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Memory Impairments Due to Binge Ethanol are Impacted by Age and Sex

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BY

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Table of Contents

List of Figures

Page

List of Tables

Page

List of Abbreviations

5-HT3	5-hydroxytryptamine 3
β1	beta 1
β4	beta 4
AAV	adeno-associated virus
AB	adult
AC	adenylyl cyclase
ACC	anterior cingulate cortex
ADH	alcohol dehydrogenase
ADME	absorption, distribution, metabolism, and elimination
ae	adolescents
AER	alcohol elimination rate
ALDH	aldehyde dehydrogenase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
ATN	anterior thalamic nuclei
AUC	area under the curve
AUD	alcohol use disorder
BAC	blood alcohol concentrations
BDNF	brain-derived neurotrophic factor
BEC	blood ethanol concentration
BK	large-conductance Ca2+ dependent K+ channel
BM	barnes maze
Br	bromodomain
CA1	cornu Ammon 1
CA2	cornu Ammon 2
CA3	cornu Ammon 3
CaM	calmodulin

CAMKII	calcium/calmodulin-dependent protein kinase II
CAMKIV	calcium/calmodulin-dependent protein kinase IV
CAMKK	CaM Kinase Kinase
cAMP	cyclic adenosine monophosphate
CBP	CREB binding protein
CD40	cluster of differentiation 40
CF	control females
CH1	cysteine/histidine-rich region 1
CI	confidence intervals
CM	control males
CNS	central nervous system
CORT	corticosterone
COVID-19	coronavirus Disease 2019
CRE	cAMP response element
CREB	cAMP response element-binding
CSF2RB	colony stimulating factor 2 receptor beta
CYP2E1	cytochrome P450 2E1
DA	dopamine
DAG	diacylglycerol
DCX	doublecortin
DG	dentate gyrus
DHEA	dehydroepiandrosterone
DHEAS	DHEA sulfate
dHPC	dorsal hippocampus
DREADD	designer receptors exclusively activated by designer drugs
EC	entorhinal cortex
EdU	5-ethynyl-2'-deoxyuridine
EF	ethanol females

EM	ethanol males
ER	estrogen receptor
ERK	extracellular signal-regulated kinases
Et-Loc	ethanol-induced locomotion
Et-Loc	ethanol-induced locomotion
fMRI	functional magnetic resonance imaging
FPM	first-pass metabolism
FSH	follicular stimulating hormone
$GABA_A$	γ-Aminobutyric acid type A
GC	gas chromatography
GFP	green fluorescent protein
GH	growth hormone
GIRK	G-protein inward rectifying K+ channel
GlyR	glycine receptors
GnRH	gonadotropin-releasing hormone
GPCR	G-protein coupled receptor
GR	glucocorticoid receptor
GSH	glutathione
HAT	histone acetyltransferase domain
HATs	histone acetyltransferase
HDAC	histone deacetylase
HDACs	histone deacetyltransferase
HPA	hypothalamus-pituitary-adrenal
HPC	hippocampus
HPG	hypothalamic-pituitary-gonadal
I.P.	intraperitoneal
IL	infralimbic cortex
IP3	inositol triphosphate

IPA	ingenuity pathway analysis
ITI	intertrial interval
Kcnmb1	potassium large conductance calcium-activated channel, subfamily M, beta member 1
kDa	kilodalton
KIX/KID	kinase inducible domain
KO	knockout
LEC	lateral EC
LET	letrozole
LH	luteinizing hormone
L-LTP	late long-term potentiation
LORR	loss of righting reflex
LTP	long-term potentiation
Mag	myelin-associated glycoprotein
Mal	myelin and lymphocyte protein
MAPK	mitogen-activated protein kinase
Mbp	myelin basic protein
MD	mediodorsal thalamic nuclei
MEC	medial EC
MEK/MAPKK	mitogen-activated protein kinase kinase
MEOS	microsomal ethanol oxidation system
MET	metyrapone
Mobp	myelin-associated oligodendrocytic basic protein
mPFC	medial PFC
MSK1	mitogen- and stress-activated protein kinase 1
MWM	morris water maze
Myrf	myelin regulatory factor gene
NAc	nucleus accumbens
nACH	nicotinic acetylcholine

NCBD	nuclear coactivator binding domain
NCOA1	nuclear receptor coactivator 1
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptor
NOR	novel object recognition
NRG1	neuregulin 1
NRID	nuclear receptors interaction domain
NTD	N-terminal domain
OR	odds ratio
pCBP	phosphorylated CBP
pCREB	phosphorylated CREB
PDGFRα	platelet-derived growth factor receptor α
PFC	prefrontal cortex
PHD	plant homeodomain
PIP2	phosphatidylinositol 4,5-biphosphate
PKA	protein kinase A
PKC	protein kinase C
PL	prelimbic cortex
PLC	phospholipase C
Plp	myelin proteolipid protein
PND	postnatal day
PRC	perirhinal cortex
PV	parvalbumin
RAM	radial arm maze
RE	reuniens nuclei
RM	repeated measures
ROS	reactive oxygen species

RSK2	ribosomal S6 protein kinase 2
RSTS	Rubinstein-Taybi
SEM	standard error of the mean
SI	social interaction
TAZ1	transcriptional-adaptor zinc-finger domain 1
TAZ2	transcriptional-adaptor zinc-finger domain 2
TBW	total body water
U.S.	United States
VTA	ventral tegmental area
ZZ	ZZ-type zinc finger domain

Abstract

Ethanol is the most consumed drug among adolescents, which can cause long-term effects on memory and changes in gene and protein expression. While both adolescents and adults engage in binge drinking, the neurological and behavioral effects differ, with adolescents showing fewer adverse physiological effects but greater memory deficits than adults. Sex differences related to ethanol occur with women showing higher BEC than men and similar cognitive deficits despite fewer years of ethanol consumption. During adolescence the mPFC and dHPC undergo maturation development with both brain regions playing a role in spatial and recognition memory. Proteins critically involved in memory and transcription, CREB and CBP, are decreased following ethanol exposure with decreased protein expression resulting in memory deficits. This dissertation examines how binge ethanol may differentially impact memory performance, myelin-related gene expression, memory-related protein expression, and memory-related protein interactions due to sex and developmental age of ethanol exposure. We hypothesized that binge ethanol would result in a recognition and spatial memory impairment in adolescent exposed animals but not adult exposed, and would be associated with decreased CBP protein expression and interactions. Additionally, we hypothesized that females would be more impacted by ethanol exposure than males due to a higher BEC. To investigate age and sex differences following binge ethanol exposure, we exposed adult and adolescent DBA/2J male and female mice to the same ethanol paradigm (intermittent oral ethanol 4g/kg) from either PND 29-42 or PND 64-77. First, we behaviorally characterized the adult and adolescent exposure model finding adolescent ethanol exposure but not adult impaired recognition memory, while age-by-ethanol effects also impacted sociability and acute ethanol locomotor tolerance. Sex

impacted ethanol sedation, but age and not sex impacted BEC with adolescents displaying lower BEC than adults. Additionally, adult ethanol exposure resulted in one myelin-related gene expression change. Next, adolescent ethanol impacted spatial learning and impaired spatial memory, while sex impacted spatial learning in the adult cohort and spatial memory in the adolescent cohort. At the same timepoint as the observed memory deficits, CBP protein expression was unchanged by ethanol, while CREB was altered by an ethanol by sex interaction in both brain regions. CREB protein interactions in the dHPC and CRE-mediated transcription in the mPFC, did not differ by ethanol in any age group. Lastly, we unbiasedly explored upstream targets of the adolescent ethanol gene list and found two potential upstream regulators of lasting gene expression changes in the PFC, NRG1and CD40, with roles in neurogenesis and synapse assembly. Therefore, dendritic spines and basal or activity dependent neurogenesis may be impaired following adolescent ethanol exposure and may play a role in the observed memory deficits. Together, these experiments provide evidence that binge ethanol differentially impacts myelination, recognition memory, and spatial memory depending on the age of ethanol exposure, but the underlying molecular differences between the two age-exposed groups remains to be understood.

Chapter 1

Introduction

1.1 Epidemiology and consequences of alcohol use in adolescence

Alcohol is the most abused drug among adolescents and is easily accessible with 75% of those 12-20 years old obtaining alcohol without needing to pay for the drug in 2021 [1]. While 48.7% of those 12 years and older consumed alcohol within the past month, 3.2% of adolescents, ages 12-20, and 22.6% of adults, ages 26 or older, engaged in binge drinking within the last month [2]. Binge drinking is the consumption of alcohol in which blood alcohol concentrations (BAC) reach above 80 mg/dL, which typically occurs when 4-5 drinks are consumed within 2 hours in females and males respectively [3]. Binge ethanol consumption occurs in adolescents in the United States (U.S.) and internationally. In countries where the legal drinking age is lower than the U.S., 21 years old, binge drinking occurs at a higher rate [4], [5]. In the U.S., binge drinking peaked from 1970-1980 with a continual decline since that period [4]. In 2020, during the Coronavirus Disease 2019 (COVID-19) global pandemic, the consumption per capita of alcohol increased by 2.9%, which was the greatest single-year change in consumption rates since 1968 [6]. Since the pandemic the percentage of people engaging in binge alcohol use in the past month has held steady around 3% for those 12-17 years old and around 22% for those 26 or older [1], [2]. Overall, binge alcohol consumption is a common behavior engaged by both adolescents and adults.

Early ethanol consumption increases the risk for later alcohol dependence [7]–[9], with those who began consumption at ages 18-21 approximately 5 times less likely to develop alcohol dependence in their lifetime than those who began before 12 years old [7], [9]. Meanwhile,

alcohol misuse, formerly termed alcohol dependence, is most prevalent in those that began alcohol consumption at age 14. In comparison, if alcohol consumption was abstained until age 21, individuals are approximately 4 times less likely to experience lifetime alcohol misuse [7]. Therefore, increasing the age of alcohol initiation is associated with lower lifetime alcohol misuse, while those who begin alcohol consumption in adolescence are more likely to experience problematic alcohol consumption later in life.

Binge drinking is associated with several behavioral changes such as motor impairments [8], memory deficits [10], and sedation [11]. The behavioral effects of alcohol depend on the concentration with binge and heavy alcohol exposures associated with BAC > 100mg/dL [11]–[13]. While alcohol causes behavioral changes in both adolescents and adults, the severity differs depending on age. Adolescent rodents show decreased sensitivity to the motor impairment and sedative effects of alcohol compared to adults [14], [15]. Meanwhile, adolescents show greater cognitive impairments as compared to a similar exposure in adults [16]–[18]. While both age groups exhibit memory impairments due to alcohol [10], [19], [20], adolescents appear more sensitive, which may be due to the ongoing developmental window of adolescence. As the brain is still undergoing maturation, binge alcohol during this critical developmental period may result in lasting changes in behavior and brain function, while less severe changes may occur following adult binge ethanol exposure.

1.2 Ethanol pharmacokinetics, influencing factors, and mechanism of action

Ethanol (CH3-CH2-OH) is a polar substance whose pharmacokinetics are impacted by several factors such as age, sex, and feed state, that act upon absorption, distribution, metabolism, and elimination, also known as ADME. Upon oral ingestion, ethanol is absorbed into the bloodstream via passive diffusion first in the stomach and later by the intestines [21].

Following absorption into the bloodstream ethanol is distributed to tissues in the body depending on the amount of blood flow and total body water (TBW), which in humans makes up 50-60% of body weight [22]. Ethanol does not bind to a transport protein within the blood and can easily cross through the plasma membrane [23] and blood-brain barrier [24]. Upon distribution into the cytosol of a cell, ethanol is metabolized by alcohol dehydrogenase (ADH). While the liver metabolizes the majority of ethanol, ADH is present in a variety of tissues such as the stomach and brain [25], [26]. Ethanol metabolism follows zero-order kinetics at high ethanol concentrations with a constant amount of ethanol metabolized regardless of the concentration [27]. ADH metabolizes ethanol into acetaldehyde, a toxic compound that causes flushing, nausea, increased heart rate, and headaches [28]. Acetaldehyde is then further metabolized by acetaldehyde dehydrogenase (ALDH) into acetate in the cytosol and mitochondria. Acetate can be used in the KREB cycle as a part of acetyl-CoA to produce ATP as well as histone acetylation (discussed further below). During ethanol metabolism, NAD+ acts as a cofactor for the oxidation reactions producing NADH. Decreases in the ratio of NAD+ to NADH can impair other important energy-producing reactions from occurring such as glycolysis, gluconeogenesis, KREB cycle, fatty acid oxidation, and pyruvate to acetyl-co-a production [29]. As there is increased NADH following ethanol metabolism, there is more H+ available to produce reactive oxygen species (ROS) in the mitochondria during the electron transport chain reaction. ROS are toxic to cells, damaging DNA and lipid membranes while also disrupting enzyme activity and protein misfolding [30]. Ethanol increases ROS in vivo and in vitro, decreases levels of antioxidant glutathione (GSH), impairs the shuttling of GSH into the mitochondria, and increases lipid degradation [30]. ROS can also be generated by CYP2E1, a member of the cytochrome P450 family, which metabolizes ethanol into acetaldehyde within microsomes known as the

microsomal ethanol oxidation system (MEOS) [31]. Although ADH has a high affinity for ethanol, when ethanol concentrations become high ADH becomes saturated enabling a lower affinity enzyme CYP2E1 to metabolize ethanol [21]. Following chronic ethanol exposure, CYP2E1 enzyme activity and quantity increases which can contribute to lower blood ethanol concentration (BEC) and may promote increased ethanol consumption [21]. Along with metabolizing ethanol, CYP2E1 metabolizes other products such as acetaminophen, with the combination of these drugs increasing the risk for liver toxicity [21]. Ethanol can also be metabolized by the catalase enzyme within peroxisomes although this metabolism pathway is limited by the quantity of the cofactor hydrogen peroxide [30]. While the majority of ethanol is metabolized, a small fraction is eliminated via urine, sweat, and exhalation [21]. Through ADME, ethanol is utilized and eliminated by the body with several enzymes playing a role in the process.

While the ADME of ethanol is known, additional factors impact the effect of ethanol such as TBW, weight, age, sex, stomach contents, ethanol concentration, enzyme isoform distribution, and ADH & ALDH isoforms. TBW impacts ethanol distribution with decreased TBW leading to fewer areas for ethanol distribution and associated with an increased BEC [21]. Weight, age, and sex all impact the TBW of an individual with those with decreased weight, elder age, and the female sex associated with increased BEC [21], [32]. While elderly age is associated with decreased metabolism and TBW resulting in increased BEC [32], no age-related differences are noted between adolescents and adults. Women have shown increased BEC compared to men due to differences in rates of ethanol elimination [33], liver volume per lean body mass [34], decreased first-pass metabolism (FPM) [33], and the impact of reproductive hormones [35]. Meanwhile, the state of the stomach, fed or empty, also impacts ethanol absorption with an

increase in the rate of absorption when the stomach is empty [29], which can lead to increased BEC. Furthermore, the concentration of ethanol consumed impacts ethanol metabolism with higher concentrations leading to ethanol metabolism by the lower affinity ADH enzyme ADH5 [36]. Baraona [33] observed decreased gastric ADH5 activity in females and a sex difference in FPM when 10-40% ethanol but not 5% was consumed. The increased ADH5 activity in males is likely involved in the increased FPM observed at the higher ethanol concentrations as ADH5 has a low affinity for ethanol, being active mainly at high concentrations [36], [37]. ADH5 is the only ADH enzyme in the brain [36], [38], while ADH 1-5 is expressed within the liver and ADH 1, 4, and 5 are expressed in the stomach [25]. Humans have 7 different ADH genes with varying affinity for ethanol, prominence in certain ethnic groups, and associated risk for alcohol use problems [25]. The ADH1B gene has 3 alleles with ADH1B*2 and ADH1B*3 show faster rates of ethanol metabolism in vitro than ADH1B*1 and are associated with a protective effect for the development of alcohol use problems, due to the rapid buildup of the toxic acetaldehyde [25]. While ADH1B*2 is prominently found in those of Asian ethnicity it is also found in other ethnicities, where the isoforms appear less protective. This difference in protective effects could be due to social factors or due to other ADH isoforms (ADH1A and ADH1C) which form homo or heterodimers [25]. Meanwhile, ALDH isoform ALDH2*2, also predominately found in those of Asian descent, displays a very low affinity for acetaldehyde and is associated with a protective effect against alcohol use [39]. While both ALDH1 and ALDH2 metabolize acetaldehyde ALDH1 resides in the cytosol while ALDH2 resides in the mitochondria [25]. Within DBA/2J mice, adult males express higher concentrations of ALDH2 and ALDH1A1 protein in whole brain tissue compared to C57BL/6 [38], which may contribute to differences in acetaldehyde accumulation and the noted drinking preference between the two strains. Overall, several

additional factors can impact ethanol pharmacodynamics further impacting how ethanol affects the body.

While several factors influence alcohol's transport within the body, once in the bloodstream ethanol can enter the brain following passive diffusion through the BBB to the extracellular matrix. Once in the extracellular matrix, the drug impacts neurons, astrocytes, oligodendrocytes, and microglia with chronic ethanol negatively impacting morphology and development of both neurons and glia [40], [41]. Within neurons, ethanol can act as both an agonist and antagonist depending on the receptor, overall acting as a depressant in the brain. Ethanol potentates several pentameric ligand gated receptors such as γ-Aminobutyric acid type A ($GABA_A$), glycine, large-conductance Ca2+ dependent K+ channel (BK), G-protein inward rectifying K+ channel (GIRK), nicotinic acetylcholine (nACH), and 5-hydroxytryptamine 3 (5-HT3) [42], [43]. Ethanol binds to $GABA_A$ receptors, between the alpha and beta subunit [44], increasing the receptor opening frequency and duration, allowing for more chloride uptake into the cell [45]. Although ethanol is an agonist for several receptors, ionotropic glutamate receptors (N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainite) are inhibited by the drug leading to decreased excitation [42], [46]. Ethanol inhibits the NMDA receptor (NMDAR) regardless of subunit composition [47] although GluN2A appearing less sensitive to ethanol inhibition than GluN2B [48]. Ethanol interacts with GluN2B and GluN1 via the N-terminal domain (NTD), but appears to have another binding site on the same subunits as ethanol continues to inhibit the receptor but to a lesser degree without the NTD [47]. As ethanol activates inhibitory neurons, the inhibitory/excitatory balance within the brain is disrupted, to which the brain compensates, following chronic ethanol exposure, by increasing the excitatory tone [49], [50]. Dopamine (DA) release is also impacted by ethanol although the drug does not

appear to directly bind to the DA receptor, but modulates cell activity via other cell types directly acting on DA neurons. Indeed, DA levels in the nucleus accumbens (NAc) are sustained by glycine receptor (GlyR) currents [43]. Meanwhile antagonism of nACH receptor also modulates DA release in the ventral tegmental area (VTA) and NAc [43]. However, in the striatum high concentrations of ethanol decrease DA release [51]. Meanwhile, 5-HT3 activation in the VTA impact alcohol consumption [52]. Additionally, subunit specificity of GlyRs impact the effect of ethanol on sedation, motor coordination, and intake [43]. Therefore, through many different receptors, ethanol impacts the brain and modulates different behaviors.

In addition to acting upon receptors, ethanol can also passively diffuse into the cell and impact cellular function by interacting with intracellular molecules including protein kinase C (PKC) and adenylyl cyclase (AC). Acute ethanol in vitro increases cyclic adenosine monophosphate (cAMP) production from AC [53], [54] but decreases PKC delta and gamma isoform activity reducing D_1 receptor phosphorylation [54]. Protein kinase A (PKA) and PKC translocation to the nucleus is also potentiated by acute ethanol [55], [56], which leads to increased transcription [57]. While ethanol interacts directly with AC [53] and PKC [54], it currently does not appear that ethanol directly interacts with PKA, but rather modulates the kinase activity through its action on AC. Indirectly, chronic ethanol administration decreases protein expression [58]–[60], protein phosphorylation [58], [61], gene expression [59], [62], [63], and the expression of epigenetic regulators [43], [47]. While chronic ethanol administration decreases PKA expression in the cerebellum [58], PKA expression in the cortex was unaltered [64] further suggesting the impact of ethanol depends on the brain region, with differences in dominate receptor signaling and AC isoform. Indeed, AC7 and AC9 activity are sensitive to ethanol while AC2 and AC3 are insensitive [53], [65]. AC expression is variable within the

mouse brain with AC7 varying in expression, lower in the cortex than cerebellum, is expressed in GABAergic neurons, and increases in activity with ethanol [53], [66]. Meanwhile, AC9 is highly expressed throughout the brain in glutamatergic neurons [66] with ethanol decreasing AC9 activity [49]. Changes in protein and gene expression are likely due to disruptions in kinase activity leading to disruptions in transcriptional activation, along with ethanol disruptions on protein folding, protein degradation, and post-translational modifications [67], [68]. Acetylation is a post translational modification often found on histones and associated with relaxed chromatin. Other post translational modifications can also occur on histones including methylation, phosphorylation, and ubiquitination [69]. Acetate from brain and liver metabolism is incorporated into histone acetylation [70] with acute ethanol increasing acetylation levels [59], while abstinence following chronic ethanol exposure decreases histone acetylation [59], [68], [71]. Epigenetic regulators such as histone acetyltransferases (HATs) and histone deacetyltransferases (HDACs) are also impacted by chronic ethanol exposure with decreases in HAT protein [59], [68], [71]) and increase in HDAC activity [68]. Meanwhile histone methylation can be associated with open or closed chromatin depending on the histone residue with marks like H3K9 tri methylation associated with chromatin repression [69], which is increased following adolescent ethanol exposure [63]. Through various receptors and intracellular proteins, ethanol has widespread effects within cells which varies with brain region and length of ethanol exposure.

1.3 Adolescent brain development and the impact of ethanol

Adolescence, approximately ages 11-25 in humans and postnatal day (PND) 25-55 in rodents, is a developmental period in which changes in behavior, the body, and the brain occur. Behaviorally, adolescents increase peer interactions and risk-taking, while in the body there are

changes in hormone activation and brain maturation [72]. Indeed, during adolescence, there is an increase in drug-seeking behavior [72] with alcohol being a drug highly consumed by adolescents due to its easy access [73]. Within the body, hormone activation includes gonadarche, increased gonadal hormone secretion during adolescence, and adrenarche, increased adrenal hormone secretion which begins before adolescence but continues through adolescence [72], [74]. During adrenarche, increases in certain androgens are associated with increased skeletal, bone, and hair growth [72], [74]. Adrenal hormones, dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEAS) act as neurosteroids in the brain where in the hippocampus they antagonize $GABA_A$ receptors and act as positive allosteric modulators on NMDA receptors [75], [76]. While humans undergo adrenarche, rodents do not, although both species produce DHEA locally in the brain [75]. However, both humans and rodents undergo gonadarche in which gonadal hormone production increases due to increases in gonadotropin-releasing hormone (GnRH) in the hypothalamus as part of the hypothalamic-pituitary-gonadal (HPG) axis activation. GnRH then acts on the pituitary to stimulate luteinizing hormone (LH) and follicle stimulating hormone (FSH). Both LH and FSH act on the respective gonadal organs with LH leading to increased testosterone or estrogen production while FSH leads to the release of inhibin and androgen binding protein [77]. While both males and females have testosterone and estrogen, each contains more of their respective sex hormone. The HPG axis is a negative feedback loop in which increases of gonadal hormones decrease GnRH production. During this developmental window, growth hormone (GH) production in the pituitary also increases, contributing to adolescent growth [77]. Adolescent males in a rehabilitation program for drug use, including alcohol, displayed decreased LH and FSH, with testosterone levels roughly half of the controls [78]. However, continued abstinence from alcohol and other drugs increased

testosterone levels [78]. In rodents, female adolescent rats exposed to chronic ethanol displayed blunted GH levels, decreased LH levels, and delayed puberty onset [79]. Similarly, chronic ethanol exposure in an adolescent primate model increased the duration between menstrual cycles, blunted GH and estrogen, and decreased LH levels [80]. Through chronic ethanol exposure, adolescent behavioral changes are exacerbated while hormonal changes are dampened due to chronic alcohol use.

Within the brain, maturation changes include increased myelination, decreased grey matter, refined synaptic connections, and changes in neurotransmitter release and firing patterns. During adolescence, white matter levels increase in both males and females [81], [82] in a linear fashion into the early 20s [81], [83], [84]. Simultaneously grey matter levels decline [81], [82], [85], with some regions declining earlier than others [85]. White matter is produced by oligodendrocytes and increases signal transduction within and between neurons. Studies have reported males to have a greater white matter than females [86]. But when body size is considered, males and females show similar proportional increases in brain size and white matter in adolescence [82]. Myelination between brain regions is critical for communication between regions and following adolescent ethanol exposure, decreases in the typical myelination process occurs [87]–[89]. Although Medina [87] found males with an alcohol use disorder (AUD) diagnosis showed increased myelination compared to controls, females with AUD had decreased myelination. Rodent studies also show decreases in myelin protein expression [90], [91], gene expression [63], density [92], and myelin structure [92] following adolescent ethanol exposure, recapitulating the observed phenotype in humans. Additional deficits on myelin thickness and integrity have been observed immediately and persistently following adolescent ethanol exposure [91], further indicating several aspects of myelin can be impaired by adolescent ethanol.

Therefore, in both rodents and humans, ethanol exposure in adolescence decreases myelination throughout the brain.

During adolescence, the brain also undergoes synaptic pruning in which synaptic terminals and dendritic spines are decreased. Within the prefrontal cortex (PFC), rodents show increased synaptic terminals [93] and density of dendritic spines [94] during adolescence which decrease in adulthood. In the dorsal hippocampus (dHPC) during adolescence, the number of mature spines increases, without altering the density, while dendritic length and intersections undergo a transient remodeling [95]. Adolescent ethanol exposure persistently (roughly 20 days post ethanol) decreases mature mushroom spine density, while increasing immature spines in the PFC and dHPC [50], [96], [97]. The PFC and dentate gyrus (DG) of the dHPC also experience decreased total spine density [50], [96] following adolescent ethanol exposure which is not found in cornu Ammon 1 (CA1) [97]. This suggests subregion specific differences in persistent dendritic spine density following adolescent ethanol exposure.

In addition to synaptic pruning during adolescence, the neurotransmitter receptor quantity within the brain changes with declines in NMDA, DA, and GABA innervations [72], [93]. Indeed, in the hippocampus (HPC) and frontal cortex, NMDA receptor density is higher in adolescence than in adulthood [98]. Within the PFC, DA receptor density also peaks in adolescence with D1 receptor changes thought to aid in NMDA transmission [99], [100]. GABAergic parvalbumin (PV) interneuron density also increases during adolescence [101], demonstrating that still maturing regions of the adolescent brain undergo many neurotransmitter shifts during this developmental period. The adolescent period also involves increased brain utilization of frontal cortical areas including the PFC. Cognitive tasks assessing working memory and spatial working memory found increased brain activation in the PFC and posterior parietal

cortex with age in adolescent participants [102], [103]. Ethanol during adolescence decreased activation in the frontal gyrus in a spatial working memory task with several other brain regions showing decreased activity in female adolescents with binge ethanol exposure but increased activity in males [104]. Overall, ethanol decreases mature dendritic spine morphology, temporal and frontal cortex volume, and frontal gyrus activation, therefore altering aspects of adolescent brain maturation. Through disruptions of both the brain and body ethanol blunts the developmental changes associated with adolescence.

1.4 Neurobiology of memory

Memories are essential for the success of many organisms from invertebrates like sea slugs (*Aplysia*), to humans. Disturbances in memory formation and recall are known to occur in many neurological disorders such as Alzheimer's Disease and with the use of drugs such as alcohol. Memories can be categorized as implicit, involving motor skills and simple reflexes, or explicit, involving people, places, things, events, and facts [105]. Implicit and explicit memories can further be subdivided into short- or long-term memories [105], [106]. While implicit and explicit memory utilize different brain regions they share many of the same intracellular signaling molecules. Additionally, explicit and implicit memory share the same sequence starting with learning/encoding, short-term memory, intermediate-term memory, long-term memory consolidation, long-term maintenance, and retrieval [105]. Implicit memory relies on motor regions such as the cerebellum, and striatum (basal ganglia, caudate, putamen), while explicit memory relies on a wider range of brain regions such as the medial temporal lobe and the frontal cortex**.** Recognition memory, spatial memory, and cognitive flexibility are forms of explicit memory that involve different circuitry, cell types, and brain regions due to their different functions. Each memory task has a temporal aspect to it depending on the inter trial interval (ITI)

between the training and testing phase to assess working (seconds), short-term (minutes) intermediate (hours), or long-term (overnight and beyond) memory. Recognition memory involves comparison of the present stimulus to the previously encountered stimuli. Spatial memory involves information regarding the environment and spatial location within said environment. Cognitive flexibility involves updating existing information about a procedure or situation. While there are many forms of memory, this next section will discuss the different brain regions, circuitry, and significant intracellular signaling players in spatial memory, recognition memory, and cognitive flexibility.

1.4.1 Brain regions and circuitry of memory

The process of memory can be broken into three main elements: encoding, consolidation, and retrieval. Encoding is also interchangeably described as learning and acquisition in the literature and this work. For a memory to be formed, first the stimulus is received through various brain regions depending on the stimulus. Various sensory information travels to the thalamus which then sorts information to appropriate brain regions. Stimuli information entering the thalamus related to explicit encoding and spatial navigation is processed by the anterior thalamic nuclei (ATN) [107] while abstract thinking, cognitive flexibility, and long-term goal behavior are processed in the mediodorsal thalamic nuclei (MD) [108]. From the ATN, projections are made to many brain regions including the subiculum (including pre, para, and post), entorhinal cortex (EC), perirhinal cortex (PRC), anterior cingulate cortex (ACC), and HPC [107], [109]. Reciprocal projections from the parasubiculum, postsubiculum, EC, and anterior cingulate cortex project back to the ANT [107]. Within the ATN, specifically the anterodorsal and anteroventral nuclei there are head direction cells which fire depending on the specific direction of the head [107]. The MD, specifically the ventrolateral region, also projects to the

PFC and receives inputs back [108], [109]. The reuniens nuclei (Re) of the thalamus also projects to the PFC, subiculum, and EC receiving reciprocal projects back to the Re [107], [110]. Overall, the thalamus projects to many brain regions and receives many reciprocal projections back.

The EC is one of many brain regions in the medial temporal lobe that is involved in memory encoding and external/internal representation. The EC can be subdivided into a medial and lateral portion due to differences in inputs and cytoarchitecture [111]. The medial EC (MEC) receives inputs from the orbitofrontal cortex, retrosplenial cortex, postrhinal cortex, and pre and parasubiculum [111]. While the lateral EC (LEC) receives inputs from the ACC, insular cortex, PRC, and orbitofrontal cortex [111]. Both the MEC and LEC receive inputs from the parietal cortex, prelimbic cortex (PL), infralimbic cortex (IL), Re, and olfactory cortex [107], [110], [111]. Of note, the EC is bidirectionally connected to the PFC [112], [113]. The EC has many specialized cell types including head direction, grid, object-trace, border, and speed cells [107], [114]–[116]. Grid cells, found in the MEC, fire in a novel environment to build a representative map of the area, with a single grid cell firing in multiple locations [114]. While the LEC has object firing cells, which fire specifically at locations with novel objects/locations, and object-trace cells, which fire for a location that previously held an object up to 3-4 hours following object presentation [115], [116]. The MEC in comparison shows little object-specific firing [115]. Border cells, which fire when an animal is near the border of a wall or the edge of an open arena are also found in the MEC and parasubiculum, with some cells firing for a single wall or multiple walls [117]. Lastly, speed cells, found in the MEC and HPC increase firing as an animal's speed increases regardless of the environment [118]. The aforementioned specialized cell types appear before weaning PND 15-18 with head direction and border cells having adult

like firing properties while very few grid cells are found, about 2-12%, with poor spatial firing but during adolescence the number of cells, spatial resolution, and field size stability increase into adulthood [119]–[121]. Both the MEC and LEC cells project to the DG and CA3, but differ in projection location within CA1[112]. The MEC projects to CA1 proximal to CA2 while LEC projects to CA1 distal to CA2 [112], [115]. The difference in projections to and from the EC are a part of the distinction between the "what" and "where" streams involved in object and spatial representation. The EC is an integral brain region for object and spatial representation in memory receiving and projecting to many brain regions.

The HPC is a critical brain region for memory encoding and pattern separation that has intrigued researchers since patient H.M. [122]. The HPC consists of a dorsal region, associated with spatial, context, and cue-associated memory, and a ventral region, associated with stress and emotion-based memories [123]. This dorsal and ventral distinction is due to behavioral and connectivity differences between the regions. In primates, the anterior region of the HPC corresponds with the ventral region in a rodent, while the posterior region corresponds with the dorsal region [123]. As the focus of the following research is the dHPC, this section will focus on dHPC circuitry. The HPC itself can be further divided into four subregions, the DG, CA3, CA2, and CA1. Previous models of the HPC suggested unilateral synapses through the HPC, from DG to CA1, in what was termed the trisynaptic loop [112]. Recent data indicates subregions of the HPC synapse unto one another with CA3 synapsing onto DG [124] and dorsal CA3 synapsing onto ventral CA1 [125]. Within the HPC, place cells fire when the animal enters a particular location, with a single cell firing for a single location [126]. Both the ventral and dHPC have place cells, although the dHPC has more place cells with a higher spatial resolution [127]. Place cells also can undergo remapping in which new spatial environments are represented

by place cells that display a new firing place reference and firing rate (global remapping) or change firing rate but constant place preference (rate remapping) [128]. The remapping ability is unique to place cells which is thought to allow for spatial updates or to create multiple spatial maps. Place, grid, border, speed, and head direction do not map topographically, resulting in neighboring cells that do not encode neighboring information [114], [117], [126], [129]. Place cells are present at PND 17, at about 40%, and increase in number and field stability during adolescence into adulthood [120]. The dHPC receives projections from many brain regions including the EC, PRC, and Re [112], [115], [123], [130], [131]. The dHPC projects to the subiculum, EC, retrosplenial cortex, anterior cingulate cortex, and Re [123], [132]. In short, the dHPC is an interconnected brain region receiving inputs that aid in spatial and context memory with indirect connections through the thalamus and ventral HPC to the frontal cortex.

Another brain region important for memory is the PFC, which plays a role in rule-set shifting, goal-directed behaviors, inhibition of mesolimbic areas, and memory [133]. The medial PFC (mPFC) can be further subdivided into the PL, IL, and ACC [113]. While the ventral HPC has a direct projection to both the PL and IL cortices, the dHPC does not [113], [125]. The mPFC receives projections from several brain regions including the Re, MD, EC, and PRC [108], [110], [111]. The mPFC does not project directly back to the HPC [130], but to nearby brain regions such as the EC and PRC [111]–[113]. Meanwhile, mPFC projections also reach the Re [110]. As the Re connects to both the dHPC and mPFC, the thalamic nuclei may play a role in communication between the two brain regions. Overall, the dHPC and mPFC are both important brain regions involved in memory with differing connectivity and indirect connectivity to one another through thalamic nuclei, the entorhinal cortex, and the ventral hippocampus.

1.4.2 Recognition memory

Recognition memory involves the encoding, consolidation, and retrieval of a stimulus such as an object, item, or face. Different behavioral tasks are used to assess recognition memory in rodents such as the novel object recognition (NOR) and object-in-place task [134]–[137]. As the NOR task was used in the subsequent studies, the following information will focus on this task. Recognition memory involves the PRC, EC, HPC, ATN, striatum, and mPFC [134], [135], [138]–[144]. While several studies have found the PRC to be necessary for NOR [134], [135], mixed results are observed in the contribution of the HPC or mPFC on NOR with some studies finding no impairment in NOR following lesions, inhibition, or excitation [134], [135], [141], [142], [145], [146]. This may be due to the intact brain region circuitry, task ITI, and the different roles the brain regions play in encoding, consolidation, and retrieval. The ITI of a task contributes to the memory task being long-term, intermediate, or short-term memory and involves different molecular processes regarding memory [106]. The PRC and EC receive projections and project to other brain regions involved in memory such as the Re, and mPFC. Additionally, the EC contains several specialized cells that encode spatial and object representation. Connectivity between the mPFC and PRC/EC through direct projections and the thalamic connection of the Re may be sufficient to enable memory following HPC lesion. Indeed, contra and ipsilateral lesions of the PRC+HPC and PFC+HPC show no change in the 5-minute short-term NOR task [134], suggesting these circuits are not required for the NOR task but other intact brain regions enable recognition memory. Meanwhile, PRC, EC, and HPC connectivity may also be sufficient for memory following PFC lesions. The dHPC is implicated in NOR memory encoding [138], consolidation [139], [142], and retrieval [143]. Meanwhile, Designer Receptors Exclusively Activated by Designer Drugs (DREADD) inhibition of both the mPFC + dHPC leads to an impairment in long-term (24 hour ITI) NOR memory consolidation

[142] further suggesting brain region circuitry plays a role depending on the ITI and time of brain region inhibition. Other studies have suggested the mPFC is involved in memory consolidation [141], [142] and retrieval [147] in the NOR task in the testing phase 24 hours following training. Meanwhile bilateral but not unilateral mPFC pharmacological inhibition with Muscimol, a $GABA_A$ receptor agonist disrupts long-term NOR memory further suggesting spared brain region activity and circuitry-enabled memory consolidation [141]. However, the mPFC does not appear to play a role in short-term (5-minute ITI) NOR memory [134], [135], [145]. Together the data suggests the PRC is necessary for NOR while the HPC and mPFC play a role in NOR depending on the ITI.

1.4.3 Spatial memory

Spatial memory involves the encoding, consolidation, and retrieval of a spatial location or environment. Several tasks are used in rodent studies to assess short-term or long-term spatial memory such as the Barnes Maze, Morris Water Maze (MWM), Radial Arm Maze (RAM), Y-maze, object-in-place, and object location [134], [148], [149]. There are also spatial working memory tasks, like spontaneous alternation, which differ in the use of no or seconds delay period as working memory involves maintained attention and manipulation of task-relevant information [150]. This section will focus on short- and long-term spatial memory tasks, mainly object location, Barnes Maze, and MWM, due to similar design set up as the Barnes Maze task which was used in the subsequent research of the dissertation. Spatial memory involves the MEC, dHPC, mPFC, striatum, and Re [134], [138], [144], [151]–[155]. Again, the timescale of memory (short, intermediate, or long) and timepoint of manipulation (before encoding, consolidation, and retrieval) show the different contributions of various brain regions to spatial memory. The dHPC is involved in encoding [138], [142], consolidation [142], and retrieval

[156] of spatial memory. Meanwhile, short-term [134], intermediate [142], and long-term [134], [138], [152] spatial memory also involves the dHPC. The mPFC does not appear to play a role in short-term consolidation [145], but impacts intermediate and long-term consolidation [142], [151] and long-term retrieval [151]. While some studies have found Re inhibition to impair short-term and intermediate spatial encoding [154], [155] and consolidation [155], others have found no impairment on the encoding of intermediate spatial memory [157], [158]. Differences in Re contribution to spatial memory between studies may be due to the method of Re inhibition or ITI between studies with one at the upper limits of short-term memory while another within the intermediate memory time frame. Overall, the dHPC plays a critical role in spatial memory while the Re and mPFC are involved in spatial memory depending on the method of inhibition, timepoint of inhibition during the task, and timescale of memory.

1.4.4 Cognitive flexibility

Cognitive flexibility involves the updating of existing information to adapt to a new environment through new encoding, reconsolidation, and retrieval [159]. Tasks such as attention set shifting, T-maze, operate conditioning, and the reversal portion of the Barnes Maze or MWM are used to assess cognitive flexibility [160]–[164]. These tasks can vary in the modality of memory and therefore would have differing brain regions involved in encoding, consolidation, and retrieval due to the memory modality. In rodent studies of cognitive flexibility, the mPFC and MD play a role [164]–[166]. Studies assessing brain region contribution to cognitive flexibility tend to use working memory tasks therefore the following information regards working memory tasks. Lesions in the MD impaired reversal learning [164] while PL and IL inhibition increases trials following the rule change [166]. Lesions in the mPFC also negatively impact attention set-shifting, requiring more trials to meet extradimensional criteria but initial

task discrimination was unaltered [165]. Meanwhile, inactivation of the dorsomedial striatum improved reversal learning [144], suggesting the brain region when active negatively impacts cognitive flexibility. Therefore, cognitive flexibility in working memory tasks utilizes the mPFC and MD with inhibition not impacting initial task encoding.

1.4.5 Cellular pathways in consolidation

Several pathways occur for memory consolidation, which involve many of the same key molecular players in different species from Aplysia to humans [105], [106], [167]. In one such pathway (Fig. 1), DA binds to D1/D5 DA receptors which are part of the G-protein coupled receptor (GPCR) family [168]. Activation of the D1/D5 receptors leads to activation of the Gαq pathway in hippocampal neurons [168]–[170]. This pathway begins with the activation of the enzyme phospholipase C (PLC) which cleaves phosphatidylinositol 4,5-biphosphate (PIP2) leading to the production of diacylglycerol (DAG) and inositol triphosphate (IP3). IP3 binds to the endoplasmic reticulum releasing calcium into the cytoplasm which can activate PKC a family of serine/threonine protein kinases that are critical in memory formation and disrupted in neurological disorders like Alzheimer's diseases [171]. PKC phosphorylates mitogen-activated protein kinase (MAPK) which phosphorylates cAMP response element-binding (CREB) protein, activating the transcription factor. CREB is necessary for long-term memory formation and, once activated via phosphorylation, recruits other factors for transcription including RNA polymerase II and CREB binding protein (CBP) [172]. CREB and CBP involvement in transcription will be discussed further below. D1/D5 receptors are also known to activate the GαS pathway which leads to the activation of AC, a plasma membrane-bound enzyme, which converts ATP to cAMP [168]. The activation of AC occurs via calmodulin (CaM) a calcium-sensitive protein, activated by calcium [173]. cAMP acts as a second messenger to activate PKA. PKA is a phosphorylating protein made

up of 4 subunits, 2 regulatory & 2 catalytic subunits, which are bound together in the absence of cAMP. Once 4 units of cAMP bind to the regulatory subunits, the catalytic subunits disassociate and move to the nucleus to phosphorylate CREB leading to transcription [167]. While D1/D5 receptor activation can release internal calcium, extracellular calcium also enters via calcium channels & NMDA receptors when DA receptors are activated [170]. These other sources of calcium lead to CaM activation and illustrate the crosstalk that can occur between molecular players in memory.

Crosstalk between PKA and the extracellular signal-regulated kinases (ERK) pathways for CREB activation is known to occur, with inhibition of kinases in the ERK pathway from a dominant-negative PKA leading to decreased phosphorylated CREB and transcription [174]. The ERK pathway, later renamed the MAPK pathway is highly conserved and activated by receptor tyrosine kinase which binds growth factors like nerve growth factor (NGF) (Fig. 2) [172]. Receptor tyrosine kinase activation turns on Ras which activates Raf leading to phosphorylation of mitogen-activated protein kinase kinase (MEK/MAPKK). MEK phosphorylates MAPK which can translocate to the nucleus to phosphorylate both mitogen- and stress-activated protein kinase 1 (MSK1) and ribosomal S6 protein kinase 2 (RSK2). Both MSK1 and RSK2 are known to phosphorylate CREB [174]. MAPK can also be phosphorylated by PKC activation due to calcium influx from internal or external sources like calcium channels and NMDA receptors. The NMDAR is activated by cell depolarization from AMPA receptor activation and binding of glycine and glutamate [106]. NMDA activation is also involved in calcium/calmodulin-dependent protein kinase II (CAMKII) and calcium/calmodulin-dependent protein kinase IV (CAMKIV) activation, both of which are implicated in long-term memory formation [167], [175]. The influx of calcium from the NMDA receptor activates CaM which phosphorylates

CAMKII and CaM Kinase Kinase (CAMKK). CAMKII activation can recruit AMPA receptors stored in the cytosol to the plasma membrane. Meanwhile, CAMKK phosphorylates CAMKIV which can translocate to the nucleus to phosphorylate CREB and CBP. Disruptions in the signaling molecules of these pathways results in memory impairments, highlighting the role of these targets in memory formation [167], [176]–[178].

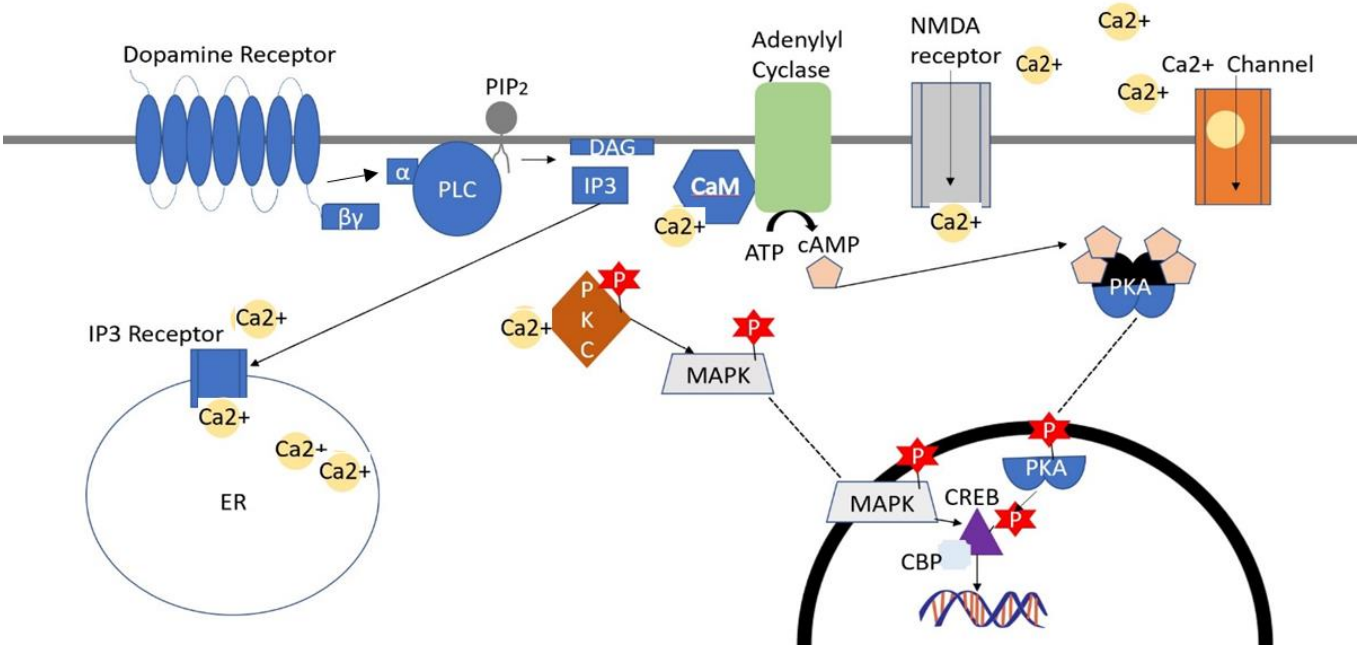

Fig 1. Dopamine receptor activation leads to GαS and Gαq pathways. Both pathways involve different signaling molecules implicated in memory and lead to CREB phosphorylation.

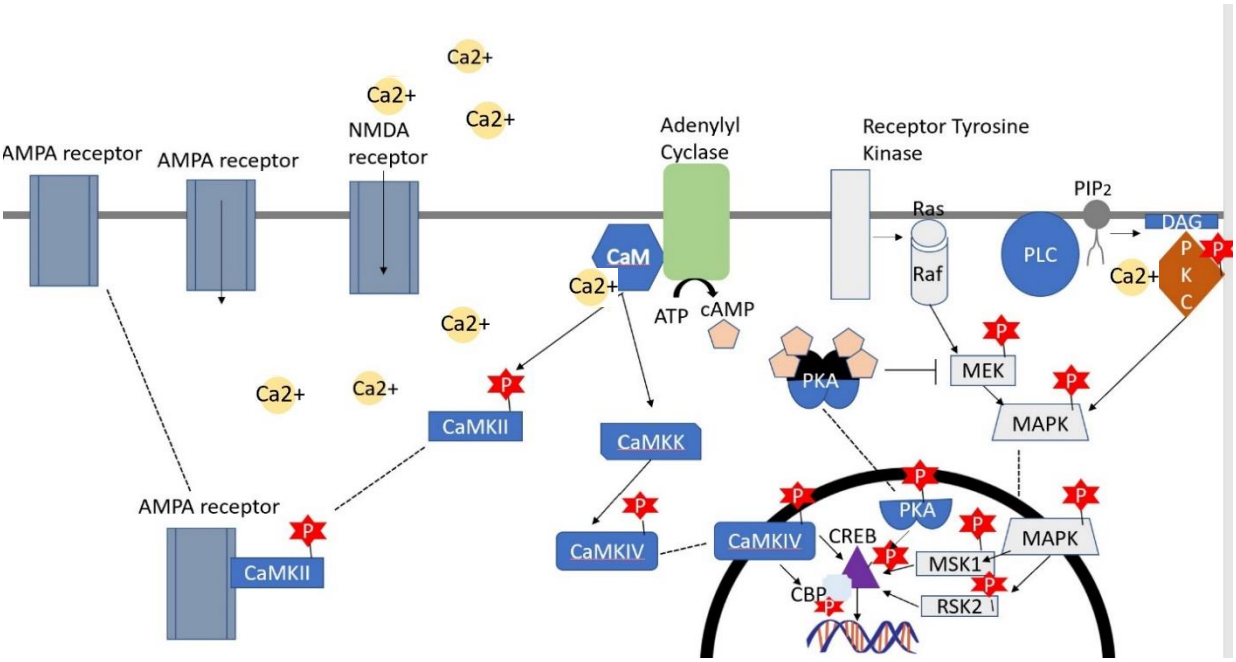

Fig 2. CREB phosphorylation can occur through the activation of CAMKIV, PKA, and PKC.

