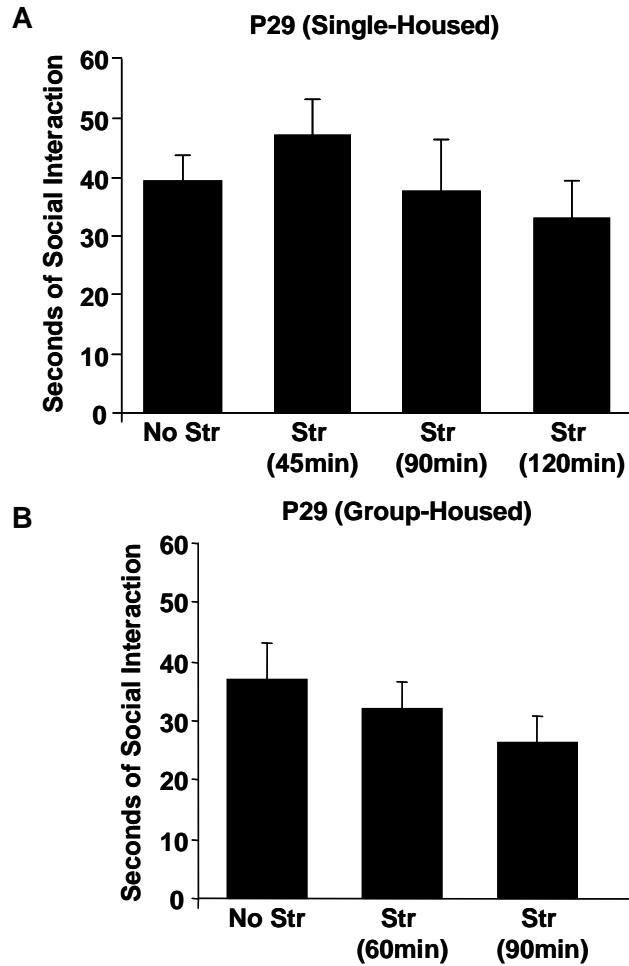


Figure 5.4 Acute effects of restraint stress on isolate and group housed adolescent rats (Panel A & B). Rats were placed on control diet (CD) until P29 in either isolate or group (3 rats/cage) housing. On P29, rats were given either 45, 60, 90, or 120 minutes of restraint stress and tested in social interaction 30 minutes following the termination of stress. Data represent means \pm SEM for 8 rats/group. Groups with different letters are significantly different from each other ($p < 0.05$).

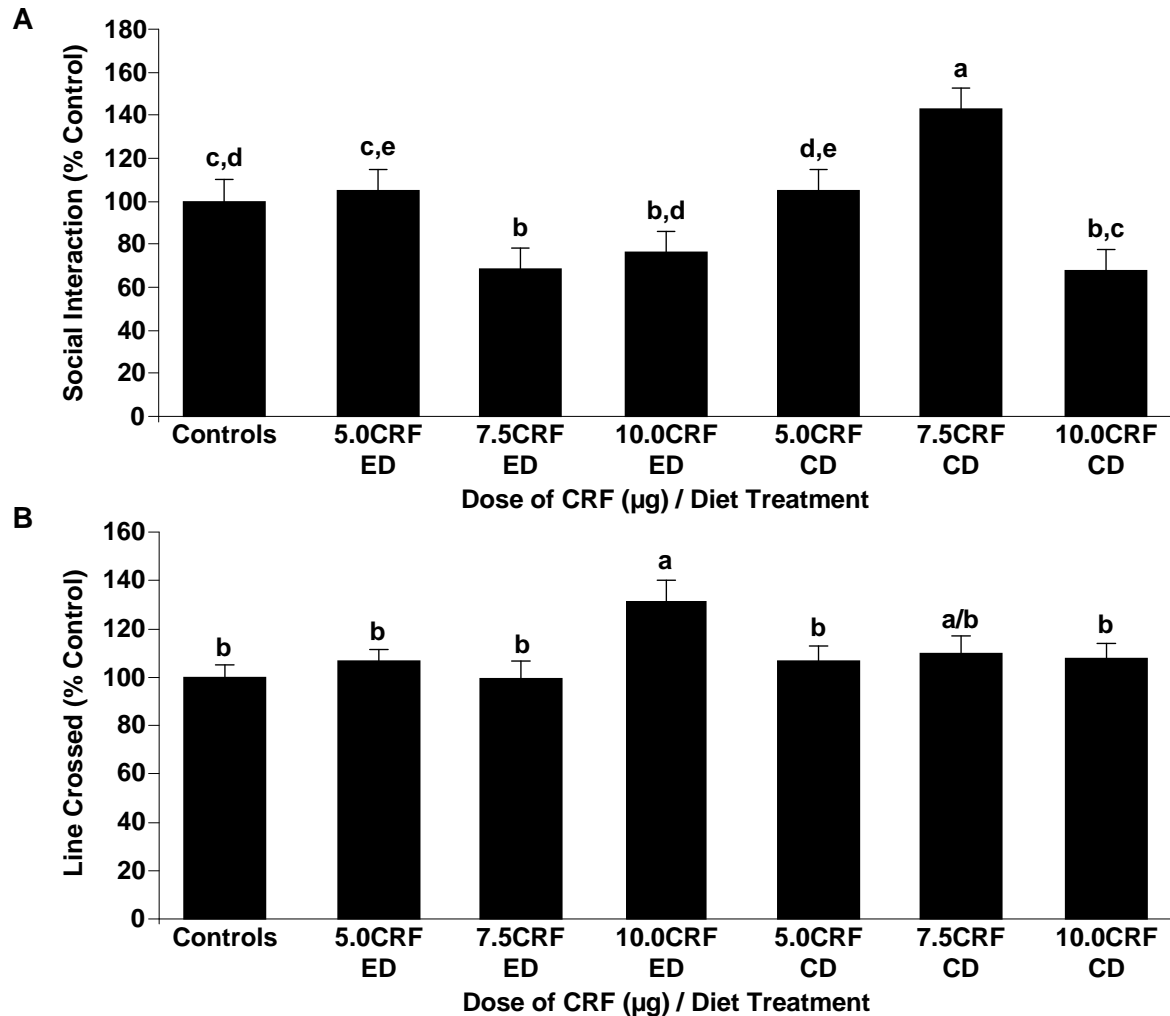


Figure 5.5 Dose-response of intracerebroventricular (icv) corticotrophin releasing factor (CRF) in adolescent rats (Panel A & B). Rats were given 14 days of control diet (CD) and two weekly microinjections of various doses of CRF icv (5.0 μ g, 7.5 μ g, or 10.0 μ g/5 μ l) or artificial cerebral spinal fluid (ACSF; given to controls). Rats were then given either 5-days of CD (CRF-CD) or 2.5% ethanol diet (CRF-ED). Controls represent rats that received either CD-ACSF or ED-ACSF. Social interaction and locomotor activity were assessed 5 hours into the ethanol withdrawal. Data represent means \pm SEM for 8 rats/group. Groups with different letters are significantly different from each other ($p < 0.05$).

