Investigating the Role of Orthodenticle Homeobox 2 in Alcohol Consumption

BY

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DISSERTATION

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I dedicate this dissertation to my sweet companion, Bindi Coles.

TABLE OF CONTENTS

CHAPTERS	PAGES
1. INTRODUCTION	1
1.1. Alcohol Use Disorder (AUD)	1
1.1.1. Definitions of Different Patterns of Ethanol Consumption	3
1.1.2. Treatments for AUD	3
1.1.3. Stages of Addiction	4
1.2. The Involvement of the Ventral Tegmental Area (VTA) in Binge Drinking	5
1.2.1. Examples of VTA Receptors and Channels that Ethanol Interacts With	6
1.2.2. VTA Circuitry in Binge Drinking	7
1.2.3. Sex Differences in How the VTA Regulates Binge Drinking	7
1.2.4. Examples of Genes in the VTA that Regulate Binge-like Ethanol Consumption	9
1.3. OTX2 Regulates the Development of VTA DA Neurons	10
1.3.1. The Role of Otx2 in Defining the Midbrain-Hindbrain Boundary	11
1.3.2. Neurogenesis	11
1.3.3. Preventing Ectopic Serotonergic Neurons in the mFP	12
1.3.4. Proliferation	13
1.3.5. Differentiation and Maturation	13
1.3.6. Neuronal Subtype	15
1.3.7. Innervation	16
1.4. Developmental Ethanol Exposure and Otx2 Expression	17
1.5. Psychiatric Disorders and Behaviors Mediated by OTX2	19
1.5.1. Stress-induced Depression	19
1.5.2. Bipolar Disorder	22
1.5.3. OTX2 and Locomotor Activity	23
1.6. Project Overview	24
2. BINGE DRINKING ALTERS OTX2 EXPRESSION IN THE MURINE VTA	26
2.1. Introduction	26
2.2. Methods and Materials	27
2.2.1. Animals	28
2.2.2. Drinking in the Dark (DID) Procedure	28
2.2.3. Tissue Collection	29
2.2.4. Nicotinamide adenine dinucleotide-alcohol dehydrogenase (NAD-ADH) enzymatic assay	29
2.2.5. Quantitative Real-Time PCR (qPCR)	30
2.2.6. Western Blot	30
2.2.7. Statistical Analysis	32

2.3. Results
2.3.1. Ethanol Intake and BALs 32
2.3.2. Binge-like ethanol drinking alters Otx2 mRNA and protein levels in the VTA 32 33
2.3.3. Binge-like ethanol drinking alters Wnt1 mRNA expression in the VTA 34
2.3.4. Binge-like ethanol drinking does not impact Th gene and protein expression levels in the VTA 35
2.3.5. Binge-like ethanol drinking alters Mdk mRNA levels in the VTA 36
2.4. Discussion
2.5. Acknowledgements 36 38
3. DEPLETING OTX2 IN THE VTA OF MALE AND FEMALE ADULT MICE DOES NOT IMPACT BINGE DRINKING 46
3.1. Introduction
3.2. Methods and Materials 46
3.2.1. Animals 47
3.2.2. Lentivirus Production 47
3.2.3. Delivery of LV vectors into the VTA of adult mice 47
3.2.4. Verifying shRNA-induced depletion of Otx2 in the VTA 48
3.2.5. qPCR 48
3.2.6. DID Test 49
3.2.7. NAD-ADH enzymatic assay 49
3.2.8. Immunohistochemistry 50
3.2.9. Quantification of OTX2 and TH fluorescence in the VTA 50
3.2.10. Statistical Analysis 51
3.3. Results 51
3.3.1. OTX2 shRNA reduces Otx2 mRNA expression in the VTA 51
3.3.2. OTX2 shRNA does not impact binge-like drinking in adult male and female mice 52 52
3.3.3. shOTX2 in the VTA does not affect sucrose consumption
3.3.4. OTX2 protein levels were reduced after the completion of drinking tests 53 54
3.4. Discussion
3.5. Acknowledgements 55 57
4. TRANSIENT REDUCTION OF OTX2 IN THE VTA FROM PNDs 15 – 24 RESULTS IN INCREASED TYROSINE HYROXYLASE WITHIN THE NAC AND VTA OF ADULT MICE, BUT DOES NOT ALTER ETHANOL CONSUMPTION, LOCOMOTOR ACTIVITY, OR ANXIETY-LIKE BEHAVIOR 65
4.1. Introduction 65

4.1.1. OTX2 and Depression 65
4.1.2. OTX2 and Locomotor Activity 66
4.1.3. OTX2 and Anxiety 67
4.1.4. OTX2 and VTA DA axon targeting 67
4.1.5. Aims of the study 68
4.2. Methods and Materials 68
4.2.1. Animals 68
4.2.2. Experimental Design 69
4.2.3. Herpes Simplex Viral Vectors 69
4.2.4. Delivery of HSV vectors into the VTA on PND 15 – 17 70
4.2.5. Verifying HSV infection of OTX2+ VTA neurons 70
4.2.6. Locomotor Activity Test 71
4.2.7. Two-bottle Choice Drinking Test 71
4.2.8. Sucrose Preference Test 71
4.2.9. Light Dark Box Test 72
4.2.10. Tissue Collecion 72
4.2.11. qPCR 72
4.2.12. Western Blot 73
4.2.13. Statistical Analysis 73
4.3. Results 73
4.3.1. Transient depletion of VTA Otx2 from PNDs 15 – 24 does not alter locomotor activity or promote the development of anxiety-like behavior in adulthood 73
4.3.2. Transient depletion of VTA Otx2 from PNDs 15 – 24 does not affect ethanol preference and intake in adulthood 75
4.3.3. Transient depletion of VTA Otx2, during PNDs 15 – 24, followed by ethanol consumption in adulthood, does not promote anhedonia. 77
4.3.4. The combination of transient depletion of Otx2 in the VTA from PNDs 15 – 24 and ethanol consumption in adulthood does not alter locomotor activity or promote the development of anxiety-like behavior 78
4.3.5. Transient depletion of VTA Otx2 from PNDs 15 – 24 and ethanol consumption in adulthood does not lead to anxiety-like behavior 79
4.3.6. Transient depletion of VTA Otx2, from PNDs 15 - 24, does not impact Otx2, Th, Sema3c, Wnt1, and Mdk mRNA expression levels in the adult male and female VTA 80
4.3.7. Transient depletion of VTA Otx2 from PNDs 15 - 24 resulted in increased TH protein levels in the adult male and female VTA 81
4.3.8. Transient depletion of VTA Otx2 from PNDs 15 - 24 resulted in a significant increase in TH protein levels in the NAC in adulthood 82
4.4. Discussion 83
4.5. Acknowledgements 85

5. CONCLUSIONS 98
5.1. Acknowledgements 105
REFERENCES 106
APPENDICES 113
Appendix A: Approval of Animal Protocols 113
VITA 116

LIST OF TABLES

TABLE	PAGE
1. Primer Sequences	45

LIST OF FIGURES

FIGURES **PAGE**

2. Ethanol consumption in the drinking in the dark protocol. 39

3. Binge drinking alters *Otx2* mRNA and protein levels in the VTA. 40

4. Binge drinking increases *Wnt1* mRNA in the VTA. 41

5. Binge drinking does not impact *Th* gene and protein levels in the VTA. 42

6. Binge drinking increases *Mdk* mRNA in the VTA. 43

7. Model of binge drinking-induced activation of the Wnt1/Otx2/Mdk pathway in the male and female mouse VTA. 44

8. Effectiveness of shOtx2 in depleting *Otx2* in the murine VTA. 58

9. Depleting *Otx2* in the VTA of adult male mice did not alter binge-like drinking. 59

10. Depleting *Otx2* in the VTA of adult female mice did not alter binge-like drinking. 60

11. Depleting *Otx2* in the VTA of adult male mice did not alter sucrose consumption. 61

12. Depleting Otx2 in the VTA of adult female mice did not alter sucrose consumption. 62

13. Anterior – Posterior spread of the infection did not differ between the SCR and OTX2 shRNA groups. 63

14. There is a significant reduction in OTX2 intensity in the OTX2+ and GFP+ neurons in the OTX2 shRNA group compared to the SCR shRNA group. 64

15. Timeline of behavioral assays. 86

16. HSV vector infection in VTA. 87

17. Transiently depleting VTA *Otx2* from PNDs 15 - 24 did not alter locomotor activity or promote anxiety-like behavior. 88

18. Transiently depleting Otx2 in the male murine VTA from PNDs 15 -24 did not alter ethanol consumption. 89

19. Transiently depleting *Otx2* in the female murine VTA from PNDs 15 -24 did not alter ethanol consumption. 90

20. Transiently depleting *Otx2* in the male murine VTA from PNDs 15 -24 did not alter sucrose consumption during adulthood. 91

21. Transiently depleting *Otx2* in the female murine VTA from PNDs 15 -24 did not alter sucrose consumption during adulthood. 92

22. Transiently depleting *Otx2* in the VTA from PNDs 15 -24 did not alter locomotor activity or promote anxiety-like behavior after ethanol exposure. 93

23. Transiently depleting *Otx2* in the male murine VTA from PNDs 15 -24 did not promote the development of anxiety-like behavior after ethanol exposure. 94

24. Transiently depleting *Otx2* in the murine VTA from PNDs 15 -24 did not alter the expression of *Otx2, Th, Mdk, Wnt1, or Sema3c* during adulthood. 95

25. Transiently depleting *Otx2* in the murine VTA from PNDs 15 -24 promotes an increase in TH protein expression levels within the adult VTA. 96

26. Transiently depleting *Otx2* in the murine VTA from PNDs 15 -24 increased TH in the adult NAC. 97

LIST OF ABBREVIATIONS

2BC	2-bottle choice
5-HT_{2C}	5-Hydroxytryptamine receptor 2C
δ-$GABA_A$Rs	δ subunit-containing $GABA_A$ receptors
ACTB	β-actin
ALHD2	Aldehyde dehydrogenase 2
ALK	Anaplastic lymphoma kinase
ANOVA	Analysis of variance
AMY	Amygdala
AP	Alar plate
aVTA	Anterior VTA
AUD	Alcohol use disorder
BALs	Blood alcohol levels
BCA	Bicinchoninic acid assay
BHLH	Basic helix loop helix
BP	Basal plate
BrdU	Bromodeoxyuridine / 5-bromo-2'-deoxyuridine
cDNA	Complementary DNA
CALB	Calbidin
CDC	Center for Disease Control and Prevention
CeA	Central amygdala
ChIP	Chromatin immunoprecipitation
CMV	Cytomegalovirus
Cola8	Collagen 8
CPu	Caudate Putamen
CRF	Corticotrophin-releasing factor
CycD1	Cyclin D1
D1R	Dopamine Receptor D1
DA	Dopamine
DAT	Dopamine Transporter
DID	Drinking in the dark
dlBNST	Dorsal lateral bed nucleus of the stria termnalis
DNA	Deoxyribonucleic acid
DOPAC	Dopamine and dihydroxyphenylacetic acid
DSM	Diagnostic and Statistical Manual of Mental Disorders
dUTP	Deoxyuridine Triphosphate
E	Embryonic Day
EGFP	Enhanced Green Fluorescent Protein
EPM	Elevated Plus Maze
ERα	Estrogen Receptor alpha
ERβ	Estrogen Receptor beta
FP	Floor plate
G	Gestation
$GABA_AR$	γ-aminobutyric acid A receptor
Gbx2	Gastrulation brain homeobox 2

GFP	Green Fluorescent Protein
GIRK	G protein-gated inwardly rectifying potassium channel
Glyco-	Glycosylated
GPCR	G protein-coupled receptor
HIPP	Hippocampus
HPLC	High performance liquid chromatography
Hprt	Hypoxanthine-guanine phosphoribosyltransferase
H	Hours
IsO	Isthmic organizer
KCNK13	Potassium two-pore domain channel subfamily K member 13
Ki-67	Marker of proliferation Ki-67
KO	Knockout
NAC	Nucleus Accumbens
NMDA	N-methyl-D-aspartate receptor
LACZ	Beta-galactosidase
LDB	Light-dark box
LMX1A	LIM homeobox transcription Factor 1α
LMX1B	LIM homeobox transcription Factor 1β
mFP	Midbrain FP
Mash1	Achaete-Scute Family Basic Helix-Loop-Helix (BHLH) transcription factor 1
MCP-1	Monocyte chemotactic protein-1
Mdk	Midkine
MesDA	Mesencephalic dopaminergic
mRNA	Messenger ribonucleic acid
MSX1A	Msh homeobox 1
NAD-ADH	Nicotinamide adenine dinucleotide-alcohol dehydrogenase
Ngn2	Neurogenin 2
NKX2-2	NK2 homeobox 2
Nrfa2	Nuclear receptor subfamily 4 group A member 2
Nrp1	Neuropilin 1
Nrp2	Neuropilin 2
Nurr1	Nuclear receptor subfamily 4 group A member 2
OB	Olfactory bulb
OCT	Optimal cutting tissue media
OF	OF
OXR	Orexin
Otd	Orthodenticle
OTX2	Orthodenticle homeobox 2
p27Kip1	Cyclin dependent kinase inhibitor 1B
P rat	Alcohol-preferring rat
Pax6	Paired box 6
PCR	Polymerase chain reaction
PFC	Prefrontal cortex
Pitx3	Paired like homeodomain 3

PND	Postnatal day
pVTA	Posterior VTA
QTL	Quantitative trait locus
Rbp3	Retinol-binding protein 3
RP	Roof plate
RI	Recombinant inbred
Rpl13a	Ribosomal protein L13a
RM	Repeated measures
SAMHSA	Substance Abuse and Mental Health Services Administration
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SCR	Scrambled
Sh	Short hairpin
Shh	Sonic hedgehog signaling molecule
Slit2	Slit guidance ligand 2
SNc	Substantia nigra
SNPs	Single-nucleotide polymorphisms
SOX6	SRY-box transcription factor 6
st	Stage
TBS	Tris-buffered saline
Th	Tyrosine hydroxylase
Thbs4	Thrombospondin 4
TLR	Toll-like receptor 4
TSS	Transcription start site
TUNEL	Terminal deoxynucletidyl transferase deoxyuridine triphosphate (dUTP) nick end labeling
UIC	University of Illinois at Chicago
UTR	Untranslated region
VTA	Ventral tegmental area
WIN	WIN 55–212,2
Wnt	Wingless-type MMTV integration site family, member 1
WT	Wild type
Y-VACs	Yale–Vermont Adverse Childhood Experiences Scale

SUMMARY

Alcohol use disorder (AUD) is the third major preventable killer in the United States. Consequently, rectifying the issues that come with reckless ethanol consumption can be quite costly to society. There are both genetic and environmental factors that encourage the development of AUD. Elucidating the underlying mechanisms that link these factors to AUD may eventually help with discovering a unique therapeutic target that can help with treating AUD.

Previous studies have demonstrated a relationship between orthodenticle homeobox 2 (OTX2) and ethanol exposure. Ethanol exposure during development can alter *Otx2* mRNA levels in the central nervous system (CNS). OTX2 is a transcription factor that is expressed by dopaminergic (DA) neurons of the ventral tegmental area (VTA), which is a brain region involved in the development of AUD. What is not known is whether ethanol consumption during adulthood can impact OTX2 within the VTA. My results have revealed that binge drinking by adult mice can increase both *Otx2* mRNA and protein expression within the VTA. Some of the transcriptional targets of OTX2 are wingless-type MMTV integration site family member 1 (*Wnt1*) and semaphorin 3c (*Sema3c).* Midkine (*Mdk*) might also be a potential transcriptional target of OTX2. I demonstrate here that binge drinking also altered the expression of *Wnt1* and *Mdk* mRNA levels in the VTA. Since I showed that Otx2 is an ethanol-responsive gene, I went on to test if *Otx2* plays a role in binge drinking. I found that knockdown of *Otx2* expression within the adult murine VTA did not impact binge drinking.

However, OTX2 within the male murine VTA around PND 21 has been implicated in the development of depression-like behavior in adulthood, and AUD is often co-morbid with depression. I hypothesized that reducing *Otx2* levels in the VTA around PND 21 would increase ethanol consumption in adult mice. I found that transient reduction of OTX2 in the VTA had no effect on ethanol drinking, but it did increase protein levels of tyrosine hydroxylase (TH), an enzyme in the DA biosynthetic pathway, in the VTA and nucleus accumbens (NAC).

In summary, this is the first study to reveal that binge drinking during adulthood can impact *Otx2* gene and protein expression levels in the mouse VTA. This work adds to the existing literature demonstrating a relationship between OTX2 and ethanol exposure. In addition, this is the first study to demonstrate that a transient reduction in male and female VTA *Otx2* around PND 15 - 24 results in increased TH protein in the VTA and NAC of adult mice. Future studies should be done to determine the behavioral consequences of these changes.

CHAPTER ONE:

INTRODUCTION

1.1. Alcohol Use Disorder (AUD)

Alcohol use disorder (AUD) is one of the top three preventable killers in the United States [1]. According to the National Institute on Alcohol Abuse and Alcoholism (NIAAA), an individual diagnosed with AUD has an insatiable craving for and uncontrollable use of ethanol, and experiences a negative emotional state in the absence of ethanol. The Diagnostic and Statistical Manual of Mental Disorders (DSM) is a handbook that contain symptoms and criteria used to diagnose mental disorders like AUD. In the past, the DSM-4 categorized individuals as having two distinct disorders, alcohol abuse and alcohol dependence. Alcohol abuse is when an individual continues to consume ethanol despite negative consequences in their lives. Alcohol dependence consists of both tolerance and withdrawal. Tolerance is defined as a person requiring a higher amount of ethanol to experience the same level of euphoria associated with lower amounts of ethanol. Withdrawal is defined as the development of negative emotional and physical states in the absence of ethanol. The DSM-5 essentially combines the symptoms associated with alcohol abuse and dependence and refers to it as AUD. An individual is diagnosed with AUD if they experience at least 2 out of the 11 criteria found in the DSM-5 [2]. If a person experiences 2 – 3, 4 – 5, or 6 or more of the criteria found in the DSM-5, they suffer from mild, moderate, or severe AUD, respectively [2].

AUD negatively impacts the quality of life by increasing the risk of getting certain diseases, potentially placing the drinker and other individuals around them in danger, and contributing to the destruction of property. It is no surprise that these damages are quite costly to the individual and society. Moderate and excessive ethanol drinkers have an increased chance of developing oral, liver, and breast cancer [3-8]. In addition, people who participate in excessive ethanol consumption may put themselves and other individuals in dangerous situations. For example, excessive ethanol consumption promotes reckless driving, dangerous firearm use, and domestic violence [9-13]. In 2006, it was reported that the cost of dealing with issues caused by AUD was about $223.5 billion and binge drinking, which is when an individual drinks enough ethanol in 2 h to produce a blood ethanol concentration of 80 mg/dL, was attributed to $170.7 billion [14, 15]. In 2010, the amount of money spent to deal with the damage associated with excessive alcohol use went up to $249 billion and ~76 % of that cost was because of binge drinking [16]. Overall, it has been established AUD has some detrimental consequences and it is quite costly to rectify the alcohol-related damages.

Environmental factors, genetics, and the interaction between the two can encourage the development of AUD. For example, family history and an early onset of ethanol consumption is associated with a higher chance of developing AUD [17]. Understanding the underlying mechanisms that link these factors to the development of AUD will help in with identifying a therapeutic target that will aid in treating AUD.

1.1.1. Definitions of Different Patterns of Ethanol Consumption

The alcohol research field has identified different patterns of ethanol consumption. According to the 2015-2020 Dietary Guidelines for Americans, a standard drink contains 0.6 fl. oz. or 14 g of pure alcohol [15, 18, 19]. Women and men that drink moderately consume 1 or 2 drinks per day, respectively. Patterns of ethanol consumption that are associated with AUD are binge and heavy drinking. According to the Substance Abuse and Mental Health Services Administration (SAMHSA), a binge drinker consumes enough ethanol in a short period of time to produce blood alcohol levels (BALs) equal to or higher than 0.08 g/dL. Binge drinking is defined by drinking 4 and 5 standard drinks within a couple of hours (h) for women and men, respectively [20, 21]. If a person is a heavy drinker they binge drink 5 or more days within a month [21].

1.1.2. Treatments for AUD

In the United States, the current treatments for AUD are disulfiram, acamprosate, naltrexone, and behavioral therapy. Disulfiram discourages alcohol consumption in patients who suffer from AUD through the inhibition of aldehyde dehydrogenase, an enzyme involved in the metabolism of ethanol. Disulfiram-induced inhibition of aldehyde dehydrogenase results in an accumulation of acetaldehyde, which is generated by the conversion of ethanol to acetaldehyde by alcohol dehydrogenase. Acetaldehyde accumulation causes several adverse reactions to alcohol, including skin flushing, nausea, and vomiting [22]. A meta-analysis revealed that disulfiram only had a significant effect in increasing abstinence from ethanol consumption in the studies in which the researcher and patient knew the drug they were getting. In addition, many of

the studies had a majority of male participants so more studies need to be done with female patients [23]. Acamprosate, which is a partial co-agonist of the N-methyl-D-aspartate receptor (NMDA) receptor, promotes a reduction in cravings for ethanol [24, 25]. Naltrexone inhibits opioid receptors promoting a reduction in ethanol intake [26, 27].There are studies demonstrating that combined treatment with naltrexone and acamprosate can further reduce ethanol consumption [28, 29]. In 2018, another meta-analysis demonstrated that acamprosate and naltrexone did not have real effect in reducing ethanol consumption [30]. In addition, behavioral therapy can be used alone or in combination with disulfiram, acamprosate, and naltrexone to help individuals suffering with AUD to reduce their ethanol consumption [31, 32]. Overall, these medications have been reported to be effective, but they also have their limitations. It is important to continue the search for an appropriate therapeutic target that will help with treating AUD.

1.1.3. *Stages of Addiction*

The development of drug addictions, such as AUD, requires the dysregulation of several circuitries and can be characterized in three stages [33]. During the binge intoxication stage, the individual takes the potential drug of abuse and experiences euphoria. The “reward” circuitry, which includes the VTA DAergic neurons projecting to the NAC and PFC, is a crucial part of processing the alcohol-induced rewarding effects during the binge intoxication stage. The underlying mechanism that links drugs of abuse to the euphoria is when VTA DAergic neurons experience phasic firing, which is one or two action potentials that occur when the stimulus first happens. There Is an increase in

DA released into the NAC followed by an interaction between DA and Dopamine receptor D1 (D1R) [34]. During the withdrawal stage, the discontinuation of drug use promotes the development of several negative emotional and physical states such as irritability, stress, difficulty processing emotions, and a preference for the drug-induced reward over natural rewards. Also, the individual taking the drug of abuse no longer experiences the same level of pleasure they once did with that amount of drug. The negative emotional states are caused by a dysregulation in the hypothalamic-pituitary-adrenal axis and the extended amygdala [33, 35]. Finally, the preoccupation/anticipation stage is when the individual seeks out the drug of abuse [36]. Many rodent and humans have demonstrated that the basolateral amygdala, prefrontal cortex (PFC), and nucleus accumbens (NAC) are the main brain regions involved in the drug-seeking behavior [37].

1.2. The Involvement of the Ventral Tegmental Area (VTA) in Binge Drinking

As mentioned before, the mesocorticolimbic dopamine system is crucial for processing the rewarding and reinforcing effects of a variety of drugs of abuse including ethanol [34, 38]. The mesocorticolimbic system consists of dopamine-producing neurons whose cell bodies are located in the VTA and their axons, which project from the VTA to the NAC and the PFC [39]. It is well known that ethanol can directly activate dopaminergic neurons within the VTA [40-42]. In addition, oral and systemic administration of ethanol increases dopamine release into the NAC [43-47]. The dopamine D1 receptor (D1R) that is expressed in the NAC is responsible for processing the rewarding effects and consumption of ethanol [48]. In contrast, the depletion of the

dopamine receptor D2 (D2R), which is an inhibitory G protein coupled receptor (GPCR) located on DAergic neurons within the VTA and neurons within the NAC, increases ethanol consumption [49, 50]. Interestingly, the dopamine receptor D3 (D3R) is also an inhibitory GPCR and reducing this receptor also promotes an increase in ethanol consumption [51, 52].

1.2.1 Examples of VTA Receptors and Channels Sensitive to Ethanol

Ethanol is capable of directly increasing VTA DA neuronal excitation [40] and the underlying mechanisms are still being understood. Studies have started to determine how ethanol is responsible for this ethanol-induced increase in neuronal excitation by investigating how it acts on and interacts with certain neuroreceptors and channels. Several studies have identified some neuronal channels and receptors that interact with ethanol. First, nuclear magnetic resonance revealed that ethanol directly binds to the G-protein inward rectifying potassium channel (GIRK) [53-55]. Ethanol can directly activate GIRK, which lowers neuronal activity [56]. In addition, GIRK3 within the VTA can impact ethanol intake [57]. Naugler *et. al.,* also revealed that ethanol can bind to the nicotinic acetylcholine receptor via nuclear magnetic resonance [58]. Interestingly, the effect ethanol has on the nicotinic receptor varies. Studies have shown that the difference in how ethanol affects the nicotinic receptor is based on the subunits that make up the nicotinic acetylcholine receptors [59]. The activation of nicotinic acetylcholine receptors within the VTA is important in the ethanol induced activation of the DA neurons [60]. In addition, receptor binding and activity assays revealed that ethanol can bind directly to the glycine and GABA receptors [61-63]. One study demonstrated that the affinity of

glycine to the glycine receptor may be lowered in the presence of ethanol [64]. This data suggests that ethanol may decrease the activity of the glycine receptor. In addition, ethanol increases the GABAergic transmission on DA neuron within the VTA [65]. Finally, ethanol is capable of inhibiting N-methyl-D-aspartate receptors [66]. Overall, it is clear that ethanol can impact the function of the neuroreceptors and channels within the VTA.

1.2.2. VTA Circuitry in Binge Drinking

So far, there are studies that have demonstrated that connections from the dorsal lateral bed nucleus of the stria terminalis (dlBNST) and hypothalamus to the VTA are important in regulating binge drinking. Olney *et. al.,* investigated how the connection between the orexin/hypocretin neurons, which reside in the hypothalamus, and VTA regulates binge-like drinking patterns. Inhibition of the orexin receptors (OXR) -1 and -2 in the male VTA promoted a decrease in binge-like drinking within the first hour of the drinking in the dark (DID) test. Their work suggests that the hypothalamic OXR neurons that project to the VTA may regulate binge-like drinking [67]. Also, several studies have examined how corticotrophin-releasing factor (CRF) receptors within the VTA regulate binge-like drinking. Immunohistochemistry revealed that one cycle of DID can increased CRF immunoreactivity within the male murine VTA [68]. These results suggested that binge drinking increases the release of CRF into the VTA. Blocking the CRF receptors in the VTA, but not the central nucleus of the amygdala, of C57BL/6J male mice, promoted a decrease in binge drinking, during the 4^{th} drinking session of the DID, which suggests the CRF receptors in the VTA are important in binge drinking [69]. Rinker *et.*

al., revealed that inhibiting CRF neurons that project from the dlBNST to the VTA, caused male mice to reduce ethanol intake during a standard DID procedure [70]. Another study revealed that some of the dlBNST CRF neurons may actually be combinatorial neurons and regulate ethanol consumption via GABA. Silencing the GABAergic and CRF neurons via chemogenetics led to a reduction in binge-like drinking [71]. These studies are just beginning to highlight the importance of communication between the VTA and other brain regions in regulating binge-like drinking.

1.2.3. Sex Differences in How the VTA Regulates Binge Drinking

Binge drinking can also be affected by biological sex. For example, there is a sex difference in how GABA receptors in the VTA can impact binge drinking. One group demonstrated that there were higher amounts of δ subunit-containing *$GABA_A$ receptors (δ-$GABA_ARs$)* in the female VTA in comparison to the male VTA. They utilized male and female mice that expressed floxed δ-$GABA_A$Rs and injected the VTA with a adeno-associated viral vector that carried GFP with or without cre-recombinase. In comparison to the control female group, the female mice that had lower *δ-$GABA_ARs$* mRNA levels in the VTA consumed lower levels of ethanol during the DID test. The male mice did not experience a reduction in *δ-$GABA_ARs$* mRNA levels within the VTA and Darnieder et. al., speculates it is because of the male VTA already has low expression levels of this receptor. In addition, there was no difference in how much ethanol the male groups consumed during the DID test [72]. This suggests that this specific $GABA_A$ subunit regulates binge drinking in female mice. Not only that, our group has shown that

depleting estrogen receptor subtypes (ERα and ERβ) in the VTA decreased binge-like drinking patterns only in female mice [73]. These studies directly demonstrate how sex can affect how the VTA regulates binge-like drinking.

1.2.4. Examples of Genes in the VTA that Regulate Binge-like Ethanol Consumption

The Lasek group was able to demonstrate the involvement of anaplastic lymphoma kinase (ALK) and midkine (Mdk) within the VTA in regulating binge-like drinking. They demonstrated that inhibition of ALK, which is a tyrosine receptor kinase, in the VTA reduces binge-like drinking in mice [74]. In addition, depleting the ALK ligand MDK in the VTA increased binge-like drinking and two-bottle choice (2BC) ethanol consumption [75].

Modulating potassium channels within the VTA also impacted binge drinking. For example, binge drinking increased the levels of potassium two-pore domain channel subfamily K member 13 (KCNK13) in the murine VTA. Depleting KCNK13 in the VTA of male mice decreased ethanol consumption during a binge-like drinking test [76]. Herman *et al*. demonstrated that GIRK 3 Knockout (KO) mice binge drank more than the wild type (WT) mice. When GIRK3 expression was rescued specifically in the male murine VTA, binge-like drinking was reduced within the first two h of the modified DID test [57]. It is clear that modulating the expression levels of KNCK13 and GIRK3, within the VTA, can impact binge drinking.

There are a couple of studies that show that toll-like receptor (TLR) regulates binge-drinking. Balan *et* al, demonstrated that the lower amounts of both TLR4 and $GABA_AR$ α2 in the VTA of male alcohol-preferring (P) rats promoted lower ethanol

consumption during their binge-drinking sessions for days. [77]. June *et al.* also revealed that Herpes simplex virus (HSV)-induced reduction of monocyte chemotactic protein-1 (MCP-1) in the VTA and TLR-4 also caused P rats to lower ethanol consumption during their binge drinking sessions [78]. Overall, modulation of the various types of proteins mentioned in this section can impact binge drinking in rodents.

