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Education

2009-2013 Trinity University, San Antonio, TX
B.S. in Neuroscience, minor in Chemistry
Advisor: Dr. Michele A. Johnson

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Professional Experience

2014 -2017	Research Assistant, Department of Pharmacology, UT Health Science Center San Antonio
2013-2014	Research Assistant, Department of Biology, Trinity University

Publications

Patton MS, Paige N. McKeon, Jonathan W. VanRyzin, Mary H. Patton, Heckman M, Brian N. Mathur (*in prep*): Chronic ethanol exposure remodels perineuronal nets and inhibitory synapses on striatal fast-spiking interneurons.

Patton MS, Sheats S, Brian N. Mathur (*in prep)*: Alcohol potentiates multiple GABAergic inputs to dorsal striatum fast-spiking interneurons.

Georgiou P, Mou TM, Potter LE… **Patton MS**, et al. (*in review*): Estradiol mediates stress-susceptibility in the male brain.

Patton MS, Mathur BN (2022)*:* Striatal Circuits. Book chapter in Neurocircuitry of Addiction. *Elsevier*

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Patton MH, Padgett KE, McKeon PN, Qadir H, **Patton MS**, Mu C, Roberts BM, Mathur BN (2019): TrkB-dependent disinhibition of the nucleus accumbens is enhanced by ethanol. *Neuropsychopharmacology* 44:1114-1122.

Patton MS, Lodge DJ, Morilak DA, Girotti M (2016): Ketamine corrects stress-induced cognitive dysfunction through JAK2/STAT3 signaling in the orbitofrontal cortex. *Neuropsychopharmacology* 42: 1220-1230.

Donnegan, JJ, **Patton MS**, Chavera TA, Berg KA, Morilak DA, Girotti M (2015): Interleukin-6 attenuates serotonin 2A receptor signaling by activating the JAK-STAT pathway. *Molecular Pharmacology.* 87: 492-500.

Robinson CD, **Patton MS**, Andre BM, Johnson MA (2015): Convergent evolution of brain morphology and communication modalities in lizards. *Current Zoology.* 61: 281-291.

Lectures and Presentations

Patton MS, McKeon PN, VanRyzin JW, Heckman M, Mathur BN (2022): Chronic ethanol exposure remodels perineuronal neuronal nets and inhibitory synapses on striatal fast-spiking interneurons. Alcohol and the Nervous System Gordon Conference, Ventura, CA (poster)*

Patton MS, Heckman M, Kim C, Mu C, Mathur BN (2020): Compulsive alcohol consumption is regulated by striatal fast-spiking interneurons. Alcohol and the Nervous System Gordon Conference, Galveston, TX (poster)*

Patton MS, Kim C, Mu C, Mathur BN (2019): Compulsive alcohol consumption is regulated by striatal fast-spiking interneurons. Greater Baltimore Chapter of the Society for Neuroscience (poster).

Patton MS, Girotti M, Morilak DA (2015): Involvement of the JAK/STAT3 Signaling Pathway in Ketamine-induced Synaptic Plasticity. Center for Biomedical Neuroscience, UTHSCSA (poster).

Patton MS, Girotti, M, Morilak DA (2015): Involvement of the JAK/STAT3 Signaling Pathway in Ketamine-induced Synaptic Plasticity. Learning and Memory Conference, UT Austin, TX (poster).

Patton MS, Girotti M, Morilak DA (2015): Involvement of the JAK/STAT3 Signaling Pathway in Ketamine-induced Synaptic Plasticity. Society for Neuroscience, Chicago (poster).

Girotti M, **Patton MS**, Morilak DA (2015): Ketamine ameliorates reversal-learning deficits induced by chronic cold stress via the JAK/STAT3 signaling pathway. Society for Neuroscience, Chicago (poster).

Stercula JM, **Patton MS**, Selznick LA, Johnson MA (2015): The role of myoblast fusion in the evolution of muscle fiber size in *Anolis* lizards. Society for Integrative and Comparative Biology, San Francisco (poster).

Patton, MS, Girotti M, Morilak DA (2014): Ketamine corrects reversal learning deficits induced by chronic cold stress – possible involvement of STAT3. UTHSCSA Graduate Student Symposium, San Antonio (poster).

Girotti M, **Patton MS**, Morilak DA (2014) Ketamine corrects reversal learning deficits induced by chronic cold stress – possible involvement of STAT3. Society for Neuroscience, Washington D.C. (poster).

Donegan J, Girotti M, Chavera T, **Patton MS**, Berg KA, Morilak DA (2014) Interleukin 6 attenuates serotonin 2A receptor signaling through the JAK-STAT pathway. Society for Neuroscience, Washington D.C. (poster).

Guest lectured for Introductory Chemistry at Trinity University (2013).

Patton MS, Megahed T, Johnson MA (2013): Lateralization of aggressive behavior and brain morphology in the green anole lizard (*Anolis carolinensis*). Brain, Behavior, and Evolution Symposium, University of Texas, Austin. (poster).

Patton MS, Megahed T, Johnson MA (2013): Lateralization of aggressive behavior and brain morphology in the green anole lizard (*Anolis carolinensis*). Meeting of the Society for Integrative and Comparative Biology, San Francisco. (poster) *Travel supported by Charlotte Mangum Student Support Award.*

Patton MS, Megahed T**,** Johnson MA (2012): Lateralization of aggressive behavior and brain morphology in the green anole lizard (*Anolis carolinensis*). Meeting of the Texas Herpetological Society, McKinney Tx. (poster).

Grants Received

2021	Ruth L. Kirschtein National Research Service Award (F31)
2012	Howard Hughes Medical Institute Summer Undergraduate Research Fellowship

Honors and Awards

2022	Poster winner Alcohol and the Nervous System Gordon Research Conference
2022	Outstanding Program in Neuroscience Student Researcher Award
2022	Research Society on Alcoholism Student Merit Award
2020	Poster winner Alcohol and the Nervous System Gordon Research Conference
2013	Outstanding senior Neuroscience student from Trinity University
2013	Charlotte Mangum Student Support: Society for Integrative and Comparative Biology

2011 Southern Collegiate Athletic Conference honor roll: intercollegiate varsity swimming

Other

2021 *ASCEND* undergraduate mentoring program with Morgan State University
2013 President of Trinity University Neuroscience Club
2013 Founder of Neuroscience Journal Club: weekly undergraduate journal club
2013 Mind Science Foundation volunteer
2013 Participant in Brain Bowl Competition: funded by UTHSCSA Graduate Program in Neuroscience and Center for Biomedical Neuroscience
2013 Behavior, Ecology, and Evolution weekly journal club
2012 Bamberger Ranch guide: middle school science mentoring program funded by Howard Hughes Medical Institute (HHMI)
2012 Officer for Trinity University Neuroscience Club

Compulsive Alcohol Consumption is Regulated by Dorsal Striatum Fast-spiking Interneurons

by
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University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
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Abbreviations List

aCSF	Artificial cerebral spinal fluid
AUD	Alcohol use disorder
BEC	Blood ethanol concentration
BC	Before Christ
BLA	Basolateral amygdala
BP	Before present
CB1	Cannabinoid type 1 receptor
CeA	Central amygdala
ChR2	Channelrhodopsin
CIE	Chronic intermittent exposure
CM	Center median nucleus of the thalamus
CPP	Conditioned place preference
CRF	corticotropin-releasing factor
DID	Drinking in the dark
DLS	Dorsolateral striatum
DMS	Dorsomedial striatum
DREADDS	Designer receptors exclusively activated by designer drugs
EAAT-2	Excitatory amino acid transporter 2
EPSC	Excitatory postsynaptic current
EtOH	Ethanol
eYFP	enhanced yellow fluorescent protein
fMRI	Functional magnetic resonance imaging

FSI	Fast-spiking interneuron
GAT-3	GABA transporter 3
GIRK	G protein-coupled inwardly-rectifying potassium channel
GP	Globus pallidus
HCN	hyperpolarization-activated cyclic nucleotide gated channel
IEI	Inter event interval
IHC	Immunohistochemistry
IPSC	Inhibitory postsynaptic current
LTD	Long-term depression
LTP	Long-term potentiation
mPFC	Medial prefrontal cortex
MSN	Medium spiny neuron
NAc	Nucleus accumbens
NSDUH	National Survey on Drug Use and Health
OFC	Orbitofrontal cortex
Pf	Parafascicular nucleus of the thalamus
PFC	Prefrontal cortex
PIT	Pavlovian instrumental transfer
PNN	Perineuronal net
PPR	Paired pulse ratio
PV	Parvalbumin
PVN	Paraventricular nucleus of the thalamus
rILN	Rostral intralaminar nucleus of the thalamus

RTN	Reticular thalamic nucleus
RuBi-GABA	ruthenium-bipyridine-triphenylphosphine caged GABA
sIPSC	Spontaneous inhibitory postsynaptic current
SNr	substantia nigra pars reticulata
TH	tyrosine hydroxylase
TrkB	Tropomyosin receptor kinase B
U.S.A	United States of America
VC	Vapor chamber
VTA	Ventral tegmental area

Chapter 1: Introduction

1.1 Historical Alcohol Use and Misuse

Society has a longstanding history of alcohol use, with the earliest documented evidence of an alcoholic beverage dating back to 7,000-6,000 BC in northern China (McGovern et al. 2004). However, earlier paintings and archeological artifacts suggest that alcohol was used even as far back as the genesis of early civilization (Braidwood et al., 1953; Slingerland 2021), where modern theorists suggest that human interest in alcohol initiated group settlement into agricultural societies. This theory, termed the "beer before bread" hypothesis, proposes that societies initially settled on fertile land to domesticate cereal grains for alcohol consumption (Braidwood et al., 1953). Support for this hypothesis comes from a recent archeological discovery at a burial site in Israel, where scientists discovered the earliest documented evidence of beer brewing dating back to 13,700-11,700 BP (Liu et al 2018). These neolithic artifacts are attributed to the Natufian culture who were semi-sedentary hunter-gatherers that "paved the way to the establishment of the first sedentary Neolithic villages" (Liu et al., 2018).

From its inception in these early civilizations, alcohol consumption quickly spread across the globe as civilizations advanced and established expansive trade networks. Documents from the ancient Sumerians in 3,000 BC to the Greco-Roman era around 2,000 BC indicate that alcoholic beverages were commonplace in ancient society, being used during religious ceremonies and festivals (Thomas 2022). Beyond its celebratory use, ancient Greek texts reference alcohol as a treatment for several ailments, including lethargy, diarrhea, childbirth pains, and keeping wounds clean (Murcia 2017). From these early documents, it is evident that the discovery of alcohol had a profoundly positive impact on society, where it not only elevated the moods of the labor force, but also exhibited life-saving medical uses.

As interest in alcohol spread throughout the globe, signs of alcohol misuse began to appear and concerns about public safety provoked laws restricting widespread alcohol consumption. In ancient Rome, celebrants of Bacchus, the god of agriculture, wine, and fertility, held Bacchanalian parties where guests drank excessively while celebrating the emotional freedom of their god. However, by 186 BC the Roman senate banned the performance of these Bacchanalian rituals believing they posed a threat to public safety (Murcia 2017).