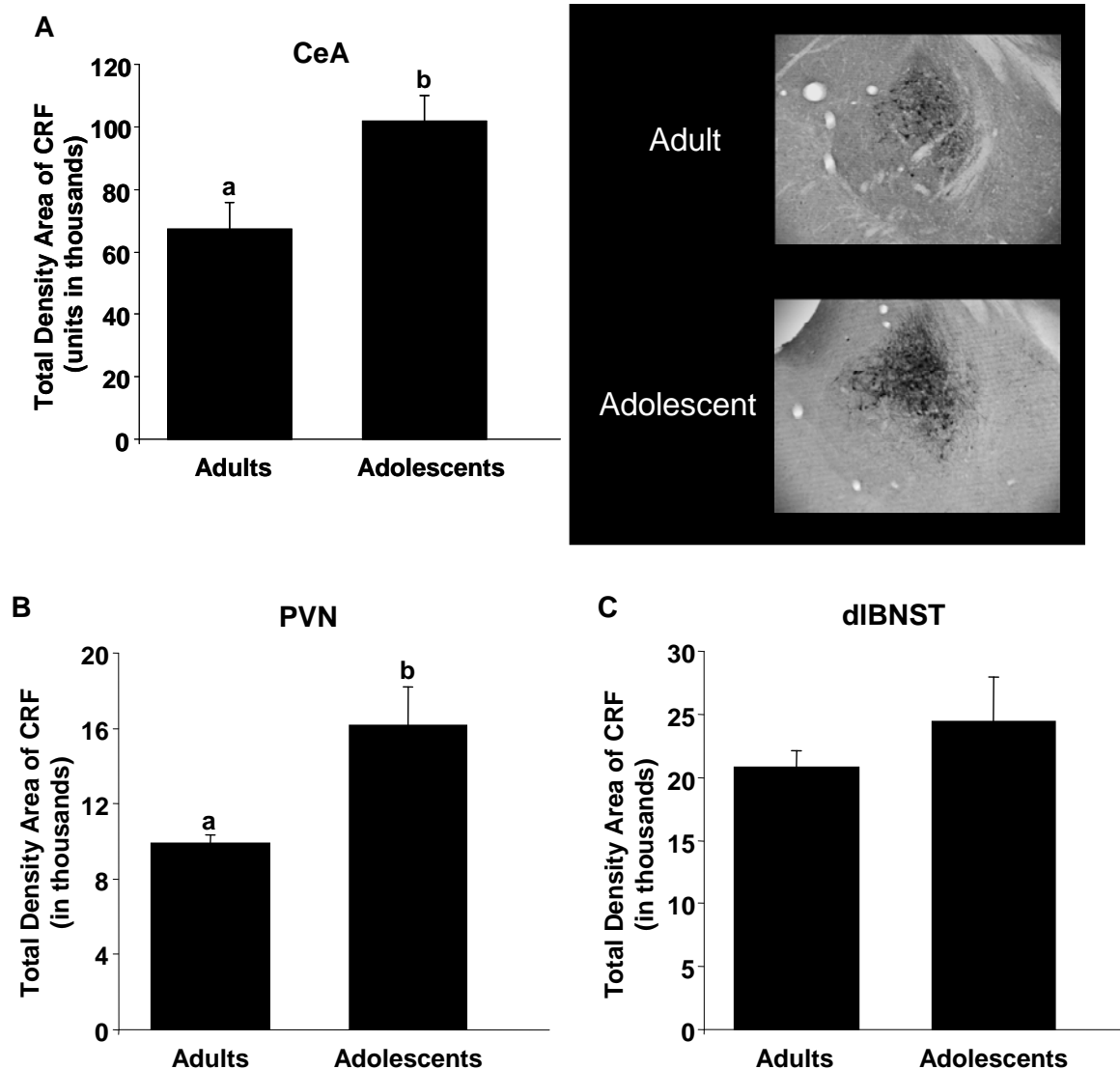


Figure 5.6 Baseline differences in corticotrophin releasing factor (CRF)-immunoreactivity within the central nucleus of the amygdala (CeA), paraventricular nucleus of the hypothalamus (PVN), and dorsal lateral bed nucleus of the stria terminalis (dlBNST) of adolescent and adult rats (Panel A, B, & C). Rats were given 19 days of control diet (CD) and brain tissue was collected immediately following social interaction. Total density measurements were made using ImageJ program for CeA, PVN, and dlBNST. Data represent means \pm SEM for 8 rats/group. Groups with different letters are significantly different from each other ($p < 0.05$).