1.3. OTX2 Regulates the Development of VTA DA Neurons

In 1984, the transcription factor orthodenticle (Otd) was discovered in fruit flies (*Drosophila melanogaster*) [79]. The predicted protein contained the homeobox domain, which is a DNA binding domain that is 60 amino acids long, and the mutants that lacked *Otd* had defects in the head region and central nervous system (CNS) [80, 81]. The *Otd*-mediated malformation of the CNS and head region is not unique to *Drosophila*. In fact, manipulation of *Otx2,* a homolog of *Otd* found in mammals, can impact the critical period and the development of the many components of the head region like the eyes, forebrain, and midbrain [82-84]. The next sections will focus on the importance of OTX2 in the development of the VTA DA neurons because of the crucial role they play in ethanol consumption.

1.3.1. The Role of Otx2 in Defining the Midbrain-Hindbrain Boundary

Beginning on embryonic day (E) 7.5, OTX2 and gastrulation brain homeobox 2 (GBX2) define midbrain and hindbrain region, respectively. *Otx2* and *Gbx2* mutually repress one another and the point at which these transcription factor meet marks the isthmic organizer (IsO) [85, 86]. Thanks to transgenic mice in which *Otx2* expression

has been modulated, we know that *Otx2* expression levels regulate the size and location of the midbrain and hindbrain region. For example, Martinez-Barbera et. al. demonstrated that in situ hybridization showed that embryos with global reduction in *Otx2* had an anterior shift in *Gbx2*+ region in comparison to the WT embryos [87]. Mice that lack *Otx2* in mesencephalic dopaminergic (mesDA) progenitors have a smaller midbrain region in comparison to control mice. In addition, the *Gbx2*+ region, which marks the hindbrain region, partially expands into what would have been the midbrain region [88]. The opposite holds true in mice that over-express *Otx2* in mesDA progenitors. Mice that over-express *Otx2* in mesDA progenitors have an enlarged midbrain region that is accompanied by a smaller hindbrain region [89]. Overall, it is clear that OTX2 is important in maintaining the size and location of the midbrain and hindbrain regions.

1.3.2. Neurogenesis

The notochord starts to secrete Sonic Hedgehog (Shh) on E8, which begins the dorsal-ventral organization of the neural tube. The region of the neural tube that is exposed to the highest concentration of this Shh gradient becomes the floor plate (FP) while the region that flanks the FP is considered basal plate (BP). The second most dorsal structure is the alar plate (AP) and the most dorsal structure is the roof plate (RF). The midbrain (m)FP, which is exposed to the highest concentration of secreted Shh, gives rise to future DA neurons [90]. The first event that occurs in the mFP is the neurogenesis of mesDA progenitors. LIM homeobox transcription factor 1 alpha (LMX1A) expression is first detected in the mFP on E9 while Msh homeobox 1 (MSX1A)

appears on E9.5 [91]. It is clear that modulating OTX2 in the mesDA progenitors can regulate the proneural markers *Lmx1a* and *Msx1*. Mice that lack Otx2 in the mesDA progenitors, have a reduction in *Msx1* and *Lmx1a* gene expression in the mFP. The mice that over-express *Otx2* in mesDA progenitors have a higher expression in *Msx1* and *Lmx1a* expression in the mFP when compared to the wildtype group [89]. This study revealed that OTX2 regulates the neurogenesis process in the mFP via *Msx1* and *Lmx1a* genes. Overall, this data suggests that the depletion of OTX2 is important in neurogenesis.

1.3.3. Preventing Ectopic Serotonergic Neurons in the mFP

Several studies have demonstrated that depleting OTX2 promotes ectopic expression of NK2 Homeobox 2+ (NKX2-2+) cells, which eventually become serotonergic neurons, into the mFP [89, 92]. Expectedly, depleting *Otx2* in the mesDA progenitors prevented the ectopic expression of NKX2-2+ cells into the mFP [89, 92]. The Prakash study also revealed that reduction of OTX2 led to a major reduction of *wingless-type MMTV integration site family, member 1* (*Wnt1*+) neurons within the mFP. Interestingly, the combination of globally depleting NKX2.2 and specifically reducing *Otx2* in mesDA progenitors promoted an increase in *Wnt1*+ neurons in comparison to the *Otx2* knockout mice [92]. These studies reveal that OTX2 inhibits ectopic expression of serotonergic neurons within the mFP. In addition, OTX2 maintains the number of *Wnt1*+ neurons through the inhibition of NKX2-2. WNT1 is necessary for the differentiation of postmitotic mesDA neurons, which will be discussed in section 1.3.5.

1.3.4. Proliferation

The mesDA progenitors proliferate within the ventricular zone, which is the region of the mFP closest to the ventricle. Modulating OTX2 expression levels within the mesDA progenitors can impact their proliferating activity. There is a lower number of bromodeoxyuridine / 5-bromo-2'-deoxyuridine+ (Brdu+) and Cyclin-D1-1+ (CycD1+) cells within the mFP of the mice that lacked *Otx2* when compared to control animals. In addition, the reduction of *Otx2* in mesDA progentiors led to more mFP cells that were cyclin dependent kinase inhibitor 1B+ (p27Kip1+), which is a marker expressed in quiescent cells. This specific mouse model reveals that reduction in OTX2 can lead to lower proliferating activity in mFP progenitors. Expectedly, over-expression of Otx2 in mesDA progenitors led to a higher amount of Brdu+, CycD1+, and marker of proliferation Ki-67+ (Ki-67+) cells [89]. The Nestin$^{Cre/+Otx2flox/flox}$ mouse model, which is another mouse model that lacks *Otx2* in the postmitotic mesDA cells, also revealed that postmitotic reduction of *Otx2* in the mesDA progenitors promotes a reduction in Brdu+ progenitors [93]. Collectively, this data strongly suggests that OTX2 regulates the proliferating activity of the mFP progenitors.

1.3.5. Differentiation and Maturation

When the mesDA progenitors exit the cell cycle, they move on to the differentiation process. The postmitotic mesDA cells move ventrally from the ventricular zone to the mantle zone and then laterally to their final destination [94-96]. *Wnt1*, *Lmx1b*, neurogenin 2 (*Ngn2*) and achaete-scute family basic helix-loop-helix (BHLH) transcription factor 1 (*Mash1*) induce the expression of markers found in differentiated

mesDA neurons, such as nuclear receptor subfamily 4 group A member 2 (Nurr1), paired like homeodomain 3 (*Pitx3*) and Tryosine Hydroxylase (*Th*) [97-99]. Many studies have demonstrated that modulating OTX2 expression levels can affect the differentiation process. OTX2 regulates the amount of *Wnt1*+ cells in the mFP [89, 92] and *Wnt1*$^{-/-}$ mice have a severe reduction in TH+ and PITX3+ neurons [92]. It is possible that the OTX2-induced changes in the differentiation of postmitotic mesDA could be mediated through *Wnt1*. In addition, higher and lower amounts of NGN2+ and MASH1+ postmitotic mesDA neurons were found in the mouse models that lack and over-express *Otx2* in the mesDA neurons, respectively [89]. Mice that lack *Otx2* in mesDA neurons also revealed a lower number of postmitotic mesDA progenitors that were *Ngn2*+ or *Mash1*+ when compared to the control group [93]. Over-expressing and depleting OTX2 in mesDA progenitors promoted an increase in LMX1B+ and a decrease in *Lmx1b*+ postmitotic cells, respectively [89, 93, 99]. One study demonstrated that LMX1B is required for the production of NURR1+ and TH+ neurons [99]. On E11.5, 12.5, 13.5, 15, and 18.5 less PITX3+ or NURR1+ postmitotic neurons were observed in the mFP of mice that lack *Otx2* in the mesDA progenitors. An increase in PITX3+ and NURR1+ postmitotic neurons were observed in the mouse model that overexpressed *Otx2* in the mesDA neurons on E11.5, 12.5 and 18.5 [89]. Reducing *Otx2* in mesDA also led to a lower amount of *Pitx3*+ and *Nr4a2*+ *(Nurr1+)* postmitotic cells in the mFP on E12.5 and 15.5 [93]. OTX2 also regulates the amount TH+ neurons found within the VTA and substantia nigra pars compacta (SNc). Reduction of OTX2 in mesDA neurons led to a lower number of TH+ postmitotic neurons found within the VTA and SNc on

E12.5 and 15.5 [93]. Collectively, these results strongly suggest that OTX2 is crucial in the differentiation and maturation process of postmitotic mesDA neurons.

1.3.6 Neuronal Subtype

There are subpopulations of dopaminergic neurons within the VTA. Immunohistochemistry has revealed that the TH+ postmitotic mesDA neurons within the VTA can also be aldehyde dehydrogenase2+ (AHD2+), calbindin+ (CALB+), glycosylated (glyco-) dopamine transporter+ (DAT+), or G-protein inwardly rectifying potassium channel+ (GIRK+). Although the OTX2+ neurons are mainly CALB+ or AHD2+, OTX2 can impact the amount of TH+ neurons that are CALB+, AHD2+, GIRK2+, or glycol-DAT+ [100-103]. At E13.5, 15.5, and 18.5, reducing *Otx2* in mesDA progenitors promoted a decrease in a subpopulation of TH+ neurons that were AHD2+ or CALB+. Interestingly, there was an increase in TH+ neurons that were glycol-DAT+ or GIRK+. When OTX2 was over-expressed in mesDA progenitors the opposite held true. The same study also demonstrated the depletion of *Otx2* in mesDA from conception can still promote a decrease in the subpopulation of TH+ neurons that were AHD2+ or CALB+ and an increase in the TH+ and GIRK2+ and glcol-DAT+ neurons at 12-14 weeks of age [103]. Postmitotic activation and inactivation of OTX2 in dopaminergic neurons revealed that OTX2 is important in maintaining neuronal subtype. To modulate OTX2 in postmitotic mesDA, Cre recombinase was put under that control of the promoter for the DAT gene, which is expressed in postmitotic mesDA neurons. At 14-16 weeks of age, over-expressing or depleting OTX2 in the DAT-expressing neurons led to no changes in the overall number of TH+ neurons or TH+ neurons that were

AHD2+ or CALB+ within the VTA. Therefore, postmitotic modulation of OTX2 did not affect the TH+ neurons that are also CALB+ or AHD2+. However, postmitotic inactivation of OTX2 promoted an increase in the TH+ neurons that are GIRK2+ or glycol-DAT+ within the VTA. In contrast, postmitotic activation of OTX2 promotes a decrease in the TH+ and GIRK2+ or glycol-DAT+ within the VTA. Glycol-DAT+ and TH+ neurons were also reduced in the SNc of mice overexpressing OTX2 in DAT-expressing neurons when compared to control animals. *Dat*+ neurons are also reduced and increased in the VTA and SNc of mouse models that lack and over-express Otx2 in DAT+ neurons, respectively [100]. Lia Panman and colleagues beautifully demonstrated that SRY-box transcription factor 6+ (SOX6+) neurons are usually found in the SNc of adult mice, while OTX2+ neurons are restricted to neurons within the VTA. The neurons within the VTA of mice that lacked *Otx2* in the mesDA progenitors, had ectopic expression of SOX6 within OTX2+ neurons [104]. Collectively, these studies demonstrate that OTX2 is important in maintaining the identity of dopaminergic neurons subpopulations within the VTA and SNc.

1.3.7. Innervation

Through the usage of mutant mice, we know that OTX2 is necessary for the development of the proper amount of innervation from the VTA to the PFC, NAC, amygdala (AMY), and olfactory bulb (OB). The NAc AMY, PFC, and OB in adult mice that lacked *Otx2* in mesDA progenitors from conception showed a reduction in TH+ axonal terminals originating from the VTA [105, 106]. Although there was a significant reduction of TH+ neurons in the SNc, reducing *Otx2* in the mesDA neurons did not

affect the number of TH+ axonal terminals in the caudate putamen (CPu). Expectedly, high performance liquid chromatography (HPLC) revealed a significant decrease in dopamine and dihydroxyphenylacetic acid (DOPAC), which is a dopamine metabolite, within the PFC and striatum [105]. Over-expression of *Otx2* in mesDA neurons promotes an increase in glycol-DAT+ innervation from the VTA to the PFC of 3 – 5 month-old mice. In accordance with what was seen in the mice that lacked *Otx2* in mesDA progenitors, over-expressing *Otx2* did not affect the CPu [107]. In 2010, the Isacson lab tried to determine the axonal guidance genes that could be regulated by OTX2. Over-expressing *Otx2* in MN9D, which is a murine dopaminergic cell line, led to an increase in *neuropilin 1* (*Nrp1*), *neuropilin 2* (*Nrp2*), and *Slit Guidance Ligand 2* (*Slit2*) gene expression. In addition, *Otx2* over-expression and reduction in primary ventral mesencephalic cultures caused an increase and decrease in *Nrp1*, *Nrp2*, and *Slit2* gene expression, respectively [106]. In conclusion, OTX2 is necessary for proper innervation of projection targets of the VTA.

1.4. Developmental Ethanol Exposure and *Otx2* Expression

Currently, there are five studies that have examined the relationship between *Otx2* and ethanol. Two studies have demonstrated that ethanol exposure during development alters the expression of the *Otx2* gene in the brain. In 2012, the Singh group allowed pregnant dams to have access to either 10% ethanol and water or just water via the 2-bottle choice ethanol consumption protocol, from gestation day (G)0 – postnatal day (PND)10. The resulting male offspring were weaned at PND 21 and once they reached PND 70, their brains were removed and analyzed for *Otx2* expression.

When compared to male offspring of pregnant dams that drank water, there was a significant reduction in *Otx2* messenger ribonucleic acid (mRNA) levels within the brains of PND 70 male offspring of dams that drank ethanol [108]. Another group revealed a decrease in *Otx2* mRNA expression in the head region of *Xenopus laevis* embryos exposed to ethanol. Embryos of *Xenopus laevis* at the stage (st) 8.5 were exposed to 2 or 2.5% ethanol. At st 26, which is 29.5 h post fertilization, embryos were washed and processed for in situ hybridization. When compared to untreated embryos, 2 and 2.5% ethanol treatment promoted a significant and dose-dependent reduction in *Otx2* mRNA levels within the head region. [109]. Interestingly, there was another study that investigated if ethanol-induced death in the neural tube could be linked to particular genes. BXD recombinant inbred (RI), and C57BL/6J and DBA/2J (parental strains) pregnant mice, were given isocaloric maltose dextrin (control) or 2 doses of 5.8 g/kg ethanol via intragastric gavage on E 9.5 and embryos were harvested 7 h later. Compared to mice that received maltose dextrin, mice that received ethanol had higher levels of cell death in the forebrain and brainstem as revealed by terminal deoxynucletidyl transferase deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL). In addition, quantitative trait locus (QTL) analysis determined that higher *Otx2* gene expression levels in the brainstem of C57BL/6J, DBA/2J, and BXD mice were associated with higher amounts of ethanol-induced cell death during embryogenesis [110]. Not all ethanol exposure during the developmental period altered *Otx2* gene expression in the brain. Another group exposed Japanese medaka fish embryos to increasing concentrations of ethanol (0-400 mm) for various time points (0 – 144 h post fertilization) to determine if ethanol exposure during development impacts several

transcription factors including *Otx2*. Quantitative real-time polymerase chain reaction (PCR) revealed that ethanol did not alter *Otx2* mRNA levels in the whole medaka embryo [111]. In another study, zebrafish embryos were kept in water or 2% ethanol solution for 6 -24 h and then *in situ* hybridization was performed. The embryos that were exposed to 2% ethanol for 6 – 24 h did not reveal a change in *Otx2* mRNA expression levels in comparison to the untreated group [112]. Overall, these studies demonstrate that ethanol consumption or exposure during the developmental period can impact *Otx2* mRNA levels in the CNS.

1.5. Psychiatric Disorders and Behaviors Mediated by OTX2

1.5.1 Stress-induced Depression

OTX2 in the VTA has been shown to play a role in stress-induced depression. In one study, a late postnatal stress protocol was used in mice to model one form of adverse childhood experiences, which is child neglect [113]. PND10 male mice underwent standard weaning or an early life stress protocol, which involved separating dams from their respective litters from PNDs 10 – 17 or 10 – 21 in combination with reducing enrichment paper in the home cages. Late postnatal stress promoted a transient reduction of *Otx2* gene expression and the number of OTX2+ neurons within the VTA. Once male mice reached PND 70, *Otx2* gene expression and the number of OTX2+ neurons within the VTA were similar between the mice that experienced standard weaned or late postnatal stress. This suggests that late postnatal stress only alters *Otx2* around PND 17 – 21. Also, this early life stress protocol led to a significant reduction in OTX2 binding to the promoter regions of the *Wnt1,* thrombospondin 4

(Thbs4), semaphorin3c *(Sema3c),* paired box 6 *(Pax6),* collagen type 8 *(Cola8),* and retinol-binding protein 3 *(Rbp3)* using chromatin immunoprecipitation (ChIP). Interestingly, this group found that both late postnatal stress and social defeat during adulthood can significantly reduce *Wnt1, Thbs4, Sema3c, Pax6, Wnt1, and Rbp3* mRNA expression within the VTA. In fact, male mice that underwent both this early life stress protocol and the social defeat during adulthood developed more depression-like behavior, in comparison to the mice that experienced standard weaning and social defeat. Male mice that underwent transient depletion of VTA *Otx2* by injecting a herpes simplex viral (HSV) vector carrying a microRNA targeting *Otx2* around PND 15 - 24 and social defeat in adulthood displayed the same increase in susceptibility to developing depression-like behavior. Over-expression of *Otx2* after the postnatal stress protocol reduced susceptibility to developing depression-like behaviors. Overall, this study demonstrated that *Otx2* is involved in the development of depression-like behavior in male mice [114].

There was a follow up study done in children. The aim of this study was to create a mediation model to identify a potential underlying mechanism that links childhood adversity to the development of depression. The postnatal stress and social defeat-induced decrease in OTX2 within the VTA could be explained by an increase in methylation, which is an epigenetic marker that usually promotes transcriptional silencing. The degree of CpG methylation at the gene body, transcriptional start site (TSS), 5’ untranslated region (UTR), and 3’ UTR, and mRNA levels of *Otx2, Sema3c, Pax6, Wnt1, Cola8, and Thbs4,* were determined via the Illumina 450 K Methylation BeadChip in saliva samples. The participants filled out the Yale–Vermont Adverse

Childhood Experiences Scale (Y- VACS), which is a test that ultimately determines the degree of adversity a child experienced. To determine the depressive signs and symptoms, children filled out the Mood and Feeling Questionnaire. To determine if childhood depression could impact regional interactions, children underwent resting-state functional magnetic resonance imaging (rsfMRI). This study demonstrated a trend for a positive correlation between that intrafamilial adversity, which is childhood adversity that happens within the family, and depression scores. In addition, there was a significant positive correlation between the depression score and the methylation at cg23706497, which is in the 5'UTR of the *OTX2* transcript. Higher levels of methylation at *OTX2* cg23706497 were associated with higher amounts of methylation found on the transcripts of *RBP3*, *WNT1, THBS4,* and *PAX6.* OTX2 cg23706497 had a positive correlation with the connectivity between the right ventromedial PFC and bilateral regions of the medial FC and the cingulate. Surprisingly, lower depression scores were associated with a higher connectivity between the right ventromedial PFC and bilateral regions of the medial FC and the cingulate. The results in this study may have identified a potential underlying mechanism that links childhood adversity to depression. Childhood intrafamilial adversity induced-increase in the methylation of OTX2 and its transcriptional targets may mediate the development of depression [115].

1.5.2. Bipolar Disorder

OTX2 has also been linked to bipolar disorder. In 2007, the Weis group wanted to determine if OTX2 could be involved in the development bipolar disorder or schizophrenia. First, they extracted genomic DNA from the postmortem visual cortex

from patients that had schizophrenia, bipolar disorder, non-psychotic depression, or no history of psychiatric issues, and the 3 exons and flanking introns were amplified and sequenced. Compared to the control group, two intronic single-nucleotide polymorphisms (SNPS), which were rs2277499 and rs28757218, were identified in the *OTX2* gene. The Soft Genetic mutation surveyor software revealed that rs28757218 had a higher probability of being identified with patients that had schizophrenia and bipolar disorder compared to the control group while rs2277499 did not. They decided to further investigate rs28757218 in blood samples from patients that had schizophrenia, bipolar disorder, or no history of psychiatric issues. Rs28757218 had a higher probability of being identified with patients that had bipolar disorder compared to the control group. Overall, this study demonstrates that the intronic SNP located in the *OTX2* gene may play a role in the development of bipolar disorder [116]. Results from another study collectively showed that mice that overexpress *Otx2* in mesDA progenitors display manic behavior linked to bipolar disorder in comparison to control mice. Male mice that over-expressed *Otx2* in mesDA spent more time in the open arms of the elevated plus maze (EPM) and light area of the light/dark box (LDB). The EPM and LDB results revealed that the male murine models that over-express *Otx2* in mesDA progenitors displayed less anxiety-like or more risk-taking behavior. In addition, the locomotor activity of both groups was tracked by telemetry probes for 30 days and there was more fluctuation in periods spent being hypo- and hyper-active compared to the control groups. Finally, the group of mice that overexpressed *Otx2* in mesDA experienced a lower preference for the sucrose solutions compared to the control

group. Overall, this study demonstrated that over-expression of Otx2 in the DA neurons can lead to behaviors associated with bipolar disorder [117].

1.5.3. Locomotor Activity and Anxiety

My project also investigates how transient depletion of OTX2 around PND 21 VTA impacts locomotor activity; therefore, I would like to summarize the findings of several studies that have started to determine how OTX2 affects locomotor activity. During a 15-minute exploratory test, a mouse model with a DBA/2 x C57BL/6 genetic background and an overexpression of OTX2 in mesDA neurons displayed lower locomotor activity and spent less time in the center of the OF apparatus when compared to the control group [107]. These results suggest that overexpressing OTX2 in mesDA progenitors promotes hypoactivity and an increase in anxiety-like behavior. Interestingly, overexpressing *Otx2* in mesDA progenitors of black swiss mice lead to prolonged periods of increased and decreased activity over a course of 30 days. In addition, over-expressing *Otx2* in the mesDA caused the black swiss mice to travel more distance and spend more time in and make more entries into the center of the OF apparatus. The results from this study suggested that overexpression of OTX2 in mesDA progenitors promoted activity fluctuations, increased locomotor activity and risk taking behavior [117]. A final study showed that both male and female C57BL/6, CD-1, and 129/SV mice, that had increased *Otx2* in the mesDA, displayed higher locomotor activity, during a 60 minute OF test, in comparison to the control group [118]. Although all the mouse models described in this section experienced an increase in *Otx2* in mesDA progenitors, the different outcomes of the OF tests could be attributed to the

different genetic backgrounds of the mice. Even with conflicting results with the same animal model it is clear that overexpression of OTX2 within mesDA neurons affects both locomotor activity and anxiety-like behaviors.

1.6. Project Overview

It has become clear that OTX2 regulates the development of VTA DA neurons, is an ethanol-responsive gene, and regulates psychiatric disorders and related behaviors. The goal of this project was to determine if VTA OTX2 plays a role in ethanol drinking in mice. I hypothesized that binge levels of ethanol drinking during adulthood would impact OTX2 expression in the VTA. I also hypothesized that decreasing OTX2 in the VTA of adult mice would alter ethanol intake. Finally, I hypothesized that transient depletion of OTX2 within the murine VTA from PND 15 - 24 would alter ethanol consumption, locomotor activity, and markers associated with dopaminergic neurons. I tested each of these hypotheses in the chapters that follow.

In the second chapter, I describe results of molecular experiments designed to answer the question of whether or not binge-like drinking, during adulthood, impacted OTX2 and some of its known transcriptional target genes in the VTA of adult male and female mice. I knew that ethanol consumption, during the developmental period, impacted OTX2 levels in the CNS, but this is the first study shows that binge drinking, during adulthood, alters OTX2 expression specifically in the VTA.

In the third chapter of this dissertation, I describe behavioral studies to determine if depleting OTX2, within the VTA, could impact binge-like drinking. I depleted VTA

OTX2 in mice by injecting a lentiviral vector carrying an shRNA that targeted *Otx2* and then tested them for binge-like ethanol consumption using the drinking in the dark test.

Finally, in the fourth chapter, I show results of molecular and behavioral tests to investigate if transient depletion of VTA OTX2 around PND 15 -24 affects ethanol consumption, locomotor activity, anxiety- and depression-like behavior, and the expression of TH and DAT.

CHAPTER TWO:

BINGE DRINKING ALTERS OTX2 EXPRESSION IN THE MURINE VENTRAL TEGMENTAL AREA

2.1 Introduction

According to the Centers for Disease Control and Prevention (CDC), alcohol misuse is the third major preventable killer worldwide and alcohol-related damages are very costly to the United States. In fact, 75% of the cost related to alcohol misuse is attributable to binge drinking. [14, 16] It is imperative to understand the relationship between environmental and genetic factors and alcohol drinking because this may help us identify underlying mechanisms that can be targeted to reduce binge drinking and associated costs.

Ethanol exposure alters the expression of the *Otx2* gene in the brain. For example, when pregnant dams drink 10% ethanol, their male offspring have a significant reduction in *Otx2* mRNA in the whole brain, compared to male offspring of pregnant dams that drank water [108]. *Xenopus laevis* embryos that have been incubated in an ethanol solution have decreased *Otx2* transcript in the developing head region in comparison to embryos incubated in PBS [109]. Interestingly, there is a positive correlation between *Otx2* gene expression in the brainstem of C57BL/6J, DBA/2J, and recombinant inbred strains of C57BL/6J and DBA/2J mice and amount of ethanol-induced cell death during embryogenesis [110]. In contrast, one study found that exposing zebrafish embryos to a 2% ethanol solution did not alter *Otx2* gene expression levels in the developing brain [112]. Overall, however, most studies have demonstrated a relationship between ethanol exposure during embryonic development

and *Otx2* mRNA expression in the CNS. The effect of alcohol drinking in adults on *Otx2* gene and protein expression in the brain has not yet been examined. The VTA, which is at the center of the reward neurocircuitry, plays a critical role in the development of AUD [119]. OTX2 is involved in the development of VTA dopamine neurons and expression of OTX2 in the VTA is maintained in adult mice [100-102, 107, 120, 121]. I hypothesized that alcohol consumption by adult mice would alter the expression of OTX2 in the VTA. The first goal of this study was therefore to examine mRNA and protein levels of OTX2 in the mouse VTA after binge levels of alcohol drinking.

OTX2 is a transcription factor that regulates the expression of many genes [122]. Within the VTA there are several known targets or OTX2 including the *Wnt1* and *Sema3c* genes [114]. Another putative OTX2 target gene is *Mdk*, which encodes a secreted growth factor involved in the development of DA neurons [75].The second goal of this study was to determine if the expression of OTX2 transcriptional targets in the VTA is altered by ethanol drinking.

To conduct these studies, I modeled binge-like drinking in adult male and female mice by using the DID test and determined the effect on *Otx2* and *Th* mRNA and protein, and *Sema3c*, *Wnt1*, and *Mdk* mRNA expression in the VTA. I demonstrate here that binge-like drinking alters *Otx2* mRNA and protein, *Wnt1* mRNA, and *Mdk* mRNA expression in the VTA of male and female mice. My results suggest that OTX2 is an important ethanol-responsive factor in the VTA, a brain region involved in the rewarding and reinforcing effects of ethanol.

2.2 Methods and Materials

2.2.1. Animals

Male and female C57BL/6J mice (n = 9 – 12 per group) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and arrived at UIC at 8 weeks of age. The mice were acclimated to the reverse dark cycle (lights off at 10 am/ lights on at 10 pm) and single housing for 2 weeks, and were tested for ethanol consumption at 10 weeks of age. Mice had access to food *ad libitum* at all times. Mice also had access to water *ad libitum*, except during the ethanol drinking sessions during DID procedure. All procedures were approved by the University of Illinois at Chicago (UIC) Animal Care Committee and animal care followed the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

2.2.2. Drinking in the Dark (DID) Procedure

The DID test is a mouse model of binge drinking that results in pharmacologically relevant blood alcohol levels (BALs) (≥ 80 mg %) [123]. Each mouse was provided with a modified sipper tube in the home cage that contained either a 20% ethanol solution (95% ethanol [Decon Labs, King of Prussia, PA, USA] diluted in tap water) or water (for the control group), 3 h into the dark cycle [75]. Mice were acclimated to the sipper tube containing water for one 2-h drinking session on Friday, 3 days prior to providing them with ethanol or water. Mice were weighed 4 hours before the start of the DID test. Regardless of the treatment and time of sacrifice, the average weight of the male groups was significantly higher than the average weight of the female groups (data not shown). When comparing between groups of each sex there was no significant difference in the average weight of the groups (data not shown). Mice then had 2 h

access to the 20% ethanol solution or water from Monday – Wednesday and the amount of fluid consumed was measured at the end of each drinking session. On the fourth day (Thursday), mice had access to the ethanol or water for 4 h. and the amount of fluid consumed was measured at both the 2- and 4-h time points.

2.2.3. Tissue Collection

Mice were euthanized by rapid decapitation immediately (referred to as the 0 h group) or 24 h after 4th drinking session. The brain was rapidly removed from the skull and the VTA was dissected by sectioning the brain into 1 mm-thick sections using a brain matrix (Zivic Instruments, Pittsburgh, PA, USA) and punched out of the section using a glass Pasteur pipette. Tissue punches were transferred to a 1.5 ml centrifuge tube, snap frozen on dry ice, and stored at -80^{o}C until processing for RNA or protein. Trunk blood was collected immediately after decapitation using heparinized capillary tubes in the group that was euthanized immediately after the drinking session in order to measure BALs. Blood samples were stored at -80^{o}C until processing for BALs measurement.