Much later in 1600 England, intoxication became widespread during the reign of James I and, shortly after, Parliament passed the Act to Repress the Odious and Loathsome Sin of Drunkenness. As the British empire expanded, they brought along alcohol to the new colonies, including the present-day U.S.A., believing drinking water to be contaminated and unsafe. However, European settlers were not the first to introduce alcohol to the Americas. In fact, evidence of alcohol use and production dates back to 300 C.E. from the Aztec empire in modern day Mexico (Wadell & Everitt 1980). Once established, alcohol use spread up to southern Arizona and New Mexico where it remained primarily confined to native tribes residing in the American Southwest (Wadell & Everitt 1980).

In the American Northeast, most documented indigenous use of alcohol coincides with European settlers in the 16^{th} and 17^{th} century (Abbot 1987), where in the 1630s, new world colonists began brewing their own beer using supplies shipped from England (Thomas 2022). As consumption spread in America, reaching an average of 5.8 gallons of alcohol per person each year by 1790 (O'Brian 2015), the Temperance Movement was formed to combat the surge in alcohol consumption and the harmful effects of excessive drinking. Later, in 1919, prohibition was enacted in the U.S. through the 18th amendment and stayed into effect until 1933 when it was recognized that legalizing alcohol use would boost the dwindling economy of the great depression and deter crime.

1.2 Modern Alcohol Use and Misuse

Today alcohol is one of the most widely used substances in the world, where in the U.S. alone, approximately 69% of adults (ages 18>) reported alcohol consumption in a 2019 National Survey on Drug Use and Health (NSDUH). Globally, the average rate of alcohol consumption varies widely across countries due to cultural and societal factors, including race, gender, socioeconomic status, and perhaps even climate. According to a recent study, countries with warmer climates exhibit lower alcohol abuse, whereas countries with colder climates with less sunlight exhibit more binge drinking behavior (Ventura-Cots et al., 2019). Eastern Europe, for example, exhibits the highest annual per capita alcohol consumption of 15.7 L per person (Popova et al., 2007), whereas North Africa and the Middle East exhibit the lowest per capita consumption of 1.0 L per person (Ventura-Cots et al., 2019). However, the authors note that cultural and religious factors are undoubtedly contributing to this effect.

One major cultural factor that influences consumption rates is how and when alcohol is introduced to adolescents, since adolescent alcohol exposure serves as a strong predictor for future alcohol consumption (Brown & Tapert 2004). In Western Europe alcohol consumption is generally introduced at an early age at family gatherings where alcohol, often wine, is diluted and served with dinner to moderate and teach responsible consumption. By contrast, in the U.S. and Eastern Europe, adolescents are generally exposed to higher alcohol content spirits like vodka that are consumed in less socially controlled settings.

The results from the National Longitudinal Alcohol Epidemiologic Survey indicate that adolescents who abuse alcohol before the age of 15 are four times more likely to develop alcohol dependence than those who begin drinking at age 21 (Grant & Dawsone 1997). In response to this staggering statistic, countries including the U.S. developed policies to regulate adolescent consumption in an attempt to mitigate this negative trajectory. However, the age at which countries permit adolescent consumption varies across countries and serves as a highly debated topic for public policy. Advocates for a higher minimum legal drinking age (e.g., 21 years) argue that restricting alcohol consumption during adolescence reduces alcohol related accidents and the risk of developing alcohol use disorder. However, opponents point to the lower rates of binge drinking and alcohol misuse in European countries with a lower minimum legal drinking age (Wechler & Nelson 2010).

In the U.S., the minimum legal drinking age was federally raised to 21 years by President Ronald Reagan in 1984 through the National Minimum Drinking Age Act to address concerns about adolescent drunk driving. However support of this federal law has since been divided, and proponents for policy change continue to voice their opinions. Starting in 2008, presidents from 136 colleges and universities across the U.S. signed the Amethyst Initiative advocating for a lower minimum legal drinking age, arguing that raising the drinking age to 21 has promoted a culture of unhealthy binge drinking on campuses resulting in higher rates of alcohol-related deaths. While the debate for legal minimum drinking age still continues in the U.S., and likely will for the foreseeable future, it is important to consider how cultural approaches to drinking influence adolescent alcohol consumption as this poses a significant risk factor for future alcohol misuse (Grant & Dawson 1997; Brown & Taper 2004).

How a country regulates media portrayal of alcohol use, through advertisements, movies, and social media, significantly shapes a nation's cultural attitude toward drinking and consequently influences consumption rates (Sudhinaraset et al., 2016). Notably, media portrayal of alcohol consumption predominantly impacts adolescent and minority communities (Moore et al., 2008; Grenard et al., 2013), where adolescents exposed to alcohol advertisements drink more than peers exposed to less alcohol marketing (Snyder et al., 2006).

Beyond culture, societal factors, including socioeconomic status, strongly predict an individual's the level of alcohol consumption. People with higher socioeconomic status tend to drink more frequently than individuals with low socioeconomic status, but lower status groups drink larger quantities of alcohol per drinking session (Huckle et al., 2010). Interestingly, this drinking trend extends out to the financial status of the country, where developed nations drink more than developing ones (Rehm et al., 2009). In addition to these financial factors, sex plays a considerable role in alcohol consumption, where across the globe men drink more than women. This topic will be covered in more detail later in this introduction, including how sex hormones influence of alcohol consumption (see **1.11**).

While the level of alcohol consumption may vary across the globe, alcohol abuse is universally evident and its detrimental impact on the human body generates serious health consequences. Individuals who abuse alcohol are at greater risk of liver disease, heart disease, stomach bleeding, and intestinal cancer (Grewal et al., 2012; Bagnardi et al., 2015). Being the primary site of ethanol metabolism, the liver bears the greatest level of damage, where during the early stages of alcohol-induced liver damage (i.e., steatosis) fat begins to deposit in hepatocytes disrupting liver function. With continued alcohol-related damage, liver damage can progress to cirrhosis characterized by liver scarring, vascular alterations, and eventually liver failure (Osna et al., 2017). According to the 2019 NSDUH survey, 1 out of 3 liver transplants in the U.S. were due to alcohol-related liver disease. Additionally, 45% of male and 39% of female liver disease deaths involved alcohol use (NSDUH).

The effect of alcohol exposure on the cardiovascular system is somewhat complex and depends on the dose of alcohol consumption and the duration use (Piano 2017). However, chronic alcohol abuse increases the risk of developing hypertension, coronary heart disease, stroke, and peripheral arterial disease (Piano 2017). The resulting healthcare cost of alcohol misuse in the U.S. alone reached to 249 billion dollars in 2010, and the financial loss from diminished workplace productivity resulted in an additional 28-billion-dollar deficit (Sacks et al., 2015). With the rates of alcohol abuse increasing in response to the COVID-19 pandemic, these numbers are projected to increase substantially over the next decade (Grossman et al., 2020).

Unfortunately, of the 69% of adults (ages 18>) who reported alcohol consumption in a 2019 NSDUH survey, approximately 6% had alcohol use disorder (9 million men, 5.5

million women), yet only a small fraction of these individuals (7.2%) received any treatment in the past year, with even fewer (4%) reporting the use of an FDA approved medication for alcohol use disorder. It is therefore imperative to study the underlying biological mechanisms driving alcohol use disorder so that we can better understand the biological complexity of this disorder and hopefully, one day, create patient-specific therapies.

The transition from moderate alcohol use to abuse involves neuroadaptations to key brain circuits governing reward learning, sensory processing, and stress responding (Koob & Nestler 1997; Koob & Volkow 2016). These adaptations ultimately manifest the maladaptive behaviors seen in substance use disorder that promote continued drug use despite efforts to abstain (Koob & Nestler 1997; Koob & Volkow 2016). Compulsive drinking, or drinking in the face of a negative consequence, is a core feature of alcohol use disorder that is inadequately addressed by current therapeutic interventions (Garbusow et al., 2014; Stock 2017; Maisto et al., 2018; Burch et al., 2019). How chronic alcohol abuse promotes compulsive drinking is not entirely understood, but significant research points to modified function of the striatum and its ability to regulate actions geared toward alcohol consumption.

1.3 The Striatum in Substance Use Disorder

The striatum is a subcortical structure that serves as the primary input nucleus of the basal ganglia system. Through its diverse arrangement of afferent and efferent connections, the striatum regulates the expression of actions by integrating motor commands with cognitive, motivational, and emotional input (Haber, 2016). By merging internal value with external cues, the striatum learns which actions, and in which context, result in the acquisition of a reward and reinforces those actions to guide future reward-driven behavior. If the reward being sought is a drug of abuse, like alcohol, the striatum pathologically promotes actions that lead to acquisition of the substance. Consider the process of drinking a few beers after a stressful week of work. Execution of this behavior requires the selection of a motor plan motivated by an internal drive (e.g., to reduce stress) and the integration of the reward history of that motor action (e.g., drinking previously was rewarding). Once this motor program is learned, through repeated execution and reinforcement, this behavior becomes engrained in striatal circuits and can be elicited almost automatically by sensory cues in the environment – even in the face of known negative consequences (e.g., drinking previously led to poor work performance). As such, the striatum plays a fundamental role in the decisions and actions we make every day, and by extension how we define ourselves through those actions.

As an action leading to the acquisition of alcohol is learned and repeatedly executed, the recurring exposure of alcohol to the brain induces maladaptive neuroplastic changes to discrete striatal circuits that ultimately promotes compulsive drug use, which is a core feature of substance use disorder. As such, striatal pathology is a core component of addiction. Therefore, it is critical to understand how drugs of abuse, like alcohol, affect striatal circuits.

The striatal complex is divided into dorsal and ventral regions. These subdivisions have distinct afferent and efferent anatomical connections and contribute to specific aspects of motivated behavior. The ventral striatum, also referred to as the nucleus accumbens, is a critical structure in the reward system, receiving dopaminergic signals from the ventral tegmental area (VTA) and providing feedback to the VTA in the form of the so-called "direct" striatal output pathway. Through these reciprocal connections, the nucleus accumbens plays an important role in the motivational drive underlying instrumental learning. The nucleus accumbens is further divided into the core and shell regions based on neurochemical staining and specific connections to limbic and cortical brain regions (Zahm, 1989). Additionally the border between the core and shell, termed the "shore", exhibits a unique dopamine repones to novel stimuli suggesting that this may additionally serve as a functional subregion (Rebec et al., 1997). However, little is known about the anatomical connections to this area.

The nucleus accumbens core receives excitatory input from the prelimbic prefrontal cortex (PFC) and sends inhibitory projections to the ventral pallidum, while the nucleus accumbens shell receives excitatory input from the infralimbic PFC and relays information to the VTA (Zahm & Brog, 1992). Nucleus accumbens core and shell subcomponents uniquely contribute to motivational learning: the nucleus accumbens core drives reward seeking and acquisition while the nucleus accumbens shell encodes associations between rewards and sensory cues (Rodd-Henricks et al., 2002; Sellings & Clarke, 2003).