1.4.6 CBP and memory

1.4.6.1 CBP domains and biological roles

CBP is a 265 kDa protein, ubiquitously expressed throughout the body and brain [176], [179] with 9 binding domains which aid in its and its homolog P300, to bind to over 400 other proteins [180]. The various binding domains enable CBP to have a variety of functions and interactions with other proteins. The 9 domains, listed in N terminus to C terminus order, are the nuclear receptor interaction domain (NRID), cysteine/histidine-rich region 1 (CH1)/ transcriptional-adaptor zinc-finger domain 1 (TAZ1), kinase inducible domain (KIX/KID), bromodomain (Br), plant homeodomain (PHD), histone acetyltransferase domain (HAT), ZZ-type zinc finger domain (ZZ), transcriptional-adaptor zinc-finger domain 2 (TAZ2), and the nuclear coactivator binding domain (NCBD) [180]. The NRID domain allows CBP to interact with nuclear receptors such as the estrogen receptor (ER) [181] while the TAZ1 and TAZ2 regions appear to interact with the glucocorticoid receptor (GR) [182]. Additionally, CAMKIV interacts with CBP phosphorylating S301 between the NRID and TAZ1 domains [175]. Meanwhile, the KIX/KID domain enables CREB interaction following CREB phosphorylation at S133 [172], [174], [183], [184]. The HAT domain enables CBP to function as a histone acetyltransferase [185], adding an acetyl group to specific H3 and H4 residues [68], [186] resulting in accessible chromatin for the transcription machinery. Lastly, the NCBD domain enables CBP interaction with coactivators such as nuclear receptor coactivator 1 (NCOA1) [187]. Due to the many interactions of CBP with other proteins, CBP is involved in a wide range of biological processes including cell growth, energy homeostasis, fertilization, neural progenitor migration, neurogenesis, cortex development, dendritic density, length, and morphology, and embryonic development [179], [180], [188]–[190]. The following section will briefly explain the

biological processes of CBP regulation in the brain. A homozygous knockout (KO) of CBP is embryonically lethal with embryo death occurring from PND 9.5-11.5 due to extensive signs of hemorrhaging and impaired hematopoiesis [179]. Homozygous CBP embryos have also shown impaired neural tube closure, decreased blood vessels, inverted mesencephalon and mesenchyme, and decreased cell density in the mesenchyme [179]. CBP is also involved in cell development in the cortex with a truncated model, amino acids 1-523, expressing smaller total brain, corpus callosum, and HPC volume in addition to decreased thickness in the aforementioned areas [188]. Furthermore, truncated CBP mice, containing the N-terminus to the KIX domain, showed decreased neural progenitor migration and decreased mature dendritic spines, density of apical dendrites, and length of apical dendrites [188]. Meanwhile altering residue 436 in the TAZ1 domain also decreases neurogenesis in adult mice [189], further indicating the role of CBP in adult brain plasticity. Through the many domains CBP is able to interact with many different proteins and impact brain development and plasticity.

1.4.6.2 CBP disease models and memory

CBP is dysregulated in many neurological disorders such as Alzheimer's disease, Rubinstein-Taybi syndrome (RSTS), and Huntington's disease [191], in which patients show memory impairments. Alzheimer's animal models or models with truncated, as in RSTS, or conditional KO of CBP show decreased CBP expression and impaired long-term memory in spatial tasks [185], [192]–[194], and novel object recognition [185], [195]. While Chen [176] showed a CBP-driven impairment in short-term memory with the novel object recognition task, others have shown no impairment in short-term memory with object location [193], [196] and fear conditioning [192] tasks. These differences may be due to the difference in brain regions utilized for the task and manipulated in the studies. Decreases in CBP expression coincide with

decreases in histone acetylation [71], [193], [195]. Histone acetylation is associated with open chromatin which would allow the transcriptional machinery access to DNA enabling transcription and is suggested to be important for memory formation [197]. Disruption of the histone acetyltransferase property of CBP results in decreased transcription and memory impairments [185], [195], [196]. Meanwhile, the enhancement of CBP acetyltransferase activity increased histone acetylation in the HPC and frontal cortex, considerably improving long-term memory [198]. Several studies have looked at the role of histone deacetylase (HDAC) inhibitors, which remove acetyl groups from histones, in improving memory impairments with conflicting results. While some studies show HDACs to improve memory following a CBP memory deficit [185], others have not [176], [178], [193]. Meanwhile, when CBP is increased via viral delivery, performance on a memory-related task is improved [194], indicating therapeutic methods via overexpression may be a promising therapeutic method for memory deficits.

1.5 Dissertation Objectives

There exists a gap in knowledge regarding the underlying mechanisms driving the difference in ethanol-induced memory impairments due to developmental age and sex. As adolescent binge drinking could disrupt developing cortical processes and result in long-lasting changes, understanding the alterations induced by ethanol will be key to developing treatment options. Our lab has shown that binge ethanol in adolescence decreases CBP gene expression in the mPFC and results in a recognition memory deficit following adolescent ethanol exposure. This dissertation explores the underlying mechanisms of ethanol-induced memory impairments due to age and sex by directly comparing the behavioral and molecular response to binge ethanol in adolescent and adult mice. We hypothesize that spatial memory, recognition memory, and cognitive flexibility will be differently impacted by development and sex following binge

ethanol, which we posit CBP protein expression and binding interactions in the mPFC and dHPC are underlying the observed behavioral differences. This research will contribute to the literature by further understanding how age of ethanol exposure and biological sex during ethanol exposure can differentially impact memory.

Chapter 2

Comparing Behavior Following Binge Ethanol in Adolescent and Adult DBA/2J Mice

This chapter is a modified version of an article published in Behavioural Brain Research (Bent et al., 2022)

2.1 Introduction

Alcohol is the most prevalent drug consumed by adolescents as of 2017 with 61% of high school students in the United States consuming alcohol in the past 30 days [73]. Unlike adults, adolescents tend to consume most of their alcohol in a binge-like fashion, consuming a large amount of alcohol in a short period, with 43.8% of adolescents binge drinking while 22.5% of adults binge drink as of 2014 [199]. While both age groups consume alcohol, the behavioral and physiological effects of ethanol differ due to differences in development. During adolescence, the brain and body undergo physical changes such as neural alterations [200] and adrenarche resulting in a different physiology than adults [72]. Adolescents are less sensitive than adults to the negative effects of ethanol, such as motor impairment and sedation [14], [63], [133], while also being more sensitive to the rewarding effects [201]. This shifted alcohol sensitivity in adolescents may enable them to consume ethanol at a higher degree than their adult counterparts, resulting in lasting changes in the brain and behavior [72]. Whereas adolescence is a developmental period of maturation, adulthood is the result of these changes, meaning disruptions such as binge ethanol differentially affect their systems.

The adolescent brain undergoes considerable changes to mature into an adult brain. During this period, brain volume and white matter increase linearly with age [81], [82] until age 20 [83]. Alcohol use in human adolescents is associated with decreased volume [87], [88], [202] and structural changes in white matter [89]. Rodent studies also show decreased myelin gene and protein expression [63], [90] and decreased myelin density in males [92]. While adolescent ethanol negatively impacts white matter, no studies to our knowledge have compared developmental differences in myelin gene expression following binge ethanol. Since active myelination is critical for learning a new motor skill [203], [204] and ethanol negatively impacts myelination, developmental differences in myelination may be an additional factor impacting the behavioral differences observed following binge ethanol treatment.

In addition to myelin changes in adolescence, a shift in connectivity among mesocortical and mesolimbic regions occurs [205] with increased connectivity between limbic regions and cortical regions taking place [206]. The Nac is involved in social [207] and reward [208] behaviors which are more negatively impacted by ethanol in adults than adolescents [209]. Additionally, the Nac integrates information from the PFC [210], a region that matures after the Nac [200], [205], and is involved in judgment, cognitive flexibility, and inhibition of impulsive behaviors [133]. Behavioral tasks which utilize these brain regions, the NOR task [211], and social interaction task [212] may show greater impairments in adolescent-exposed mice than adults. Developmental age also impacts sensitivity to sedative doses of ethanol in the loss of righting reflex (LORR) task [14] and locomotor activation following low-dose ethanol administration [60]. While these studies assessed age differences shortly after ethanol exposure, it is unclear if these behavioral differences persist following a long period of abstinence or in adult animals following binge ethanol exposure in adolescence.

Persistent behavioral differences may be due to underlying age or sex differences in BEC. Adolescent rodents display lower BEC compared to adult counterparts [14], although age effects are not found when gastric first pass metabolism is bypassed [16], [209], [213]–[215]. Furthermore, sex differently impacts BEC in humans [33], [216] and rodents [26] following oral administration with females resulting in a greater BEC than males. The difference in BEC may result in greater sensitivity to cognitive deficits in females than in males. Indeed, alcoholic men and women have similar cognitive deficits despite women reporting fewer years of alcohol use [217]. These studies suggest females may more sensitive than males to the chronic effects of ethanol due to the higher BEC, while adolescents may be reaching similar or lower BEC than adults.

The purpose of this study was to assess the impact of binge ethanol in adolescence or adulthood on changes in myelin gene expression, social approach, memory, ethanol sensitivity and BECs in DBA/2J mice. We hypothesized that adolescent ethanol exposure would more severely impact gene expression and memory than adult exposure. Meanwhile, sociability and ethanol sensitivity would be altered more severely in adult-exposed mice. Lastly, BEC would differ by sex and age with females displaying higher ethanol levels than males and adolescent mice having lower BEC compared to adults. In this study, adolescent and adult male and female mice were exposed to binge levels of ethanol using the same intermittent exposure paradigm where we previously observed decreases in myelin-related gene expression and lasting deficits in recognition memory [63]. Binge ethanol in adulthood decreased social interaction while having little impact on myelin gene expression. Additionally, recognition memory was unchanged by previous adult binge ethanol exposure, while adult females showed ethanol-locomotor sensitivity. Meanwhile, adolescent ethanol resulted in a persistent memory deficit and locomotor

tolerance in males. Developmental age but not sex impacted the duration and peak of ethanol in the blood, with adults having a greater BEC than adolescents. This study suggests that developmental age impacts BEC, social interaction, and ethanol sedation with ethanol additionally impacting memory and ethanol locomotion sensitivity differentially due to age and sex.

2.2 Materials and Methods

2.2.1 Animals and Binge Ethanol Paradigm

DBA/2J males and females from Jackson Laboratory (Bar Harbor, ME) arrived at 7-8 weeks for experiments 1, 2, & 4 and at PND 19-21 for experiment 3 & 4. All mice were habituated to the Virginia Commonwealth University vivarium for one week, housed in same-sexed cages (4-5/cage) on a 15/9 light-dark cycle (15 hours light/9 hours dark cycle) for experiments 1-3 and a 12/12 light-dark cycle for experiment 4 with food and water available *ad libitum*. Mice in experiments 1-3 were dosed with ethanol (25% w/v, by gavage) as described [63]. Adult (AB, PND 62-77) and adolescent (ae, PND 27-42) mice were habituated to gavage with 0.1% saccharin in tap water for two days and then gavaged with tap water or 4 g/kg ethanol in a two days on/two days off paradigm for eight total ethanol binges (Fig. 3). We used DBA2/J mice in these studies as they have more robust behavioral responses [29–31] and myelin-related gene expression changes to acute ethanol [32] as compared to the traditionally used C57BL/6 strain. Mice in Experiment 4 were ethanol and behaviorally naïve until the start of the experiment. In experiment 1, adult male and female mice (n=64, 6-8/group) were administered binge ethanol or water from PND 64-77 which resulted in 4 groups; control males (CM), ethanol males (EM), control females (CF), and ethanol females (EF). PFC tissue was harvested 24 hours or 3 weeks (PND 99) after the last ethanol dose. Three EF mice were lost from this cohort due to

issues affiliated with gavage. In experiment 2, adult male and female mice (n=80) were similarly dosed with binge ethanol and tested for social, cognitive, and ethanol sensitive behaviors. Separate groups of mice were tested at an early phase (within 1 week of dosing, 8-10/group) or a late phase (more than 3 weeks following binge ethanol, 7-10/group). Two EM mice were lost due to issues affiliated with gavage. In experiment 3, adolescent mice (n=84) were binged with ethanol from PND 29-42 and used for adolescent early and late phase behavioral assessments. Three mice (2 EM & 1 EF) were lost from this cohort due to issues affiliated with gavage. Behavioral assessments were separated by 72 hours. In experiment 4, mice were left undisturbed until PND 35 for the adolescent cohort (n=6/sex) and PND 65 for the adult cohort (n=6/sex) to assess naïve BEC. No mice were lost from the experiment or analysis. All animal housing and care were conducted with the approval of the Virginia Commonwealth University IACUC Committee and in accordance with the NIH Guide for the Care and Use of Laboratory Animals [218]. In all cases, mice were habituated to the behavioral testing room each day for at least one hour before testing. Observers were blinded to the sex and treatment groups and equipment was cleaned with 10% ethanol between animals.

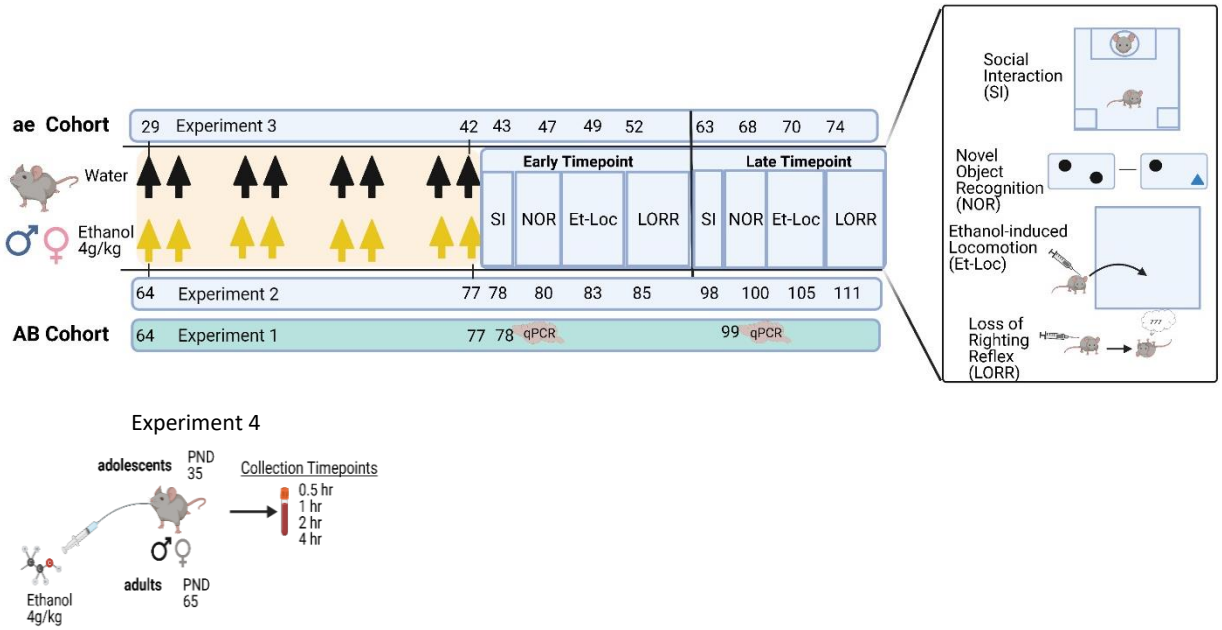

Fig 3. Timeline of chapter 2 experimental procedure. Adolescent (ae) and adult (AB) DBA/2J mice underwent the same intermittent ethanol dosing paradigm from either postnatal (PND) 29-42 or PND 64-77. Following binge ethanol, mice were assessed for behavioral changes in the social interaction (SI), novel object recognition (NOR), ethanol-induced locomotion (Et-Loc), and loss of righting reflex (LORR) tasks. Changes in myelin-related genes were assessed in PFC tissue using qPCR in a behaviorally naïve cohort. Separate groups of mice were used for the early and late behavioral time points. No mice were re-tested in any task. A separate ethanol naïve cohort was given ethanol to assess BEC over 4 hours. Figure created in Biorender.

2.2.2 Social Interaction

Animals were tested for social interactions 24 hours or 21 days after their last ethanol dose as described [219], [220]. Male and female mice were habituated to an open field locomotor activity box (41 x 41 x 31 cm, Omnitech Electronics, Inc, Columbus, OH) with an inverted wire mesh cup (8.3 X 8.3 X 10.1 cm) on a raised platform (12.8 X 9 X 5.1 cm) for 3 minutes. Animals were returned to their home cage for a 30-second intertrial interval (ITI) while a same-sexed, same-age stimulus mouse was placed under the wire cup. Activity tracking software (Fusion v5.3; Omnitech Electronics Inc., Columbus, OH) recorded movement of the test animal in the interaction zone (25 x 7.5 cm) surrounding the stimulus mouse, and two corner zones (10 x 10 cm) far away from the interaction zone for 3 minutes. Animals who failed to enter at least 1 other zone (ae: 1 EM, 1C; AB: 1 CM) or cases when the stimulus mouse briefly escaped the jail cell or a test mouse was sitting on top of the jail cell during the task were excluded from analysis (ae: 2 CF, 2 EM).

2.2.3 Novel Object Recognition

NOR was performed as described [63] using a 5-minute ITI to assess short-term recognition memory [221]. Animals were tested for NOR either 3-5 days or 22-25 days after the last ethanol dose. Mice were habituated to an empty mouse cage (28.5 x 17.5 x 12.5 cm) for 30 minutes twice before testing and then interacted with two identical objects for 5 minutes for training. After a 5-minute ITI, one object was replaced with a novel object of similar size. Time in close contact with nose oriented towards the familiar or novel object (<2 cm) was recorded for 5 minutes. A discrimination index was calculated by subtracting the familiar object interaction

time from the novel object interaction time divided by the total interaction time. Animals who did not investigate the training objects for at least 10 seconds were excluded (AB: 1EF; ae: 1EM).

2.2.4 Ethanol-Induced Locomotion

Separate cohorts of animals were tested for ethanol-induced locomotion either 6-8 days or 27-28 days after their last dose as described [222]. Mice were habituated to the locomotor boxes (25.4 x 12.7 x 20.3 cm, Omnitech Electronics Inc., Columbus, OH) for 15 minutes, before injection with 0.9% saline or 2g/kg ethanol (20% v/v ethanol in 0.9% saline, I.P.) and returned to the activity chambers. Locomotor activity was recorded via infrared photo beam breaks (Fusion v5.3) for 15 minutes. No animals were excluded, nor were they repeatedly tested in the locomotor boxes.

2.2.5 Loss of Righting Reflex

Animals were tested for LORR as described [63], [222] and modified from [223] either 8-12 days or 30-33 days after their last dose. Animals were administered 4g/kg ethanol (25% w/v in saline, I.P.) to test the effects of a sedating dose of ethanol. LORR onset was recorded as the time the mouse could not right itself from the supine position after 5 seconds. LORR duration was measured as the time when the mouse righted to the prone position, twice within 30 seconds. If a mouse did not experience LORR onset within 5 minutes, suggestive of a missed injection [223] they were excluded from further analysis (AB: 1 EF, 1 CF, 1 EM and 2 CM; ae: 3 EF,1 EM and 2 CM).

2.2.6 Blood Ethanol Concentration

Male and female mice in adolescence (PND 35) or adulthood (PND 65) were given ethanol (4g/kg by gavage), followed by collection of blood via retro-orbital sinus blood (80 μL) at four different time points (0.5, 1.0, 2.0 and 4.0 hours). Samples were collected into BD Microtainer® Tubes K2E EDTA (Becton, Dickinson and Company, Franklin Lakes, NJ), vortexed and centrifuged at 2,000 x g for 15 minutes. Plasma was transferred into new tubes and stored at -80°C until analysis. Aliquots of 20-μL plasma were prepared in 20 mL glass GC vials, along with 960 μL of ultrapure water, 500 mg of NaCl and 20 μL 1-propanol (0.1 mg/mL). The 1-propanol was used as an internal standard while a standard curve (0.5, 1.0, 2.5, 5.0, and 10.0 mg/mL) was run prior to loading samples (Shelton, 2010). BECs were determined via headspace gas chromatography (GC) using an Agilent model 6980 GC with CTC Combi-Pal static headspace autosampler (LEAP Technologies, Inc., Carrboro, NC). Ethanol concentrations were normalized to the internal standard (1-propanol). No animals were excluded from the analysis.

2.2.7 RNA Isolation, cDNA synthesis, and qPCR analysis

Total RNA from adult PFC (PFC, a midline wedge shaped cut from 1.4 mm to 0.5 mm from Bregma [224]) was isolated using STAT 60 Reagent (Tel-Test, Friendswood, TX) and Rneasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. PFC total RNA was reverse transcribed into cDNA using the iScript cDNA kit according to the manufacturer's protocol (Bio-Rad, Hercules, CA). To determine expression changes in myelin-related genes, real-time PCR was performed using the CFX System (Bio-Rad) and SYBR Green-based detection (SsoAdvanced Universal SYBR Green Supermix, Bio-Rad). The following myelin-related genes (*Mag*, *Mal*, *Mbp*, *Mobp*, and *Plp*) were chosen due to their role in myelin formation [225], [226], myelin integrity [227], or oligodendrocyte processes [228]. Primer sequences (from 5' to 3') are as follows: *Mag* (Forward: GGTCTCTACCCGGGATTGTC

Reverse: CTGTCCTTGGTGGGTCGTTT), *Mal* (Forward: TCTTCACCACCTTCCCTGAC Reverse: GCCACAAAGCAGAACACAGA), *Mbp* (Forward: ACACACGAGAACTACCCATTATGG Reverse: AGAAATGGACTACTGGGTTTTCATCT), *Mobp* (Forward: AACTCCAAGCGTGAGATCGT Reverse: CTCGGTCACTTCTTCCTTGG), and *Plp* (Forward: TGAGCGCAACGGTAACAGG Reverse: CCCACAAACTTGTCGGGATG). Samples were run in triplicate with cycle thresholds normalized to housekeeping genes *Ppp2r2b* and *Ublcp1* [63]. Relative changes in gene expression were normalized to a control male.

2.2.8 Statistics

Two-way ANOVAs were performed with treatment and sex as factors using GraphPad Prism (GraphPad Software, San Diego, CA). Tukey's post hoc tests were performed when a significant interaction was observed ($p<0.05$). Repeated measures (RM) three-way ANOVAs were used to assess ethanol-induced locomotion within an age group and for BEC data between age groups. To assess area under the curve (AUC) for the BEC data, a linear regression was computed for the four timepoints and then run on a one-way ANOVA. P-values less than $\alpha=0.05$ were considered significant. Data is presented as mean $\pm$ standard error of the mean (SEM).

2.3 Results

2.3.1 Social interactions were decreased in adults following binge ethanol, but not adolescents.

The social interaction task was used to measure social anxiety phenotypes [229], [230], as well as social motivation [231], [232]. In adolescent mice, binge ethanol did not alter performance in the social interaction task at either 24 hours or 3 weeks after the last exposure (Fig. 4). Time in the interaction zone [$F_{treatment}$ (1, 38) = 0.1936, P=0.6624] or in the corner zone

[$F_{treatment}$ (1, 38) = 1.149, P=0.2906] was not changed by adolescent ethanol (Fig. 4 A&B). Likewise, sex did not alter time in the interaction zone [F_{sex} (1, 38) = 0.1438, P=0.7067], or in the corner zone [F_{sex} (1, 38) = 0.1104, P=0.7415]. Not surprisingly, adolescent-exposed mice, 3 weeks following ethanol, performed comparably with the early phase data, and showed no significant differences in social interactions (Fig. 4 C&D). No main effects of treatment on time spent in the interaction zone [$F_{treatment}$ (1, 33) = 0.5776, P=0.4527], or on time spent in the corner zone [$F_{treatment}$ (1, 33) = 2.368, P=0.1334], were found. Sex did not significantly affect the time in the interaction zone [F_{sex} (1, 33) = 0.1667, P=0.6857], or in the corner zone [F_{sex} (1, 33) = 1.043, P=0.3146].

In adults, social interaction was dramatically reduced by binge ethanol, but this did not persist 3 weeks following exposure. Twenty-four hours after the last ethanol dose, adult ethanol-exposed mice spent significantly less time in the interaction zone than control mice [$F_{treatment}$ (1, 35) = 17.72, P=0.0002] (Fig. 4E). Likewise, time in the corner zones was increased in ethanol-exposed adults [$F_{treatment}$ (1, 35) = 5.409, P=0.0260] (Fig. 4F). Sex did not significantly alter the time in the interaction zone [F_{sex} (1, 35) = 0.03410, P=0.8546], or corner zone [F_{sex} (1, 35) = 0.2166, P=0.6445]. At the late phase, neither treatment nor sex altered performance in the adult-dosed animals in the interaction zone ($F_{treatment}$ (1, 32) = 1.24, P=0.2731; F_{sex} (1, 32) = 0.1451, P=0.7058; Fig. 4G), or corner zone ($F_{treatment}$ (1, 32) = 2.509, P=0.1230]; F_{sex} (1, 32) = 3.266, P=0.0801; Fig. 4H). No significant interactions between treatment and sex were found in either age group.

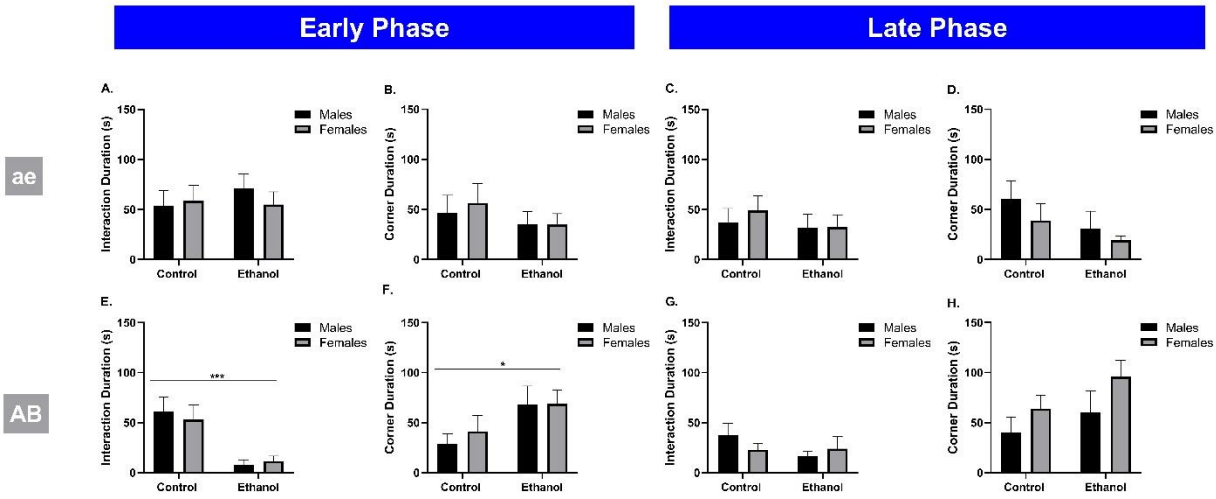

Fig 4. Social interactions differed between adolescent and adult-exposed mice. In adolescent mice, duration in the A) interaction zone and B) corner zone was unchanged 24 hours after the last dose (n: CM=10, EM=11, CF=10, EF=11). Three-weeks after the last dose, duration in the C) interaction zone and D) corner zone duration remained similar between treatment groups (n: CM=12, EM=6, CF=9, EF=10). Meanwhile, ethanol treatment in adulthood impacted social interaction at the early phase with E) decreased interaction time and F) increased duration in the corners (n: CM=10, EM=9, CF=10, EF=10). At the late phase, adult mice showed no sex or treatment differences in the G) interaction duration or the H) corner duration (n: CM=9, EM=8, CF=9, EF=9). Data is presented as mean +/- SEM. (* $p \leq 0.05$, *** $p \leq 0.001$)

2.3.2 Recognition memory is impaired long-term by adolescent binge ethanol but not adult binge ethanol.

The novel object recognition task was used to assess short-term recognition memory mediated by the PFC, perirhinal cortex, and hippocampus [135], [211]. When tested in the early phase, neither adolescents nor adults showed memory deficits. Two-way ANOVA revealed no significant differences in the discrimination index in adolescent mice due to treatment [$F_{treatment}$ (1, 40) = 0.5353, P=0.4687], or sex [F_{sex} (1, 40) = 0.1328, P=0.7174] (Fig. 5A). Similarly, adult-exposed mice showed no differences in the discrimination index due to treatment [$F_{treatment}$ (1, 34) = 0.1309, P=0.7197], or sex [F_{sex} (1, 34) = 0.1470, P=0.7038] (Fig. 5C).

Testing at the late phase revealed long-term deficits in novel object recognition memory in adolescent-exposed mice (PND 68), but not adult-exposed (PND 99/100). As previously reported [63] and seen again here, mice exposed to binge ethanol in adolescence showed a main effect of treatment [$F_{treatment}$ (1, 36) = 4.384, P=0.0434], where binge ethanol significantly decreased the discrimination index as compared to controls (Fig. 5B). Sex did not alter recognition memory [F_{sex} (1, 36) = 0.8887, P=0.3521] in the adolescent-exposed group. Adults exposed to binge ethanol showed no differences in NOR performance due to treatment [$F_{treatment}$ (1, 33) = 0.01681, P=0.8976], or sex [F_{sex} (1, 33) = 0.2129, P=0.6475] (Fig. 5D). No significant interactions were found for any age group.

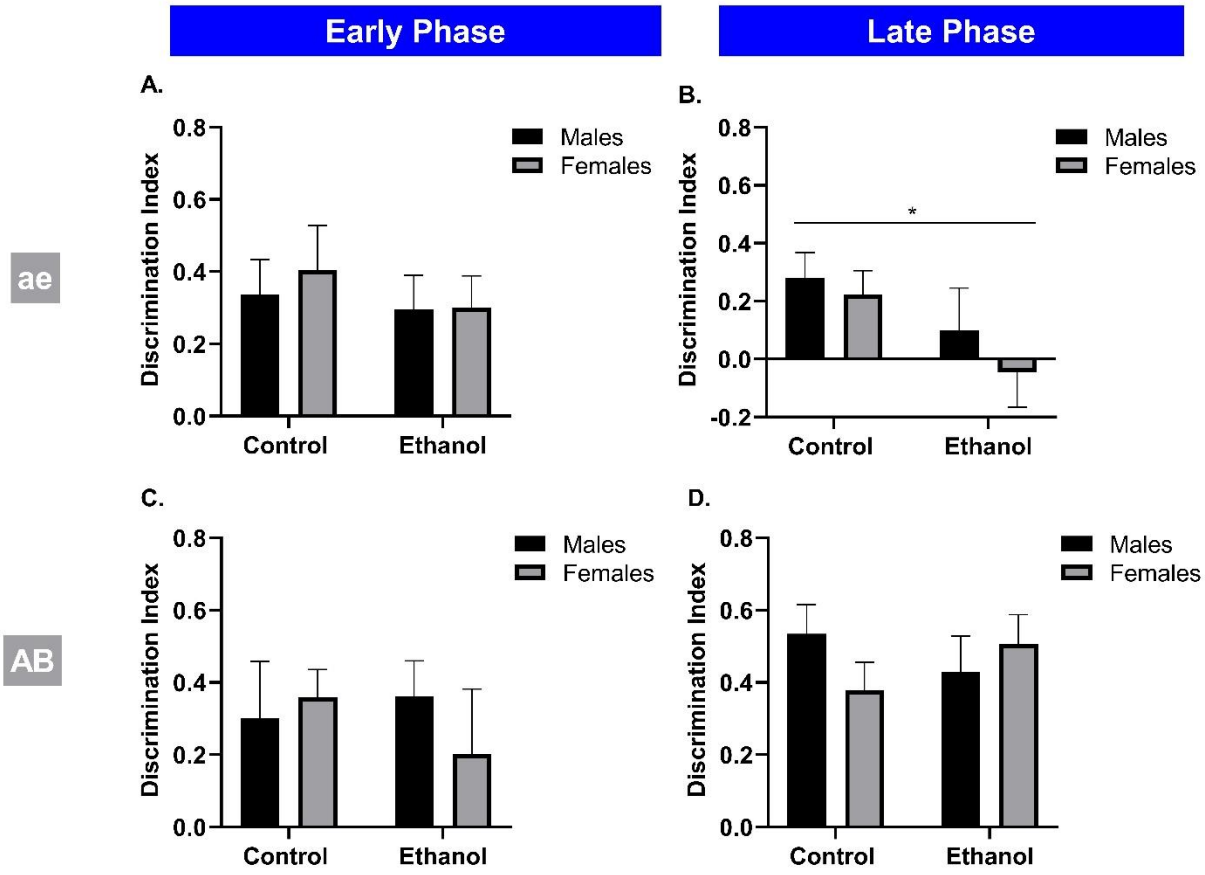

Fig 5. Adolescent binge ethanol alters adult recognition memory. Novel object recognition memory was unchanged at the early phase by either sex or treatment in the A) adolescent-exposed cohort (n: CM=10, EM=12, CF=10, EF=12) or B) adult-exposed cohort (n: CM=10, EM=9, CF=10, EF=9). However, at the late phase when all mice were adults, recognition memory was impaired in C) adolescent-exposed mice given ethanol (n: CM=12, EM=7, CF=11, EF=10). No difference was observed in D) adult-exposed mice due to sex or treatment (n: 9/group). Data is presented as mean +/- SEM. (* $p \leq 0.05$)

2.3.3 Ethanol-exposed adolescent males and adult females show locomotor tolerance at the early phase.

Ethanol-induced locomotion was used to assess acute ethanol sensitivity following a history of binge ethanol. In all cases, acute ethanol (2g/kg, I.P.) on the test day induced locomotor activity in both adolescent and adult cohorts (ae early phase: males [$F_{\text{test-day treatment}}$ (1, 21) = 69.71, P<0.0001]; females [$F_{\text{test-day treatment}}$ (1, 21) = 39.91, P<0.0001], ae late phase: males [$F_{\text{test-day treatment}}$ (1, 48) = 42.27, P<0.0001]; females [$F_{\text{test-day treatment}}$ (1, 51) = 132.0, P<0.0001], AB early phase: males [$F_{\text{test-day treatment}}$ (1, 45) = 158.4, P<0.0001]; females [$F_{\text{test-day treatment}}$ (1, 48) = 111.4, P<0.0001], AB late phase: males [$F_{\text{test-day treatment}}$ (1, 45) = 18.30, P<0.0001]; females [$F_{\text{test-day treatment}}$ (1, 42) = 21.90, P<0.0001]). Additionally, sex did not impact total distance traveled [ae cohort: early phase (F_{sex} (1, 14) = 0.02105, P=0.8867); late phase (F_{sex} (1, 33) = 2.210, P=0.1466); AB cohort: early phase (F_{sex} (1, 31) = 2.604, P=0.1167), late phase (F_{sex} (1, 29) = 2.622, P=0.1162), by 3-way ANOVA on total distance, data not shown]. Within each sex, three-way RM ANOVA were used to assess test-day treatment, previous binge treatment, and time on locomotor activity (see Table 1). At the early phase, ethanol-induced locomotion was depressed in binge ethanol adolescent males as compared to control males (treatment*test-day treatment interaction [$F_{\text{interaction}}$ (1, 21) = 19.38, P=0.0002]; Fig. 6A). Additionally, a time*test-day treatment interaction [$F_{\text{interaction}}$ (2, 21) = 4.406, P=0.0252] was found in the adolescent-exposed males. Tukey's post hoc revealed that control males given acute ethanol traveled more than control males given saline at 10 & 15 minutes and ethanol males given acute ethanol at 15 minutes. In contrast, adolescent-exposed females were not significantly affected by adolescent binge treatment [$F_{\text{treatment}}$ (1, 21) = 0.7761, P=0.3883] (Fig. 6B). Both males and females showed

a main effect of time (males [F_{time} (2, 21) = 20.04, P<0.0001]; females [F_{time} (2, 21) = 6.605, P=0.0060].

In adult-exposed females at the early phase, a three-way RM ANOVA revealed a significant difference due to adult binge treatment [$F_{treatment}$ (1, 48) = 10.03, P=0.0027] (Fig. 6D). A time*test-day treatment interaction [$F_{interaction}$ (2, 48) = 3.234, P=0.0481] was found where females given acute ethanol traveled farther than their saline counterparts at 10 & 15 minutes. Meanwhile, in males, an adult treatment*test-day treatment interaction [$F_{interaction}$ (1, 45) = 7.938, P=0.0072] (Fig. 6C) was observed with mice given ethanol on test-day traveling further than their saline counterparts at 5, 10, and 15 minutes.

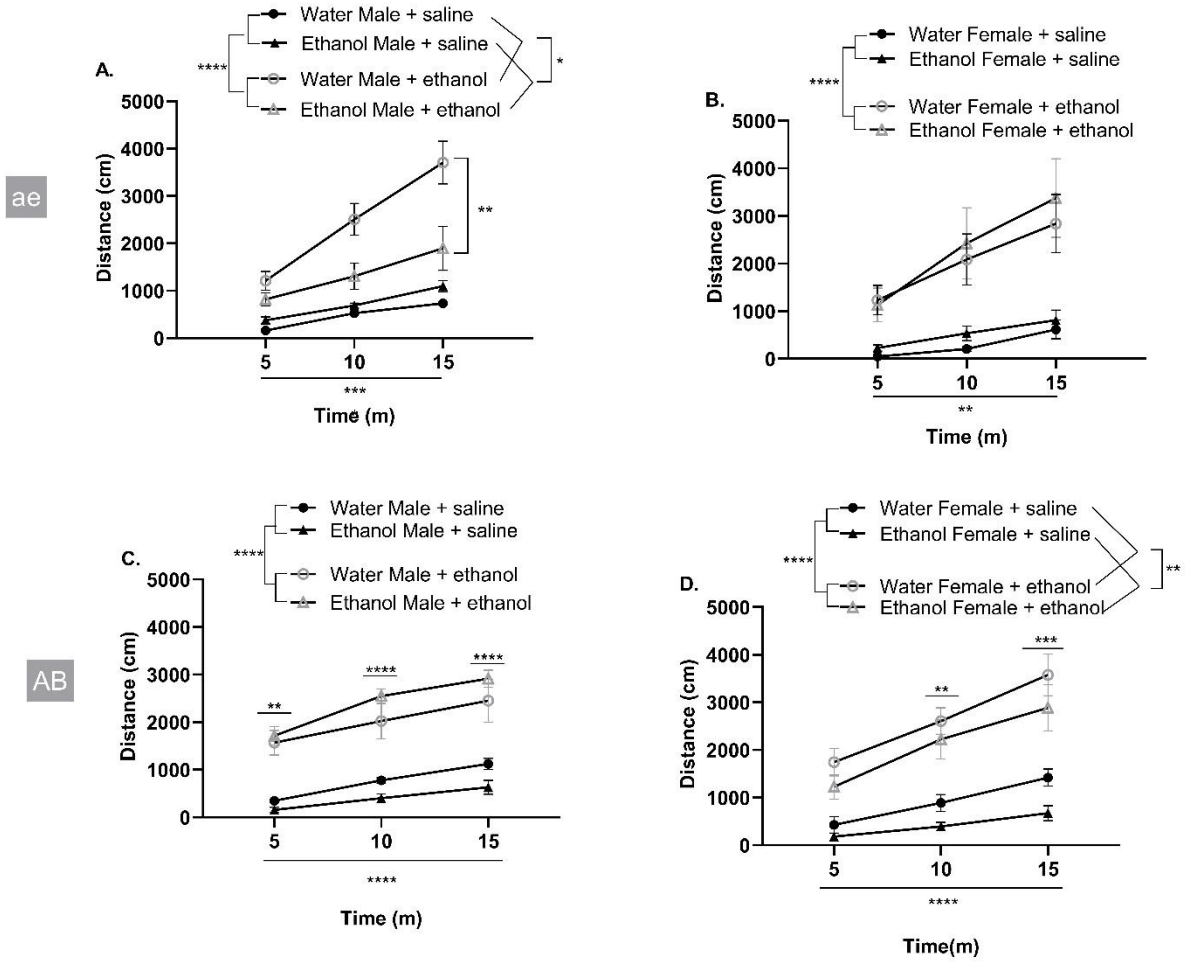

Fig 6. Adolescent males and adult females show locomotor tolerance at the early phase. At the early phase ethanol-induced cumulative distance was impacted by A) adolescent treatment in males and test-day treatment (n: CM=5, EM=6). B) Females also showed locomotor differences due to test-day treatment (n: CF=5, EF=6). Adult mice revealed that test-day treatment impacted C) male distance (n: CM=10, EM=9). D) Adult female distance (CF=10, EF=10) was significantly impacted by adult treatment and test-day treatment. Data is presented as mean +/- SEM. (**p≤0.01, *** p≤0.001, ****p≤0.0001)

2.3.4 Persistently, only adult females display a locomotor effect due to a history of ethanol.

As seen in the early phase for ethanol-induced locomotion, in all cases, acute ethanol (2 g/kg, ip) increased locomotor activity (Table 1). At the late phase, adolescent-exposed mice showed increased locomotion due to time (males [F_{time} (2, 48) = 11.84, P<0.0001]; females [F_{time} (2, 51) = 21.81, P<0.0001]; Fig. 7 A&B). Additionally, a time*test-day treatment interaction [$F_{interaction}$ (2, 51) = 4.028, P=0.0238] was found in adolescent-exposed females with test-day ethanol administration increasing total distance at 10 & 15 minutes compared to saline. Meanwhile, adult females with a history of binge ethanol showed locomotor tolerance to acute ethanol as compared to water control mice (Fig. 7D). A main effect of adult binge treatment [$F_{treatment}$ (1, 42) = 4.741, P=0.0351], and an adult treatment*test-day treatment interaction [$F_{interaction}$ (1, 42) = 4.756, P=0.0348] were found in females. Adult males showed no ethanol effect [$F_{treatment}$ (1, 45) = 3.835, P=0.0564] but a significant main effect of time [F_{time} (2, 45) = 20.32, P<0.0001] (Fig. 7C).

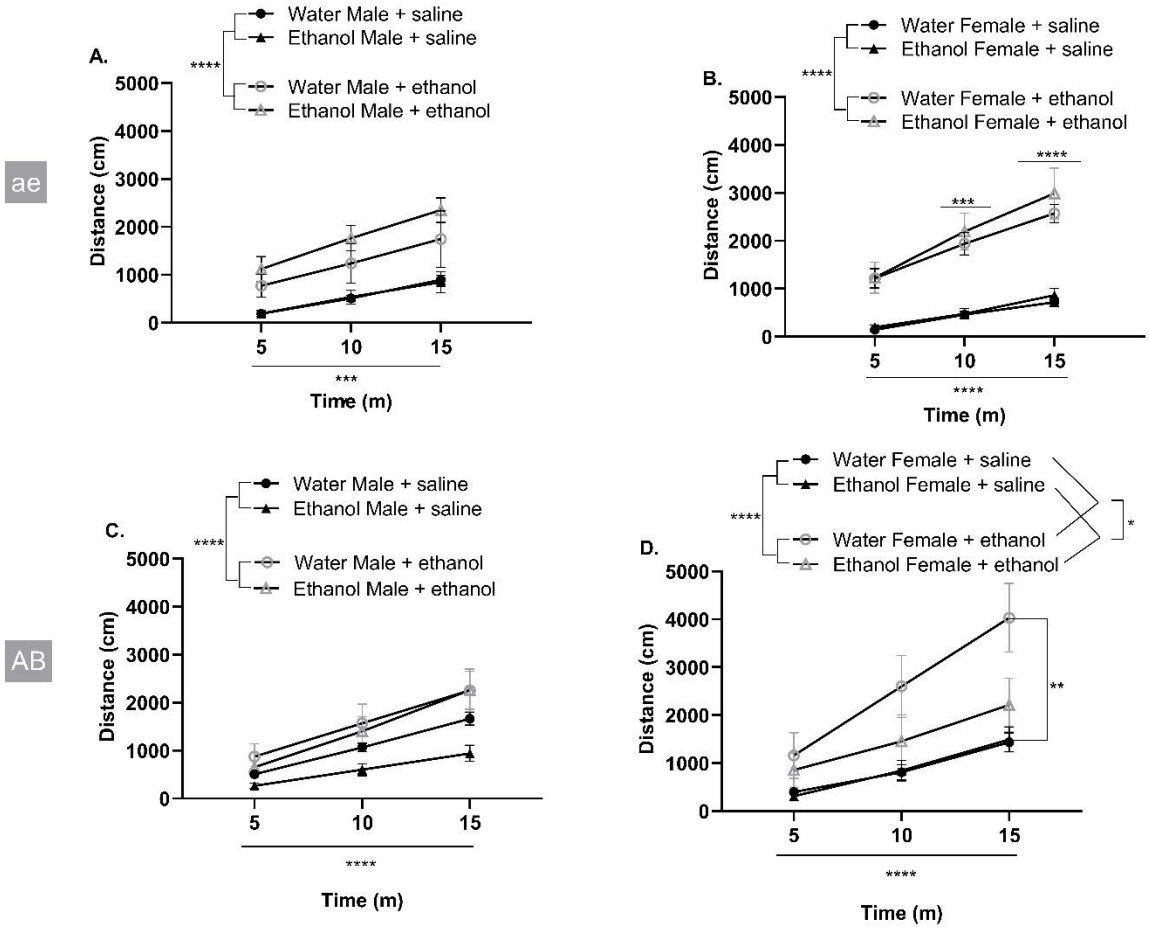

Fig 7. Adult females with a history of binge ethanol display locomotor tolerance.

Adolescent-exposed mice at the late phase were revealed test-day treatment impacted cumulative distance in A) males (n: CM=12, EM=8) and B) females (CF=11, EF=10). C) Male locomotor distance (n: CM=10, EM=9) was increased by acute test-day ethanol. D) Adult females previously exposed to ethanol displayed locomotor tolerance to acute ethanol. Test-day treatment also impacted female cumulative distance (CF=10, EF=8). Data is presented as mean +/- SEM. (**p≤0.01, *** p≤0.001, ****p≤0.0001)

Table 1. Locomotor distance in the ethanol-induced locomotion task was significantly impacted by time and test day treatment in both age cohorts.

Ethanol-induced locomotion Cumulative Distance									
		Early Phase				Late Phase			
		Males		Females		Males		Females	
Table 1	Factor	F (DFn, DFd)	P value	F (DFn, DFd)	P value	F (DFn, DFd)	P value	F (DFn, DFd)	P value
ae Cohort	**Time**	F (2, 21) = 20.04	**P<0.0001**	F (2, 21) = 6.605	**P=0.0060**	F (2, 48) = 11.84	**P<0.0001**	F (2, 51) = 21.81	**P<0.0001**
	Adolescent Treatment	F (1, 21) = 7.991	**P=0.0101**	F (1, 21) = 0.7761	NS P=0.3883	F (1, 48) = 2.668	NS P=0.1089	F (1, 51) = 1.247	NS P=0.2693
	Test Day Treatment	F (1, 21) = 69.71	**P<0.0001**	F (1, 21) = 39.91	**P<0.0001**	F (1, 48) = 42.27	**P<0.0001**	F (1, 51) = 132.0	**P<0.0001**
	Time x Adolescent Treatment	F (2, 21) = 1.438	NS P=0.2598	F (2, 21) = 0.1344	NS P=0.8750	F (2, 48) = 0.05066	NS P=0.9507	F (2, 51) = 0.2839	NS P=0.7540
	Time x Test Day Treatment	F (2, 21) = 4.406	**P=0.0252**	F (2, 21) = 1.965	NS P=0.1650	F (2, 48) = 0.6614	NS P=0.5208	F (2, 51) = 4.028	**P=0.0238**
	Adolescent Treatment x Test Day Treatment	F (1, 21) = 19.38	**P=0.0002**	F (1, 21) = 0.002102	NS P=0.9639	F (1, 48) = 2.775	NS P=0.1023	F (1, 51) = 0.3336	NS P=0.5661
	Time x Adolescent Treatment x Test Day Treatment	F (2, 21) = 2.082	NS P=0.1497	F (2, 21) = 0.1027	NS P=0.9028	F (2, 48) = 0.08710	NS P=0.9167	F (2, 51) = 0.1312	NS P=0.8773
AB Cohort	**Time**	F (2, 45) = 13.92	**P<0.0001**	F (2, 48) = 19.55	**P<0.0001**	F (2, 45) = 20.32	**P<0.0001**	F (2, 42) = 13.92	**P<0.0001**
	Adult Treatment	F (1, 45) = 0.01056	NS P=0.9186	F (1, 48) = 10.03	**P=0.0027**	F (1, 45) = 3.835	**P=0.0564**	F (1, 42) = 4.741	**P=0.0351**
	Test Day Treatment	F (1, 45) = 158.4	**P<0.0001**	F (1, 48) = 111.4	**P<0.0001**	F (1, 45) = 18.30	**P<0.0001**	F (1, 42) = 21.90	**P<0.0001**
	Time x Adult Treatment	F (2, 45) = 0.06175	NS P=0.9402	F (2, 48) = 0.4144	NS P=0.6631	F (2, 45) = 0.06017	NS P=0.9417	F (2, 42) = 0.6238	NS P=0.5408
	Time x Test Day Treatment	F (2, 45) = 0.9220	NS P=0.4051	F (2, 48) = 3.234	**P=0.0481**	F (2, 45) = 1.158	NS P=0.3232	F (2, 42) = 1.353	NS P=0.2694
	Adult Treatment x Test Day Treatment	F (1, 45) = 7.938	**P=0.0072**	F (1, 48) = 0.01201	NS P=0.9132	F (1, 45) = 1.266	NS P=0.2664	F (1, 42) = 4.756	**P=0.0348**
	Time x Adult Treatment x Test Day Treatment	F (2, 45) = 0.5816	NS P=0.5632	F (2, 48) = 0.1358	NS P=0.8734	F (2, 45) = 0.4440	NS P=0.6442	F (2, 42) = 0.9268	NS P=0.4038

2.3.5 Ethanol sensitivity in the LORR task is altered by age and sex, but not prior ethanol exposure.

In mice that experienced LORR, females, regardless of treatment, showed decreased sensitivity to the sedative effects of ethanol as compared to their male counterparts. In adolescent-exposed mice tested at the early phase, LORR onset differed in males and females with females showing a faster onset [F_{sex} (1, 36) = 5.468, P=0.0250] (Table 2). Binge ethanol did not affect LORR onset in adolescent mice [$F_{treatment}$ (1, 36) = 2.771, P=0.1047]. No difference in LORR onset was observed in the adult-exposed group ($F_{treatment}$ (1, 28) = 0.9183, P=0.3461, F_{sex} (1, 28) = 0.05003, P=0.8246, Table 2). LORR duration was also significantly impacted by sex with adolescent-exposed females spending less time sedated than males [F_{sex} (1, 36) = 6.477, P=0.0154] (Fig. 8A). Adolescent ethanol exposure did not alter LORR duration [$F_{treatment}$ (1, 36) = 0.1265, P=0.7241]. Similarly, LORR duration was decreased in adult females [F_{sex} (1, 28) = 12.85, P=0.0013] as compared to males, but binge ethanol had no significant effect [$F_{treatment}$ (1, 28) = 0.09634, P=0.7586, Fig. 8C).