2.2.4. Nicotinamide adenine dinucleotide-alcohol dehydrogenase (NAD-ADH) enzymatic assay

The protocol used for measuring BALs was based off of the NAD-ADH enzymatic assay described in Zapata et al [124]. For cell lysis and deproteinization,10 µl of whole blood samples and ethanol standards were incubated with 40 µl 3.4% of perchloric acid and centrifuged at 4^{o}C for 6 minutes @ 2000 rpm. The supernatant (7 µl) was removed

and plated in triplicate wells within a 96 well plate. Samples were incubated in 0.43 M Tris-HCl buffer (pH 8.8) containing 2.36 µg/ml of ADH (Sigma-Aldrich, St. Louis, MO) and 0.43 mg/ml β-NAD (Sigma-Aldrich) for 40 min. at room temperature. Accumulation of β-NADH was measured by reading sample absorbance at 340 nm (Sigma-Aldrich)

2.2.5. Quantitative Real-Time PCR (qPCR)

RNA was isolated using the miRNeasy Micro Kit (Qiagen, Germantown, MA, USA) as per manufacturers' instructions. Total RNA (80 ng/µl) underwent first strand complementary (c) DNA synthesis using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Foster City, CA, USA). The final concentration of primers used was 200 nM. SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) was used for qPCR on cDNA samples that were diluted 1:5. Hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) and ribosomal protein L13a (*Rpl13a*) were used as reference genes. Primer sequences for all genes are shown in Table 1. Relative gene expression was calculated via the $\Delta\Delta$ cycle quantification value (Cq) method. Enrichment for VTA tissue was determined by analyzing expression of *Th*. Sample exclusion criteria were *Th* Cq values that were identified as outliers by Grubbs' test. 2 out of 96 samples were excluded.

2.2.6. Western Blot

VTA punches were homogenized in 50 µL of RIPA buffer (Cell Signaling Technology, Danvers, MA: 150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) containing Halt™phosphatase and

protease inhibitor cocktails (Thermo Fisher Scientific, Waltham, MA, USA). Protein concentrations were quantified using the Bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific). Equal amounts of protein (20 µg) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were separated by size using the Novex™ 4-12% Tris-Glycine Mini Gels, WedgeWell™ format, 15-well (Thermo Fisher Scientific) and transferred to nitrocellulose membranes (Bio-Rad Laboratories). Membranes were blocked with 5% (w/v) nonfat dry milk in Tris-buffered saline (TBS) (25 mM Tris-HCl and 137 mM NaCl) and incubated with primary antibodies overnight at 4°C (anti-OTX2, 1:1000; anti-TH, 1:10,000; and anti- β-actin (ACTB), 1:10,000). The OTX2 antibody was a rabbit polyclonal antibody (Proteintech, Cat# 13497-1-AP, RRID: AB_2157176). The TH antibody was a mouse monoclonal antibody (Millipore, Cat# AB1542, RRID: AB_90755). The ACTB antibody was a mouse monoclonal antibody (Santa Cruz Biotechnology, Cat# sc-47778, RRID: AB_626632). The membranes were then incubated with secondary antibodies conjugated with infrared (IR) dyes at room temperature for 1 h (IRDye 680RD donkey anti-mouse IgG, cat# 925-68072, RRID: AB_2814912; IRDye 800CW donkey anti-rabbit IgG, cat# 925-32213, RRID: AB_2715510, LI-COR Biosciences, Lincoln, NE, USA). Bands were visualized using the Odyssey Fc system (LI-COR Biosciences). Blots were quantified using the Image Studio Lite software (LI-COR Biosciences) and protein levels were normalized to the average density of the β-actin band for each sample. Samples with very low or undetectable TH band intensities were excluded because this suggested that the tissue punches were not DA neuron-enriched.

2.2.7. Statistical Analysis

Prism version 8 (GraphPad, San Diego, CA, USA) was used for statistical analysis. Student's t-test was used for comparison between two groups. For comparison between two different independent variables, a two-way analysis of variance (ANOVA) or repeated measures (RM) ANOVA was used. Holm-Sidak's multiple comparisons test were performed if a significant interaction was found by ANOVA. A *p*-value less than 0.05 was considered statistically significant. Data are presented as the mean ± S.E.M.

2.3 Results

2.3.1. Ethanol Intake and BALs

Adult male and female C57BL/6 mice underwent the 4 day DID procedure and were euthanized immediately after the last drinking session (0 h group) or 24 h after the last drinking session. Fig. 1A-B, show the average daily ethanol intake during the 2 h drinking sessions in the 0 h (23 female and 24 male mice) and 24 h (24 female and 24 male mice) groups. Female mice drank significantly more 20% ethanol than male mice (Fig. 1A, 0 h group, 2-way RM ANOVA: sex, $F(1, 45) = 20.72$, $P<0.0001$; time, $F(3, 135) = 3.23$, $P=0.024$; interaction, $F(3, 135) = 0.71$ $P=0.546$; Fig. 1B, 24 h group, 2-way RM ANOVA: sex, $F(1, 46) = 7.09$, $P=0.011$; time, $F(3, 138) = 9.24$, $P<0.0001$; interaction, $F(3, 138) = 3.29$, $P=0.023$). Female mice also drank more ethanol than males during the 4 h drinking session (Fig. 1A-B, Student's t-test, $t(46) = 5.41$, $P<0.0001$). Total ethanol consumed between the 0 h and 24 h groups did not differ, but females drank more total ethanol than males (Fig. 1C, 2-way ANOVA: sex, $F(1, 91) = 33.59$, $P<0.0001$; group, $F(1, 91) = 0.95$, $P=0.333$; interaction, $F(1, 91) = 0.61$,

P=0.435). Consistent with higher levels of ethanol consumption in females, BALs were also higher in female compared to male mice (Fig. 1D, male, 65.3 ± 16.3 mg%; female, 135.0 ± 14.8 mg%; Student's t-test, t (45) =3.16, P=0.003). BALs significantly correlated with ethanol consumed during the 4 h drinking session in both sexes (Fig. 1E, males, R^2 = 0.476, P=0.0002; females, R^2 = 0.214, P=0.026).

2.3.2. Binge-like ethanol drinking alters Otx2 mRNA and protein levels in the VTA

The VTA was dissected from mice that underwent the DID test described above at 0 and 24 h after the 4th drinking session. Mice in the control group drank water instead of ethanol. Immediately after the last drinking session, *Otx2* mRNA levels were not significantly altered by ethanol exposure (Fig. 2A, n=9 per sex per group, 2-way ANOVA: ethanol, F (1, 32) = 3.93, P=0.056; sex, F (1, 32) = 0.19, P=0.665; interaction, F (1, 32) = 0.59, P=0.449). However, 24 h after the 4th drinking session *Otx2* mRNA levels in the VTA were increased in the ethanol-drinking group compared to the control group (Fig. 2B, n=11-12 per sex per group, 2-way ANOVA: ethanol, F (1, 43) = 6.35, P=0.016; sex, F (1, 43) = 5.64, P=0.022; interaction, F (1, 43) = 3.76, P=0.059). In addition, *Otx2* expression differed by sex, with *Otx2* levels higher in female mice compared to males. This appeared to be driven by a greater increase in *Otx2* in female mice drinking ethanol, although the sex by ethanol interaction did not quite reach statistical significance.

OTX2 protein levels were increased immediately after the drinking session in female mice consuming ethanol (Fig. 2C, n=10-12 per sex per group, 2-way ANOVA: ethanol, F (1, 40) = 0.99, P=0.327; sex, F (1, 40) = 2.88, P=0.097; interaction, F (1, 40)

= 5.17, P=0.029). Holm Sidak's multiple comparison test revealed increased OTX2 protein in the VTA of the female ethanol group in comparison to the female control group that nearly reached significance (P=0.051). At 24 h after the 4th drinking session, OTX2 protein levels were higher in mice consuming ethanol compared to control mice, regardless of sex (Fig. 2D, n=8-9 per sex per group, 2-way ANOVA: ethanol, F (1, 30) = 6.11, P=0.019; sex, F (1, 30) = 0.89, P=0.353; interaction, F (1, 30) = 1.27, P=0.269). Taken together, these data indicate that binge levels of ethanol drinking impact *Otx2* gene and protein expression in the VTA of male and female mice.

2.3.3. Binge-like ethanol drinking alters Wnt1 mRNA expression in the VTA

I next investigated whether the expression of transcriptional targets of OTX2 (*Wnt1*, *Sema3c*) were altered by binge levels of ethanol intake [7]. *Sema3c* levels did not change after ethanol drinking either immediately after the last drinking session (Fig. 3A, n=10-11 per sex per group, 2-way ANOVA: ethanol, F (1, 38) = 9.69 x 10^{-006}, P=0.998; sex, F (1, 38) = 1.49, P=0.229; interaction, F (1, 38) = 0.0192, P=0.891) or 24 h after the last drinking session (Fig. 3B, n=9-12 per sex per group, 2-way ANOVA: ethanol, F (1, 37) = 0.76, P=0.389; sex, F (1, 37) = 0.08, P=0.780; interaction, F (1, 37) = 0.44, P=0.513). In contrast to *Sema3c*, *Wnt1* was increased immediately after the drinking session (Fig. 3C, n= 7-8 per sex per group, 2-way ANOVA: ethanol, F (1, 26) = 7.63, P=0.010; sex, F (1, 26) = 1.26, P=0.273; interaction: F (1, 26) = 0.28, P=0.602), but returned to control levels at 24 h after the 4th drinking session (Fig. 3D, n=9-12 per sex per group, 2-way ANOVA: ethanol, F (1, 38) = 0.24, P=0.629; sex, F (1, 38) = 0.11, P=0.737; interaction: F (1, 38) = 0.02, P=0.893). These experiments demonstrate that

binge drinking increased *Wnt1* gene expression in the VTA, immediately after the drinking test.

2.3.4. Binge-like ethanol drinking does not impact Th gene and protein expression levels in the VTA

Next, I determined if binge drinking would alter tyrosine hydroxylase *(Th)* gene and protein expression in the VTA because there are some studies demonstrating that OTX2 can impact TH levels. For example, one study demonstrated that overexpression of OTX2 in mesDA neurons during development increased the innervation of VTA TH+ neuron projection targets [107]. *Th* gene expression was not changed after ethanol consumption, either immediately after the 4th drinking session (Fig. 4A, n=9 per sex per group, 2-way ANOVA: ethanol, $F(1, 32) = 2.91$, $P=0.098$; sex, $F(1, 32) = 0.10$, $P=0.753$; interaction, $F(1, 32) = 0.01$, $P=0.927$) or 24 h later (Fig. 4B, n=11-12 per sex per group, 2-way ANOVA: ethanol, $F(1, 43) = 0.49$, $P=0.487$; sex, $F(1, 43) = 1.08$, $P=0.306$; interaction, $F(1, 43) = 2.28$, $P=0.138$). Consistent with the mRNA results, TH protein levels were also not altered by ethanol consumption (Fig. 4C, 0 h group, n=10-12 per sex per group, 2-way ANOVA: ethanol, $F(1, 40) = 0.67$, $P=0.417$; sex, $F(1, 40) = 1.67$, $P=0.204$; interaction, $F(1, 40) = 0.94$, $P=0.338$; Fig. 4D, 24 h group, n=8-9 per sex per group; ethanol, $F(1, 30) = 1.65$, $P=0.210$; sex, $F(1, 30) = 0.19$, $P=0.667$; interaction, $F(1, 30) = 3.54$, $P=0.070$). Interestingly, OTX2 and TH protein levels were correlated in females in the group euthanized immediately after the 4th drinking session ($R^2 = 0.531$, $P=0.011$). These results suggest that binge drinking does not significantly

impact *Th* gene and protein levels, but that OTX2 and TH protein levels are correlated in female mice after ethanol consumption.

2.3.5. Binge-like ethanol drinking alters Mdk mRNA levels in the VTA

Next, I determined if binge-like ethanol consumption could impact *Mdk* gene and protein expression in the murine VTA. I analyzed the promoter of the *Mdk* gene and found that it had consensus binding sites for OTX2 (Fig. 5A), suggesting that it could be regulated by OTX2 in response to ethanol exposure. Ethanol did not affect *Mdk* mRNA levels immediately after the last drinking session (Fig. 5B, n=9 per sex per group, 2-way ANOVA: ethanol, $F(1, 32) = 0.48$, $P=0.495$; sex, $F(1, 32) = 0.08$, $P=0.776$; interaction, $F(1, 32) = 1.02$, $P=0.320$). However, *Mdk* mRNA levels were elevated in the ethanol-drinking group 24 h after the last drinking session (Fig. 5B, n=11-12 per sex per group, 2-way ANOVA: ethanol, $F(1, 43) = 4.14$, $P=0.048$; sex, $F(1, 43) = 1.68$, $P=0.202$; interaction: $F(1, 43) = 0.037$, $P=0.848$).

2.4 Discussion

Overall, my results demonstrate that binge-like drinking alters *Otx2* mRNA and protein, *Wnt1* mRNA, and *Mdk* mRNA expression in the VTA of male and female mice. Although there are studies demonstrating ethanol can impact *Otx2* gene expression in the CNS, I have demonstrated here that binge drinking during adulthood increases *Otx2* gene and protein expression levels in the VTA of male and female mice. I also investigated if binge drinking could alter some potential transcriptional targets of OTX2. A previous study used ChIP with an antibody to OTX2 and found that postnatal stress

decreased OTX2 binding at the promoter regions of the *Sema3c* and *Wnt1* genes [114], demonstrating that OTX2 does interact with the promoter region of these genes and may be responsible for the decrease in transcriptional activity for these genes. I found that immediately and 24 h after 4 days of DID, there was no difference in *Sema3c* mRNA levels between the water and ethanol groups. Interestingly, immediately after the last drinking session the male and female ethanol group had significantly higher *Wnt1* mRNA expression levels in the VTA, which had normalized 24 h later. These data indicate that *Wnt1* expression is affected by binge drinking. I did not observe a significant change in OTX2 protein expression levels at this timepoint which suggests that OTX2 may not be responsible for the increase in *Wnt1* mRNA levels. One possibility is that WNT1 signaling could mediate the increase in OTX2 in the VTA of the 24 h male and female ethanol groups because phospho-ß-catenin (part of the WNT signaling pathway) can bind to the promoter region of *Otx2*.

I also found increased *Mdk* mRNA in the VTA of both the 24 h male and female ethanol groups. Using JASPAR, a database of transcription factor binding profiles, I found potential functional OTX2 binding sites at the promoter of the *Mdk* gene, so it is possible that the increase in OTX2 protein expression observed 24 h after ethanol drinking is responsible for the increase in *Mdk* transcription at this time point [125]. In addition, our group previously demonstrated that depletion of *Mdk* in the VTA increased binge-like drinking in male and female mice [75]. It is possible that the increase in *Mdk* and OTX2 is a protective response to ethanol exposure.

I also investigated if binge drinking could impact *Th* gene and protein expression levels within the VTA. The data revealed that binge drinking did not impact *Th* gene and

protein expression levels in the VTA. TH activity is regulated by phosphorylation and dopamine, therefore it is possible that binge drinking can impact TH activity levels without changing its expression [126]. In the future, studies should be done to determine if binge drinking can impact TH activity levels.

Figure 6 is a working model illustrating how a Wnt1/Otx2/Mdk pathway, within the murine VTA, could be activated by binge-like drinking. 1) Immediately post the 4th drinking session there is an increase in *Wnt1* mRNA, within the male and female VTA. 2) Presumably, the increase in *Wnt1* mRNA levels is followed by an increase in WNT1 protein expression levels. Studies have shown that the interaction between WNT1 and the receptor Frizzled ultimately increase the presence of the transcription factor β-catenin by preventing it from being marked for degradation [127]. β-catenin binds to the promoter region of *Otx2* promoter, which mediates an increase in *Otx2* mRNA levels. 3) 24 h after the 4th drinking session, there is an increase in OTX2 protein expression levels that may be followed by a potential increase in binding to the *Mdk* promoter, resulting in increased expression of *Mdk* transcript. The next steps are to verify if this model will hold true and to determine the behavioral consequences linked to these mechanistic changes.

2.5 Acknowledgements

Kana Hamada, Dr. Elisa Hildebrand, and Dr. Hu Chen assisted in administering ethanol and harvesting the VTA and trunk blood for BALs.

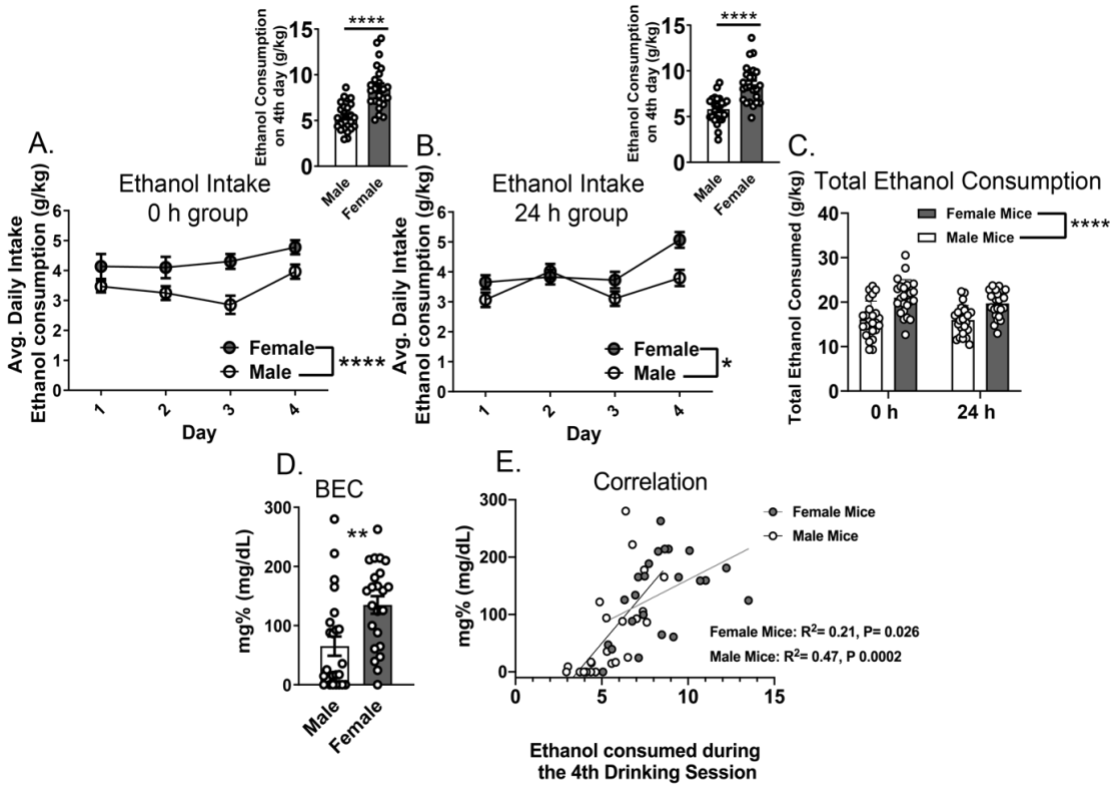


Figure 1. Ethanol consumption in the drinking in the dark protocol. (A, B) Average daily ethanol intake (g/kg/2 h) by the 0 and 24 h groups, respectively. Graphs on top show ethanol consumed during the 4 h drinking session on the 4th day. (C) Total ethanol consumed over 4 days by the 0 and 24 h groups. (D) Blood ethanol concentrations (BECs) (mg/dL) in the 0 h group. (E) Correlations between BECs and the amount of ethanol consumed during the 4th drinking session. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Data are presented as the mean ± SEM. Animal numbers for 0 h group: male: n = 24, female: n = 23. Animal numbers for 24 h group: male: n = 24, female: n = 24.

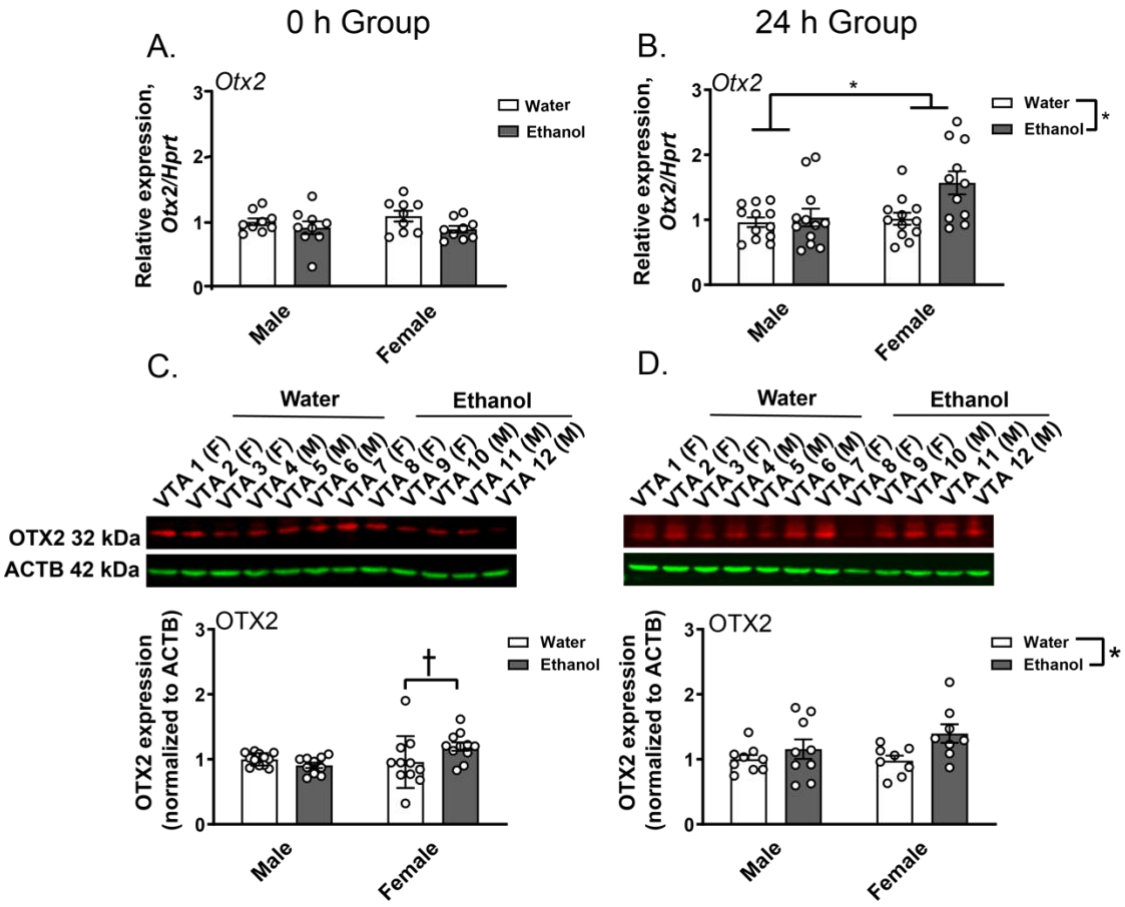


Figure 2. Binge drinking alters *Otx2* mRNA and protein levels in the VTA. Mice had access to water (white bars) or ethanol (grey bars) in the DID protocol and VTA tissue was harvested immediately (0 h, A, C) or 24 h (B, D) after the 4th drinking session. (A, B) qPCR of *Otx2* transcript relative to *Hprt*. (C, D) Top: Representative images of western blots used to quantify OTX2 levels in VTA tissue. Bottom: Protein levels of OTX2 relative to ACTB as measured by Western blot. *P < 0.05 by two-way ANOVA. Data are presented as the mean ± SEM. Animal numbers for 0 and 24 h *Otx2* groups: n = 9-12. Animal numbers for 0 and 24 h OTX2 groups: n = 8-12.

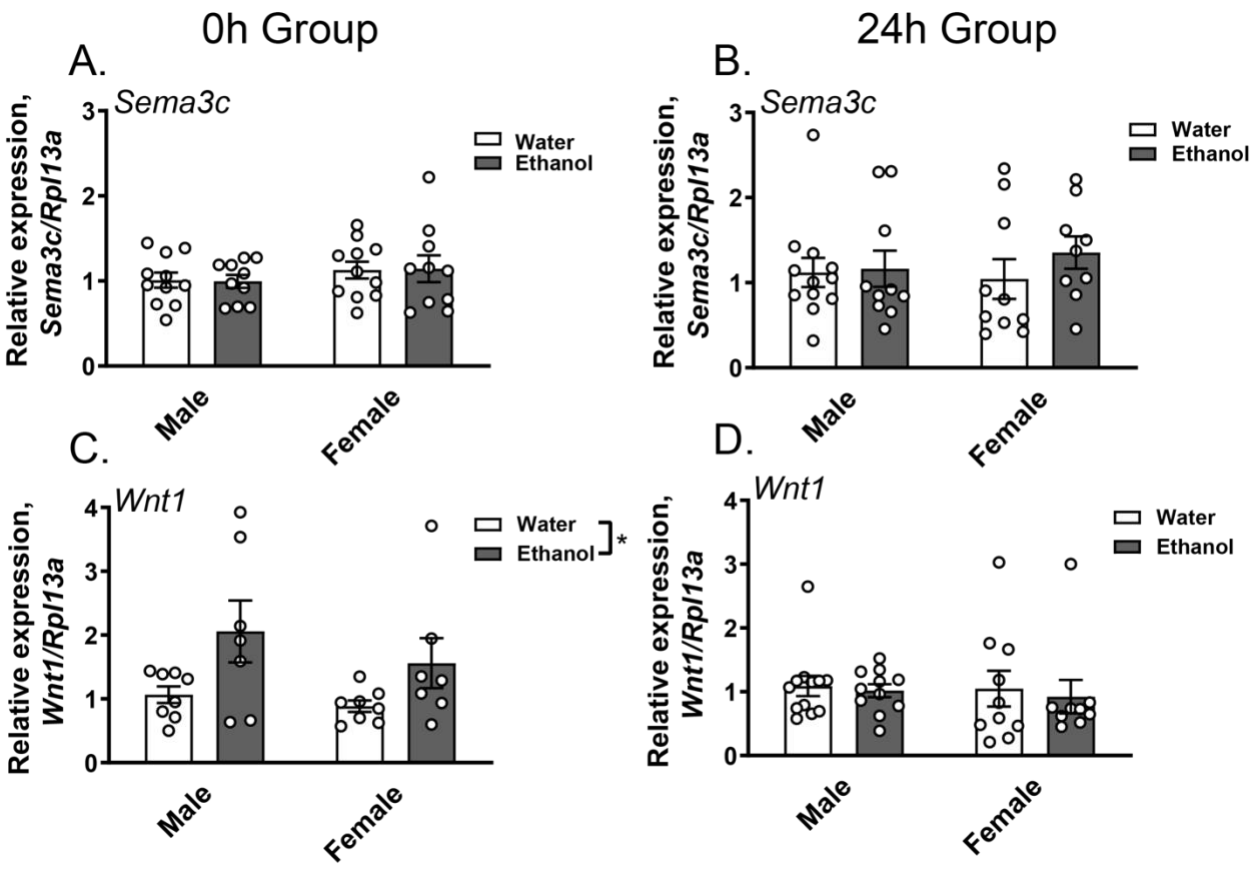
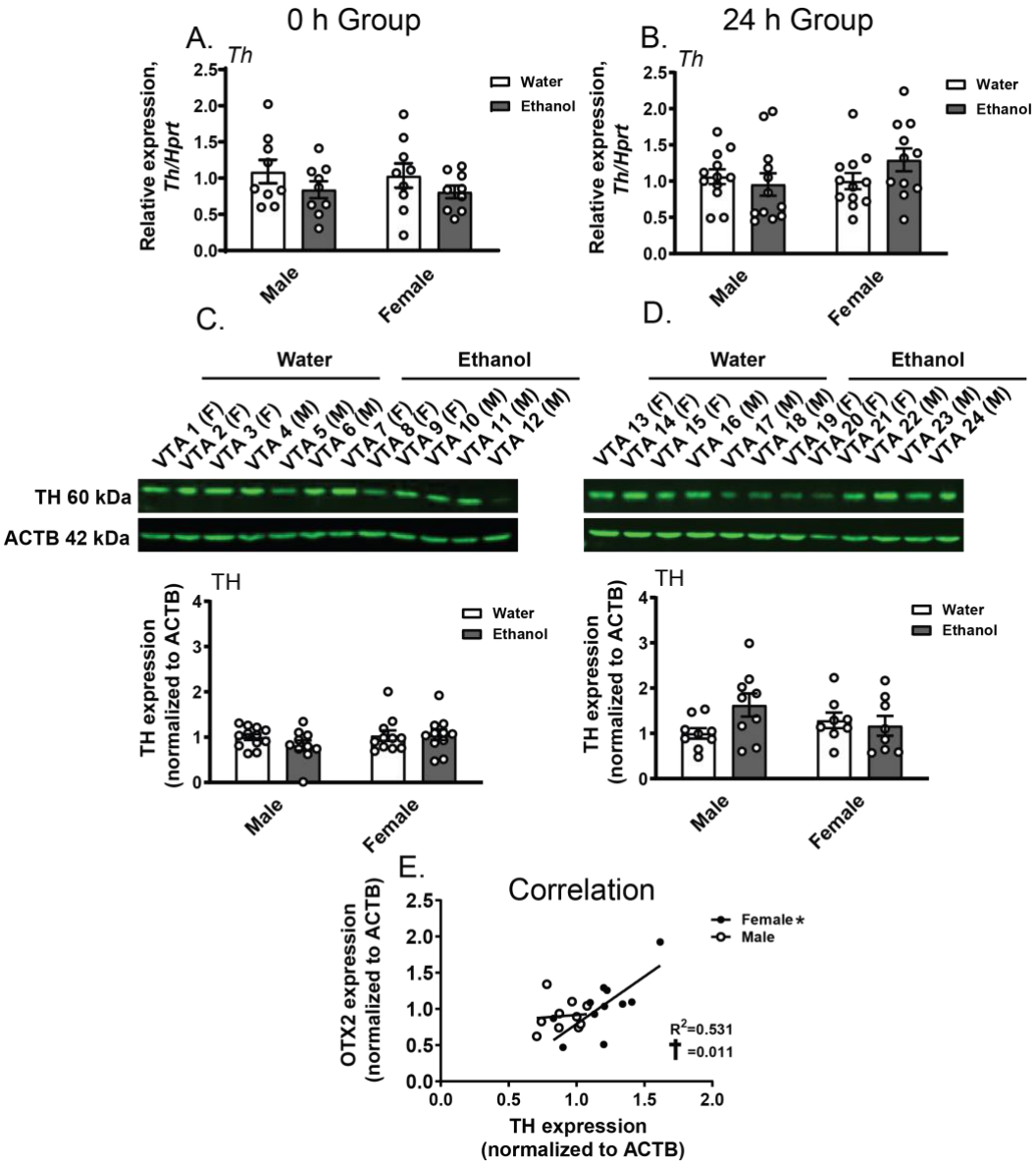


Figure 3. Binge drinking increases *Wnt1* mRNA in the VTA. Mice had access to ethanol or water in the DID protocol and VTA was harvested at 0 h (A, C) and 24 h (B, D) after the 4th drinking session. mRNA levels were determined by qPCR and are shown relative to reference gene *Rpl13a*. (A, B) *Sema3c.* (C, D) *Wnt1*. *P < 0.05 by two-way ANOVA.