While the nucleus accumbens provides the incentive value for goal-directed actions, the dorsal striatum encodes the motor programs that allow us to perform complex actions required for reward acquisition in complex environments. Working together, the nucleus accumbens communicates reward incentive information to the dorsal striatum to shape the expression of goal-relevant motor skills (**Fig. 1.1**). For example, as an action is initially learned, the nucleus accumbens recruits the involvement of dorsal striatum through recurrent connections with midbrain dopamine neurons (Haber et al., 2000). The nucleus accumbens continues to influence the dorsal striatum as a motor skill is learned and mastered, at which point the activity in the dorsal striatum eventually dominates behavioral expression (Everitt & Robbins, 2016). This concept follows an early reinforcement learning model that likens the role of the nucleus accumbens to a critic that directs an actor's actions to acquire a reward (O'Doherty et al., 2004). In addition to the shift in activity from the ventral to dorsal striatum, there is a medial to lateral shift in activity within the dorsal striatum as actions transition from initial learning to mastery (**Fig. 1.1**). The dorsomedial striatum (DMS), or the caudate nucleus in humans and primates, initially dominates behavioral expression and encodes motor programs that allow us to perform complex, yet reversible, actions for the acquisition of a reward. However, as an action is repeatedly executed and reinforced, the motor program is stored in the dorsolateral striatum (DLS) (putamen in humans and non-human primates) (Wolf et al., 2019; Dhawale et al., 2019) and DLS activity dominates behavioral expression (**Fig. 1.1**; Everitt & Robbins, 2016).

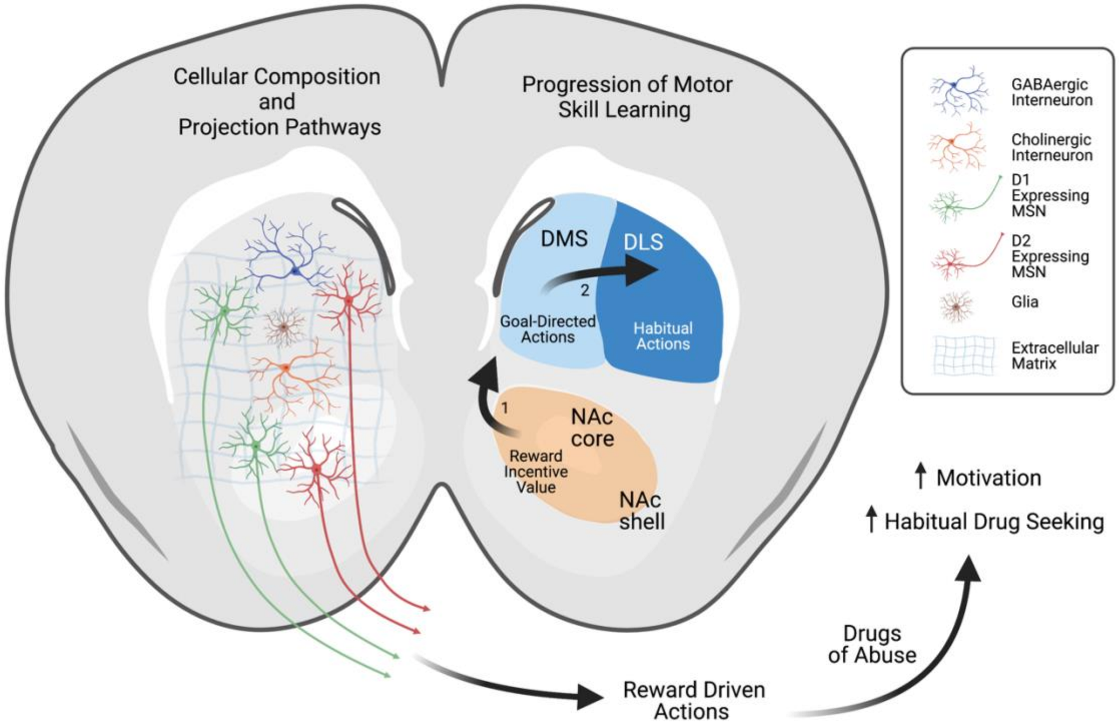

Figure 1.1 Cellular composition and projection pathways of the nucleus accumbens (NAc) dorsomedial striatum (DMS) and dorsolateral striatum (DLS) (left). D1 dopamine receptor-expressing medium spiny neurons (MSNs) transmit 'go' signals through the basal ganglia along the direct pathway (green). D2 dopamine receptor-expressing MSNs transmit 'no go' signals through the basal ganglia along the indirect pathway (red). The activity of striatal MSNs are locally modulated by striatal interneurons (blue, orange), glia (brown), and the dynamic regulation of the extracellular matrix. Coinciding output signals from both direct and indirect pathway MSNs enable activation of goal-directed actions and the simultaneous suppression of unwanted actions. The progression of activity in the striatum during motor skill learning (right). In the initial stages of motor skill learning, the nucleus accumbens (NAc) integrates reward information to incentivize and shape motor actions. This reward information is then relayed to the dorsal striatum (1) to produce learned goal-directed actions. Through repeated execution, motor sequences are refined to efficiently acquire reward as activity shifts from the dorsomedial to the dorsolateral striatum (2). Once mastered, these motor skills are habitually expressed with little executive oversight. Activity between these striatal subregions is highly dynamic and enables adaptive behavioral responses to sensory feedback from the environment. However, drugs of abuse impair this dynamic activity resulting in increased motivation for drug reward and an overreliance for habitual actions aimed to acquire drugs.

In the context of drug use, addictive substances reinforce behavior, in part, by positively modulating VTA dopamine release into the nucleus accumbens. For example, cocaine inhibits dopamine re-uptake by blocking the dopamine transporter at dopaminergic axon terminals (Kuhar, Rits & Boja, 1991), nicotine directly depolarizes dopamine neurons in male rats (Lüscher & Ungless, 2006), and ethanol facilitates action potential firing of VTA dopamine neurons in male rats likely through modulation of G protein-coupled inwardly-rectifying potassium (GIRK) channels and hyperpolarization-activated cyclic nucleotide gated (HCN) channels (Deehan et al., 2016; Abrahao, Salinas, & Lovinger, 2017). In part by increasing synaptic concentrations of dopamine in the striatum, these drugs of abuse reinforce drug acquisition-related actions. Significant evidence from clinical and preclinical studies indicates that as recreational drug use transitions to addiction (i.e., alcohol and drug use disorders), there is a coinciding shift toward DLS-mediated behaviors and an increase in DLS neural activity (Lüscher et al., 2020). This bias toward DLS output (relative to DMS and ventral striatum output) indicates that chronic drug exposure alters the balance of neural activity in striatal subregions, resulting in preferential engagement of the DLS encoding habitual behaviors that support highly efficient, compulsive drug acquisition.

Compulsive drug-seeking and -taking behavior, hallmarks of substance use disorder, manifest, at least in part, by profound drug-induced adaptations to broader corticostriatal circuitry. Exacerbating this effect is the role of stress in modulating corticostriatal circuits and associated behaviors. Chronically stressed male rats exhibit a bias for habitual responding and a corresponding reduction of medial PFC (mPFC), an area that projects to the DMS, neuronal volume and enhanced dendritic branching of DLS medium spiny neurons (Dias-Ferreira et al. 2009). Thus, stress and drug exposure tag-team to deliver a two-hit blow exerting a loss of top-down control from medial prefrontal cortex (PFC) and a failure to disengage DLS circuits during reward-driven tasks (Lüscher et al., 2020). To make matters worse, repeated drug exposure elevates stress levels in substance use disorder (Koob & Volkow 2016). Thus, the intimate relationship between stress and drug abuse creates a positive feedback loop, whereby stress promotes habitual actions that increase drug consumption, which in turn elevates stress hormones that further bias DLS-mediated behavior. The nature of this positive feedback relationship and its intricate actions upon corticostriatal systems are highly complex. Therefore, it is important to understand the nuts and bolts of striatal anatomy and physiology, and how drugs of abuse modulate these components to promote compulsive drug use within a broader physiological context that includes stress.

1.4 The Striatum: cells and circuits

The striatum integrates cortical and limbic signals and relays these signals to downstream basal ganglia nuclei to guide motivated behavior. Processing this input in the striatum are multiple neuron types that are categorized into two distinct groups: the projection neurons and the interneurons. The projection neurons are the most prominent cell type and account for approximately 90-95% of the neuronal population (Haber, 2016). These projection neurons are the so-called medium spiny neurons (MSNs) that possess relatively medium-sized somata (15-20 μM) and dendrites studded with spines. MSNs are further categorized into two subpopulations based on the expression of neuropeptides, dopamine receptor subtypes, and their axonal projections that course either directly or indirectly to the output nucleus of the basal ganglia. Ultimately, these two MSN subpopulations serve distinct roles that collectively allow for proper execution of motivated behavior.

While MSNs express GABA as their primary neurotransmitter, the MSN subpopulation that forms the direct pathway contains the neuropeptides substance P and dynorphin, and preferentially expresses the D1 dopamine receptor. In contrast, the indirect pathway neurons express enkephalin and the D2 dopamine receptor (Gerfen et al., 1990; Le Moine et al., 1990,1991; Surmeier et al., 1996; Lobo et al. 2006). D1 dopamine receptors are coupled to the $G_{\alpha s/olf}$ family of G proteins that activate adenylyl cyclase to stimulate cAMP production. cAMP production subsequently activates downstream signaling cascades through cAMP-dependent protein kinases that ultimately regulate MSN synaptic strength and gene expression (Sassone-Corsi, 2012). The D2 dopamine receptors are coupled to $G_{\alpha i/o}$ proteins that inhibit adenylyl cyclase and cAMP production, thereby also exerting effects on synaptic strength and an opposing influence on gene transcription. It is important to note that while MSNs generally fall into these two neurochemical subcategories, there is a small subpopulation (approximately 10%) of neurons that express both D1 and D2 dopamine receptors (Surmeier et al., 1996).

In the dorsal striatum, D1 dopamine receptor-expressing MSNs send axonal projections directly to the basal ganglia output nucleus, the substantia nigra pars reticulata (SNr), while D2 dopamine receptor-expressing MSNs send their projections indirectly to the output nucleus through the globus pallidus external segment and, subsequently, the subthalamic nucleus. In the classical basal ganglia model, activation of the direct pathway inhibits the SNr to, in turn, disinhibit the thalamus, thereby activating the cortex. In contrast, indirect pathway activation leads to (indirect) activation of the SNr and, therefore, inhibition of the thalamus, leading to decreased cortical drive. Ultimately this striato-thalamo-cortical circuit results in the activation of cortical neurons that send projections along the pyramidal tract to the brainstem and spinal cord to control the muscles of the body, neck, and face. Basal ganglia output from the SNr additionally projects to the superior colliculus to influence orienting movements of the head, eyes, and body (Liu & Basso, 2008; Hikosaka 2007; Hikosaka et al. 2019; Lee & Sabatini 2021), to the pedunculopontine nucleus to influence cholinergic neurons projecting to the cortex, thalamus, the basal ganglia, the cerebellum and spinal cord (French & Muthusamy, 2018), and to the brainstem reticular formation to affect muscle tone (Manetto & Lidsky, 1987). There is an additional pathway called the 'hyper-direct pathway' where cortical neurons bypass the striatum and project to the subthalamic nucleus (Kita, 1992). This hyper-direct pathway likely enables general inhibition of movement (Mink, 1996; Nambu et al., 2002).