When tested at the late phase, LORR onset did not differ due to sex (ae [F_{sex} (1, 35) = 2.488, P=0.1237]; AB[F_{sex} (1, 29) = 1.661, P=0.2077]; Table 1) or treatment (ae [$F_{treatment}$ (1, 35) = 0.2003, P=0.6573]; AB[$F_{treatment}$ (1, 29) = 0.05097, P=0.8230]) in either age cohort. Similar to the early phase, LORR duration was impacted by sex in the adolescent-exposed mice at PND 74/75 [F_{sex} (1, 35) = 9.671, P=0.0037], but not by treatment [$F_{treatment}$ (1, 35) = 0.06280, P=0.8036] (Fig. 8B). Likewise, adult-exposed mice at PND 111 showed a main effect of sex [F_{sex} (1, 29) = 25.36, P<0.0001], but not treatment [$F_{treatment}$ (1, 29) = 0.6860, P=0.4143] (Fig. 8D) for LORR duration.

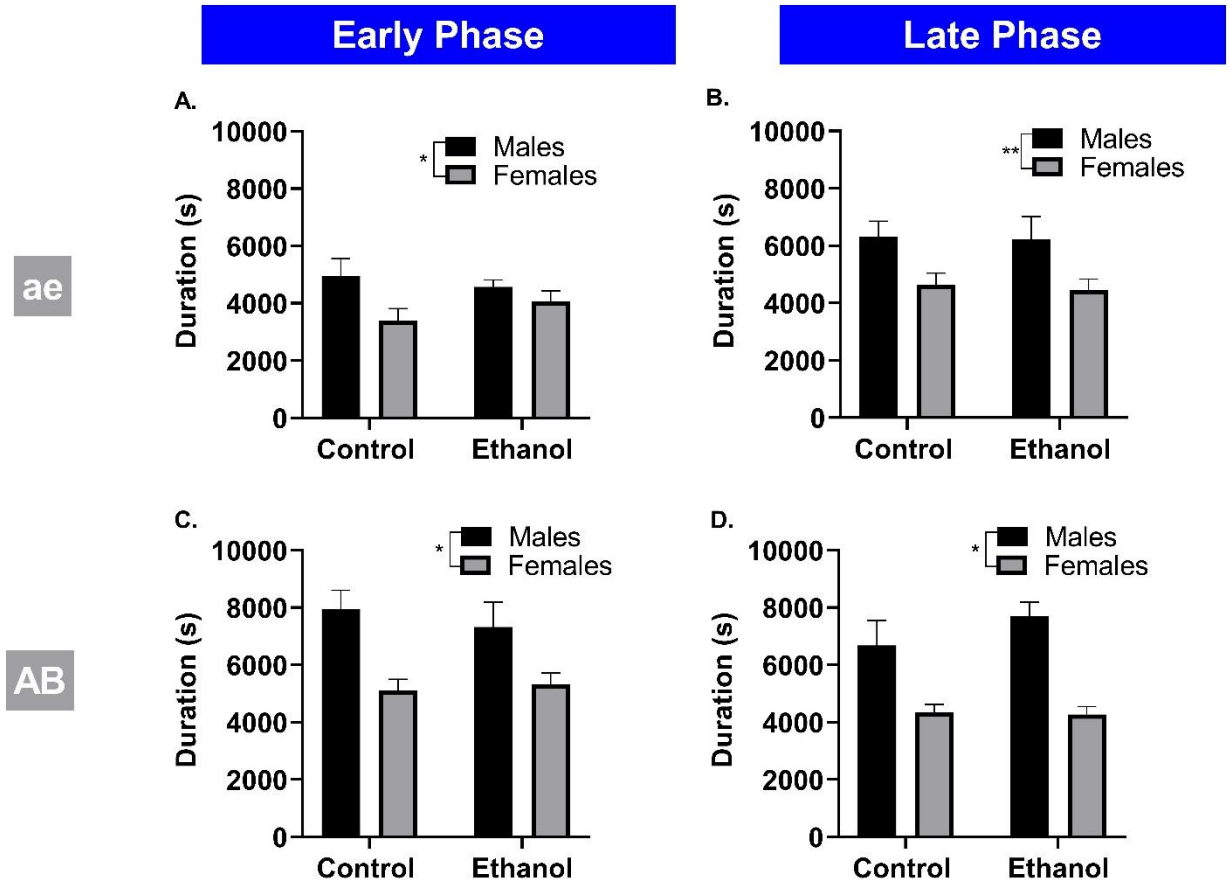

Fig 8. Ethanol sedation is altered by sex, but not history of ethanol. At the early phase, LORR duration was impacted by sex in A) adolescent-exposed (n: CM=8, EM=11, CF=10, EF=11) and C) adult-exposed mice with females waking earlier than males (n: CM=9, EM=9, CF=9, EF=8). The late phase also revealed sex differences in LORR duration in B) adolescent-exposed (n: CM=12, EM=8, CF=11, EF=8) and D) adult-exposed mice (n: CM=9, EM=8, CF=9, EF=7). Data is presented as mean +/- SEM. (*$p \leq 0.05$, ** $p \leq 0.01$).

Table 2. LORR onset was impacted by sex at the early phase in the ae cohort.

Table 2		LORR Onset	
		Ethanol vs Control	
		Early Phase	Late Phase
ae Cohort	Ethanol Effect	NS P=0.1047	NS P=0.6573
	Sex Effect	**M > F P=0.0250**	NS P=0.1237
AB Cohort	Ethanol Effect	NS P=0.3461	NS P=0.8230
	Sex Effect	NS P=0.8246	NS P=0.2077

2.3.6 Blood ethanol differs due to age but not sex

Changes in blood ethanol concentration were assessed over time and due to age and sex. Following analysis with a three-way ANOVA, plasma levels showed a significant timepoint*age interaction ($F_{interaction}$ (3, 60) = 9.781 P<0.0001). However, no meaningful timepoint*age interactions were significant following Tukey's post hoc test (Fig. 9A). A Tukey multiple comparison test revealed an effect of time within an age with adolescent females at 0.5 hours differed significantly from the 4-hour timepoint (P = 0.0024). Adult females also showed a significant difference between their 1 and 4-hour timepoint (P<0.0001) and their 2 and 4 hour timepoint (P = 0.0005). Additionally, the AUC was assessed to determine if age and sex impacted the quantity of ethanol and length of time ethanol was in the blood. A one-way ANOVA revealed a significant difference between the 4 groups (adult males, adult females, adolescent males, and adolescent females) (F_{group} (3, 20) = 8.348 P=0.0009), with adults showing a larger AUC than adolescents (Fig. 9B). Within each age group, males and females showed a similar AUC.

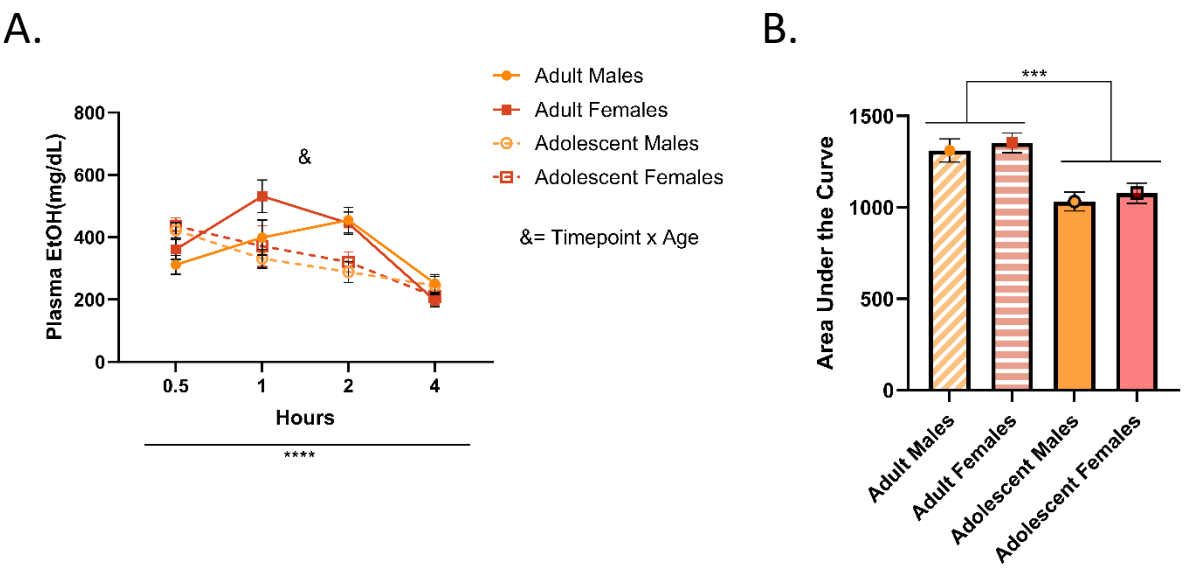

Fig 9. Blood ethanol concentration is higher in naïve adults than adolescents. A) A four hour time course showed BEC changed due to time and age ($\&p < 0.0001$) (n=6/group). Further analysis of AUC showed the quantity and duration of ethanol in blood differed by age with adults having a higher and prolonged BEC than adolescents (*** $p < 0.001$).

2.3.7 Binge ethanol during adulthood does not robustly alter myelin gene expression.

We previously reported that binge ethanol during adolescence (PND 29-42) dramatically reduced expression of the myelin-related genes, *Mag*, *Mbp*, *Mobp*, and *Plp*, 24 hours after the last ethanol dose [63]. Sex also influenced adolescent expression levels of *Mag*, *Mal*, and *Mbp* with females having lower expression levels than males. Three weeks after the last ethanol dose, myelin gene expression returned to control levels [63] and sex differences were no longer observed. Here, we asked whether our binge ethanol paradigm also altered PFC myelin gene expression in adult mice. Twenty-four hours following binge ethanol in adulthood (PND 70-83), only one myelin-related gene was affected by ethanol. Two-way ANOVA revealed a main effect of treatment for *Mal* expression which was decreased in ethanol-exposed adult mice [$F_{treatment}$ (1, 27) = 4.599, P=0.0411] (Fig. 10A). No other genes differed due to treatment (*Mag* [$F_{treatment}$ (1, 27) = 3.626, P=0.0676]; *Mbp* [$F_{treatment}$ (1, 27) = 3.629, P=0.0675]; *Mobp* [$F_{treatment}$ (1, 27) = 3.297, P=0.0805]; *Plp* [$F_{treatment}$ (1, 27) = 2.633, P=0.1163]). Meanwhile, *Mag* expression was lower in adult females as compared to males, similar to our findings in adolescents [F_{sex} (1, 27) = 5.758, P=0.0236] (Fig. 10A). No other genes were changed due to sex (*Mal* [F_{sex} (1, 27) = 0.1251, P=0.7263]; *Mbp* [F_{sex} (1, 27) = 2.007, P=0.1680]; *Mobp* [F_{sex} (1, 27) = 0.1465, P=0.7049]; *Plp* [F_{sex} (1, 27) = 0.002267, P=0.9624]). No changes in gene expression were observed 3 weeks following binge exposure (*Mag* [$F_{treatment}$ (1, 26) = 0.4143, P=0.5254]; *Mal* [$F_{treatment}$ (1, 26) = 0.7026, P=0.4095]; *Mbp* [$F_{treatment}$ (1, 26) = 0.7785, P=0.3857]; *Mobp* [$F_{treatment}$ (1, 26) = 0.9131, P=0.3481]; *Plp* [$F_{treatment}$ (1, 26) = 1.329, P=0.2595]), or differed by sex (*Mag* [F_{sex} (1, 26) = 1.060, P=0.3127]; *Mal* [F_{sex} (1, 26) = 0.02254, P=0.8818]; *Mbp* [F_{sex} (1, 26) = 1.105, P=0.3027]; *Mobp* [F_{sex} (1, 26) = 0.1196, P=0.7323]; *Plp* [F_{sex} (1, 26) = 0.2993, P=0.5890]; Fig. 10B).

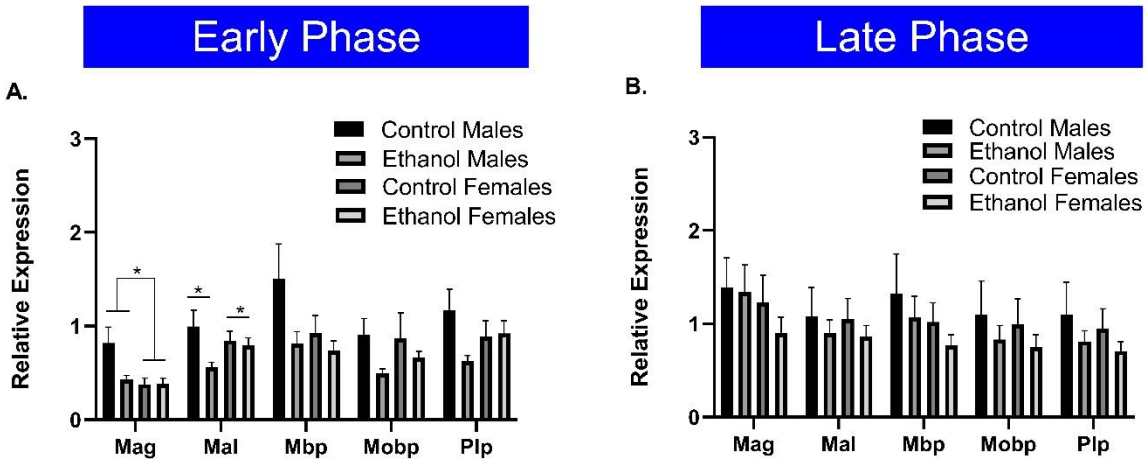

Fig 10. Binge ethanol in adulthood did not alter myelin-related gene expression. A) Twenty-four hours after the last ethanol dose, only *Mal* showed significant decreased mRNA expression due to ethanol (n: CM=8, EM=6, CF=8, EF=7, *p $\leq$0.05). Meanwhile *Mag* showed lower expression in females compared to males regardless of treatment (*p$\leq$0.05). B) Three weeks after the last dose, no differences in mRNA expression were observed between control and ethanol treated mice or between sexes (n: CM=8, EM=8, CF=8, EF=6). Data is presented as mean +/- SEM.

2.4 Discussion

Prolonged and binge ethanol consumption in adolescence leads to decreased myelination [87], impaired structural integrity of white matter in the frontal cortex [89], memory impairments [233], and tolerance in tasks when given an ethanol challenge [10] in humans. Similar changes in white matter [92] and memory [16], [160], [234] are also seen in rodents following binge ethanol in adolescence. Sex impacts BEC following oral administration leading to higher ethanol concentrations in females in humans [216] and rodents [26]. While both adolescents and adults consume ethanol, the ages are differently impacted by the drug [14], [16], [18]. The current study found that the same ethanol dosing paradigm in adult and adolescent mice resulted in different outcomes when assessed for memory, ethanol-induced locomotion, sociability, and myelin gene expression. Meanwhile, BEC in ethanol naïve mice was impacted by age, but not sex. Together, our study suggests adolescent binge ethanol, despite lower and shorter duration of BEC, leads to more robust behavioral and molecular changes than adult ethanol exposure and some of these persist long after ethanol is removed. A summary of the behavioral results following binge ethanol exposure can be found in Table 3.

Table 3. Summary of chapter 2 experimental results including the time and number of animals when the tests were conducted.

		Early Phase			Late Phase		
Table 3	**Test Type**	**Age on Test Day**	**Main Effect of Treatment**	**Main Effect of Sex**	**Age on Test Day**	**Main Effect of Treatment**	**Main Effect of Sex**
ae Cohort	Social Interaction	PND 43	NS	NS	PND 63/64	NS	NS
	NOR	PND 47	NS	NS	PND 68	Ethanol < Control	NS
	Ethanol-induced locomotion	PND 49/50	Males: ethanol <control Females: NS	NS	PND 70/71	NS	NS
	LORR	PND 52/54	NS	M>F	PND 74/75	NS	Duration: M>F Onset: NS
AB Cohort	Social Interaction	PND 78	Total Distance: NS Time in Corners: ethanol > control Time in Interaction Zone: ethanol < control	NS	PND 98	NS	Total Distance: M>F Time in Corners or Interaction Zone: NS
	NOR	PND 80	NS	NS	PND 100/101	NS	NS
	Ethanol-induced locomotion	PND 83	Males: NS Females: ethanol < control	NS	PND 105	Males: NS Females: ethanol < control	NS
	LORR	PND 85	NS	Duration: M>F Onset: NS	PND 111	NS	Duration: M>F Onset: NS

The NOR task, which measures short-term recognition memory, revealed a persistent deficit in mice treated with ethanol in adolescence which was not present in mice treated in adulthood (Fig. 5). Similar deficits are reported in the literature where ethanol exposure in adolescence more severely impacted NOR memory than adult exposure [16], [235], but not always [236]. Lacaille [235] found that acute binge ethanol (2-2.5 g/kg, I.P.) did not alter adolescent or adult memory, but repeated binging in adolescence (PND 30-45) impaired NOR short-term memory, suggesting repeated ethanol exposure impairs memory. Intriguingly, both studies found a deficit in NOR memory when tested at PND 45, a similar timepoint as our study that found no changes in adolescent memory (Fig. 5). The difference between these studies may be due to differences in mouse strain and route of administration. Oral gavage and I.P. ethanol administration can cause different levels of inflammatory markers in the brain [237], possibly leading to differences in memory tasks. Conflicting results are observed in NOR performance following adult ethanol. While some studies, including our own, show no impairment in adult memory [16], [235], others show adult memory impairments [236]. This difference is likely due to the ethanol paradigm used by Cippitelli [236] where ethanol was administered several times throughout the day via gavage and liquid diet, leading to a total of 9-15 g/kg of ethanol per day. While adolescents utilize the same brain regions as adults in recognition memory tasks, fMRI studies show changes in activity during adolescence. While activation is often associated with successful memory task performance, deactivation of certain brain regions also occurs with correct performance [238], [239]. During recognition memory, deactivation of the mPFC is greater in those 13-24 years than 8-12 years, while adolescents (13-17 years) show less deactivation in parietal cortices compared to young adults (18-24 years) [238]. The literature suggests a shift in brain region deactivation occurs with age. Together, our memory data suggests

that adolescents are more sensitive to the lasting effects of ethanol-induced memory impairments than adults and this is likely due to ethanol impacting the developing brain including the frontal cortex.

Age of ethanol exposure also significantly impacted performance in the social interaction task and in ethanol-induced locomotion. A history of binge ethanol did not alter the amount of time spent engaging in social interaction in adolescent mice. However, adult mice with a history of binge ethanol dramatically decreased their sociability when tested twenty-four hours after the last ethanol dose. Indeed, adolescent rats are less sensitive to ethanol-induced social inhibition than adults [240] and our findings in DBA/2J mice are in line with these previous studies in rats, when tested twenty-four hours after the last dose. When adults were tested three weeks later, these differences disappeared, which could be due to the waning effects of ethanol withdrawal, or due to the lower overall social interaction in older (PND 98) adult mice. While few studies address age differences within a month range but in the same developmental phase, Shoji [241] found age differences in active social contact, where 2 to 3 month-old mice show greater social contact compared to all other age groups. These studies further support the age-dependent ethanol differences in our data (Fig. 4), and suggest that young adult animals may maintain adolescent-like increases in social interaction. Adolescent mice are also less sensitive to the locomotor sensitizing effects of ethanol [242]. Here, we show that a history of binge ethanol led to persistent tolerance in ethanol-induced locomotion, but only in adult female mice. Overall, these effects could be due to differences in ethanol pharmacokinetics between age groups, as younger adolescent rodents previously showed lower BEC at the same time point as adults [14], suggesting that adolescents develop acute/within-session ethanol tolerance allowing for attenuated sensitivity to the aversive effects of ethanol [243]. Indeed, our BEC study (Fig. 9B)

found adolescent mice had a lower duration and quantity than adults. The present study did not find treatment effects on LORR duration (Fig. 8) within the adolescent-exposed cohort which is contrary to those observed in our previous study [63], in which mice given ethanol in adolescence had a greater LORR duration when tested in adulthood as compared to their controls. These differences may be due to the mice in this study being older at both LORR time points (PND 74/74 & 111) than the previous paper (PND 64). However, both studies did find a sex difference at the later time point with females showing lower LORR duration than males, which may be due to pharmacokinetic differences (discussed below). Together these studies illustrate that developmental age and age when administered ethanol impacts many different behavioral tasks.

Blood ethanol concentration and duration were also impacted by age, but not sex, within ethanol naïve animals showing adolescent mice had a lower BEC and smaller AUC than adults (Fig. 9A & B). This is the first study to our knowledge that assessed BEC in adolescents and adults of both sexes following oral administration. Our data is in line with other literature showing no age effect at 30 minutes but an age effect at 2 hours when compared at individual timepoints as in other studies (data not shown)[14], [244]. Age differences in ethanol concentration may be due to differences in gonadal hormones. In males, castration increased ADH activity in the liver [245], while in females, administration of estradiol decreased liver ADH activity [246]. As our adolescent mice were still undergoing puberty at the time of testing (PND 35) they likely had lower levels of gonadal hormones than adults. Therefore, the adolescent mice could have higher ADH activity in the liver than their adult counterparts leading to lower BECs. However, several studies have found no age differences in BEC following an I.P. route of administration which bypasses gastric first-pass metabolism [16], [209], [213]–[215]. As

the adolescent developmental window includes increased metabolism and growth**,** gastric metabolism may be increased leading to an effect in our data that would not be observed with different routes of administration. Furthermore, rodent strain impacts BEC, gastric, and liver ADH activity with DBA/2J mice displaying higher gastric ADH activity than C57BL/6J mice [26] but lower hepatic ADH activity [247]. Our data did not show a sex difference in BEC in adolescents or adults. This is in line with Melon and Boehm [215] where no sex effects were observed in DBA/2J following I.P administration. However, human studies do show a sex difference in BEC and AUC following oral administration [33], [216]. Rodent studies also observe a sex difference with females showing lower BEC than males when ethanol is administered I.P. [26], [209], [248]. However, following oral gavage DBA/2J adult females display a higher BEC than males [26]. This sex difference based on the route of administration is likely due to sex differences in ADH activity in the stomach and liver. DBA/2J adult females have higher hepatic ADH [247] but lower gastric ADH activity than males[26]. Therefore, when female DBA/2J mice are given ethanol via I.P., increased ADH activity in the liver leads to increased ethanol metabolism and decreased BEC. This likely contributed to the decreased sedation observed in our LORR task (Fig. 8) As ADH activity also differs by strain, sex differences between studies may also vary. Therefore, differences in age and sex effects between studies may be due to the rodent strain used, route of administration, and timeframe until blood collection. Additionally, the age difference in BEC could be due to age difference in ethanol absorption with adult animals showing an increase in BEC hitting a peak at 1 hour in females and 2 hours in males, whereas adolescents peak at 30 minutes. This may be due to increased metabolism in adolescents that led to decreased stomach contents which can impact ethanol absorption and BEC. This is the first data to our knowledge that suggest ethanol absorption

differs by age which may be due to some studies only looking at BEC at a single timepoint [26], [209], [215]. Meanwhile, other studies have looked over a time course [14], [248] but found no differences in BEC possibly due to difference in prior ethanol exposure or strain of rodent. Our data shows that the adolescent brain is exposed to lower levels of ethanol for a shorter timeframe which could be due to hormonal differences between adolescents and adults. Sex does not impact BEC in DBA/2J mice following oral administration in our model.

To further understand age-dependent ethanol effects within the brain, we measured myelin-related gene expression in adult PFC following binge ethanol. Using the same dosing paradigm, we previously found that adolescent binge ethanol dramatically decreased the expression of several myelin-related genes in the PFC twenty-four hours after the last dose [63]. Here, we show that myelin-related gene expression was relatively insensitive to adult binge ethanol. Adult-exposed mice showed only 1 gene decreased due to ethanol (*Mal*) which was observed in male mice (Fig. 10). *Mal* is a gene observed in mature oligodendrocytes and is integral for myelin integrity in the CNS [227]. The other myelin-related genes in which no differences were observed are involved in myelin formation (*Mbp* & *Plp*) [225], axon-glia interaction (*Mag*) [249], and oligodendrocyte morphology and extension of processes (*Mobp)* [228]. Meanwhile, sex impacted *Mag* expression resulting in decreased expression in females compared to males. When myelin levels were assessed persistently after binge ethanol, no differences were observed in either age group (Fig. 10) [63]. This data further suggests that adolescents are more sensitive to ethanol-induced changes than adults, at least in the PFC. As myelination increases during adolescence [81], [82] but not adulthood, ethanol likely disrupts the typical brain white matter maturation process. Indeed, other studies found disruptions in white matter integrity or volume following adolescent ethanol intake [88], [89], [92], [202]. However,

remyelination following short periods without ethanol was observed in rodents [92]. While our study did not address remyelination, our data suggests brief ethanol binges will not severely alter white matter in a developed adult brain. While this study utilized PFC, including prelimbic and infralimbic regions, future directions may focus on a particular mPFC region in relation to ethanol changes in behavior.

This study was limited by the number of personal able to run the experiment and lack of qPCR data in the adolescent exposed cohort. The behavioral studies were separated by age cohort with behavioral testing occurring at separate times. Meanwhile the myelin related gene expression was only run in the adult exposed cohort as a pilot study. Future studies could increase the number of people and locomotor boxes to allow both aged cohorts to be tested at the same time. Additionally, the myelin related gene expression could be conducted in both age cohorts again at the same time to reproduce the data and directly compare the cohorts.

2.5 Conclusion

This study found that adolescent binge ethanol more severely impacted memory and PFC myelination compared to adult binge ethanol. Future studies are needed to address the role of remyelination due to the factors of ethanol and age. Developmental age impacted every behavioral task. Additionally, sex impacted ethanol sedation and may impact ethanol-induced locomotion, although underlying mechanistic sex differences were not specifically evaluated. Furthermore, age but not sex impacted blood ethanol duration and quantity in ethanol naïve following oral administration. Studies addressing the molecular or mechanistic differences underlying age and ethanol-related differences are currently being explored. Gene ontology analysis from our previous study [63] suggested that transcription factor activity was negatively impacted by adolescent ethanol. Future studies may address differences in transcription factor

activity due to age and treatment. In conclusion, this study found age and prior ethanol treatment persistently impacted memory and ethanol sedation while both cohorts were in adulthood.

Chapter 3

Binge ethanol exposure impairs spatial memory in an age and sex specific manner

3.1 Introduction

Alcohol negatively impacts memory formation following acute [235], [250] and chronic use [16], [160], [235], [236], [251]–[253]. Developmental age also impacts the effect of alcohol on memory with some studies finding a memory deficit in adolescent mice, but not adult mice following the same ethanol exposure [16], [17], [235], [254]. Meanwhile, a study with only adult rodents show memory impairments from ethanol [236]. Spatial learning and memory are important cognitive domains that are impaired persistently by ethanol in adolescents [251]–[253] and adults [236]. However, conflicting data does show no ethanol effect on spatial learning in some studies [18], [160], [162], [163], [255], [256]. Meanwhile, cognitive flexibility, the ability to update behavior based on new information, also shows conflicting results with some studies finding an impairment following a history of adolescent ethanol [160], [162], [163] or adult ethanol exposure [162],while others find no effect [244]. Sex may also differentially impact spatial memory performance with some studies finding increased locomotion in females [257], [258][259], but other studies finding improved performance in males[152], [260]. Overall conflicting data exists regarding persistent ethanol effects and sex effects on spatial learning, spatial memory, and cognitive flexibility. As the adolescent brain is still developing, ethanol

exposure during this period may more negatively impact the aforementioned behaviors than adult ethanol exposure.

The Barnes Maze is a spatial learning and memory task that can be adapted to also assess cognitive flexibility [261]. The test is less stressful than the MWM, but behavioral testing in the open field, elevated plus maze, and Barnes Maze all increase corticosterone (CORT) levels [262], [263]. Aversive stimuli like fans, noises, or bright lights can be used to motivate the performance of the task [261]. The hypothalamus-pituitary-adrenal (HPA) axis is a negative feedback loop that increases CORT in response to HPA axis activation [77]. Chronic ethanol in adolescence leads to decreased CORT basally in adulthood, but following a stressor, is elevated [264]. Chronic ethanol may also lead to an anxiety-like phenotype with some studies finding an anxiety-like phenotype in the open field task and elevated plus maze[160], [265] two weeks after ethanol exposure [160], [265]. Meanwhile, other studies find no ethanol effect in the elevated plus maze or light-dark box two to three weeks after ethanol exposure [16], [18], [63]. Sex also impacts anxiety-like behaviors based on the task, with sex differences appearing in some tasks but not others [266], [267]. Furthermore, anxiety and depressive-like behaviors are strain-specific [266], [267] with DBA/2J mice showing sex differences in open field test, but no sex effect in the elevated plus maze [267]. Therefore, chronic ethanol may negatively impact CORT levels during behavioral testing or result in an anxiety-like phenotype in the open field.

The purpose of this study was to assess sex, age, and the persistent impact of intermittent binge ethanol on spatial memory, cognitive flexibility, and basal anxiety. Adolescent exposed mice displayed ethanol effects during training with decreased latency and increased speed, yet spent less time in the target quadrant during the spatial probe test. No difference in cognitive flexibility was observed in the adolescent-exposed cohort. In the adult exposed cohort, sex, but

not ethanol exposure, impacted performance on the Barnes Maze task with females reaching the goal box sooner than males. No difference in basal anxiety was observed in the open-field task in either age group. Together, this data suggests developmental age of ethanol exposure differentially impacts spatial learning and the stressful environment of the Barnes Maze may contribute to performance differences between the age groups.

3.2 Materials and Methods

3.2.1 Animals and Binge Ethanol Paradigm

DBA2/J mice, males and females, were ordered from Jackson Laboratory (Bar Harbor, ME) arriving at PND 19-21 for experiments 1 & 3, PND 43 for experiment 2, and PND 50 for experiment 4. Mice were housed 4-5/cage with same sex conspecifics on a 15:9 light-dark cycle, due to an issue with light timer, for experiments 3 & 4 and a 12:12 light-dark cycle for experiments 1 & 2 with food and water available *ad libitum*. Both age cohorts underwent the same chronic intermittent ethanol paradigm [63], [268] with mice habituated to the oral gavage method with 2 days of 0.1% saccharine administration. Following habituation, mice were given 4g/kg ethanol or water via oral gavage for 2 days followed by 2 days of abstinence. The ethanol/water window for the adolescent and adult exposed cohort was PND 29-42 and PND 64-77 respectively, resulting in 4 groups per age cohort, CM, EM, CF, and EF (Fig.11). Behavioral testing was conducted 3 weeks after the last ethanol administration with investigators blind to the sex and treatment groups. In all cases, mice were habituated to the testing room each day for at least one hour before testing and behavioral equipment was cleaned with 70% ethanol between animals. All animal housing and care was conducted with the approval of the Virginia Commonwealth University IACUC Committee and in accordance with the NIH Guide for the Care and Use of Laboratory Animals [218].

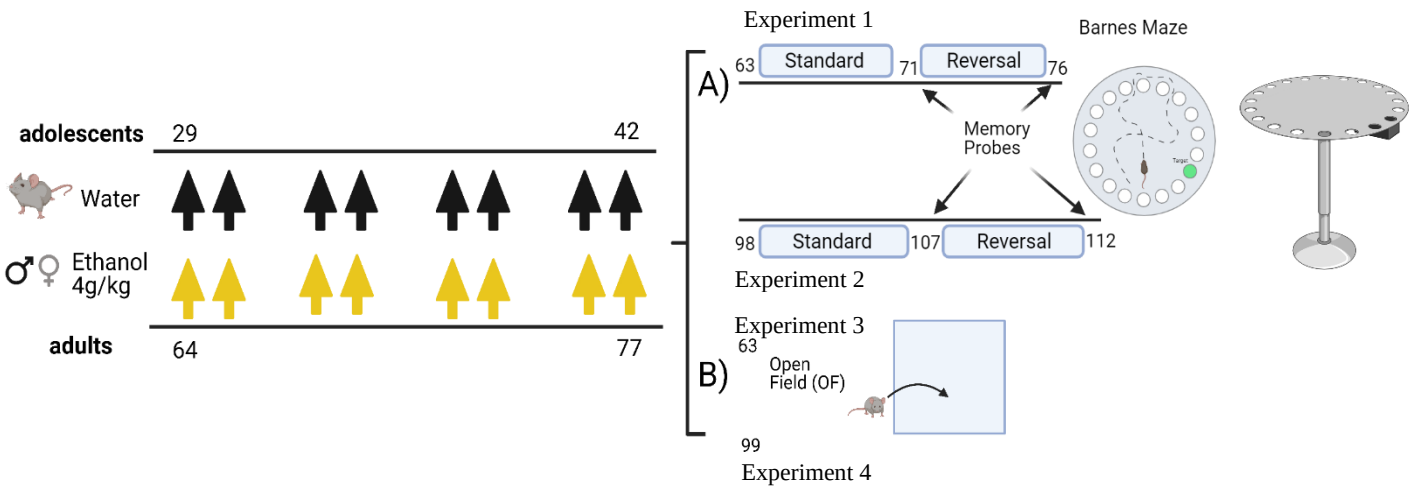

Fig 11. Timeline of Chapter 3 experimental procedure. Adolescent and adult DBA/2J mice underwent the same intermittent ethanol dosing paradigm from either postnatal (PND) 29-42 or PND 64-77. Three weeks following binge ethanol exposure, mice were assessed for behavioral changes in the open field and Barnes Maze tasks. A separate cohort of mice was used for each behavioral task. Figure created in Biorender.

3.2.2 Barnes Maze

The Barnes Maze task was adapted from [269] with adolescent exposed mice undergoing testing from PND 63-77 (n=34,6-10/group) while adult exposed mice (n=28, 7/group) were tested from PND 98-114. Mice were habituated to the testing room at least 1 hour prior to testing. The Barnes Maze was 95 cm high and 92 cm in diameter with 20 holes (5 cm in diameter) with 1 escape box (20cm x 10cm) and placed in a brightly lit room (3000 lux) with distinct cues on each wall. Mice were given 2 trials a day each 3 minutes long with an ITI of 5 minutes. On the first training day, the mouse was placed in a 150 mL glass beaker with a metal slab (16cm x 8cm) over the top, transported to the arena, and placed in the middle of the arena. The beaker was held in place for 15 seconds after which the beaker was removed and the mouse was freely able to explore the maze. The mouse was considered to have entered the goal box after it was no longer detected by the computer software (AnyMaze Software, Stoelting Co., Wood Dale, IL), at which the investigator turned off the room lights and placed a metal slab over the goal box entrance. The mouse remained in the goal box for 2 minutes and then was returned to its home cage for 3 minutes before starting the second trial. All objects and the maze were cleaned with 70% ethanol between each trial. On all subsequent training days, the beaker was immediately removed after the mouse was placed in the center of the maze. Mice were trained on the maze continuously until all groups reached a training criteria of latency $\leq$ 35 seconds and errors $\leq$ 5 to the goal box for 2 consecutive days. Once criteria were met on standard training, mice underwent a memory probe trial, twenty-four hours later, where the goal box was removed while the mouse spent 3 minutes on the maze. The next day, the goal box was moved 180 degrees and mice began reversal training as described above. After meeting criteria again, a reversal probe trial was performed. During standard and reversal training the latency, speed, and

distance to the goal box were measured by the AnyMaze Software. The number of errors, visits to a hole that was not the goal box, and search strategy during training was recorded by a blind investigator either live or via video playback. Search strategy categories were adapted from [270] and consisted of 3 strategies seen in Table 4. Search strategy was live scored or by video playback with an investigator blind to sex and treatment. Any trials which did not import data but had videos were re-analyzed with AnyMaze and an investigator blind to sex and treatment groups.

Table 4. Search Strategy categories and definitions

Table 4	
Search Strategy	**Definition**
Direct	Moving directly to goal box or adjacent holes prior to goal box or visiting less than 2 holes prior to goal box in a non-consecutive fashion
Serial	Visits 2 or more holes prior to goal box in a consecutive fashion
Random or Mixed	Moving in an unorganized fashion, or demonstrating a combination of search patterns

3.2.3 Open Field

The open field task was performed to assess anxiety-like behavior by measuring time spent in the center and periphery with increased time in the center associated with decreased anxiety [271]. An adolescent exposed (n= 25, 5-8/group) and adult exposed cohort (n=30, 6-8/group) were assessed three weeks after the last ethanol dose. Mice were habituated to the behavior room 1 hour prior to testing then placed in a square locomotor activity box (41 x 41 x 31 cm, Omnitech Electronics, Inc, Columbus, OH) with a center zone (25.63 x 25.63 x 31 cm) and periphery zone (41 x 7.69 x 31 cm). Mice were left undisturbed in the arena for 5 minutes and then returned to their home cage. One animal was excluded from the analysis due to technical issues during the test.

3.2.4 Statistics

All behavioral data was analyzed within each age cohort as the experiments were run at different times. Barnes Maze training data from the first trial was analyzed for latency, speed, distance, and errors via a three-way repeated measure ANOVAs with sex, treatment, and day as factors. Meanwhile, for probe day, two-way ANOVAs were performed within quadrant with treatment and sex as factors. Additionally, for reversal memory probe day, a three-way ANOVA was performed with quadrant, sex, and treatment as factors to assess time spend in the new target quadrant compared to the opposite quadrant. A mixed-effects model ANOVA was run if data was missing due to technical issues. The open field data was analyzed via two-way ANOVAs for duration, distance, and speed in the center and periphery zones. If significant interactions occurred in an ANOVA ($p<0.05$), a Tukey's post hoc test was performed. P-values less than $\alpha=0.05$ were considered significant. Behavioral data was analyzed with GraphPad Prism (GraphPad Software, San Diego, CA) with all data presented mean + standard error of the mean

(SEM). Search strategy was categorized into strategy type and analyzed via a Generalized Estimate Equation Poisson model with sex, treatment, and strategy as factors using the geepack package in R studio (Rstudio, PBC, Boston, MA URL http://www.rstudio.com/). Odds ratios (OR) and 95% confidence intervals (CI) were obtained using the "exp(cbind(OR=coef()" and "confint.default" function in R studio with statistical significance set at $p<0.05$ for all R studio analysis.

3.3 Results

3.3.1 Adolescent ethanol altered standard training on the maze, while the adult exposed cohort is impacted by sex.

Adolescent exposed mice began testing on the Barnes Maze to evaluate spatial learning at PND 63, 3 weeks since the last ethanol administration. Data was separated by trial, as the focus of the study was on long-term memory changes due to ethanol. Trial 1 occurred following overnight consolidation (roughly 24 hours) after the last training session, but trial 2 occurred 5 minutes after trial 1. As there was a focus on long term memory, the following data presented is from trial 1 rather than averaging data between the two trials. Latency to the goal box showed a significant main effect of ethanol [$F(1, 30) = 6.774$, $p=0.0141$] where ethanol treated mice reached the goal box sooner than controls (Fig. 12A). This ethanol effect was also reflected in speed to the goal box [$F(1, 30) = 15.33$, $p=0.0005$] with ethanol treated mice displaying a faster speed than controls (Fig. 12B). A history of ethanol did not impact the number of errors [$F(1, 30) = 3.275$, $p= 0.0804$] (Fig. 12C) or distance traveled [$F(1, 30) = 1.616$, $p=0.2134$] (Fig. 12D) on the maze. Sex did not impact errors [$F(1, 30) = 0.03614$, $p= 0.8505$], latency [$F(1, 30) = 2.839$, $p=0.1024$], speed [$F(1, 30) = 0.4339$, $p=0.5151$], or distance [$F(1, 30) = 0.3521$, $p=0.5574$] on the maze. Over the training days, all groups improved their performance, which

was observed as a main effect of day in latency [F (6, 180) = 67.13, p<0.0001], speed [F (6, 180) = 16.09, p<0.0001], errors [F (6, 179) = 3.646, p= 0.0019], and distance [F (6, 180) = 10.92, p<0.0001] (Fig. 12A-D).

Adult mice underwent training on the Barnes Maze three weeks after the last ethanol administration on PND 98 with a four-day break taken between day 2 and 3 of training due to COVID -19. A prior treatment of ethanol did not impact latency [F (1, 24) = 0.004543, p=0.9468], speed [F (1, 24) = 0.6363, p=0.4329], the number of errors [F (1, 24) = 3.361, p=0.0792], or distance [F (1, 24) = 1.060, p=0.3135] (Fig. 13A-D). Sex impacted adult performance on the maze with females showing a significant main effect of speed [F (1, 24) = 5.471, p=0.0280], reaching the goal box faster than males (Fig. 13B). Meanwhile, no sex effect was found on latency [F (1, 24) = 1.790, p=0.1934], the number of errors [F (1, 24) = 0.02210, p=0.8831], or distance [F (1, 24) = 0.06207, p=0.8054]. Maze performance was similarly improved with training as seen by a main effect of day on latency [F (8, 192) = 53.06, p<0.0001], speed [F (8, 192) = 12.99, p<0.0001], and distance [F (8, 192) = 10.83, p<0.0001] (Fig. 13A,B,D). The number of errors to the goal box showed no effect by day [F (8, 192) = 1.829, p=0.0738] (Fig. 13C).

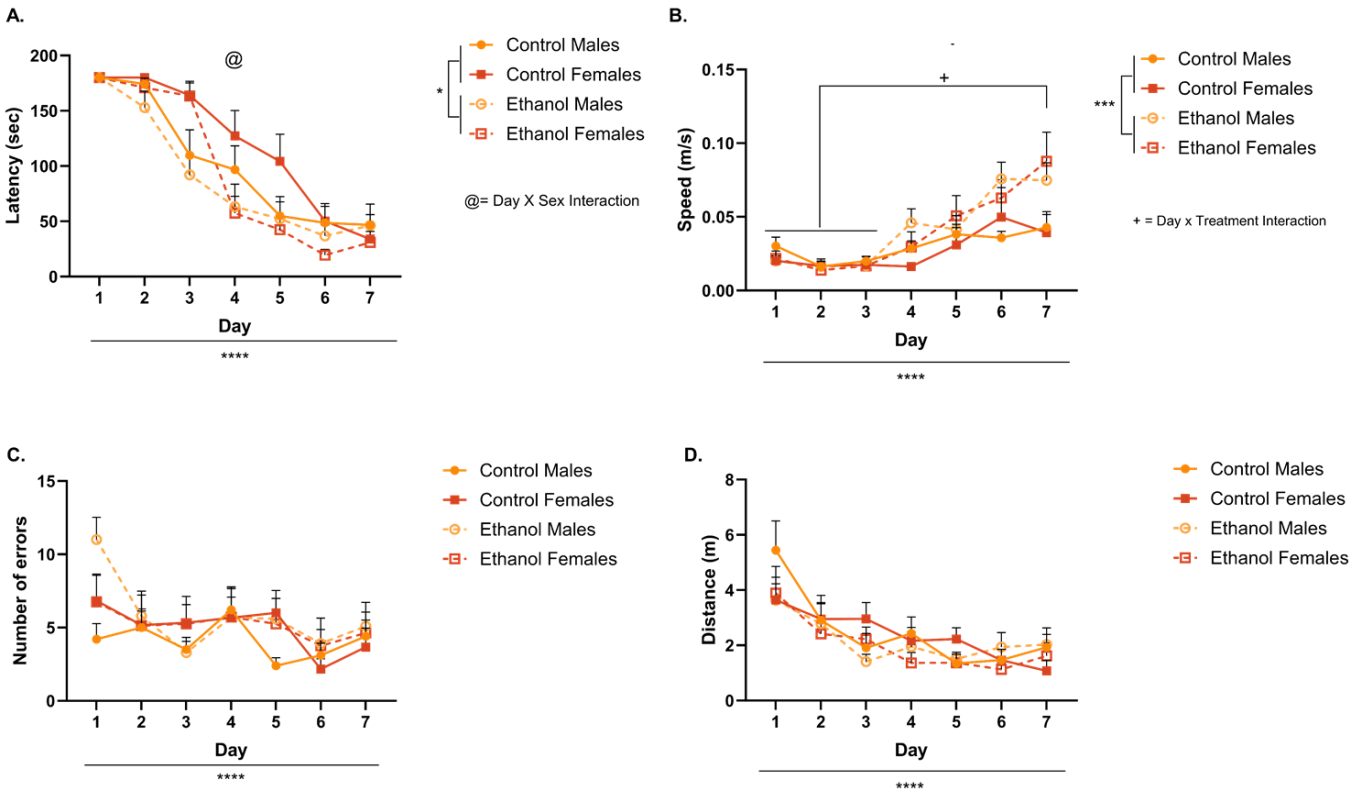

Fig 12. Adolescent ethanol exposure decreases latency and increases speed on the Barnes Maze. Performance during standard training was altered by adolescent ethanol with ethanol exposed mice having a A) shorter latency and B) greater speed (n: CM=10, EM=10, CF=6, EF=8). Both C) total errors and D) distance on the maze was not impacted by prior ethanol exposure. Performance on the maze changed over day in each metric listed above. Data is presented as mean +/- SEM. (*, $p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$, ****$p \leq 0.0001$)

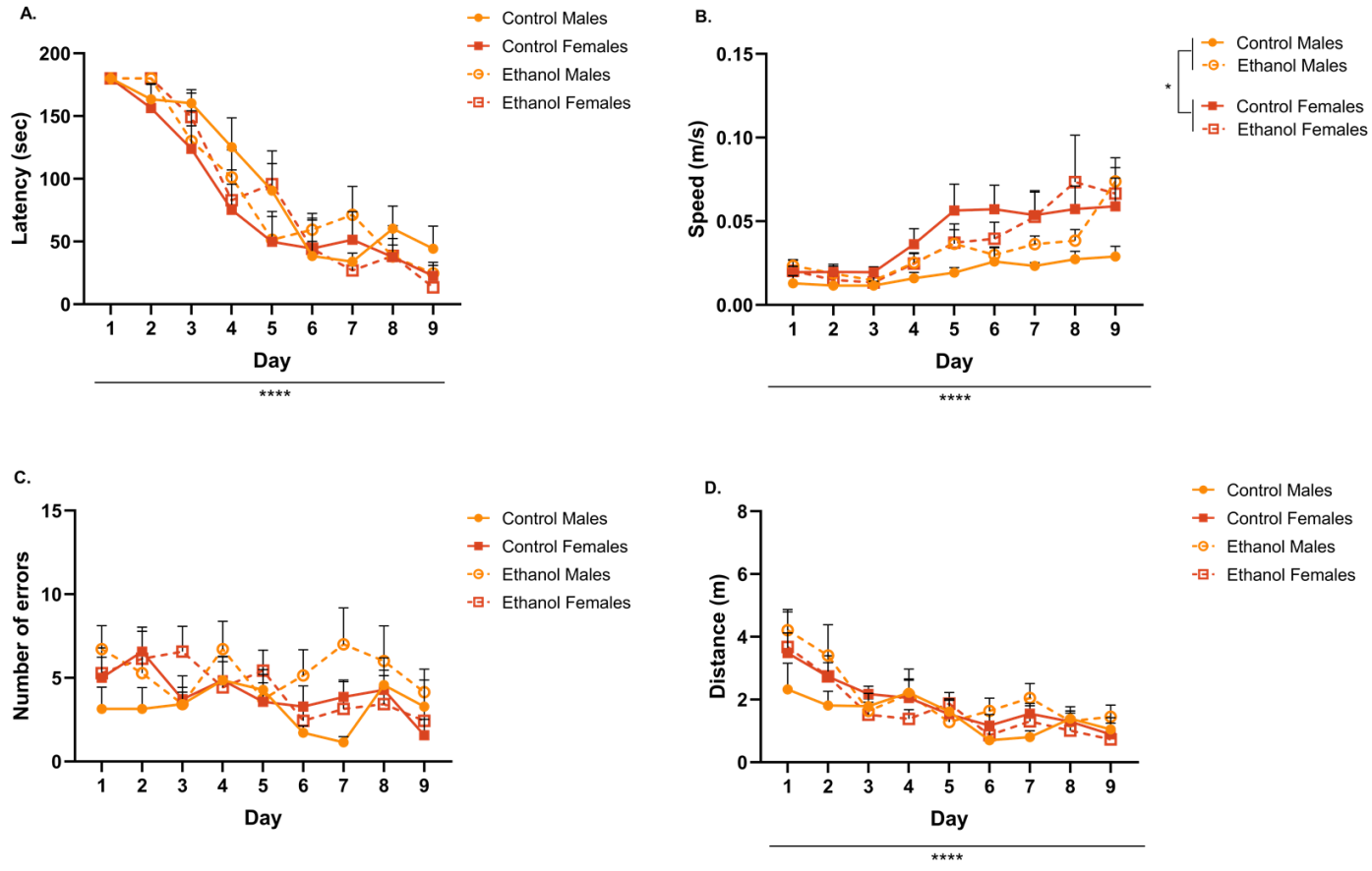

Fig 13. Sex, but not adult ethanol exposure alters speed on the Barnes Maze. During standard training adult ethanol exposure did not impact A) latency, B) speed, C) errors, or D) distance (n:7/group). However, sex altered B) speed with females travelling faster on the maze than males. Performance on the maze changed over day in latency, speed, and distance but not errors. Data is presented as mean +/- SEM. (*, $p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$, ****$p \leq 0.0001$)

3.3.2 Spatial memory is impaired by adolescent binge ethanol but not adult binge ethanol.

Once all groups reached the training criteria of a latency $\leq$ 35 seconds and errors $\leq$ 5 for 2 consecutive days, both cohorts underwent a probe trial 24 hours after the last training day (on day 8 in adolescents and day 10 in adults) in which the goal box was removed from the maze to test spatial memory. Time spent in the target quadrant significantly differed in the adolescent exposed cohort due to a treatment*sex interaction [$F(1, 30) = 4.991$, $p=0.0331$] where control females spent more time in the target quadrant compared to all other groups. A main effect of treatment [$F(1, 30) = 7.797$, $p=0.0090$] was also observed with ethanol treated mice spending less time in the target quadrant than controls (Fig. 14A). Additionally, a significant sex effect was observed [$F(1, 30) = 5.426$, $p=0.0268$] with females spending more time in the target quadrant, but this appears to be driven by the control females. In the adult exposed cohort, time spent in the target quadrant did not differ by treatment [$F(1, 24) = 0.2439$, $p=0.6259$] or sex [$F(1, 24) = 0.8242$, $p=0.3730$] (Fig. 14B).