Data are presented as the mean ± SEM. Animal numbers for 0 and 24 h *Sema3c* groups: n = 9-12. Animal numbers for 0 and 24 h *Wnt1* groups: n = 7-12.

Figure 4. Binge drinking does not impact *Th* gene and protein levels in the VTA. Mice had access to water (white bars) or ethanol (grey bars) in the DID protocol and VTA was harvested 0 h (A, C) or 24 h (B, D) after the 4th drinking session. (A, B) Levels of *Th* mRNA by qPCR relative to reference gene *Hprt*. (C, D) Top: Representative images of western blots used to quantify TH levels in VTA tissue. Bottom: Levels of TH protein relative to ACTB by western blot. (E) Correlation between OTX2 and TH protein levels in VTA samples obtained immediately after the 4th drinking session. Data are presented as the mean ± SEM. Animal numbers for 0 and 24 h *Th* groups: n = 9-12. Animal numbers for 0 and 24 h TH groups: n = 8-12.

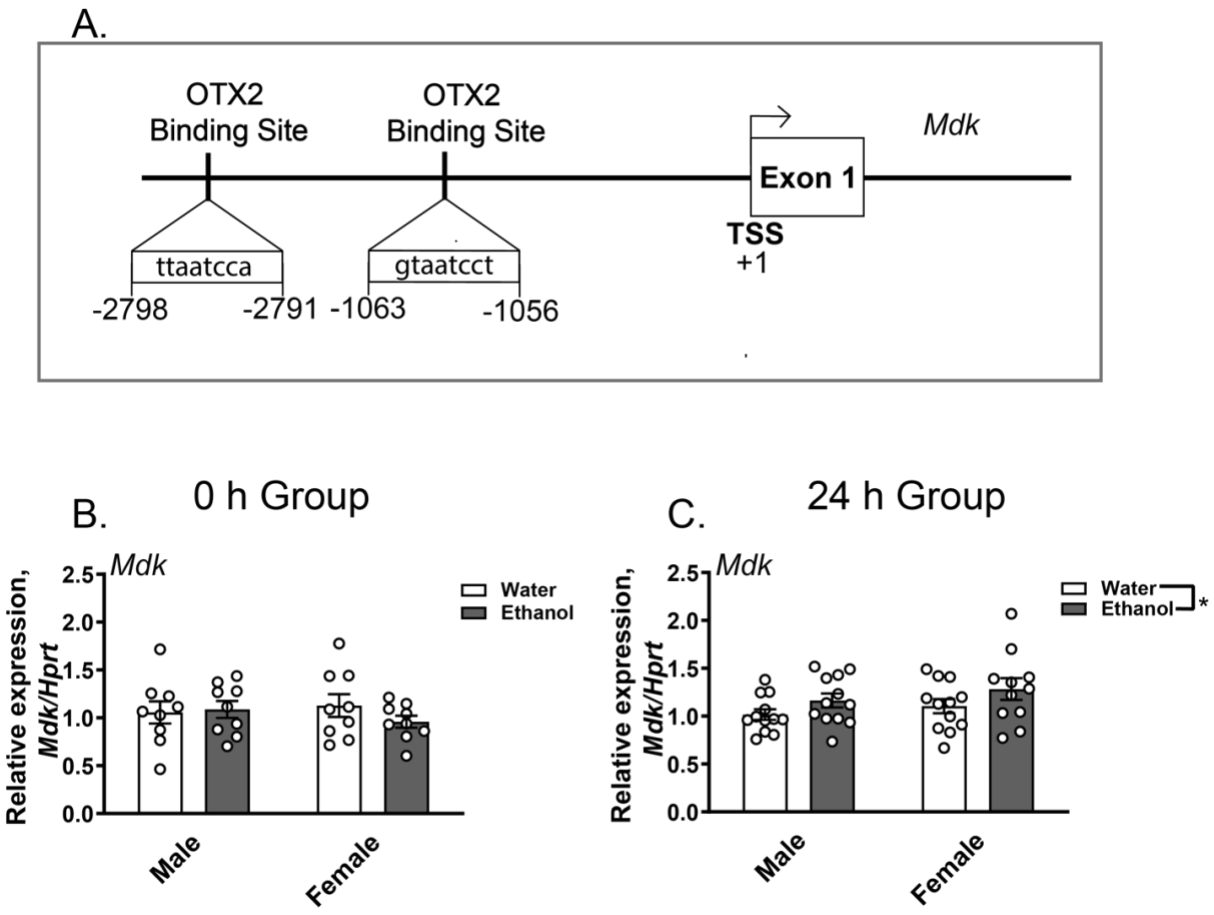


Figure 5. Binge drinking increases *Mdk* mRNA in the VTA. (A) Potential OTX2 binding sites upstream from the TSS of *Mdk.* Mice had access to water (white bars) or ethanol (grey bars) the DID protocol and VTA was harvested 0 h (B) or 24 h (C) after the 4th drinking session. (B, C) *Mdk* mRNA levels relative to reference gene *Hprt* by qPCR. *P < 0.05 by two-way ANOVA. Data are presented as the mean ± SEM. Animal numbers for 0 and 24 h *Mdk* groups: n = 9-12.

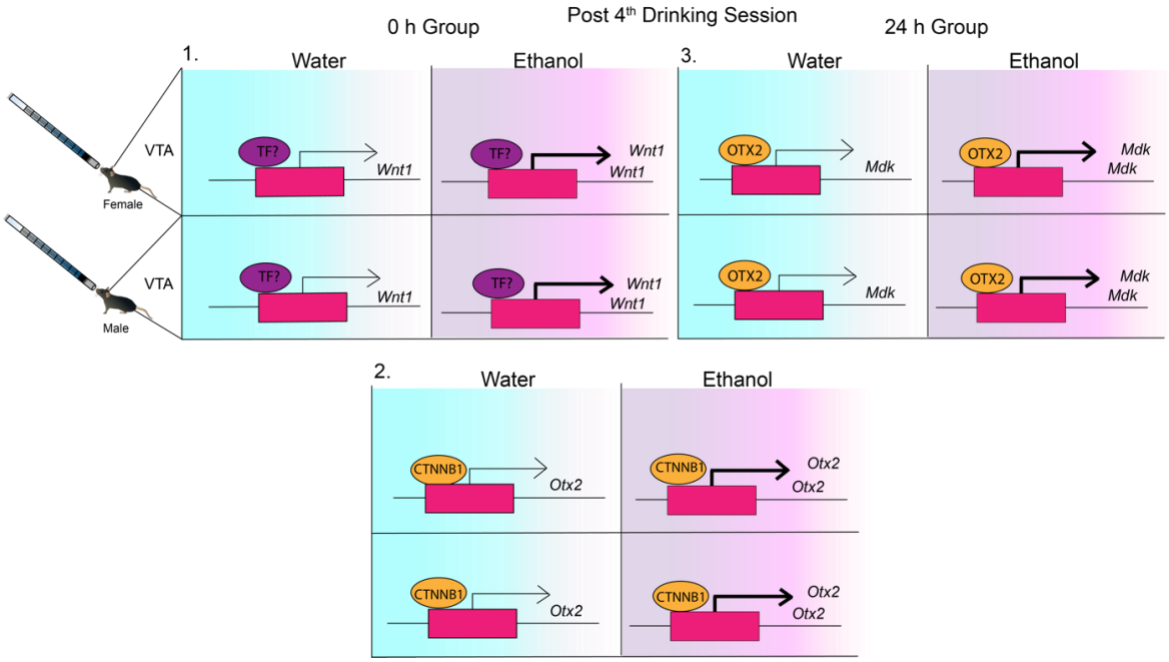


Figure 6. Model of binge drinking-induced activation of the Wnt1/Otx2/Mdk pathway in the male and female mouse VTA. 1) *Wnt1* mRNA, within the male and female VTA, is increased immediately after ethanol drinking. 2) Presumably, the increase in *Wnt1* mRNA levels is followed by an increase in WNT1 protein expression levels. The presence of WNT1 ultimately increases the activity of the transcription factor β-catenin, which binds to the promoter region of *Otx2* and increases *Otx2* mRNA levels. 3) 24 h after the 4th drinking session, there is an increase in OTX2 protein levels that may be followed by a potential increase in binding to the *Mdk* promoter, resulting in increased expression of *Mdk* transcript.

Table 1: Primer Sequences

Gene	Forward primer (5’ -3’)	Reverse primer (5’ -3’)
Hprt	GTTGGGCTTACCTCACTGCT	TCATCGCTAATCACGACGCT
Mdk	CCCTGCAACTGGAAGAAGGAAT	TTGGAGGTGCAGGGCTTAGT
Otx2	TGAGGCCTGCCAAGAAGAAG	GACAAGGGGTCAGACAGTGG
Rpl13a	TACCAGAAAGTTTGCTTACCTGGG	TGCCTGTTTCCGTAACCTCAAG
Sema3c	TGGCAAAGGACGATGCTCTT	GCGTCCACAAACATGGGTTC
Th	TCTTGAAGGAACGGACTGGC	GAGTGCATAGGTGAGGAGGC
Wnt1	GATGGTGGGGCATCGTGAA	GATGAACGCTGTTTCTCGGC

CHAPTER THREE:

DEPLETING OTX2 IN THE VTA OF MALE AND FEMALE ADULT MICE DOES NOT IMPACT BINGE DRINKING

3.1. Introduction

In Chapter 2, I demonstrated that binge drinking during adulthood increased *Otx2* gene and protein expression in the male and female VTA. In addition, binge-like ethanol consumption, during adulthood, can impact *Wnt1* and *Mdk,* which are some potential VTA specific transcriptional target of OTX2. Based on the data I acquired in Chapter two, there is a relationship between OTX2 and its transcriptional targets within the VTA, and binge drinking. What is not known is if OTX2, within the VTA, regulates ethanol consumption.

Based on previous studies from the Lasek lab, the reduction of *Mdk* within the adult VTA promoted an increase in moderate and binge-like ethanol consumption [75]. Using JASPAR, I was able to identify two potential OTX2 binding sites at the promoter region of the Mdk gene. Therefore, it is possible that OTX2 is responsible for that increase in VTA *Mdk* mRNA expression levels. I hypothesized that the depletion of OTX2 expression levels in the adult VTA would promote an increase in ethanol consumption. In this chapter, I tested whether depleting *Otx2* in the VTA of adult male and female mice would increase binge drinking by injecting mice with lentiviral vectors carrying the gene for green fluorescent protein (GFP) and a control non-targeting short hairpin (sh)RNA or a shRNA targeting *Otx2*, followed by a repeated binge ethanol drinking test.

3.2 Methods and Materials

3.2.1. Animals

Male and female C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and arrived at UIC at 8 weeks of age. Stereotaxic surgeries were done at 8 weeks of age and mice went through a 3-week recovery period and acclimation to single housing and the reverse dark cycle (lights off at 10 am/lights on at 10 pm). Ethanol administration began at 11 weeks of age. Mice had access to food *ad libitum*. During the ethanol and sucrose drinking sessions, the mice were restricted to either the 20% ethanol or 1% sucrose solution, but at all other times, the mice had access to water *ad libitum*. All procedures were approved by the UIC Animal Care Committee and animal care followed the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

3.2.2. Lentivirus Production

Replication-deficient lentiviruses expressing a short-hairpin RNA (shRNA) targeting *Otx2* (shOtx2) or the non-targeting shRNA control (shScr) were produced as described previously using the lentiviral vector pLL3.7, which expresses the shRNA from the U6 promoter and enhanced green fluorescent protein (EGFP) from the cytomegalovirus (CMV) promoter [128]. Lasek *et. al.*, demonstrated that this lentiviral vector is capable of infecting cell culture and depleting the mu-opiod receptor protein expression levels within 72 hours. In addition, only 7 days was needed to see a depletion in the mu-opiod receptor protein expression levels within the dorsal striatum in comparison to the side injected with the shScr. [128] The 23-nucleotide targeting

sequence for *Otx2* was 5′-TTGCAAATGATTGATCAAATATA-3′, corresponding to positions 1549-1571 on the *Otx2* transcript (Genbank accession number NM_001286481.1).

3.2.3. Delivery of LV vectors into the VTA of adult mice

Stereotaxic delivery of the lentiviral vector (1 µl of 4 x 10^7 pg/mL p24gag antigen for shScr and 3x10^7 pg/mL for shOtx2) bilaterally into the VTA was performed as described previously [128, 129]. Briefly, 8-week old male and female C57BL/6J mice were anesthetized with ketamine and xylazine (100/8 mg/kg, IP) and placed in a stereotaxic alignment instrument for viral injections (David Kopf Instruments, Tujunga, CA, USA). The coordinates for bilateral infusion of virus were −3.2 mm anterior/posterior (in reference to bregma), +/− 0.5 mm medial/lateral, and −4.7 mm dorsal/ventral. The virus was injected at a rate of 0.2 µl/min and cannulas remained lowered for an additional 7 minutes to prevent backflow of the virus. After the infusion step, the scalp was closed with nylon monofilament suture (Ethicon, Somerville, NJ, USA) and tissue glue (Vetclose, Dublin, OH, USA) and the mice injected subcutaneously with 2 mg/kg meloxicam for analgesia. There was a 3-week period in which the mice had time to recover from the stereotaxic surgery and get used to the reverse light/dark cycle and single housing.

3.2.4. Verifying shRNA-induced depletion of Otx2 in the VTA

Three cohorts of mice (n = 18 – 19) were used to verify knockdown of VTA *Otx2* before the behavioral tests. Male and female mice were injected with a lentivirus

carrying the gene for GFP and shScr or shOtx2. After the 3-week period of recovering from the stereotaxic surgery and acclimation to the reverse light dark cycle and single housing, brains were rapidly removed from the cranium and frozen at -80^{o}C until sectioning to a thickness of 300 µm using a cryostat (Microm HM 550, Thermo Fisher Scientific, Kalamazoo, MI, USA). Sections were mounted on Superfrost Microscope Slides (Thermo Fischer Scientific, Waltham, MA, USA) The fluorescent signal from GFP was visualized with a flashlight paired with filter glasses that match the royal blue excitation color (NightSea, Lexington, MA, USA). The green fluorescent area on the section was punched out with a 1mm biopsy punch with plunger (Militex, Plainsboro, NJ, USA) and processed for RNA isolation and qPCR.

3.2.5. qPCR

Refer to Chapter Two, section *2.2.5.* for details of the qPCR procedure.

3.2.6. DID Test

DID was conducted as described in Chapter Two (section *2.2.2.*), except that mice underwent 3 cycles of the 4 day procedure, in which they drank ethanol for 4 days (Mon-Thurs) and had 3 days off between each cycle. All groups were weighed 4 hours before a new cycle. I observed no significant difference between the average weights of the shScr and shOtx2 groups (data not shown). Three days after completion of the ethanol consumption test, mice were tested for consumption of a 2% (w/v) sucrose solution, 3 h into the dark cycle for 2 h per day for 4 days. Mice were euthanized 1 day following the completion of the last sucrose drinking test.

3.2.7. NAD-ADH enzymatic assay

Refer to Chapter Two, section *2.2.4.* for details of the NAD-ADH enzymatic assay to measure BALs. After the 3rd 4 h drinking session, tail vein blood was collected and used to measure BALs.

3.2.8. Immunohistochemistry

Mice were euthanized with Somnasol (sodium pentobarbital 390 mg and sodium phenytoin 50 mg/ml, IP) and transcardially perfused with ice-cold 0.01M PBS and 4% paraformaldehyde. Brains were removed, snap frozen on dry ice, and stored at -80°C until processing for immunohistochemistry. Snap frozen brains embedded in Optimal Cutting Temperature compound (OCT) were sliced into 40 μm-thick coronal sections. Free-floating sections were blocked and permeabilized for 1 hour in blocking solution (10% donkey serum [Jackson ImmunoResearch, West Grove, PA, USA], 0.25% Triton X-100 [Fisher Scientific, Pittsburgh, PA, USA] and 0.01M PBS). Sections were then incubated overnight in TH (1:1000), OTX2 (1:1000), or GFP (1:1000) primary antibodies in diluted blocking solution (2.5% donkey serum and 0.0625% Triton X-100). The OTX2 antibody used is a rabbit polyclonal antibody from Proteintech (Proteintech Cat# 13497-1-AP, RRID: AB_2157176). I co-stained the slices in which I probed for OTX2 with the GFP antibody, which is a mouse monoclonal antibody from Thermo Fisher (Molecular Probes Cat# A-11120, RRID: AB_221568). The TH antibody used is a mouse polyclonal antibody from Millipore (Millipore Cat# AB1542, RRID: AB_90755). I co-stained the slices in which I probed for TH with the GFP antibody, which is a rabbit monoclonal

antibody from Thermo Fisher (Thermo Fisher Scientific Cat# G10362, RRID: AB_2536526). The following day, the brain slices were washed 3 times with PBS and then incubated with Donkey anti-Mouse IgG secondary antibody conjugated to Alexa Fluor 488 (Jackson ImmunoResearch, cat# 715-545-150, RRID: AB_2340846) and Donkey anti-Rabbit IgG secondary antibody conjugated to Alexa Fluor 594 (Jackson ImmunoResearch Labs Cat# 711-585-152, RRID: AB_2340621). Sections were mounted on Superfrost Microscope Slides (Thermo Fischer Scientific, Waltham, MA, USA) using Vectashield antifade mounting medium (Vector Laboratories #H-1200, Burlingame, CA, USA). Images of the VTA were taken using the Zeiss LSM 710 Confocal Microscope (Zeiss, Oberkochen, Germany). I excluded 3 animals because of incorrect placements.

3.2.9. Quantification of OTX2 and TH fluorescence in the VTA

ImageJ software (National Institutes of Health) was used to calculate corrected total cell fluorescence. OTX2 intensity was measured in OTX2+ and GFP+ cells. Regions with no infections were used as background signal. The equation used for calculating corrected total cell fluorescence is CTCF = Integrated Density – (Area of selected cell X Mean fluorescence of background readings).

3.2.10. Statistical Analysis

Refer to Chapter Two, section *2.2.7.* for details on Statistical Analysis.

3.3. Results

3.3.1. OTX2 shRNA reduces Otx2 mRNA expression in the VTA

To determine if the shOtx2 was able to reduce expression of *Otx2* in the VTA of mice, lentiviral vectors carrying the genes for GFP and shScr or shOtx2 were injected into the VTA via stereotaxic surgery. Mice recovered for 3 weeks after surgery. Transduced VTA, as observed by GFP fluorescence, was punched out of brain sections. RNA was isolated from the punches and analyzed by qPCR for *Otx2* mRNA levels. shOtx2 reduced expression of *Otx2* mRNA in the VTA by ~20% when compared to the shScr group (Figure 7A, n = 18-19, Students' t-test, t (35) = 2.31, P= 0.028). This effect was specific to *Otx2*, as shOtx2 did not impact levels of *Th* mRNA in the VTA (Figure 7B, n = 18-19, Students' t-test, t (35) = 0.96, P=0.342).

3.3.2. OTX2 shRNA does not impact binge-drinking in adult male and female mice

To determine if depleting OTX2 in the VTA would alter binge drinking, male and female mice were injected in the VTA with a lentiviral vector expressing either shScr or shOtx2. Three weeks after surgery, mice were tested for ethanol consumption using the DID protocol for 4 days per week for 3 weeks. The amount of ethanol consumed during the 2 h sessions was not affected by OTX2 depletion in the VTA of male mice (Figure 8 A, n = 8, 2-way ANOVA, effect of shRNA: F (1, 161) = 0.60, P=0.441, effect of time: F (11, 161) = 2.92, P=0.002, shRNA by time interaction: F (11, 161) = 0.51, P=0.898). Likewise, depleting OTX2 in the VTA of female mice also had no effect on ethanol consumption (Figure 9A, n = 6, 2-way ANOVA, effect of shRNA: F (1, 116) = 0.003, P=0.960, effect of time: F (11, 116) = 2.51, P=0.007, shRNA by time interaction: F (11, 116) = 0.7 1, P=0.725). Figures 8B and 9B show the amount of ethanol consumed

during the 4 hour drinking sessions on the 4th day of each week. Similar to the 2 h drinking sessions, there was no significant difference in the amount of ethanol consumed during the 4 h drinking sessions between the male shScr and shOtx2 groups (Figure 8B, n = 8, student's t-test, day 4: t (14) = 0.67, P=0.512; day 8: t (14) = 1.26, P=0.126; day 12: t (14) = 1.20, P=0.291). In addition, OTX2 depletion in the VTA of female mice did not affect the amount of ethanol consumed during the 4 h drinking sessions (Figure 9B, n = 6, Student's t-test, day4: t (10) = .49, P=0.633; day 8: t (10) = 1.26, P=0.237; day 12: t (10) = 1.97, P=0.077). BALs were measured immediately after the 4 hour drinking session on day 12 in the shScr and shOtx2 groups. There was no difference in the BALs between the shScr and shOtx2 groups (Student's t-test, Figure 8C, males: t (14) = 0.26, P=0.802; Figure 9C, females: t (10) = 0.57, P=0.579).

3.3.3. shOTX2 in the VTA does not affect sucrose consumption

Although *Otx2* depletion in the VTA did not alter ethanol drinking, it was important to determine if this could impact intake of another rewarding substance. After the completion of the ethanol drinking tests, shScr- and shOtx2-expressing mice were tested for consumption of a 2% sucrose solution using the DID protocol. Figures 10 and 11 (A) show the amount of 2% sucrose solution the male and female shOtx2 and shScr groups consumed during the 2 h drinking sessions. There was no difference in the amount of sucrose solution consumed between the male shScr and shOtx2 groups (Figure 10A, n = 8, 2-way ANOVA, effect of shRNA: F (1, 56) = 0.47, P=0.495, effect of time: F (3, 56) = 4.82, P=0.005, shRNA by time interaction: F (3, 56) = 0.09, P=0.965). In addition, the male shScr and shOtx2 groups drank similar amounts of sucrose

solution during the 4h drinking session (Figure 10B, n = 8 Students' t-test, t (15) = 0.03, P=0.974). OTX2 depletion in the VTA of females also did not affect the amount of sucrose consumed during the 2 h sessions (Figure 11A, n = 6, 2-way ANOVA, effect of shRNA: F (1, 40) = 0.3909, P=0.535, effect by time: F (3,40) = 3.81, P=0.017, shRNA by time interaction: F (3, 40) = 0.31, P=0.821). The amount of sucrose solution consumed during the 4 h drinking session also did not differ between the female shScr and shOtx2 groups (Figure 11B, n = 6, Students' t-test, t (12) = 0.44, P=0.666).

3.3.4. Reductions of OTX2 intensity in OTX2+ and GFP+ neurons within the VTA after the completion of drinking tests

I investigated whether or not the anterior – posterior spread of the virus was similar between the shScr and shOtx2 groups. Figure 12A is an illustration of the VTA which is where I aimed to inject the lentiviral vectors. Figures 12B and C demonstrate that I successfully injected lentivirus carrying the gene for GFP and the shScr or shOtx2 into the VTA, which is the TH+ (magneta) region. Table 2 shows no difference in anterior – posterior spread between the shScr and shOtx2 shRNA groups. Three samples (two female and one male) were excluded because the viral placement did not hit the VTA.

One explanation for the lack of an effect on drinking in the mice expressing shOtx2 in the VTA could be due to ineffective reduction of OTX2 protein expression. I measured the intensity of OTX2 in GFP-expressing neurons in the VTA to determine the extent of OTX2 reduction using immunohistochemistry in mouse brain sections after the completion of the drinking tests. Figures 13A and B show neurons in the VTA that have

been successfully infected with the lentivirus (green) and are OTX2+ (magneta). Figures 13C and D show TH+ and GFP+ neurons in the VTA and that strongly suggests they have been successfully infected with the lentiviral vector. The average reduction in OTX2 intensity with the OTX2+ and GFP+ neurons that were successfully infected with the lentivirus carrying the gene for GFP and shRNA targeting Otx2 was 66% in comparison to the shScr group (Figure 13E, n = 9-10, Student's t-test, t (17) = 0.42, P=0.001). This was not accompanied by any difference in TH intensity in GFP+ neurons between the shScr and shOtx2 groups (Figure 13F, n = 9-10, Students' t-test, t (5) = 0.49, P=0.491), demonstrating the specificity of the effect. These results indicate that the VTA was effectively transduced by lentivirus, resulting in a reduction of OTX2 protein in the VTA.

3.4. Discussion

In conclusion, my data demonstrate that depletion of *Otx2* in the VTA of adult male and female mice does not alter binge-like drinking. There are several limitations to this study. Before the behavioral test, I quantified the reduction in *Otx2* mRNA levels in the whole VTA and the VTA is made up of several cell populations, like dopaminergic, GABA, and glutamatergic neurons. I do not know what and how many specific cell types actually experienced a loss in *Otx2* mRNA or protein expression levels before the behavioral tests. I do have an idea of how much OTX2 would be reduced within each cell. After behavioral tests, I quantified and compared the intensity of OTX2 within OTX2+ and GFP+ neurons in the shScr and shOtx2 groups and found a ~70% reduction in OTX2 intensity in the group injected with the shRNA targeting *Otx2.* These

results only demonstrate the efficiency of the shRNA targeting *Otx2* to deplete OTX2 to about 70% in the cell it infects. In the future, it would be good to determine the degree of OTX2 knockdown in each cell population within the VTA before and after behavioral tests. This can be done by injecting the adult male and female VTA with lentivirus carrying the gene for GFP and the shRNA targeting OTX2 or a non-targeting shRNA, identification of the different cell populations within the VTA by immunohistochemistry, and quantifying the average degree of knockdown within each cell population. This future experiment will help with revealing if knockdown of OTX2 within each VTA cell population can impact binge ethanol consumption during adulthood. It is also worth mentioning that OTX2 is mostly expressed in DA neurons residing in the VTA [101]. It is possible that the depletion of OTX2 in multiple cell populations may be the reason for not seeing a change in the amount of ethanol consumed during the DID tests. Future experiments should be done to deplete OTX2 specifically in the different cell types within the VTA.

One study demonstrated that the combination of temporarily reducing *Otx2* in the male murine VTA around PND 21 and social defeat during adulthood increased susceptibility to the development of depression-like behaviors in comparison to mice that only experienced social defeat during adulthood [114]. Since AUD and depression are often comorbid [130, 131], and that VTA OTX2 has been implicated in the development of depression [114], it is possible that modulation of OTX2, from PND 15 - 24, could be implicated in the development of AUD. In the next Chapter, I investigated if reduction of *Otx2* in the VTA from PND 15 - 24 could impact ethanol consumption.

3.5. Acknowledgements

Donghong He produced the lentiviral vectors.

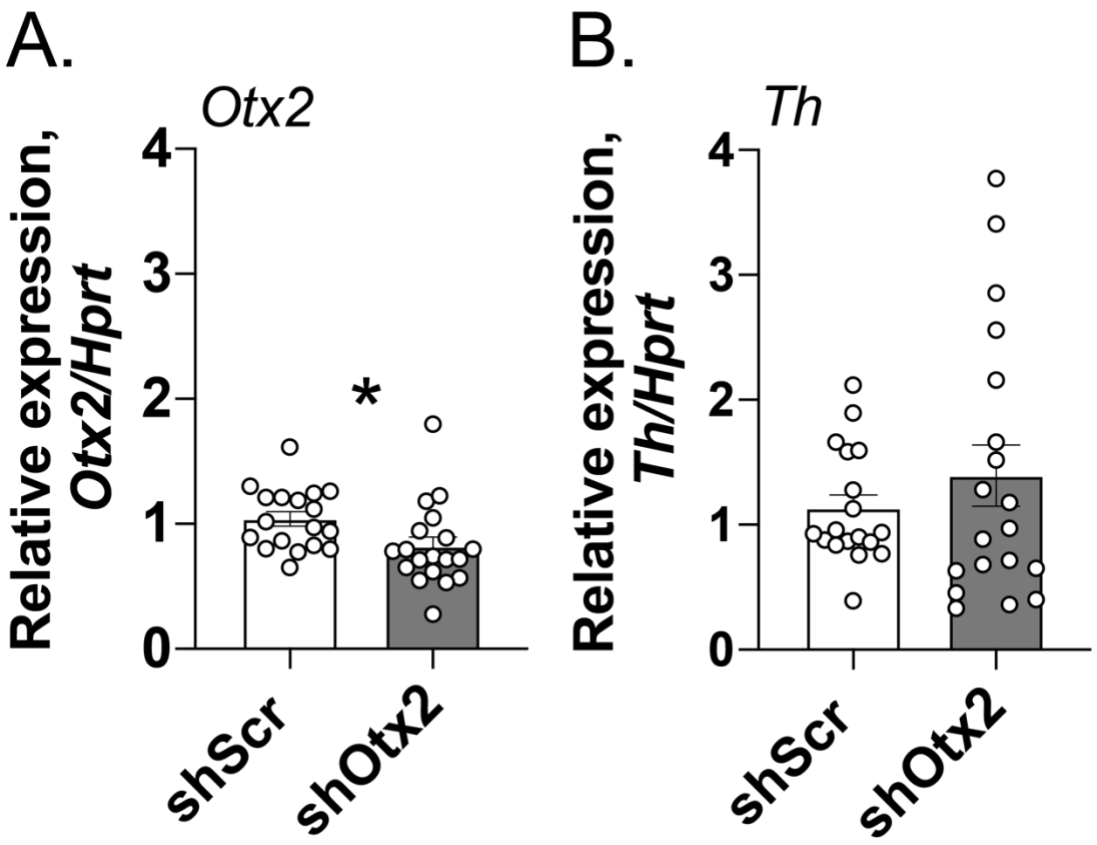


Figure 7. Effectiveness of shOtx2 in depleting *Otx2* mRNA in the murine VTA. PND 56 mice received bilateral VTA injections of lentivirus expressing shScr or shOtx2. After a 3-week recovery, transduced VTA was dissected and *Otx2* and *Th* gene expression levels were measured by qPCR. A) *Otx2* gene expression in the VTA between shScr and shOtx2 groups. B) Levels of *Th* mRNA between shScr and shOtx2 groups. *P<0.05 by t-test. Data are presented as the mean ± SEM. Animal numbers for shRNA groups: shScr: n = 18-19, shOtx2 groups: n = 19.