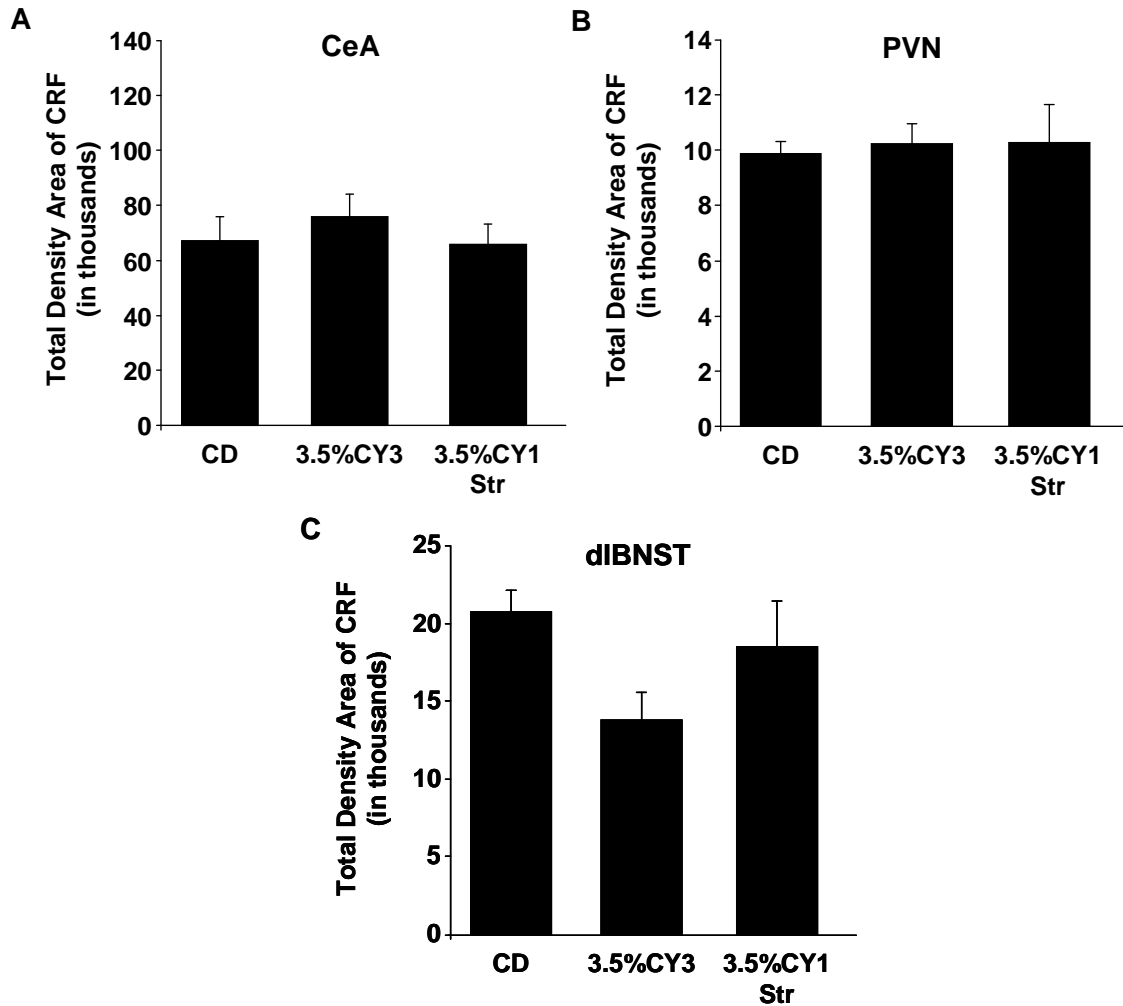


Figure 5.7 Adult corticotrophin releasing factor (CRF)-immunoreactivity within the central nucleus of the amygdala (CeA), paraventricular nucleus of the hypothalamus (PVN), and dorsal lateral bed nucleus of the stria terminalis (dIBNST) following repeated ethanol withdrawal and stress/withdrawal paradigms (Panel A, B, & C). Adult rats were given either 19 days of control diet (CD), three 5-day cycles of 3.5% ethanol diet (ED) interspersed with two 2-day withdrawal period (3.5%CY3), or 14 days of CD followed by 5 days of 3.5%ED with stress (3.5%CY1-Str). Stress is one hour of restraint stress given at weekly intervals. Brain tissue was collected immediately following social interaction five hours into ethanol withdrawal. Total density measurements were made using ImageJ program for CeA, PVN, and dIBNST. Data represent means \pm SEM for 8 rats/group. Groups with different letters are significantly different from each other ($p < 0.05$).