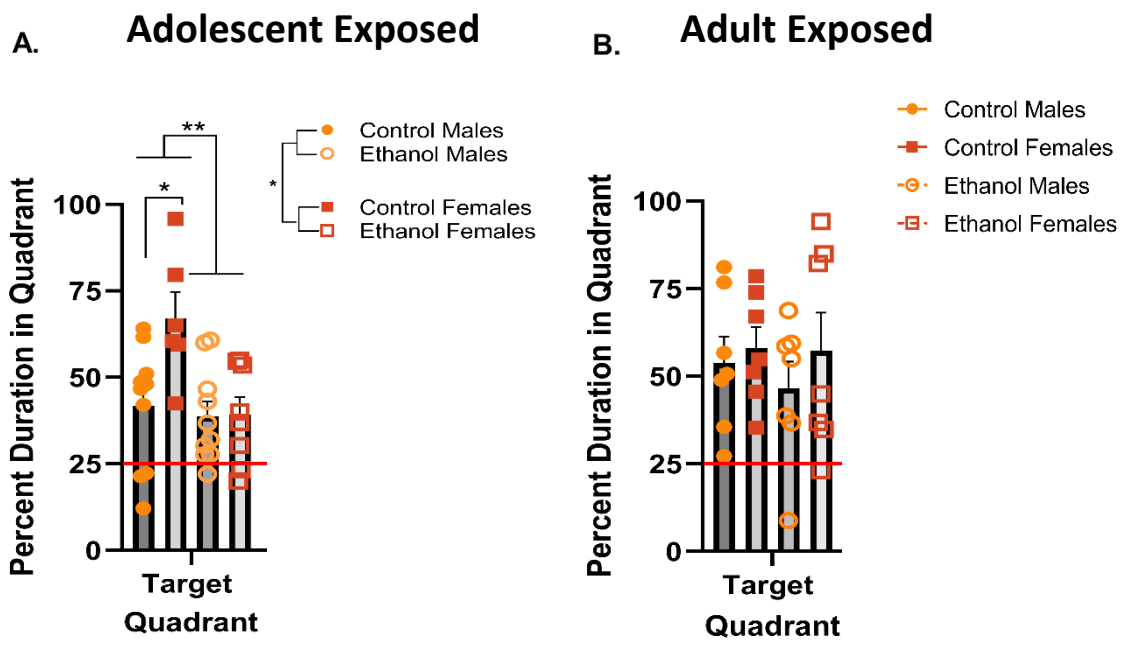

Fig 14. Adolescent ethanol exposure decreases time in the target quadrant. The standard probe trial revealed spatial memory was impacted by ethanol in the A) adolescent exposed cohort but not in the B) adult exposed cohort (n:7/group). Mice exposed to ethanol in adolescence spend less time in the target quadrant than control animals. A treatment*sex interaction was found in the adolescent exposed cohort with control females performing better than every other group (n: CM=10, EM=10, CF=6, EF=8). Data is presented as mean +/- SEM. (*, $p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$, ****$p \leq 0.0001$)

3.3.3 Reversal training is altered by ethanol in adolescent exposed mice but sex in adult exposed mice.

Following the probe trial, the goal box was moved 180 degrees and mice began training for the new location. Adolescent exposed mice displayed a significant effect of ethanol on latency [$F(1, 30) = 17.67$, $p=0.0002$] and speed [$F(1, 30) = 23.81$, $p<0.0001$] with ethanol treated mice reaching the goal box sooner and faster than controls (Fig. 15A & B). However, the number of errors [$F(1, 30) = 0.0001385$, $p=0.9907$] and distance on the maze did not differ due to treatment [$F(1, 149) = 0.3435$, $p= 0.5587$]) in the adolescent ethanol-exposed cohort (Fig. 15C & D). Sex did not impact the performance on latency [$F(1, 30) = 1.296$, $p=0.2639$], speed [$F(1, 30) = 1.007$, $p=0.3237$], errors [$F(1, 30) = 1.768$, $p=0.1936$], or distance [$F(1, 149) = 0.9977$, $p= 0.3195$]. A main effect of day was observed on latency [$F(4, 120) = 31.04$, $p<0.0001$], speed [$F(4, 119) = 12.94$, $p<0.0001$], and distance [$F(4, 149) = 6.316$, $p<0.0001$] but no day effect in errors [$F(4, 120) = 2.079$, $p=0.0878$] (Fig. 15A-D).

The adult exposed cohort underwent the same reversal paradigm as the adolescent exposed cohort where ethanol treatment did not alter the performance in latency [$F(1, 24) = 0.09860$, $p=0.7562$], speed [$F(1, 24) = 0.02219$, $p= 0.8828$], errors [$F(1, 24) = 0.7858$, $p=0.3842$], or distance [$F(1, 24) = 0.01535$, $p= 0.9024$] (Fig. 16A-D). A main effect of sex was observed on latency [$F(1, 24) = 12.52$, $p=0.0017$] and speed [$F(1, 24) = 12.17$, $p= 0.0019$] with females again performing better than males (Fig. 16A & B). The number of errors [$F(1, 24) = 0.05506$, $p=0.8165$] and distance traveled not differ due to sex [$F(1, 24) = 0.4973$, $p=0.4875$]. A significant main effect of day was observed for latency [$F(4, 96) = 35.22$, $p<0.0001$], speed [$F(4, 95) = 12.60$, $p<0.0001$], the number of errors [$F(4, 96) = 13.08$, $p<0.0001$], and distance [$F(4, 95) = 16.16$, $p<0.0001$] (Fig. 16A-D).

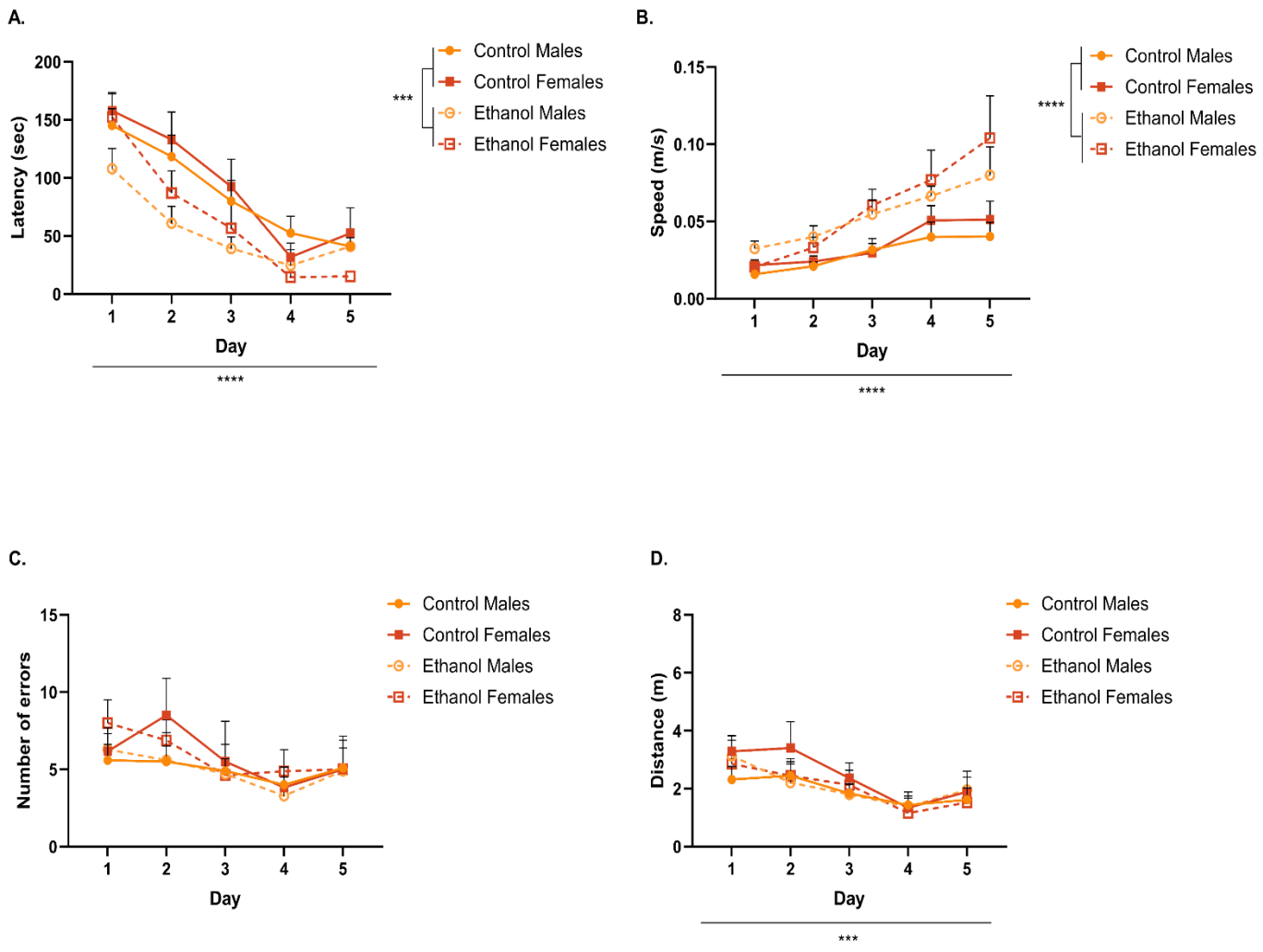

Fig 15. Reversal training latency and speed are impacted by adolescent ethanol exposure. During reversal training A) latency and B) speed were still impacted by adolescent ethanol exposure with ethanol exposed mice making decreased latency and increased speed (n: CM=10, EM=10, CF=6, EF=8). No ethanol effect was observed on C) total errors or D) distance. A main effect of day was found for A) latency, B) speed, and D) distance but no effect observed for C) errors. Data is presented as mean +/- SEM. (*, $p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$, ****$p \leq 0.0001$)

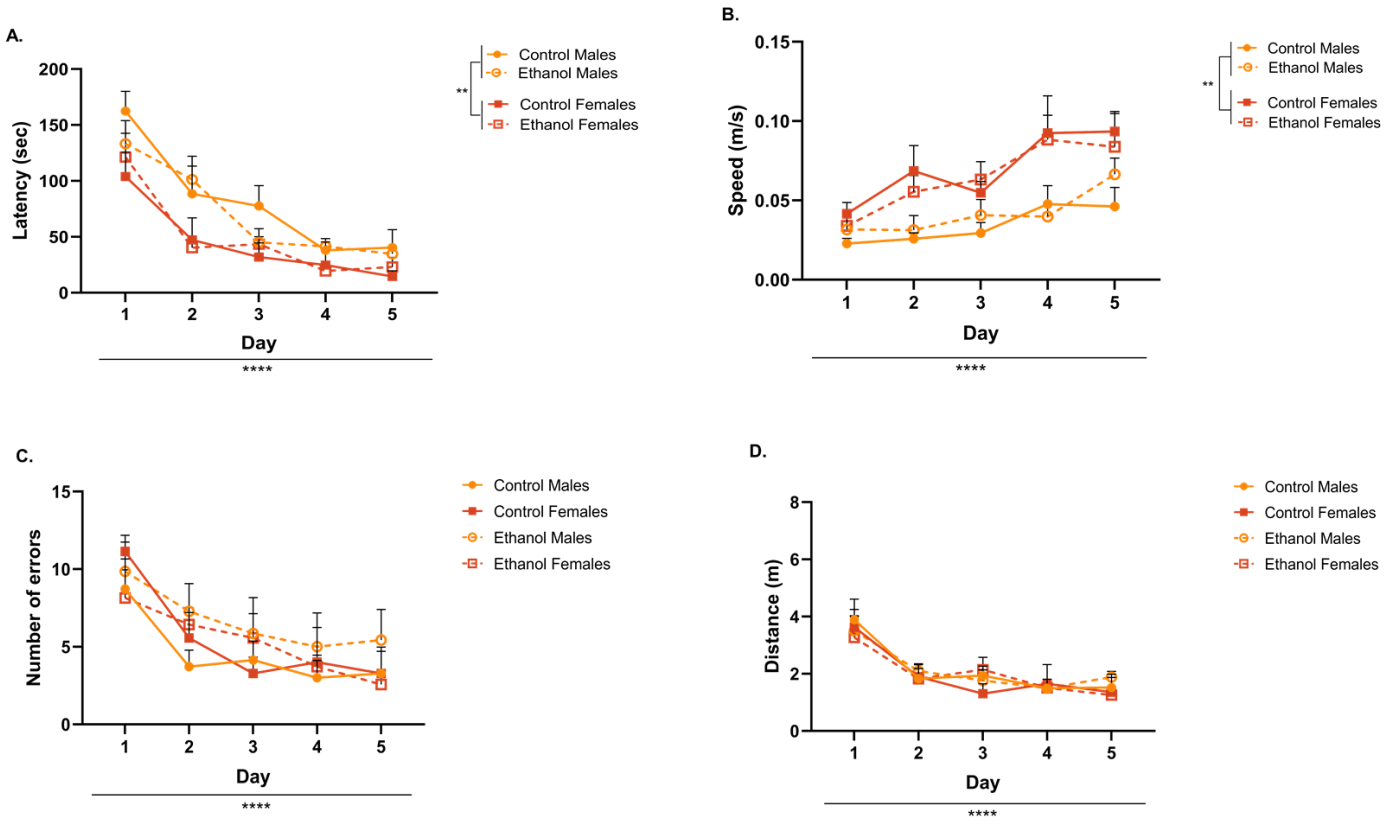

Fig 16. Sex impacts speed in adult exposed cohort during reversal training. During reversal training B) speed was still impacted by sex within the adult exposed cohort (n:7/group). No ethanol effect was observed on A) latency, B) speed, C) total errors, or D) distance. A main effect of day was found all metrics above. Data is presented as mean +/- SEM. (*, $p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$, ****$p \leq 0.0001$)

3.3.4 Second probe trial unchanged by ethanol in both cohorts.

Twenty-four hours after the last training a second probe trial was performed to assess if time spend in the new quadrant would differ due to sex or treatment. In the adolescent exposed cohort, time spent in the target quadrant did not differ by treatment [F (1, 30) = 0.007665, p=0.9308] or sex [F (1, 30) = 0.05106, p=0.8228] (Fig. 17A). The adult exposed cohort similarly showed no treatment [F (1, 24) = 0.02648, p=0.8721] or sex [F (1, 24) = 0.05900, p=0.8102] effect (Fig. 17B) with all groups spending a similar time in the target quadrant. A three-way ANOVA was used to compare time spent in the opposite quadrant, the standard target quadrant, to the new reversal target quadrant. A main effect of quadrant was found in the adolescent [F (1, 30) = 10.47, p=0.0030] and adult [F (1, 24) = 4.471, p=0.0450] exposed cohorts where more time was spent in the new target quadrant (Fig. 17A & B) regardless of sex or history of ethanol. The second probe trial showed that both age groups spend more time in the target quadrant and showed no negative effect on memory due to ethanol.

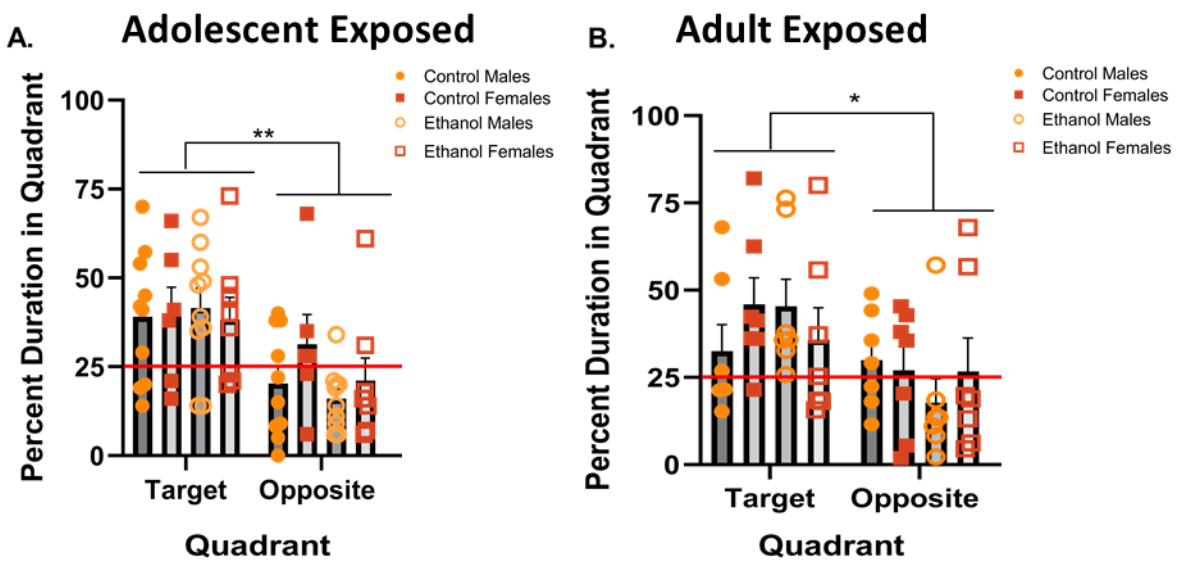

Fig 17. No effect of ethanol or sex on the second probe trial. Following reversal training a second probe trial showed both A) adolescent exposed (n: CM=10, EM=10, CF=6, EF=8) and B) adult exposed mice (n:7/group) spent more time in the new target quadrant compared to the opposite target quadrant. No effect of ethanol or sex was observed on duration in the target quadrant. Data is presented as mean +/- SEM. (*, $p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$, ****$p \leq 0.0001$)

3.3.5 Search strategy is not impacted by ethanol or sex in either age cohort.

Search strategy was used to assess how search of the goal box location occurred over time, due to sex, and a history of ethanol exposure within each age cohort. In the adolescent exposure cohort during standard training, strategy use changed over time with higher odds of the serial (OR=1.5603, 95% CI= 1.30290;1.869, p= 0.0000013) and spatial (OR= 1.5109, 95% CI= 1.16342;1.962, p= 0.00197) search strategy (Fig. 18). To investigate if treatment or sex impacted the odds of a used strategy, a separate analysis was conducted within a strategy. No difference in serial or spatial strategy was found due to sex or treatment. Within the adult, cohort the odds of using the spatial search strategy increased over time (OR= 1.4958, 95% CI=1.20636;1.8547, p=0.00024) (Fig.19). Treatment and sex did not alter the count of mice using the serial or spatial search strategy.

In reversal training, the adolescent exposed cohort showed the same changes in strategy with increased odds of serial (OR=, 1.6811 95% CI= 1.4438;1.957, p= 0.000000000022) and spatial (OR= 2.3153, 95% CI= 1.7420;3.077, p= 0.0000000073) compared to random over time (Fig. 20). Within the serial and spatial categories, strategy use did not differ by treatment or sex. Meanwhile, the adult exposed cohort continued to show increased odds of spatial search strategy over time (OR= 2.1068, 95% CI= 1.5598;2.846, p= 0.00000119). However, this did not differ by sex or treatment (Fig. 21).

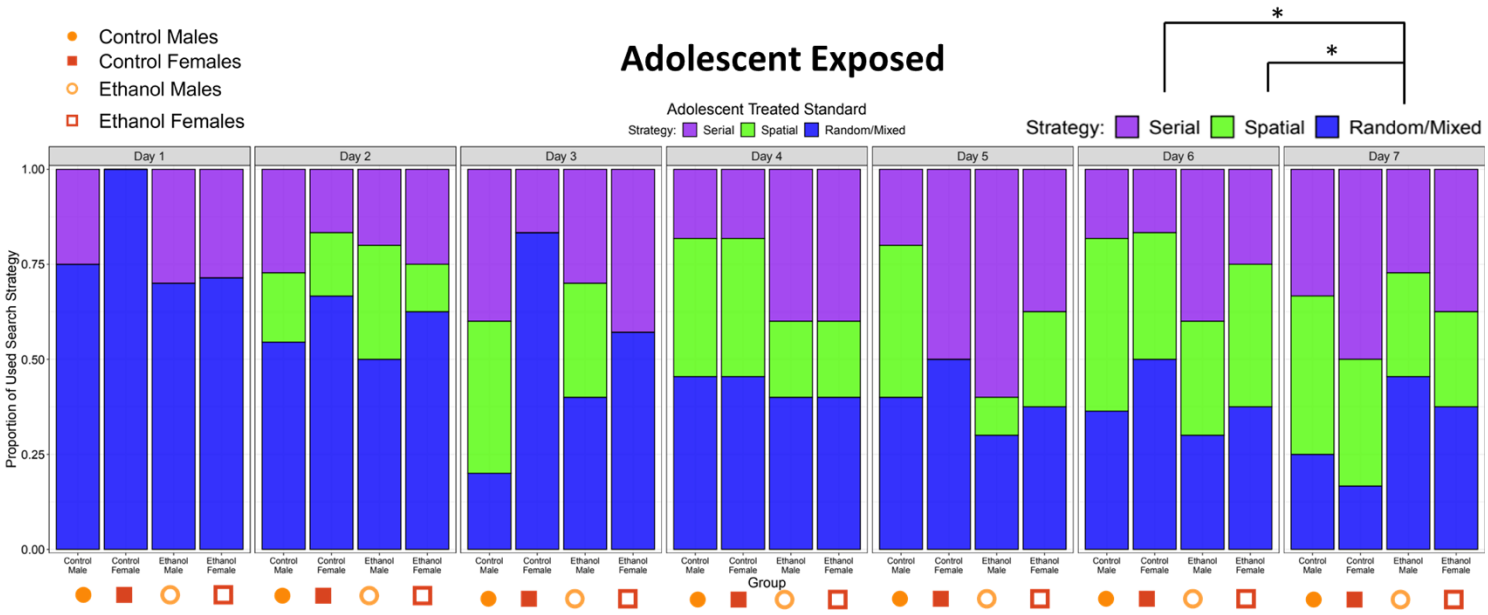

Fig 18. Search strategy during standard training unchanged by adolescent ethanol exposure. During standard training all groups updated the strategy used to search the maze. All groups increased the use of the serial and spatial search strategy over time (n: CM=10, EM=10, CF=6, EF=8). Data is presented as proportion of used strategy. (*, $p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$, ****$p \leq 0.0001$)

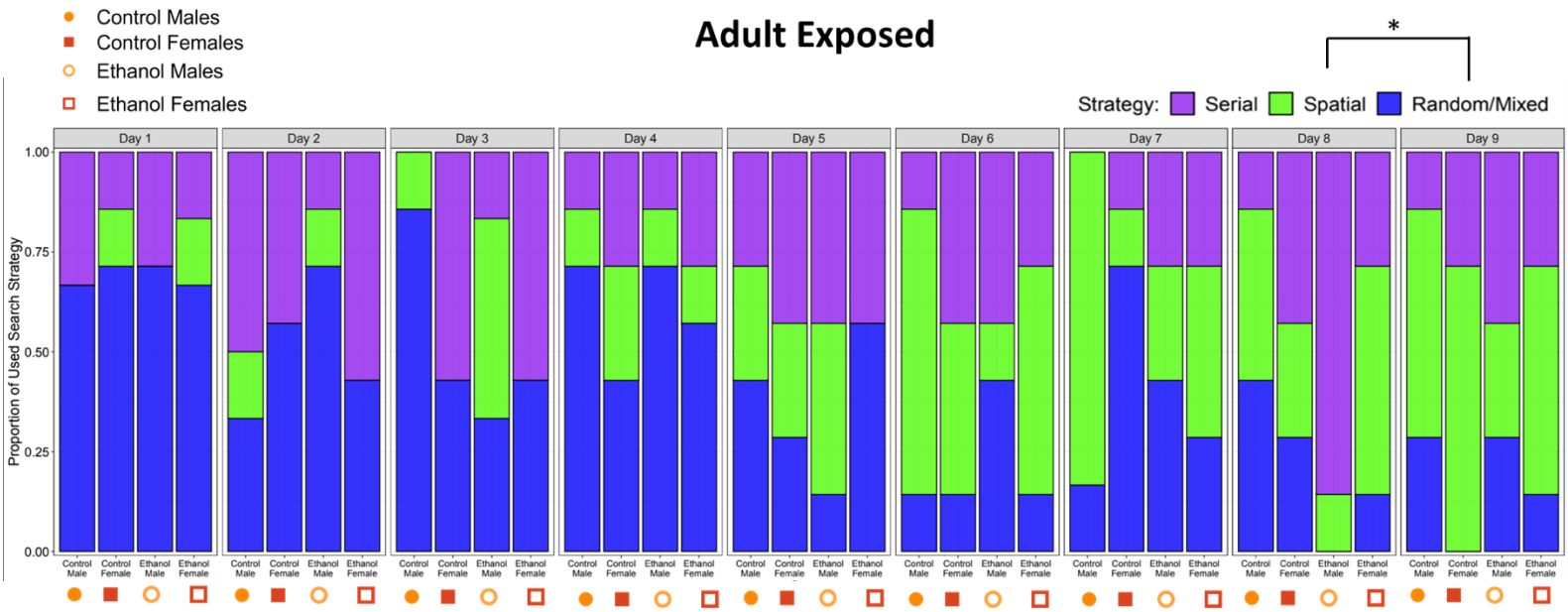

Fig 19. Adult exposed cohort increases use of spatial search strategy over time during standard training. During standard training both sexes and treatment groups increased the use of the spatial search strategy over time. The use of the serial strategy did not change over time (n:7/group). Data is presented as proportion of used strategy. (*, $p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$)

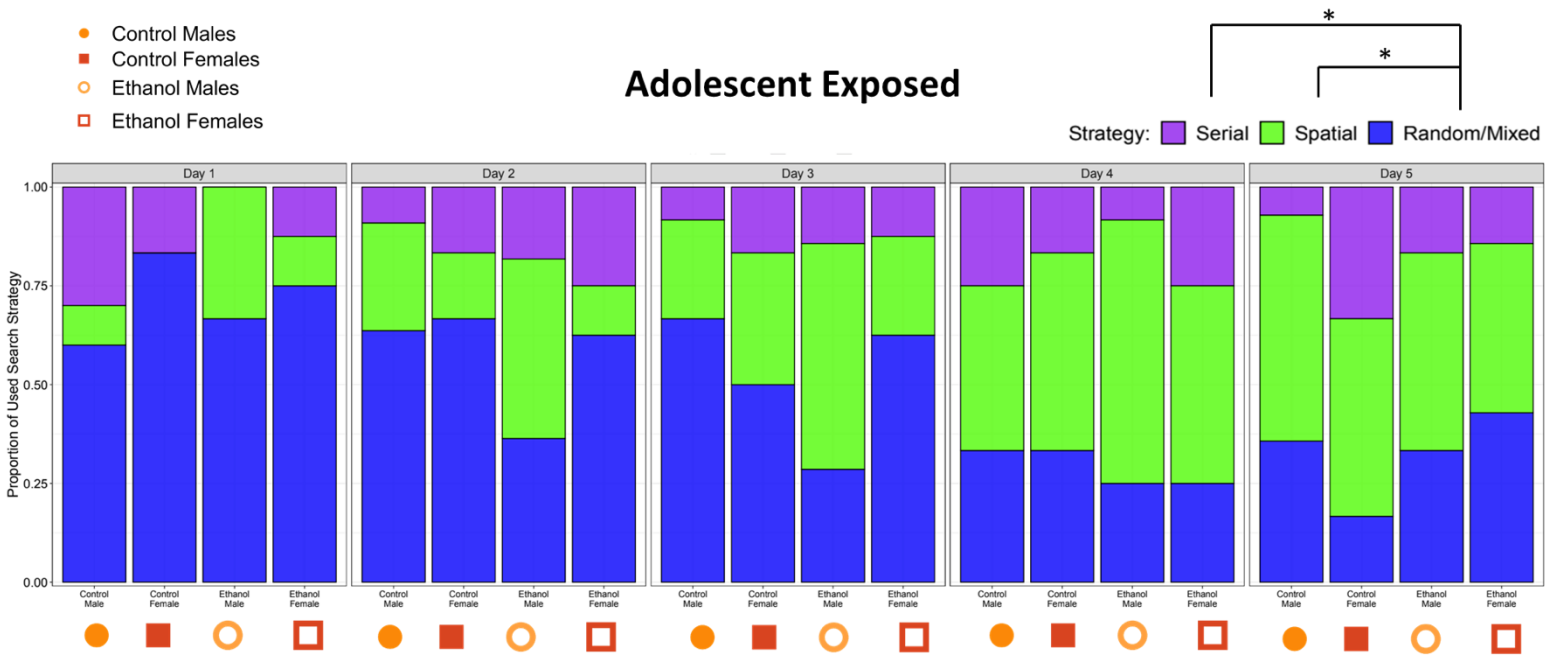

Fig 20. Sex and adolescent ethanol do not impact reversal search strategy. Search strategy during reversal training is not impacted by sex or treatment in the adolescent exposed cohort. Both serial and spatial search strategy use is increased over time in all groups (n: CM=10, EM=10, CF=6, EF=8). Data is presented as proportion of used strategy. (*, $p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$, ****$p \leq 0.0001$)

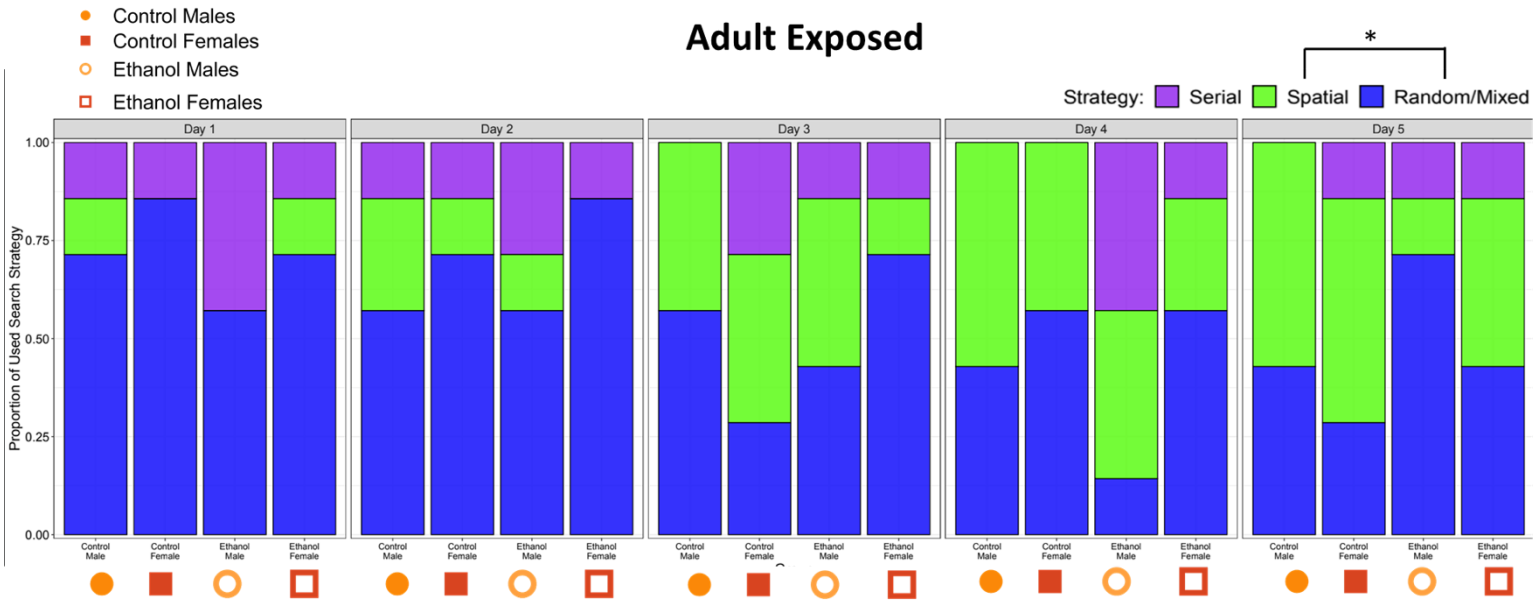

Fig 21. Spatial search strategy increased over time in adults during reversal training. The adult exposed cohort regardless of sex or ethanol treatment increased the use of the spatial search strategy (n:7/group). Data is presented as proportion of used strategy. (*, $p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$, ****$p \leq 0.0001$)

3.3.6 Open field performance is unaltered by sex or treatment in either age group

The open field task was used to assess general locomotion and anxiety-like phenotypes in a separate adolescent exposed and adult exposed cohort following a 3-week abstinence period from the ethanol paradigm. The adolescent exposed cohort showed no difference in the duration in the center of the arena due to treatment [F (1, 21) = 0.5654, p=0.4604] or sex [F (1, 21) = 0.0003842, p=0.9845] (Fig. 22A). Similarly, the duration in the peripheral zone of the arena was unchanged by treatment [F (1, 21) = 0.4953, p=0.4893] or sex [F (1, 21) = 0.04716, p=0.8302] (Fig. 22B). Distance in the center showed no ethanol [F (1, 21) = 3.949, p=0.0601] or sex effect [F (1, 21) = 1.191, p=0.2875] (Figure 22 C). Meanwhile, distance in the periphery was unchanged by ethanol treatment [F (1, 21) = 0.6082, p=0.4442] and sex [F (1, 21) = 0.3357, p=0.5685] (Fig. 22D). Velocity in the center and peripheral zones were also unchanged by treatment (center: [F (1, 21) = 0.4077, p=0.5300], periphery [F (1, 21) = 1.169, p=0.2920]) or sex (center: [F (1, 21) = 0.006229, p=0.9378], periphery: [F (1, 21) = 0.07838, p=0.7822]) (Fig. 22 E-F).

The adult exposed cohort also showed no difference in performance, with duration in center and periphery not differing due to treatment (center: [F (1, 26) = 1.142, p= 0.2951], periphery [F (1, 26) = 1.224, p= 0.2787]) or sex (center: [F (1, 26) = 0.07497, p= 0.7864], periphery: [F (1, 26) = 0.2993, p= 0.5890]) (Fig. 23 A-B). Likewise, distance in the center and peripheral zones did not differ due to ethanol treatment (center: [F (1, 26) = 0.08681, p= 0.7706], periphery: [F (1, 26) = 0.001813, p= 0.9664]) or sex (center: [F (1, 26) = 0.8490, p= 0.3653], periphery: [F (1, 26) = 0.01310, p= 0.9098]) (Fig. 23 C-D). Lastly, speed during the test was similarity unchanged in both zones by adult ethanol exposure (center: [F (1, 26) = 0.3533,

p=0.5574], periphery [F (1, 26) = 0.02399, p=0.8781]) or sex (center: [F (1, 26) = 0.3904, p=0.5375], periphery [F (1, 26) = 0.01563, p=0.9015]) (Fig. 23 E-F).

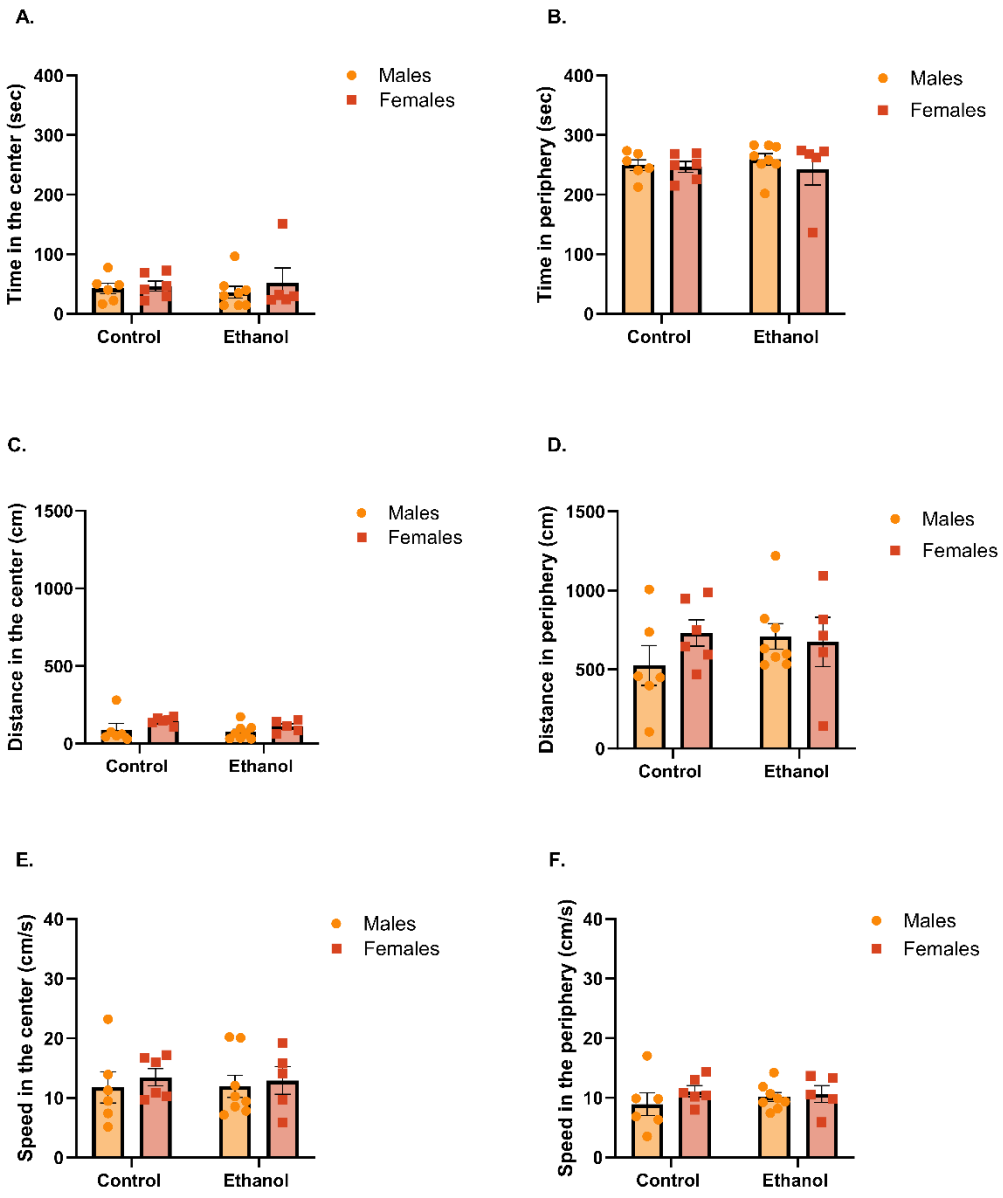

Fig 22. Adolescent ethanol exposure nor sex alters open field performance. No effect of adolescent ethanol or sex was observed on A) time in the center, B) time in the periphery, C) distance in the center, D) distance in the periphery, E) speed in the center, and F) speed in the periphery (n: CM=6, EM=8, CF=6, EF=5). Data is presented as mean +/- SEM. (*, $p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$, ****$p \leq 0.0001$)

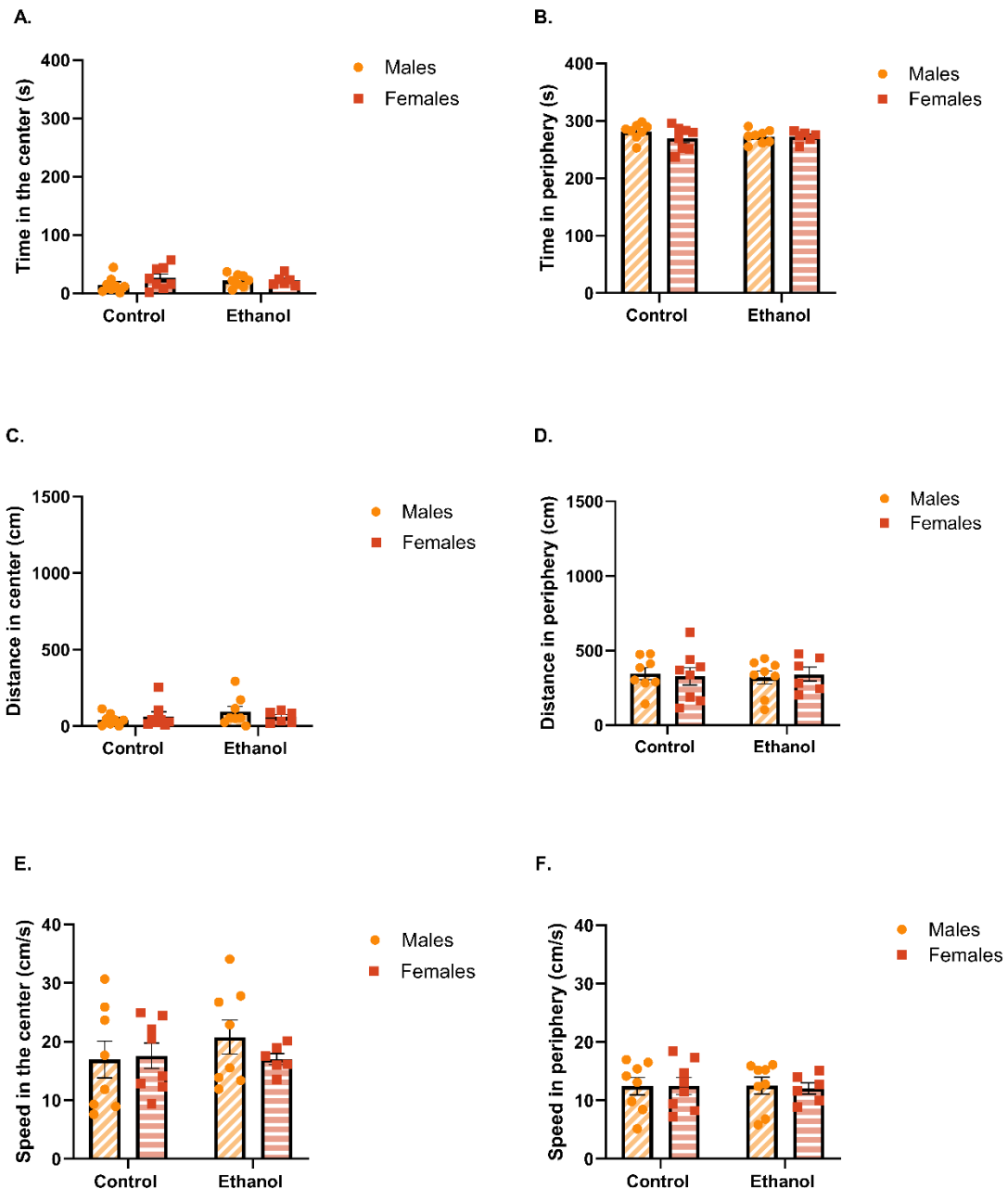

Fig 23. Open field performance not impacted by adult ethanol exposure or sex. Three weeks after ethanol exposure, no effect of ethanol or sex was observed on A) time in the center, B) time in the periphery, C) distance in the center, D) distance in the periphery, E) speed in the center, and F) speed in the periphery (n: CM=8, EM=8, CF=8, EF=6). Data is presented as mean +/- SEM. (*, $p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$, ****$p \leq 0.0001$)

3.4 Discussion

Ethanol exposure has shown to result in memory impairments when ethanol is actively in the body [17], [254], but also following prolonged exposure when ethanol is no longer circulating in the body [16], [235], [236], [251]–[253]. The Barnes Maze is a commonly used apparatus to test spatial learning, spatial memory, and cognitive flexibility. While the Barnes Maze is less stressful than the MWM, the task still increases CORT without the use of bright lights or other aversive stimuli [263]. The use of mildly aversive stimuli, such as bright lights, is done to motivate completion of the task rather than increased exploration and thereby latency, errors, and distance to the goal [261]. Ethanol exposure may also lead to an anxiety-like phenotype [160], [214], [265] which may impact other tasks like the Barnes Maze. The present study found that the developmental age of ethanol exposure differentially impacted spatial memory and spatial learning. While adolescent ethanol exposure impacted performance on the maze, adult ethanol exposure did not alter any portion of testing. However, sex impacted both cohorts with adolescent exposure mice showing sex effects during standard probe, while the adult exposed cohort sex impacted standard and reversal training. A basal anxiety test, using the open field, revealed no effect of ethanol in either age group. Together this data shows that, within a stressful environment, adolescent ethanol exposure increases speed and decreases latency to the goal box, while negatively impairing spatial memory; yet the same ethanol exposure in adulthood results in no lasting behavioral changes. A summary of the Barnes Maze results following binge ethanol exposure can be found in Table 5.

Table 5. Summary of chapter 3 Barnes Maze results.

			Standard Training				Standard Probe	Reversal Training				Reversal Probe
Table 5	Age on Test Day	Main Effect	Latency (sec)	Speed (m/s)	Number of Errors	Distance	Duration in Target Quadrant	Latency (sec)	Speed (m/s)	Number of Errors	Distance	Duration in Target Quadrant
Adolescent Exposed Cohort	PND 63-76	Main Effect of Treatment	**(ethanol < control)**	**(ethanol > control)**	NS		**(ethanol < control)**	**(ethanol < control)**	**(ethanol > control)**	NS		NS
	PND 63	Main Effect of Sex	NS				**(M< F)**	NS				NS
Adult Exposed Cohort	PND 98-112	Main Effect of Treatment	NS				NS	NS				NS
	PND 99	Main Effect of Sex	NS	**(M< F)**	NS		NS	**(M< F)**		NS		NS

The standard probe test, which assessed spatial memory, revealed an ethanol*sex interaction only in the adolescent exposed cohort with the control female group performed better on the spatial probe task than any other group (Fig. 14). Meanwhile, adult ethanol exposure had no effect on performance in the standard probe test. Similar age differences are observed in studies when ethanol is in the body finding adolescents perform worse than adults on spatial memory [17], [254]. This is the first study to our knowledge to investigate persistent changes in spatial memory following binge ethanol in adolescents and adults. While some studies have also conducted the Barnes Maze test following adolescent ethanol exposure, those studies have not included a probe session to assess spatial memory. Our data is in line with other single trial tests showing impaired spatial memory following adolescent ethanol exposure [251]–[253]. The ethanol*sex interaction effect may be due to the hormonal effects and/or the absence of the goal box as a location cue. The control females performed better than any other group in the task, although no difference in estrus stage was confirmed via visual assessment (data not shown). While visual estrus assessment is less invasive than lavage/ cytology, visual assessment is less accurate [272]. Some control females may have been in the proestrus stage, rather than the observed diestrus stage, which has peak estrogen levels [273]. Studies have shown improved spatial memory on the probe trial of MWM [152] and increased dendritic spine density in CA1 of the hippocampus [274] during proestrus. As our animals were not staged, it is possible differences in estrus stage contributed to control females spending significantly more time in the target quadrant. Furthermore, chronic ethanol exposure during adolescence can delay puberty onset, alter menstrual cycling, and blunt the estrogen rise typically seen in adolescence [79], [80]. Therefore, our adolescent ethanol female group may have dysregulated or decreased circulating estrogen levels, and may not have the cognitive benefits of increased estrogen at the

time of testing. Our ethanol animals may also have spent less time in the target quadrant due to using the goal box as a location cue rather than the cues places around the arena. Chow [152] found decreased spatial memory when animals are trained on a cued version of the MWM compared to a spatial version. As our ethanol mice had decreased latency and increased speed during training, it is possible the ethanol mice transverse the maze in search of the goal box rather than using the spatial cues around the arena to orientate themselves. Additionally, adolescent ethanol decreases hippocampal neurogenesis [68], [235], [252] while spatial learning increases hippocampal neurogenesis [152]. Meanwhile, reversal probe data showed no effect of adolescent or adult ethanol exposure (Fig.17). As our ethanol mice showed worse performance on the standard probe trial, suggesting impaired spatial memory, our second probe trial may be considered a measure of continued training rather than cognitive flexibility. Continued training has shown to improve memory [275]. Our reversal probe data is in line with Vetreno and Crews [255] finding no effect of adolescent ethanol on reversal probe performance. While Coleman [256] found ethanol treated mice spent less time in the target quadrant during MWM, their controls spent 25% of their time in the quadrant, therefore showing no preference for the target quadrant. The lack of a preference for a target quadrant suggests impaired memory formation in the controls and subsequently the effect of ethanol is questionable. Overall, the two probe trials suggest adolescent ethanol exposure and sex interact to impair spatial memory possibly due to estrogen levels, learning pattern, or impaired neurogenesis during spatial learning, which can be overcome with continued training.

The standard training portion of the Barnes Maze assessed spatial learning while the reversal portion typically measures cognitive flexibility to the new goal box location. The adolescent exposed cohort revealed a persistent effect of ethanol on both standard and reversal training with

ethanol decreasing latency to the goal box and increasing speed (Fig. 12 & 15). While the adolescent data could be interpreted as an improvement in spatial learning, we do not think this is occurring, nor have we observed supporting data in the literature. Rather, the stressful environment of the maze in combination with adolescent ethanol exposure may lead to an exacerbated stress response leading to the increased speed and decreased latency. Chronic ethanol exposure in adolescence may dysregulate the HPA axis as ethanol increases CORT [264], [276], [277]. When examined in adulthood following a history of adolescent ethanol exposure basal CORT levels are decreased, while following an acute stressor, acute dose of ethanol, CORT levels increase compared to controls [264]. However, others have found adolescent ethanol exposure via vapor inhalation to blunt CORT levels when given ethanol, via intragastric catheter, in adulthood [276] or to result in no difference in CORT [277]. Differences between these studies may be due to differences in route of ethanol administration or length of ethanol exposure. While these studies varied in the effect of adolescent ethanol exposure on CORT levels they all noticed cellular differences due to ethanol with increases in corticotrophin-releasing hormone, decreases in arginine vasopressin, and decreased cytokine mRNA expression in the hypothalamic paraventricular nucleus or blood [264], [276], [277] Meanwhile, behavioral testing alone increases CORT [262], [263] which is further exacerbated in rodents with a history of stress with increased CORT compared to non-stressed matched rodents [262], [278]. Therefore, in our study a history of adolescent ethanol may have led to elevated CORT levels compared to controls during the Barnes Maze. Our open field data (Fig. 22) further suggests ethanol effects on locomotion are dependent on the environment with no differences observed in the open field (distance, time, or speed) with normal lighting conditions. Other studies have found an ethanol effect on the open field task [160], [265] and elevated plus maze [265] which

may be due to differences in testing procedure, ethanol dose, and ethanol exposure. While Coleman [160] used a higher ethanol dose (5g/kg) than our study, Fernandes [265] exposed their mice to 3g/kg of ethanol for a longer timeframe, PND 35-86. Therefore, greater exposure to ethanol either via a higher initial concentration or longer timeframe of ethanol exposure may lead to an anxiety like phenotype. Overall our open field data does not suggest an anxiety like phenotype following adolescent or adult ethanol exposure. Therefore, increased latency on the Barnes Maze may be due to environmental differences between the open field and Barnes Maze arena.