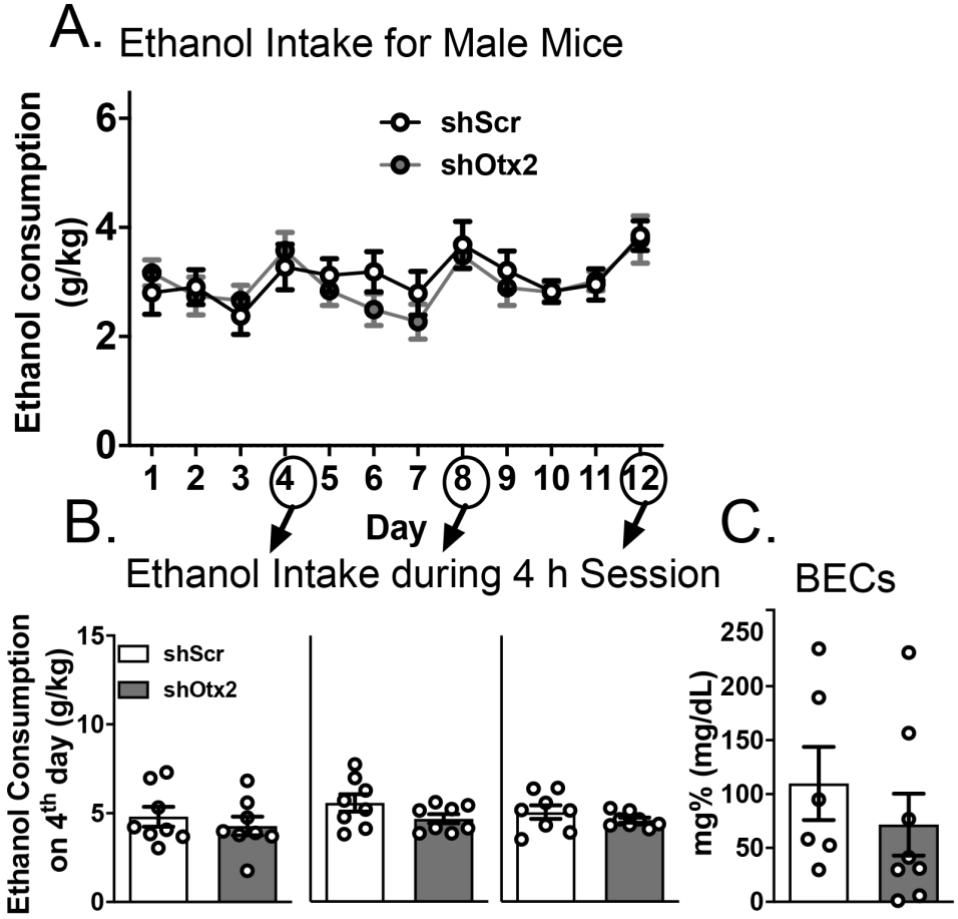


Figure 8. Depleting *Otx2* in the VTA of adult male mice did not alter binge-like drinking. Male mice were injected with shScr- or shOtx2-expressing lentivirus and were tested for DID for 3 weeks. A) Average daily ethanol intake (g/kg/2hr) for shScr and shOtx2 groups. B) Amount of ethanol consumed during the 4th drinking session each week. C) Blood ethanol concentrations (BECs) (mg/dL) in the shScr and shOtx2 groups after the final 4 hour drinking session. Data are presented as the mean ± SEM. Animal numbers for shRNA groups: shScr: n = 8, shOtx2: n = 8.

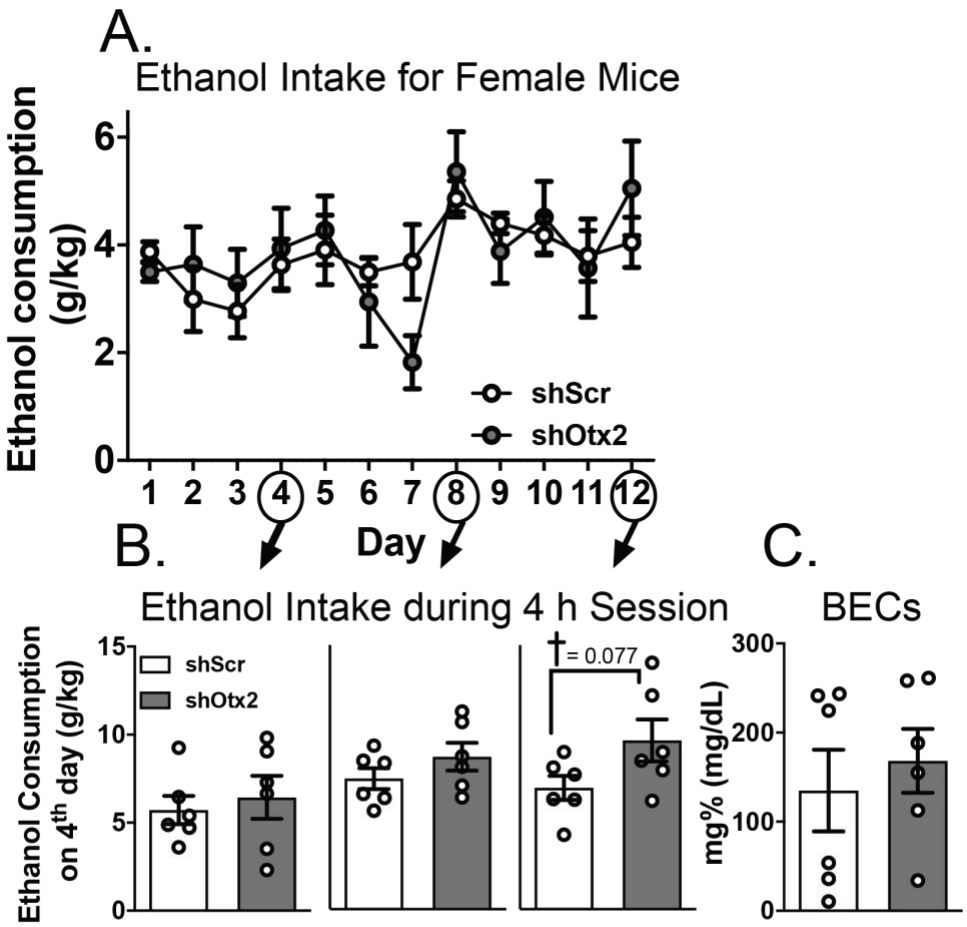


Figure 9. Depleting *Otx2* in the VTA of adult female mice did not alter binge-like drinking. Female mice were injected with lentivirus expressing shScr or shOtx2 and were tested for DID for 3 weeks A) Average daily ethanol intake (g/kg/2hr) for the shScr and shOtx2 groups. B) Amount of ethanol consumed during the 4th drinking session each week. C) Blood ethanol concentrations (BECs) (mg/dL) in the shScr and shOtx2 groups immediately after the final 4 hour drinking session. Data are presented as the mean ± SEM. Animal numbers for shRNA groups: shScr: n = 6, shOtx2: n = 6.

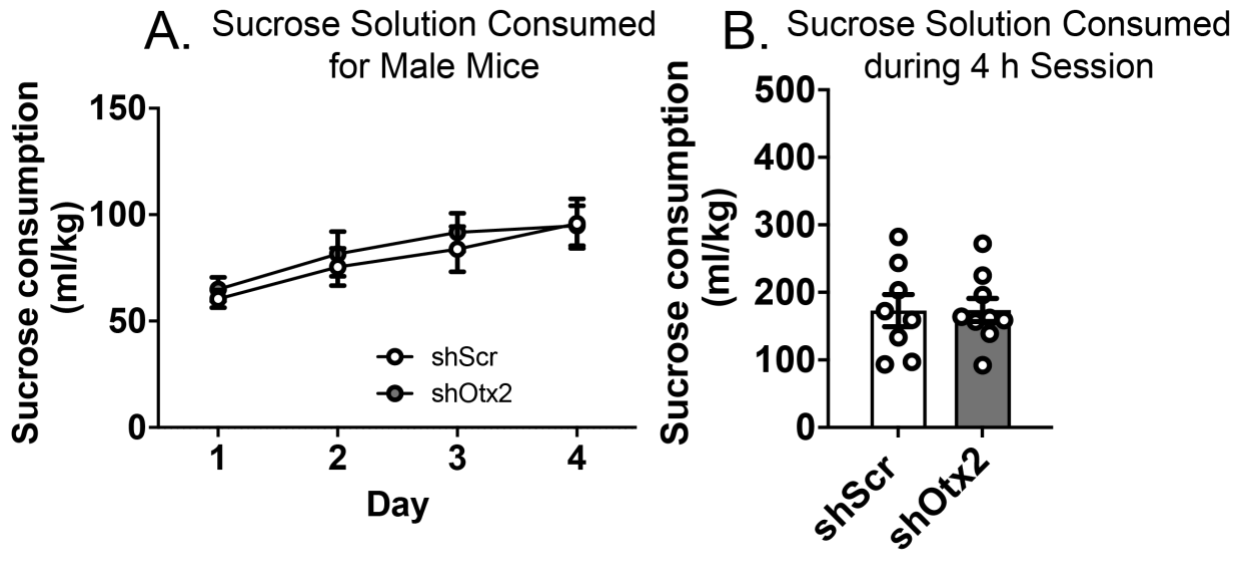


Figure 10. Depleting *Otx2* in the VTA of adult male mice did not alter sucrose consumption. Mice were injected with lentivirus expressing shScr or shOtx2. After the completion of the ethanol drinking test, they were tested for consumption of 2% sucrose for 4 days in the DID protocol. A) Average daily sucrose solution intake (ml/kg/2hr) for the shScr and shOtx2 groups. B) Amount of sucrose consumed during 4 h in the 4th drinking session. Data are presented as the mean ± SEM. Animal numbers for shRNA groups: shScr: n = 8, shOtx2: n = 8.

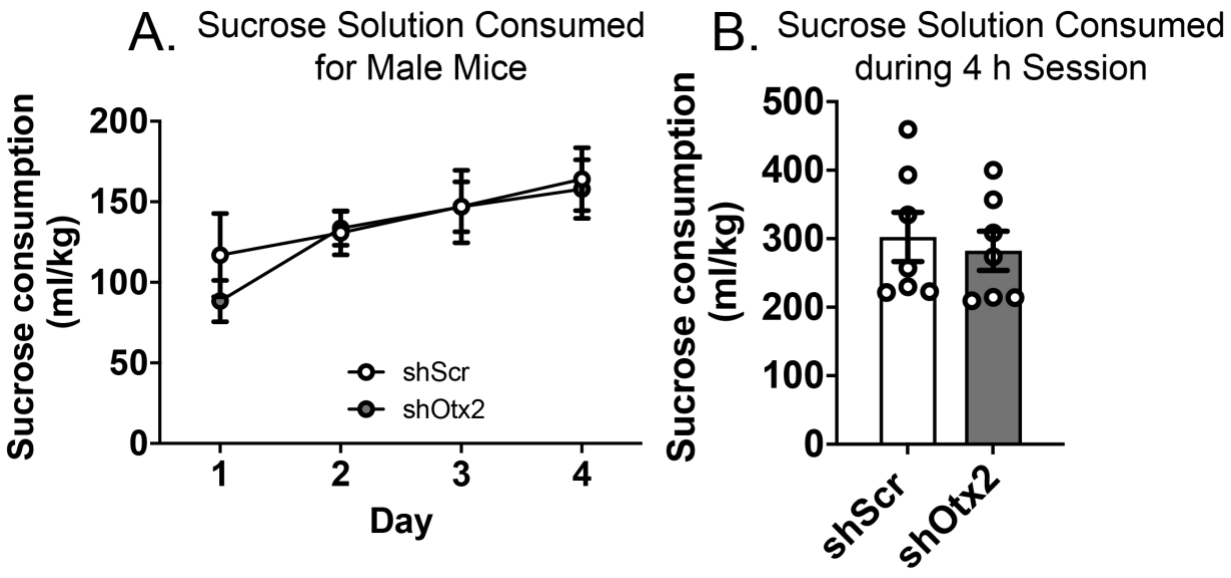
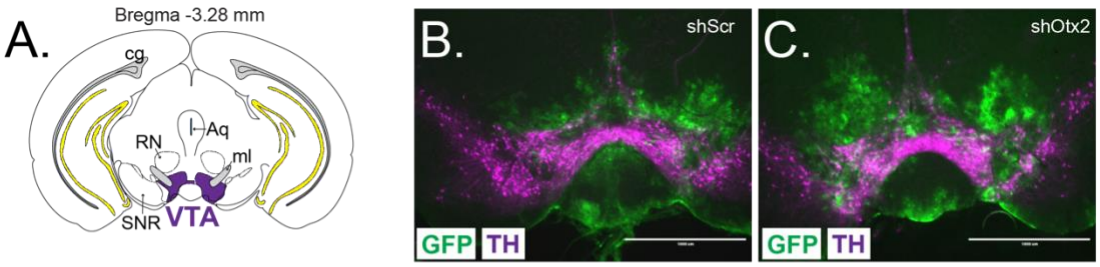


Figure 11. Depleting Otx2 in the VTA of adult female mice did not alter sucrose consumption. Mice were injected with lentivirus expressing shScr or shOtx2. After the completion of the ethanol drinking test, they were tested for intake of a 2% sucrose solution for 4 days in the DID protocol. A) Average daily sucrose solution intake (ml/kg/2hr) for the female shScr and shOtx2 groups. B) Amount of sucrose consumed in 4 h during the 4th drinking session. Data are presented as the mean ± SEM. Animal numbers for shRNA groups: shScr: n = 6, shOtx2: n = 6.



D. **Average A/P spread of Lentivirus**

shRNA	Anterior (mm)	Posterior (mm)	Average A/P Spread (mm)
SCR	-2.99	-3.31	0.33
OTX2	-3.03	-3.38	0.35

Figure 12. Anterior – Posterior spread of the infection did not differ between the SCR and OTX2 shRNA groups. After the behavioral tests, I determined the A/P spread of the infections of the shScr and shOtx2. A) Illustration of coronal section containing the VTA (purple), which is the site of injection. B) Representative image of GFP (green) and TH (magenta) immunostaining in the VTA after injection of lentivirus carrying shScr and gene for GFP. C) Representative image of GFP (green) and TH (magenta) immunostaining in the VTA after injection of lentivirus carrying shOtx2 and gene for GFP. D) Table demonstrates that there is no significant difference in the A/P spread of the infections. Animal numbers for shRNA groups: shScr: n = 9. shOtx2: n = 10.

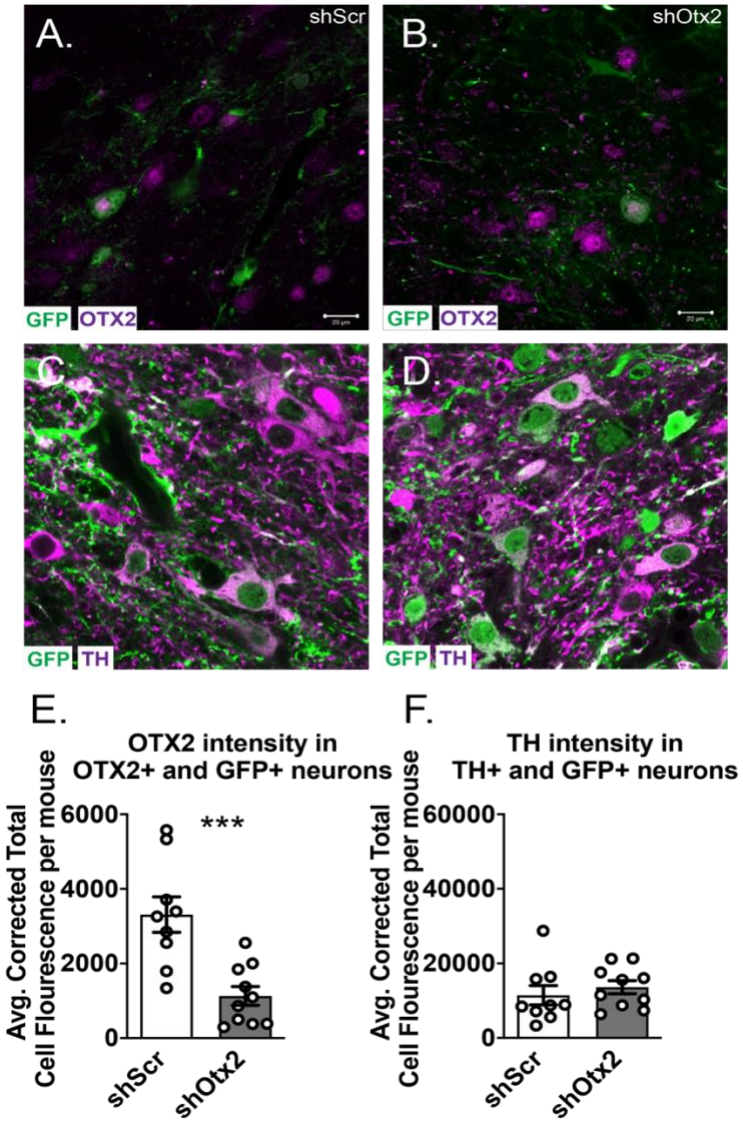


Figure 13. There is a significant reduction in OTX2 intensity in the OTX2+ and GFP+ neurons in the OTX2 shRNA group compared to the SCR shRNA group. After the final behavioral test, OTX2 intensity in the OTX2+ and GFP+ and TH intensity in the GFP+ and TH+ neurons was measured via immunohistochemistry. A) Representative image of GFP+ (green) and OTX2+ (magenta) neurons infected in the VTA of a shScr mouse. B) Representative image of GFP+ (green) and OTX2+ (magenta) neurons infected in the VTA of a shOtx2 mouse. C) Representative image of GFP+ (green) and TH+ (magenta) neurons in the VTA of a shScr mouse. D) Representative image of GFP+ (green) and TH+ (magenta) neurons infected in the VTA of a shOtx2 mouse. E) The intensity of OTX2 between the SCR and OTX2 shRNA groups. F) The intensity of TH in the TH+ and GFP+ VTA neurons in the SCR and OTX2 shRNA groups. ***P<0.001. Data are presented as the mean ± SEM. Animal numbers for shRNA groups: shScr: n = 9. shOtx2: n = 10.

CHAPTER FOUR:

TRANSIENT REDUCTION OF OTX2 IN THE VTA FROM PND 15 – 24 RESULTS IN INCREASED TYROSINE HYROXYLASE WITHIN THE NAC AND VTA OF ADULT MICE, BUT DOES NOT ALTER ETHANOL CONSUMPTION, LOCOMOTOR ACTIVITY, OR ANXIETY-LIKE BEHAVIOR

4.1 Introduction

In the previous chapters, I demonstrated that binge-like drinking by mice altered *Otx2* mRNA and protein levels within the VTA. In addition, binge-like drinking altered potential transcriptional targets of OTX2 within the VTA. However, long-term knockdown of OTX2 in the VTA of adult male and female mice did not affect binge-like drinking. There are several potential reasons for this. One reason is that it is possible that downregulation of VTA OTX2 expression was not conducted at the right age. Indeed, one study demonstrated that transient reduction of OTX2 in the murine VTA around PND 21 is an important contributor to the development stress-induced depression-like behavior in adult mice [114]. In addition, mice in which OTX2 expression levels were modulated specifically in DA neurons throughout brain development displayed altered locomotor activity, anxiety-like behavior, and innervation of DA neurons in VTA projection regions [107, 117, 118]. Therefore, I hypothesized that reducing OTX2 levels in the murine VTA around PND 21 might impact ethanol consumption and other behaviors, and innervation of DA neurons in VTA projection targets such as the NAC.

4.1.1. OTX2 and Depression

OTX2 has been implicated in the development of depression. Combining 7-10 days of 4 h of maternal separation per day with reduced enrichment (nesting material) starting on PND 10, resulted in a temporary reduction in *Otx2* gene expression and the number of OTX2-expressing neurons in the in the VTA of PND 21 male mice when compared to mice that experienced standard weaning. When male mice that experienced postnatal stress from PND 10 – 17 or PND 10 - 21 also experienced social defeat during adulthood, they displayed higher levels of depression-like behavior compared to mice that went through standard weaning and a second hit of stress during adulthood. These results were recapitulated by microRNA-induced transient reduction of *Otx2* in the murine VTA around PND 15 - 24 and a second hit of social defeat during adulthood, suggesting that a reduction of OTX2 in the murine VTA around PND 21 contributes to susceptibility to stress-induced depression in adulthood. Another study in human subjects found a correlation between peripheral methylation at the *OTX2* gene and depression [114]. These studies have demonstrated a relationship between OTX2 and depression. A comorbidity of AUD and depression is well established [132-134]. Because OTX2 levels in the murine VTA around PND 21 are implicated in the development of stress-induced depression in adulthood, altering OTX2 levels in the VTA at this time period could contributing to the development of AUD in adulthood.

4.1.2. OTX2 and Locomotor Activity

Over-expressing OTX2 in mesDA neurons in mice resulted in aberrant locomotor activity. One study found that overexpressing OTX2 in mesDA neurons led to a reduction in locomotor activity compared to the control group [107]. In contrast, another

laboratory observed that overexpressing OTX2 actually increased locomotor activity [118]. Another study used a telemetry to track the locomotor activity for 30 days, of a control group and a group in which mesDA neurons overexpressed Otx2. The data revealed that overexpressing OTX2 in the mesDA caused fluctuations in locomotor activity when compared to the control group. The mutant mice had prolonged periods of hyper- and hypo- activity in comparison to the control group [117].

4.1.3. OTX2 and Anxiety

Modulating OTX2 within mesDA neurons also affected anxiety-like behavior. The first study found that mice overexpressing of OTX2 in mesDA neurons spent less time in the center of the OF apparatus compared to the control group [107]. This suggests that over-expressing OTX2 in mesDA promotes the development of anxiety-like behavior. In contrast, another study revealed that over-expressing OTX2 in mesDA neurons resulted in increased time in the middle of the OF [117], which reflects reduced anxiety-like behaviors.

4.1.4. OTX2 and VTA DA axon targeting

OTX2 regulates the amount of innervation from the VTA to several brain regions, including the PFC and NAC. Two studies have shown that reducing OTX2 within DA neurons led to decreased TH+ axonal terminals in the PFC and NAC [105, 106]. Over-expression of OTX2 in mesDA neurons resulted in increased glycosylated (glyco) - dopamine transporter+ (DAT+) in the PFC of adult mice [105, 106].

4.1.5. Aims of the study

Based on my results from the previous two chapters and other studies, I know that OTX2 is an ethanol-responsive gene. In addition, other studies have shown that OTX2, within the mesDA, can impact locomotor activity and the innervation from the VTA to its projection regions. Finally, OTX2 has been linked to the development of depression- and anxiety- like behavior. It is currently not known if transiently depleting VTA OTX2 around PND 21 can impact ethanol consumption, locomotor activity, and anxiety-like behavior. Also, it isn't known if transiently reducing OTX2 in the murine VTA around PND 21 can impact the VTA DA axons which terminate in the NAC, PFC, and HIPP [39, 135]. Therefore, I investigated if temporarily modulating VTA OTX2 around PND 15 - 24 can impact ethanol preference and ethanol consumption using the 2BC ethanol consumption test. In addition, I tested whether down-regulating *Otx2* could alter locomotor activity, both before and after exposure to ethanol. Also, I determined whether transient reduction of *Otx2* in the VTA promoted the development of anxiety-like behavior in adulthood. Finally, I measured the effect of transient knockdown of VTA *Otx2* on the expression of putative OTX2 target genes in the VTA, and TH and DAT levels in the PFC, HIPP, and NAC in adult mice.

4.2 Methods and Materials

4.2.1. Animals

Male and female WT C57BL/6J mice were purchased from the Jackson Laboratory, arrived at 8 weeks of age, and were used as breeders. One male mouse was paired with two female mice for approximately 18 days. On the 18th day, male and

female mice were placed in separate cages. Pregnant dams were checked daily until pups were born. The day pups were born was considered PND 0. Stereotaxic surgeries were performed on PND 15 -17. Pups remained with the dam until PND 21. All procedures were approved by the UIC Animal Care Committee, and animal care followed the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

4.2.2. Experimental Design

Figure 14 demonstrates the battery of tests that 2 – 3 month old mice went through so I could assess locomotor activity, ethanol preference and consumption, and depression- or anxiety-like behavior. Time between each assays was 24 h.

4.2.3. Herpes Simplex Viral (HSV) Vectors

We purchased the HSV vectors expressing either a microRNA to *Otx2* or a microRNA to *LacZ* (as a control) from the Gene Delivery Technology Core at Massachusetts General Hospital. These viral vectors were the same as those previously described and characterized [114]. HSVs were bicistronic and expressed both the fluorescent reporter GFP and the microRNA which targeted *LacZ* or *Otx2*. I used HSV to transiently deplete *Otx2* in the VTA of pups from PND 15 – 24. The HSV vector is expressed within 12 h of injection, has the greatest expression levels between days 2-4, and is reduced expression thereafter, being undetectable by day 7. The time *Otx2* is injected into the VTA is from PND 15 – 17 and the HSV-induced reduction of

Otx2 happens from PND 15 -24. The greatest reduction in *Otx2,* within the VTA, happened between PNDs 17 – 21.

4.2.4. Delivery of HSV vectors into the VTA on PND 15 - 17

Stereotaxic delivery of HSVs bilaterally into the VTA was performed as described previously [128]. Before the surgeries, mice were acclimated to the biohazard room, which is where the stereotaxic surgeries were done. Three days before the surgeries, dams and their litters were moved into the biohazard room and a paper towel with a drop of the tissue glue was placed in the cage. At PND 15 – 17, male and female C57BL/6J pups were anesthetized with ketamine (100 mg/kg) and xylazine (8 mg/kg) and placed in a stereotaxic alignment instrument for viral injections (David Kopf Instruments, Tujunga, CA, USA). The coordinates for bilateral infusions were anterior/posterior, -3.2 mm; medial/lateral, +/- 0.5 mm; and dorsal/ventral -4.5 mm. The virus was injected at a rate of 0.1 μl/min and cannulas remained lowered for an additional 7 minutes to prevent backflow of the virus. After the infusion step, the wounds were closed with tissue glue (Vetclose, Dublin, OH, USA) and the mice injected with meloxicam (2 mg/kg). Pups were returned to their home cages with dams when they were fully awake. At PND 21, mice were weaned and transferred to the reverse light/dark cycle room to get acclimated to the reverse light dark cycle and single housing until they reached 2 months age.

4.2.5. Verifying HSV infection of OTX2+ VTA neurons

Refer to Chapter Three, section *3.2.9.* for details of the immunohistochemistry protocol.

4.2.6. Locomotor Activity Test

To determine if transient depletion of *Otx2* within the VTA OTX2 alone or in combination with moderate ethanol consumption can impact locomotor activity, mice were subjected to the locomotor activity test before the drinking test and after the sucrose preference test. Locomotor activity was measured in mouse OF apparatus (27.3 cm L x 27.3 cm W x 20.3 cm H) with 48-channel infrared beam detectors and Activity Monitor software (Med Associates, St. Albans, VT). For all locomotor studies, animals were moved to the testing room for 5 min prior to testing. During the test, the movement of the mice were tracked for 30 minutes.

4.2.7. Two-bottle Choice (2BC) Drinking Test

To determine if transient depletion of Otx2 in the VTA from PND 15 – 24 will impact ethanol consumption, PND 15 – 17 pups were injected with a HSV carrying the gene for GFP and an *Otx2* or *LacZ* (control) miRNA via stereotaxic surgery followed by a 2BC test. Two month old male and female mice had 24 hour access to increasing concentrations of ethanol and water. Mice were weighed every 7 days and regardless of treatment there was no significant difference in the average weights between all groups (data not shown). The concentrations of the ethanol solutions were 3, 6, 10, 15, and 20%, and were sequentially increased every 4 days. The position of the tubes were switched every 2 days to control for any side preference.

4.2.8. Sucrose Preference Test

To determine whether transient depletion of VTA *Otx2* promoted the development of anhedonia, mice were subjected to a sucrose preference test. Two month old male and female mice had 48 hour access to two bottles, one containing 1% sucrose and the other containing water. The position of the tubes were switched every day to control for side preference. Water and sucrose solution consumption was measured at both the 2 and 24 h timepoints.

4.2.9. Light Dark Box Test

Anxiety-like behavior was measured via the LDB test. Animals were moved to the testing room 1 hour prior to testing. The LDB apparatus contains two regions connected by a small hole. The dimly illuminated region is called the dark zone and the dark zone is enclosed and opaque. Both zones have the same dimensions (19.69 cm L x 40 cm W x 34.29 cm H). Mice faced the entrance to the dark zone when placed in the apparatus. The mice were allowed to explore the box for 5 minutes. ANY-Maze (Stoelting Co, Wood Dale, IL) was used to quantify the amount of time mice spent in the light zone.

4.2.10. Tissue Collection

Refer to Chapter Two, section *2.2.3.* for details of the PFC, NAC, HIPP, and VTA tissue collection.

4.2.11. qPCR

qPCR was used to measure mRNA levels of *Otx2, Wnt1, Mdk*, and *Sema3c*. Refer to Chapter Two, section *2.2.5.* for details of the qPCR procedure.

4.2.12. Western Blot

Refer to Chapter Two, section *2.2.5.* for details of the western blot procedure. The DAT antibody used was a rat monoclonal antibody from Santa Cruz (Santa Cruz Biotechnology Cat# sc-32258, RRID:AB_627400). The secondary antibody used was a goat anti-rat IgG-HRP antibody (Santa Cruz Biotechnology Cat# sc-2032, RRID:AB_631755). The TH antibody was a mouse monoclonal antibody (Millipore, Cat# AB1542, RRID: AB_90755). The secondary antibody was a donkey anti-mouse IgG IRDye 680RD antibody (IRDye 680RD donkey anti-mouse IgG, cat# 925-68072, RRID: AB_2814912).

4.2.13. Statistical Analysis

Refer to Chapter Two, section *2.2.7.* for details on Statistical Analysis.

4.3 Results

4.3.1. Transient depletion of Otx2 from PND 15 - 24 VTA does not alter locomotor activity or promote the development of anxiety-like behavior in adulthood

First, a couple of mice were used to verify infection of HSV vector carrying the gene for GFP and the miRNA for *Otx2* (mi*Otx2*) or *LacZ* (mi*LacZ*) at PNDs 18 – 20. Figure 15 demonstrates the infection (green) from the HSV vector carrying the gene for GFP within the VTA.