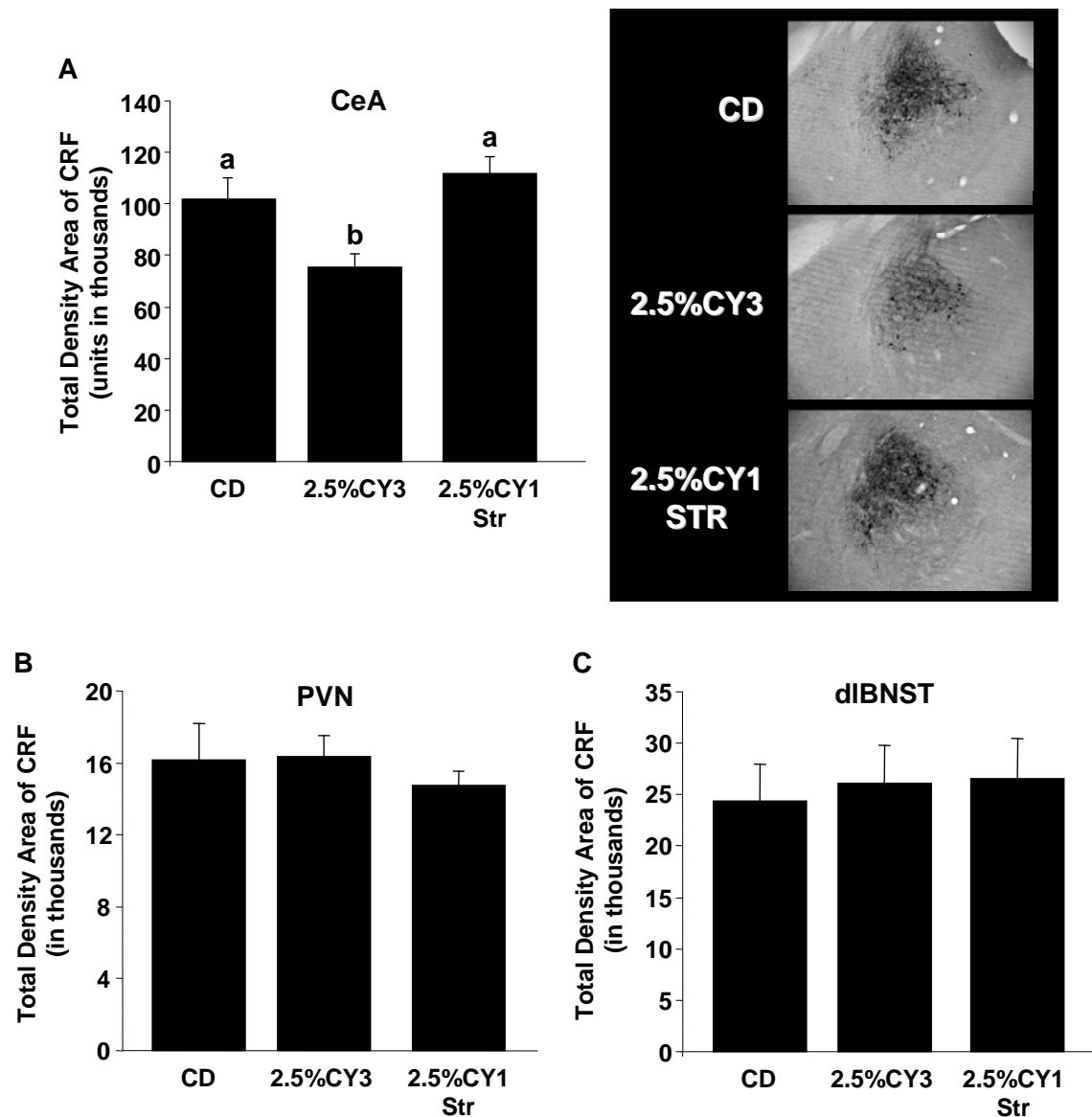


Figure 5.8 Adolescent corticotrophin releasing factor (CRF)-immunoreactivity within the central nucleus of the amygdala (CeA), paraventricular nucleus of the hypothalamus (PVN), and dorsal lateral bed nucleus of the stria terminalis (dlBNST) following repeated ethanol withdrawal and stress/withdrawal paradigms (Panel A, B, & C). Adolescent rats were given either 19 days of control diet (CD), three 5-day cycles of 2.5% ethanol diet (ED) interspersed with two 2-day withdrawal period (2.5%CY3), or 14 days of CD followed by 5 days of 2.5%ED with stress (2.5%CY1-Str). Stress is one hour of restraint stress given at weekly intervals. Brain tissue was collected immediately following social interaction five hours into ethanol withdrawal. Total density measurements were made using ImageJ program for CeA, PVN, and dlBNST. Data represent means \pm SEM for 8 rats/group. Groups with different letters are significantly different from each other ($p < 0.05$).

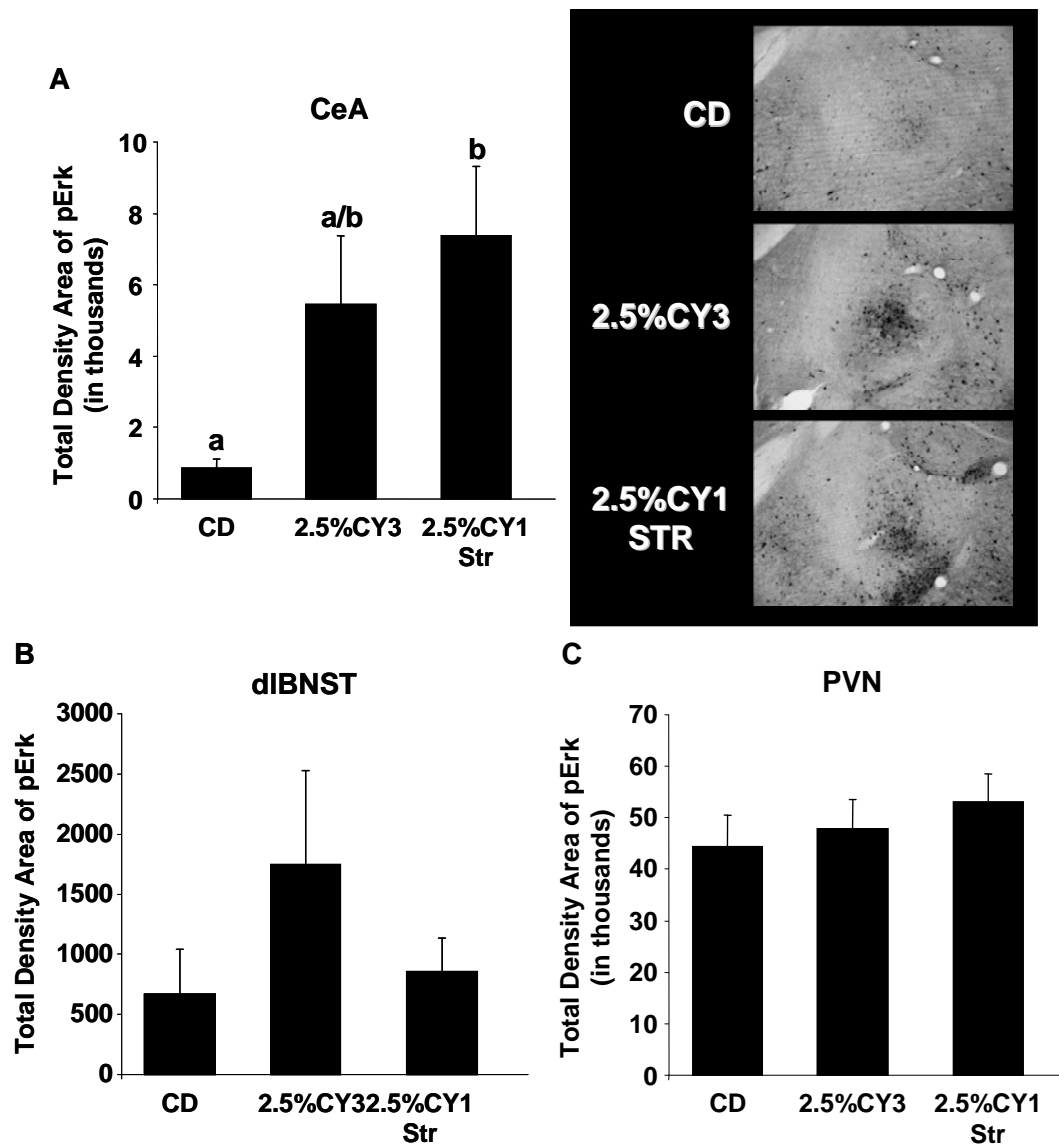


Figure 5.9 Adolescent phosphorylated extracellular signal-regulated kinase 1/2 (pErk)-immunoreactivity within the central nucleus of the amygdala (CeA), paraventricular nucleus of the hypothalamus (PVN), and dorsal lateral bed nucleus of the stria terminalis (dIBNST) following repeated ethanol withdrawal and stress/withdrawal paradigms (Panel A, B, & C). Adolescent rats were given either 19 days of control diet (CD), three 5-day cycles of 2.5% ethanol diet (ED) interspersed with two 2-day withdrawal period (2.5%CY3), or 14 days of CD followed by 5 days of 2.5%ED with stress (2.5%CY1-Str). Stress is one hour of restraint stress given at weekly intervals. Brain tissue was collected immediately following social interaction five hours into ethanol withdrawal. Total density measurements were made using ImageJ program for CeA, PVN, and dIBNST. Data represent means \pm SEM for 8 rats/group. Groups with different letters are significantly different from each other ($p < 0.05$).