Our adolescent exposed data is not in line with literature in which adolescent ethanol exposure persistently did not impacted spatial acquisition in the Barnes Maze, Radial Arm Maze, or MWM [18], [160], [163], [244], [255], [256]. The differences between our study and others may be due to a lower ethanol dose in other studies [163], [244] or differences in lighting intensity and therefore stress and motivation of the stimulus. Lighting conditions between Barnes Maze studies can vary with many studies not stating the lighting intensity [160], [263] and those that do using office room lighting (around 500 lux) [244]. As our lighting intensity (around 3000 lux) is between outside overcast lighting and full day light, the stress from the brighter environment may be greater than of office lighting. The MWM does increase CORT levels more than the Barnes Maze task with unknown likely office light [263], but the use of bright light may further increase CORT levels. Conflicting data on the impact of adolescent ethanol on reversal training in a spatial task shows persistently negative impacts the first day of reversal learning [160], [163] or no effect [244], [256]. Negative effects of ethanol on reversal training may be due to a higher ethanol dose in the Coleman and Matthews study or due to paradigm differences between the studies. While our reversal training occurred 24 hours after standard probe day,

Coleman [160] gave their animals a 4-day break before beginning reversal training. As our 1st day of reversal training followed probe day, in which no goal box was present, exploration patterns may have been different than the Coleman study, in which the goal box location moved following the break. Furthermore CORT levels following stress differ by strain with elevated CORT in DBA/2J but not C57BL/6J following the elevated plus maze [278]. As Coleman [160] used C57BL/6J, CORT levels in their study may have been lower than our study which used DBA/2J. Therefore, differences in strain used, schedule of testing, lighting intensity, and ethanol dose may lead to differences in ethanol effect in training during the Barnes Maze. Overall our study shows the combination of adolescent ethanol exposure and a brightly lit (3000 lux) environment increases locomotion and speed in DBA/2J which is possibly due to elevated CORT levels.

While training was impacted by ethanol in the adolescent exposed cohort, the adult exposed cohort only showed a sex effect in both standard and reversal with adult exposed females, regardless of treatment, displaying decreased latency and increased speed compared to males (Fig. 13 & 16). Our adult data is in line with other studies finding decreased latency in females [257]–[259] and increased speed [258] in the Barnes Maze or MWM. However conflicting data exists finding females with increased latency [152] or a trend towards increased latency [260] in the Barnes Maze or MWM. Sex differences between studies may be due to arena or strain differences. While O'Leary, Savoie, and Brown [260] used adult DBA/2J mice in the Barnes Maze task, the mice were individually housed and the arena used a buzzer as an aversive stimulus after thirty seconds into the task. Our task used bright lights to make the arena roughly 3000 lux which may have motivated or stressed our animals more than the O'Leary study as our animals after 4 days of training reached the goal box sooner than the O'Leary study. Studies

have found C57BL/6J and ICR female mice to have higher CORT levels than male counterparts basally and following a minor stressor (saline injection) or behavioral testing (open field) [262], [279]. Therefore, our sex effect in latency may reflect a strain difference that is exacerbated by bright light as an aversive stimulus.

Search strategy used during the Barnes Maze changed over time, but not due to sex or ethanol treatment in either age cohort during standard and reversal training phases. While the adolescent exposed cohort showed increased serial and spatial training over time (Fig. 18-19) the adult exposed cohort only showed an increase in spatial strategy (Fig. 20-21). This data is in line with Youn [280] finding increased use of the spatial strategy over time in adult male mice. Overall, this study found no difference due to sex or ethanol on strategy but that the use of serial and spatial strategies increased over time.

This study has several limitations due to the testing paradigm, issues obtaining animals, animal health issues, and COVID-19. First this study is limited by not testing mice that ran on the Barnes Maze for an anxiety like phenotype or collected blood to assess circulating CORT. This can be mitigated in the future by performing an anxiety test prior to the Barnes Maze, collecting saliva during the task, collecting blood after the task, or manipulating CORT levels during the task. Additionally, the testing criteria used to ensure the mice learned the goal box location before the rule set shift limited the ability to combine cohorts as different cohorts trained for a different number of days. Meanwhile, the length of the Barnes Maze paradigm and availability of 1 arena limited the number of animals that could be tested per day. Therefore, the adolescent exposed and adult exposed cohorts had to be run at separate times limiting statistical comparison between the two aged cohorts. These procedural limitations can be reduced by switching to a set number of training days, increasing the number of Barnes Maze arenas, and

increasing the number of personal conducting the study. Furthermore, the adolescent exposed spatial memory data (Fig. 14) is driven by the control females with a n=6, which is lower than the planned n=13/group. This study occurred in 2021 in which the lab was still struggling to obtain adolescent animals from Jackson Laboratory (Bar Harbor, ME). Additionally, another adolescent cohort was unable to be used due to health issues with a female pseudopregnancy, and incorrect sex identification. Future studies can reduce these limitations by increasing the number of animals per group, increasing the number of arenas, dividing testing based on sex, or choosing a set number of training days. Lastly, the adult exposed cohort used in this study had a 4-day break in training after day 2, due to COVID-19 in the lead investigator. Future studies can include additional personal trained on the task that have handled the mice to avoid breaks in testing due to illness.

3.5 Conclusion

This study found binge ethanol during adolescence negatively impacted spatial memory performance while promoting increased latency and speed during training when tested in adulthood. Furthermore, continued training during the reversal phase resulted in improved memory performance in the second probe trial. Meanwhile, adult ethanol exposure did not impact any portion of Barnes Maze testing, further highlighting the impact developmental age has on ethanol induced memory deficits. Additionally, sex impacted spatial memory and spatial learning with females performing better than males, which may have been impacted by the interaction of stress, sex, and ethanol. Future studies may address the impact of estrogen modulation and ethanol on memory performance. Additional studies may address changes in CORT due to sex and ethanol on the Barnes Maze. In conclusion, this study found age of ethanol exposure differentially impacted spatial memory and spatial learning on the Barnes Maze.

Chapter 4

Binge ethanol exposure alters CREB protein expression but not CRE-based gene expression

4.1 Introduction

Memory is a complex process involving stimuli encoding, consolidation, and retrieval which aids in an animal's everyday needs. The modality of memory such as recognition, spatial, or emotion-based memory, requires different brain region contributions and circuitry [136]. Furthermore, the ITI of a memory task can make the same modality of memory a working, short-term, or long-term memory task, involving different brain regions [105], [106], [136]. The short-term NOR task previously used by Bent [268] involves the dHPC [138], [139], [142], [143]. While long-term memory in the Barnes Maze task of Chapter 3 involves the mPFC [142], [151] and the dHPC [134], [138], [152]. Adolescent ethanol exposure impairs short-term NOR [16], [235] and long-term spatial memory [251]–[253]. Meanwhile, adult ethanol exposure does not lead to the same NOR [16], [235], [268] and spatial memory deficits [254]. Differences in ethanol memory impairments in adolescents and adults may be due to the ongoing adolescent brain development with the frontal and temporal cortices experiencing grey matter pruning later in adolescence than other brain regions [85]. Additionally, place and grid cells that aid in spatial representation mature during adolescence [119], [120]. As the adolescent brain is still developing, ethanol during the developmental timepoint may have more lasting effects on the dHPC and mPFC which play a role in object recognition and spatial memory.

Various proteins are involved in successful memory performance with CBP involved in both short-term and long-term spatial and recognition memory [176], [185], [192], [193], [196]. CBP is a 265 kDa protein that plays a wide role in cellular processes including neurogenesis, dendritic morphology and density, histone acetylation, and transcription due to its 9 binding domains [188]–[190], [281]. Studies have found impairing various binding domains of CBP, thus inactivating certain protein-protein interactions, impairs memory performance [178], [185], [192]. During transcription, CBP can interact with several transcription factors, such as CREB following phosphorylation by upstream kinases like CAMKIV, PKC, or PKA, which are activated by calcium and also implicated in memory [167], [175] (Fig. 24). Other transcriptional machinery is also recruited for transcription such as CREB-regulated transcription coactivator 1 (CRTC1) which interacts with CREB, and NCOA1 which interacts with CBP [180], [187]. Both CRTC1 and NCOA1 are implicated in long-term memory with overexpressing CRTC1 increasing CREB mediated transcription and memory performance [282] while decreasing NCOA1 decreases spine density and impairs spatial memory [283]. While the aforementioned factors interact together for transcription, they are also implicated in synaptic plasticity and memory with changes in total protein expression impacting these functions. Additionally, while intermediate and long-term memory includes the effects of transcription and protein synthesis, short-term memory does not allow time for protein synthesis to occur [105], [106], [284]. Ethanol in adolescence or early life decreases CBP protein expression in the amygdala and cerebellum [59], [71] and CREB protein expression in the amygdala, PFC, and HPC [59], [60]. Meanwhile, Wolstenholme [63] found decreased gene expression of *Cbp*, *Ncoa1*, and *Crtc1* and increased expression of potassium large conductance calcium-activated channel, subfamily M, beta member 1 (*Kcnmb1)*, the beta 1 (β1) subunit of the BK channel, 3 weeks after adolescent

ethanol exposure in the mPFC. The BK channel is a calcium and voltage sensitive potassium channel with channel activation differing by voltage, intracellular calcium, intracellular magnesium, and subunit composition [285]. The β1 subunit, found in smooth muscle tissue and vasculature but limitedly in the brain, is associated with increased calcium but decreased voltage sensitivity [286]. In myocytes ethanol effects differ with subunit composition, where KO of β1 potentiates channel opening, while wild type controls with native BK channels displayed channel inhibition [286]. Channel inhibition by β1 is further driven by calcium levels in oocytes with additional β1 switching the channel from open to closed at lower calcium concentrations (about 3 μM) than wild type controls [286]. Meanwhile in the brain, BK channels play a role in action potential hyperpolarization and refractory period with BK channels generally closed at rest [285], [287]. Differences in BK channel state and activity appears to differ by the factors mentioned above but also brain region and the presence of other channels involved in calcium and potassium signaling [285]. Deletion of the β1 subunit is associated with elevated risk for alcohol dependence [285], accelerated ethanol escalation [288], and reduced tolerance to ethanol sedation [289]. While several studies have explored β1 channel properties in relation to ethanol or ethanol behaviors, the Wolstenholme [63] study is the first study to our knowledge showing altered β1 subunit gene expression in the brain following ethanol exposure. Within the Nac, BK channels occasionally open at resting membrane potential while as the membrane depolarizes the channel opens more frequently towards to state of permanently open [287]. BK channels also increase channel potentiation at -40 mV as calcium concentration increases [287]. Ethanol potentiates BK channels in the Nac in the soma but not the dendrites likely mediated by beta 4 (β4) [287]. Indeed Martin [287] found β4 increased open probability with ethanol, while β1 did not. Therefore, the increase in β1 following adolescent ethanol may be a means of decreasing

ethanol sensitivity in the brain but may also alter BK channel potentiation in the absence of ethanol due to increased calcium sensitivity. As CBP manipulation in the dHPC and mPFC impairs spatial and recognition memory [185], [193], [195], [196], understanding the protein landscape of both brain regions may provide further insights into ethanol-induced changes in memory performance.

The purpose of this study was to investigate the impact of binge ethanol in adolescence or adulthood on the expression of proteins involved in memory, the protein-protein interactions of CREB, and whether CREB-mediated gene expression was altered by adolescent ethanol. We hypothesized that CBP, NCOA1, and CRTC1 protein expression would be decreased by adolescent but not adult ethanol exposure and that other signaling proteins (CREB, CAMKIV, CAMKII, CAM, PKA, PKC) may also be decreased in the adolescent cohort (Fig. 24). Meanwhile, we hypothesized that CREB protein interactions and CREB-mediated gene expression would be decreased by adolescent ethanol exposure. This study found that CREB, but not CBP, protein expression was altered by an interaction of ethanol exposure and sex in the mPFC and dHPC, but CREB protein interaction with CAMKIV and CREB-mediated gene expression was not altered by ethanol exposure. Lastly, upstream regulator analysis revealed that genes related to cell proliferation, immune response, neuronal synapse assembly, and hippocampal dendritic length are mediating gene expression changes following adolescent ethanol exposure. This study suggests that CREB protein expression is altered by ethanol exposure in a sex-dependent manner, but gene expression changes following adolescent ethanol is mediated by other genes besides CREB.

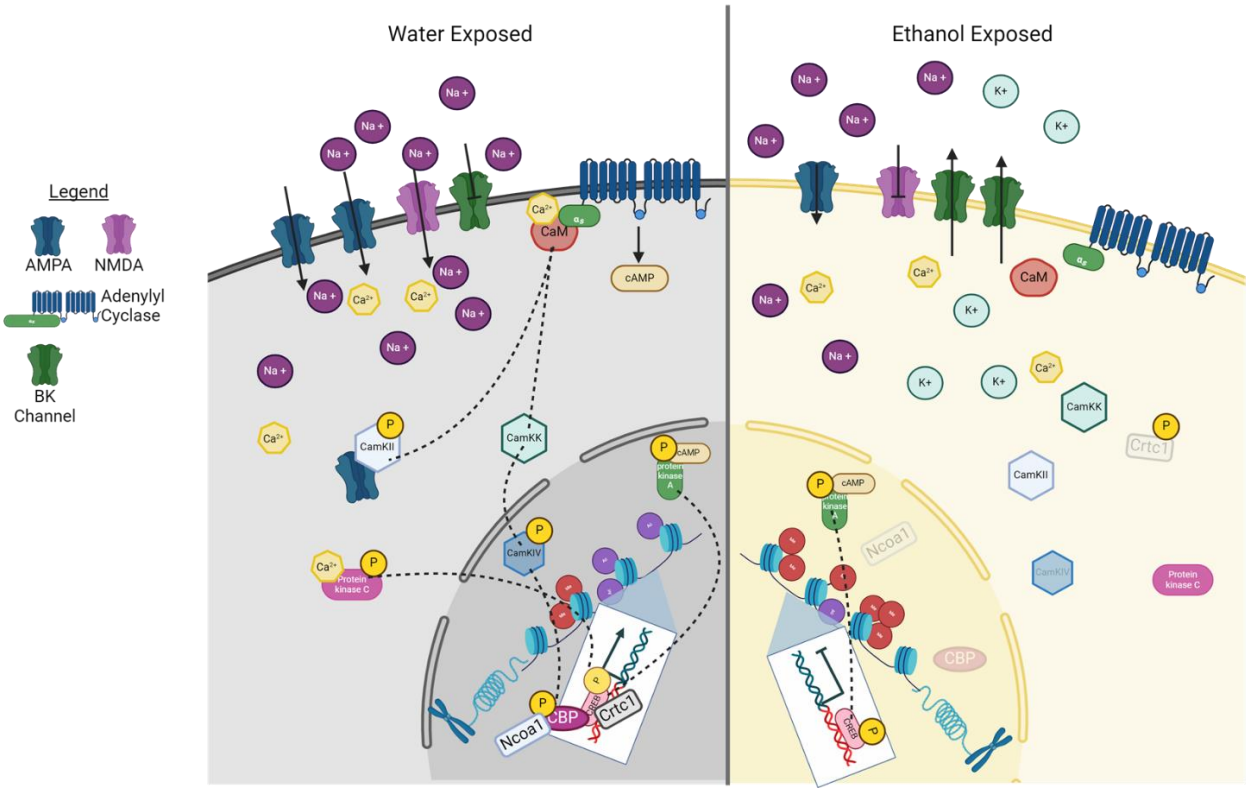

Fig 24. Hypothetical cellular cascade in a glutamatergic neuron following adolescent ethanol exposure. Following a three-week ethanol abstinence period from adolescent ethanol exposure, we hypothesize DBA/2J mice will have decreased CBP, NCOA1, and CRTC1 protein expression which may disrupt CREB mediated transcription. Meanwhile increases in the β1 subunit of the BK channel may have altered the receptor leading to potentiation, and potassium efflux, in a low calcium enviornment. Therefore, the NMDAR may not open leading to decreased calcium internally inhibiting calcium mediated kinase activation of CAMKII, CAMKIV, CAM, PKA, and PKC in the ethanol exposure animals. Meanwhile, we hypothesize water exposed mice have uninterrupted calcium signaling and normal CBP, NCOA1, and CRTC1 protein expression leading to CREB mediated transcription.

4.2 Materials and Methods

4.2.1 Animals and Binge Ethanol Paradigm

DBA2/J male and female mice were ordered from Jackson East Laboratory (Bar Harbor, ME) and were housed in facilities at Virginia Commonwealth University with food and water available ad libitum until tissue collection. The adolescent cohort was ordered at PND 19-21 while the adult cohort was ordered at PND 58-60. Mice were housed 4-5/cage with same-sex conspecifics on a 12:12 light-dark cycle. Both age cohorts underwent the same chronic intermittent ethanol paradigm [63], [268] in which ethanol (25% w/v) or water was administered by oral gavage for 2 days followed by 2 days of abstinence. Mice were habituated to the oral gavage method with 2 days of 0.1% saccharine administration. The ethanol/water dosing window for the adolescent cohort was PND 29-42 while the adult cohort was dosed from PND 64-77 and resulted in 4 groups per age cohort, control males (CM), ethanol males (EM), control females (CF), and ethanol females (EF). Following the last ethanol, dose mice were left undisturbed for three weeks at which point brain tissue was collected for further analysis (Fig. 25). In experiment 1, the adolescent-exposed and adult-exposed cohorts consisted of 8/group (n=32). The adolescent-exposed cohort lost 1CF due to issues affiliated with gavage, while the adult-exposed cohort lost two mice (1 CF, 1 EM). Experiment 2 used the same dHPC samples as experiment 1 but, due to use in the prior experiment and needing a large volume of lysate, the adolescent-exposed cohort contained 6-8/group, while the adult-exposed cohort included 3-6/group. Experiment 3 used the gene lists from [63] DBA2/J male and female mice (n=39) in which tissue was collected at PND 66 following the same adolescent binge ethanol paradigm described above with 1 CF lost during the microarray process. All animal housing and care were conducted with

the approval of the Virginia Commonwealth University IACUC Committee and in accordance with the NIH Guide for the Care and Use of Laboratory Animals [218].

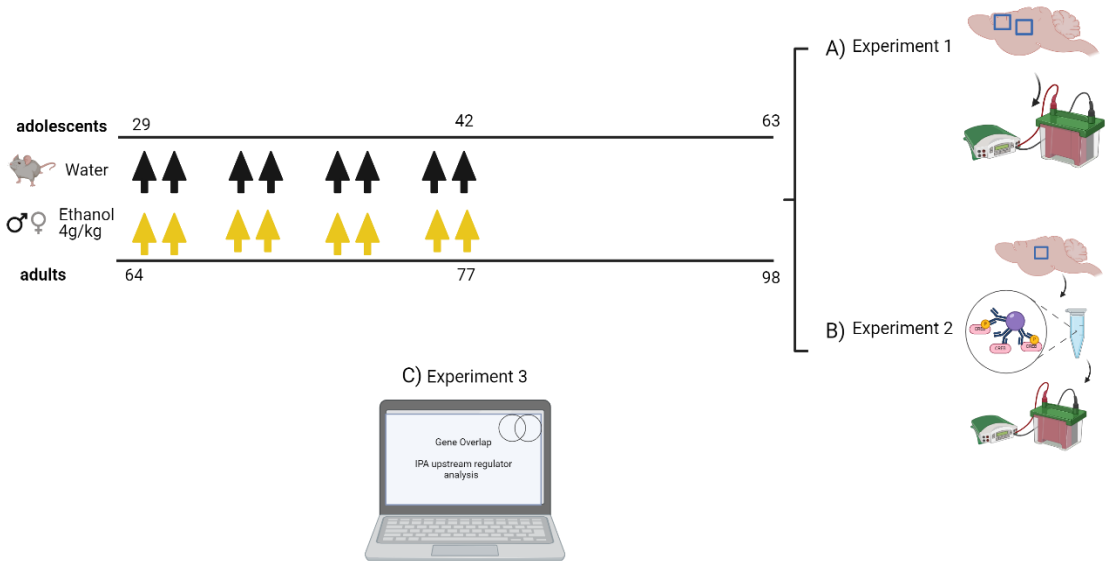

Fig 25. Timeline of Chapter 4 experimental procedure. Adolescent and adult DBA/2J mice underwent the same intermittent ethanol dosing paradigm from either postnatal (PND) 29-42 or PND 64-77. Three weeks following binge ethanol exposure, mPFC and dHPC tissue were collected for A) western blot analysis, while dHPC tissue was only used in B) co-immunoprecipitation analysis. C) A gene list from Wolstenholme *et al* 2017 was used for the bioinformatics analysis. Figure created in Biorender.

4.2.2 Western Blot Analysis

Following the last ethanol dose mice were left undisturbed for three weeks, until PND 63 or 98 for the adolescent-exposed and adult-exposed cohort, respectively. At the respective ages, whole brains were collected, following cervical dislocation, rapid decapitation, and flash freezing, for further analysis in experiment 1. Animal dosing and tissue collection occurred at separate times for the adult and adolescent cohorts, after which all western blot analysis was performed for both age cohorts at the same time. Whole brains were stored in a -80-degree freezer until mPFC and dHPC tissues were dissected from the frozen brain using a cryostat to maintain the tissue at -16°C. mPFC was collected from a midline wedge shaped cut from 1.4 mm to 0.5 mm from Bregma and from dHPC collected from -1.5 mm to -3.5 mm [224]. Frozen tissue was homogenized using sonication in a lysis buffer (1M Tris, pH 8.0, 5M NaCl, 1% NP 40). The Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, Rockford, IL) was used to determine protein concentration. Ten microgram samples were run with 10% NuPage 10X Sample Reducing Agent (ThermoFisher Scientific, Rockford, IL), 25% LiCor 4X Sample Loading Buffer (LI-COR Biosciences, Lincoln, NE), lysis buffer, and 1X Halt™ Protease Inhibitor Cocktail (ThermoFisher Scientific, Rockford, IL). Samples were heated for 10 minutes at 70°C then samples were separated with gel electrophoresis. To assess proteins > 100 kDa, samples were run on 3-8% tris-acetate gels (ThermoFisher Scientific, Rockford, IL) with the Spectra™ Multicolor High Range Protein Ladder (ThermoFisher Scientific, Rockford, IL). Meanwhile, proteins < 100 kDa were run on 4-12% bis-tris gels (ThermoFisher Scientific, Rockford, IL) with LiCor Chameleon Kit Pre-Stained Protein Ladder (LI-COR Biosciences, Lincoln, NE). Gels were run at 125V for approximately 1 hour then transferred wet overnight (14-16 hours) onto an Immobilon®-FL PVDF Membrane (Millipore, Merck KgaA, Darmstadt, Germany) at 4°C at

10V. Membranes were blocked for 1 hour at room temperature with LiCor Intercept (TBS) Blocking Buffer (LI-COR Biosciences, Lincoln, NE), then incubated in primary antibody in LiCor Intercept (TBS) Blocking Buffer (LI-COR Biosciences, Lincoln, NE) rotating for 19 hours at 4°C. The following antibodies and concentrations used in this experiment can be found in Table 6. While phosphorylated CREB (pCREB) or CBP (pCBP) are involved in transcription and is measured to determine activity of said protein, this study did not include these phosphorylated proteins as the focus was not on protein activity. Rather the focus of the study was to investigate protein expression changes following ethanol that may be associated with both the short-term memory, protein synthesis independent, and long-term memory, protein synthesis dependent, memory deficits [284]. Additionally, the literature has observed decreased CREB protein expression in the amygdala and cingulate gyrus [59], [64] following adolescent ethanol exposure indicating ethanol can decrease CREB protein expression. Membranes were then washed and incubated with a Licor IRDye® 680RD Donkey anti-Rabbit IgG Secondary Antibody, IRDye® 800CW Donkey anti-Rabbit IgG Secondary, or IRDye® 800CW goat anti-Mouse IgG Secondary Antibody (LI-COR Biosciences, Lincoln, NE) in LiCor Intercept (TBS) Blocking Buffer (LI-COR Biosciences, Lincoln, NE) for 1 hour at room temperature at a 1:15,000 concentration. Membranes were imaged for fluorescence with a Li-Cor Odyssey Scanner (LI-COR Biosciences, Lincoln, NE). Proteins were quantified for integrated density via ImageJ (NIH, Bethesda, Maryland) [290] and normalized to Beta-actin as the reference control. An age difference was observed in beta-actin, as well as GAPDH and total protein density via three-way ANOVA, so an adjustment factor per blot was used to correct for the age effect. For each set of blots, the average adult-exposed and adolescent-exposed beta-actin integrated density was determined. Then, the difference between the two age cohorts was determined. The

integrated density for each adolescent-exposed beta-actin well was then divided by the age density difference to create a new age-adjusted beta- actin integrated density. Adolescent-exposed proteins of interest were then normalized to the newly adjusted beta-actin. Some samples did not express a quantifiable band for the various 9 antibodies resulting in a different number of total animals per target but resulted in 6-8/group in the mPFC samples and 4-8/group in the dHPC.

Table 6. List of primary antibodies

Table 6				
Protein	**Manufacturer**	**Catalogue Number**	**Host Species**	**Concentration**
CBP	Invitrogen	PA5-27369	Rabbit	1:500
NCOA1	Invitrogen	PA1-840	Rabbit	1ug/mL
CRTC1	Invitrogen	PA5-17365	Rabbit	1:500
CREB	Proteintech	12208-1-AP	Rabbit	1:500
CAMKIV	Invitrogen	PA1-542	Rabbit	1:500
CAMKII	Invitrogen	PA5-17095	Rabbit	1:500
CAM	Proteintech	10541-1-AP	Rabbit	1:8000
PKC	Thermo Fisher Scientific	PA5-17551	Rabbit	1:500
PKA	Proteintech	24503-1-AP	Rabbit	1:500

4.2.3 Co-immunoprecipitation Analysis

In experiment 2, the same dHPC tissue used for immunoblots was used following the sample preparation and protein quantification in experiment 1. Due to the quantity of protein needed, multiple aliquots from a single animal were pooled and, in some cases, there was not enough lysate to run a sample. To preserve the number of biological replicates, samples were not pooled. This led to n=6-8/group in the adolescent-exposed cohort, while the adult-exposed cohort included 3-6/group. The Pierce™ Protein A/G Magnetic Beads manual immunoprecipitation protocol (ThermoFisher Scientific, Rockford, IL) was followed with the following modifications. 500 ug of protein lysate was combined with 0.5 ug/ul of CREB1 Polyclonal antibody (Proteintech Group, Inc, Rosemont, IL) in a total volume of 500 ul of lysis buffer (1M Tris, pH 8.0, 5M NaCl, 1% NP 40) and 1X Halt™ Protease Inhibitor Cocktail (ThermoFisher Scientific, Rockford, IL) and rotated for 24 hours at 4°C. The samples were washed as indicated in the manufacturer's protocol and eluted at room temperature for 10 minutes in 50 ul of elution buffer (5 ul of NuPage 10X Sample Reducing Agent (ThermoFisher Scientific, Rockford, IL), 12.5 ul of LiCor 4X Sample Loading Buffer (LI-COR Biosciences, Lincoln, NE), and 32.5 ul of lysis buffer with 1X Halt™ Protease Inhibitor Cocktail). Following elution, samples were separated with gel electrophoresis following 10 minutes on a heat block at 70°C. Samples were run on a tris-acetate gel (ThermoFisher Scientific, Rockford, IL) with the Spectra™ Multicolor High Range Protein Ladder (ThermoFisher Scientific, Rockford, IL) at 120 V for 1 hour. Proteins were then transferred wet overnight (14 hours) onto an Immobilon®-FL PVDF Membrane (Millipore, Merck KgaA, Darmstadt, Germany) at 4°C at 10V. Following the transfer, membranes were cut into 4 horizontal strips cut just between the ladder mark for 150 kDa, 65 kDa, and 50 kDa. Membranes were blocked for 1 hour at room temperature with LiCor Intercept

(TBS) Blocking Buffer (LI-COR Biosciences, Lincoln, NE) then incubated in primary antibody rotating for 19 hours at 4°C. The following antibodies and concentrations used in this experiment can be found in Table 6. Membranes were then washed and incubated with a Licor IRDye® 680RD Donkey anti-Rabbit IgG Secondary Antibody or IRDye® 800CW Donkey anti-Rabbit IgG Secondary (LI-COR Biosciences, Lincoln, NE) for 1 hour at room temperature. Membranes were imaged for fluorescence with a Li-Cor Odyssey Scanner (LI-COR Biosciences, Lincoln, NE) and bands were quantified with ImageJ (NIH, Bethesda, Maryland) using integrative density. Phosphorylated CREB was not part of this analysis as CREB interactions with CRTC1 and CAMKIV is not dependent on phosphorylation [175], [291] and the observed ethanol effect was occurred in total CREB. In the adult cohort, 3 EF did not express CREB following the co-immunoprecipitation process. Meanwhile, following CAMKIV primary antibody hybridization, the adolescent-exposed and adult-exposed cohort showed no band, due to a lack of antibody binding, and was not used for further analysis (adolescent-exposed: 5 CM, 2 EM, 2 CF, 3EF; adult-exposed: 1 EM, 1 CF, 2 EF).

4.2.4 Bioinformatics Analysis

To investigate if CREB-mediated gene expression was altered due to changes in protein expression, a gene overlap analysis was performed using a gene list from Wolstenholme [63] which found genes that differed due to adolescent ethanol exposure from Signal space transformation RMA (sstRMA) analysis. Genes changed due to adolescent ethanol from Wolstenholme [63] (n=624) and Zhang [292] (n=3,554) list of genes with full (TGACGTCA) or half CRE (TGACG/CGTCA) sequences upstream genes on the mouse genome (mm3 released in 2002) sequence was compared using the Bioconductor package GeneOverlap v1.32.0. We chose the Zhang [292] CRE dataset as other datasets used microarrays, and therefore only examined a

subset of genes, or conducted in other tissue samples than the brain. Genes found to overlap between the two lists were assessed for molecular function, biological processes, and cellular components using the gene ontology over-representation analysis from ToppFun compared to the human genome [293]. A false discovery rate of 1 for a gene limit of ≥ 2 and ≤ 1000 were included in the analysis with the top 5 molecular function, biological process, and cellular components are represented later below. Next, to investigate if CREB was a potential upstream regulator of the differentially expressed genes in Wolstenholme [63] and to also identify other upstream genes which may impact the differential gene profiles observed in Wolstenholme [63] due to adolescent ethanol exposure, an upstream regulator analysis using software from Ingenuity Pathway Analysis (IPA) (Qiagen, Redwood City, CA) was conducted [294]. The upstream regulator analysis uses IPA's ingenuity knowledge base of approximately 5 million findings to determine an overlap p-value and activational Z-score. The overlap p-value measures enrichment of the network regulated genes in the investigator's database by measuring a significant overlap between the investigator's database and genes regulated by a regulator in IPA's database using a Fisher's exact test. While the Z-score finds likely regulating targets based on IPA database of a significant up or down regulation. In some cases, the database has an equal weight of evidence predicting up and down regulation resulting in a Z-score=0.

4.2.6 Statistics

The western blot data was analyzed via three-way ANOVSs with sex, treatment, and age as the factors. While 9 proteins were assessed in the analysis, a multiple test correction was not performed as the method is typically used when assessing -omics data with thousands of targets of interest. Additionally, in these analyses, each protein assessed is considered an independent experiment. The multiple correction on such a low number of targets in this study could

overcorrect occluding possible biological effects due to ethanol. Co-immunoprecipitation data was analyzed between age cohorts using three-way ANOVAs with ethanol treatment, age, and sex as factors. If a significant interaction occurred ($p<0.05$), a Tukey's post hoc test was performed. P-values less than $\alpha=0.05$ were considered significant. Data was analyzed with GraphPad Prism version 10.0.2 (GraphPad Software, San Diego, CA) with all data presented as mean + standard error of the mean (SEM).

4.3 Results

4.3.1 Binge ethanol increased CRTC1 and CAMKII protein expression in the mPFC, while ethanol and sex alter CREB expression.

We previously reported the gene expression of *Cbp, Ncoa1,* and *Crtc1* were decreased by adolescent ethanol three weeks after the last ethanol exposure [63]. To assess changes in protein expression of these targets, CREB, and the upstream kinases for CREB and CBP activation, the dHPC and mPFC were dissected three weeks following the last ethanol exposure in adolescent and adult exposed mice of both sexes. Within the mPFC, two proteins showed a main effect of ethanol with ethanol increasing CRTC1 [$F (1, 49) = 4.136$, $P=0.0474$] and CAMKII expression [$F (1, 50) = 4.625$, $P=0.0364$] (Fig. 26 D&H). Meanwhile, CREB expression showed a treatment*sex interaction [$F (1, 47) = 9.576$, $P=0.0033$] (Fig. 26 E). A Tukey's multiple comparison test revealed that within the control animals, adult-exposed males were significantly different from the adult-exposed females and adolescent-exposed males. The other quantified proteins did not show a main effect of ethanol treatment on protein expression (CBP [$F (1, 47) = 0.6301$, $P=0.4313$], NCOA1 [$F (1, 48) = 0.8732$, $P=0.3547$], CAMKIV [$F (1, 51) = 1.345$, $P=0.2516$], CAM [$F (1, 50) = 0.08833$, $P=0.7675$], PKC [$F (1, 49) = 0.09406$, $P=0.7604$], PKA [$F (1, 50) = 1.268$, $P=0.2655$]). Several proteins showed a main effect of age in which adult-

exposed animals had a greater protein expression than adolescent exposed mice (CRTC1 [F (1, 49) = 11.47, P=0.0014], CREB [F (1, 47) = 14.89, P=0.0003], CAMKIV [F (1, 51) = 23.98, P<0.0001], CAMKII [F (1, 50) = 23.52, P<0.0001], PKC [F (1, 49) = 12.51, P=0.0009], and PKA [F (1, 50) = 23.46, P<0.0001]). CAMKIV also showed an age*sex interaction [F (1, 51) = 7.564, P=0.0082], in which control adult-exposed males had greater CAMKIV expression than control adult-exposed females and adolescent-exposed males (Fig. 26 F). CBP [F (1, 47) = 2.704, P=0.1067], NCOA1 [F (1, 48) = 3.594, P=0.0640], and CAM [F (1, 50) = 1.108, P=0.2976] did not show a main effect of age (Fig. 26 B, C, &G). Lastly, sex impacted the expression of two proteins CREB and CAMKIV via a treatment*sex and age*sex interaction respectively mentioned above. No other proteins displayed a sex effect (CBP [F (1, 47) = 0.4053, P=0.5275], NCOA1 [F (1, 48) = 0.06127, P=0.8056], CRTC1 [F (1, 49) = 4.010, P=0.0508], CAM [F (1, 50) = 0.01002, P=0.9207], CAMKII [F (1, 50) = 0.6934, P=0.4090], PKC [F (1, 49) = 0.2552, P=0.6157], and PKA [F (1, 50) = 1.361, P=0.2488]) (Fig. 25 B-J).

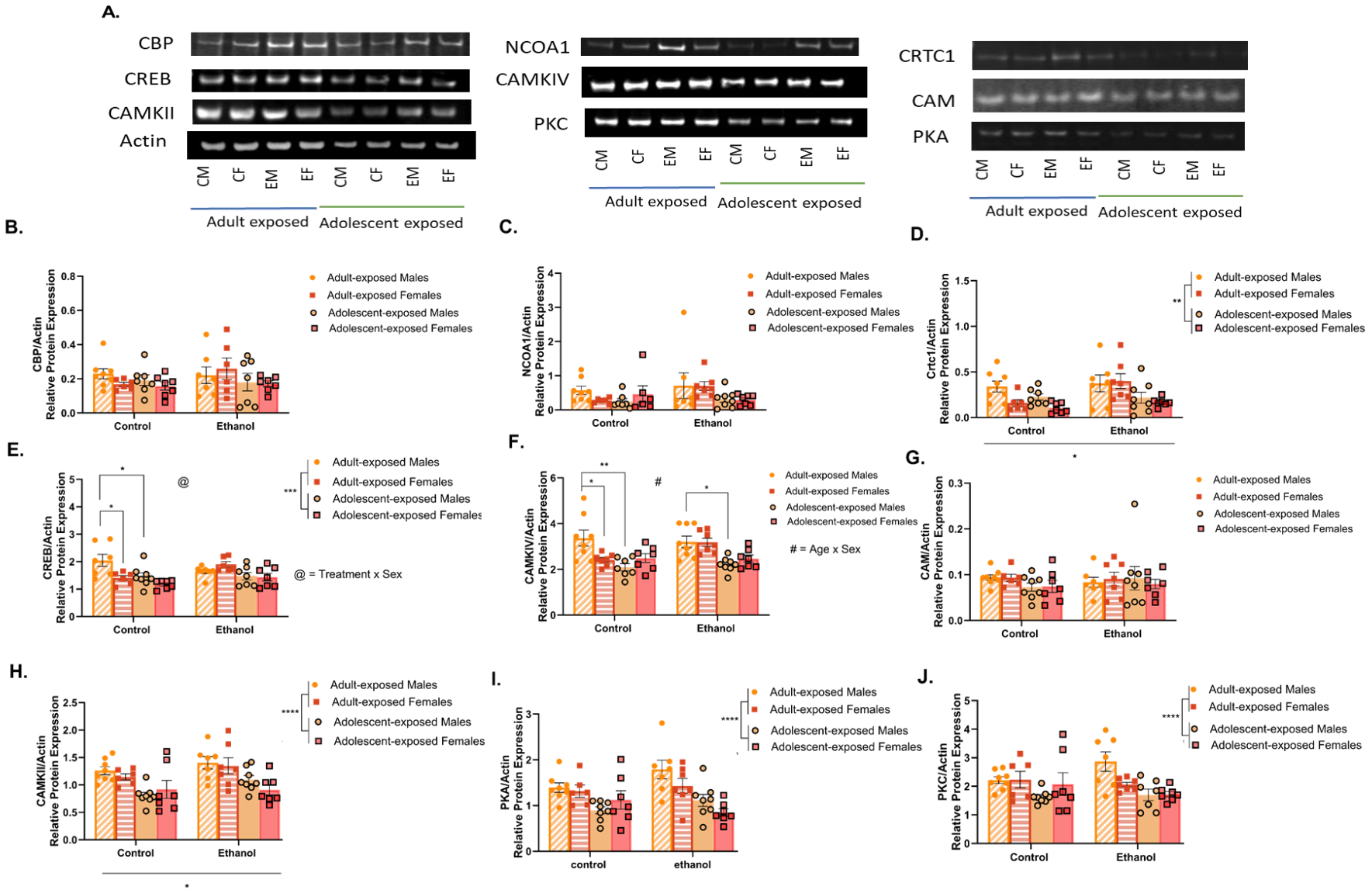

Fig 26: mPFC protein expression changes in adolescent and adult-exposed mice impact CREB, CRTC1, and CAMKII. E) CREB protein expression showed a treatment*sex interaction in all groups with control adult-exposed males showing greater expression than control adult-exposed females or adolescent-exposed males. D) CRTC1 and H) CAMKII also showed an ethanol effect with increased protein expression in ethanol-exposed animals from both age cohorts. F) CAMKIV also showed an age*sex effect with adolescent-exposed males showing decreased expression compared to their adult-exposed counterparts. Several proteins differed by age but not ethanol treatment or sex. Adult-exposure cohort and adolescent-exposed cohort (n=6-8/group). Data is presented as mean +/- SEM. (*, $p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$, ****$p \leq 0.0001$)

4.3.2 CREB protein expression in the dHPC was modulated by ethanol and sex.

Next, we examined the protein expression in the dHPC as the brain region plays a role in spatial and object recognition. One protein, CREB, showed an ethanol effect via a treatment*sex interaction [F (1, 51) = 4.767, P=0.0336] with no significant posthoc comparisons (Fig. 27 D). Meanwhile, the other proteins showed no effect due to ethanol (CBP [F (1, 49) = 1.086, P=0.3024], NCOA1 [F (1, 51) = 0.8680, P=0.3559], CRTC1 [F (1, 34) = 0.09151, P=0.7641], CAMKIV [F (1, 50) = 0.4668, P=0.4976], CAM [F (1, 51) = 0.3934, P=0.5333], CAMKII [F (1, 48) = 0.1714, P=0.6807], PKC [F (1, 48) = 0.8075, P=0.3733], and PKA [F (1, 50) = 1.313, P=0.2573]). Age again impacted several proteins with the adult-exposed animals showing increased expression compared to the adolescent-exposed cohort (CBP [F (1, 49) = 4.514, P=0.0387], NCOA1 [F (1, 51) = 8.234, P=0.0060], CAMKIV [F (1, 50) = 16.88, P=0.0001], CAMKII [F (1, 48) = 5.319, P=0.0254], and PKC [F (1, 48) = 4.485, P=0.0394]) (Fig. 27 B-J). CRTC1 showed an age*sex interaction [F (1, 34) = 5.339, P=0.0271] in which no significant Tukey's comparisons were found (Fig. 27 D). Three proteins, CREB [F (1, 51) = 3.385, P=0.0716], CAM [F (1, 51) = 0.7925, P=0.3775], and PKA [F (1, 50) = 1.536, P=0.2210] showed no effect due to age. Lastly, two proteins, CBP [F (1, 49) = 5.990, P=0.0180] and CRTC1 [F (1, 34) = 8.023, P=0.0077], showed a main effect of sex with males expressing more protein than females (Fig. 27 B&D). Sex interactions impacted NCOA1 and CREB with an age*sex [F (1, 51) = 4.418, P=0.0405] and treatment*sex interaction, respectively (Fig. 27 C). No other proteins showed a sex effect (CAMKIV [F (1, 50) = 0.1631, P=0.6880], CAM [F (1, 51) = 0.0001596, P=0.9900], CAMKII [F (1, 48) = 0.2044, P=0.6532], PKC [F (1, 48) = 0.02129, P=0.8846], and PKA [F (1, 50) = 0.4796, P=0.4918]).

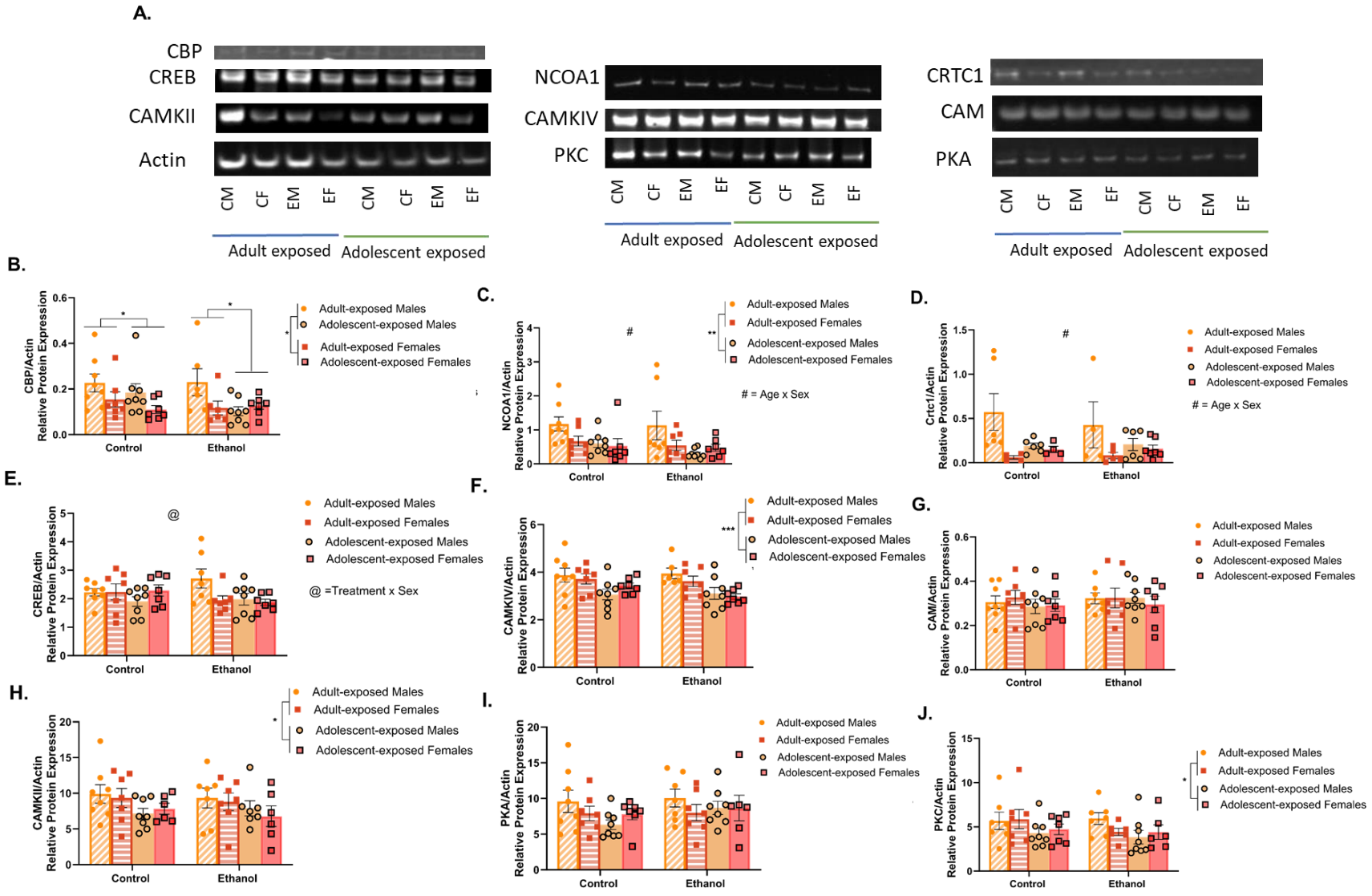

Fig 27. CREB protein expression impacted by ethanol and sex in the dHPC. E) CREB protein expression showed a treatment*sex interaction in all groups. While B) CBP, C) NCOA1, and D) CRTC1 show a sex effect. Several proteins differed by age but not ethanol treatment or sex. adult-exposure cohort (n=4-8/group) adolescent-exposed cohort (n=6-8/group). Data is presented as mean +/- SEM. (*, p≤0.05, **p≤0.01, *** p≤0.001, ****p≤0.0001)

4.3.3 CREB-CAMKIV protein interaction was not impacted by binge ethanol in any group.

As the protein expression data had shown that CREB expression was modulated by an ethanol*sex interaction, we next investigated if CREB protein interactions with CBP, CRTC1, or CAMKIV were altered due to ethanol, age, or sex in the dHPC. CBP and CRTC1 were not found bound to CREB or in the input samples (data not shown) suggesting protein degradation. Meanwhile, CAMKIV expression was present in some samples (Fig. 28 A) and a subsequent three-way ANOVA was performed on the ratio of CAMKIV to CREB. We determined no main effect of treatment [F (1, 25) = 0.0002194, P=0.9883], age [F (1, 25) = 0.006026, P=0.9387], or sex [F (1, 25) = 0.4158, P=0.5249] (Fig 28 B). A separate three-way was run on the pull-down on CREB and CAMKIV to assess the protein expression change following the pull-down process. The CREB pull-down showed an age*sex interaction effect [F (1, 38) = 8.636, P=0.0056] but no significant group differences were observed following a Tukey's post hoc test (Fig 28 A & C). Meanwhile, no main effect was found due to ethanol treatment [F (1, 38) = 2.064, P=0.1590], age [F (1, 38) = 1.131, P=0.2943] or sex [F (1, 38) = 1.809, P=0.1867] on CREB immunoprecipitation expression. Lastly, CAMKIV expression following CREB pull down found no effect due to treatment [F (1, 26) = 2.480, P=0.1274], age [F (1, 26) = 1.281, P=0.2680], or sex [F (1, 26) = 0.9429, P=0.3405] (Fig 28 A & D).

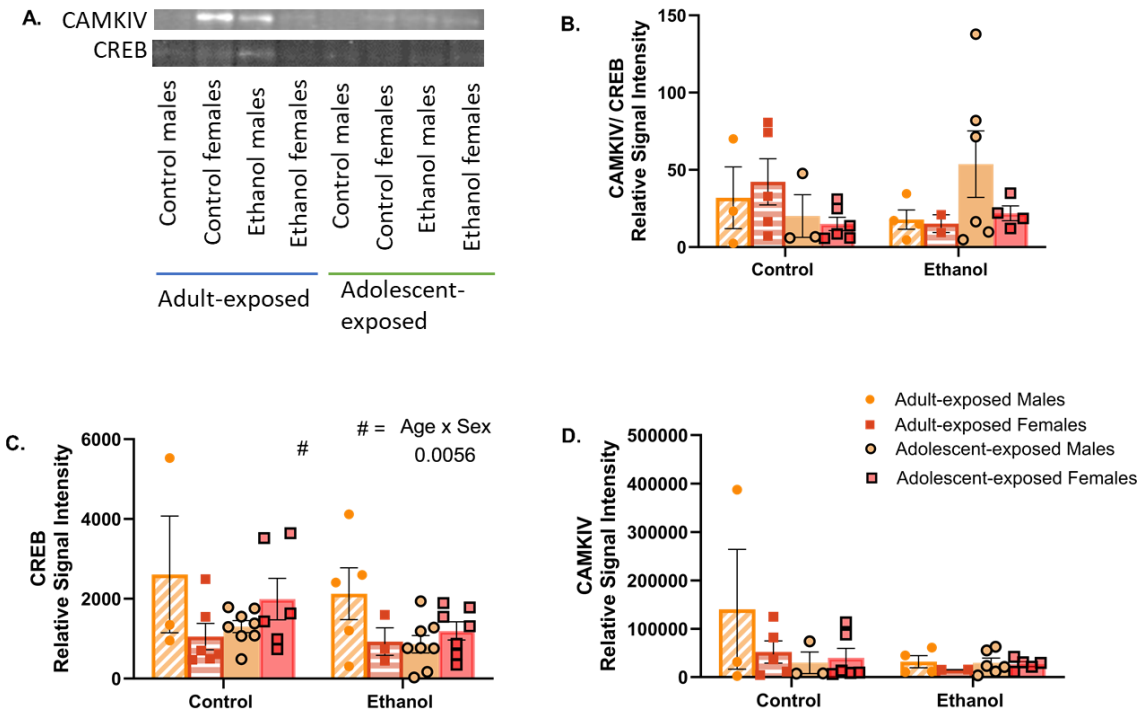

Fig 28. Ethanol does not impact CREB protein interaction with CAMKIV. Following CREB co-immunoprecipitation A) CREB and CAMKIV were detected in all assessed groups. B) The ratio of CAMKIV relative to CREB pull-down and D) CAMKIV immunoprecipitation was not altered by prior ethanol exposure, age, or sex. C) CREB immunoprecipitation showed an age*sex interaction but no difference due to ethanol. CAMKIV adult-exposure cohort (n: CM=3, EM=4, CF=5, EF=2), adolescent-exposed cohort (n: CM=3, EM=6, CF=6, EF=5). CREB adult-exposure cohort (n: CM=3, EM=5, CF=6, EF=3), adolescent-exposed cohort (n: CM=8, EM=8, CF=6, EF=7). Data is presented as mean +/- SEM. (*, $p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$, ****$p \leq 0.0001$)

4.3.4 Genes changed by adolescent ethanol exposure are not CRE-mediated.

CREB is a transcription factor responsible for regulating about 16% of genes in the mouse genome [292]. To determine if CREB-mediated genes significantly overlapped with genes changed by adolescent ethanol from Wolstenholme [63], we compared the Wolstenholme dataset to a gene list from Zhang [292] which found the full CRE sequence (TGACGTCA) and half CRE sequence (TGACG/CGTCA) on the mouse genome using a Fisher's exact test. We found there was no significant overlap (p=1) between the two datasets (Wolstenholme genes=624, Zhang genes=3,554) suggesting that genes changed by adolescent ethanol exposure were not significantly CRE mediated (Fig. 29). Gene ontology analysis of the 59 common genes between the two lists revealed molecular functions and cellular components related to transcription while biological processes related to learning and synaptic signaling (Table 7).