Initial models of basal ganglia function proposed that the striatum is composed of a homogenous pool of projection neurons that provide a single output signal to control motor actions. While this model served as a useful neurological framework for early studies of basal ganglia disorders, closer examination of this model revealed contradictions in the predicted outcomes of specific basal ganglia manipulations (Albin, Young, & Penney, 1989). Upon presentation of the histochemical and fiber tracing data previously mentioned, the model was updated to include two separate subpopulations of projection neurons that are differentially regulated by dopamine (Albin, Young, & Penney, 1989). These two populations, as mentioned above, form the direct and indirect pathways and provide signals to initiate and suppress motor actions (**Fig. 1.1**; Kravitz et al., 2010). These two pathways, referred to as the "go" and "no-go" pathways, collectively coordinate the initiation of task-relevant behavior and simultaneous suppression of unwanted actions (Cui et al., 2013). One significant way in which drugs of abuse alter striatal function is by shifting the balance in activity of the go and no-go pathways to promote reward-related behavior. In the classic model, synaptic dopamine binds to and activates D1 dopamine receptor-expressing MSNs and suppresses D2 dopamine receptor-expressing MSNs through their respective G-protein signaling cascades. The net result is preferential activation of the 'go' pathway and suppression of the 'no go' pathway, which strongly biases the striatum to engage in reward-driven behavior including drug seeking and consumption. While this model is not without merit, we shall see later that the picture is much more complex than this.

In rats (Kawaguchi, Wilson, & Emson, 1990) and in monkeys (Parent et al., 1995), dorsal striatum MSNs do not exclusively send their axons to either the globus pallidus or to the substantia nigra, but rather the density of the axonal arbor is greater in one of these targets and the collaterals to the other region is sparse. For example, direct pathway neurons send the majority of their axon fibers to the substantia nigra and only sparsely innervate the globus pallidus. However, the difference in density of these projections suggests that activation of direct pathway neurons still ultimately leads to activation of thalamus and cortex, while activation of the indirect pathway neurons predominantly suppresses activity of the thalamus and cortex. Additional regulatory control of action may arise from these sparse collateral fibers as well as collateral MSN projections within the striatum forming synapses onto other MSNs and interneurons (Smith et al., 1998).

In the ventral striatum, it was originally assumed that the nucleus accumbens core exhibits a similar projection pattern, where D1 dopamine receptor-expressing MSNs project directly to the ventral tegmental area (VTA), while D2 dopamine receptor-expressing MSNs project indirectly through the ventral pallidum to the VTA. However, further investigation of these projection pathways indicates that there is considerably more convergence of D1 and D2 dopamine receptor-expressing MSNs with regard to their output projections as compared to the dorsal striatum in mice (Kupchik & Kalivas, 2017). Retrograde tracing studies demonstrate that both D1 and D2 dopamine receptor expressing nucleus accumbens MSNs project to the ventral pallidum (Lu, Ghasemzadeh, & Kalivas, 1998). Following this study, Kupchik et al. (2015) demonstrated that ventral pallidum neurons respond to optical stimulation of both D1 and D2 dopamine receptor-expressing nucleus accumbens MSNs, though the majority of D2 dopamine receptor-expressing MSNs and only a subset of D1 dopamine receptor-expressing MSNs elicited responses. While these data indicate a significant convergence of projection fibers from D1 and D2 dopamine receptor expressing MSNs, the electrophysiology data suggests that, like the dorsal striatum, information from D1 and D2 dopamine receptor-expressing MSNs are predominately relayed through the direct and indirect pathways, respectively. Thus, conventional models of the direct and indirect pathway hold value in the nucleus accumbens. However, further investigation into the functional significance of these collateral projections will likely reveal novel regulatory actions on the expression of motivated behavior.

In the nucleus accumbens shell, there is a similar proposed projection scheme where D1 dopamine receptor-expressing MSNs of the shell project directly to VTA while D2 dopamine receptor-expressing neurons project indirectly through the ventral pallidum (Heimer et al., 1991; Scofield et al., 2016). The classical interpretation of this projection model posits that D1 dopamine receptor-expressing MSNs convey reward signals by increasing VTA-derived accumbal dopamine release, while D2 dopamine receptor-expressing MSNs encode aversion by inhibiting VTA-derived accumbal dopamine release via the indirect pathway (Soares-Cunha et al., 2019). Modulation of VTA dopamine neuron activity occurs through MSN projections onto VTA interneurons whereby direct pathway activation disinhibits VTA dopamine neurons, and indirect pathway activation inhibits VTA dopamine neurons (Xia et al., 2011). Supporting the reward/aversion model of the nucleus accumbens, Lobo et al. (2010) demonstrated that optogenetic stimulation of D1 dopamine receptor-expressing MSNs enhances cocaine-conditioned place preference in male mice, while optogenetic stimulation of D2 dopamine receptor-expressing MSNs decreases cocaine-conditioned place preference. While this study supports the notion that reward and aversion are encoded through selective activation of direct and indirect pathways, respectively, Soares-Cunha et al. (2019) demonstrated that both D1 and D2 dopamine receptor-expressing MSNs drive both reward and aversion in male and female mice depending on the pattern of neuronal stimulation. An important consideration of these data, again, is the potential interaction of MSN output pathways through their collateral inhibitory projections.

Beyond MSNs, the remaining 5-10% of neurons in the striatal complex are interneurons and can be categorized into several subclasses by their neurochemical profiles (Kawaguchi et al., 1995). Classically there are three distinct populations of GABAergic interneurons in the striatum: the parvalbumin expressing fast-spiking interneurons; the somatostatin/neuropeptide Y expressing low-threshold spiking interneurons; and the calretinin expressing interneurons (Tepper & Bolam, 2004). An additional subset of GABAergic interneurons, first identified in the primate brain (Dubach et al., 1987) recently received considerable attention because of their expression of tyrosine hydroxylase (TH), the rate-limiting enzyme for the synthesis of dopamine. However, it was later discovered that these striatal TH-positive interneurons do not release dopamine (Ibáñez-Sandoval et al., 2010; Xenias et al., 2015), but instead release GABA to provide widespread inhibition of both D1 and D2 dopamine receptor-expressing MSNs. The other major class of interneuron is the tonically active cholinergic interneuron that provide the primary source of acetylcholine in the striatum and are by far the most studied striatal interneuron population (Calabresi et al., 2000). Collectively, these interneurons shape striatal function by modulating presynaptic input to MSNs, as well as MSN output (Ding et al., 2010; Koos and Tepper, 1999).

In addition to neurons, glia, which are approximately three times as abundant as neurons in the brain (von Bartheld, Bahney, & Herculano-Houzel, 2016), play a critical role in striatal function (Khakh, 2019). While the exact role of glia in striatal microcircuits is only now beginning to unfold, initial studies clearly demonstrate their important function in the regulation of striatal MSN activity and subsequently striatal dependent behavior. For example, astrocytes are known to regulate neuronal function, in part, through fluxes in intracellular calcium at distal astrocytic processes causing release of transmitters that act on neighboring neurons (Bazargani & Atwell 2016). Recently, Yu et al. (2018) demonstrated that selectively altering astrocyte calcium influx impacts neighboring MSN activity and the expression of a repetitive motor action sequence, self-grooming, in male and female mice. This mechanism likely involves GABAergic transmission, as the effect was reversed by blocking astrocyte GABA transporter 3 (GAT-3). Notably, GABAergic communication between astrocytes and striatal MSNs is bi-directional, as Nagai et al.

(2019) demonstrated that MSNs communicate with astrocytes through $GABA_B$ receptors to modulate the influx of astrocyte calcium. Selectively activating the Gi pathway in striatal astrocytes with DREADDS, thereby mimicking the signaling cascade induced by $GABA_B$ receptor activation, induces hyperactivity and attentional deficits in male and female mice.

Astrocytes additionally modulate neuronal function by regulating the concentration of extracellular glutamate through their dynamic expression of glutamate transporters (Williams et al., 2005) and cystine-glutamate exchangers (Malarkey & Parpura, 2008). In the striatum, the expression level of these astrocytic proteins significantly impacts MSN function and striatum-dependent behavior. For example, after repeated instrumental training, the glutamate transporter EAAT-2 is upregulated in the DLS, and that preventing this upregulation blocks habitual responding for food reward in male mice (Boender et al.. 2020). The promotion of habitual behavior through EAAT-2 expression likely occurs through modifications in glutamatergic synaptic plasticity, as EAAT-2 function modulates spike time-dependent plasticity in the dorsal striatum (Valtcheva & Venance, 2015). Drugs of abuse, including psychostimulants and opioids disrupt astrocytic regulation of striatal glutamate resulting in shifts in striatal glutamate levels that promote addiction-like behavior (Kruyer & Kalivas, 2021). Chronic heroin and cocaine exposure each downregulate the expression of EAAT-2 on astrocytes in the nucleus accumbens of male rats (Roberts-Wolfe & Kalivas, 2015; Kim et al., 2018), resulting in increased synaptic glutamate spillover. Notably, this increase in nucleus accumbens glutamate promotes drug-seeking behavior (Shen et al., 2015) that can be reversed by pharmacological treatments that upregulate EAAT-2 expression in male rats (Kruyer & Kalivas, 2021). Collectively, these studies demonstrate the significant, but understudied, role that striatal glia play in drug addiction. Further research will likely broaden the depth of their contribution to include modulation of other transmitter systems, including dopamine (Fouyysac & Belin, 2018).

Lastly, the extracellular matrix, a collection of structural molecules that surround and support the branched network of neurons and glia cells is modulated by drugs of abuse to influence striatal-dependent behavior. The extracellular matrix comprises approximately 20% of the volume of the mature rat brain (Nicholson & Sykova, 1998) that in addition to providing structural support for neurons and glia, acts as a signaling domain that regulates neurotransmission, plasticity, and cellular apoptosis (Barros, Franco, & Müller, 2011). The extracellular matrix consists of two main classes of proteins: proteoglycans and fibrous proteins including laminin, collagen, elastin, and fibronectin. Communication between neurons and this extracellular matrix is bidirectional, whereby changes in the extracellular space are signaled to the neuron and changes in the internal state of the neuron can be transmitted to the extracellular matrix.

Pharmacologically blocking neuronal activity, including action potential firing and synaptic transmission through calcium permeable AMPA receptors, dramatically decreases extracellular matrix structures *in vitro*, suggesting that extracellular matrix formation is dependent on neuronal activity (Dityatev et al. 2006). Additionally, changes in synaptic strength signal structural modifications to the extracellular matrix to enable morphological plasticity of dendritic spines (Lu et al., 2011). Communication is transmitted in part through cell adhesion molecules, which are transmembrane proteins expressed on neurons that bind to extracellular matrix glycoproteins (Shinoe & Goda, 2015). Collectively, this bidirectional communication between neurons and the extracellular matrix help to maintain neuronal homeostasis and promote dynamic synaptic transmission.