CHAPTER VI: GENERAL DISCUSSION

The purpose of these studies was to evaluate several key factors that are thought to be important in the progression of ethanol dependence in a vulnerable population, adolescence. These studies have focused on determining the effects of cyclic ethanol exposure on withdrawal related behaviors, especially anxiety. In addition, the interactions between stress and ethanol were also investigated in relation to these anxiety-like behaviors. Finally, this work explored the role of CRF in the adaptations that occur from repeated withdrawals, which lead to this anxiety-like behavior. Throughout this work, there are a number of conclusions and observations that can be made about ethanol withdrawal in adolescents.

Methodological Considerations for Adolescent Experiments

A great deal of the work accomplished in this dissertation relied heavily on addressing the numerous methodological confounds that arise when evaluating differences between adolescent and adult rats. The first efforts focused on the time frames of ethanol exposures and behavioral assessment. In many chronic ethanol treatments (like ours) in rodents, ethanol is generally administered throughout the adolescent period (P28-42). This limited window of opportunity makes it nearly impossible to establish the effects of chronic ethanol on adolescent behavior because by the time chronic ethanol paradigms are complete the rats are no longer adolescents. Therefore, in our experiments behavioral assessment does not occur until around P45 when adolescent rats are considered young adults. The results from these experiments can

only illustrate the effects of ethanol treatment during adolescence on ethanol withdrawal in young adulthood. They are not able to provide information about the effects of these procedures on ethanol withdrawal while rats are still adolescents. Another aspect that requires thought is the proper adult comparison group. In these studies, we gave identical treatments to rats who were already adults, so all ethanol treatments and behavioral tests were completed during adulthood. This comparison has the advantage of identical ethanol exposures and withdrawal periods; however, a disadvantage is that rats were of different ages at the time of test. Optimizing the advantages to disadvantages is the key in finding the appropriate adult controls. The take home message from these considerations is that one needs to be aware of the time frame limitations of adolescent rodents and make appropriate conclusions about these results.

Another factor that can complicate the interpretations of results is differences in normal (baseline) behaviors between adolescent and adult rats. Hopefully, it has been made clear that adolescents are not merely little adults. Adolescents have been shown to display a whole host of unique behavioral responses under baseline conditions and following ethanol exposure. These baseline differences were found for social interaction and locomotor activity. These differences lead to additional steps (corrections for baseline) in comparing the ethanol related effects between adolescent and adult rats. If these corrections are not made, then conclusions regarding the effects of ethanol between these age groups can be misinterpreted.

Further, the most formidable methodological factor in these studies was the use of liquid diet as a route of ethanol administration. There are a number of reasons why this route of administrations was challenging in adolescent rats. Most of challenges stem from

the fact that given a certain concentration of ethanol diet, adolescent rats had increased consumption (ethanol intake). This increased consumption was likely caused by the rapid growth rate that occurs during adolescence. In these liquid diet procedures, calories are solely derived from diet. During times of rapid growth, more calories are needed than normal and, therefore more diet will be consumed. Since ethanol is administered within this diet, ethanol intake is also rapidly increased in adolescent rats. The data on ethanol intake in Chapter III and IV illustrated that if adolescent and adult rats are given the same ethanol diet concentrations then adolescents consistently had higher g/kg ethanol intake compared to adults. This factor complicates the interpretations of behavioral results since it is unclear whether differences arise because of age or merely ethanol intake. In these studies, considerable effort was made to equalize ethanol intake. Another factor that affects ethanol intake in these chronic ethanol diet administrations is the decline in ethanol intake that occurs with age. As the adolescent rats age into adults, there is a decline in ethanol intake. This is most likely due again to a decrease in growth rate that occurs as adolescents become adults. This feature makes it more difficult to maintain consistent ethanol intake throughout chronic treatments in adolescent rats.

It was also necessary to make sure that BECs were comparable between ages. Again the results from Chapter III and IV illustrate that even when ethanol intake is equalized (4.5% adolescent and 7% adult or 2.5% adolescent and 3.5% adult) BECs were not always the same. Other researchers have evaluated differences in ethanol metabolism and found similar metabolism at both ages (Silveri & Spear, 2000). Therefore, the most likely explanation for these differences in BECs is that the drinking patterns between adolescent and adult rodents are not the same. For example, studies have shown that

adolescent rats are less entrained to the light/dark cycle (Brunell & Spear, 2005). Adult rodents are known to be nocturnal and, therefore, have higher levels of eating, drinking, and activity during the dark cycle. However, adolescent rats seem to drink throughout both the light and dark cycle (Brunell & Spear, 2005). In our studies, BECs were measured at the end of the dark cycle, which most likely captures the peak BEC in adult rats but might not represent the peak BEC in adolescent rats. The studies presented here used adolescent and adult diet concentrations with the most closely matched ethanol intake and BEC but the comparisons were not perfect. Future work is needed to investigate ethanol intake and BECs throughout a 24-hour period to fully understand these differences between adult and adolescent rats. These methodological issues illustrate that careful considerations and adjustments are needed when conducting ethanol studies in adolescent rats and in interpreting their results.

Similar Responses between Adolescent and Adult Rats

Previous work in adolescents illustrated that they can have very different responses to ethanol than adults. However, many of experiments presented here showed that, after corrections for ethanol intake were made, many of the withdrawal related behaviors from repeated ethanol withdrawals were similar between ages. Adolescent rats were shown to have similar reductions in social interactions and seizure thresholds. Additionally, it was determined that sensitization of anxiety-like behavior from repeated ethanol withdrawal and stress/withdrawal paradigms were similar between adolescent and adults. Even though many of these responses were similar, they still provide valuable information about the use of chronic ethanol during adolescence. Research from other labs showed that withdrawal from acute ethanol is reduced in adolescents,

specifically seizure thresholds and anxiety-like behavior (Acheson et al., 1999; Doremus et al., 2003; Varlinskaya & Spear, 2004). Therefore, this work might suggest that since these withdrawal-related behaviors are reduced then adolescents may be susceptible to processes that underlie addiction. The work provided here demonstrates that this is not the case. Our work shows that, even though adolescents may have reduced withdrawal related behaviors to acute ethanol, they still show equal sensitization of anxiety-like behavior from repeated withdrawal paradigms. This outcome indicates that the adaptations producing this sensitized behavioral response can occur across different age groups. More importantly, this outcome shows that adolescents are at least equally susceptible for the development of ethanol addiction as adults.