Fig 29: CRE-based genes do not make up a significant portion of genes changed by adolescent ethanol. Fisher's exact test determined there was there was not a significant overlap of genes between CRE sequence genes and ethanol-altered genes.

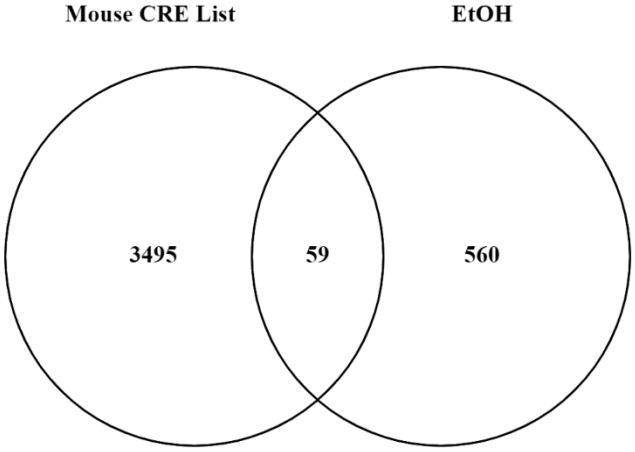

Table 7. Gene ontology for overlapping CRE-mediated genes altered by adolescent ethanol exposure

Table 7	
Go: Moleular Function	
Name	**p-value**
DNA-binding transcription activator activity, RNA polymerase II-specific	4.87E-08
DNA-binding transcription activator activity	5.36E-08
transcription factor binding	1.13E-06
RNA polymerase II-specific DNA-binding transcription factor binding	2.95E-06
DNA-binding transcription factor binding	5.43E-06
Go: Biological Process	
Name	**p-value**
learning	2.84E-05
anterograde trans-synaptic signaling	4.55E-05
chemical synaptic transmission	4.55E-05
trans-synaptic signaling	4.87E-05
synaptic signaling	6.81E-05
Go: Cellular component	
Name	**p-value**
transcription regulator complex	4.07E-05
transcription repressor complex	1.76E-03
ATF1-ATF4 transcription factor complex	5.54E-03
CHOP-ATF4 complex	5.54E-03
RNA polymerase II transcription regulator complex	7.02E-03

4.3.5 Upstream regulator analysis finds CD40 and NRG1 involved in neuronal synapse assembly and dendritic length.

As there was not a significant overlap between CRE mediated genes [292] and genes changed by adolescent ethanol exposure [63], we next investigated which upstream genes may be involved in the adolescent ethanol gene profile observed in Wolstenholme [63]. Using the IPA software specifically the upstream regulator analysis, we filtered for targets that were genes or proteins and selected the top 5 targets with the lowest p-value of overlap, which is a metric of enrichment of the particular regulator. The 5 upstream targets, their roles, and the genes they regulate in our dataset can be found in Table 8. Three of the 5 upstream targets, Colony stimulating factor 2 receptor beta (CSF2RB), Cluster of differentiation 40 (CD40), and Neuregulin 1 (NRG1) are involved in cell proliferation and the immune response, while 2 (CD40, NRG1) are also involved in neuronal synapse assembly and dendritic length. Overall, the upstream regulator analysis revealed targets with roles in cell proliferation, immune response, neuronal synapse assembly, and dendritic length that are impacting the ethanol adolescent-exposed mPFC in adulthood.

Table 8. Upstream regulators of genes changed by adolescent ethanol exposure

Table 8			
p-value of overlap	**Upstream Target**	**Function**	**Genes in Wolstenholme dataset**
2.06E-04	CSF2RB	Role in cell survival, proliferation, and differentiation	BCL2L1,ITGAM,JUN,SNAP23
5.88E-04	Cdkal1	Role in translation and tRNA methylthiolation	CHGA,PTPRN
8.10E-04	miR-122-5p	Role in tumor suppression and cardiac function	BCKDK,EGLN3,MAPK11,NUMBL,SLC7A1,TTYH3,UBAP2
1.17E-03	NRG1	Role in synapse assembly and neurogensis	ASCL1,ATF4,BCL2L1,CAPN1,DAD1,DUSP6,HMGCR,ITGAM,JUN,JUND,L1CAM,NR4A1, NR4A3,PDCD4,UCN
1.32E-03	CD40	Role in immune and inflammation response, cell survival, and dendritic length	ATF4,BCL2L1,CR2,CX3CL1,DUSP6,IL10RA,IL27,ITGAM,JUN,Ly6a,REL,SMAD7

4.4 Discussion

Binge ethanol exposure is known to impair spatial and object recognition memory [16], [235], [251]–[253], which utilize the dHPC and mPFC depending on the temporal timing of the paradigm [138], [139], [142], [143], [151]. Successful memory performance depends on a combination of factors including the existing structural components of neurons as well as the cellular composition. CBP is a multifunctional protein involved in spatial and object recognition tasks when short or long-term memory is tested [176], [185], [192], [193], [196]. During intracellular calcium-triggered transcription, CBP interacts with other proteins related to memory, CREB and NCOA1 [167], [175], [180], [187]. Ethanol exposure in early life decreases CBP protein and gene expression [59], [63], [71] which may lead to memory impairments later in life due to disrupted transcription or CBP's role in histone acetylation, neurogenesis, and dendritic morphology [68], [188]–[190]. This study found that CREB, but not CBP protein expression, was altered due to ethanol exposure in a sex-dependent manner in both the dHPC and mPFC. Meanwhile, CRE-mediated genes did not significantly overlap with genes changed by adolescent ethanol exposure, at least in comparison to the Zhang [292] dataset. CREB protein interaction with an upstream kinase, CAMKIV, was also not altered due to ethanol. Lastly, the upstream regulator analysis suggests CSF2RB, CD40, and NRG1 are regulating the gene expression change in the adolescent ethanol-exposed brain. Together, this data suggests changes in CREB protein expression occur in adolescent and adult exposed cohorts following binge ethanol but that CREB is not an upstream regulator of gene expression changes following adolescent binge ethanol exposure. A summary of protein expression changes can be found in Table 9.

Table 9. Summary of Chapter 4 protein expression results

Table 9	Prefrontal Cortex			Hippocampus		
Protein	**Main Effect of Treatment**	**Main Effect of Age**	**Main Effect of Sex**	**Main Effect of Treatment**	**Main Effect of Age**	**Main Effect of Sex**
CBP	NS			NS	adult-exposed > adolescent-exposed	males > females
NCOA1	NS			NS	adult-exposed > adolescent-exposed	Age X Sex interaction
CRTC1	**ethanol > control**	adult-exposed > adolescent-exposed	NS	NS	Age X Sex interaction	males > females
CREB	**Treatment X Sex interaction**	adult-exposed > adolescent-exposed	Treatment X Sex interaction	**Treatment X Sex interaction**	NS	Treatment X Sex interaction
CAMKIV	NS	adult-exposed > adolescent-exposed	Age X Sex interaction	NS	adult-exposed > adolescent-exposed	NS
CAM	NS			NS		
CAMKII	**ethanol > control**	adult-exposed > adolescent-exposed	NS	NS	adult-exposed > adolescent-exposed	NS
PKC	NS	adult-exposed > adolescent-exposed	NS	NS	adult-exposed > adolescent-exposed	NS
PKA	NS	adult-exposed > adolescent-exposed	NS	NS		

We hypothesized that CBP, NCOA1, and CRTC1 protein expression would be decreased due to adolescent but not adult ethanol exposure, while CREB and upstream kinases might also be decreased due to ethanol. This study showed three proteins, CREB, CRTC1, and CAMKII, were altered in the mPFC due to an ethanol*sex interaction or ethanol treatment (Fig.26 D,E,& H), while only CREB had a treatment*sex interaction in the dHPC (Fig. 27 D). While CRTC1 and CAMKII were increased by ethanol in the mPFC, no significant ethanol-related comparisons were found for CREB. This data is not in line with other studies finding decreased or no change in protein expression following chronic ethanol exposure [58], [59], [61], [64], [295], [296]. This may reflect increased protein-mediated function of CAMKII, CRTC1, and CREB as a homeostasis process following chronic ethanol exposure or may reflect dysregulation due to ethanol. CAMKII is known to translocate AMPA receptors and is interacts with many proteins in the post synaptic density, while CRTC1 is involved in dendritic lengthening, in addition to aiding CREB in transcriptional activation [297]–[299]. Chronic ethanol exposure also decreases complex dendritic spine morphology but not length [97] which may be in the process of restoration at the timepoint the tissue was collected for this study. CRTC1 is localized to the dendrites in neurons and transports to the nucleus to interact with CREB following activity-dependent dephosphorylation [300]. Following calcineurin dephosphorylation and translocation, CRTC1 can interact with CREB as a cofactor to mediate transcription. Increased CRTC1 expression due to ethanol may suggest increased CREB-mediated transcription in the mPFC. However, our data showed there was not a significant overlap between CRE-mediated genes and genes changed by adolescent ethanol exposure suggesting CRE-mediated transcription was not altered in the mPFC by adolescent ethanol exposure. Additionally, these samples were processed at the basal state without a prior behavioral task that would depolarize glutamatergic neurons

leading to pCREB. Furthermore, this study is limited by the use of bulk tissue used rather than glutamatergic cell type specific samples. Other studies have found changes in CREB following adolescent ethanol exposure without a task [59], [64] suggesting a behavioral task is not necessary to observe ethanol induced protein changes in memory related proteins. Meanwhile our study did not observe a decrease in CREB protein expression which may be due to the timepoint of our study (3 weeks after ethanol) for the adolescent exposed cohort compared to the Pandey [64] which looked immediately after the last ethanol exposure or Zhang [59] who looked 2 months after their ethanol exposure paradigm. Additionally, the studies looked at different brain regions, cingulate gyrus in the Pandey study and amygdala in the Zhang study, both without specifically looking at behavioral effects in their studies. Furthermore Pandey [64] looked at frontal, parietal, and piriform cortex finding decreased pCREB in ethanol withdrawal animals in those regions without changes in total CREB indicating changes in pCREB differ by the brain region. Lastly the Zhang study administered ethanol via I.P which would lead to a higher BEC due to bypassing gastric ethanol metabolism which may also account for their ethanol effect in addition to the selected brain region. Together, this data suggests CREB, CRTC1, or CAMKII mediated functions may be increased as a part of restoring homeostasis or due to ethanol induced dysregulation.

CREB's role as a transcription factor may be impacting memory performance via changes in synaptic plasticity. CREB has a role in late long-term potentiation (L-LTP), neurogenesis, and transcription of factors associated with synaptic plasticity like brain-derived neurotrophic factor (BDNF) [172], [301]–[304]. Although the role of CREB in L-LTP appears to be stimulation method and stimulation location specific while CREB mediated BDNF transcription is brain region specific [303]–[305]. While CREB plays a role in LTP it does not appear to be necessary

for synaptic plasticity with other members of the CREB family of transcription factors compensating for the loss of CREB [303], [306]. CREB also plays a role in neurogenesis through its expression and phosphorlyation levels, and the transcription of factors like BDNF [172], [306]. BDNF plays a role in neurogenesis by aiding in cell proliferation and cell survival [307], [308] and increasing dendritic branching and spine density [309]–[311]. CREB also plays a role in dendritic length with decreasing pCREB resulting in decreased dendritic length and complexity [306]. BDNF stimulates pCREB in neurons through multiple kinase pathways and impacts neuronal plasticity due to myelin inhibition [309]. Therefore, CREB also plays a role in synaptic plasticity through neurite length modulation. Through changes in neurogenesis, synaptic plasticity, and LTP, CREB can modulate memory and brain plasticity that may alter memory performance.

CREB also plays a role in myelination with the number of CREB and pCREB-positive cells increasing in oligodendrocytes as maturation occurs [312]. Additionally, in an oligodendrocyte culture, the application of a cAMP analog increases the number of MBP-positive cells and complex morphology of MBP-positive cells [313]. CREB appears to play a role in myelination in early development with decreased pCREB and CREB in oligodendrocytes in rodents from adolescence (PND 21) compared to neonate (PND 4 and 11) [314]. Therefore, changes in CREB expression by a treatment*sex interaction may reflect changes in synaptic plasticity, neurogenesis, or oligodendrocyte maturation that may have played a role in the memory deficits three weeks following ethanol. Importantly, these changes were observed in both the adolescent and adult exposed cohort suggesting CREB is not the molecular difference between the cohorts associated with the difference in memory performance or myelin-related gene expression.

Our ethanol data also suggests the mPFC and dHPC react differently to ethanol and age. These results may reflect differences in protein functionality in different brain regions or differences in brain development between the two age cohorts. CREB-mediated transcription differs by tissue type with differences in CREB promoter gene binding between the frontal cortex and hippocampus with 74 overlapping promoters while 81 unique frontal cortex and 185 unique HPC promoters [315]. While other studies have reported protein expression differences in adult and elderly rodents [316] or adolescents and adults [317], this is the first study to our knowledge examining age effects between 2-month (adolescent-exposed) and 3-month (adult-exposed) old mice. This data suggests that in early adulthood the mouse brain is developing and has increased protein expression. Indeed, human studies have shown increased myelin protein expression from adolescence into early adulthood (the early to mid-20s) [81], [83], [84]. Memory performance also improved over the adolescent to adult period [318]. Therefore, memory-related proteins may also increase during early adulthood. Sex also impacted the protein expression of CBP, NCOA1, and CRTC1 in the dHPC and CREB in both brain regions with males showing higher protein levels than females (Fig. 26 E & Fig. 27 B & D). Our data is in line with one study, but not another. Auger [319] found increased CBP protein levels in males in brain regions with high steroid hormone receptor density, ventromedial hypothalamus, and medial preoptic area, but not in the cortex of PND 0 rats. The hippocampus is also a region with many steroid hormone receptors [320], [321], indicating it could also be another region for CBP sex differences in expression. CRTC1 was also found to have a sex difference in the dHPC that may be driven by the low number of animals tested or low band visibility. A limitation of this study is the low sample size, which may have led to an underpowered study. Indeed, a power analysis was conducted and found a small effect size and low power for most proteins in which the study

would need hundreds to thousands of animals for the study to be properly powered (Table 10 & Table 11). Therefore, we would not recommend increasing the sample size for these targets in future studies as the posthoc power analysis suggests the selected proteins are unlikely to be changed by ethanol. Meanwhile, studies have not shown CREB protein expression to differ by sex at birth, but pCREB levels do show a sex difference with increased pCREB in males [322]. This is the first study to our knowledge reporting a sex difference in the expression of CBP, CRTC1, or CREB possibly highlighting the lack of prior sex difference studies or brain region-specific effects. Overall, our data shows that memory-related proteins increase during the early adulthood period in the mouse brain possibly due to continued brain maturation, while sex impacts select memory-related proteins possibly due to the specific brain regions examined and the inclusion of both sexes in these studies.

Table 10. Power analysis of chapter 4 mPFC protein expression results.

	Prefrontal Cortex						
	3 way ANOVA						
	Main Effects			Interactions			
Table 10	Treatment	Age	Sex	Treatment x Age	Treatment x Sex	Age x Sex	Treatment x Age x Sex
Protein Name	Power (effect size) [n=power at 0.8]						
CBP	0.0931759 (0.1157902) [800]	0.2674823 (0.2398533) [200]	0.0770129 (0.0928641) [1250]	0.0898902 (0.1115938) [850]	0.1511991 (0.1707512) [375]	0.0523233 (0.0279394) [n>2000]	0.0836339 (0.1030117) [1000]
NCOA1	0.1114009 (0.1348894) [600]	0.3474621 (0.2736134) [150]	0.0538737 (0.0356597) [n>2000]	0.1919359 (0.1965363) [300]	0.0511811 (0.0197523) [n>2000]	0.1274844 (0.1499481) [500]	0.1319678 (0.1538074) [500]
CRTC1	0.3952913 (0.2904742) [125]	**0.8503576** (0.4837713)	0.3841923 (0.2860383) [145]	0.1915511 (0.1944133) [300]	0.2759598 (0.2392699) [200]	0.0504005 (0.0114025) [n>2000]	0.0980829 (0.1193743) [750]
CREB	0.1246896 (0.1489340) [500]	**0.9343858** (0.5628295)	0.3470761 (0.2761595) [150]	0.0660296 (0.0722992) [n>2000]	0.7748644 (0.4512861) [58]	0.0520537 (0.0262769) [n>2000]	0.2995360 (0.2551003) [170]
CAMKIV	0.1485203 (0.1623803) [425]	**0.9943720**(0.6856003)	0.0674540 (0.0725086) [n>2000]	0.0987061 (0.1178571) [800]	0.1832780 (0.1857722) [325]	0.6603794 (0.3850675) [80]	0.3172058 (0.2532274) [180]
CAM	0.0556022 (0.0419893) [n>2000]	0.1295448 (0.1488638) [500]	0.0506283 (0.0141436) [n>2000]	0.1136246 (0.1345046) [600]	0.0514167 (0.0212180) [n>2000]	0.0622659 (0.0616800) [n>2000]	0.0699333 (0.0780181) [1800]
CAMKII	0.4376457 (0.3041417) [120]	**0.9936395** (0.6859471)	0.0976627 (0.1177699) [790]	0.0529398 (0.0305101) [n>2000]	0.0966349 (0.1165859) [800]	0.0569633 (0.0467414) [n>2000]	0.1608905 (0.1727875) [375]
PKA	0.1423839 (0.1592391) [440]	**0.9935185** (0.6850015)	0.1500379 (0.1650210) [410]	0.2006791 (0.1979863) [280]	0.3571702 (0.2724293) [150]	0.1207947 (0.1412084) [550]	0.0675991 (0.0734778) [2000]
PKC	0.0560034 (0.0438599) [n>2000]	**0.8822011** (0.5052446)	0.0666223 (0.0721594) [n>2000]	0.1551554 (0.1703476) [380]	0.3697855 (0.2802070) [144]	0.3765042 (0.2829374) [140]	0.0758802 (0.0892351) [1375]

Table 11. Power analysis of chapter 4 dHPC protein expression results.

	Hippocampus						
	3 way ANOVA						
	Main Effects			Interactions			
Table 11	Treatment	Age	Sex	Treatment x Age	Treatment x Sex	Age x Sex	Treatment x Age x Sex
Protein Name	Power (effect size)						
CBP	0.1279366 (0.1488989) [495]	0.4285243 (0.3035133) [125]	0.5500648 (0.3496374) [94]	0.0562576 (0.0447661) [n>2000]	0.0748072 (0.0874531) [1430]	0.2035685 (0.2015590) [280]	0.2145938 (0.2078434) [257]
NCOA1	0.1107563 (0.1304799) [650]	0.7017633 (0.4018466) [72]	0.3057365 (0.2482488) [180]	0.0572285 (0.0471693) [n>2000]	0.0561040 (0.0433998) [n>2000]	0.4193473 (0.2943627) [130]	0.0737246 (0.0840208) [73]
CRTC1	0.0559756 (0.0518382) [n>2000]	0.1846614 (0.2253295) [220]	0.7022412 (0.4857378) [50]	0.0625902 (0.0747059) [2000]	0.0649940 (0.0813231) [1650]	0.5092418 (0.3962225) [74]	0.0686270 (0.0903105) [1340]
CREB	0.0538738 (0.0346618) [n>2000]	0.3275726 (0.2576476) [170]	0.0955283 (0.1142281) [840]	0.1053932 (0.1250635) [700]	0.4493201 (0.3057283) [120]	0.3048047 (0.2478400) [182]	0.0648956 (0.0671584) [n>2000]
CAMKIV	0.0812248 (0.0966248) [1172]	**0.9600262** (0.5811069)	0.0604724 (0.0571016) [n>2000]	0.0743896 (0.0859309) [1480]	0.1037507 (0.1488989) [500]	0.1431526 (0.1598323) [430]	0.0651868 (0.0684238) [n>2000]
CAM	0.076055 (0.0878586) [1420]	0.1049962 (0.1246504) [705]	0.0500096 (0.0017321) [n>2000]	0.05617 (0.0436305) [n>2000]	0.0736889 (0.0839604) [1500]	0.067315 (0.0722293) [n>2000]	0.0518624 (0.0240902) [n>2000]
CAMKII	0.0610585 (0.0597722) [n>2000]	0.4971326 (0.3328887) [103]	0.0632408 (0.0652538) [n>2000]	0.0535966 (0.0343714) [n>2000]	0.0684292 (0.0765780) [1865]	0.0526144 (0.0293384) [n>2000]	0.0695542 (0.0787928) [1760]
PKA	0.1460244 (0.1620232) [420]	0.164481 (0.1752579) [360]	0.0821279 (0.0979342) [1140]	0.0938728 (0.1133263) [855]	0.0704859 (0.0790494) [1750]	0.2429896 (0.2209337) [225]	0.0577778 (0.0493550) [n>2000]
PKC	0.1063511 (0.1296849) [655]	0.4264008 (0.3056305) [125]	0.051333 (0.0209808) [1100]	0.0521252 (0.0264668) [700]	0.0838533 (0.1023155) [1045]	0.1311236 (0.1530905) [470]	0.0981568 (0.1206177) [750]

While CREB protein expression differed by ethanol treatment and sex, CREB protein interaction with CAMKIV showed no effect of ethanol, age, or sex (Fig. 28 B). This is the first study to our knowledge to investigate CREB and CAMKIV interaction. We also probed for CBP and CRTC1 which did not show bands in the immunoprecipitated or input sample suggesting protein degradation. A limitation of this study is the inclusion of previously thawed aliquots, possibly leading to protein degradation that obscured effects on protein interaction. Another limitation is the low number of biological replicates, due to limited lysate, which influenced focus on the dHPC. Additionally, our samples were collected basally meaning without memory performance and therefore particular calcium signaling cascades may have not been stimulated prior to tissue collection resulting in dephosphorylated CBP or phosphorylated CRTC1 inhibiting interaction with CREB. Lastly, the study was limited by the choice to not include pCREB or pCBP, limiting the ability to look at the interaction between these two factors. If future studies assess protein interactions a separate cohort of animals should be used to increase the sample size and include analysis of the PFC, as well as include pCREB and pCBP. Our data shows that without cell stimulation CREB does interact with the kinase CAMKIV, suggesting transient ongoing CREB S133 phosphorylation within the limited sample size present.

While we investigated if gene expression changes by adolescent ethanol exposure included a significant overlap of CRE-based genes, we found no significant overlap. This may be due in part to the reference database used from Zhang [292], in which using the 2002 mouse genome the lab compared the genome sequence to the full or half CRE sequence. Given the genome used was approximately 20 years old and the mouse genome has been updated with an expanded identification of genes there may be genes from both gene list that are not identified as common genes due to the use of different gene symbols. Still 59 genes, including some Riken gene

symbols, were commonly identified between the two data sets suggesting common gene symbols between the two lists. Next, we performed an upstream regulator analysis in the adolescent cohort, and identified several target candidates based on p-overlap value. We further investigated the top 5 targets which included CD40 and NRG1 (Table 8). Meanwhile, CREB was not on the list of upstream regulators. CD40 is a member of the TNF receptor family, expressed on immune cells including microglia, and when KO, decreases dendritic length in the hippocampus during development [323]. Meanwhile, NRG1 binds to a tyrosine kinase receptor, ErbB, which increases hippocampal cell excitability and when inhibited decreases object exploration and long-term object recognition [324]. While we had a microarray dataset from an adolescent exposed cohort, we do not have a matching dataset for the adult exposed cohort, limiting our ability to investigate differences in upstream regulators based on age of ethanol exposure. As both NRG1 and CD40 were implicated in regulating several genes altered by adolescent ethanol exposure and are involved in recognition memory or synaptic plasticity they may be promising candidates for future studies, although we are limited by the lack of adult gene expression data.

4.5 Conclusion

We hypothesized that the memory impairments observed in the NOR and Barnes Maze task were due to an underlying basal difference in CBP protein expression. This study found no ethanol induced changes in CBP protein expression but ethanol induced increased expression in CRTC1 and CAMKII in the mPFC with CREB expression altered by ethanol and sex in both brain regions. Furthermore, age and sex effects were found in both brain regions suggesting continued brain development in early adulthood and novel sex differences. Meanwhile CREB-CAMKIV protein interaction was unchanged by ethanol and CRE mediated gene transcription did not significantly overlap with genes changed by adolescent ethanol. However, two candidate

upstream regulators, CD40 and NRG1, were identified that are known to have a role in dendritic length, cell excitability, and long-term object recognition memory. This suggests that ethanol induced memory impairments at least in adolescent exposed mice, may be changed by CD40 or NRG1, which have a role in memory and plasticity, but not CBP or CREB protein interaction or mediated transcription. Future studies may address the impact of ethanol in both cohorts on basal or activity dependent dendritic length. Meanwhile, CD40 and NRG1 appear as possible upstream targets of adolescent ethanol induced memory impairments that may be explored in future studies. Overall, this study found protein expression changes in CREB not CBP at the basal level depending on ethanol exposure and sex in both the adolescent and adult-exposed cohorts but it is unclear if CREB play a role in the previously observed memory impairments.

Chapter 5

Discussion

5.1 Rationale and Summary of Overall Hypothesis

Binge alcohol consumption affects both adolescents and adults in the United States with alcohol the most commonly used drug among adolescents as it is easily obtained despite being illegal for those under 21. The adolescent brain undergoes several changes including synaptic pruning, increased white matter, and improved memory performance [81], [82], [238] as it matures into an adult brain. Ethanol can impair memory while present in the body [18], [254] and during abstinence following chronic ethanol exposure [16], [235], [236]. Additionally, ethanol paradigms in rodents have been shown to impair memory more severely following adolescent ethanol exposure than adult ethanol exposure [16], [235]. Meanwhile, factors like sex may also play a role in differences in ethanol-induced memory impairments as females have increased BEC compared to males [33], [34]. The mechanisms behind chronic ethanol-induced memory impairments that differ by developmental age have yet to be fully understood. Our lab found a behavioral deficit in NOR at the same timepoint as we observed a decrease in *Cbp* gene expression. CBP, a protein expressed throughout the brain with roles in memory, neurogenesis, and CREB-mediated transcription [175], [179], [188], [292]. To understand developmental and sex differences in ethanol-induced memory impairments, this dissertation aimed to examine forms of memory, memory-related protein expression, and memory-related protein interaction. We hypothesized that adolescent ethanol exposure would be more impactful than adult ethanol exposure on recognition memory, spatial memory, and cognitive flexibility due to changes in CBP protein expression in the dHPC and mPFC.

5.2 Data Integration, Interpretation, and Future Directions

This research examined behavioral and molecular differences due to ethanol exposure, developmental age, and sex, finding adolescent ethanol exposure, but not adult ethanol exposure, negatively impacted short-term recognition memory, and long-term spatial memory, and decreased expression of more myelin-related genes. Meanwhile, only adult ethanol exposure decreased social interaction. Both cohorts experienced ethanol-induced locomotor tolerance (males in the adolescent exposed cohort and females in the adult exposed cohort), and ethanol-induced changes in memory-related protein expression. Age differentially impacted BEC and memory-related protein expression, while sex impacted ethanol sedation, spatial memory performance, spatial learning during training, and memory-related protein expression. Together, these experiments show that adolescent ethanol exposure more negatively impacts memory-related behaviors and myelin-related gene expression than adult ethanol exposure, but this is not due to differences in ethanol-induced expression changes in our predicted set of memory-related proteins, or greater blood ethanol concentration from acute ethanol exposure. However, factors such as sex and stress during behavioral testing play a role in learning and memory performance. Age effects in memory-related protein expression also intersect with ethanol exposure to produce different results in the adolescent-exposed and adult-exposed cohorts. More research is required to determine what structural deficits may impact short-term memory, if long-term spatial memory on the Barnes Maze is impacted by estrogen and CORT levels, and if behavioral activity and ethanol alter neurogenesis or de novo myelination.

This dissertation modeled binge ethanol drinking which NIAAA defines as ethanol consumption that reaches above 80 mg/dL following 4-5 drinks within roughly 2 hours. Our ethanol model achieves a large dose of ethanol (4g/kg) following a single oral gavage, resulting

in a BEC between 600-400 mg/dL therefore modeling a high BEC within a short time period. However, unlike in humans our animal model has limitations with controlled administration of ethanol via gavage, very high BEC, and altered timeline of ethanol consumption than in humans. While ethanol exposure in human adolescents is associated with later ethanol consumption in life, our model stops ethanol exposure during mid adolescence. Additionally, adolescents tend to consume alcohol on the weekends [325] approximately a week apart, where as our model involved a 2-day break between ethanol exposure. Overall while our ethanol model does not exactly mimic binge ethanol in humans, our model achieves delivering a large volume of ethanol within a short time span during adolescence. This dissertation aimed to examine memory deficits due to the age of ethanol exposure and which memory-related proteins may be differentially altered following binge ethanol and abstinence. We found that memory performance in the NOR and Barnes Maze task differed by ethanol and sex depending on the age of ethanol exposure. Short-term recognition memory and long-term spatial memory were impaired by adolescent, but not adult ethanol exposure, while spatial learning was impacted by ethanol exposure in the adolescent cohort and sex in the adult cohort. The adolescent exposed cohort showed an ethanol effect in the spatial probe trial with decreased time in the target quadrant compared to the controls and an ethanol effect in spatial learning with decreased latency to the goal box during training. Sex also impacted the adolescent exposed spatial probe trial with females, driven by the control females, performing better than all the other groups. In the adult exposed cohort, sex affected spatial learning with females displaying decreased latency to the goal box and increased speed as compared to the males during training. However, no ethanol or sex effect was observed in the number of errors suggesting an altered search strategy but no difference in strategy was observed with all groups increasing use of the serial and spatial search strategy. Deficits in short-

term NOR memory task suggests an existing structural deficit due to adolescent but not adult ethanol exposure. Meanwhile, the long-term spatial memory deficit in the Barnes Maze task in the adolescent cohort suggests an activation-related deficit that may be impacted by changes in circulating estrogen or CORT. The adult cohort's performance during spatial learning may also be impacted by circulating estrogen or CORT in the brightly lit arena. We also found 1 myelin-related gene, *Mal*, whose expression changed 24 hours after the last ethanol administration by adult ethanol exposure, while a previous study utilizing adolescent ethanol exposure found 4 genes (*Mag, Mbp, Mobp, Plp*) were decreased by ethanol. However, no difference in myelin-related gene expression was observed 3 weeks after the last ethanol administration in both aged cohorts. This suggests that during the abstinence period in the adolescent exposed cohort, myelin gene expression recovered, but due to the decreases in gene expression related to myelin formation (*Mbp & Plp*), axon-glia interactions (*Mag*), myelin stabilization/compaction (*Mobp & Plp*), and oligodendrocyte morphology and extension (*Mobp*) previously, the myelin three weeks in to abstinence may be less thick, compact, or less extensive in the adolescent cohort than the adult cohort possibly leading to subsequent changes in neuronal transduction and behavioral performance. Additionally, at the 3-week timepoint, this study found ethanol in both age groups increased CRTC1 and CAMKII protein expression in the mPFC while CREB was modulated by ethanol and sex in the mPFC and dHPC. While we found impaired spatial and recognition memory performance only in the adolescent ethanol-exposed cohort, both cohorts showed the same ethanol changes in memory-related protein expression. Therefore, the memory-related proteins we investigated are not likely involved in the adolescent-specific memory impairments. Rather, following adolescent ethanol exposure but not adult ethanol exposure, there may be

decreased structural synaptic plasticity or decreased activity-dependent plasticity that is impacted by estrogen and CORT levels.

To determine if structural deficits that may impact short-term memory are present following ethanol exposure, we could perform stereotaxic surgery following our standard ethanol paradigm in both age cohorts to label hSyn1 positive glutamatergic neurons with a green fluorescent protein (GFP) adeno-associated virus (AAV) in the mPFC and dHPC. Then at the three-week timepoint, we could collect tissue to examine dendritic morphology and density in the two age groups. We hypothesize that adolescent ethanol exposure will decrease the density of mature spines found in both dHPC and mPFC, although more so in the mPFC, while adult ethanol exposure will either cause no change or increase mature spine density. We are interested in changes in dendritic spine morphology and density as they are associated with synaptic plasticity involved in memory [326]–[328], while adolescent ethanol has shown to decrease mature spine density [50], [96], [97] but increase mature spine density in adult ethanol exposed animals [50]. The use of viral injections to label neurons would allow us to co-label the tissue with other markers of interest (discussed below). Many of the proposed studies described in this chapter could be run together on the same animals but have been separated out to thoroughly explain rationale and interpretations. Ethanol effects in dendritic morphology and density may suggest deficits in synaptic plasticity or cell-to-cell connectivity which may explain deficits in short-term recognition memory. If our hypothesis, that adolescent binge ethanol but not adult binge ethanol exposure negatively impacts mature dendritic spine density, is incorrect this would still add to the literature by showing similar ethanol disruptions between adolescent and adults, that have previously not been reported. This data will clarify if basal dendritic structural changes

occur following binge ethanol exposure that may decrease cell-to-cell connectivity and impact memory performance.

Next, we could investigate if long-term spatial memory on the Barnes Maze is impacted by estrogen or CORT levels following a 6-day Barnes Maze task in which saliva is collected following task performance daily and blood is collected on the last day following the probe trial. We hypothesize that estrogen levels will positively correlate with time in the target quadrant during the probe trial in both age cohorts, while CORT levels will be increased in the adolescent ethanol exposed but not adult exposed mice. We are interested in estrogen levels as other studies have found improved spatial memory [152] and a greater number of spines and synaptic connections in the HPC in proestrus than non-proestrus rats [274]. Meanwhile, CORT levels are increased following behavioral testing [262], [263] with adolescent ethanol exposure dysregulating CORT basally and following an acute challenge [264]. Therefore, examining CORT levels during the task and estrogen following the probe trial may further explain ethanol and sex effects during training and the probe trial. CORT levels have been detected in saliva in rodents and birds following anesthesia and in awake animals [329], [330]. Therefore, we may be able to collect CORT throughout the multiday testing process in awake animals. Ethanol effects on CORT levels following a stressor may suggest an alteration in the stress response due to altered HPA axis activity. Meanwhile an ethanol effect in estrogen on probe day may suggest changes in dendritic morphology, synaptic plasticity, and cell-to-cell connectivity that may be associated with memory performance. If our hypothesis, that adolescent binge ethanol but not adult binge ethanol exposure negatively impacts CORT levels, is incorrect this would still add to the literature by showing similar changes stress responses between the ages despite ongoing HPA axis development during adolescence. Additionally, if our hypothesis that estrogen levels

will positively correlate with memory performance is incorrect, this will add evidence to the literature that low estrogen animals can perform memory tasks as well as high estrogen animals suggesting estrogen mediated effects on dendritic morphology may not impact memory performance. This data will clarify if estrogen or activity dependent changes in CORT may occur following binge ethanol exposure that may alter performance on the learning and memory task.

To causally determine if modulating estrogen or CORT alters performance in the Barnes Maze, we could use pharmacological tools to manipulate our factors of interest. First, to inhibit estradiol synthesis we can administer letrozole (LET) via I.P. to half of the water-exposed and ethanol-exposed female mice daily for 1 week before Barnes Mazes testing similar to Liu [331]. While we could also decrease estradiol via ovariectomy surgery this method is more invasive, would require ovary removal during puberty, and in comparison to LET administration has shown to impact dendritic spine density and spatial memory to a lesser extent [331]. This will result in 4 groups within the female cohort (water/vehicle, water/LET, ethanol/vehicle, ethanol/LET). We hypothesize that administering LET will decrease circulating estradiol and impair memory performance on the Barnes Maze in both water and ethanol-exposed animals. In both aged cohorts, we expect LET will result in increased latency to the goal box and decreased time in the target quadrant. To investigate the relationship between CORT and Barnes Maze performance, we can use metyrapone (MET), a CORT synthesis inhibitor, via I.P. 30 minutes before Barnes Maze testing in both sexes as seen in Dominguez [332]. This will result in 4 groups within the males (water/vehicle, water/MET, ethanol/vehicle, ethanol/MET). The male mice will also be exposed to vehicle I.P. injections a week prior to Barnes Maze testing while the females undergo LET injections. As both CORT and estrogen have shown to impact memory performance, there may be a synergistic effect impacting the adolescent ethanol exposed females

with this group showing worse spatial memory performance despite reached the goal box faster during training in the Barnes Maze. In the female mice, we can use the same LET administered mice and further divide the 4 groups into 8 groups (water/vehicle/vehicle, water/vehicle/MET, water/LET/vehicle, water/LET/MET, ethanol/vehicle/vehicle, ethanol/vehicle/MET, ethanol/LET/vehicle, ethanol/LET/MET). These experiments will also include a non-injected group within the ethanol and water-exposed groups within each sex to control for the stress impact of I.P. injections, for a total of 6 groups in males and 10 groups in females. We hypothesize that CORT inhibition via MET will increase latency during training, and time in the target quadrant in the ethanol-exposed mice, resulting in a similar performance as the water/vehicle group. This experiment would be analyzed separately by sex with a 3-way RM ANOVA for the males, while a mixed-model RM ANOVA would be used for the female data, with a Tukey's post hoc test used for both sexes. A sample size of approximately 15/group would be desired for these experiments as determined from a power analysis using Barnes maze data. To support the overarching hypothesis that CORT levels impact the adolescent ethanol animals' performance during Barnes Maze we would need supporting data from this proposed experiment where adolescent ethanol animals administered MET performed similarly to the water/vehicle animals in latency, speed, errors, and time in the target quadrant. Meanwhile, we hypothesize ethanol/vehicle mice would perform as seen in this work reaching the goal box sooner and faster than water controls. Additionally, we hypothesize water/MET animals would have decreased circulating CORT than the water/vehicle and worse performance on the aforementioned independent variables of interest suggesting a certain level of CORT may be beneficial for task performance. If we see no difference in latency, speed, errors, and time in the target quadrant with CORT inhibition in the ethanol exposed animals, this would suggest that

CORT is not necessary in altering these behaviors in the Barnes Maze following ethanol. Meanwhile, to support the overarching hypothesis that estradiol levels impact the adolescent ethanol animals' performance during Barnes Maze, we would need data that shows ethanol/LET animals performing worse than the vehicle counterparts in latency, speed, errors, and time in the target quadrant. We hypothesize there is also a synergistic effect of CORT and estradiol synthesis inhibition. Therefore, we expect LET/MET mice to perform worse than the respective LET and MET only counterparts. Additionally, we expect ethanol LET/MET mice would perform worse in the aforementioned independent variables than the water LET/MET counterparts. If no difference in latency, speed, errors, and time in the target quadrant occurs following LET administration, this would suggest estradiol is not involved in Barnes Maze performance.

Next, we can assess activity-dependent neurogenesis, oligodendrogenesis, and de novo myelination by comparing a behaviorally naïve cohort to a behaviorally active cohort following a 6-day Barnes Maze task. We can label newly dividing cells using 5-ethynyl-2'-deoxyuridine (EdU) administered in drinking water for a week starting immediately after the last ethanol administration day. Tissue can be collected following the probe trial and co-labeled with doublecortin (DCX) and platelet-derived growth factor receptor α (PDGFRα) to differentiate whether new cells are neurons or oligodendrocyte precursors. Meanwhile, another cohort can be used to determine de novo myelination via electron microscopy. We hypothesize activity dependent neurogenesis will be decreased in the dHPC following adolescent ethanol exposure but unchanged in adult ethanol exposed animals. Meanwhile, oligodendrogenesis and de novo myelination would be decreased in both the mPFC and dHPC in the adolescent ethanol exposed animals but unchanged in adult ethanol exposed animals. Meanwhile, the behaviorally naïve

cohort will show decreased neurogenesis, oligodendrogenesis, and de novo myelination due to adolescent ethanol which will be similar to the behaviorally active ethanol exposed cohort. Although we hypothesize adult ethanol exposure will not alter neurogenesis, oligodendrogenesis and de novo myelination in the behaviorally naïve cohort, we expect a decrease compared to the behaviorally active cohort. We are interested in activity-dependent cell proliferation and myelination as other studies have found the MWM task increases neurogenesis in the HPC and neuronal activation [152] while adolescent ethanol exposure decreases neurogenesis in the HPC in adolescence and adulthood [68], [235], [252], [333]. Meanwhile, complex wheel running [203] and the MWM [334] increase oligodendrocyte progenitor cells and oligodendrocytes. Furthermore, Steadman [334] found maze training also increased de novo myelination. Ethanol effects in neurogenesis, oligodendrogenesis, and de novo myelination may suggest changes in neuronal connectivity, neuronal transduction, and synaptic plasticity. If our hypothesis, that adolescent ethanol exposure decreases basal and activity dependent cell proliferation and myelination, is incorrect this would suggest that neurogenesis and myelination are not involved in age differentiated memory deficits. Overall, this study would add data regarding persistent changes in cell proliferation and myelination following binge ethanol exposure in an age dependent manner.

Lastly, to determine if myelin integrity and thickness differs due to adolescent ethanol exposure, we can assess changes in myelination in another behaviorally naïve cohort using electron microscopy. We hypothesize that myelin integrity and thickness will be decreased in adolescent ethanol exposed animals, but not adult ethanol exposed mice. We are interested in the basal myelin state as myelin protein expression, gene expression, and density are decreased by adolescent ethanol exposure [63], [90]–[92] and myelination is associated with memory task

performance [203], [334]. Additionally, myelin deficits in thickness and integrity were found immediately and three weeks following adolescent ethanol exposure [91]. Meanwhile, chronic adult ethanol exposure has also shown to decrease oligodendrocyte progenitor cell and mature oligodendrocytes density in the mPFC immediately following three weeks of continual ethanol self-administration [335]. Ethanol effects on myelin integrity and thickness may suggest changes in neuronal transduction that could impact cell-to-cell communication involved in the observed memory deficit. If our hypothesis is not correct, that adolescent but not adult ethanol exposure persistently impacts myelin integrity and thickness, this would suggest short-term memory is not impacted by differences in myelination in the dHPC and mPFC. This study would clarify if changes in myelin integrity and thickness differ by age of ethanol exposure and are found persistently following ethanol exposure. Together, these proposed future studies could provide new insights into several molecular metrics that may differ between adolescents and adults exposed to binge ethanol that may underlie the difference in memory performance.

5.3 Conclusions and Implications

Overall, this dissertation contributes to understanding the behavioral and molecular impacts and differences of binge ethanol in adolescence and early adulthood in the development of memory impairments. This study identified persistent ethanol-induced memory impairments in a developmental age and sex-dependent manner, but did not identify a molecular-related difference between the two age cohorts that may contribute to understanding the behavioral difference. This data contributes to a further understanding of which molecular areas to investigate in the future to provide a therapeutic intervention for persistent adolescent ethanol-induced memory impairments.

References

[1] Substance Abuse and Mental Health Services Administration (SAMHSA), “Key substance use and mental health indicators in the United States: Results from the 2021 National Survey on Drug Use and Health (HHS Publication No. PEP19-5068, NSDUH Series H-54),” 2021.

[2] Substance Abuse and Mental Health Services Administration (SAMHSA), “Key substance use and mental health indicators in the United States: Results from the 2022 National Survey on Drug Use and Health,” 2022, [Online]. Available: https://www.samhsa.gov/data/.

[3] Niaaa, “What Colleges Need to Know Now - An Update on College Drinking Research,” pp. 1–12, 2007, [Online]. Available: papers2://publication/uuid/0EB0AE02-4DB6-43F0-B72C-1D1DA48944C2.

[4] T. Chung, K. G. Creswell, R. Bachrach, D. B. Clark, and C. S. Martin, “Adolescent Binge Drinking Developmental Context and Opportunities for Prevention,” *Alcohol Res.*, vol. 39, no. 1, pp. 5–15, 2018, [Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6104966/.

[5] B. Simons-Morton, W. Pickett, W. Boyce, T. ter Bogt, and W. Vollebergh, “Cross-national comparison of adolescent drinking and cannabis use in the United States, Canada, and the Netherlands,” *Int. J. Drug Policy*, vol. 21, no. 1, pp. 64–69, 2010, doi: 10.1016/j.drugpo.2009.02.003.Cross-national.

[6] M. E. Slater and H. R. Alpert, “SURVEILLANCE REPORT #120 APPARENT PER

CAPITA ALCOHOL CONSUMPTION: NATIONAL, STATE, AND REGIONAL TRENDS, 1977–2021," 2023, [Online]. Available: https://www.niaaa.nih.gov/publications/surveillance-reports/surveillance120.

[7] B. F. Grant and D. A. Dawson, "Age at onset of alcohol use and its association with DSM-IV alcohol abuse and dependence: Results from the national longitudinal alcohol epidemiologic survey," *J. Subst. Abuse*, vol. 9, no. 1, pp. 103–110, 1997, doi: 10.1016/S0899-3289(97)90009-2.

[8] R. W. Hingson, T. Heeren, and M. R. Winter, "Age at drinking onset and alcohol dependence: Age at onset, duration, and severity," *Arch. Pediatr. Adolesc. Med.*, vol. 160, no. 7, pp. 739–746, 2006, doi: 10.1001/archpedi.160.7.739.

[9] K. Guttmannova *et al.*, "Sensitive periods for adolescent alcohol use initiation: Predicting the Lifetime occurrence and chronicity of alcohol problems in adulthood," *J. Stud. Alcohol Drugs*, vol. 72, no. 2, pp. 221–231, 2011, doi: 10.15288/jsad.2011.72.221.

[10] C. Vinader-Caerols, A. Talk, A. Montañés, A. Duque, and S. Monleón, "Differential effects of alcohol on memory performance in adolescent men and women with a binge drinking history," *Alcohol Alcohol.*, vol. 52, no. 5, pp. 610–616, 2017, doi: 10.1093/alcalc/agx040.

[11] M. L. Langhan, "Acute alcohol intoxication in adolescents: Frequency of respiratory depression," *J. Emerg. Med.*, vol. 44, no. 6, pp. 1063–1069, 2013, doi: 10.1016/j.jemermed.2012.11.041.

[12] H. Wang *et al.*, "Forensic appraisal of death due to acute alcohol poisoning: three case reports and a literature review," *Forensic Sci. Res.*, vol. 5, no. 4, pp. 341–347, 2020, doi:

10.1080/20961790.2019.1572259.

[13] H. R. Alpert, M. E. Slater, Y. H. Yoon, C. M. Chen, N. Winstanley, and M. B. Esser, "Alcohol Consumption and 15 Causes of Fatal Injuries: A Systematic Review and Meta-Analysis," *Am. J. Prev. Med.*, vol. 63, no. 2, pp. 286–300, 2022, doi: 10.1016/j.amepre.2022.03.025.

[14] P. Little, C. Kuhn, W. A. Wilson, and H. S. Swartzwelder, "Differential effects of ethanol in adolescent and adult rats," *Alcohol. Clin. Exp. Res.*, vol. 22, no. 2, pp. 416–421, 1996, doi: 10.1111/j.1530-0277.1996.tb01133.x.

[15] L. C. Ornelas, A. Novier, C. E. Van Skike, J. L. Diaz-Granados, and D. B. Matthews, "The effects of acute alcohol on motor impairments in adolescent, adult, and aged rats," *Alcohol*, vol. 49, no. 2, pp. 121–126, 2015, doi: 10.1016/j.alcohol.2014.12.002.

[16] G. Beaudet *et al.*, "Long-Lasting Effects of Chronic Intermittent Alcohol Exposure in Adolescent Mice on Object Recognition and Hippocampal Neuronal Activity," *Alcohol. Clin. Exp. Res.*, vol. 40, no. 12, pp. 2591–2603, 2016, doi: 10.1111/acer.13256.

[17] R. Sircar and D. Sircar, "Adolescent rats exposed to repeated ethanol treatment show lingering behavioral impairments," *Alcohol. Clin. Exp. Res.*, vol. 29, no. 8, pp. 1402–1410, 2005, doi: 10.1097/01.alc.0000175012.77756.d9.

[18] A. M. White, A. J. Ghia, E. D. Levin, and H. Scott Swartzwelder, "Binge pattern ethanol exposure in adolescent and adult rats: Differential impact on subsequent responsiveness to ethanol," *Alcohol. Clin. Exp. Res.*, vol. 24, no. 8, pp. 1251–1256, 2000, doi: 10.1111/j.1530-0277.2000.tb02091.x.

[19] J. Sneider, J. Cohen-Gilbert, D. Crowley, M. Paul, and M. Silveri, “Differential effects of binge drinking on learning and memory in emerging adults,” *J. Addict. Res. Ther.*, vol. 23, no. 1, pp. 1–7, 2006, doi: 10.1038/jid.2014.371.

[20] W. V. Lechner, A. M. Day, J. Metrik, A. M. Leventhal, and C. W. Kahler, “Effects of alcohol-induced working memory decline on alcohol consumption and adverse consequences of use,” *Psychopharmacology (Berl).*, vol. 233, no. 1, pp. 83–88, 2016, doi: 10.1007/s00213-015-4090-z.

[21] A. W. Jones, “Alcohol, its absorption, distribution, metabolism, and excretion in the body and pharmacokinetic calculations,” *WIREs Forensic Sci.*, vol. 1, no. 5, pp. 1–26, 2019, doi: 10.1002/wfs2.1340.