To determine if transient depletion of VTA *Otx2* could impact locomotor activity and promote the development of anxiety-like behavior, HSV vectors carrying the gene

for GFP and the mi*LacZ* or mi*Otx2* were injected into the VTA on PND 15 -17 via stereotaxic surgery. Mice recovered from the surgery, and were single housed until they were 2 – 3 months old, at which point they began a series of behavioral tests (Fig 14). Mice underwent the OF test for 30 minutes. Transient depletion of VTA *Otx2* on PND 15 – 24 did not impact locomotor activity in male mice (Figure 16A, n=7 per group, 2-way ANOVA, effect of miRNA: $F(5, 60) = 1.38$, $P=0.245$, effect of time: $F(5, 60) = 32.67$, $P<0.0001$, miRNA by time interaction: $F(1, 12) = 2.32$, $P=0.154$). Likewise, the transient depletion of VTA *Otx2* did not impact locomotor activity (Figure 16B, n=7 per group, 2-way ANOVA, effect of miRNA: $F(5, 60) = 1.80$, $P=0.127$, effect of time: $F(5, 60) = 28.25$, $P<0.0001$, miRNA by time interaction: $F(1, 12) = 2.72$, $P=0.125$). In addition, I determined that depleting *Otx2* in the male VTA on PND 15 - 24 did not change the amount of time spent in the center of the apparatus (Figure 16C, n=7 per group, Student's t-test, $t(12) = 1.48$, $P=0.164$). Also, transient depletion of *Otx2* in the female VTA did not change the amount of time spent in the center of the OF apparatus (Figure 16D, n=7 per group, Student's t-test, $t(12) = 0.48$, $P=0.962$). Finally, I determined that transiently reducing *Otx2* in the VTA did not significantly impact the total distanced travelled throughout the 30 minute locomotor activity test, although there was a trend towards reduced distance travelled in the mice that received mi*Otx2* (Figure 16E, n=7 per group, 2-way ANOVA, effect of miRNA: $F(1, 24) = 4.04$, $P=0.056$, effect of sex: $F(1, 24) = 2.22$, $P=0.150$, miRNA by sex interaction: $F(1, 24) = 0.06$, $P=0.815$). Overall, these results suggest that transient depletion of VTA *Otx2* from PND 15- 24 does not significantly alter locomotor activity or result in the development of anxiety-like behavior, as measured using the OF test, during adulthood.

4.3.2. Transient depletion of VTA Otx2 from PND 15 -24 does not affect ethanol preference and intake in adulthood

To determine if transient depletion of VTA *Otx2* could affect the preference or consumption of ethanol in adulthood, mice underwent the 2BC drinking test with different concentrations of ethanol (3, 6, 10, 15, and 20%), with 4 days of access at each concentration. Ethanol preference was not affected by the transient reduction of *Otx2* in the male murine VTA around PND 15 – 24 (Figure 17A, n = 14 – 19 per group, 2-way ANOVA, effect of miRNA: $F (1, 155) = 0.02$, $P=0.892$, effect of concentration: $F (4, 155) = 11.74$, $P<0.0001$, miRNA by concentration interaction: $F (4, 155) = 0.21$, $P=0.932$). Similarly, depleting *Otx2* in the female murine VTA did not alter ethanol preference in comparison to the control group (Figure 18A, n = 15 – 16 per group, 2-way ANOVA, effect of miRNA: $F (1, 29) = 0.778$, $P=0.892$, effect of concentration: $F (1.922, 55.26) = 9.68$, $P=0.0003$, miRNA by concentration interaction: $F (4, 115) = 2.56$), $P=0.042$). Holm Sidak's multiple comparison tests revealed that the female mi*LacZ* group on average drank significantly more 6% ethanol solution than the 3% solution ($P=0.003$). Holm Sidak's multiple comparison also revealed that the female mi*Otx2* group on average drank more 6% ($P=0.014$), 10% ($P=0.001$), 15% ($P=0.001$), and 20% ($P=0.010$) ethanol solution in comparison to the 3% ethanol solution they drank. The amount of ethanol consumed (in g/kg body weight) during the 2BC test was not affected by transient *Otx2* depletion in the VTA of male mice (Figure 17B, n = 14 – 19 per group, 2-way ANOVA, effect of miRNA: $F (1, 155) = 2.83$, $P=0.095$, effect of concentration: $F (4, 155) = 135.0$, $P<0.0001$, miRNA by concentration interaction: $F (4, 155) = 0.98$,

P=0.419). There was no difference in the amount of ethanol consumed between the mi*LacZ* and mi*Otx2* female mice (Figure 18B, n = 15 – 16 per group, 2-way ANOVA, effect of miRNA: F (1, 29) = 0.17, P=0.688, effect of concentration: F (1.731, 49.76) = 236.9, P<0.0001, miRNA by concentration interaction: F (4, 115) = 0.58, P=0.680). Transiently reducing *Otx2* in the male VTA did not change water intake (Figure 17C, n = 14 – 19 per group, 2-way ANOVA, effect of miRNA: F (1, 31) = 0.57, P=0.455, effect of concentration: F (4, 124) = 15.66, P<0.0001, miRNA by concentration interaction: F (4, 124) = 2.67, P=0.035). Holm Sidak's multiple comparison revealed that the male mi*LacZ* mice drank significantly lower amounts of water when given 6% (P<0.0001), 10% (P<0.0001), and 15% (P<0.0001) ethanol solutions compared to when the 3% ethanol solution was present in the cage. In addition, Holm Sidak's multiple comparison revealed that the male mi*Otx2* mice drank significantly lower amounts of water when given 6% (P=0.005), 10% (P=0.001), 15% (P<0.0001), and 20% (P=0.002) ethanol solutions compared to the time they had the 3% ethanol solution. In addition, water intake in female mice was not affected (Figure 18C, n = 15 – 16 per group, 2-way ANOVA, effect of miRNA: F (1, 29) = 0.20, P=0.658, effect of concentration: F (4, 115) = 6.29, P<0.0001, miRNA by concentration interaction: F (4, 115) = 0.34, P=0.850. These results suggest that transient depletion of *Otx2* in the murine VTA from PND 15 - 24 does not alter ethanol consumption during adulthood. Finally, total liquid intake was not affected by the reduction of *Otx2* in the male and female VTA (males, Figure 17D, n = 14 – 19 per group, 2-way ANOVA, effect of miRNA: F (1, 155) = 0.69, P=0.409, effect of concentration: F (4, 155) = 0.85, P=0.496, miRNA by concentration interaction: F (4, 155) = 0.85, P=0.496; females, Figure 18D, n = 15 – 16, 2-way ANOVA, effect of

miRNA: F (1, 29) = 0.41, P=0.529, effect of concentration: F (4, 115) = 8.18, P<0.0001, miRNA by concentration interaction: F (4, 115) = 0.71, P=0.585). Overall, these results suggest that transient reduction of *Otx2* in the VTA from PND 15 – 24 does not affect ethanol consumption and preference.

4.3.3. Transient depletion of VTA OTX2, during PND 15 - 24, followed by ethanol consumption in adulthood, does not promote anhedonia.

Next, I wanted to determine if transient reduction of *Otx2* in the VTA of male and female mice would promote the development of anhedonia. After the 2BC test, I measured anhedonia via the sucrose preference test. Adult male and female mice had access to a 1% sucrose solution and water in a 2BC format for 48 h. Fluid intake was measured at the 2 and 24 h timepoints. Transient depletion of VTA *Otx2* did not impact sucrose preference at the 2 h timepoint (Figure 19A, n = 7 – 12 per group, Student's t-test, t (17) = 0.58, P=0.571). In addition, sucrose preference at the 2 h timepoint was not affected by transient depletion of *Otx2* in the female VTA (Figure 20A, n = 7 – 8 per group, Student's t-test, t (13) = 1.15, P=0.270). At the 24 h timepoint, there was no difference in sucrose preference between the male mi*LacZ* and mi*Otx2* groups (Figure 19B, n = 7 – 12 per group, Student's t-test, t (16) = 0.01, P=0.644). Likewise, transiently reducing VTA *Otx2* from PND 15 – 24 did not affect sucrose preference at the 24 h timepoint (Figure 20B, n = 7 – 8 per group, Student's t-test, t (13) = 0.628, P=0.541). Transient depletion of *Otx2* in the male murine VTA did not impact sucrose consumption (in ml/kg body weight) at the 2 h timepoint (Figure 19C, n = 7 – 12 per group, Student's t-test, t (17) = 0.60, P=0.556). In addition, transient depletion of *Otx2* in the VTA of

female mice did not impact sucrose consumption at the 2 h timepoint (Figure 20C, n = 7 – 8 per group, Student's t-test, t (13) = 0.924, P=0.372). Finally, transient depletion of *Otx2* in the male murine VTA from PN 15- 17 did not impact sucrose consumption at the 24 h timepoint (Figure 19D, n = 7 – 12 per group, Student's t-test, t (16) = 0.006, P=0.995). In addition, transient depletion of *Otx2* in the female murine VTA did not lead to a difference in sucrose consumption at the 24 h timepoint (Figure 20D, n = 7 – 8 per group, Student's t-test, t (13) = 0.04, P=0.970).These results suggest that transient depletion of *Otx2* in the male and female murine VTAs does not promote the development of anhedonia in adulthood.

4.3.4. The combination of transient depletion of Otx2 in the VTA from PND 15 - 24 and ethanol consumption in adulthood does not alter locomotor activity or promote the development of anxiety-like behavior

To determine if the combination of transient depletion of *Otx2* in the male and female VTAs around PND 15 - 24 and ethanol consumption could impact locomotor activity or promote the development of anxiety-like behavior, mice underwent the locomotor activity test after the sucrose preference test. Transient depletion of VTA *Otx2* did not impact locomotor activity in male mice (Figure 21A, n = 7 per group, 2-way ANOVA, effect of miRNA: F (1, 16) = 0.410, P=0.531, effect of time: F (5, 80) = 10.87, P<0.0001, miRNA by time interaction: F (5, 80) = 1.02, P=0.412). Likewise, the transient depletion of *Otx2* in the female VTA did not significantly impact locomotor activity (Figure 21B, n = 7 per group, 2-way ANOVA, effect of miRNA: F (1, 19) = 1.41, P=0.250, effect of time: F (5, 95) = 31.36, P<0.0001, miRNA by time interaction: F (5,

95) = 2.08, P=0.075). In addition, I determined that depleting *Otx2* in the male VTA did not change the amount of time spent in the center of the apparatus (Figure 21C, n = 7 per group, Student's t-test, t (16) = 0.02, P=0.986). Also, transient depletion of *Otx2* in the female VTA did not change the amount of time spent in the center of the OF apparatus (Figure 21D, n = 7 per group, Student's t-test, t (19) = 0.70, P=0.494). In addition, temporarily depleting *Otx2* in the VTA did not impact the total distanced travelled throughout the 30 minute locomotor activity test in adulthood (Figure 21E, n = 7 per group, 2-way ANOVA, effect of miRNA: F (1, 24) = 2.04, P=0.166, effect of sex: F (1, 24) = 2.64, P=0.117, miRNA by sex interaction: F (1, 24) = 2.00, P=0.170). Overall, these results suggest the combination of the transient depletion of VTA *Otx2,* during PND 15 - 24, followed by ethanol consumption in adulthood does not alter locomotor activity or promote the development of anxiety-like behaviors.

4.3.5. Transient depletion of VTA Otx2 from PND 15 – 24 and ethanol consumption in adulthood does not lead to anxiety-like behavior

To investigate if the depletion of *Otx2* in the murine VTA from PND 15 – 24 promoted the development of anxiety-like behavior after ethanol exposure, mice underwent the LDB test after the locomotor activity test. Transient reduction of VTA *Otx2* in male mice around PND 15 – 24 did not lead to a difference in percentage of time spent in the light zone (Figure 22A, n = 6 – 12 per group, Student's t-test, t (16) = 0.74, P=0.468). Similarly, there was no difference in the percentage of time the female mi*LacZ* and mi*Otx2* groups spent in the light zone (Figure 22D, n = 7 – 9 per group, Student's t-test, t (14) = 0.24, P=0.814). Transient reduction of *Otx2* in the male murine

VTA did not affect the amount of distance travelled in the light zone (Figure 22B, n = 6 – 12 per group, Student's t-test, t (16) = 0.84, P=0.412). Similarly, there was no difference in the distance travelled between the female mi*LacZ* and mi*Otx2* groups (Figure 22E, n = 7 – 9 per group, Student's t-test, t (14) = 0.67, P=0.514). Finally, transient depletion of *Otx2* in the male VTA around PND 15 - 24 did not affect the number of entries into the light zone in comparison to the control group (Figure 22C, n = 6 – 12 per group, Student's t-test, t (16) = 0.97, P=0.349). Likewise, transient depletion of *Otx2* in the female VTA did not alter the amount of entries into the light zone (Figure 22D, n = 7 – 9 per group, Student's t-test, t (14) = 0.87, P=0.401). The results from the LDB test suggest that transient depletion of VTA *Otx2* from PND 15 – 24 does not promote the development of anxiety-like behavior in adult male and female mice.

4.3.6. Transient depletion of VTA OTX2, from PND 15 - 24, does not impact Otx2, Th, Sema3c, Wnt1, and Mdk mRNA expression levels in the adult male and female VTA

I next tested if transiently depleting VTA *Otx2* regulated *Otx2, Th, Sema3c, Wnt1, and Mdk mRNA expression levels* in the male and female adult VTA. Transient depletion of *Otx2* did not significantly alter *Otx2* mRNA levels in the adult male and female VTA (Figure 23A, n = 6 – 11 per sex per group, 2-way ANOVA, effect of miRNA: F (1, 30) = 0.002, P=0.962, effect of sex: F (1, 30) = 0.03, P=0.858, miRNA by sex interaction: F (1, 30) = 0.31, P=0.582). In addition, *Th* mRNA expression, within the adult male and female VTA, was not affected by the transient reduction of VTA *Otx2* (Figure 23B, n = 6 – 11 per sex per group, 2-way ANOVA, effect of miRNA: F (1, 30) = 1.35, P=0.254, effect of sex: F (1, 30) = 0.87, P=0.358, miRNA by sex interaction: F (1,

30) = 0.49, P=0.489). *Mdk* expression within the adult VTA was also not affected by the depletion of *Otx2* (Figure 23C, n = 6 – 11 per sex per group, 2-way ANOVA, effect of miRNA: F (1, 30) = 0.43, P=0.518, effect of sex: F (1, 30) = 0.37, P=0.549, miRNA by sex interaction: F (1, 30) = 0.86, P=0.361). Similarly, transiently reducing *Otx2* in the murine VTA did not impact VTA *Wnt1* levels (Figure 23D, n = 6 – 11 per sex per group, 2-way ANOVA, effect of miRNA: F (1, 30) = 0.97, P=0.332, effect of sex: F (1, 30) = 3.01, P=0.093, miRNA by sex interaction: F (1, 30) = 0.28, P=0.598). Finally, *Sema3c* mRNA levels within the adult male and female VTA was not affected by the transient depletion of *Otx2* but regardless of treatment female groups had more *Sema3c* compared to the male groups Figure 23E, n = 6 – 11 per sex per group, 2-way ANOVA, effect of miRNA: F (1, 30) = 1.88, P=0.181, effect of sex: F (1, 30) = 5.41, P=0.027, miRNA by sex interaction: F (1, 30) = 1.16, P=0.291). These results suggest that transiently depleting *Otx2* in the VTA from PND 15 – 24 does not affect the mRNA levels of *Otx2, Th, Sema3c, Wnt1, and Mdk* mRNA expression levels in the adult male and female VTA.

4.3.7. Transient depletion of VTA OTX2 from PND 15 - 24 resulted in increased TH protein levels in the adult male and female VTA

I investigated whether or not the transient reduction in VTA *Otx2* from PND 15 - 24 had long-term effects on OTX2, TH, and DAT protein expression. Transient depletion of *Otx2* did not significantly impact OTX2 protein levels within the adult VTA of both sexes (Figure 24A, n = 6 – 7 per sex per group, 2-way ANOVA, effect of miRNA: F (1, 21) = 0.13, P=0.717, effect of sex: F (1, 21) = 0.001, P=0.969, miRNA by sex

interaction: F (1, 21) = 9.11, P=0.007). Holm Sidak's multiple comparison test revealed decreased OTX2 protein in the VTA of the male mi*Otx2* group, compared to the male mi*LacZ* group, that did not reach significance (P=0.070). In contrast, Holm Sidak's multiple comparison revealed an increase in OTX2 protein expression in the VTA of the female miOtx2, when compared to the miLacZ group, that did not reach significance (P=0.056). Interestingly, the transient depletion of VTA *Otx2* from PND 15 - 24 resulted in increased in TH protein levels in adult VTA, regardless of sex (Figure 24B, n = 6 – 7 per sex per group, 2-way ANOVA, effect of miRNA: F (1, 21) = 4.86, P=0.039, effect of sex: F (1, 21) = 0.45, P=0.512, miRNA by sex interaction: F (1, 21) = 0.11, P=0.744). DAT protein expression levels were not affected by the transient reduction in VTA *Otx2* from PND 15 - 24 (Figure 24C, n = 6 – 7 per sex per group, 2-way ANOVA, effect of miRNA: F (1, 21) = 0.37, P=0.551, effect of sex: F (1, 21) = 0.35, P=0.562, miRNA by sex interaction: F (1, 21) = 1.71, P=0.205). These experiments suggest that the transient reduction in *Otx2* promotes an increase in TH protein levels in the VTA of both sexes.

4.3.8. Transient depletion of VTA Otx2 from PND 15 - 24 resulted in a significant increase in TH protein levels in the NAC in adulthood

I determined whether transient depletion of *Otx2* in the VTA would alter TH or DAT protein levels in DA axonal terminals in the NAC, HIPP, and PFC, which are projection targets of DA neurons in the VTA. After the behavioral tests, I dissected the NAC, HIPP, and PFC from the mouse brains, and then measured the amount of DAT and TH in the NAC, HIPP, and PFC via western blotting. The mi*Otx2* group had

significantly more TH in the NAC compared to the control group (Figure 25A, , n = 13 - 19 per sex per group 2-way ANOVA, effect of miRNA: F (1, 57) = 5.70, P=0.020, effect of sex: F (1, 57) = 0.35, P=0.557, miRNA by sex interaction: F (1, 57) = 0.004, P=0.951). Transient depletion of VTA *Otx2* from PND 15 - 24 did not impact DAT in the adult male and female NAC (Figure 25B, n = 10 - 13 per sex per group 2-way ANOVA, effect of miRNA: F (1, 43) = 1.31, P=0.260, effect of sex: F (1, 43) = 1.76, P=0.192, miRNA by sex interaction: F (1, 43) = 1.71, P=0.198). Also, temporarily depleting VTA *Otx2* from PND 15 - 24 did not impact DAT protein expression levels in the PFC (Figure 25C, n = 6 – 10 per sex per group, 2-way ANOVA, effect of miRNA: F (1, 29) = 0.30, P=0.588, effect of sex: F (1, 29) = 0.30, P=0.588, miRNA by sex interaction: F (1, 29) = 0.03, P=0.860). Finally, DAT in HIPP of adult mice was not affected by the transient depletion of VTA *Otx2* from PND 15 - 24 (Figure 25D, n = 6 – 10 per sex per group, 2-way ANOVA, effect of miRNA: F (1, 31) = 0.13, P=0.725, effect of sex: F (1, 31) = 1.16, P=0.290, miRNA by sex interaction: F (1, 31) = 4.30, P=0.047). Holm's Sidak multiple comparison test revealed the female miLacZ mice had lower amounts of DAT in the HIPP in comparison to the male miLacZ mice that did not reach significance (P=0.079). Overall, these results suggest that transient depletion VTA *Otx2* from PND 15 - 24 promotes an increase in TH protein levels in the NAC of both sexes.

4.4 Discussion

In conclusion, the depletion of VTA *Otx2* from PND 15 - 24 did not alter ethanol preference or consumption, promote the development of depression or anxiety like-behavior, or affect locomotor activity. The modulation of *Otx2* alone or in combination

with ethanol drinking did not alter the locomotor activity or promote anxiety like-behavior. Although there is evidence that modulating OTX2 in mesDA does impact locomotor activity, those experiments were done with overexpression of *Otx2* throughout brain development and not specifically from PND 15 - 24. My study is the first to demonstrate that the temporary depletion of VTA *Otx2* from PND 15 - 24 does not impact locomotor activity or promote the development of anxiety like-behavior in adult mice. The LDB test also revealed that modulating VTA *Otx2* from PND 15 - 24 did not promote anxiety-like behavior. Similarly, modulating VTA *Otx2* from PND 15 - 24 did not impact sucrose preference or intake. The Nestler group showed that only depleting VTA *Otx2* from PND 15 - 24 did not lead to an increase in depression-like behavior. It took exposing male mice to either postnatal stress (which was found to reduce VTA *Otx2* on PND 21) or a miRNA-mediated reduction of *Otx2* in the VTA from PND 15 - 24, plus social defeat in adulthood, to promote depression-like behavior [114], indicating that it is necessary to expose mice to two hits of stress in order to encourage the development of depression-like behavior. Thus, it is possible that knockdown of *Otx2* from PND 15 - 24, plus an additional stressor in adulthood, would be required to reveal a role for *Otx2* in ethanol consumption. It is also possible that *Otx2*, although an ethanol-responsive gene in the VTA (as I demonstrated in Chapter 2), does not play a role in ethanol consumption at all. Finally, one of the limitations of my study is that I was unable to check HSV transduction in the VTA of adult animals after the conclusion of the behavioral testing, because of the transient nature of the HSV infection. It is possible that I did not accurately target the VTA or get sufficient knockdown of *Otx2* in all of my mice.

Interestingly, transiently depleting *Otx2* in the VTA from PND 15 -24 resulted in increased TH protein levels in the VTA and NAC of both sexes. When OTX2 is depleted in murine mesDA neurons from conception, there is a reduction in innervation from the VTA to other brain regions like the PFC and NAC, as demonstrated by reduced TH immunoreactivity in these brain regions [105, 106]. The increase in TH protein expression levels in the VTA could be because of an increase in the number of TH+ neurons in the adult VTA or an increase in TH protein levels within VTA DA neurons. The increase in TH protein expression levels in the NAC could be explained by an increase in TH protein expression levels in the VTA axonal terminals or an increase in the number of VTA TH+ axonal terminals. The increase in TH may suggest an increase in DA production in the VTA which is being released into the NAC. It is important to note that TH activity is regulated by phosphorylation and dopamine therefore, it is important to determine if transient reduction of VTA *Otx2* from PND 15 - 24 could impact TH activity [126]. My results suggest that transiently reducing VTA *Otx2*, from PND 15 -24, leads to long-term change in TH protein expression levels in the NAC and VTA.

4.5 Acknowledgements

Dr. Subhash Pandey graciously allowed me to use their LDB setup. Dr. Amynah Pradhan graciously allowed me to use the ANY-Maze software to analyze my LDB data. Dr. Eric Nestler graciously provided us with the HSV that carried the gene for GFP and the microRNA that targeted *LacZ* or *Otx2*.

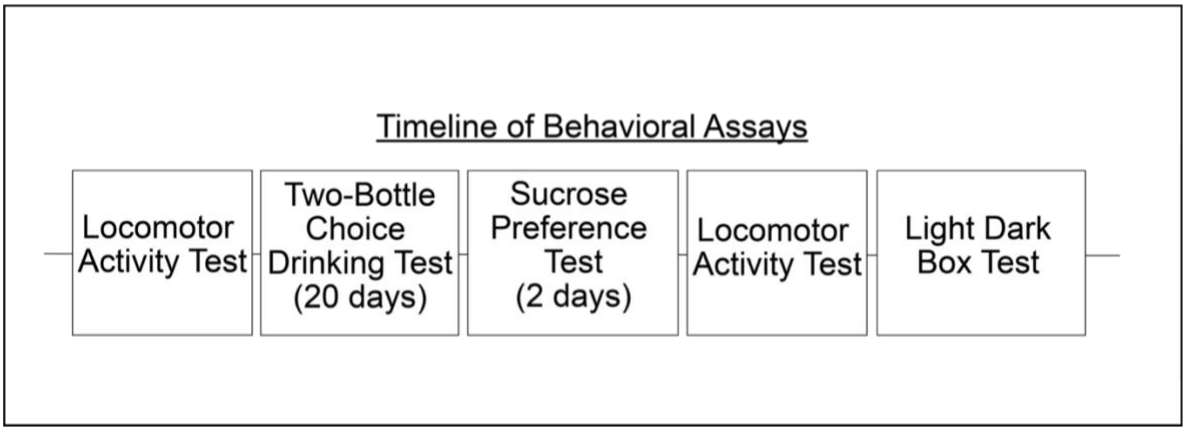


Figure 14. Timeline of behavioral assays.
At 2-3 months of age, mi*LacZ* and mi*Otx2* mice went through a series of tests to determine how transient depletion of Otx2 within the VTA affected locomotor activity, ethanol preference and consumption, and anxiety- and depression-like behavior.

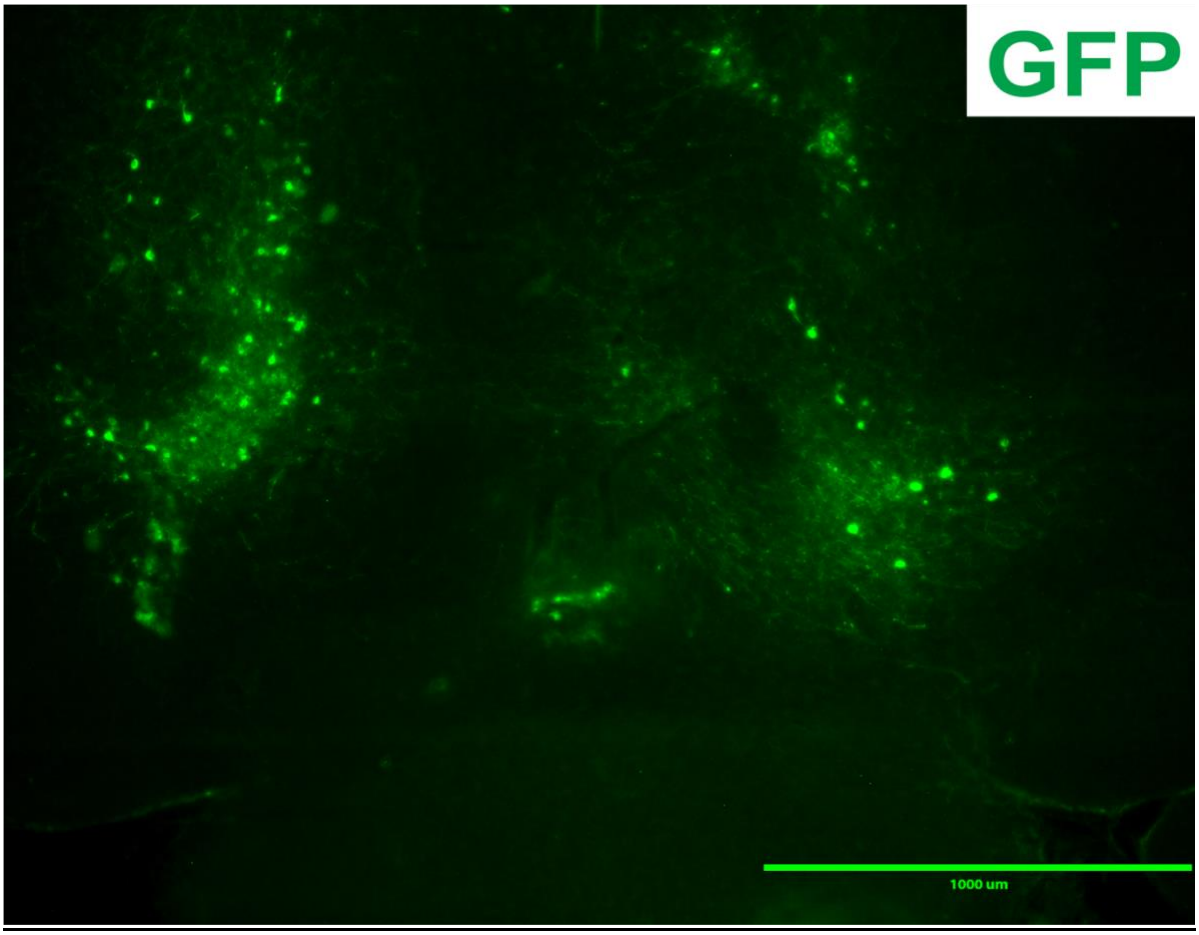


Figure 15. HSV vector infection in VTA. Representative image showing GFP (green) in the murine VTA at PND 18.

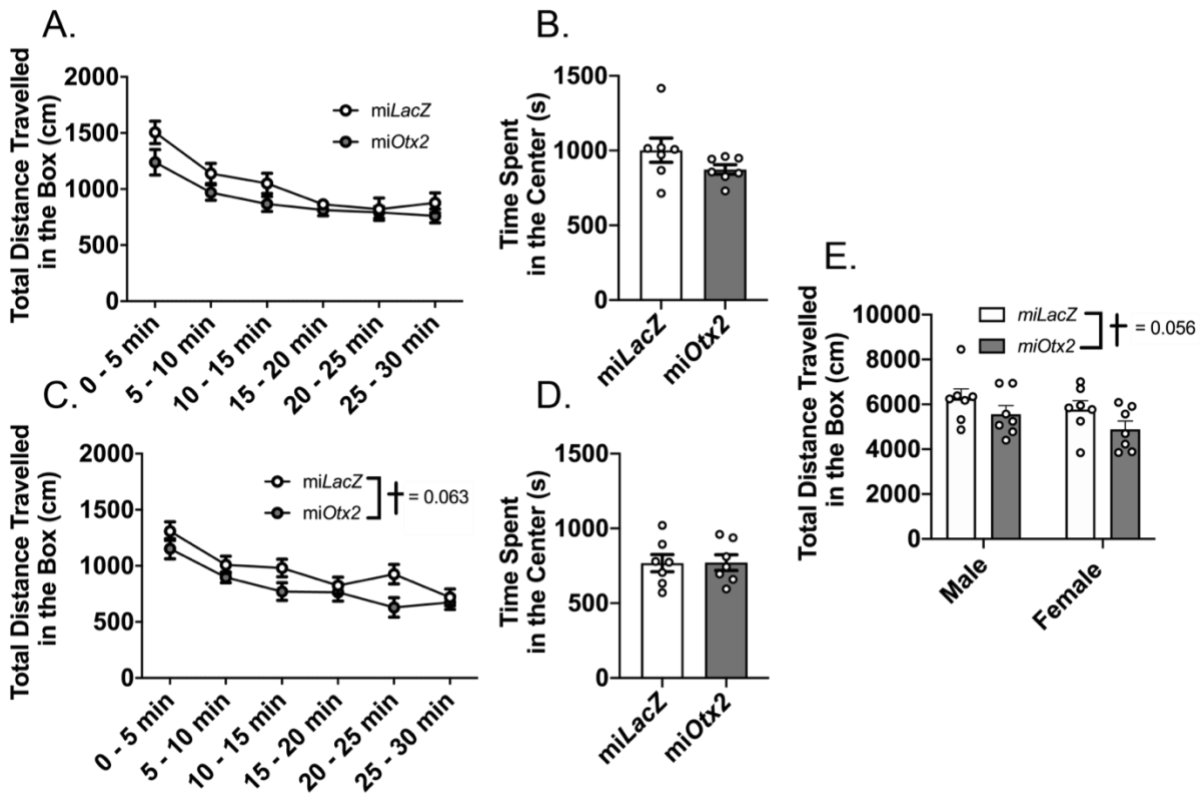


Figure 16.Transiently depleting VTA *Otx2* from PND 15 - 24 did not alter locomotor activity or promote anxiety-like behavior. Male (A,B) and female (B,C) at PND 15 mice were injected with HSV vector expressing mi*LacZ* (white bars) or mi*Otx2* (grey bars). Locomotor activity and anxiety-like behavior were tested in the ethanol naïve group. (A and C) Total distance travelled in the OF test apparatus. (B and D) Time spent in the center of the OF test apparatus.(E) Total distance travelled during OF test. Data are presented as the mean ± SEM. Animal numbers for miRNA groups: miLacZ: n = 7, miOtx2: n = 7.