Extended Duration in Adolescent Rats

Despite the many similarities in withdrawal-related behaviors, there were also aspects of this sensitized anxiety-like behavior that are different between ages. Specifically, this research showed anxiety-like behavior following repeated ethanol withdrawals in adolescent rats had an extended duration compared to adult rats. Duration was defined as the ability to measure anxiety-like behavior (by reduction in SI) at different time points into the final withdrawal. In adolescents, this behavior could be detected up to a week after the final withdrawal, whereas in adults it is recovered within 24 hours (Figure 4.4 and 4.5). This extended duration of anxiety in adolescent rats would suggest that this population may be more vulnerable to adaptations involved in addiction. It was illustrated previously that negative affect (anxiety) during withdrawal is likely responsible for continued drinking behavior and relapse (Annis et al., 1998; Roberts et al., 2000; Valdez et al., 2002).

In addition to differences in the duration of anxiety-like behavior post-withdrawal, studies in adult rats have also shown that re-exposure to normally non-anxiogenic (“subthreshold”) amounts of chronic ethanol can re-elicite the anxiety-like response caused by repeated withdrawals (Overstreet et al., 2002). These experiments attempt to model persistent changes in the responsiveness to future ethanol after baseline anxiety from acute withdrawal has returned to normal. Similar studies were attempted in adolescent rats but were fraught with methodological issues that prevented clear interpretations of results. In these studies, adolescents were exposed to repeated ethanol withdrawals (as described in previous chapters) and then were re-exposed to 5 days of ethanol diet (a non-anxiogenic regiment; see Figure 4.2) 16, 21, or 32 days later (data not shown). In the experiment using a 16-day re-exposure, it was shown that anxiety-like behavior was still present in rats that received only control diet (CD). These data illustrated that the “duration” of anxiety-like behavior described in the previous paragraph could reappear with even a minor disruption (simply the re-introduction to CD). Therefore even though behavior in adolescents seems to have returned to baseline by 14-days (Figure 4.5), these data suggest that small changes in the environment can re-elicite this anxiety response.

Further studies used extended time points (21 and 32 days) and encountered additional methodological issues. These studies demonstrated that re-exposure to 5 days of ethanol diet (ED) did not produce anxiety-like behaviors. One of the reasons these studies might have failed to produce anxiety-like behavior was that ethanol intake during this cycle was low. This low ethanol intake was caused by the decrease in ethanol diet consumption that occurs as the rats age. To overcome this problem, higher (4.5%ED)

concentrations of ED were used during re-exposure and also showed no anxiety-like behavior. Studies previously performed in adults used 4.5%ED and 7%ED in the repeated withdrawal paradigm (Overstreet et al., 2002; unpublished data) so the negative results in these adolescent tests are most likely a result of insufficient ethanol intake. Studies discussed here used 2.5%ED during the adolescent repeated withdrawal procedure. This diet concentration produces ethanol intake much lower than that which would be produced from either 4.5%ED or 7%ED in adult rats (compare g/kg intake in Figure 3.5/3.6 and Table 4.3). Therefore, these methodological issues compromise our ability to interpret results from these studies in adolescent rats and to determine whether this model of withdrawal provides comparable results across age.

Overall these experiments with repeated ethanol withdrawals in rats indicate that binge drinking episodes, which are known to occur in human adolescent populations, produce anxiety-like behavior. Further, this behavior seems to be much longer lasting in adolescents compared to adults. These results suggest that teenage drinking patterns very likely set the stage for future ethanol dependence.

Reduced Sensitivity to Stress in Adolescent Rats

The stress/withdrawal paradigm in adolescent rats illustrated that stress in combination with ethanol withdrawal sensitized anxiety-like behavior. These data indicate that both stress and ethanol withdrawal have common mechanisms (possibly release of CRF), which interact to produce this sensitized anxiety. Therefore, these data indicated that not only are binge drinking episodes (that produce repeated withdrawals) able to set the stage for later addiction but that episodes of stress can also contribute. While these data illustrated a clear interaction of stress and ethanol withdrawal in

adolescent rats, it was also evident that these effects were less robust than those of repeated ethanol withdrawal. Specifically, it was shown that the duration of anxiety-like behavior recovered with 24hrs with stress/withdrawal paradigm (Figure 5.3) while this behavior was present up to a week following repeated ethanol withdrawals (Figure 4.5).

An explanation for this reduced response following the stress/withdrawal paradigm is that the acute effects of stress are lower in adolescent rats. Our data showed that this was in fact the case with various periods of restraint stress unable to produce an acute anxiety-like phenotype (Figure 5.4A). There are a number of variables that might have played a role in this reduced anxiety-like behavior from restraint stress. One of the variables that was evaluated in these experiments was housing conditions. Isolate housing in adolescent rats, which is used in our experiments, has been used as a social isolation stress specifically in adolescent rats (Hall, 1998). For this reason, we also evaluated the acute effects of stress in group housed adolescents (Figure 5.4B). These rats still demonstrated an anxiety-like response. It is, therefore, unlikely that this variable was the source of reduced sensitivity to stress in adolescents.

Another variable that could have contributed to this result was the type of stress used. It has been shown that restraint stress (60 minutes) produces acute increases in anxiety-like behavior in adult rats (Breese et al., 2005b). It is possible that confinement is not perceived as a stressor in adolescent rats and, therefore, less effective at producing anxiety. This explanation is unlikely since multiple studies have shown that restraint stress produced increases in ACTH and corticosterone (Romeo et al., 2006; Schramm-Sapota et al., 2008), which demonstrate HPA activation and the effectiveness of restraint as a stressor.