[22] H. H. Mitchell, T. S. Hamilton, F. R. Steggerda, and H. W. Bean, “the Chemical Composition of the Adult Human Body and Its Bearing on the Biochemistry of Growth,” *J. Biol. Chem.*, vol. 158, no. 3, pp. 625–637, 1945, doi: 10.1016/s0021-9258(19)51339-4.

[23] G. Cooper, *The Cell, A Molecular Approach 2nd edition*, 2nd ed. Sinauer Associates, 2000.

[24] W. M. Pardridge, “Transport of small molecules through the blood-brain barrier: biology and methodology,” *Adv. Drug Deliv. Rev.*, vol. 15, no. 1–3, pp. 5–36, 1995, doi: 10.1016/0169-409X(95)00003-P.

[25] H. J. Edenberg, J. McClintick, and M. Genetics, “Alcohol dehydrogenases, aldehyde dehydrogenases and alcohol use disorders: a critical review,” *Alcohol Clin Exp Res*, vol. 42, no. 12, pp. 2281–2297, 2018, doi: 10.1111/acer.13904.

[26] D. Desroches, C. Orevillo, and D. Verina, “Sex- and strain-related differences in first-pass alcohol metabolism in mice,” *Alcohol*, vol. 12, no. 3, pp. 221–226, 1995, doi: 10.1016/0741-8329(94)00098-X.

[27] C. Winek and K. Murphy, “THE RATE AND KINETIC ORDER OF ETHANOL ELIMINATION,” *Forensic Sci. Int.*, vol. 25, no. 3, pp. 159–166, 1984, [Online]. Available: https://doi.org/10.1016/0379-0738(84)90189-0.

[28] L. Siqueira and V. C. Smith, “Binge Drinking,” *Pediatrics*, vol. 136, no. 3, 2015, doi: 10.1542/peds.2015-2337.

[29] A. I. Cederbaum, “Alcohol Metabolism,” *Clin Liver Dis*, vol. 16, pp. 667–685, 2012, doi: 10.1016/j.cld.2012.08.002.

[30] D. Wu and A. I. Cederbaum, “Alcohol, Oxidative Stress, and Free Radical Damage,” *Alcohol Res. Heal. Heal.*, vol. 27, no. 4, pp. 853–853, 2003, doi: 10.1007/978-3-642-16712-6_100356.

[31] T. M. Leung and N. Nieto, “CYP2E1 and oxidant stress in alcoholic and non-alcoholic fatty liver disease,” *J. Hepatol.*, vol. 58, no. 2, pp. 395–398, Feb. 2013, doi: 10.1016/j.jhep.2012.08.018.

[32] H. Seitz *et al.*, “Human gastric alcohol dehydrogenase activity: effect of age, sex, and alcoholism,” *Gut*, vol. 34, pp. 1433–1437, 1993, doi: 10.1136/GUT.34.10.1433.

[33] E. Baraona *et al.*, “Gender differences in pharmacokinetics of alcohol,” *Alcohol. Clin. Exp. Res.*, vol. 25, no. 4, pp. 502–507, 2001, doi: 10.1111/j.1530-0277.2001.tb02242.x.

[34] P. Y. Kwo *et al.*, “Gender Differences in Alcohol Metabolism: Relationship to Liver

Volume and Effect of Adjusting for Body Mass," *Gastroenterology*, vol. 115, pp. 1552–1557, 1998.

[35] M. S. Mumenthaler, J. L. Taylor, R. O'hara, and J. A. Yesavage, "Gender Differences in Moderate Drinking Effects," *Alcohol Res. Heal.*, vol. 23, no. 1, pp. 55–64, 1999.

[36] T. B. Beisswenger, B. Holmquist, and B. L. Vallee, "χ-ADH is the sole alcohol dehydrogenase isozyme of mammalian brains: Implications and inferences," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 82, no. 24, pp. 8369–8373, 1985, doi: 10.1073/pnas.82.24.8369.

[37] H. J. Edenberg, "The Genetics of Alcohol Metabolism: Role of Alcohol Dehydrogenase and Aldehyde Dehydrogenase Variants," *Alcohol Res. Heal. Heal.*, vol. 30, no. 1, pp. 5–13, 2007.

[38] S. V. Bhave *et al.*, "Gene array profiles of alcohol and aldehyde metabolizing enzymes in brains of C57BL/6 and DBA/2 mice," *Alcohol. Clin. Exp. Res.*, vol. 30, no. 10, pp. 1659–1669, Oct. 2006, doi: 10.1111/j.1530-0277.2006.00201.x.

[39] F. Tanaka, Y. Shiratori, O. Yokosuka, F. Imazeki, Y. Tsukada, and M. Omata, "High incidence of ADH2*1/ALDH2*1 genes among Japanese alcohol dependents and patients with alcoholic liver disease," *Hepatology*, vol. 23, no. 2, pp. 234–239, 1996, doi: 10.1053/jhep.1996.v23.pm0008591846.

[40] W. A. Koss, R. N. Sadowski, L. K. Sherrill, J. M. Gulley, and J. M. Juraska, "Effects of ethanol during adolescence on the number of neurons and glia in the medial prefrontal cortex and basolateral amygdala of adult male and female rats," *Brain Res.*, vol. 1466, pp. 24–32, 2012, doi: 10.1016/j.brainres.2012.05.023.

[41] K. N. Nwachukwu, D. M. King, K. L. Healey, H. S. Swartzwelder, and S. A. Marshall, "Sex-specific effects of adolescent intermittent ethanol exposure-induced dysregulation of hippocampal glial cells in adulthood," *Alcohol*, vol. 100, pp. 31–39, 2022, doi: 10.1016/j.alcohol.2022.02.002.

[42] K. P. Abrahao, A. G. Salinas, and D. M. Lovinger, "Alcohol and the Brain: Neuronal Molecular Targets, Synapses, and Circuits," *Neuron*, vol. 96, no. 6, pp. 1223–1238, Dec. 2017, doi: 10.1016/j.neuron.2017.10.032.

[43] B. Söderpalm, H. H. Lidö, and M. Ericson, "The Glycine Receptor—A Functionally Important Primary Brain Target of Ethanol," *Alcohol. Clin. Exp. Res.*, vol. 41, no. 11, pp. 1816–1830, 2017, doi: 10.1111/acer.13483.

[44] S. Ueno, M. J. Wick, Q. Ye, N. L. Harrison, and R. A. Harris, "Subunit mutations affect ethanol actions on GABA(A) receptors expressed in Xenopus oocytes," *Br. J. Pharmacol.*, vol. 127, no. 2, pp. 377–382, 1999, doi: 10.1038/sj.bjp.0702563.

[45] H. Tatebayashi, H. Motomura, and T. Narahashi, "Alcohol modulation of single GABA(A) receptor-channel kinetics," *Neuroreport*, vol. 9, no. 8, pp. 1769–1775, 1998, doi: 10.1097/00001756-199806010-00018.

[46] D. M. Lovinger, G. White, and F. F. Weight, "NMDA Receptor-Mediated Synaptic Excitation Selectively Inhibited by Ethanol in Hippocampal Slice from Adult Rat," *J. Neurosci.*, vol. 10, no. 4, pp. 1372–1379, 1990.

[47] C. T. Smothers, C. Jin, and J. J. Woodward, "Deletion of the N-terminal domain alters the ethanol inhibition of N-methyl-D-aspartate receptors in a subunit-dependent manner," *Alcohol. Clin. Exp. Res.*, vol. 37, no. 11, pp. 1882–1890, 2013, doi: 10.1111/acer.12168.

[48] Y. Zhao, H. Ren, D. S. Dwyer, and R. W. Peoples, “Different sites of alcohol action in the NMDA receptor GluN2A and GluN2B subunits,” *Neuropharmacology*, vol. 97, pp. 240–250, 2015, doi: 10.1016/j.neuropharm.2015.05.018.

[49] C. Fernando Valenzuela, “Alcohol and neurotransmitter interactions,” *Alcohol Res. Heal.*, vol. 21, no. 2, pp. 144–148, 1997, Accessed: Dec. 19, 2020. [Online]. Available: /pmc/articles/PMC6826822/?report=abstract.

[50] E. Galaj, C. Guo, D. Huang, R. Ranaldi, and Y. Ma, “Contrasting Effects of Adolescent and Early-Adult Ethanol Exposure on Prelimbic Cortical Pyramidal Neurons,” pp. 1–24, 2021, doi: 10.1016/j.drugalcdep.2020.108309.Contrasting.

[51] A. N. Karkhanis, K. N. Huggins, J. H. Rose, and S. R. Jones, “Switch from excitatory to inhibitory actions of ethanol on dopamine levels after chronic exposure: Role of kappa opioid receptors,” *Neuropharmacology*, vol. 110, pp. 190–197, 2016, doi: 10.1016/j.neuropharm.2016.07.022.

[52] Z. A. Rodd *et al.*, “Serotonin-3 receptors in the posterior ventral tegmental area regulate ethanol self-administration of alcohol-preferring (P) rats,” *Alcohol*, vol. 44, pp. 245–255, 2010, doi: 10.1016/j.alcohol.2010.01.002.

[53] M. Yoshimura, S. Pearson, Y. Kadota, and C. E. Gonzalez, “Identification of ethanol responsive domains of adenylyl cyclase,” *Alcohol. Clin. Exp. Res.*, vol. 30, no. 11, pp. 1824–1832, 2006, doi: 10.1111/j.1530-0277.2006.00219.x.

[54] E. B. Rex, M. L. Rankin, M. A. Ariano, and D. R. Sibley, “Ethanol regulation of D1 dopamine receptor signaling is mediated by protein kinase C in an isozyme-specific manner,” *Neuropsychopharmacology*, vol. 33, no. 12, pp. 2900–2911, 2008, doi:

10.1038/npp.2008.16.

[55] D. P. Dohrman, I. Diamond, and A. S. Gordon, “Ethanol causes translocation of cAMP-dependent protein kinase catalytic subunit to the nucleus,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 93, pp. 10217–10221, Sep. 1996, doi: 10.1073/pnas.93.19.10217.

[56] A. S. Gordon, L. Yao, D. P. Dohrman, and I. Diamond, “Ethanol Alters the Subcellular Localization of CAMP-Dependent Protein Kinase and Protein Kinase C,” *Alcohol. Clin. Exp. Res.*, vol. 22, no. 5, pp. 238–242, 1998, doi: 10.1111/j.1530-0277.1998.tb04009.x.

[57] O. Asher, T. D. Cunningham, L. Yao, A. S. Gordon, and I. Diamond, “Ethanol Stimulates cAMP-Responsive Element (CRE)-Mediated Transcription via CRE-Binding Protein and cAMP-Dependent Protein Kinase,” 2002, Accessed: Sep. 16, 2020. [Online]. Available: http://jpet.aspetjournals.org.

[58] X. Yang, K. Horn, G. S. Wand, J. M. Baraban, and G. S. Wand, “Chronic Ethanol Administration Decreases Phosphorylation of Cyclic AMP Response Element-Binding Protein in Granule Cells of Rat Cerebellum,” *Alcohol. Clin. Exp. Res.*, vol. 22, no. 2, pp. 382–390, Apr. 1998, doi: 10.1111/j.1530-0277.1998.tb03664.x.

[59] H. Zhang, E. J. Kyzar, J. P. Bohnsack, D. M. Kokare, T. Teppen, and S. C. Pandey, “Adolescent alcohol exposure epigenetically regulates CREB signaling in the adult amygdala,” *Sci. Rep.*, vol. 8, no. 1, pp. 1–10, Dec. 2018, doi: 10.1038/s41598-018-28415-9.

[60] S. L. Soares-Simi *et al.*, “Changes in CREB activation in the prefrontal cortex and hippocampus blunt ethanol-induced behavioral sensitization in adolescent mice,” *Front. Integr. Neurosci.*, vol. 7, no. DEC, pp. 1–12, 2013, doi: 10.3389/fnint.2013.00094.

[61] X. Yang, K. Horn, and G. S. Wand, “Chronic ethanol exposure impairs phosphorylation of CREB and CRE- binding activity in rat striatum,” *Alcohol. Clin. Exp. Res.*, vol. 22, no. 2, pp. 382–390, Apr. 1998, doi: 10.1111/j.1530-0277.1998.tb03664.x.

[62] A. Contreras, L. Morales, and N. Del Olmo, “The intermittent administration of ethanol during the juvenile period produces changes in the expression of hippocampal genes and proteins and deterioration of spatial memory,” *Behav. Brain Res.*, vol. 372, p. 112033, Oct. 2019, doi: 10.1016/j.bbr.2019.112033.

[63] J. T. Wolstenholme, T. Mahmood, G. M. Harris, S. Abbas, and M. F. Miles, “Intermittent Ethanol during Adolescence Leads to Lasting Behavioral Changes in Adulthood and Alters Gene Expression and Histone Methylation in the PFC,” *Front. Mol. Neurosci.*, vol. 10, pp. 1–14, 2017, doi: 10.3389/fnmol.2017.00307.

[64] S. C. Pandey, A. Roy, and N. Mittal, “Effects of Chronic Ethanol Intake and Its Withdrawal on the Expression and Phosphorylation of the CREB Gene Transcription Factor in Rat Cortex,” *J. Pharmacol. Exp. Ther.*, vol. 296, no. 3, pp. 857–868, Mar. 2001, Accessed: Sep. 12, 2020. [Online]. Available: http://jpet.aspetjournals.org.

[65] E. Qualls-Creekmore, R. Gupta, and M. Yoshimura, “The effect of alcohol on recombinant proteins derived from mammalian adenylyl cyclase,” *Biochem. Biophys. Reports*, vol. 10, pp. 157–164, 2017, doi: 10.1016/j.bbrep.2017.03.011.

[66] C. Sanabra and G. Mengod, “Neuroanatomical distribution and neurochemical characterization of cells expressing adenylyl cyclase isoforms in mouse and rat brain,” *J. Chem. Neuroanat.*, vol. 41, no. 1, pp. 43–54, 2011, doi: 10.1016/j.jchemneu.2010.11.001.

[67] T. M. Donohue and N. A. Osna, “Intracellular proteolytic systems in alcohol-induced

tissue injury," *Alcohol Res. Heal.*, vol. 27, no. 4, pp. 317–324, 2003.

[68] A. J. Sakharkar, R. P. Vetreno, H. Zhang, D. M. Kokare, F. T. Crews, and S. C. Pandey, "A role for histone acetylation mechanisms in adolescent alcohol exposure-induced deficits in hippocampal brain-derived neurotrophic factor expression and neurogenesis markers in adulthood," *Brain Struct. Funct.*, vol. 221, pp. 4691–4703, Dec. 2016, doi: 10.1007/s00429-016-1196-y.

[69] B. Li, M. Carey, and J. L. Workman, "The Role of Chromatin during Transcription," *Cell*, vol. 128, no. 4, pp. 707–719, 2007, doi: 10.1016/j.cell.2007.01.015.

[70] P. Mews *et al.*, "Alcohol metabolism contributes to brain histone acetylation," *Nature*, vol. 574, no. 7780, pp. 717–721, 2019, doi: 10.1038/s41586-019-1700-7.

[71] W. Guo *et al.*, "Alcohol exposure decreases CREB binding protein expression and histone acetylation in the developing cerebellum," *PLoS One*, vol. 6, no. 5, 2011, doi: 10.1371/journal.pone.0019351.

[72] L. Spear, "The adolescent brain and age-related behavioral manifestations," *Neurosci. Biobehav. Rev.*, vol. 24, no. 4, pp. 417–463, 2000, doi: 10.1016/S0149-7634(00)00014-2.

[73] L. D. Johnston, R. A. Miech, P. M. O'malley, J. G. Bachman, J. E. Schulenberg, and M. E. Patrick, "Monitoring the Future National Survey Results on Drug Use, 1975-2017: Overview, Key Findings on Adolescent Drug Use," Institute for Social Research. University of Michigan, P.O. Box 1248, 426 Thompson Street, Ann Arbor, MI 48106. Tel: 734-764-8354; Fax: 734-647- 4575; e-mail: isr-info@isr.umich.edu; Web site: http://www.isr.umich.edu, Ann Arbor, 2018. Accessed: Aug. 07, 2020. [Online]. Available: http://www.monitoringthefuture.org/pubs/monographs/mtf-overview2017.pdf.

[74] N. Vijayakumar, Z. Op de Macks, E. A. Shirtcliff, and J. H. Pfeifer, “Puberty and the human brain: Insights into adolescent development,” *Neurosci. Biobehav. Rev.*, vol. 92, pp. 417–436, 2018, doi: 10.1016/j.neubiorev.2018.06.004.

[75] N. Maninger, O. M. Wolkowitz, V. I. Reus, E. S. Epel, and S. H. Mellon, “Neurobiological and neuropsychiatric effects of dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEAS),” *Front. Neuroendocrinol.*, vol. 30, no. 1, pp. 65–91, 2009, doi: 10.1016/j.yfrne.2008.11.002.

[76] D. S. Reddy, *Neurosteroids. Endogenous role in the human brain and therapeutic potentials*, vol. 186, no. C. Elsevier B.V., 2010.

[77] N. Rachdaoui and D. Sarkar, “Effects of Alcohol on the Endocrine System,” *Endocrinol Metab Clin North Am.*, vol. 42, no. 3, pp. 593–615, 2013, doi: 10.1016/j.ecl.2013.05.008.Effects.

[78] F. Diamond *et al.*, “Effects of drug and alcohol abuse upon pituitary-testicular function in adolescent males,” *J. Adolesc. Heal. Care*, vol. 7, no. 1, pp. 28–33, 1986, doi: 10.1016/S0197-0070(86)80091-2.

[79] W. L. Dees and C. W. Skelley, “Effects of ethanol during the onset of female puberty,” *Neuroendocrinology*, vol. 51, no. 1, pp. 64–69, 1990, doi: 10.1159/000125317.

[80] W. L. Dees, G. A. Dissen, J. K. Hiney, F. Lara, and S. R. Ojeda, “Alcohol ingestion inhibits the increased secretion of puberty-related hormones in the developing female rhesus monkey,” *Endocrinology*, vol. 141, no. 4, pp. 1325–1331, 2000, doi: 10.1210/endo.141.4.7413.

[81] J. N. Giedd *et al.*, “Brain development during childhood and adolescence: A longitudinal MRI study,” *Nat. Neurosci.*, vol. 2, no. 10, pp. 861–863, 1999, doi: 10.1038/13158.

[82] E. R. Sowell, D. A. Trauner, A. Gamst, and T. L. Jernigan, “Development of cortical and subcortical brain structures in childhood and adolescence: a structural MRI study,” *Dev. Med. Child Neurol.*, vol. 44, pp. 4–16, 2002, doi: 10.1111/j.1469-8749.2002.tb00253.x.

[83] A. Pfefferbaum, D. H. Mathalon, E. V Sullivan, J. M. Rawles, R. B. Zipursky, and K. O. Lim, “A Quantitative Magnetic Resonance Imaging Study of Changes in Brain Morphology,” *JAMA Neurol.*, vol. 51, no. 9, pp. 874–887, Sep. 1994, doi: 10.1001/archneur.1994.00540210046012.

[84] R. K. Lenroot and J. N. Giedd, “Brain development in children and adolescents: Insights from anatomical magnetic resonance imaging,” *Neurosci. Biobehav. Rev.*, vol. 30, no. 6, pp. 718–729, 2006, doi: 10.1016/j.neubiorev.2006.06.001.

[85] N. Gogtay *et al.*, “Dynamic mapping of human cortical development during childhood through early adulthood,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 101, no. 21, pp. 8174–8179, 2004, doi: 10.1073/pnas.0402680101.

[86] B. J. Nagel, K. L. Medina, J. Yoshii, A. D. Schweinsburg, I. Moadab, and S. F. Tapert, “Age-related changes in prefrontal white matter volume across adolescence,” *Neuroreport*, vol. 13, pp. 1427–1431, 2006, doi: 10.1038/jid.2014.371.

[87] K. L. Medina, T. McQueeny, B. J. Nagel, K. L. Hanson, A. D. Schweinsburg, and S. F. Tapert, “Prefrontal Cortex Volumes in Adolescents With Alcohol Use Disorders: Unique Gender Effects,” *Alcohol. Clin. Exp. Res.*, vol. 32, no. 3, pp. 386–394, 2008, doi: 10.1111/j.1530-0277.2007.00602.x.

[88] M. D. De Bellis, A. Narasimhan, D. L. Thatcher, M. S. Keshavan, P. Soloff, and D. B. Clark, “Prefrontal cortex, thalamus, and cerebellar volumes in adolescents and young adults with adolescent-onset alcohol use disorders and comorbid mental disorders,” *Alcohol. Clin. Exp. Res.*, vol. 29, no. 9, pp. 1590–1600, 2005, doi: 10.1097/01.alc.0000179368.87886.76.

[89] T. McQueeny *et al.*, “Altered white matter integrity in adolescent binge drinkers,” *Alcohol. Clin. Exp. Res.*, vol. 33, no. 7, pp. 1278–1285, 2009, doi: 10.1111/j.1530-0277.2009.00953.x.

[90] M. Pascual, A. Pla, J. Miñarro, and C. Guerri, “Neuroimmune Activation and Myelin Changes in Adolescent Rats Exposed to High-Dose Alcohol and Associated Cognitive Dysfunction: A Review with Reference to Human Adolescent Drinking,” *Alcohol Alcohol.*, vol. 49, no. 2, pp. 187–192, Mar. 2014, doi: 10.1093/alcalc/agt164.

[91] J. Montesinos *et al.*, “TLR4 elimination prevents synaptic and myelin alterations and long-term cognitive dysfunctions in adolescent mice with intermittent ethanol treatment,” 2014, doi: 10.1016/j.bbi.2014.11.015.

[92] E. R. Tavares, A. Silva-Gotay, W. V. Riad, L. Bengston, and H. N. Richardson, “Sex differences in the effect of alcohol drinking on myelinated axons in the anterior cingulate cortex of adolescent rats,” *Brain Sci.*, vol. 9, no. 7, Jul. 2019, doi: 10.3390/brainsci9070167.

[93] C. M. Drzewiecki and J. M. Juraska, “The structural reorganization of the prefrontal cortex during adolescence as a framework for vulnerability to the environment,” *Pharmacol. Biochem. Behav.*, vol. 199, p. 173044, 2020, doi: 10.1016/j.pbb.2020.173044.

[94] W. A. Koss, C. E. Belden, A. D. Hristov, and J. M. Juraska, “Dendritic remodeling in the adolescent medial prefrontal cortex and the basolateral amygdala of male and female rats,” *Synapse*, vol. 68, no. 2, pp. 61–72, 2014, doi: 10.1002/syn.21716.

[95] Y. W. Chen, A. Akad, R. Aderogba, T. G. Chowdhury, and C. Aoki, “Dendrites of the dorsal and ventral hippocampal CA1 pyramidal neurons of singly housed female rats exhibit lamina-specific growths and retractions during adolescence that are responsive to pair housing,” *Synapse*, vol. 72, no. 7, pp. 1–17, 2018, doi: 10.1002/syn.22034.

[96] P. J. Mulholland, T. L. Teppen, K. M. Miller, H. G. Sexton, S. C. Pandey, and H. S. Swartzwelder, “Donepezil Reverses Dendritic Spine Morphology Adaptations and Fmr1 Epigenetic Modifications in Hippocampus of Adult Rats After Adolescent Alcohol Exposure,” *Alcohol. Clin. Exp. Res.*, vol. 42, no. 4, pp. 706–717, 2018, doi: 10.1111/acer.13599.

[97] M. L. Risher *et al.*, “Adolescent Intermittent Alcohol Exposure: Persistence of Structural and Functional Hippocampal Abnormalities into Adulthood,” *Alcohol. Clin. Exp. Res.*, vol. 39, no. 6, pp. 989–997, 2015, doi: 10.1111/acer.12725.

[98] T. R. Insel, L. P. Miller, and R. E. Gelhard, “The ontogeny of excitatory amino acid receptors in the rat forebrain-II. Kainic acid receptors,” *Neuroscience*, vol. 35, no. 1, pp. 45–51, 1990, doi: 10.1016/0306-4522(90)90118-N.

[99] S. L. Andersen, A. T. Thompson, M. Rutstein, J. C. Hostetter, and M. H. Teicher, “Dopamine receptor pruning in prefrontal cortex during the periadolescent period in rats,” *Synapse*, vol. 37, pp. 167–169, 2000, doi: 10.1002/1098-2396(200008)37:2<167::AID-SYN11>3.0.CO;2-B.

[100] E. Flores-Barrera, D. R. Thomases, L. J. Heng, D. K. Cass, A. Caballero, and K. Y. Tseng, "Late adolescent expression of GluN2B transmission in the prefrontal cortex is input-specific and requires postsynaptic protein kinase A and D1 dopamine receptor signaling," *Biol. Psychiatry*, vol. 75, pp. 508–516, 2014, doi: 10.1016/j.biopsych.2013.07.033.

[101] A. Caballero, E. Flores-Barrera, D. K. Cass, and K. Y. Tseng, "Differential regulation of parvalbumin and calretinin interneurons in the prefrontal cortex during adolescence," *Brain Struct. Funct.*, vol. 219, pp. 395–406, 2014, doi: 10.1007/s00429-013-0508-8.

[102] A. D. Schweinsburg, B. J. Nagel, and S. F. Tapert, "fMRI reveals alteration of spatial working memory networks across adolescence," *J. Int. Neuropsychol. Soc.*, vol. 11, no. 5, pp. 631–644, 2005, doi: 10.1017/S1355617705050757.

[103] H. Kwon, A. L. Reiss, V. Menon, and C. E. by Eleanor Maccoby, "Neural basis of protracted developmental changes in visuo-spatial working memory," *PNAS*, vol. 99, no. 20, pp. 13336–13341, 2002, doi: 10.1073/pnas.162486399.

[104] L. M. Squeglia, A. D. Schweinsburg, C. Pulido, and S. F. Tapert, "Adolescent binge drinking linked to abnormal spatial working memory brain activation: Differential gender effects," *Alcohol. Clin. Exp. Res.*, vol. 35, no. 10, pp. 1831–1841, Oct. 2011, doi: 10.1111/j.1530-0277.2011.01527.x.

[105] E. R. Kandel, Y. Dudai, and M. R. Mayford, "The molecular and systems biology of memory," *Cell*, vol. 157, no. 1, pp. 163–186, Mar. 2014, doi: 10.1016/j.cell.2014.03.001.

[106] E. R. Kandel, "The molecular biology of memory: CAMP, PKA, CRE, CREB-1, CREB-2, and CPEB," *Mol. Brain*, vol. 5, no. 1, p. 14, 2012, doi: 10.1186/1756-6606-5-14.

[107] M. M. Jankowski *et al.*, “The anterior thalamus provides a subcortical circuit supporting memory and spatial navigation,” *Front. Syst. Neurosci.*, vol. 7, pp. 1–12, 2013, doi: 10.3389/fnsys.2013.00045.

[108] A. S. Mitchell, “The mediodorsal thalamus as a higher order thalamic relay nucleus important for learning and decision-making,” *Neurosci. Biobehav. Rev.*, vol. 54, pp. 76–88, 2015, doi: 10.1016/j.neubiorev.2015.03.001.

[109] W. O. Guldin, M. Pritzelb, and H. J. Markowitsch, “Prefrontal Cortex of the Mouse Defined as Cortical Projection Area of the Thalamic Mediodorsal Nucleus,” vol. 19, pp. 93–107, 1981.

[110] Y. B. Saalmann, “Intralaminar and medial thalamic influence on cortical synchrony, information transmission and cognition,” *Front. Syst. Neurosci.*, vol. 8, no. MAY, pp. 1–8, 2014, doi: 10.3389/fnsys.2014.00083.

[111] M. P. Witter, T. P. Doan, B. Jacobsen, E. S. Nilssen, and S. Ohara, “Architecture of the entorhinal cortex a review of entorhinal anatomy in rodents with some comparative notes,” *Front. Syst. Neurosci.*, vol. 11, pp. 1–12, 2017, doi: 10.3389/fnsys.2017.00046.

[112] J. J. Knierim, “The hippocampus,” *Curr. Biol.*, vol. 25, no. 23, pp. R1116–R1121, 2015, doi: 10.1016/j.cub.2015.10.049.

[113] H. Eichenbaum, “Prefrontal-hippocampal interactions in episodic memory,” *Nat. Rev. Neurosci.*, vol. 18, pp. 547–558, 2017, doi: 10.1038/nrn.2017.74.

[114] M. Fyhn, S. Molden, M. P. Witter, E. I. Moser, and M. B. Moser, “Spatial representation in the entorhinal cortex,” *Science (80-.).*, vol. 305, no. 5688, pp. 1258–1264, Aug. 2004,

doi: 10.1126/science.1099901.

[115] S. S. Deshmukh and J. J. Knierim, “Representation of non-spatial and spatial information in the lateral entorhinal cortex,” *Front. Behav. Neurosci.*, vol. 5, 2011, doi: 10.3389/fnbeh.2011.00069.

[116] A. Tsao, M. B. Moser, and E. I. Moser, “Traces of experience in the lateral entorhinal cortex,” *Curr. Biol.*, vol. 23, no. 5, pp. 399–405, 2013, doi: 10.1016/j.cub.2013.01.036.

[117] T. Solstad, C. N. Boccara, E. Kropff, M. Moser, and E. I. Moser, “Representation of geometric borders in the Entorhinal Cortex,” *Science (80-.).*, vol. 322, 2008, doi: 10.1126/science.1166466.

[118] E. Kropff, J. E. Carmichael, M. B. Moser, and E. I. Moser, “Speed cells in the medial entorhinal cortex,” *Nature*, vol. 523, no. 7561, pp. 419–424, 2015, doi: 10.1038/nature14622.

[119] T. L. Bjerknes, E. I. Moser, and M. B. Moser, “Representation of geometric borders in the developing rat,” *Neuron*, vol. 82, no. 1, pp. 71–78, 2014, doi: 10.1016/j.neuron.2014.02.014.

[120] R. F. Langston *et al.*, “Development of the Spatial Representation System in the Rat,” *Science (80-.).*, vol. 328, 2010, doi: 10.1126/science.1188210.

[121] T. Wills, F. Cacucci, N. Burgess, and J. O’Keefe, “Development of the Hippocampal Cognitive Map in Preweanling Rats,” *Science (80-.).*, vol. 328, 2010, doi: 10.1126/science.1188224.

[122] W. B. Scoville and B. Milner, “Loss of recent memory after bilateral hippocampal

lesions," *J. Neurol. Neurosurg. Psychiatry*, vol. 20, no. 1, pp. 11–21, 1957, doi: 10.1136/jnnp.20.1.11.

[123] M. S. Fanselow and H. W. Dong, "Are the Dorsal and Ventral Hippocampus Functionally Distinct Structures?," *Neuron*, vol. 65, no. 1, pp. 7–19, Jan. 2010, doi: 10.1016/j.neuron.2009.11.031.

[124] H. E. Scharfman, "The CA3 'backprojection' to the dentate gyrus," *Prog. Brain Res.*, vol. 163, no. 07, pp. 627–637, 2007, doi: 10.1016/S0079-6123(07)63034-9.

[125] K. F. Messanvi, K. Berkun, A. Perkins, and Y. Chudasama, "Parallel Pathways Provide Hippocampal Spatial Information to Prefrontal Cortex," *J. Neurosci.*, vol. 43, no. 1, pp. 68–81, 2023, doi: 10.1523/JNEUROSCI.0846-22.2022.

[126] J. O'Keefe, J. Dostrovsky, and J. D. J. O'Keefe, "The hippocampus as a spatial map . Preliminary evidence from unit activity in the freely-moving rat," *Brain Res.*, vol. 34, no. 1, pp. 171–175, Nov. 1971, doi: 10.1016/0006-8993(71)90358-1.

[127] M. W. Jung, S. I. Wiener, and B. L. McNaughton, "Comparison of spatial firing characteristics of units in dorsal and ventral hippocampus of the rat," *J. Neurosci.*, vol. 14, no. 12, pp. 7347–7356, 1994, doi: 10.1523/jneurosci.14-12-07347.1994.

[128] S. Leutgeb, J. K. Leutgeb, C. A. Barnes, E. I. Moser, B. L. McNaughton, and M. B. Moser, "Neuroscience: Independent codes for spatial and episodic memory in hippocampal neuronal ensembles," *Science (80-.).*, vol. 309, no. 5734, pp. 619–623, 2005, doi: 10.1126/science.1114037.

[129] J. Ye, M. P. Witter, M. B. Moser, and E. I. Moser, "Entorhinal fast-spiking speed cells

project to the hippocampus," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 115, no. 7, pp. E1627–E1636, 2018, doi: 10.1073/pnas.1720855115.

[130] R. P. Vertes, *Major diencephalic inputs to the hippocampus: Supramammillary nucleus and nucleus reuniens. Circuitry and function*, 1st ed., vol. 219. Elsevier B.V., 2015.

[131] S. Tao *et al.*, "Whole-Brain Mapping the Direct Inputs of Dorsal and Ventral CA1 Projection Neurons," *Front. Neural Circuits*, vol. 15, pp. 1–16, 2021, doi: 10.3389/fncir.2021.643230.

[132] S. Ohara, M. Rannap, K. I. Tsutsui, A. Draguhn, A. V. Egorov, and M. P. Witter, "Hippocampal-medial entorhinal circuit is differently organized along the dorsoventral axis in rodents," *Cell Rep.*, vol. 42, no. 1, 2023, doi: 10.1016/j.celrep.2023.112001.

[133] S. Alfonso-Loeches and C. Guerri, "Molecular and behavioral aspects of the actions of alcohol on the adult and developing brain," *Crit. Rev. Clin. Lab. Sci.*, vol. 48, no. 1, pp. 19–47, 2011, doi: 10.3109/10408363.2011.580567.

[134] G. R. I. Barker and E. C. Warburton, "When is the hippocampus involved in recognition memory?," *J. Neurosci.*, vol. 31, no. 29, pp. 10721–10731, Jul. 2011, doi: 10.1523/JNEUROSCI.6413-10.2011.

[135] G. R. I. Barker, F. Bird, V. Alexander, and E. C. Warburton, "Recognition Memory for Objects, Place, and Temporal Order: A Disconnection Analysis of the Role of the Medial Prefrontal Cortex and Perirhinal Cortex," *J. Neurosci.*, vol. 27, no. 11, pp. 2948–2957, 2007, doi: 10.1523/jneurosci.5289-06.2007.

[136] O. Y. Chao, S. Nikolaus, Y. M. Yang, and J. P. Huston, "Neuronal circuitry for

recognition memory of object and place in rodent models," *Neurosci. Biobehav. Rev.*, vol. 141, 2022, doi: 10.1016/j.neubiorev.2022.104855.

[137] M. Antunes and G. Biala, "The novel object recognition memory: Neurobiology, test procedure, and its modifications," *Cogn. Process.*, vol. 13, no. 2, pp. 93–110, 2012, doi: 10.1007/s10339-011-0430-z.

[138] N. J. Broadbent, L. R. Squire, and R. E. Clark, "Spatial memory, recognition memory, and the hippocampus," *PNAS*, vol. 101, no. 40, pp. 14515–14520, 2004, doi: 10.1073/pnas.0406344101.

[139] N. J. Broadbent, S. Gaskin, L. R. Squire, and R. E. Clark, "Object recognition memory and the rodent hippocampus," *Learn. Mem.*, vol. 17, pp. 5–11, 2010, doi: 10.1101/lm.1650110.

[140] J. Basu *et al.*, "Gating of hippocampal activity, plasticity, and memory by entorhinal cortex long-range inhibition," *Science (80-.).*, vol. 351, no. 6269, 2016, doi: 10.1126/science.aaa5694.

[141] A. B. de Landeta, M. Pereyra, M. Miranda, P. Bekinschtein, J. H. Medina, and C. Katche, "Functional connectivity of anterior retrosplenial cortex in object recognition memory," *Neurobiol. Learn. Mem.*, vol. 186, 2021, doi: 10.1016/j.nlm.2021.107544.

[142] J. J. Tuscher, L. R. Taxier, A. M. Fortress, and K. M. Frick, "Chemogenetic inactivation of the dorsal hippocampus and medial prefrontal cortex, individually and concurrently, impairs object recognition and spatial memory consolidation in female mice," *Neurobiol. Learn. Mem.*, vol. 156, pp. 103–116, 2018, doi: 10.1016/j.nlm.2018.11.002.

[143] T. Raam, K. M. McAvoy, A. Besnard, A. Veenema, and A. Sahay, “Hippocampal oxytocin receptors are necessary for discrimination of social stimuli,” *Nat. Commun.*, vol. 8, pp. 1–14, 2017, doi: 10.1038/s41467-017-02173-0.

[144] Y. Qiao, X. Wang, L. Ma, S. Li, and J. Liang, “Functional inactivation of dorsal medial striatum alters behavioral flexibility and recognition process in mice,” *Physiol. Behav.*, vol. 179, pp. 467–477, 2017, doi: 10.1016/j.physbeh.2017.07.026.

[145] A. Benn *et al.*, “Optogenetic stimulation of prefrontal glutamatergic neurons enhances recognition memory,” *J. Neurosci.*, vol. 36, no. 18, pp. 4930–4939, 2016, doi: 10.1523/JNEUROSCI.2933-15.2016.

[146] R. F. Langston and E. R. Wood, “Associative recognition and the hippocampus: Differential effects of hippocampal lesions on object-place, object-context and object-place-context memory,” *Hippocampus*, vol. 20, pp. 1139–1153, 2010, doi: 10.1002/hipo.20714.

[147] C. Wang *et al.*, “Hippocampus–prefrontal coupling regulates recognition memory for novelty discrimination,” *J. Neurosci.*, vol. 41, no. 46, pp. 9617–9632, 2021, doi: 10.1523/JNEUROSCI.1202-21.2021.

[148] C. V. Vorhees and M. T. Williams, “Assessing spatial learning and memory in rodents,” *ILAR J.*, vol. 55, no. 2, pp. 310–332, Jan. 2014, doi: 10.1093/ilar/ilu013.

[149] M. Pitts, “Barnes Maze Procedure for Spatial Learning and Memory in Mice,” *Bio-Protocol*, vol. 8, no. 5, pp. 1–11, 2018, doi: 10.21769/bioprotoc.2744.

[150] A. Baddeley, “Working Memory,” *Sc*, vol. 255, no. 5044, pp. 556–559, 2008, doi:

10.1126/science.1736359.

[151] W. C. Leon, M. A. Bruno, S. Allard, K. Nader, and A. C. Cuello, “Engagement of the PFC in consolidation and recall of recent spatial memory,” *Learn. Mem.*, vol. 17, no. 6, pp. 297–305, 2010, doi: 10.1101/lm.1804410.

[152] C. Chow, J. R. Epp, S. E. Lieblich, C. K. Barha, and L. A. M. Galea, “Sex differences in neurogenesis and activation of new neurons in response to spatial learning and memory,” *Psychoneuroendocrinology*, vol. 38, pp. 1236–1250, 2013, doi: 10.1016/j.psyneuen.2012.11.007.

[153] S. A. Tennant *et al.*, “Stellate Cells in the Medial Entorhinal Cortex Are Required for Spatial Learning,” *Cell Rep.*, vol. 22, no. 5, pp. 1313–1324, 2018, doi: 10.1016/j.celrep.2018.01.005.

[154] D. Jung, Y. Huh, and J. Cho, “The ventral midline thalamus mediates hippocampal spatial information processes upon spatial cue changes,” *J. Neurosci.*, vol. 39, no. 12, pp. 2276–2290, 2019, doi: 10.1523/JNEUROSCI.2127-18.2019.

[155] M. R. Schwabe, C. M. Lincoln, M. M. Ivers, and K. M. Frick, “Chemogenetic inactivation of the nucleus reuniens impairs object placement memory in female mice,” *Neurobiol. Learn. Mem.*, vol. 185, p. 107521, 2021, doi: 10.1016/j.nlm.2021.107521.

[156] J. B. Kahn, R. G. Port, C. Yue, H. Takano, and D. A. Coulter, “Circuit-based interventions in the dentate gyrus rescue epilepsy-associated cognitive dysfunction,” *Brain*, vol. 142, no. 9, pp. 2705–2721, 2019, doi: 10.1093/brain/awz209.

[157] G. Barker, S. Tran, K. Gilroy, Z. Bashir, and E. Warburton, “Encoding and retrieval of

associative recognition memory engage different sub-networks within a hippocampal-thalamo-cortical memory circuit," *Res. Sq.*, pp. 0–50, 2021.

[158] G. R. I. Barker and E. C. Warburton, "A critical role for the nucleus reuniens in long-term, but not short-term associative recognition memory formation," *J. Neurosci.*, vol. 38, no. 13, pp. 3208–3217, 2018, doi: 10.1523/JNEUROSCI.1802-17.2017.

[159] L. Q. Uddin, "Cognitive and behavioural flexibility: neural mechanisms and clinical considerations," *Nat. Rev. Neurosci. 2021 223*, vol. 22, no. 3, pp. 167–179, Feb. 2021, doi: 10.1038/s41583-021-00428-w.

[160] L. G. Coleman, W. Liu, I. Oguz, M. Styner, and F. T. Crews, "Adolescent binge ethanol treatment alters adult brain regional volumes, cortical extracellular matrix protein and behavioral flexibility," *Pharmacol. Biochem. Behav.*, vol. 116, no. 1, pp. 142–151, 2014, doi: 10.1038/jid.2014.371.

[161] J. M. Barker, L. J. Chandler, K. G. Bryant, and J. I. Osborne, "Age and Sex Interact to Mediate the Effects of Intermittent, High-Dose Ethanol Exposure on Behavioral Flexibility," *Front. Pharmacol.*, vol. 8, 2017, doi: 10.3389/fphar.2017.00450.

[162] G. M. Fernandez, B. J. Lew, L. C. Vedder, and L. M. Savage, "Chronic intermittent ethanol exposure leads to alterations in brain-derived neurotrophic factor within the frontal cortex and impaired behavioral flexibility in both adolescent and adult rats," *Neuroscience*, vol. 348, pp. 324–334, 2017, doi: 10.1016/j.neuroscience.2017.02.045.

[163] D. B. Matthews *et al.*, "Chronic Intermittent Ethanol Administration during Adolescence Produces Sex Dependent Impairments in Behavioral Flexibility and Survivability," *Brain Sci.*, vol. 12, no. 5, p. 606, May 2022, doi: 10.3390/brainsci12050606.

[164] P. R. Hunt and J. P. Aggleton, “Neurotoxic lesions of the dorsomedial thalamus impair the acquisition but not the performance of delayed matching to place by rats: A deficit in shifting response rules,” *J. Neurosci.*, vol. 18, no. 23, pp. 10045–10052, 1998, doi: 10.1523/jneurosci.18-23-10045.1998.

[165] J. M. Birrell and V. J. Brown, “Medial frontal cortex mediates perceptual attentional set shifting in the rat,” *J. Neurosci.*, vol. 20, no. 11, pp. 4320–4324, 2000, doi: 10.1523/jneurosci.20-11-04320.2000.

[166] M. E. Ragozzino, S. Detrick, and R. P. Kesner, “Involvement of the prelimbic-infralimbic areas of the rodent prefrontal cortex in behavioral flexibility for place and response learning,” *J. Neurosci.*, vol. 19, no. 11, pp. 4585–4594, 1999, doi: 10.1523/jneurosci.19-11-04585.1999.

[167] K. P. Giese and K. Mizuno, “The roles of protein kinases in learning and memory,” *Learn. Mem.*, vol. 20, pp. 540–52, Sep. 2013, doi: 10.1101/lm.028449.112.

[168] C. Missale, S. R. Nash, S. W. Robinson, M. Jaber, and M. G. Caron, “Dopamine Receptors: From Structure to Function,” *Physiol. Rev.*, vol. 78, no. 1, 1998, doi: 10.1152/physrev.1998.78.1.189.

[169] N. Lezcano and C. Bergson, “D1/D5 Dopamine Receptors Stimulate Intracellular Calcium Release in Primary Cultures of Neocortical and Hippocampal Neurons,” *J Neurophysiol*, vol. 87, pp. 2167–2175, 2002, doi: 10.1152/jn.00541.2001.

[170] Y. Ming, H. Zhang, L. Long, F. Wang, J. Chen, and X. Zhen, “Modulation of Ca2+ signals by phosphatidylinositol-linked novel D1 dopamine receptor in hippocampal neurons,” *J. Neurochem.*, vol. 98, no. 4, pp. 1316–1323, Aug. 2006, doi: 10.1111/j.1471-

4159.2006.03961.x.

[171] M. K. Sun and D. L. Alkon, “The ‘memory kinases’: Roles of PKC isoforms in signal processing and memory formation,” in *Progress in Molecular Biology and Translational Science*, 2014.

[172] S. Ortega-Martínez, “A new perspective on the role of the CREB family of transcription factors in memory consolidation via adult hippocampal neurogenesis,” *Front. Mol. Neurosci.*, vol. 8, no. 46, 2015, doi: 10.3389/fnmol.2015.00046.

[173] A. R. Means and J. R. Dedman, “Calmodulin-an intracellular calcium receptor,” *Nature*, vol. 285, pp. 73–77, 1980, doi: 10.1038/285073a0.

[174] S. Impey *et al.*, “Cross talk between ERK and PKA is required for Ca2+ stimulation of CREB-dependent transcription and ERK nuclear translocation,” *Neuron*, vol. 21, pp. 869–883, Oct. 1998, doi: 10.1016/S0896-6273(00)80602-9.

[175] S. Impey *et al.*, “Phosphorylation of CBP mediates transcriptional activation by neural activity and CaM kinase IV,” *Neuron*, vol. 34, pp. 235–244, Apr. 2002, doi: 10.1016/S0896-6273(02)00654-2.

[176] G. Chen, X. Zou, H. Watanabe, J. M. Van Deursen, and J. Shen, “CREB binding protein is required for both short-term and long-term memory formation,” *J. Neurosci.*, vol. 30, no. 39, pp. 13066–13077, 2010, doi: 10.1523/JNEUROSCI.2378-10.2010.

[177] K. Takao *et al.*, “Comprehensive behavioral analysis of calcium/calmodulin-dependent protein kinase IV knockout mice,” *PLoS One*, vol. 5, no. 3, Mar. 2010, doi: 10.1371/journal.pone.0009460.

[178] C. G. Vecsey *et al.*, “Histone deacetylase inhibitors enhance memory and synaptic plasticity via CREB: CBP-dependent transcriptional activation,” *J. Neurosci.*, vol. 27, no. 23, pp. 6128–6140, 2007, doi: 10.1523/JNEUROSCI.0296-07.2007.

[179] Y. Tanaka *et al.*, “Extensive brain hemorrhage and embryonic lethality in a mouse null mutant of CREB-binding protein,” *Mech. Dev.*, vol. 95, no. 1–2, pp. 133–145, Jul. 2000, doi: 10.1016/S0925-4773(00)00360-9.

[180] D. C. Bedford and P. K. Brindle, “Is histone acetylation the most important physiological function for CBP and p300?,” *Aging (Albany. NY).*, vol. 4, no. 4, pp. 247–255, 2012, doi: 10.18632/aging.100453.

[181] Y. Kamei *et al.*, “A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors,” *Cell*, vol. 85, no. 3, pp. 403–414, 1996, doi: 10.1016/S0092-8674(00)81118-6.

[182] K. A. Sheppard *et al.*, “Nuclear integration of glucocorticoid receptor and nuclear factor-κB signaling by CREB-binding protein and steroid receptor coactivator-1,” *J. Biol. Chem.*, vol. 273, no. 45, pp. 29291–29294, 1998, doi: 10.1074/jbc.273.45.29291.

[183] J. Chrivia, R. P. S. Kwok, N. Lamb, M. Haglwara, M. R. Montminy, and R. H. Goodman, “Phosphorylated CREB binds specifically to the nuclear protein CBP,” *Nature*, vol. 365, pp. 855–859, 1993.

[184] J. A. Kim, J. Lu, and P. G. Quinn, “Distinct cAMP response element-binding protein (CREB) domains stimulate different steps in a concerted mechanism of transcription activation,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 97, no. 21, pp. 11292–11296, Oct. 2000, doi: 10.1073/pnas.97.21.11292.

[185] E. Korzus, M. G. Rosenfeld, and M. Mayford, “CBP histone acetyltransferase activity is a critical component of memory consolidation,” *Neuron*, vol. 42, pp. 961–972, Jun. 2004, doi: 10.1016/j.neuron.2004.06.002.

[186] R. A. Henry, Y. M. Kuo, and A. J. Andrews, “Differences in specificity and selectivity between CBP and p300 acetylation of histone H3 and H3/H4,” *Biochemistry*, vol. 52, no. 34, pp. 5746–5759, 2013, doi: 10.1021/bi400684q.

[187] H. M. Sheppard, J. C. Harries, S. Hussain, C. Bevan, and D. M. Heery, “Analysis of the Steroid Receptor Coactivator 1 (SRC1)-CREB Binding Protein Interaction Interface and Its Importance for the Function of SRC1 Downloaded from,” *Mol. Cell. Biol.*, vol. 21, no. 1, pp. 39–50, 2001, doi: 10.1128/MCB.21.1.39-50.2001.

[188] M. Schoof *et al.*, “The transcriptional coactivator and histone acetyltransferase CBP regulates neural precursor cell development and migration,” *Acta Neuropathol. Commun.*, vol. 7, no. 1, p. 199, Dec. 2019, doi: 10.1186/s40478-019-0849-5.

[189] A. Gouveia *et al.*, “The aPKC-CBP Pathway Regulates Adult Hippocampal Neurogenesis in an Age-Dependent Manner,” *Stem Cell Reports*, vol. 7, no. 4, pp. 719–734, 2016, doi: 10.1016/j.stemcr.2016.08.007.

[190] J. P. Lopez-Atalaya *et al.*, “CBP is required for environmental enrichment-induced neurogenesis and cognitive enhancement,” *EMBO J.*, vol. 30, no. 20, pp. 4287–4298, Oct. 2011, doi: 10.1038/emboj.2011.299.

[191] T. Tully, R. Bourtchouladze, R. Scott, and J. Tallman, “Targeting the creb pathway for memory enhancers,” *Nat. Rev. Drug Discov.*, vol. 2, no. 4, pp. 267–277, 2003, doi: 10.1038/nrd1061.