This raises the possibility that drugs of abuse may influence the extracellular matrix to alter striatal circuit function and, therefore, drug-seeking behavior. Indeed, emerging evidence indicates that drugs of abuse alter extracellular matrix proteins to influence striatal synaptic transmission in rodents (Smith et al., 2015). Heroin reinstatement transiently increases matrix metalloproteinase-9 activity, an enzyme that degrades the extracellular matrix, around D1 dopamine receptor-expressing MSNs in the accumbens of male and female rats (Chioma et al. 2021). Similarly, cocaine withdrawal increases nucleus accumbens matrix metalloproteinase activity, which is necessary for cocaine relapse in male rats (Smith et al. 2014). In line with these preclinical studies, RNA sequencing data from men and women diagnosed with opioid use disorder depict an enrichment of transcripts that regulate extracellular matrix signaling in the nucleus accumbens (Seney et al. 2021). Collectively, these data indicate that multiple drugs of abuse impact striatal extracellular matrix. Continued exploration of these drug-induced changes will expand our understanding of the pathophysiology of substance use disorder and may lead to novel therapeutic targets for substance use disorder.

1.5 Striatum in Action Selection

So, with knowledge of these molecular, cellular and circuit components intact, what does the striatum do? The striatum integrates executive and sensorimotor information from cortical centers with ascending reinforcement signals from midbrain dopamine neurons to allow for selection of actions that result in reward. Thus, while excitatory input to the striatum informs the content of the action, dopamine input provides an action selection and vigor signal (Klaus et al., 2017). An early model of basal ganglia function posited that activation of, particularly dorsal striatal, direct pathway MSNs produces pro-kinetic effects, while activation of indirect pathway MSNs produces anti-kinetic effects (Albin et al., 1995; Nelson & Kreitzer, 2014). In support of this model, optogenetic activation of D1 dopamine receptor-expressing MSNs results in action expression, while activating D2 dopamine receptor-expressing MSNs results in action inhibition in male and female mice (Kravitz et al., 2010). This along with other data support the "rate model": that is, the striatum serves as the gas and brake pedals of the motor system whereby the degree of activation of the direct pathway concomitantly drives action, while the degree of activation of the indirect pathway concomitantly inhibits action.

However, the rate model does not fully explain how selective actions are encoded and elicited by the striatum. Addressing this, Mink (1996) and, later, Hikosaka et al. (2000) proposed a model where direct and indirect pathway MSNs are concurrently activated to initiate a selective action and simultaneously suppress unwanted actions. This model is described as the support/suppress model and gained support by advances in genetic engineering that enable simultaneous measurement of direct and indirect MSN activity during motor behavior. Using two different optical indicators specifically expressed in D1 and D2 dopamine receptor-expressing MSNs, Cui et al. (2013) revealed that both direct and indirect pathway neurons are activated in male and female mice during a given action. This discovery supports the notion that both neuron populations are necessary for the proper execution of a motor behavior. Additional evidence for the support/suppress model came from data demonstrating that ensembles of MSNs activate in male and female mice for specific components of a motor action (Klaus et al., 2017; Parker et al., 2018): for example, a neural ensemble consisting of both direct and indirect pathway MSNs encode leftward head movement while a separate MSN ensemble encode rightward head movements.

These neural ensembles likely develop as a motor behavior is learned and refined over time, as chronic striatal electrophysiology recordings in male rats reveal that MSN activity becomes more organized as a motor skill is learned (Barnes, 2005). Again, activity of both direct and indirect pathway MSNs would be required for this refinement. One can imagine that to perform a particularly refined action, such as playing a scale on a piano, the motor movements unnecessary for accurate performance of the action must be inhibited just as the motor movements necessary for the action itself must be promoted. In summary, as a motor skill is acquired, dynamic events in the striatum result in the organization of neuronal ensembles consisting of direct and indirect pathway MSNs that harmoniously activate to properly execute a motor skill. Significant changes to this balance result in overt motor impairments, exemplified by Parkinson's disease and Huntington's disease patients, but as we will discuss in the next section, changes induced by drugs of abuse selectively promote actions related to drug seeking and acquisition.

1.6 The Ventral Striatum: motivated behavior and addiction

A prominent view of the ventral striatum, or nucleus accumbens, is that it serves as the pleasure center of the brain, a notion that stems from numerous studies implicating the nucleus accumbens in the acquisition of rewarding stimuli, such as food, sex, or drugs of abuse. Indeed, animals will elect to press a lever that electrically stimulates the nucleus accumbens (Mogenson, 1979). In humans, the nucleus accumbens activates in response to the presentation of rewarding stimuli, including monetary gain (Knutson et al., 2001), a pleasant taste (O'Doherty, 2004) and smiling faces (Spreckelmeyer et al., 2009). Collectively, these studies support the notion that the nucleus accumbens is simply the area of brain that encodes reward. However, this notion deviates from the original idea put forth by Mogenson, who proposed that the nucleus accumbens serves as a limbic-motor interface that integrates emotional signals from the limbic system to bias the direction and intensity of behavior to obtain a reward.

Many of these early studies describing the rewarding effects of nucleus accumbens activation led the field to believe that the nucleus accumbens is responsible for encoding positive valence, while other limbic structures, like the amygdala, are responsible for negative emotional processing. However, subsequent studies revealed that the nucleus accumbens also plays a role in aversive behavior and negative reinforcement learning (Reynolds & Berridge, 2002). This has led to an updated model of the nucleus accumbens where both rewarding and aversive information are integrated to promote an appropriate behavioral response, particularly one that reduces the likelihood of negative outcomes and increases the likelihood of positive rewards. For example, activation of D1 dopamine receptor-expressing MSNs drives reward-related behaviors while activation of D2 dopamine receptor-expressing MSNs drives aversion in male mice (Lobo et al., 2010). Further supporting this idea, inactivation of D1 dopamine receptor-expressing MSNs in the nucleus accumbens with Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) prevents reward-seeking behavior in male mice (Calipari et al., 2016). These studies provide an important foundation to understand how parallel MSN circuits are influenced by drugs of abuse to, in turn, influence drug-seeking behavior.

Looking into the mechanism underlying incentivized behavior, the nucleus accumbens integrates reward signals from midbrain dopamine neurons to regulate the association of a motor action with a rewarding outcome (Dickinson & Balleine, 1994). A core associative learning type requiring this system is Pavlovian learning (also referred to as classical conditioning), which involves associating a sensory cue with the delivery of a reward. If an animal initially trained to associate a sensory cue with a reward is then trained to perform an action (e.g., a lever press) in order obtain the associated reward, the reward incentive from the original conditioned stimulus is transferred to the motor action required to receive the reward. This process is referred to as Pavlovian instrumental transfer and lesioning the nucleus accumbens in male rats significantly impairs the acquisition of this lever behavior (Balleine & Killcross, 1994; Chang et al., 2012). In other words, the nucleus accumbens encodes the reward value of an outcome and provides the incentive for an animal to perform an action in order to receive the reward.

In addition to mediating the association of cues, rewards, and the actions that elicit rewards, the nucleus accumbens also encodes information about the context in which cues are presented. This contextual information is relayed from the hippocampus fibers that project to the nucleus accumbens and will be covered in more detail later (see **1.9.6**). This reward-context association is evidenced by a commonly used behavioral paradigm that utilizes classical Pavlovian conditioning to study the rewarding effects of abused drugs called conditioned place preference (CPP). In this paradigm an animal is placed in a two-chamber arena where each side is discriminated by distinct visual symbols (e.g., stripes or dots). The animal is administered a dose of a rewarding substance on only one side of the chamber where it learns that being in that side results in a reward. Given the chance to explore either side of the arena, the animal will exhibit a preference for the drug-associated side. Most drugs of abuse, including cocaine, amphetamines, nicotine, and ethanol induce a dose dependent, and sometimes species dependent, conditioned place preference (Tzschentke, 2007). In line with the role of D1 dopamine receptor-expressing MSNs, but not D2 dopamine receptor-expressing MSNs, in reward-related behavior, Cervo & Samanin (1995) demonstrated that cocaine CPP is blocked in male rats by administration of a D1 receptor antagonist, but not a D2 dopamine receptor antagonist. Additionally, nicotine CPP is prevented by blocking D1 dopamine receptors in the nucleus accumbens in male rats (Spina et al., 2006). This drug pairing is attributed to release of dopamine in the nucleus accumbens from VTA projections, as selective activation of these dopaminergic terminals enhances conditioned place preference for morphine in male mice (Koo et al., 2012). These studies demonstrate that drugs of abuse result in preferential activation of D1 dopamine receptor-expressing MSNs in the nucleus accumbens and that this activation underlies reward-related behavior.

The cyclical nature of repeated drug use and withdrawal activates the brain's stress response system, which in turn impacts how the nucleus accumbens encodes reward/aversion. corticotropin-releasing factor (CRF), a neuropeptide released in response to arousing environmental stimuli and stress, acutely enhances dopamine release in the nucleus accumbens to promote reward-cue associations, as measured by CPP (Lemos et al., 2012). This enhanced dopamine release is mediated by CRF activating cholinergic interneurons through the CRF type 1 receptors, which increases their firing rate of cholinergic interneurons (Lemos et al. 2019). The resulting release of acetylcholine activates local type 5 muscarinic acetylcholine receptors in the nucleus accumbens that is necessary for the enhanced dopamine release induced by CRF (Lemos et al. 2019). This CRF signal likely comes from one or more projections to the nucleus accumbens, including the paraventricular nucleus, the central amygdala and/or the bed nucleus of the stria terminalis where CRF-expressing neurons are concentrated (Swanson & Simmons 1989; Makino et al., 1994a, b; Itoga et al., 2019; Pomrenze et al., 2015).

While acute stress enhances dopamine release in the nucleus accumbens, chronic stress, which elevates CRF levels in the nucleus accumbens (Zorilla, Logrip, & Koob 2014) prevents acute CRF-mediated potentiation of dopamine release and diminishes CRF-induced CPP in male mice (Lemos et al. 2012). This stress induced transition from reward signaling to aversion likely underlies the negative affective state seen in substance use disorder that promotes drug seeking and consumption aimed at alleviating negative affective symptoms of drug withdrawal (Zorilla, Logrip, & Koob 2014).