Another possible explanation for these results is the time frames that were measured. Romeo et al. (2006) used a 30 minute restraint stress in both adolescent and adult rats and showed that corticosterone levels peaked at the termination of stress. These levels declined from this peak and were back to basal levels in adult rats but still slightly elevated in adolescents after 45 minutes. These data indicated that 30 minutes of restraint stress produces peak corticosterone response which is significantly reduced by 45 minutes. It is, therefore, likely in our studies with longer lengths of stress that corticosterone response and anxiety may have returned to baseline by the time of test. Additionally, Romeo et al. (2006) also illustrated that when stress was given chronically (30 minutes/day for 1 week) corticosterone levels still peaked at stress termination but 45 minutes following stress adolescent's corticosterone returned to basal levels and adult levels were still elevated. These data illustrate that corticosterone response is longer in duration in adolescents following acute stress but shorter following chronic stress. These data also suggest that corticosterone levels peak 30 minutes into stress. In our work, it has been shown that adult rats have elevated corticosterone response 1 hour following the termination of 1 hour restraint stress. However, preliminary work in adolescent rats has shown no elevation of corticosterone response at this time point (data not shown). Since the corticosterone response in adolescent rats seems to have a shorter duration than in adult rats, this would suggest that our conditions have induced chronic stress. This chronic stress condition could be caused by a combination of isolate housing and liquid diet, which are both thought to be moderately stressful. Future work needs to evaluate earlier time points in order to determine which of these factors is at work.

Overall this evidence indicates a number of reasons why acute stress was unable to produce anxiety. Any one of these factors might have lead to the somewhat less robust effects of the stress/withdrawal paradigm compared to repeated ethanol withdrawals. However, it is important to emphasize that stress in adolescents did substitute for early withdrawals to sensitize anxiety-like behavior and is therefore critical to the understanding of the development of ethanol addiction in adolescents.

Reduced Sensitivity to CRF Substitution in Adolescent Rats

In these experiments, it was shown that intracerebroventricular (icv) administration of CRF substituted for early ethanol withdrawals to sensitize anxiety-like behavior (Figure 5.5). As described above, a common mechanism responsible for the sensitized anxiety-like behavior between stress and ethanol could be CRF and these studies lend support to that claim. Further, drug pretreatment experiments specifically determined that blocking CRF type 1 receptors (CRF₁R) during early withdrawals prevented development of anxiety-like behavior (Figure 4.6). Taken together, these data suggest that ethanol withdrawal and stress act through CRF at CRF₁R to produce this withdrawal induced anxiety. These data agree with previous work conducted in adult rats, which also showed these same adaptations (Breese et al., 2004; 2005a/b; Overstreet et al., 2004a).

One aspect of these studies that was unique to adolescents was a reduced sensitivity to CRF (higher dose required) to produce anxiety. In these experiments with adolescent rats, a dose of 7.5µg was needed to substitute for early ethanol withdrawals or stress compared to the 5µg dose that is effective in adult rats. There are a number of possible explanations for this insensitivity of adolescent rats found in these experiments.

One possibility is that CRF receptors in adolescents are less sensitive to CRF than the same receptors in adults. This effect could be caused by desensitization of these receptors or decreased receptor expression in adolescent versus adult rats. A second possibility is a difference in the distribution of CRF₁R and CRF type 2 receptors (CRF₂R). There is evidence in the literature showing that these receptors may have opposing actions in the production of anxiety-like behaviors, where activation of CRF₁R seem to have anxiogenic actions and CRF₂R seem to have anxiolytic actions (Bale et al., 2000; Timpl et al., 1998). The dichotomy between these two receptors was also illustrated in our model, where it was discovered that CRF₁R antagonist can block the development of anxiety-like behavior but CRF₂R antagonist was ineffective (Overstreet et al., 2004a). Since these receptors have opposite functions, it is possible that adolescents may have a higher CRF₂R versus CRF₁R distribution than adult rats. Therefore, it would require higher doses of CRF to produce anxiety through CRF₁R. A third possibility is that adolescents have higher basal CRF levels than adult rats. If this is the case then it might take higher doses of CRF to disrupt the basal tone of CRF in these adolescent rats. A higher basal CRF could also desensitize receptors and make them less sensitive to later CRF administrations (as discussed above).

Basal Differences in CRF between Adolescent and Adult Rats

The current work evaluated one of these possibilities, differences in basal levels of CRF between adolescent and adult rats. These studies illustrated that there was a higher density of CRF immunoreactivity within the PVN and CeA of adolescent rats compared to adults (Figure 5.6). Cell counts of these areas also illustrated a higher number of cells with CRF immunoreactivity in adolescents compared to adults. These

data indicate that under control conditions (isolate housed rats maintained on control diet for entire experimental period) adolescent rats seem to have a larger population of cells in the PVN and CeA containing CRF than adults do. It is also possible that the density of CRF within a given cell is higher in adolescents than adults. Regardless, these data show that the basal amount of CRF in these regions is increased in adolescent rats.

Furthermore, this increase was region specific with no changes in CRF immunoreactivity found in dIBNST. These changes in CRF are either due specifically to age or a combination of age and treatment. Viau et al. (2005) found that basal levels of CRF mRNA within the PVN were not different between 30-day old and 60-day old male rats. Additionally, they showed that basal levels of CRF mRNA actually increased in 60-day old rats compared to 30-day old rats in the CeA. These studies quantified only changes in CRF mRNA and not protein levels. Additionally, the ages examined in those studies (P30 & P60) were somewhat distinct from our work (P45 & 73). The differences between these studies make it unclear how to interpret these distinct results. Furthermore, no other studies could be found to either confirm or deny the results found in these studies, increased basal CRF immunoreactivity in adolescents.

An alternative explanation for this basal increase of CRF in adolescents may be a consequence of control conditions as well as age. It was discussed above that there is evidence that certain housing conditions can be particularly disruptive during adolescence as may be the case with social isolation stress. In one version of this social isolation stress, 1 hour of isolation stress was administered daily followed by return to group housing from P30-45. This isolation stress produced increases in basal CRF mRNA with the PVN but not the CeA in P45 rats (McCormick et al., 2006). In a different version of

social isolation stress, male rats were isolate housed from P16-76. These studies showed no change in CRF-immunoreactivity under basal or stressed conditions (Sanchez et al., 1998). Therefore, it is unclear whether the basal changes detected in our experiments were the result of isolate housing conditions. Future studies in more naturalistic controls (chow fed and group housed) are needed to determine the source of these age related differences in CRF.

Despite the undetermined source of these age related changes in CRF, the elevated CRF levels in adolescents might help to explain the reduced behavioral sensitivity to stress and CRF that were found in adolescent rats. The increased levels of CRF within the CeA could be responsible for the reduced sensitivity of CRF to produce anxiety. CRF within the CeA is known to be critical in the production of anxiety and, as described above, higher basal levels of CRF might account for reduced sensitivity of microinjected CRF. This effect might be a result of either an inability to overcome basal tone or desensitization of CRF₁R. In addition, the changes in basal levels of CRF within the PVN could be responsible differences in the effects of stress between ages. CRF neurons within the PVN are a critical part of the physiological response to stress. CRF is released from this region and triggers the release of ACTH and corticosterone. Therefore, these changes in CRF within the PVN may be a cause of the reduced acute effects of stress on anxiety.