[192] M. A. Wood *et al.*, “Transgenic mice expressing a truncated form of CREB-binding protein (CBP) exhibit deficits in hippocampal synaptic plasticity and memory storage,” *Learn. Mem.*, vol. 12, no. 2, pp. 111–119, Mar. 2005, doi: 10.1101/lm.86605.inhibitory.

[193] R. M. Barrett *et al.*, “Hippocampal focal knockout of CBP affects specific histone modifications, long-term potentiation, and long-term memory,” *Neuropsychopharmacology*, vol. 36, no. 8, pp. 1545–1556, Apr. 2011, doi: 10.1038/npp.2011.61.

[194] A. Caccamo, M. A. Maldonado, A. F. Bokov, S. Majumder, and S. Oddo, “CBP gene transfer increases BDNF levels and ameliorates learning and memory deficits in a mouse model of Alzheimer’s disease,” *PNAS*, vol. 107, no. 52, pp. 22687–22692, 2010, doi: 10.1073/pnas.1012851108.

[195] L. M. Valor, M. M. Pulopulos, M. Jimenez-Minchan, R. Olivares, B. Lutz, and A. Barco, “Ablation of CBP in Forebrain Principal Neurons Causes Modest Memory and Transcriptional Defects and a Dramatic Reduction of Histone Acetylation But Does Not Affect Cell Viability,” *J. Neurosci.*, vol. 31, no. 5, 2011, doi: 10.1523/JNEUROSCI.4737-10.2011.

[196] P. A. Vieira and E. Korzus, “CBP-Dependent Memory Consolidation in the Prefrontal Cortex Supports Object-Location Learning,” *Hippocampus*, vol. 25, pp. 1532–1540, Dec. 2015, doi: 10.1002/hipo.22473.

[197] B. Starkman, A. J. Sakharkar, and S. Pandey, “Epigenetics — Beyond the genome in Alcoholism,” *Alcohol Res. Curr. Rev.*, vol. 34, no. 3, pp. 293–305, 2012.

[198] S. Chatterjee *et al.*, “A novel activator of CBP/p300 acetyltransferases promotes

neurogenesis and extends memory duration in adult mice," *J. Neurosci.*, vol. 33, no. 26, pp. 10698–10712, 2013, doi: 10.1523/JNEUROSCI.5772-12.2013.

[199] C. for B. H. S. and Quality, "Behavioral health trends in the United States: Results from the 2014 National Survey on Drug Use and Health," Rockville, 2015. [Online]. Available: http://www.samhsa.gov/ data/.

[200] F. T. Crews, R. P. Vetreno, M. A. Broadwater, and D. L. Robinson, "Adolescent alcohol exposure persistently impacts adult neurobiology and behavior," *Pharmacol. Rev.*, vol. 68, pp. 1074–1109, 2016, doi: 10.1124/pr.115.012138.

[201] R. M. Philpot, L. Wecker, and C. L. Kirstein, "Repeated ethanol exposure during adolescence alters the developmental trajectory of dopaminergic output from the nucleus accumbens septi," *Int. J. Dev. Neurosci.*, vol. 27, pp. 805–815, 2009, doi: 10.1016/j.ijdevneu.2009.08.009.

[202] H. El Marroun *et al.*, "Alcohol use and brain morphology in adolescence: A longitudinal study in three different cohorts," *Eur. J. Neurosci.*, vol. 54, pp. 6012–6026, Sep. 2021, doi: 10.1111/ejn.15411.

[203] I. A. McKenzie *et al.*, "Motor skill learning requires active central myelination," *Science (80-.).*, vol. 346, no. 6207, pp. 318–322, Oct. 2014, doi: 10.1126/science.1254960.

[204] L. Xiao *et al.*, "Rapid production of new oligodendrocytes is required in the earliest stages of motor skill learning Europe PMC Funders Group," *Nat. Neurosci.*, vol. 19, no. 9, pp. 1210–1217, 2016, doi: 10.1038/nn.4351.

[205] L. P. Spear, "Adolescent Alcohol Exposure : Are There Separable," *Physiol. Behav.*, vol.

148, pp. 122–130, Sep. 2015, doi: 10.1016/j.physbeh.2015.01.027.

[206] L. Yetnikoff, R. A. Reichard, Z. M. Schwartz, K. P. Parsely, and D. S. Zahm, "Protracted Maturation of Forebrain Afferent Connections of the Ventral Tegmental Area in the Rat," *J. Comp. Neurol.*, vol. 522, pp. 1031–1047, 2014, doi: 10.1002/cne.23459.

[207] A. Manduca, M. Servadio, R. Damsteegt, P. Campolongo, L. J. M. J. Vanderschuren, and V. Trezza, "Dopaminergic neurotransmission in the nucleus accumbens modulates social play behavior in rats," *Neuropsychopharmacology*, vol. 41, pp. 2215–2223, 2016, doi: 10.1038/npp.2016.22.

[208] G. F. Koob, P. P. Sanna, and F. E. Bloom, "Neuroscience of addiction," *Neuron*, vol. 21, pp. 467–476, Sep. 1998, doi: 10.1016/S0896-6273(00)80557-7.

[209] E. I. Varlinskaya and L. P. Spear, "Acute effects of ethanol on social behavior of adolescent and adult rats: Role of familiarity of the test situation," *Alcohol. Clin. Exp. Res.*, vol. 26, no. 10, pp. 1502–1511, Oct. 2002, doi: 10.1111/j.1530-0277.2002.tb02449.x.

[210] Y. Goto and A. A. Grace, "Limbic and cortical information processing in the nucleus accumbens," *Trends Neurosci.*, vol. 31, no. 11, pp. 552–558, Nov. 2008, doi: 10.1016/j.tins.2008.08.002.

[211] L. M. DeVito and H. Eichenbaum, "Distinct contributions of the hippocampus and medial prefrontal cortex to the 'what-where-when' components of episodic-like memory in mice," *Behav. Brain Res.*, vol. 215, no. 2, pp. 318–325, Dec. 2010, doi: 10.1016/j.bbr.2009.09.014.

[212] L. K. Bicks, H. Koike, S. Akbarian, and H. Morishita, “Prefrontal cortex and social cognition in mouse and man,” *Front. Psychol.*, vol. 6, pp. 1–15, 2015, doi: 10.3389/fpsyg.2015.01805.

[213] M. Pascual, J. Boix, V. Felipo, and C. Guerri, “Repeated alcohol administration during adolescence causes changes in the mesolimbic dopaminergic and glutamatergic systems and promotes alcohol intake in the adult rat,” *J. Neurochem.*, vol. 108, pp. 920–931, Feb. 2009, doi: 10.1111/j.1471-4159.2008.05835.x.

[214] H. S. Swartzwelder, A. Hogan, M. L. Risher, R. A. Swartzwelder, W. A. Wilson, and S. K. Acheson, “Effect of sub-chronic intermittent ethanol exposure on spatial learning and ethanol sensitivity in adolescent and adult rats,” *Alcohol*, vol. 48, no. 4, pp. 353–360, 2014, doi: 10.1016/j.alcohol.2014.02.003.

[215] L. C. Melón and S. L. Boehm, “Role of Genotype in the Development of Locomotor Sensitization to Alcohol in Adult and Adolescent Mice: Comparison of the DBA/2J and C57BL/6J Inbred Mouse Strains,” *Alcohol. Clin. Exp. Res.*, vol. 35, no. 7, pp. 1351–1360, Jul. 2011, doi: 10.1111/j.1530-0277.2011.01471.x.

[216] M. Frezza, C. di Padova, G. Pozzato, M. Terpin, E. Baraona, and C. Lieber, “High Blood Alcohol Levels in Women — The Role of Decreased Gastric Alcohol Dehydrogenase Activity and First-Pass Metabolism,” *N. Engl. J. Med.*, vol. 322, no. 2, pp. 977–986, 1990, doi: 10.1056/NEJM199309303291401.

[217] S. J. Nixon, “Cognitive Deficits in Alcoholic Women,” *Alcohol Health Res. World*, vol. 18, no. 3, pp. 228–232, 1994, Accessed: Aug. 06, 2020. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/31798100.

[218] National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals., *Guide for the Care and Use of Laboratory Animals 8th edition*. 2011.

[219] A. B. Pais, A. C. Pais, G. Elmisurati, S. H. Park, M. F. Miles, and J. T. Wolstenholme, "A novel neighbor housing environment enhances social interaction and rescues cognitive deficits from social isolation in adolescence," *Brain Sci.*, vol. 9, no. 12, 2019, doi: 10.3390/brainsci9120336.

[220] S. A. Golden, H. E. Covington, O. Berton, and S. J. Russo, "A standardized protocol for repeated social defeat stress in mice," *Nat. Protoc.*, vol. 6, pp. 1183–1191, 2011, doi: 10.1038/nprot.2011.361.

[221] E. Warburton and M. Brown, "Neural circuitry for rat recognition memory," *Behav. Brain Res.*, vol. 285, pp. 131–139, 2015, doi: 10.1016/j.bbr.2014.09.050.

[222] J. T. Wolstenholme *et al.*, "Dietary Omega-3 Fatty Acids Differentially Impact Acute Ethanol-Responsive Behaviors and Ethanol Consumption in DBA/2J Versus C57BL/6J Mice," *Alcohol. Clin. Exp. Res.*, vol. 42, no. 8, pp. 1476–1485, 2018, doi: 10.1111/acer.13780.

[223] I. Ponomarev and J. C. Crabbe, "A novel method to assess initial sensitivity and acute functional tolerance to hypnotic effects of ethanol," *J. Pharmacol. Exp. Ther.*, vol. 302, no. 1, pp. 257–263, Jul. 2002, doi: 10.1124/jpet.302.1.257.

[224] P. R. Hof, *Comparative cytoarchitectonic atlas of the C57BL/6 and 129/Sv mouse brains* . Amsterdam: Elsevier, 2000.

[225] J. M. Boggs, “Myelin basic protein: a multifunctional protein,” *Cell. Mol. life Sci.*, vol. 63, pp. 1945–1961, Sep. 2006, doi: 10.1007/S00018-006-6094-7.

[226] P. E. Knapp, “Proteolipid protein: is it more than just a structural component of myelin?,” *Dev. Neurosci.*, vol. 18, no. 4, pp. 297–308, 1996, doi: 10.1159/000111420.

[227] N. Schaeren-Wiemers *et al.*, “The raft-associated protein MAL is required for maintenance of proper axon-glia interactions in the central nervous system,” *J. Cell Biol.*, vol. 166, no. 5, pp. 731–742, Aug. 2004, doi: 10.1083/jcb.200406092.

[228] I. Schäfer, C. Müller, H. Luhmann, and R. White, “MOBP levels are regulated by Fyn kinase and affect the morphological differentiation of oligodendrocytes,” *J. Cell Sci.*, vol. 129, no. 5, pp. 930–942, 2016, doi: 10.1242/JCS.172148.

[229] S. E. File, “The use of social interaction as a method for detecting anxiolytic activity of chlordiazepoxide-like drugs,” *J. Neurosci. Methods*, vol. 2, no. 3, pp. 219–238, Jun. 1980, doi: 10.1016/0165-0270(80)90012-6.

[230] S. E. File and P. Seth, “A review of 25 years of the social interaction test,” *Eur. J. Pharmacol.*, vol. 463, pp. 35–53, Feb. 2003, doi: 10.1016/S0014-2999(03)01273-1.

[231] G. D. Greenberg *et al.*, “Sex differences in stress-induced social withdrawal: role of brain derived neurotrophic factor in the bed nucleus of the stria terminalis,” *Front. Behav. Neurosci.*, vol. 7, no. 233, 2014, doi: 10.3389/fnbeh.2013.00223.

[232] B. C. Trainor *et al.*, “Sex Differences in Social Interaction Behavior Following Social Defeat Stress in the Monogamous California Mouse (Peromyscus californicus),” *PLoS One*, vol. 6, no. 2, p. e17405, Feb. 2011, doi: 10.1371/journal.pone.0017405.

[233] J. L. Winward, K. L. Hanson, S. F. Tapert, and S. A. Brown, “Heavy alcohol use, marijuana use, and concomitant use by adolescents are associated with unique and shared cognitive decrements,” *J. Int. Neuropsychol. Soc.*, vol. 20, no. 8, pp. 784–795, Aug. 2014, doi: 10.1017/S1355617714000666.

[234] M. Pascual, A. M. Blanco, O. Cauli, J. Miñarro, and C. Guerri, “Intermittent ethanol exposure induces inflammatory brain damage and causes long-term behavioural alterations in adolescent rats,” *Eur. J. Neurosci.*, vol. 25, pp. 541–550, Jan. 2007, doi: 10.1111/j.1460-9568.2006.05298.x.

[235] H. Lacaille, D. Duterte-Boucher, D. Liot, H. Vaudry, M. Naassila, and D. Vaudry, “Comparison of the deleterious effects of binge drinking-like alcohol exposure in adolescent and adult mice,” *J. Neurochem.*, vol. 132, pp. 629–641, 2015, doi: 10.1111/jnc.13020.

[236] A. Cippitelli *et al.*, “Reversibility of object recognition but not spatial memory impairment following binge-like alcohol exposure in rats,” *Neurobiol. Learn. Mem.*, vol. 94, pp. 538–546, 2010, doi: 10.1016/j.nlm.2010.09.006.

[237] A. Gano, R. M. Pautassi, T. L. Doremus-Fitzwater, and T. Deak, “Conditioned effects of ethanol on the immune system,” *Exp. Biol. Med.*, vol. 242, pp. 718–730, Apr. 2017, doi: 10.1177/1535370217694097.

[238] X. J. Chai, N. Ofen, J. D. E. Gabrieli, and S. Whitfield-Gabrieli, “Development of deactivation of the default-mode network during episodic memory formation,” *Neuroimage*, vol. 84, pp. 932–938, 2014, doi: 10.1016/j.neuroimage.2013.09.032.

[239] S. M. Daselaar, S. E. Prince, and R. Cabeza, “When less means more: Deactivations

during encoding that predict subsequent memory," *Neuroimage*, vol. 23, pp. 921–927, 2004, doi: 10.1016/j.neuroimage.2004.07.031.

[240] E. I. Varlinskaya and L. P. Spear, "Acute Ethanol Withdrawal (Hangover) and Social Behavior in Adolescent and Adult Male and Female Sprague-Dawley Rats," *Alcohol. Clin. Exp. Res.*, vol. 28, no. 1, pp. 40–50, Jan. 2004, doi: 10.1097/01.ALC.0000108655.51087.DF.

[241] H. Shoji, K. Takao, S. Hattori, and T. Miyakawa, "Age-related changes in behavior in C57BL/ 6J mice from young adulthood to middle age," *Mol. Brain*, vol. 9, no. 11, 2016, doi: 10.1186/s13041-016-0191-9.

[242] R. Stevenson, J. Besheer, and C. Hodge, "Comparison of ethanol locomotor sensitization in adolescent and adult DBA/2J mice," *Psychopharmacology (Berl).*, vol. 197, pp. 361–370, Apr. 2008, doi: 10.1007/S00213-007-1038-Y.

[243] L. P. Spear, "Adolescents and alcohol: Acute sensitivities, enhanced intake, and later consequences," *Neurotoxicol. Teratol.*, vol. 41, pp. 51–59, Jan. 2014, doi: 10.1016/j.ntt.2013.11.006.

[244] G. M. Fernandez and L. M. Savage, "Adolescent binge ethanol exposure alters specific forebrain cholinergic cell populations and leads to selective functional deficits in the prefrontal cortex," *Neuroscience*, vol. 361, pp. 129–143, 2017, doi: 10.1016/j.neuroscience.2017.08.013.

[245] E. Mezey and J. J. Potter, "Effect of Dihydrotestosterone on Rat Liver Alcohol Dehydrogenase Activity," *Hepatology*, vol. 2, no. 3, pp. 359S-365S, 1982, doi: 10.1002/hep.1840020312.

[246] M. Qulali, R. A. Ross, and D. W. Crabb, “Estradiol induces class I alcohol dehydrogenase activity and mRNA in kidney of female rats,” *Arch. Biochem. Biophys.*, vol. 288, no. 2, pp. 406–413, 1991, doi: 10.1016/0003-9861(91)90213-3.

[247] U. N. Rao, M. Aravindakshan, V. Satyanarayan, and P. S. Chauhan, “Genotype- and gender-dependent hepatic alcohol dehydrogenase (ADH) activity in developing mice,” *Alcohol*, vol. 14, no. 6, pp. 527–531, 1997, doi: 10.1016/S0741-8329(97)00040-2.

[248] D. N. Linsenbardt, E. M. Moore, C. D. Gross, K. J. Goldfarb, L. C. Blackman, and S. L. Boehm, “Sensitivity and tolerance to the hypnotic and ataxic effects of ethanol in adolescent and adult C57BL/6J and DBA/2J mice,” *Alcohol. Clin. Exp. Res.*, vol. 33, no. 3, pp. 464–476, Mar. 2009, doi: 10.1111/j.1530-0277.2008.00857.x.

[249] R. H. Quarles, “Myelin-associated glycoprotein (MAG): Past, present and beyond,” *J. Neurochem.*, vol. 100, pp. 1431–1448, Mar. 2007, doi: 10.1111/j.1471-4159.2006.04319.x.

[250] R. Sircar, “Estrogen Modulates Ethanol-Induced Memory Deficit in Postpubertal Adolescent Rats,” *Alcohol. Clin. Exp. Res.*, vol. 43, pp. 61–68, 2019, doi: 10.1111/acer.13921.

[251] P. Sampedro-Piquero, R. D. Moreno-Fernández, A. Begega, M. López, and L. J. Santín, “Long-term consequences of alcohol use in early adolescent mice: Focus on neuroadaptations in GR, CRF and BDNF,” *Addict. Biol.*, vol. 27, no. 2, pp. 1–13, 2022, doi: 10.1111/adb.13158.

[252] N. L. Reitz, P. T. Nunes, and L. M. Savage, “Adolescent Binge-Type Ethanol Exposure in Rats Mirrors Age-Related Cognitive Decline by Suppressing Cholinergic Tone and

Hippocampal Neurogenesis," *Front. Behav. Neurosci.*, vol. 15, 2021, doi: 10.3389/fnbeh.2021.772857.

[253] I. Rico-Barrio *et al.*, "Cognitive and neurobehavioral benefits of an enriched environment on young adult mice after chronic ethanol consumption during adolescence," *Addict. Biol.*, vol. 24, no. 5, pp. 969–980, 2019, doi: 10.1111/adb.12667.

[254] B. J. Markwiese, S. K. Acheson, E. D. Levin, W. A. Wilson, and H. S. Swartzwelder, "Differential Effects of Ethanol on Memory in Adolescent and Adult Rats," *Alcohol. Clin. Exp. Res.*, vol. 22, no. 2, pp. 416–421, Apr. 1998, doi: 10.1111/j.1530-0277.1998.tb03668.x.

[255] R. P. Vetreno and F. T. Crews, "Adolescent binge drinking increases expression of the danger signal receptor agonist HMGB1 and toll-like receptors in the adult prefrontal cortex," *Neuroscience*, vol. 226, pp. 475–488, 2012, doi: 10.1016/j.neuroscience.2012.08.046.

[256] L. G. Coleman, J. He, J. Lee, M. Styner, and F. T. Crews, "Adolescent Binge Drinking Alters Adult Brain Neurotransmitter Gene Expression, Behavior, Brain Regional Volumes, and Neurochemistry in Mice," *Alcohol. Clin. Exp. Res.*, vol. 35, no. 4, pp. 671–688, 2011, doi: 10.1111/j.1530-0277.2010.01385.x.

[257] C. S. Rosenfeld and S. A. Ferguson, "Barnes maze testing strategies with small and large rodent models," *J. Vis. Exp.*, vol. 84, pp. 1–15, 2014, doi: 10.3791/51194.

[258] M. M. Hyer *et al.*, "Chronic adolescent stress causes sustained impairment of cognitive flexibility and hippocampal synaptic strength in female rats," *Neurobiol. Stress*, vol. 14, 2021, doi: 10.1016/j.ynstr.2021.100303.

[259] J.-F. Ge, C.-C. Qi, J.-P. Qiao, C.-W. Wang, J.-N. Zhou, and J.-N. Zhou, “Sex Differences in ICR Mice in the Morris Water Maze Task,” *Physiol. Res.*, vol. 62, pp. 107–117, 2013, doi: 10.33549/physiolres.932371.

[260] T. P. O’Leary, V. Savoie, and R. E. Brown, “Learning, memory and search strategies of inbred mouse strains with different visual abilities in the Barnes maze,” *Behav. Brain Res.*, vol. 216, pp. 531–542, Jan. 2011, doi: 10.1016/j.bbr.2010.08.030.

[261] K. Gawel, E. Gibula, M. Marszalek-Grabska, J. Filarowska, and J. H. Kotlinska, “Assessment of spatial learning and memory in the Barnes maze task in rodents—methodological consideration,” *Naunyn. Schmiedebergs. Arch. Pharmacol.*, vol. 392, pp. 1–18, Jan. 2019, doi: 10.1007/s00210-018-1589-y.

[262] M. Aoki, M. Shimozuru, T. Kikusui, Y. Takeuchi, and Y. Mori, “Sex differences in behavioral and corticosterone responses to mild stressors in ICR mice are altered by ovariectomy in peripubertal period,” *Zoolog. Sci.*, vol. 27, no. 10, pp. 783–789, 2010, doi: 10.2108/zsj.27.783.

[263] F. E. Harrison, A. H. Hosseini, and M. P. McDonald, “Endogenous anxiety and stress responses in water maze and Barnes maze spatial memory tasks,” *Behav. Brain Res.*, vol. 198, pp. 247–251, 2009, doi: 10.1016/j.bbr.2008.10.015.

[264] M. M. Przybycien-Szymanska, N. N. Mott, C. R. Paul, R. A. Gillespie, and T. R. Pak, “Binge-pattern alcohol exposure during puberty induces long-term changes in HPA axis reactivity,” *PLoS One*, vol. 6, no. 4, 2011, doi: 10.1371/journal.pone.0018350.

[265] L. M. P. Fernandes *et al.*, “Repeated cycles of binge-like ethanol exposure induce immediate and delayed neurobehavioral changes and hippocampal dysfunction in

adolescent female rats," *Behav. Brain Res.*, vol. 350, pp. 99–108, Sep. 2018, doi: 10.1016/j.bbr.2018.05.007.

[266] C. Pitzer, B. Kurpiers, and A. Eltokhi, "Sex Differences in Depression-Like Behaviors in Adult Mice Depend on Endophenotype and Strain," *Front. Behav. Neurosci.*, vol. 16, pp. 1–8, 2022, doi: 10.3389/fnbeh.2022.838122.

[267] G. L. Ryherd, A. L. Bunce, H. A. Edwards, N. E. Baumgartner, and E. K. Lucas, "Sex differences in avoidance behavior and cued threat memory dynamics in mice: Interactions between estrous cycle and genetic background," *Horm. Behav.*, vol. 156, 2023, doi: 10.1016/j.yhbeh.2023.105439.

[268] M. A. M. Bent, A. C. Pais, and J. T. Wolstenholme, "Comparing behavior following binge ethanol in adolescent and adult DBA/2 J mice," *Behav. Brain Res.*, vol. 419, p. 113703, Feb. 2022, doi: 10.1016/J.BBR.2021.113703.

[269] G. A. Shaw *et al.*, "Traumatic stress history interacts with sex and chronic peripheral inflammation to alter mitochondrial function of synaptosomes," *Brain. Behav. Immun.*, vol. 88, pp. 1–17, 2020, doi: 10.1016/j.bbi.2020.05.021.

[270] G. B. Fox, L. Fan, R. A. Levasseur, and A. I. Faden, "Effect of traumatic brain injury on mouse spatial and nonspatial learning in the Barnes circular maze," *J. Neurotrauma*, vol. 15, no. 12, pp. 1037–1046, 1998, doi: 10.1089/neu.1998.15.1037.

[271] K. R. Bailey, "Anxiety-Related Behaviors in Mice," in *Methods of Behavior Analysis in Neuroscience*, 2nd ed., J. Buccafusco, Ed. Boca Raton: CRC Press/Taylor & Francis, 2009.

[272] S. L. Byers, M. V. Wiles, S. L. Dunn, and R. A. Taft, “Mouse estrous cycle identification tool and images,” *PLoS One*, vol. 7, no. 4, pp. 1–5, 2012, doi: 10.1371/journal.pone.0035538.

[273] K. Staley and H. Scharfman, “A woman’s prerogative,” *Nat. Neurosci.*, vol. 8, no. 6, 2005, [Online]. Available: http://www.nature.com/natureneuroscience.

[274] C. S. C. Woolley and B. S. B. McEwen, “Estradiol mediates fluctuation in hippocampal synapse density during the estrous cycle in the adult rat,” *J. Neurosci.*, vol. 12, no. 7, pp. 2549–2554, 1992, doi: 10.1523/jneurosci.12-07-02549.1992.

[275] K. A. Ericsson, W. G. Chase, S. Faloon, S. Science, N. Series, and N. Jun, “Acquisition of a Memory Skill,” *Science (80-.).*, vol. 208, no. 4448, pp. 1181–1182, 1980.

[276] C. D. Allen, J. S. Grigoleit, J. Hong, S. Bae, J. Vaughan, and S. Lee, “Exposure to alcohol during adolescence exerts long-term effects on stress response and the adult brain stress circuits,” *Neuroscience*, vol. 339, pp. 64–71, 2016, doi: 10.1016/j.neuroscience.2016.09.031.

[277] A. S. Vore, T. Doremus-Fitzwater, A. Gano, and T. Deak, “Adolescent ethanol exposure leads to stimulus-specific changes in cytokine reactivity and Hypothalamic-Pituitary-Adrenal axis sensitivity in adulthood,” *Front. Behav. Neurosci.*, vol. 11, no. 78, pp. 1–15, 2017, doi: 10.3389/fnbeh.2017.00078.

[278] K. Mozhui *et al.*, “Strain differences in stress responsivity are associated with divergent amygdala gene expression and glutamate-mediated neuronal excitability,” *J. Neurosci.*, vol. 30, no. 15, pp. 5357–5367, 2010, doi: 10.1523/JNEUROSCI.5017-09.2010.

[279] K. Nguyen, K. Kanamori, C. S. Shin, A. Hamid, and K. Lutfy, “The impact of sex on changes in plasma corticosterone and cotinine levels induced by nicotine in c57bl/6j mice,” *Brain Sci.*, vol. 10, no. 705, pp. 1–14, 2020, doi: 10.3390/brainsci10100705.

[280] J. Youn, B. A. Ellenbroek, I. van Eck, S. Roubos, M. Verhage, and O. Stiedl, “Finding the right motivation: Genotype-dependent differences in effective reinforcements for spatial learning,” *Behav. Brain Res.*, vol. 226, pp. 397–403, 2012, doi: 10.1016/j.bbr.2011.09.034.

[281] R. P. S. Kwok *et al.*, “Nuclear protein CBP is a coactivator for the transcription factor CREB,” *Nature*, vol. 370, pp. 223–226, 1994.

[282] M. J. Sekeres *et al.*, “Increasing CRTC1 Function in the Dentate Gyrus during Memory Formation or Reactivation Increases Memory Strength without Compromising Memory Quality,” *J. Neurosci.*, vol. 32, no. 49, pp. 17857–17868, 2012, doi: 10.1523/JNEUROSCI.1419-12.2012.

[283] C. Bian, Y. Huang, H. Zhu, Y. Zhao, J. Zhao, and J. Zhang, “Steroid Receptor Coactivator-1 Knockdown Decreases Synaptic Plasticity and Impairs Spatial Memory in the Hippocampus of Mice,” *Neuroscience*, vol. 377, pp. 114–125, May 2018, doi: 10.1016/j.neuroscience.2018.02.034.

[284] L. R. Squire and S. H. Barondes, “Variable decay of memory and its recovery in cycloheximide-treated mice.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 69, no. 6, pp. 1416–1420, 1972, doi: 10.1073/pnas.69.6.1416.

[285] C. Contet, S. P. Goulding, D. A. Kuljis, and A. L. Barth, “BK Channels in the Central Nervous System,” in *International Review of Neurobiology*, vol. 128, La Jolla: Elsevier

Inc., 2016, pp. 281–342.

[286] A. N. Bukiya, J. Liu, and A. M. Dopico, “The BK channel accessory β1 subunit determines alcohol-induced cerebrovascular constriction,” *FEBS Lett.*, vol. 583, pp. 2779–2784, 2009, doi: 10.1016/j.febslet.2009.07.019.

[287] G. Martin, S. Puig, A. Pietrzykowski, P. Zadek, P. Emery, and S. Treistman, “Somatic localization of a specific large-conductance calcium-activated potassium channel subtype controls compartmentalized ethanol sensitivity in the nucleus accumbens,” *J. Neurosci.*, vol. 24, no. 29, pp. 6563–6572, 2004, doi: 10.1523/JNEUROSCI.0684-04.2004.

[288] M. Kreifeldt, D. Le, S. N. Treistman, G. F. Koob, and C. Contet, “BK channel β1 and β4 auxiliary subunits exert opposite influences on escalated ethanol drinking in dependent mice,” *Front. Integr. Neurosci.*, vol. 7, no. 1 DEC, pp. 1–6, 2013, doi: 10.3389/fnint.2013.00105.

[289] M. Kreifeldt, C. Cates-Gatto, A. J. Roberts, and C. Contet, “BK Channel β1 Subunit Contributes to Behavioral Adaptations Elicited by Chronic Intermittent Ethanol Exposure,” *Alcohol. Clin. Exp. Res.*, vol. 39, no. 12, pp. 2394–2402, 2015, doi: 10.1111/acer.12911.

[290] C. A. Schneider, W. S. Rasband, and K. W. Eliceiri, “NIH Image to ImageJ: 25 years of image analysis,” *Nat. Methods*, vol. 9, no. 7, pp. 671–675, 2012, doi: 10.1038/nmeth.2089.

[291] M. D. Conkright *et al.*, “TORCs: Transducers of regulated CREB activity,” *Mol. Cell*, vol. 12, pp. 413–423, 2003, doi: 10.1016/j.molcel.2003.08.013.

[292] X. Zhang *et al.*, “Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues,” *PNAS*, vol. 102, no. 12, pp. 4459–4464, 2005, doi: 10.1073/pnas.0501076102.

[293] J. Chen, E. E. Bardes, B. J. Aronow, and A. G. Jegga, “ToppGene Suite for gene list enrichment analysis and candidate gene prioritization,” *Nucleic Acids Res.*, vol. 37, pp. 305–311, 2009, doi: 10.1093/nar/gkp427.

[294] A. Krämer, J. Green, J. Pollard, and S. Tugendreich, “Causal analysis approaches in ingenuity pathway analysis,” *Bioinformatics*, vol. 30, no. 4, pp. 523–530, 2014, doi: 10.1093/bioinformatics/btt703.

[295] M. Xu, L. J. Chandler, and J. J. Woodward, “Ethanol inhibition of recombinant NMDA receptors is not altered by coexpression of CaMKII-α or CaMKII-β,” *Alcohol*, vol. 42, no. 5, pp. 425–432, 2008, doi: 10.1016/j.alcohol.2008.04.007.

[296] Y. Avchalumov *et al.*, “Chronic ethanol exposure differentially alters neuronal function in the medial prefrontal cortex and dentate gyrus,” *Neuropharmacology*, vol. 185, 2021, doi: 10.1016/j.neuropharm.2020.108438.

[297] S. Li, C. Zhang, H. Takemori, Y. Zhou, and Z. Q. Xiong, “TORC1 regulates activity-dependent CREB-target gene transcription and dendritic growth of developing cortical neurons,” *J. Neurosci.*, vol. 29, no. 8, pp. 2334–2343, 2009, doi: 10.1523/JNEUROSCI.2296-08.2009.

[298] A. Parra-Damas, L. Rubió-Ferrarons, J. Shen, and C. A. Saura, “CRTC1 mediates preferential transcription at neuronal activity-regulated CRE/TATA promoters OPEN,” *Sci. Rep.*, vol. 7, 2017, doi: 10.1038/s41598-017-18215-y.

[299] G. Zalcman, N. Federman, and A. Romano, “CaMKII isoforms in learning and memory: Localization and function,” *Front. Mol. Neurosci.*, vol. 11, pp. 1–14, 2018, doi: 10.3389/fnmol.2018.00445.

[300] T. H. Ch’Ng, B. Uzgil, P. Lin, N. K. Avliyakulov, T. J. O’Dell, and K. C. Martin, “Activity-dependent transport of the transcriptional coactivator CRTC1 from synapse to nucleus,” *Cell*, vol. 150, pp. 207–221, Jul. 2012, doi: 10.1016/j.cell.2012.05.027.

[301] B. E. Lonze and D. D. Ginty, “Function and regulation of CREB family transcription factors in the nervous system,” *Neuron*, vol. 35, pp. 605–623, 2002, doi: 10.1016/S0896-6273(02)00828-0.

[302] S. Impey, M. Mark, E. C. Villacres, S. Poser, C. Chavkin, and D. R. Storm, “Induction of CRE-mediated gene expression by stimuli that generate long-lasting LTP in area CA1 of the hippocampus,” *Neuron*, vol. 16, pp. 973–982, 1996, doi: 10.1016/S0896-6273(00)80120-8.

[303] C. Pittenger *et al.*, “Reversible inhibition of CREB/ATF transcription factors in region CA1 of the dorsal hippocampus disrupts hippocampus-dependent spatial memory,” *Neuron*, vol. 34, pp. 447–462, 2002, doi: 10.1016/S0896-6273(02)00684-0.

[304] S. L. Patterson, L. M. Grover, P. A. Schwartzkroin, and M. Bothwell, “Neurotrophin expression in rat hippocampal slices: A stimulus paradigm inducing LTP in CA1 evokes increases in BDNF and NT-3 mRNAs,” *Neuron*, vol. 9, pp. 1081–1088, 1992, doi: 10.1016/0896-6273(92)90067-N.

[305] M. Hofer, S. R. Pagliusi, A. Hohn, J. Leibrock, and Y. A. Barde, “Regional distribution of brain-derived neurotrophic factor mRNA in the adult mouse brain.,” *EMBO J.*, vol. 9, no.

8, pp. 2459–2464, 1990, doi: 10.1002/j.1460-2075.1990.tb07423.x.

[306] C. Giachino *et al.*, "cAMP response element-binding protein regulates differentiation and survival of newborn neurons in the olfactory bulb," *J. Neurosci.*, vol. 25, no. 44, pp. 10105–10118, 2005, doi: 10.1523/JNEUROSCI.3512-05.2005.

[307] J. Lee, W. Duan, and M. P. Mattson, "Evidence that brain-derived neurotrophic factor is required for basal neurogenesis and mediates, in part, the enhancement of neurogenesis by dietary restriction in the hippocampus of adult mice," *J. Neurochem.*, vol. 82, pp. 1367–1375, 2002, doi: 10.1046/j.1471-4159.2002.01085.x.

[308] R. Katoh-Semba *et al.*, "Riluzole enhances expression of brain-derived neurotrophic factor with consequent proliferation of granule precursor cells in the rat hippocampus," *FASEB J.*, vol. 16, no. 10, pp. 1328–1330, 2002, doi: 10.1096/fj.02-0143fje.

[309] Y. Gao *et al.*, "Activated CREB Is Sufficient to Overcome Inhibitors in Myelin and Promote Spinal Axon Regeneration In Vivo," *Neuron*, vol. 44, pp. 609–621, 2004, doi: 10.1016/j.neuron.2004.10.030.

[310] H. W. Horch and L. C. Katz, "BDNF release from single cells elicits local dendritic growth in nearby neurons," *Nat. Neurosci.*, vol. 5, no. 11, pp. 1177–1184, 2002, doi: 10.1038/nn927.

[311] M. Alonso, J. H. Medina, and L. Pozzo-Miller, "ERK1/2 Activation Is Necessary for BDNF to Increase Dendritic Spine Density in Hippocampal CA1 Pyramidal Neurons," *Learn. Mem.*, vol. 11, pp. 172–178, 2004, doi: 10.1101/lm.67804.

[312] H. Shiga, Y. Yamane, M. Kubo, Y. Sakurai, H. Asou, and E. Ito, "Differentiation of

immature oligodendrocytes is regulated by phosphorylation of cyclic AMP-response element binding protein by a protein kinase C signaling cascade," *J. Neurosci. Res.*, vol. 80, pp. 767–776, 2005, doi: 10.1002/jnr.20513.

[313] C. Sato-Bigbee and G. H. DeVries, "Treatment of oligodendrocytes with antisense deoxyoligonucleotide directed against CREB mRNA: Effect on the cyclic AMP-dependent induction of myelin basic protein expression," *J. Neurosci. Res.*, vol. 46, pp. 98–107, 1996, doi: 10.1002/(SICI)1097-4547(19961001)46:1<98::AID-JNR12>3.3.CO;2-G.

[314] C. Sato-Bigbee, S. Pal, and A. K. Chu, "Different neuroligands and signal transduction pathways stimulate CREB phosphorylation at specific developmental stages along oligodendrocyte differentiation," *J. Neurochem.*, vol. 72, pp. 139–147, 1999, doi: 10.1046/j.1471-4159.1999.0720139.x.

[315] K. Q. Tanis, R. S. Duman, and S. S. Newton, "CREB Binding and Activity in Brain: Regional Specificity and Induction by Electroconvulsive Seizure," *Biol. Psychiatry*, vol. 63, pp. 710–720, 2008, doi: 10.1016/j.biopsych.2007.08.003.

[316] J. Cosgrove, J. Atack, and S. Rapoport, "REGIONAL ANALYSIS OF RAT BRAIN PROTEINS DURING SENESCENCE," *Exp. Gerontol.*, vol. 22, pp. 187–198, 1987, doi: 10.1016/0531-5565(87)90039-8.

[317] A. E. Agoglia, S. E. Holstein, A. T. Small, M. Spanos, B. M. Burrus, and C. W. Hodge, "Comparison of the adolescent and adult mouse prefrontal cortex proteome," in *PLoS ONE*, 2017, vol. 12, no. 6, pp. 1–31, doi: 10.1371/journal.pone.0178391.

[318] K. H. C. Choy *et al.*, "Cognitive behavioral markers of neurodevelopmental trajectories in

rodents," *Transl. Psychiatry*, vol. 11, pp. 1–12, 2021, doi: 10.1038/s41398-021-01662-7.

[319] A. P. Auger, T. S. Perrot-Sinal, C. J. Auger, L. A. Ekas, M. J. Tetel, and M. M. McCarthy, "Expression of the nuclear receptor coactivator, cAMP response element-binding protein, is sexually dimorphic and modulates sexual differentiation of neonatal rat brain," *Endocrinology*, vol. 143, no. 8, pp. 3009–3016, 2002, doi: 10.1210/endo.143.8.8975.

[320] R. Ahima, Z. Krozowski, and R. Harlan, "Type I corticosteroid receptor-like immunoreactivity in the rat CNS: distribution and regulation by corticosteroids," *J. Comp. Neurol.*, vol. 313, pp. 522–538, 1991, doi: 10.1002/cne.903130312.

[321] R. S. Ahima and R. E. Harlan, "Charting of Type II glucocorticoid receptor-like immunoreactivity in the rat central nervous system," *Neuroscience*, vol. 39, no. 3, pp. 579–604, 1990, doi: 10.1016/0306-4522(90)90244-X.

[322] A. P. Auger, D. P. Hexter, and M. M. Mccarthy, "Sex difference in the phosphorylation of cAMP response element binding protein (CREB) in neonatal rat brain," *Brain Res.*, vol. 890, pp. 110–117, 2001, doi: 10.1016/s0006-8993(00)03151-6.

[323] P. Carriba and A. M. Davies, "CD40 is a major regulator of dendrite growth from developing excitatory and inhibitory neurons," *Elife*, vol. 6, pp. 1–22, 2017, doi: 10.7554/eLife.30442.

[324] A. Ledonne *et al.*, "Neuregulin 1/ErbB signalling modulates hippocampal mGluRI-dependent LTD and object recognition memory," *Pharmacol. Res.*, vol. 130, pp. 12–24, 2018, doi: 10.1016/j.phrs.2018.02.003.

[325] E. Crane and R. Cai, "The CBHSQ Report: Temporal Patterns of Emergency Department

Visits Related to Underage Drinking," Rockville, 2017. [Online]. Available: https://www.ncbi.nlm.nih.gov/books/NBK425084/.

[326] L. Restivo, G. Vetere, B. Bontempi, and M. Ammassari-Teule, "The formation of recent and remote memory is associated with time-dependent formation of dendritic spines in the hippocampus and anterior cingulate cortex," *J. Neurosci.*, vol. 29, no. 25, pp. 8206–8214, 2009, doi: 10.1523/JNEUROSCI.0966-09.2009.

[327] H. Kasai, M. Fukuda, S. Watanabe, A. Hayashi-Takagi, and J. Noguchi, "Structural dynamics of dendritic spines in memory and cognition," *Trends Neurosci.*, vol. 33, no. 3, pp. 121–129, 2010, doi: 10.1016/j.tins.2010.01.001.

[328] M. Segal, "Dendritic spines: Morphological building blocks of memory," *Neurobiol. Learn. Mem.*, vol. 138, pp. 3–9, 2017, doi: 10.1016/j.nlm.2016.06.007.

[329] I. Aota, C. Yatsuda, and E. I. Izawa, "Salivary corticosterone measurement in large-billed crows by enzyme-linked immunosorbent assay," *J. Vet. Med. Sci.*, vol. 85, no. 1, pp. 71–75, 2023, doi: 10.1292/jvms.22-0383.

[330] M. Nohara, A. Tohei, T. Sato, and H. Amao, "Evaluation of response to restraint stress by salivary corticosterone levels in adult male mice," *J. Vet. Med. Sci.*, vol. 78, no. 5, pp. 775–780, 2016, doi: 10.1292/jvms.15-0610.

[331] M. Liu *et al.*, "Letrozole induces worse hippocampal synaptic and dendritic changes and spatial memory impairment than ovariectomy in adult female mice," *Neurosci. Lett.*, vol. 706, pp. 61–67, 2019, doi: 10.1016/j.neulet.2019.05.006.

[332] D. Gaelle *et al.*, "Sustained corticosterone rise in the prefrontal cortex is a key factor for

chronic stress-induced working memory deficits in mice," *Neurobiol. Stress*, vol. 10, pp. 1–10, 2019, doi: 10.1016/j.ynstr.2019.100161.

[333] M. A. Broadwater, W. Liu, F. T. Crews, L. P. Spear, and M. A. Broadwater, "Persistent loss of hippocampal neurogenesis and increased cell death following adolescent, but not adult, chronic ethanol exposure," *Dev. Neurosci.*, vol. 36, no. 0, pp. 297–305, 2014, doi: 10.1159/000362874.

[334] P. E. Steadman *et al.*, "Disruption of Oligodendrogenesis Impairs Memory Consolidation in Adult Mice," *Neuron*, vol. 105, pp. 150–164, 2020, doi: 10.1016/j.neuron.2019.10.013.

[335] F. Guo *et al.*, "Chronic Exposure to Alcohol Inhibits New Myelin Generation in Adult Mouse Brain," *Front. Cell. Neurosci.*, vol. 15, 2021, doi: 10.3389/fncel.2021.732602.

Vita

Maria Alexis Margaux Bent was born in Durham, North Carolina on April 25th, 1992. She was raised in North Carolina, California, and Virginia completing high school in 2010 in Fairfax Virginia. Maria then went to college in Wilmington North Carolina completing a Bachelor of Science in Marine Biology with a minor in International Affairs in 2014. Following college Maria taught marine science at a nonprofit, Reef Relief, and at the Virginia Aquarium and Marine Science Center. In 2017 Maria was accepted into the Post Baccalaureate Research Education Program (PREP) at Virginia Commonwealth University to study neuroscience. In 2018 Maria was accepted into the Biomedical Sciences Doctoral Portal (BSDP) and joined the lab of Dr. Jennifer Wolstenholme. While at VCU, Maria earned numerous awards including a F31 award for her dissertation plan (NRSA F31 AA029305), acceptance into the Summer Program in Neuroscience, Excellence and Success (SPINES) Fellow at Woods Hole, and acceptance into the Society for Neuroscience's (SfN) Neuroscience Scholars Program (NSP) as an associate. She also served on 2 student organizations (Black Graduate Student Association and Neuronerds), participated as a BSDP recruiter, served on VCU's F31 workshop panel, and the graduate student luncheon with VCU's President Rao and Provost Sotiropoulos. Maria has also presented numerous poster presentations, first authored 1 paper, and coauthored 2 papers.

Publications

1. **Bent MAM**, Pais AC, Wolstenholme JT. Comparing behavior following binge ethanol in adolescent and adult DBA/2 J mice. Behav Brain Res. 2022 Feb 15;419:113703. DOI: 10.1016/j.bbr.2021.113703. PMID: 34864163.
2. Shaw GA, **Bent MAM**, Council KR, Pais AC, Amstadter A, Wolstenholme JT, Miles MF, Neigh GN. Chronic repeated predatory stress induces resistance to quinine adulteration of ethanol in male mice. Behav Brain Res. 2020 Mar 16; 382: 112500. DOI: 10.1016/j.bbr.2020.112500. PMID: 31978491.
3. Rowson SA, Bekhbat M, Kelly SD, Binder EB, Hyer MM, Shaw G, **Bent MA**, Hodes G, Tharp G, Weinshenker D, Qin Z, Neigh GN. Chronic adolescent stress sex-specifically alters the hippocampal transcriptome in adulthood. Neuropsychopharmacology. 2019 Jun;44(7):1207-1215. DOI: 10.1038/s41386-019-0321-z. PMID: 30710108.

Honors and Awards

2024 Invited Graduate Student Lunch with Provost Sotiropoulos

2023 Invited Graduate Student Lunch with President Rao

2023 Invited talk – "Memory impairments due to binge ethanol are impacted by age and sex" – MIT, Cambridge, MA

2022 School of Medicine Travel Award for SPINES

2022 Society for Neuroscience's (SfN) Neuroscience Scholars Program (NSP) Associate

2022 Summer Program in Neuroscience, Excellence and Success (SPINES) Fellow at Woods Hole MA

2022 VCU School of Medicine Travel Award for OSSD

2022 Jan F. Chlebowski Memorial Travel Award from Women in Science at VCU

2021 Scholarship for AAAS membership

2021 Scholarship for WISDM Leadership Conference

2021 OSSD Virtual Travel Award

2019 Invited Talk "Alcohol and Your Brain" - Near Peers Summer Seminar Series, Richmond VA

2018 NIH Initiative for Maximizing Student Diversity Associate

2018 CVCSN Poster Finalist for Graduate Student Category

2017 Postbaccalaureate Research Education Program (PREP) Scholar

Poster Presentations

1. **Bent MAM**, Betancourt, I, and Wolstenholme JT. *(*November 2022*)* Adolescent vs. Adult Binge Ethanol: Impacts on Memory, Ethanol Metabolism, and Protein Expression. Society for Neuroscience (SfN) San Diego, California
2. **Bent MAM**, Betancourt, I, and Wolstenholme JT. (June 2022) Binge Ethanol in Adolescence vs Adulthood: Differences in Spatial Memory and Ethanol Metabolism. Research Society on Alcoholism (RSA) Orlando, Florida.
3. **Bent MAM**, Betancourt, I, Pais AC, and Wolstenholme JT. (May 2022) Binge Ethanol in Adolescence vs Adulthood: Differences in Spatial Memory, Ethanol Sedation, and Ethanol Metabolism. Organization for the Study of Sex Differences (OSSD) Marina del Rey, California
4. **Bent MAM**, Betancourt I, and Wolstenholme JT. (April 2022) The Role of CBP in Memory Deficits Following Adolescent Binge Ethanol. VCU 25th Annual Graduate Research Symposium, Richmond VA.
5. **Bent MAM**, Betancourt I, and Wolstenholme JT. (April 2022) Assessing the Impact of Sex, Age, and Ethanol on Spatial Memory and Ethanol Metabolism. 16th Annual Women's Health Research Day, Richmond VA.
6. **Bent MAM** and Wolstenholme JT. (November 2021) Adolescent vs Adult Binge Ethanol: Impacts on Behavior and Protein Expression. NIDA-NIAAA Frontiers in Addiction Research Mini-Convention Virtual.
7. **Bent MAM**, Pais AC, and Wolstenholme JT. (June 2021) Age Differences in Memory Following Intermittent Binge Ethanol in Mice. Research Society on Alcoholism (RSA) Virtual.
8. **Bent MAM**, Pais AC, and Wolstenholme JT. (May 2021) Sex, Age, & Ethanol: Impacts on Sedation in DBA/2J Mice. Organization for the Study of Sex Differences (OSSD) Virtual.
9. **Bent MAM**, Lodha, J, and Wolstenholme JT. (March 2021) Multiple Memory Deficits Following Adolescent Binge Ethanol in DBA/2J mice. Central Virginia Chapter of Society for Neuroscience Annual Meeting (CVCSN) Virtual.
10. **Bent MAM**, Pais AC, Blay E, and Wolstenholme JT. (August 2020) Age and Sex Impact Ethanol Sensitivity & Socialization in DBA/2J mice. International Behavioral Neuroscience Society (IBNS) Virtual.
11. **Bent MAM**, Pais AC, Blay E, and Wolstenholme JT. (June 2020) Intermittent binge ethanol exposure differently alters myelin gene expression and working memory in DBA/2J adults and adolescents. Research Society on Alcoholism (RSA) Virtual.
12. **Bent MAM**, Pais AC, Elmisurati GA, Pais AB, Miles MF, and Wolstenholme JT. (April 2019) Social Stress induced Drinking and Gene Expression Differences in NAc in a Sex-Dependent Manner. The Central Virginia Chapter of Society for Neuroscience Annual Meeting (CVCSN), Charlottesville VA.
13. **Bent MAM**, Pais AC, Elmisurati GA, Pais AB, Miles MF, and Wolstenholme JT. (April 2019) The Effect of Neighbor Housing on Social Stress-Induced Drinking, Behavior, and Gene Expression in a Sex-Dependent Manner. 15th Annual Women's Health Research Day, Richmond VA.

14. **Bent MAM**, Howell P, Bekhbat M, Hyer M, Neigh GN. (March 2018) The effect of chronic adolescent stress on expression of stress-related genes in reward circuitry of male rats. Central Virginia Chapter of Society for Neuroscience Annual Meeting (CVCSN), Richmond VA.
15. **Bent MA**, Neigh GN, Hyer M. (November 2017) The Effect of Chronic Adolescent Stress on Recognition Memory and Vascularization. Annual Biomedical Research Conference for Minority Students (ABRCMS), Phoenix AZ.