As mentioned above, the nucleus accumbens can be divided into core and shell regions based on neurochemical staining, connections to limbic and cortical structures, and their role in distinct components of motivated behavior (Zahm ,1999). The nucleus accumbens core, which comprises the population of MSNs closely encompassing the anterior commissure, receives more dense projections from prelimbic, anterior cingulate, and insular cortices. On the other hand, the shell, which surrounds the core, receives projections from infralimbic prefrontal cortex and piriform cortex (Zahm & Brog, 1992). Evidence of behavioral distinctions between these sub-compartments come from site-specific lesion studies (Corbit et al., 2001) and site-specific intracranial self-administration studies (Rodd-Henricks et al., 2002; Engleman et al., 2009) where female rats will self-administer cocaine or ethanol directly into the accumbens shell, but not the accumbens core

(Rodd-Henricks et al., 2002; Engleman et al., 2009). Additionally, lesions to the nucleus accumbens shell produce a deficit in stimulus-guided action selection in female rats (Corbit et al., 2001), which taken together with the self-administration studies demonstrates its role in cue-reward associations. In contrast, lesions to the nucleus accumbens core, but not the shell, reduce the acquisition rate of lever-pressing behavior, indicating its role in reward acquisition (Corbit et al., 2001).

The division between the nucleus accumbens core and shell is further characterized by the expression of distinct classes of opioid receptors, where the core predominately expresses the mu opioid receptor, and the shell predominately expresses the delta opioid receptor (Mansour et al. 1995; Hipólito et al. 2008). Delivering a mu opioid receptor antagonist into the nucleus accumbens core decreases outcome-guided responses, while a delta opioid receptor antagonist administered into the nucleus accumbens shell impairs cue-guided actions in male mice (Laurent et al., 2012).

Recent investigation into the anatomical divisions of the nucleus accumbens has revealed that the story is, in fact, more complex with further sub-compartmental organization evident (Gangarossa et al., 2013; Yang et al., 2018). This warrants further characterization to better understand how these sub-compartments specifically contribute to motivated behavior. However, while it is likely that each nucleus accumbens circuit encodes a distinct component of motivated behavior, it is important to note that the nucleus accumbens core and shell work in concert to properly integrate reward information to guide goal-directed actions.

1.7 The Dorsal Striatum: motivated behavior and addiction

The nucleus accumbens encodes reward incentive and, through a series of circuit loops, transfers information to the dorsal striatum to guide and refine the complex motor actions necessary to acquire rewards (**Fig. 1.1**). The dorsal striatum is divided into medial and lateral regions based on distinct excitatory inputs and the specific function each region contributes to motivated behavior (Burton, Nakamura & Roesch, 2015). Again, these subregions are the dorsomedial (DMS) and dorsolateral striatum (DLS), which in the human and primate brain are referred to as the caudate and putamen, respectively. The DMS receives excitatory input from the orbitofrontal cortex, the medial prefrontal cortex, and the intralaminar nuclei of the thalamus (Gerfen, 1988; Hunnicutt et al., 2016). Integrating these inputs, the DMS subserves the expression of reversible, goal-directed action strategies that require executive oversight (Yin et al., 2005b; Yin et al., 2009). DMS circuit activity is critical for the formation of action-outcome associations during initial drug use and ingrain the actions performed for that drug reward (Everitt & Robbins, 2013). On the other hand, the DLS receives excitatory input from somatosensory and motor cortices as well as the intralaminar thalamus (Gerfen, 1988; Hunnicutt et al., 2016) and plays a critical role in organizing motor elements into complex action sequences that are executed in response to sensory cues with high precision and efficiency under low executive oversight (Bailey & Mair, 2006; Yin, 2010). These so-called stimulus-response behaviors underlie habitual action strategies that may lead to compulsive behavior (Graybiel, 2005; Everitt & Robbins, 2016).

As an action is learned, activation of the DMS predominates to reversibly associate a reward outcome with an action (Yin, 2010). The presentation of a reward reinforces that action and thereby encourages repeated execution of that action. If, however, the reward is devalued, by satiation, a noxious additive, omission of the reward or increased effort to perform the task, then the action is no longer reinforced and therefore, not performed. This goal-directed behavior is said to be *sensitive* to reward feedback, which is attributed to the upstream prefrontal cortex (Laskowski et al., 2016; Le Merre et al., 2018).

This reward sensitivity benefits an organism by promoting behavioral flexibility to respond accordingly to changes in the environment. Consider the following example: you are hungry so decide to drive to your local fast-food restaurant. Your goal is to purchase a savory cheeseburger with fries, but, along the way, you hear on the radio that the fast-food restaurant received a poor cleanliness rating so you decide to head home, still hungry. In this example, consciously and flexibly changing your actions away from food seeking due to reward devaluation (e.g., tainted food), allowed for a scenario ensuring optimal chances at survival, albeit at the expense of time. When this flexible behavior is disrupted however, due to dysregulation of the medial prefrontal cortex or the DMS, for example, individuals struggle to flexibly change their behavior to match a change in the environment and instead exhibit rigid, perseverative behavior. So instead of avoiding the fast-food restaurant that received a poor cleanliness rating, you go ahead and eat the potentially contaminated burger regardless of this information. Inflexible behavior is evident in numerous neuropsychiatric conditions including obsessive compulsive disorder (Vaghi et al., 2017, Remijnse et al., 2013) major depressive disorder (Remijnse et al., 2013, Miranda et al., 2013), and as we will discuss in more depth within this section, substance use disorder.

While goal-directed behavior is influenced by the outcome of an action, repeated training shifts the association from an action-outcome pairing (e.g., press lever = reward) to a stimulus-response association (cue light signaling the action = press lever), where now the actions are performed in response to the presentation of sensory cues. In this state, these actions may continue to be expressed despite changes in the reward outcome, such as devaluation. This is operationally defined as habitual behavior. Take, for example, typing on a keyboard. When a person first learns to type, they must identify where each letter is placed on the keyboard and, subsequently, slowly type each letter to construct a word. However, after many hours of practice, the sequenced finger movements necessary to generate each word improve in speed and accuracy. After years of practice, the DLS can package these individual motor elements into readily releasable motor sequences (Yin et al., 2004, 2005a). Thus, the DLS saves considerable cognitive energy that can be used instead for more demanding, parallel tasks (e.g., maintaining a conversation as you type your name). These automated behaviors play a significant role in our daily lives, and are engaged when we brush our teeth, tie our shoes, or drive a car to work.

The specific contribution of the DMS and DLS to goal-directed and habitual behavior was originally discovered through site-specific lesion studies revealing the respective contributions of each striatal subregion to motivated behavior (Yin et al., 2004, 2005a,b). Following these foundational studies, chronic electrophysiology recordings from the dorsal striatum of rats learning to perform a motor task revealed that as an animal initially learns an operant task, activity in the DMS predominates and this behavior is sensitive to reward devaluation. However, after repeated training, the behavior becomes habitual and activity in the DMS diminishes while the DLS remains active (Thorn et al., 2010).

As the striatum is driven by excitatory projections from the cortex, one might predict that transitioning from early to late skill learning is met with concomitant changes in cortical afferents. Indeed, Yin et al. (2009) demonstrated that corticostriatal LTD is enhanced in the DLS of male rats that have mastered a motor skill compared to naïve rats and those that are in the early stages of motor training. Supporting this result, Kupferschmidt et al. (2017) demonstrated that cortical terminals from the medial prefrontal cortex are robustly active during the acquisition of a new motor skill, but as the motor skill is learned, this activity is reduced. Importantly, recordings from the cell bodies of the cortical neurons projecting to the striatum revealed that while the terminals experienced learning-dependent changes in activity, the response of cell bodies in the cortex did not change during the learning paradigm. These studies indicate that synaptic plasticity in the striatum may subserve the mastery of a motor skill and subsequently the transition from goal-directed to habitual behaviors. Notably, drugs of abuse hijack these synaptic mechanisms (and others) to ultimately tip the balance of behavior toward habitual action strategy expression.

It is now evident that in both humans and rodent models of substance use disorder that chronic drug exposure promotes hyperactivation of the DLS and subsequently biases behavior from goal-directed to habitual action strategies on instrumental tasks. In men and women, the duration of alcohol dependence correlates with alcohol-cue induced responses in the putamen (i.e., DLS) (Sjoerds et al., 2014). Additionally and alcohol dependent men and women exhibit an overreliance on habitual responses on a computer-based instrumental learning task (Sjoerds et al., 2013). In rodent models of addiction, the same pattern of habitual behavior is present (Everitt & Robbins, 2016). Chronic cocaine exposure produces habitual cocaine seeking in male rats (Zapata et al., 2010). Ethanol seeking behavior is driven by stimulus-response action strategies in male rats (Dickinson et al., 2002), and habitual responding occurs in male rats self-administering nicotine (Clemens et al., 2014).

Notably, chronic drug exposure does not simply produce habitual actions for the drug itself, but also generalizes habitual responding to natural rewards and facilitates the habit-learning process. Repeated amphetamine treatment rapidly produces habitual responding for sucrose in male rats (Nelson & Killcrose, 2006); chronic ethanol exposure accelerates habitual control of natural reward seeking in male rats (Corbit et al., 2012); and cocaine administration facilitates habitual responding for a natural reward in male rats (Schmitzer-Torbert et al., 2014). Together, these studies demonstrate that drugs of abuse broadly impact habit circuitry to promote a dominance of habitual responding for both drugs and natural rewards (Everitt & Robins, 2016).

This notion is supported by a diffusion tensor imaging study of healthy men and women showing that white matter connectivity within these corticostriatal pathways predicts individual differences in the balance of goal-directed and habitual control (de Wit et al., 2012). Tract strength between the medial prefrontal cortex and the caudate (i.e., DMS) predicts goal-directed action, while tract strength between the premotor cortex and the putamen (i.e., DLS) predicts habitual behavior (de Wit et al., 2012). Additionally, brain imaging studies reveal that chronic drug abuse negatively impacts the function of the prefrontal cortex (Perry et al., 2011). Male cocaine-dependent individuals exhibit reduced prefrontal cortical volume and functional deficits (Fein et al., 2002). Even an acute alcohol exposure reduces prefrontal cortical activity in healthy male subjects (Kähkönen et al., 2003), while chronic exposure results in a significant reduction in gray matter in the prefrontal cortex of men and women (Yang et al., 2016). Provided the importance of the prefrontal cortex in DMS-mediated behavior, these studies suggest that chronic drug abuse promotes habitual behaviors by disengaging prefrontal cortex thereby shifting the balance toward DLS-driven habitual behavior.

1.8 Stress, Habits, and Addiction

Further biasing striatal function toward habitual actions is stress. This occurs through the action of stress hormones and catecholamine transmitters that bind to and activate their respective receptors expressed throughout the brain. However, stress hormone receptors are expressed in high density within the prefrontal cortex, as evidenced in male and female squirrel monkey brains, suggesting predominate effects of stress on executive control (Patel et al. 2000). Indeed, stress produces dramatic deficits in prefrontal cortex driven behavior (Moghaddam & Jackson 2004), including cognitive flexibility in male rats (Bondi et al. 2007). This deficit likely arises from stress-induced morphological changes in the prefrontal cortex, as chronically stressed male rats exhibit dendritic atrophy on pyramidal neurons in the medial prefrontal cortex (Cook & Wellman 2004). Thus, stress may profoundly dysregulate prefrontal cortex function and reduce executive control over goal-directed actions, therefore biasing DLS-mediated habitual behaviors.