Changes in CRF following Repeated Withdrawals in Adolescent Rats

In addition to these basal changes in CRF between adolescent and adult rats, it was also shown that there were age-dependent changes in CRF from ethanol treatments. Specifically, these studies found a significant chronic ethanol-dependent decrease of CRF

immunoreactivity in the CeA of adolescent rats (Figure 5.7). This result was interesting because these rats were the only group to display an extended anxiety response (described above). We hypothesize that this decrease in immunoreactivity of CRF is a result of increased CRF release from cell bodies during withdrawals.

In the adult literature, there has been an enormous amount of work to determine the role of CRF in ethanol dependence. During protracted withdrawals, dependent rats have increased stress-induced anxiety (Valdez et al., 2003), ethanol self-administration (Funk et al., 2006), and voluntary ethanol consumption (Valdez et al., 2002). All of these behaviors can be prevented or blocked by CRF receptor antagonists (either non-specific or CRF₁R). Furthermore, microinjections of a CRF receptor antagonist within the CeA blocked increased self-administration in withdrawal (Funk et al., 2006). These data illustrate that the actions of CRF in adults may be responsible for many behaviors in extended withdrawal that contribute to enhanced ethanol intake and relapse. These data suggest that the changes in CRF within the CeA found in our adolescent rats might also be related to this increased risk for future ethanol dependence.

Future Work

Many potential directions of future work have already been presented throughout this dissertation; however, some additional areas still need to be addressed. More work clearly is needed to understand some of the sensitivity differences between ages in response to CRF and stress. Immunochemistry experiments illustrated the need to determine the source of decreased CRF in the CeA of adolescent rats following repeated withdrawals. While it can be suggested that this change represents an increase in release, additional experiments will be needed to validate this hypothesis. Further, it is still

unknown if this change in CRF with the CeA is actually responsible for the extended behavioral response found in adolescents.

There are likely many mechanisms which are involved in the sensitization of this anxiety-like behavior from repeated ethanol withdrawals in adolescent rats. In this dissertation, a number of experiments focused on understanding how CRF systems might modulate this behavior but it is clear that other systems also play a role. In drug pretreatment studies with adolescent rats (Figure 4.6), it was shown that administration of either a benzodiazepine antagonist or a 5-HT_{1A} receptor agonist also prevent sensitization of anxiety-like behavior. Therefore, understanding these adaptations and how all of these pathways work together will lead to greater understanding of this disease and more effective treatments.

In addition to these mechanistic questions, the data herein showed that adolescents had extended anxiety-like behavior from repeated ethanol withdrawals. It would be interesting to evaluate other behaviors during this extended withdrawal in these adolescents. Some of most relevant behaviors to measure first are ethanol self-administration, voluntary ethanol consumption, and stress-induced anxiety. The list of additional experiments could be extensive given the lack of understanding that still remains surrounding the effects of adolescent alcohol use and future alcoholism.

Conclusion

This work illustrated the importance of characterizing the adaptations that underlie key facets of addiction during adolescence. In support of the original hypothesis, evidence showed that binge ethanol exposure, which produces repeated withdrawals, sensitized anxiety-like behavior in both adult and adolescent rats. Further

support arose from work showing that stress can interact with ethanol withdrawal to produce this anxiety-like phenotype in both ages, and that this effect seems to be produced through modulation of the CRF system. Throughout this dissertation, it has been emphasized that this anxiety behavior from ethanol withdrawal (also referred to as negative affect) is a large contributor to continued ethanol use and relapse during addiction. Therefore, the extended duration of this anxiety-like response in adolescents would indicate an increased potential for the development of future addiction. This work would also strongly encourage the development and use of treatments during adolescence, specifically those that modulate CRF. It is likely that the key step in preventing the progression to alcoholism would be to start early treatments in those adolescents that experience high levels of problematic drinking.

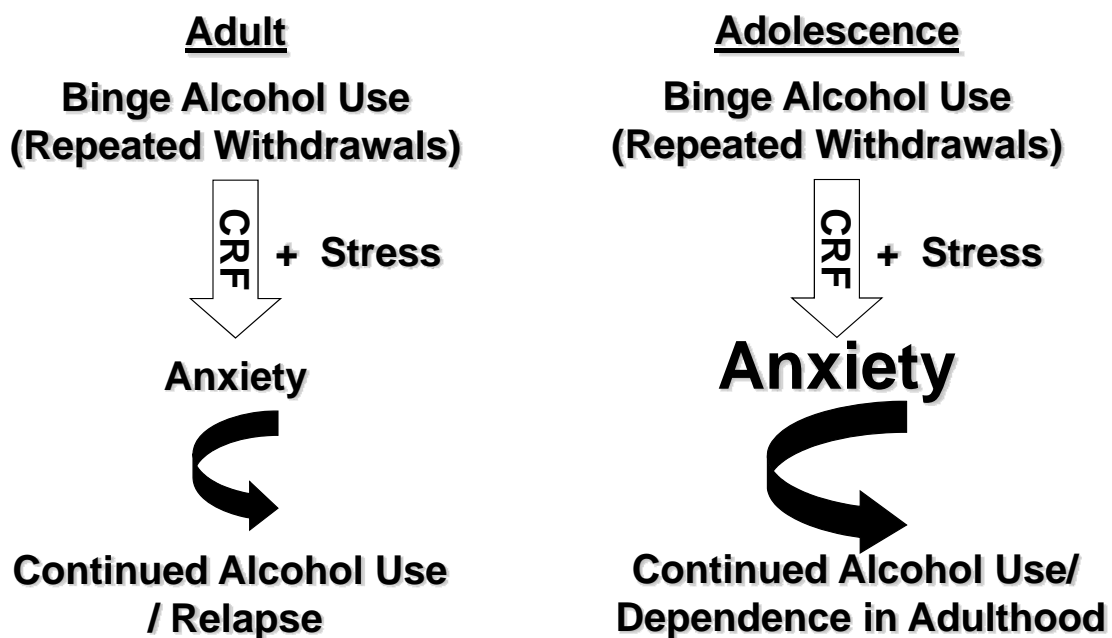


Figure 6.1 Summary of Dissertation Findings

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