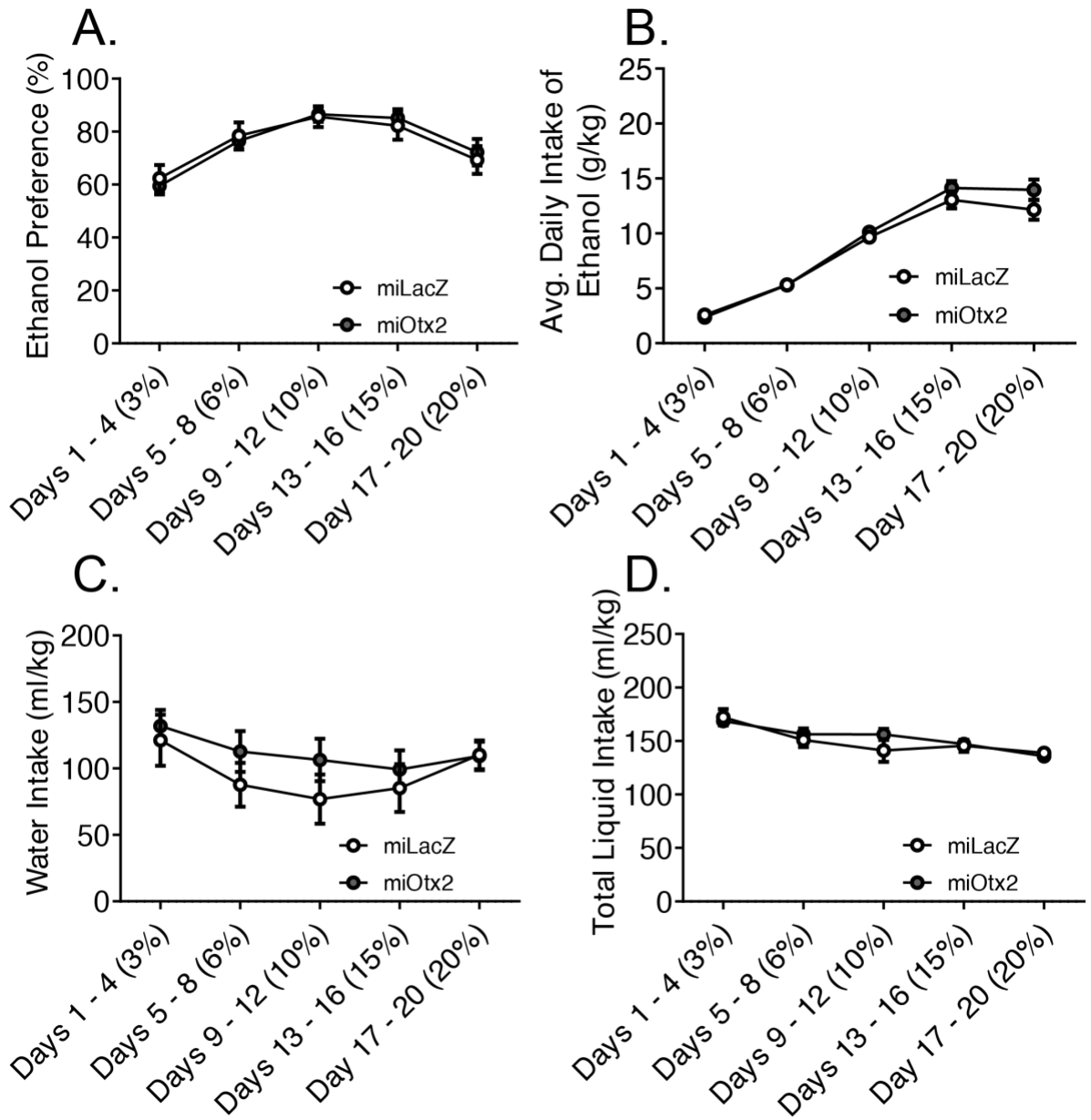


Figure 17. Transiently depleting Otx2 in the male murine VTA from PND 15 -24 did not alter ethanol consumption. Male mi*LacZ* (white circles) or mi*Otx2* (grey circles) groups underwent a 2-bottle choice test for water or 3, 6, 10, 14, and 20% (v/v) ethanol with four days of 24 h access at each ethanol concentration. (A) Average preference for each ethanol solution. (B) Average consumption of ethanol solution (g/kg body weight). (C) Average water intake. (D) Average of the total amount of liquid consumed throughout 2BC test. Data are presented as the mean ± SEM. Animal numbers for miRNA groups: miLacZ: n = 14, miOtx2: n = 19.

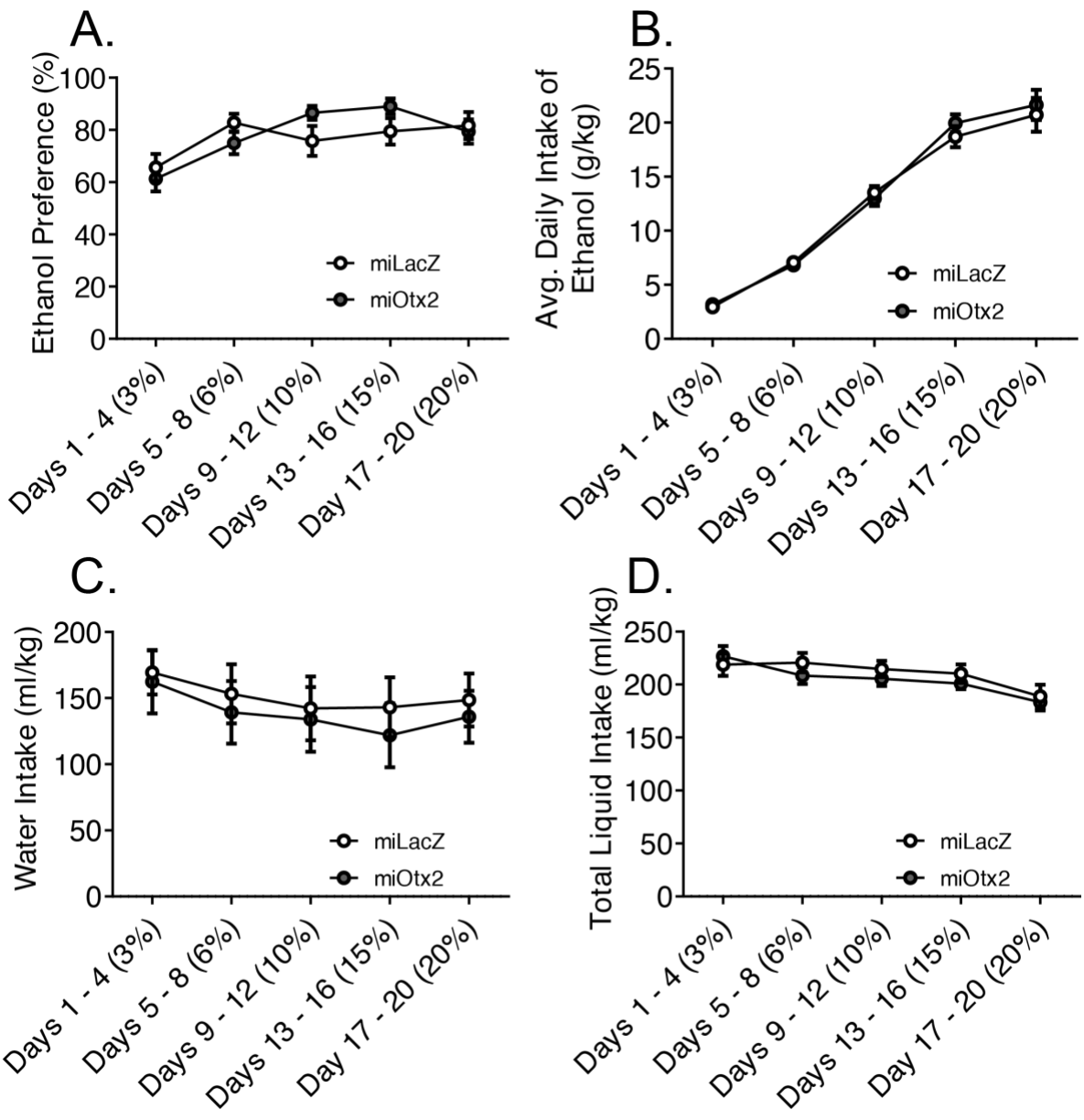


Figure 18. Transiently depleting *Otx2* in the female murine VTA from PND 15 -24 did not alter ethanol consumption. Female mi*LacZ* (white circles) or mi*Otx2* (grey circles) groups underwent a 2-bottle choice test for water or 3, 6, 10, 14, and 20% (v/v) ethanol with four days of 24 h access at each ethanol concentration. (A) Average preference for each ethanol solution. (B) Average consumption of ethanol solution (g/kg body weight). (C) Average water intake. (D) Average of the total amount of liquid consumed throughout 2BC test. Data are presented as the mean ± SEM. Animal numbers for miRNA groups: miLacZ: n = 16, miOtx2: n = 15.

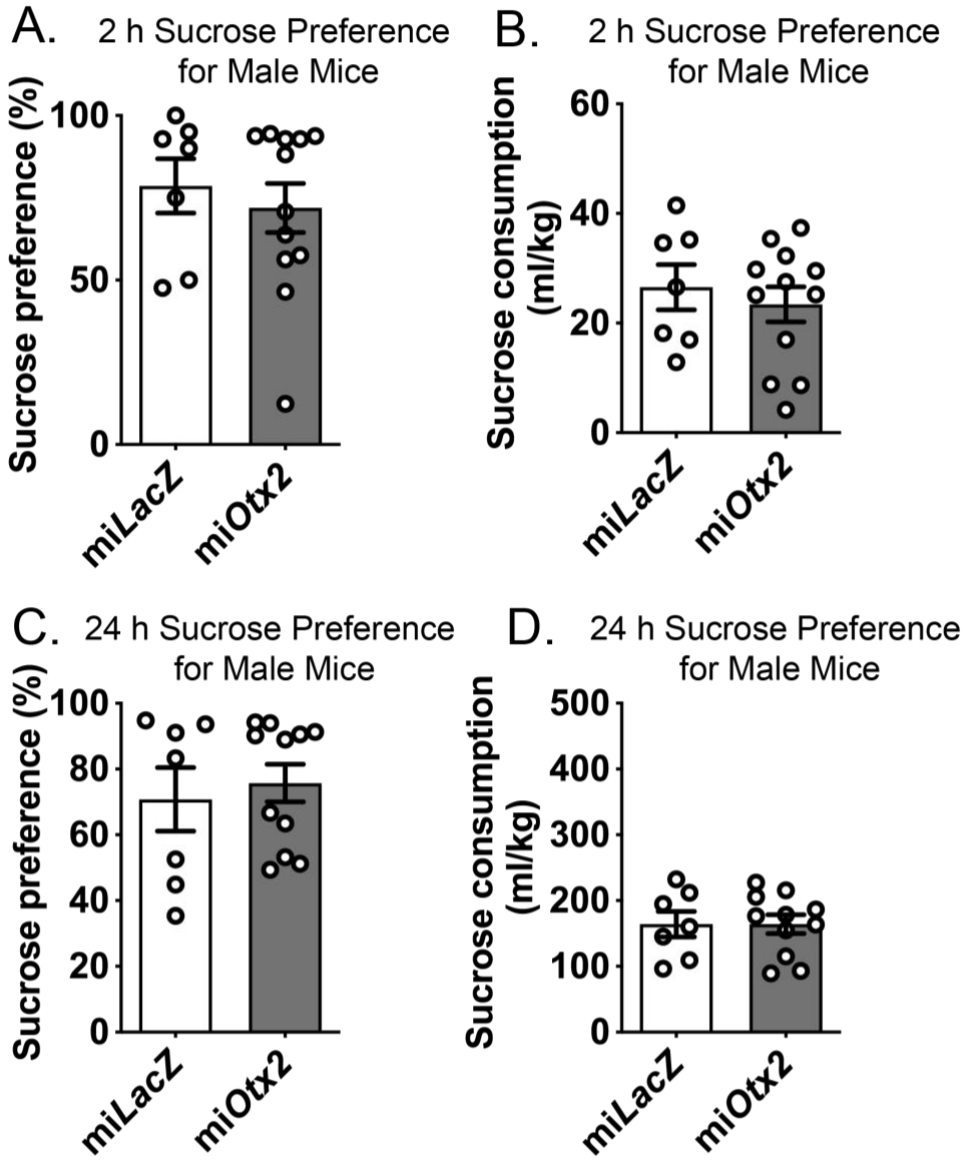


Figure 19. Transiently depleting *Otx2* in the male murine VTA from PND 15 -24 did not alter sucrose consumption during adulthood. At PND 15 male mice were injected with HSV vector expressing mi*LacZ* (white bars) or mi*Otx2* (grey bars). After the completion of the 2BC test, they were tested for intake of a 1% sucrose solution for 2 days. (A) Preference for sucrose solution over water at the 2 h timepoint. (B) Consumption of sucrose solution (in ml/kg body weight) at the 2 h timepoint. (C) Preference for sucrose solution over water at the 24 h timepoint. (D) Consumption of sucrose solution (in ml/kg body weight) at the 24 h timepoint. Data are presented as the mean ± SEM. Animal numbers for miRNA groups: miLacZ: n = 7, miOtx2: n = 11.

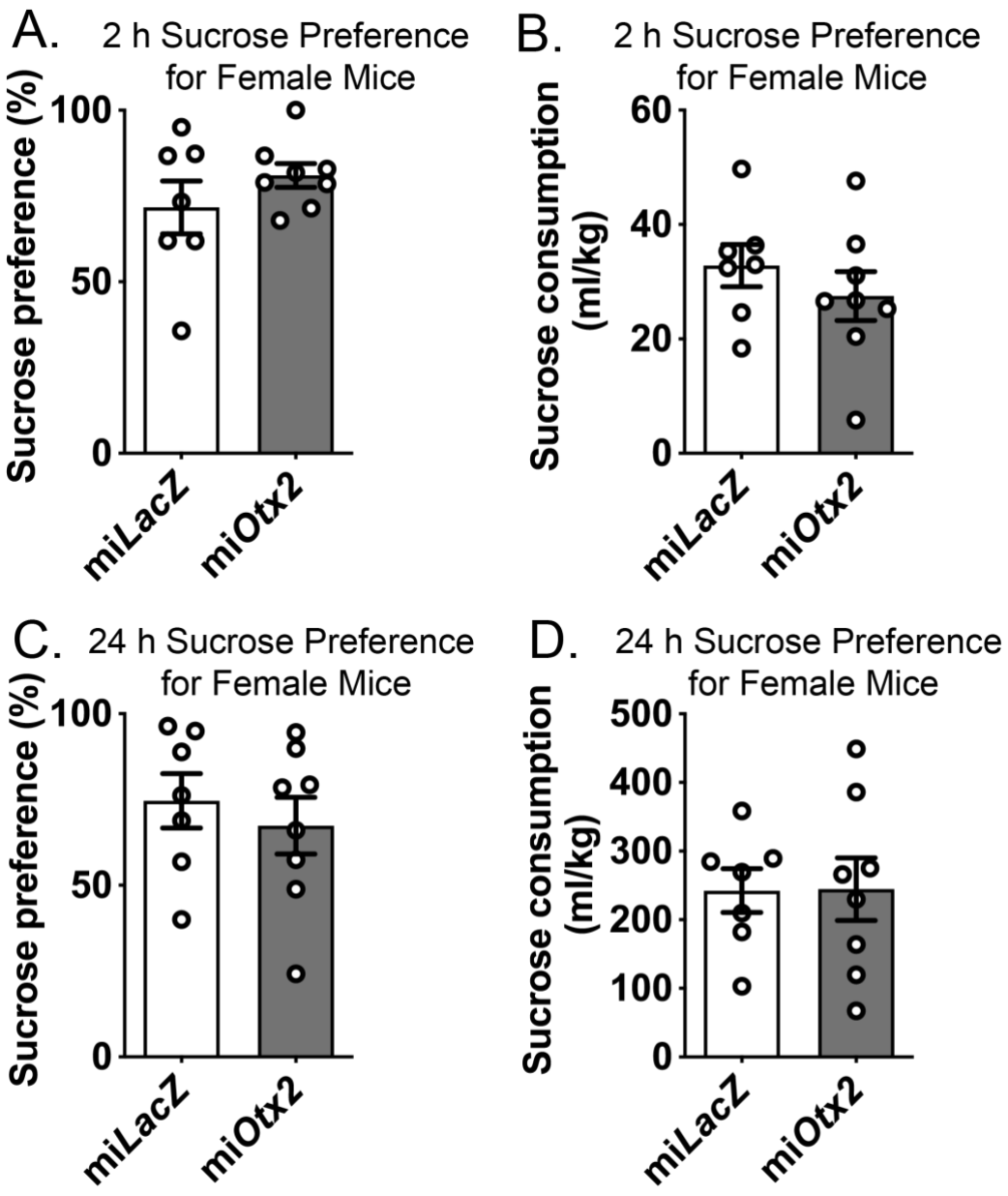


Figure 20. Transiently depleting *Otx2* in the female murine VTA from PND 15 -24 did not alter sucrose consumption during adulthood. At PND 15 the VTA of female mice was injected with HSV vector expressing mi*LacZ* (white bars) or mi*Otx2* (grey bars). After the completion of the 2BC test, they were tested for intake of a 1% sucrose solution for 2 days (A) Preference for sucrose solution over water at the 2 h timepoint. (B) Consumption of sucrose solution (in ml/kg) at the 2 h timepoint. (C) Preference for sucrose solution over water at the 24 h timepoint. (D) Consumption of sucrose solution (in ml/kg) at the 24 h timepoint. Data are presented as the mean ± SEM. Animal numbers for miRNA groups: miLacZ: n = 7, miOtx2: n = 8.

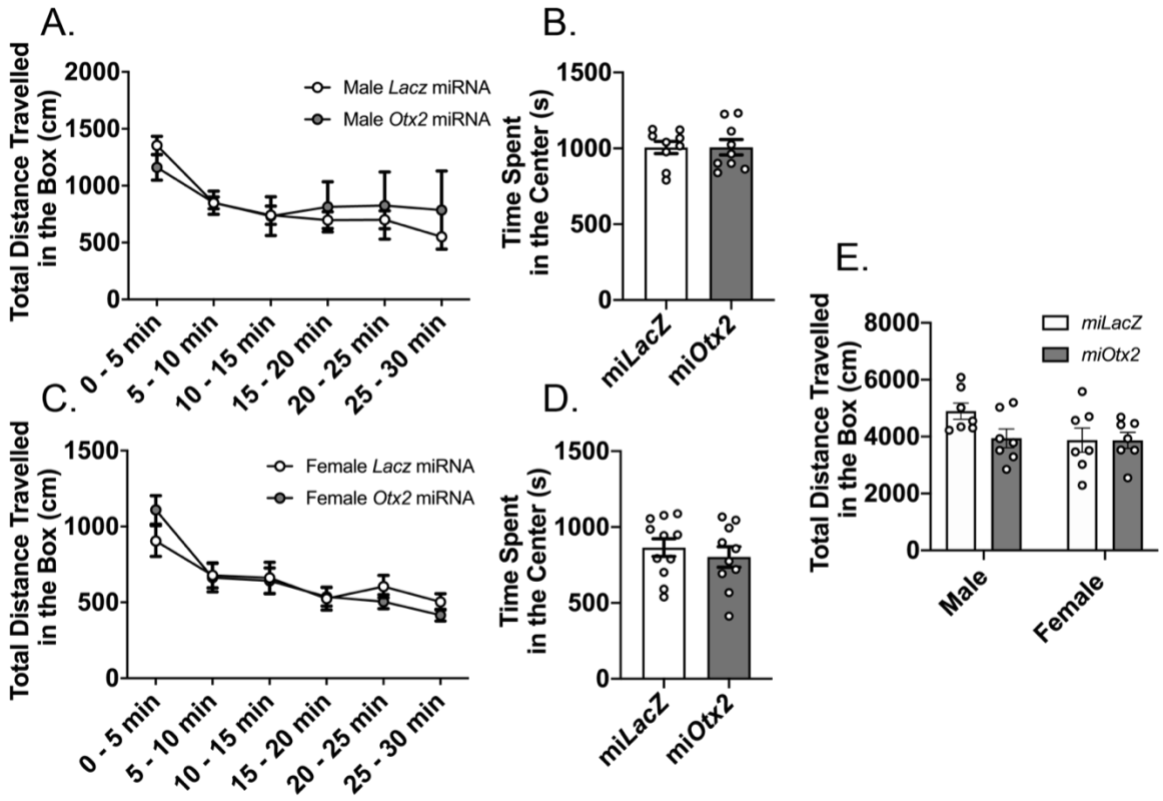


Figure 21. Transiently depleting *Otx2* in the VTA from PND 15 -24 did not alter locomotor activity or promote anxiety-like behavior after ethanol exposure. Male (A,B) and female (B,C) mice were injected in the VTA with HSV vector expressing mi*LacZ* (white bars) or mi*Otx2* (grey bars) at PND 15. Locomotor activity and anxiety-like behavior were tested via the OF test. (A and C) Five minute bins of distance travelled throughout the OF test apparatus. (B and D) Time spent in the center of the OF test apparatus.(E) Total distance travelled during OF test. Data are presented as the mean ± SEM. Animal numbers for miRNA groups: miLacZ: n = 9-11, miOtx2: n = 9-10.

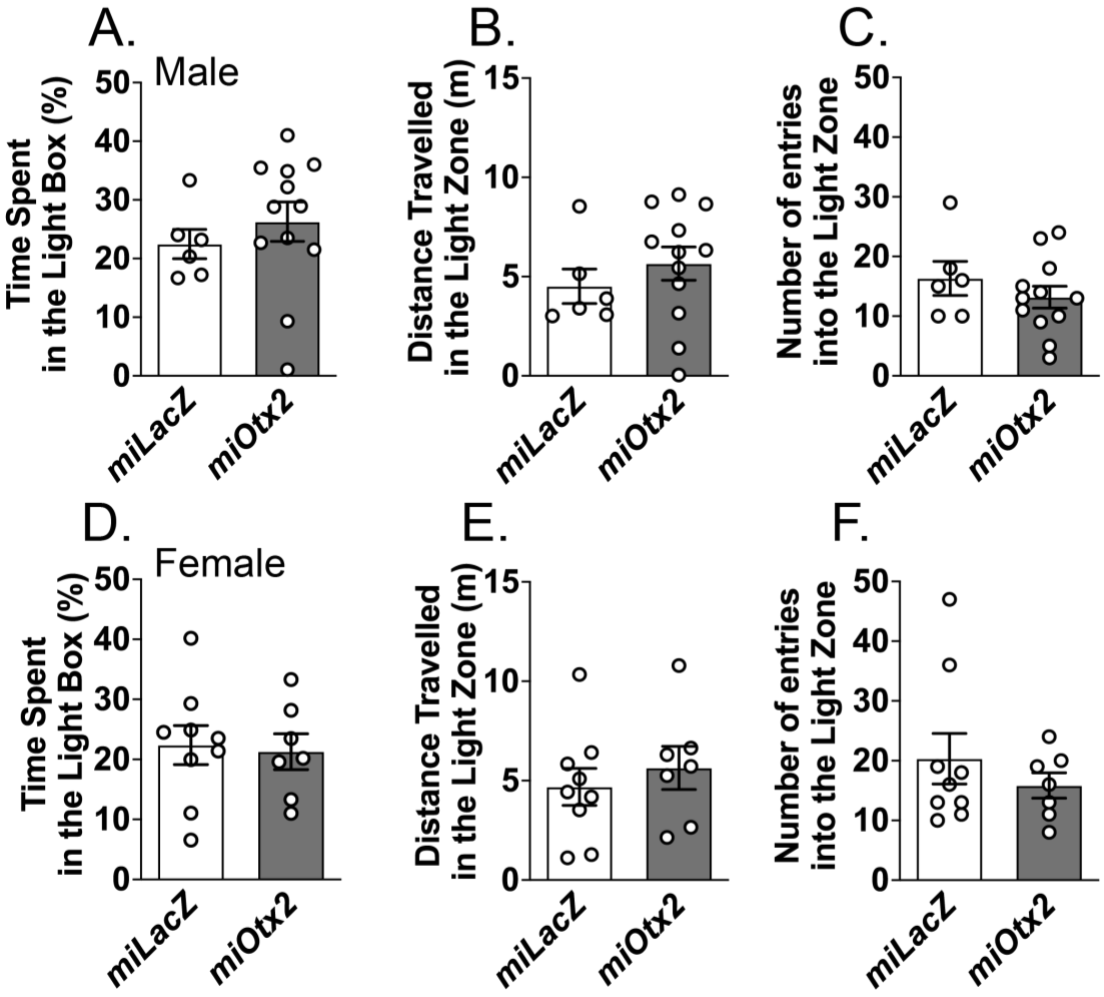


Figure 22. Transiently depleting *Otx2* in the male murine VTA from PND 15 -24 did not promote the development of anxiety-like behavior after ethanol exposure. Male (A – C) and female (D – F) mice were injected in the VTA with HSV vector expressing mi*LacZ* (white bars) or mi*Otx2* (grey bars) at PND 15. After the completion of the locomotor activity test, they were tested for changes in anxiety-like behavior. (A and D) Percentage of time spent in the light zone. (B and D) Distance travelled in the light zone. (C and F) Number of entries into the light zone. Data are presented as the mean ± SEM. Animal numbers for miRNA groups: miLacZ: n = 6-8, miOtx2: n = 7-12.

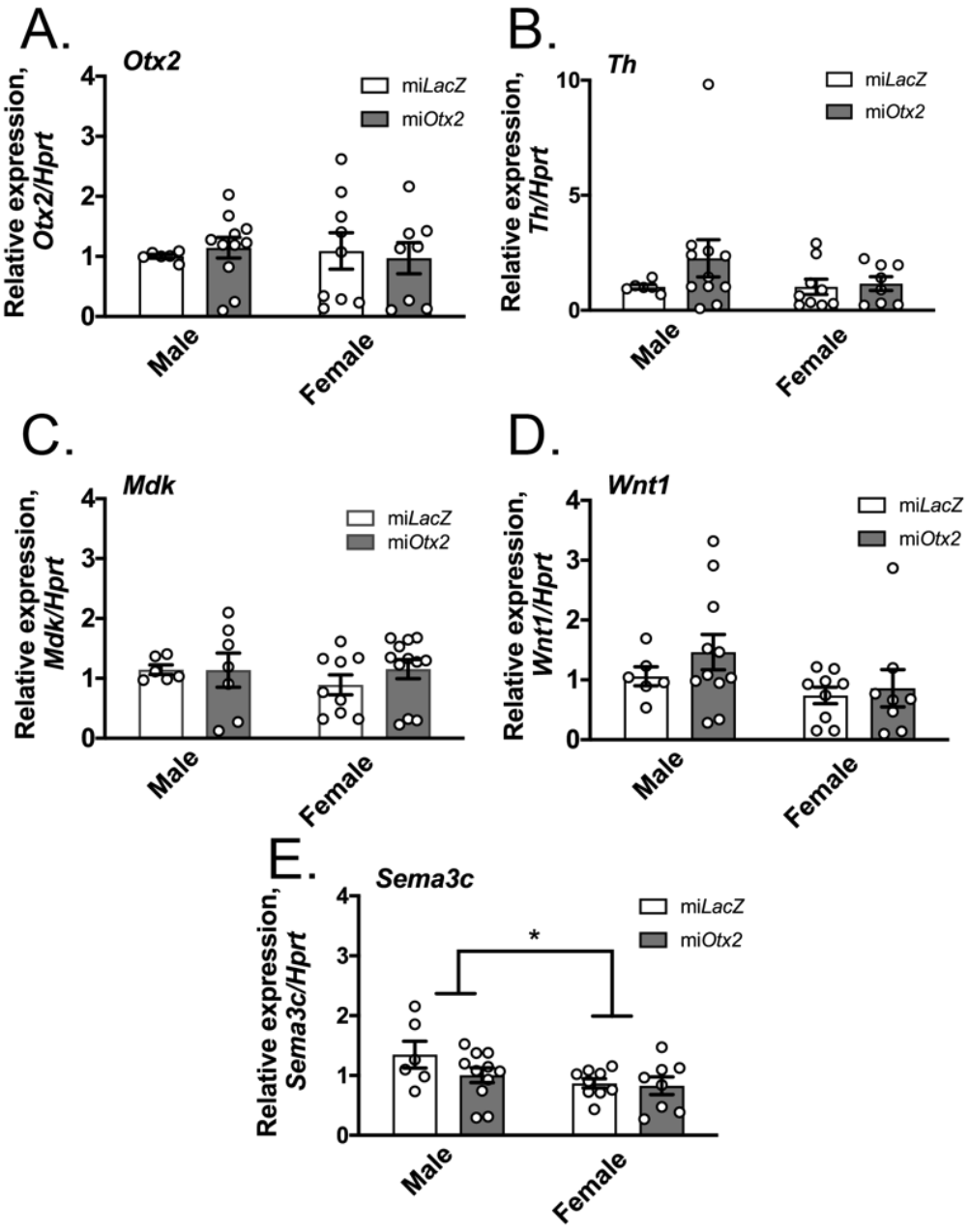


Figure 23.Transiently depleting *Otx2* in the murine VTA from PND 15 -24 did not alter the expression of *Otx2, Th, Mdk, Wnt1, or Sema3c* during adulthood. VTA samples from the mi*LacZ* (white bars) or mi*Otx2* (grey bars) groups samples were collected after behavioral tests and analyzed using qPCR to determine expression of *Otx2, Th, Mdk, Wnt1, or Sema3c* relative to *Hprt*. (A) qPCR of *Otx2* transcript relative to *Hprt*. (B) qPCR of *Th* transcript relative to *Hprt .* (C) qPCR of *Mdk* transcript relative to *Hprt.* (D) qPCR of *Wnt1* transcript relative to *Hprt .* E) qPCR of *Sema3c* transcript relative to *Hprt.* *P < 0.05 by two-way ANOVA. Data are presented as the mean ± SEM. Animal numbers for miRNA groups: miLacZ: n = 6-9, miOtx2: n = 7-12.