The stress-induced enhancement of DLS-dependent behaviors is evident from several studies. Male and female participants exposed to a stress protocol that combines physical and psychosocial stressors exhibit a shift from goal-directed action to habitual control on an instrumental learning task (Schwabe & Wolf 2009; 2011). Expanding on this work, Zerbes et al. (2020) demonstrated that the level of the stress response to an acute stressor, measured by cortisol, correlates with the activity in the dorsal striatum and the relative bias toward habitual actions in healthy men and women. These findings from human fMRI studies are further supported by preclinical models revealing that chronic stress accelerates DSL-dependent response learning and impairs hippocampus-dependent place learning in male rats, indicating that stress biases habitual actions over more cognitive-based learning strategies (Goodman & McIntyre 2017). This bias is mediated by the action of stress hormones acting locally in the striatum, as local administration of glucocorticoids into the DLS, but not the DMS, accelerates habit learning in male rats (Siller-Pérez et al. 2017). Glucocorticoid signaling in the striatum likely promotes chronic stress-induced medium spiny neuron dendritic atrophy in the DMS but enhanced dendritic complexity in DLS medium spiny neurons in male rats (Dias-Ferreira et al. 2009).

In addition to the corticosteroid response, stress also elicits an acute surge of norepinephrine in the brain that plays an important role in promoting habitual actions. Healthy men and women carrying a loss of function variant of the gene encoding the a2b-adrenoceptor (ADRA2B) exhibit a reduced bias toward habit memories after stress (Wirz et al. 2017). In rats, stress cues bias DLS-driven habitual actions over hippocampus-dependent 'cognitive' actions in a procedural memory task, which can be blocked by systemic administration of the beta-adrenergic receptor antagonist propranolol in male rats (Goode et al. 2016). Providing further interest in the adrenergic stress mechanism, Goodman et al. (2015) revealed that glucocorticoid enhancement of habitual behavior in male rats requires concurrent activation of the beta-adrenergic system, suggesting that norepinephrine receptors are important therapeutic targets for stress-related disorders.

In addition to dysregulating corticostriatal function, chronic stress impacts functional connectivity between the amygdala and striatum to further promote habitual actions. Healthy men and women exposed to an acute stressor exhibit increased functional connectivity between the amygdala and the putamen (Wirz et al., 2017). Notably, acute administration of the mineralocorticoid receptor antagonist spironolactone prevents this increased connectivity and blocks the shift toward habitual actions in healthy men (Vogel et al. 2017). In male rats, stress-induced habitual responding is blocked by administering the beta-adrenergic receptor antagonist propranolol into the basolateral amygdala (Goode et al. 2016). These studies have led many in the field to believe that stress induced changes in the amygdala orchestrate the shift to habitual actions through its connections with the striatum. Supporting this claim, individuals carrying the aforementioned a2b-adenoceptor mutation, which prevents stress-induced habitual bias, exhibit increased functional connectivity between the amygdala and entorhinal cortex instead of the increased connectivity between the amygdala and the putamen seen in healthy controls (Wirz et al., 2017). Thus, a complex interaction of glucocorticoid, noradrenergic, and extended striatal circuitry mediate a shift toward habitual action strategy selection in the face of stress.

When drugs of abuse are added to the equation, stress-induced habitual action strategy selection is kicked into high gear. In substance use disorder, periods of alcohol abstinence induce negative affective states including anxiety, irritability, emotional pain, and a loss of motivation for natural rewards that escalate drug intake (Koob et al., 2014; Uhl et al. 2019). These negative states motivate continued drug use to achieve an alleviation of these negative feelings (i.e., negative reinforcement) and are incited by drug withdrawal induced stress. Indeed, chronic administration of all major drugs of abuse elevate stress hormone responses, including adrenocorticotropic hormone, corticosterone, and CRF during acute withdrawal (Koob et al. 2014). Accordingly, administration of a CRF receptor antagonist blocks escalated self-administration of cocaine (Specio et al. 2008), nicotine (George et al. 2007) heroin (Greenwell et al. 2009), and alcohol in male rats (Funk et al. 2007). Therefore, the cycle of drug use and acute withdrawal induce key neuroadaptations within striatal circuits that ultimately promote continued, elevating drug use that traps an individual in a cycle of stress-fueled habitual drug use.

1.9 Excitatory Striatal Circuits in Motivated Behavior and Addiction

1.9.1 Orbitofrontal cortex→dorsal striatum

The orbitofrontal cortex (OFC) regulates cognitive flexibility, or the ability to change behavior in response to a change in reward contingency. Dysfunction of the OFC is implicated in the pathophysiology of obsessive-compulsive disorder and other compulsive behaviors (Saxena et al., 1998; Joel et al., 2005a, b; Reminjse et al., 2006) including compulsive drug use (Everitt & Robins, 2005). Here we focus on the excitatory projections from OFC to the striatum, discuss how this circuit regulates goal-directed actions, and describe how drugs of abuse disrupt this circuit to promote continued drug use despite a desire to achieve sobriety.

The OFC sends direct projections to the DMS (Pan et al., 2010; Hoover & Vertes, 2011; Schilman et al., 2008) that predominantly arise from ventral/ventrolateral orbital areas (**Fig. 1.2**). Additionally, projections from lateral/dorsolateral orbital areas sparsely terminate in DLS and lateral regions of the nucleus accumbens (Schilman et al., 2008). Independent lesion studies of both the OFC or the DMS shed light on the functional role of this corticostriatal circuit. As inactivating the DMS results in impaired goal-directed behavior (Yin et al., 2005a), lesioning the OFC in male rats results in compulsive lever-pressing behavior (Schilman et al., 2010), where an animal will continue to press an incorrect lever even though the reward is not delivered. These results suggest that the OFC→striatum circuit regulates the ability to shift behavior in response to updated environmental signals that enable an organism to maintain a goal-directed action. Recall the example of the late-night drive to the fast-food restaurant. In this scenario, the tainted food report is likely processed by the OFC and updated information is imported to the DMS to alter the action plan to return home. What we have learned from simultaneous *in vivo* electrophysiology recordings from both OFC and DMS is that indeed both of these structures become more active during goal-directed behavior, but also that activating this circuit optogenetically results in goal-directed action expression (Gremel & Costa, 2013). Interestingly, the ability to dissect the roles of specific OFC-striatum circuits has shed light on the unique contributions of OFC sub-circuits to flexible or adaptive decision making. OFC projections onto basolateral amygdala neurons that, in turn, project to the striatum mediate stabilization of action values and ablating this circuit enhances reversal learning performance (Groman et al., 2019). Further investigation of these sub-circuits will undoubtedly clarify the broader mechanisms that collectively shape goal-directed actions.

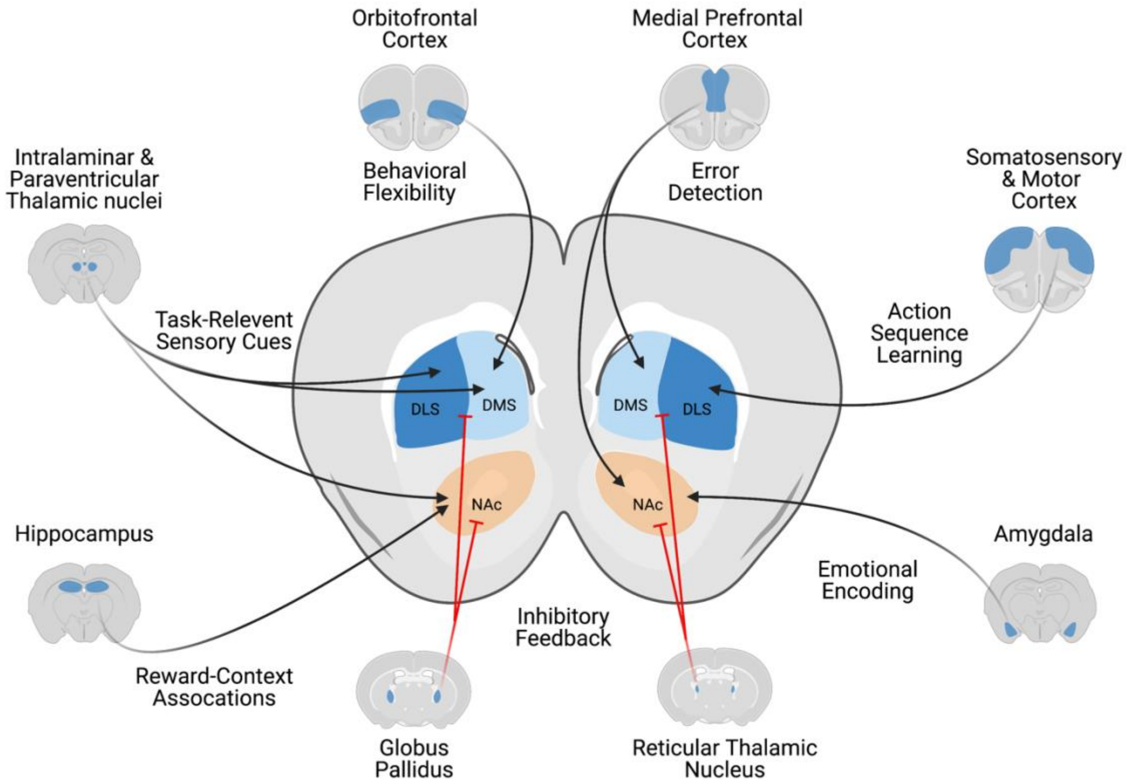

Figure 1.2 Circuit diagram of excitatory and inhibitory projections to the striatum and their respective behavioral correlates. The medial prefrontal cortex sends excitatory afferents to the DMS to encode error detection during motivated behavior. The orbitofrontal cortex sends excitatory afferents to the DMS and DLS to promote flexible actions to attain a reward. The somatosensory and motor cortices provide excitatory projections that import action sequence information to be stored in the DLS, while thalamic structures including the intralaminar nuclei and the paraventricular thalamus, send excitatory, integrated sensory inputs to all subregions of the striatum to update goal-directed actions (NAc and DMS) or initiate learned actions (DLS). The amygdala sends excitatory projections to the NAc to provide emotional context for goal-directed actions. The hippocampus sends excitatory projections to the NAc to form associations with environmental cues with reward. The globus pallidus and reticula thalamic nuclei both send inhibitory signals to all subregions of the striatum to suppress motor actions.

Drugs of abuse engage goal-directed circuitry to promote further acquisition of drugs and the corresponding rewarding sensation. This goal-directed action can be recapitulated in rodent models in which rodents are administered a drug reward acutely. Male rats robustly self-administer acute doses of cocaine that, like sucrose self-administration, diminishes in response to reward devaluation (Olmstead, 2001). The enhancement of goal-directed actions for drug acquisition is, in part, mediated through increased dopamine release in the striatum, which may preferentially activate direct, over indirect, pathway MSNs. This notion is supported by slice electrophysiology studies showing that long-term potentiation of corticostriatal synapses is dependent on D1 receptor activation whereas D2 receptor activation on indirect pathway neurons results in long-term depression (Shen et al., 2008, Surmeier et al., 2009). Acute cocaine and amphetamine exposure leads to increases in striatal dopamine levels (Di Chiara & Imperato, 1988) that result in increased activity of direct pathway MSNs and the suppression of indirect pathway MSNs (Lahiri & Bevan, 2020). By biasing the system toward direct pathway activation, cocaine, and other dopamine-enhancing drugs, act as psychomotor stimulants to increase motor activity. Examining the specific circuits mediating these stimulatory effects, Bariselli et al. (2020) demonstrated that acute cocaine administration enhances OFC transmission to the DMS in male and female mice, and that optogenetically inducing long-term depression of local field potential responses *in vivo* attenuates cocaine-induced hyperactivity.