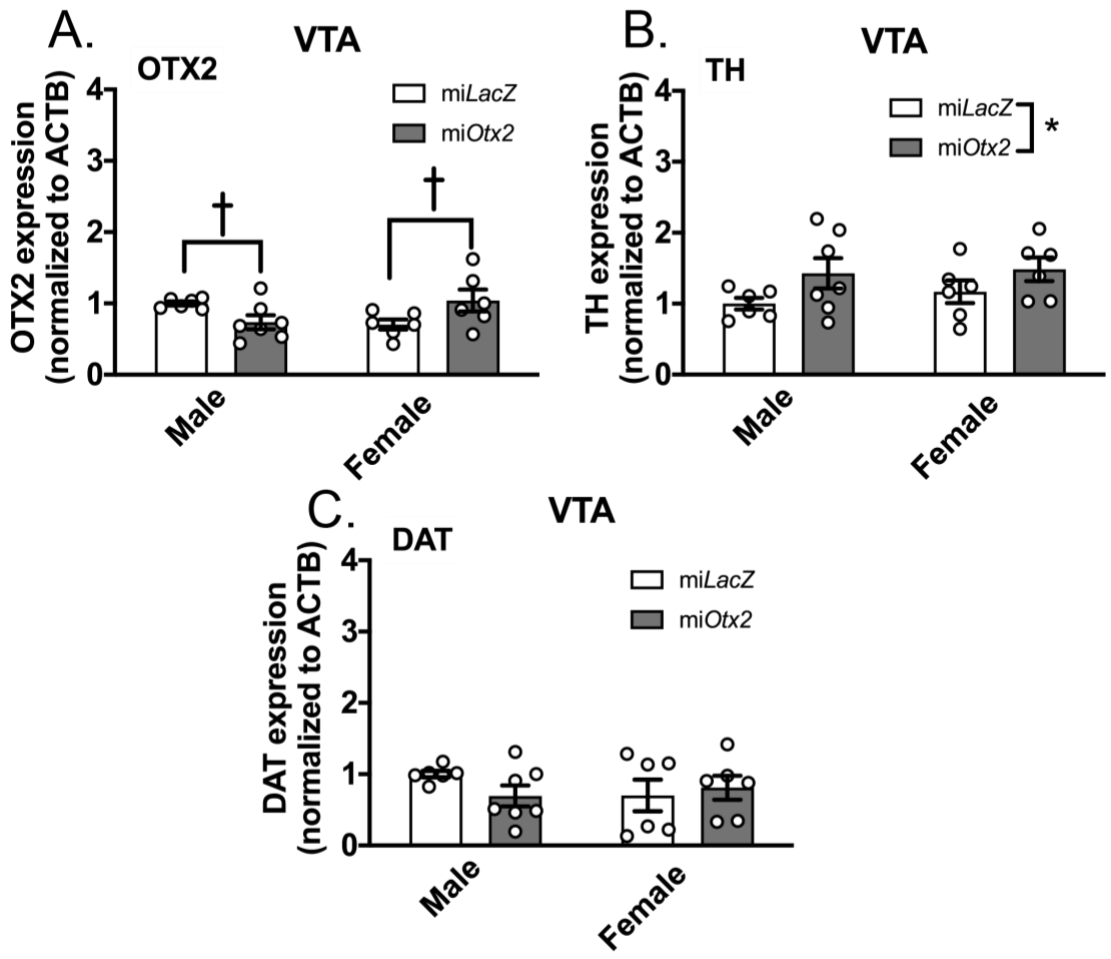


Figure 24. Transiently depleting *Otx2* in the murine VTA from PND 15 -24 promotes an increase in TH protein expression levels within the adult VTA. VTA samples from the mi*LacZ* (white bars) or mi*Otx2* (grey bars) groups samples were collected after behavioral tests and analyzed via western blotting to determine expression of OTX2, TH, and DAT relative to ACTB. (A) Protein levels of OTX2 relative to ACTB. (B) Protein levels of TH relative to ACTB. (C) Protein levels of DAT relative to ACTB. *P < 0.05 by two-way ANOVA. Data are presented as the mean ± SEM. Animal numbers for miRNA groups: miLacZ: n = 6-7, miOtx2: n = 6.

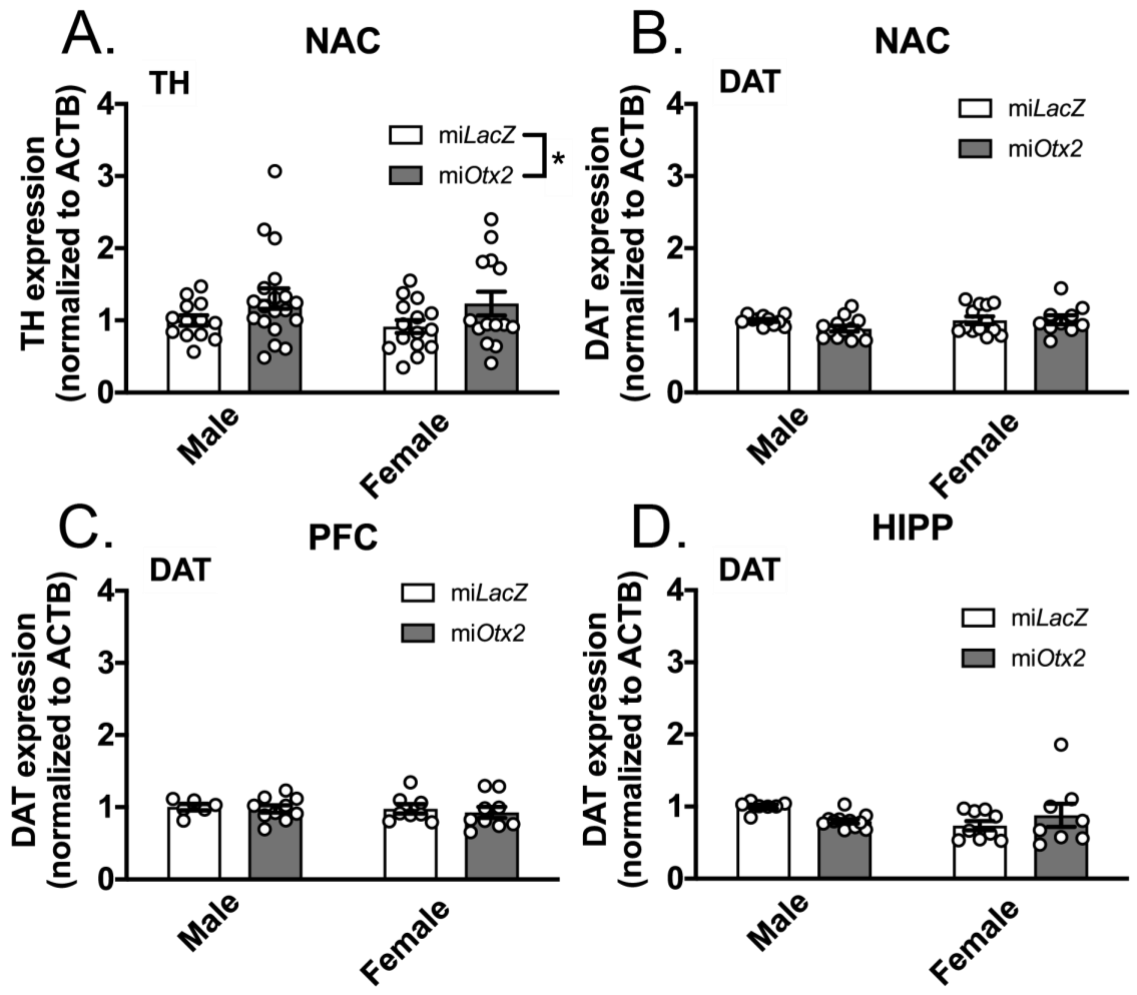


Figure 25. Transiently depleting *Otx2* in the murine VTA from PND 15 -24 increased TH in the adult NAC. NAC, PFC, and HIPP samples from the mi*LacZ* (white bars) or mi*Otx2* (grey bars) groups samples were collected after behavioral tests and analyzed via western blotting to determine expression of TH and DAT relative to ACTB. (A) Protein levels of TH relative to ACTB. B) Protein levels of DAT relative to ACTB. (C) Protein levels of DAT relative to ACTB. (D) Protein levels of DAT relative to ACTB. *P < 0.05 by two-way ANOVA. Data are presented as the mean ± SEM. Animal numbers for miRNA groups: miLacZ: n = 6-15, miOtx2: n = 8-19.

CHAPTER FIVE:

CONCLUSIONS

Individuals who suffer from AUD are unable to control their desire for and intake of ethanol regardless of the negative impacts it has on their own lives and the lives of other people. There are many genetic and environmental factors that are linked to an individual having higher risk for developing AUD. Identifying the underlying mechanisms that connect these factors to the development of AUD may help the field reveal an unique therapeutic target that will help with treating AUD. I researched the role of OTX2, which is produced and expressed in the VTA, in ethanol consumption.

There were several reasons why OTX2, within the VTA, was an attractive candidate. Studies have revealed that ethanol exposure, during the developmental period, can impact *Otx2* mRNA levels [108, 109]. In addition, OTX2 is important in the development of murine VTA DA neurons, which are important in processing the rewarding effects of ethanol and the development of AUD [88, 89, 92, 100, 103, 136]. Finally, there is a known comorbidity of depression and AUD, and OTX2 has been implicated in the development of stress-induced depression [114, 130]. The goal of my research was to determine the relationship between OTX2 in the murine VTA and ethanol consumption. I hypothesized that ethanol consumption, during adulthood, would impact VTA OTX2 expression. In addition, I hypothesized that depleting VTA OTX2 would increase ethanol consumption.

In the second chapter, I demonstrated, using qPCR and western blot methods, that binge drinking can alter *Otx2* mRNA and protein levels within the male and female

VTA. Not only that, *Mdk* and *Wnt1* mRNA levels, one potential and one known transcriptional target of OTX2, were also affected by binge drinking. This is the first study to demonstrate that binge-like drinking in adult animals can impact OTX2 and the mRNA levels of some of its transcriptional targets. In the future, the protein levels of MDK and WNT1 should be investigated after binge drinking. In addition, ChIP can be done to determine if binge drinking, during adulthood, increases OTX2 binding at the promoter region of *Mdk.* Finally, another good experiment would be to determine which transcription factor is responsible for the increase in *Wnt1* mRNA levels. Our group has demonstrated that depletion of *Mdk* within the adult male VTA leads to an increase in ethanol consumption [75]. Because it is known that reduction of OTX2 within the adult VTA induced an increase in ethanol consumption, I hypothesized that reducing OTX2 in the adult VTA would promote an increase in binge drinking. This is the reason behind my study in Chapter 3.

In the third chapter, I used a lentiviral vector to knockdown *Otx2* in the adult VTA and demonstrated that a 20% reduction in *Otx2* mRNA within the whole VTA and the average reduction in OTX2 intensity within an infected VTA neuron was 66% before and after behavioral tests, respectively. It is possible that OTX2 in the VTA of adult mice does not regulate binge drinking, or that a 20% reduction in *Otx2* was insufficient to alter binge-like ethanol consumption. One way to address these possibilities is by quantifying the average reduction in OTX2 within each cell population within the VTA. Another important experiment is to specifically deplete OTX2 from each cell population and determine how binge drinking is affected. Also, OTX2 is produced in other brain regions, during adulthood, like the medial habenula. OTX2 is important in the development of the

medial habenula, which has been implicated in the development of addiction [137-139]. Another future direction would be to knockdown OTX2 in these brain regions, during adulthood, determine how that impacts different types of drinking patterns and behaviors.

The Nestler lab has revealed that a combination of postnatal stress (which induced a transient reduction in *Otx2* mRNA levels within the male murine VTA from PND 15 -24), and social defeat during adulthood promoted the development of depression-like behavior [114]. Because of that study, I hypothesized that perhaps modulating *Otx2* in the murine VTA from PND 15 -24 could impact ethanol consumption during adulthood. This was the rationale for conducting the experiments described in Chapter 4.

In Chapter 4, I demonstrated that transiently depleting *Otx2* in the murine VTA from PND 15 -24 did not impact ethanol consumption and preference during the 2-BC test. Although the viral vectors that I used have been previously validated [114], a limitation to this study is that I did not quantify the knockdown, so it is possible that the knockdown was not enough to cause a behavioral change. One future experiment would be to inject the murine VTA from PND 15 -24 with the HSV carrying the gene for GFP and the miRNA that targets *LacZ* or *Otx2* and quantify knockdown within the different cell populations in the VTA, using immunohistochemistry within 3-4 days of injection. The other technical limitation was that I was not able to determine if all animals that were tested for behavior had the virus properly targeted to the VTA because of the transient nature of the HSV that I used. In the future it would be good to

come up with a construct that allows for a transient reduction in OTX2 while the expression of GFP is maintained up until adulthood.

It is possible that the transient depletion of OTX2 requires a second hit of stress, during adulthood, in order for there to be an impact on ethanol consumption and preference. The Nestler group demonstrated that the postnatal stress- or HSV- induced transient reduction of VTA *Otx2* on PND 21 alone did not promote the development of depression-like behaviors. It was the combination of transiently reducing *Otx2* in the VTA, around PND 21, and social defeat during adulthood that made mice more susceptible to developing depression-like behavior [114]. Based on the results I obtained in Chapter 4, I now know that transiently reducing *Otx2* in the VTA around PND 21 promotes an increase in TH protein expression levels in the VTA and NAC. It is possible that the increase in TH alone or other changes that I did not get to observe during my study could not increase ethanol preference and consumption. The second hit of stress during adulthood may alter another component of the neurons residing in the VTA or neurons that are a part of the reward circuitry. Both the increase in VTA TH levels and changes caused by social defeat, during adulthood, could ultimately cause dysregulation in communication within the reward circuitry promoting the increase in ethanol consumption and preference during adulthood. Recently, a protocol for female mice was developed in which a subset of female mice become aggressive when housed with a castrated male mouse [140]. In the future, studies can be done to see if the combination of transiently depleting VTA OTX2 and social defeat in male and female mice can increase ethanol consumption and preference. I also found that transient depletion of OTX2 in the VTA from PND 15 -24 does not impact locomotor activity or

promote the development of anxiety- or depression- like behaviors. This study is the first to show that transient depletion of VTA OTX2 from PND 15 -24 does not affect ethanol consumption or impact locomotor activity.

Finally, transient depletion of OTX2 in the murine VTA from PND 15 -24 did not impact DAT protein expression levels or *Otx2, Mdk, Th, Sema3c,* or *Wnt1* mRNA expression levels in the adult VTA. In addition, the temporary reduction in VTA *Otx2* did not impact TH or DAT protein expression levels in the HIPP or PFC. The transient reduction of VTA OTX2 did result in increased TH protein levels in the VTA and NAC. This study is the first to show the transient reduction of *Otx2* in the murine VTA from PND 15 -24 increases TH in the adult VTA regardless of sex. The results suggest that transiently modulating *Otx2* from PND 15 - 24 may have long-lasting effects on the dopaminergic system. In the future, it would be good to do immunohistochemistry to determine if there is an increase in TH protein expression levels in the VTA neurons or if there is an increase in TH+ neurons projecting from the VTA to the NAC. In addition, an experiment should be done to determine if the transient reduction of *Otx2* in the VTA from PND 15 -24 could increase VTA dopamine production and release into the NAC of adult mice.

Future experiments should also be done to determine if modulating OTX2 expression levels in other brain regions at different time points may also have an impact on ethanol consumption during adulthood. For example, many studies have modulated OTX2 in mesDA progenitors and demonstrated long-term changes in the dopaminergic system, which is important for processing reward related to ethanol consumption and implicated in the development of AUD. Therefore, future studies should be done to

investigate if modulating OTX2 in mesDA progenitors could impact ethanol consumption and preference during adolescence and adulthood. It would also be interesting to determine if modulating OTX2 in the medial habenula and interpeduncular nucleus could impact ethanol consumption. OTX2 is important for the proper development of the medial habenula and interpeduncular nucleus [137]. In addition, the medial habenula and interpeduncular nucleus have been implicated in the development of addiction [138]. Therefore, future studies should determine if depletion of OTX2, during the gestational period, within the medial habenula or interpeduncular nucleus could impact ethanol consumption, during adulthood. Another study should be done to modulate OTX2 in the adult insula. The Lasek group has demonstrated that 6 cycles of binge drinking, during adulthood, can increase the amount of perineuronal nets in the insular cortex [141]. The interaction between the chondroitin sulfates in the perineuronal nets and OTX2 is necessary for the uptake of OTX2 into targeted cells, such as parvalbumin neurons [142]. A future study should be done to determine if modulating OTX2, within the insula of adults, before and after binge drinking could impact ethanol preference and consumption. Finally, another future study can be done to determine if modulating OTX2 in the PFC of adolescent mice can impact ethanol consumption and preference during adulthood. The PFC reaches maturity during the adolescent period [143]. In addition, it is well known that OTX2 plays an important role in the development of other brain regions in the cortex [144]. Therefore, it is possible that modulating OTX2 in the adolescent PFC could impact ethanol intake and preference. Overall, future studies should focus on modulating OTX2 in different brain regions at different time-points to determine if that could impact ethanol consumption, during adolescence and adulthood.

Another future study should be done to determine how modulating OTX2 could impact signaling within the reward circuitry. For example, over-expressing OTX2 promotes a reduction in GIRK2+ neurons in the DAT+ neurons within the VTA [100]. Another study demonstrated that depletion of GIRK channels in dopaminergic neurons lowered the inhibitory effect of the autoinhibitory receptor [145]. The changes in the number of GIRK2+ and DAT+ neurons in the VTA could impact signaling within the dopaminergic neurons.

Finally, in Chapters two – four I determined how modulating OTX2 in the VTA could impact TH expression levels. TH activity level is regulated by phosphorylation and dopamine itself [126]. It would be interesting to investigate if the modulation of OTX2 could impact TH activity levels within the VTA.

In conclusion, my studies have revealed that binge drinking, during adulthood, does regulate OTX2 expression levels within the murine VTA. There are studies that have shown that TH+ neurons, within the human VTA, do express *Otx2* mRNA but do not express OTX2 protein [106, 146]. It may be worth it to re-investigate whether or not OTX2 is expressed in the human VTA and if it is linked to ethanol consumption. It may also be worth investigating the function of OTX2 in behavior and gene expression in other brain regions in mice.

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APPENDIX A:
APPROVAL OF ANIMAL PROTOCOLS

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

Office of Animal Care and Institutional
Biosafety Committee (OACIB) (M/C 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612

8/20/2020

Amy Lasek
Psychiatry
M/C 912

Dear Dr. Lasek:

The protocol indicated below was reviewed in accordance with the Animal Care Policies and Procedures of the University of Illinois at Chicago and **renewed on 8/20/2020.**

Title of Application: ALK and Midkine as Novel Neuroimmune Regulators of Alcohol Consumption
ACC NO: 19-132
Original Protocol Approval: 8/27/2019 **(3 year approval with annual continuation required).**
Current Approval Period: 8/20/2020 **to** 8/20/2021

Funding: ***Portions of this protocol are supported by the funding sources indicated in the table below.***
Number of funding sources: 1

Funding Agency	**Funding Title**			**Portion of Funding Matched**
NIH	*5/13 ALK and Midkine as Novel Neuroimmune Regulators of Alcohol Consumption*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
U01AA020912	*Funded*	*2016-05433*	*UIC*	*Amy Lasek*

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.**

Thank you for complying with the Animal Care Policies and Procedures of the UIC.

Sincerely,

Sou Soura
Associate Director, OACIB
SS/*kg*
cc: BRL, ACC File, Hu Chen

Phone (312) 996-1972 • Fax (312) 996-9088

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

August 27, 2019

Amy Lasek
Psychiatry
M/C 912

Office of Animal Care and
Institutional Biosafety Committees (MC 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

Dear Dr. Lasek:

The protocol indicated below was reviewed at a convened ACC meeting in accordance with the Animal Care Policies of the University of Illinois at Chicago on **08-20-2019**. *The protocol was not initiated until final clarifications were reviewed and approved on* **08-27-2019**. *The protocol is approved for a period of* ***3*** years with annual continuation.

Title of Application: ALK and Midkine as Novel Neuroimmune Regulators of Alcohol Consumption

ACC Number: 19-132

Initial Approval Period: 08-27-2019 to 08-20-2020

Current Funding: ***Portions of this protocol are supported by the funding sources indicated in the table below.***
Number of funding sources: 1

Funding Agency	**Funding Title**			**Portion of Proposal Matched**
NIH	*5/13 ALK and Midkine as Novel Neuroimmune Regulators of Alcohol Consumption*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
U01AA020912	*Funded*	*201605433*	*UIC*	*Amy Lasek*

This institution has Animal Welfare Assurance Number D16-00290 (A3460.01) on file with the Office of Laboratory Animal Welfare (OLAW), NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the funding proposal are matched to this ACC protocol.**

In addition, all investigators are responsible for ensuring compliance with all federal and institutional policies and regulations related to use of animals under this protocol and the funding sources listed on this protocol. Please use OLAW's "*What Investigators Need to Know about the Use of Animals*" (http://grants.nih.gov/grants/olaw/InvestigatorsNeed2Know.pdf) as a reference guide. Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,

Mary B. Bowman, PhD
Director, Animal Care Committee
MBB/ss
cc: BRL, ACC File, Hu Chen

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

September 27, 2019

Amy Lasek
Psychiatry
M/C 912

Office of Animal Care and Institutional
Biosafety Committee (M/C 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612

Dear Dr. Lasek:

The modifications requested in modification indicated below pertaining to your approved protocol indicated below have been reviewed and approved in accordance with the Animal Care Policies of the University of Illinois at Chicago on ***'Modification Approval Date'***. *The modification was not initiated until final clarifications were reviewed and approved on 'Final Approval Date'.*

Title of Application: ALK and Midkine as Novel Neuroimmune Regulators of Alcohol Consumption

ACC Number: 2019132

Modification Number: *'Modification Number'*

Nature of Modification:
1) Request for additional 102 C57 mice for the following:
- **a. Knockdown OTX2 in juvenile mouse VTA and test ethanol drinking in adulthood (females only). 12 mice per group x 2 groups (HSV LacZ-control and HSV-OTX2 shRNA) =24 mice. Subtract 10 mice that were done previously =14 mice (part was done under ACC protocol 16-144)**
- **b. Knockdown OTX2 in adult mice and test ethanol consumption (males and females). 12 mice per group x 2 groups (HSV LacZ-control and HSV-OTX2 shRNA) x 2 sexes = 48 mice.**
- **c. Addition of breeding for C57 mice**

2) Request a change for stereotaxic surgical procedure, younger animals postnatal Day 15-17 mice will be used to infuse HSV vectors as was done under previous protocol)
3) Addition of behavior test for anxiety: light-dark box test

Protocol Approved: *8/27/2019*

Current Approval Period: *8/27/2019* to ***8/20/2020. Protocol is eligible for 2 additional years of renewal prior to expiration and resubmission.***

Current Funding: *Portions of this protocol are supported by the funding sources indicated in the table below.*
Number of funding sources: 1

Funding Agency	**Funding Title**			**Portion of Funding Matched**
NIH	*5/13 ALK and Midkine as Novel Neuroimmune Regulators of Alcohol Consumption*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
U01AA020912	*Funded*	*201605433*	*UIC*	*Amy Lasek*

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PUBLICATIONS: **Coles C,** Shentu TP, Sheng Y, Fancher IS, Ng C, Christoforidis T, Subbaiah PV, Berdyshev E, Qain Z, Eddington DT, Lee J, Cho M, Fang Y, Minshall RD, Levitan I. Proatherogenic Flow Increases Endothelial Stiffness via Enhanced CD36-Mediated Uptake of Oxidized Low-Density Lipoproteins. Arterioscler Thromb Vasc Biol. 2018 Jan;38(1):64-75. doi: 10.1161/ATVBAHA.117.309907. Epub 2017 Oct 12. PMID: 29025707; PMCID: PMC5746473.

Vandegrift BJ, Hilderbrand ER, Satta R, Tai R, He D, You C, Chen H, Xu P, **Coles C**, Brodie MS, Lasek AW. Estrogen Receptor α Regulates Ethanol Excitation of Ventral Tegmental Area Neurons and Binge Drinking in Female Mice. J Neurosci. 2020 Jul 1;40(27):5196-5207. doi: 10.1523/JNEUROSCI.2364-19.2020. Epub 2020 Jun 1. PMID: 32482639; PMCID: PMC7329299.

ABSTRACTS:

Oral Presentations

Local/Regional Meetings

Cassandre Coles BS, and Amy Lasek, PhD, Binge Drinking Alters the Expression of the Orthodenticle Homeobox 2 (OTX2) Transcription Factor in the Ventral Tegmental Area, University of Illinois at Chicago, Annual Anatomy and Cell Biology Student Forum, Chicago, IL, 2020*

Cassandre Coles BS, Zhenlong Chen, PhD, Elizabeth LeMaster BS, Suellen Oliveira, PhD, Irena Levitan, PhD, and Richard D. Minshall PhD, Mechanistic insight into OxLDL and disturbed flow-induced endothelial hyperpermeability associated with atherogenesis, Goethe-Institut, Eta Science Talks, Chicago, IL, 2016

Cassandre Coles BS, Zhenlong Chen, PhD, Elizabeth LeMaster BS, Suellen Oliveira, PhD, Irena Levitan, PhD, and Richard D. Minshall PhD, Transcellular Transport vs Paracellular Leakage of LDL and OxLDL in Laminar vs Disturbed Flow-conditioned Human ECs, University of Illinois at Chicago, Annual Pharmacology Student Forum, Chicago, IL, 2016

∗Also presented at the Psychiatric Institute & Center for Alcohol Research in Epigenetics Neuroscience Seminar, Chicago, IL, 2019 and 2020

Poster Presentations

National Meetings

Cassandre Coles BS, and Amy Lasek, PhD, Transient Depletion of Otx2 in the juvenile VTA Alters Adult Locomotor Activity and Expression of Dopaminergic Neuronal Markers but not Alcohol Consumption, 43rd Annual Research Society on Alcoholism Scientific Meeting, New Orleans, LA, 2020

Cassandre Coles BS, and Amy Lasek, PhD, Binge Drinking Alters the Expression of the Orthodenticle Homeobox 2 (OTX2) Transcription Factor in the Ventral Tegmental Area, Early Career Investigator Poster Session on Alcohol and Drug Use Disorders 127th Annual Convention of the American Psychological Association, Chicago, IL, 2019 ∗ϕ

∗ Also presented at the 49th Annual Society for Neuroscience Meeting, Chicago, IL, 2019

ϕ Also presented at the 42nd Annual Research Society on Alcoholism Scientific Meeting, Minneapolis, MN, 2019

Local/Regional Meetings

Cassandre Coles BS, and Amy Lasek, PhD, Binge Drinking Alters the Expression of the Orthodenticle Homeobox 2 (OTX2) Transcription Factor in the Ventral Tegmental Area, Chicago Chapter of the Society for Neuroscience meeting, Chicago, IL, 2019 ∗ϕ†

Cassandre Coles BS, and Amy Lasek, PhD, Binge Drinking Alters Orthodenticle Homeobox 2 (OTX2) Gene and Protein Expression in the Ventral Tegmental Area, University of Illinois at Chicago, 2019 College of Medicine Research Forum, Chicago, IL, 2019

∗ Also presented at the University of Illinois at Chicago, Psychiatry's 9th and 10th Annual Research Forum, Chicago, IL, 2019

ϕ Also presented at the University of Illinois at Chicago, 4th Annual Center for Alcohol Research in Epigenetics retreat, Chicago, IL, 2019

† Also presented at the Northwestern University, 22nd Annual Black Graduate Student Association Conference, Chicago, IL, 2019

Cassandre Coles BS, Zhenlong Chen, PhD, Elizabeth LeMaster BS, Suellen Oliveira, PhD, Irena Levitan, PhD, and Richard D. Minshall PhD, Mechanistic insight into OxLDL and disturbed flow-induced endothelial hyperpermeability associated with atherogenesis, Starved Rock Lodge, Bridge to the Doctorate Retreat, Chicago, IL, 2016 ∗ϕ

* Also presented at the University of Illinois at Chicago, SFRBM Redox Symposium, Chicago, IL, 2016

ϕ Also presented at the Northwestern University, Society for the Advancement of Chicanos/Hispanics in the Sciences Symposium, Chicago, IL, 2016

Cassandre Coles BS, and Richard D. Minshall, PhD. Role of Caveolin-1 in β-Catenin-dependent Stabilization of Endothelial Cell Adherens Junctions. Abstract for Poster Presentation, University of Illinois at Chicago, College of Medicine Research Day, Chicago, IL, 2015

Cassandre Coles BS, and Richard D. Minshall, PhD. VEGF Signaling Dynamics in Endothelial Cells: Potential Mechanisms Regulating Adherens Junction Integrity during Sprouting Angiogenesis. Abstract for Poster Presentation, Bridge to the Doctorate Retreat, Starved Rock Lodge, Chicago, IL. 2015

Cassandre Coles BS, Peggy Wheeler MD, Gina Votta-Velis MD, PhD, and Richard D. Minshall, PhD. Inhibition of Morphine-induced Angiogenesis by Amide-linked Local Anesthetics. Abstract for Poster Presentation, University of Illinois at Chicago, College of Medicine Research Day, Chicago, IL, 2014