While initial drug use is characterized by goal-directed actions, repeated drug exposure impairs goal-directed behavior and biases toward habitual action expression mediated by the DLS. This phenomenon is thought to arise, in part, through a decrease in prefrontal cortical control of the DMS. Men and women with substance use disorder exhibit inflexible behavior on decision-making tasks (Bechara et al., 2001; Everitt et al., 2008) that parallel the cognitive deficits seen in patients with damage to the OFC (Bechara et al., 2000, 2004). This cognitive dysfunction diminishes the control over flexible goal-directed actions that would otherwise enable an individual to discontinue maladaptive behaviors such as drug taking. Instead, chronic drug exposure produces dysfunction of the OFC, which promotes impulsive decision making and perseverative actions for stimuli with diminishing reward value. For example, chronic cocaine use reduces gray matter volume in the OFC of men (Franklin et al., 2002), a phenomenon that correlates with the extent of drug use (Ersche et al., 2011). In addition to the structural damage induced by chronic drug use, OFC function, measured by fMRI, is markedly reduced in men and women that chronically abuse alcohol or cocaine (Goldstein & Volkow, 2002). Notably, hypofunction of the OFC can, in some cases, persist up to 10 years following the last drug exposure (Moorman, 2018), illustrating the profound impact chronic drug exposure has on the brain.

Focusing on the projection from the OFC to the striatum, Renteria et al. (2018) demonstrated that chronic ethanol exposure decreases excitatory synaptic transmission from the OFC to the DMS in male and female mice. Specifically, chronic ethanol exposure reduces OFC excitatory transmission onto direct pathway MSNs but not indirect pathway MSNs through an unknown pre-synaptic mechanism. In line with previous studies, these chronically exposed mice exhibit perseverative lever-pressing behavior for natural rewards, suggesting a disruption in top-down control over goal-directed actions. The authors also demonstrate that goal-directed behavior is restored by activating the OFC with excitatory DREADDS (Renteria et al., 2018). Similarly, chronic amphetamine exposure reduces activity in direct pathway MSNs, but not indirect pathway MSNs, in male rats (Furlong et al., 2017). This shift in MSN activity is associated with deficits in goal-directed behavior. As expected, restoring the balance in direct versus indirect pathway MSNs by inhibiting indirect pathway MSNs with an adenosine 2A receptor antagonist restores goal-directed behavior (Furlong et al. 2015). These studies not only identify specific impairments in OFC-striatum circuit function induced by chronic drug exposure, but they also implicate the OFC→DMS circuit as a potential target for therapeutic intervention in substance abuse disorders.

1.9.2 Medial prefrontal cortex→nucleus accumbens/dorsomedial striatum

The medial prefrontal cortex (mPFC) located in the frontal lobe of the brain has a prominent role in executive functions including decision making, error detection, and cognitive control (Bahlmann et al., 2015; Euston et al., 2012; Modirrousta & Fellows, 2008). In rodents, the mPFC includes the infralimbic prefrontal cortex, the prelimbic prefrontal cortex, and the anterior cingulate cortex. Early neuronal tract-tracing studies revealed that the mPFC sends dense excitatory projections to both the DMS and the nucleus accumbens (**Fig. 1.2**; Sesack et al., 1989) where they synapse onto and excite MSNs (O'Donnel & Grace, 1995). These corticostriatal projections predominantly arise from layer V/VI and, to some extent, deep layer III pyramidal neurons (Goldman-Rakic & Selemon, 1986; McFarland & Haber, 2000). However, the nucleus accumbens core additionally receives cortical input from layer II (Ding et al., 2001).

To determine the functional role of corticostriatal transmission, initial studies utilized e*x vivo* slice electrophysiology to characterized synaptic plasticity and elucidate key molecules driving striatal dependent behaviors. The mPFC pathway to the DMS is the predominant glutamatergic input to this area and, post-synaptically, both AMPA and NMDA receptors are expressed on MSNs. These seminal studies revealed that the predominant form of plasticity in the DMS, presumably at corticostriatal synapses, is long-term synaptic potentiation (LTP) that is dependent on NMDA glutamate receptors (Partridge et al., 2000; Dang et al., 2006). Notably, blocking the NMDA receptor, specifically in the DMS, impairs instrumental learning (Yin et al., 2005a).

Later, with the advent of circuit-specific monitoring and manipulation tools such as fiber photometry and optogenetics, respectively, scientists began to investigate how exactly this corticostriatal system governs goal-directed behavior *in vivo*. We now know that as an animal learns an instrumental task, the mPFC→DMS circuit becomes engaged (Kupferschmidt et al., 2017) and through positive reinforcement, synaptic transmission from the cortex to the striatum is potentiated (Reynolds et al., 2001). Additionally, the degree to which this synaptic potentiation occurs correlates with the acquisition of the instrumental task (Reynolds et al., 2001), suggesting that potentiation of corticostriatal synaptic transmission may serve as an important biological mechanism driving goal-directed actions. Testing this hypothesis, Ma et al. (2018) optogenetically stimulated cortical inputs to the DMS in a manner that produced synaptic potentiation or synaptic depression. When corticostriatal synapses were potentiated, this produced long-lasting reward-seeking behavior, but depression of these synapses prevented reward-seeking behavior. This corticostriatal plasticity may be specifically expressed on direct and indirect pathway neurons, as Shan et al. (2014) demonstrated that acquisition of a goal-directed action elicits potentiation of cortical synapses onto direct pathway neurons, and depression at cortical synapses onto indirect pathway neurons.

The NMDA receptor is critical for mediating the ability of drugs of abuse, especially ethanol, to shape action strategies that enhance drug seeking. As ethanol enters the brain, it interacts with NMDA receptors to modulate ion conductance, thereby impacting forms of glutamatergic synaptic transmission that is critical for striatal based learning (Lovinger, White, & Weight, 1989). While modulation of NMDA receptor function varies across brain regions and likely depends on ethanol exposure duration and receptor subunit composition (Kumari & Ticku, 2000; Ron, 2004), acute ethanol administration in the dorsal striatum facilitates NR2B-NMDA receptor function in male rodents (Wang et al., 2007). In contrast, chronic ethanol exposure converts NMDA receptor-dependent LTP to LTD, which likely underlies the deficits in goal-directed behavior seen in chronic alcohol users (Yin et al., 2007).

In addition to the effects of ethanol on NMDA receptor function in the striatum, opiate exposure indirectly modulates striatal NMDA receptor function by acting on neighboring opioid receptors. The resulting opioid activation of G-protein signaling cascades regulate NMDA receptor function through a mechanism referred to as receptor crosstalk (Martin et al., 1996). For example, application of a mu-opioid agonist increases the amplitude of postsynaptic NMDA currents through G-protein activation of protein kinase C (Chartoff & Connery 2014). Additionally, activation of kappa-opioid receptors in the dorsal striatum prevents LTP in direct pathway neurons (Hawes et al., 2017) while activation of mu-opioid receptor produces lasting synaptic depression (Muñoz et al., 2020). Notably, this so-called mu-opioid-LTD is sensitive to ethanol exposure, suggesting a convergent effect of ethanol and the endogenous opioid system or, possibly, an interaction of ethanol and opiates. Therefore, the crosstalk between opioid and NMDA receptors may have important implications for opiate and alcohol dependence (Glass, 2011).

The exact functional role of the mPFC projection to the nucleus accumbens is less clear than that of the DMS projection. This is due in part to the additional projections made by mPFC neurons to the VTA that ultimately modulate dopamine release in both the cortex and nucleus accumbens (Tzschentke & Schmidt, 2000). While considerable attention is paid to the influence of mPFC activity on dopamine release in the nucleus accumbens and the subsequent impact on motivated behavior (Thompson & Moss, 1995), few studies have delineated the influence of the direct mPFC→nucleus accumbens projection. However, recently it was discovered that this circuit plays an important role in integrating internal body cues, or interoceptive cues, for drug-seeking behavior (Randall et al., 2019).

Interoception, or sensation of one's self, enables an individual to maintain a homeostatic balance with the environment. For example, a person will approach a heat source when they feel cold but avoid one if they are too warm. Similarly, rewarding stimuli, including drugs of abuse, elicit an interoceptive response that guides future motivated behavior (Paulus & Stewart, 2014). In preclinical models, interoceptive responses to drugs of abuse can be studied using a drug discrimination task, where an animal learns to press one lever when it feels the effects of a drug, or a second lever when it feels a vehicle injection. Through this task, the animal demonstrates the ability to discriminate the interoceptive feeling of a drug over a vehicle injection. Chemogenetically inhibiting mPFC projections to the nucleus accumbens prevents nicotine and ethanol discrimination responding in male rats (Randall et al., 2019).

The interoceptive response to drug administration is thought to arise from increased glutamate release from cortical afferents into the nucleus accumbens that activate local glutamate receptors. Supporting this idea, cocaine administration increases glutamate release in the nucleus accumbens, which is prevented by lesioning the mPFC (Pierce et al., 1998). This drug-induced surge in glutamate release binds to glutamate receptors in the nucleus accumbens, including mGluR5, to elicit an interoceptive response. For example, Besheer et al. (2009) demonstrated that as inhibiting mGluR5 in the nucleus accumbens prevents ethanol discrimination while activating mGluR5 receptors enhance discrimination of a sub-threshold ethanol dose.

1.9.3 Somatosensory and Motor cortex→dorsolateral striatum

Pyramidal neurons in the deep layers of the somatosensory and motor cortices send excitatory projections to the DLS to integrate and transpose cortical information into selected actions (**Fig. 1.2**). These excitatory inputs converge onto the same MSNs to elicit action potential firing (Charpier et al., 2020), suggesting that somatosensory and motor information integrate to produce a selected motor response. Indeed the topographical organization seen in the somatosensory and motor cortices extend to the striatum in primates (Flaherty & Graybiel, 1994; McFarland & Haber, 2000), rats (Berendse et al.,1992) and mice (Hintiryan et al., 2016). However, more recent evidence suggests that the topographic compartmentalization in the striatum is more complicated and varies across cell type and cortical area (Hooks et al., 2018).

High frequency activation of somatomotor cortical afferents to the DLS elicits LTD onto MSNs (Calabresi et al., 1992; Lovinger, Tyler, & Merritt, 1993). This is a phenomenon that likely underlies the learning adaptations governing action automatization and, if taken further, habit formation. Supporting this notion, Yin et al. (2009) and Hawes et al. (2015) demonstrated that as a motor skill is learned, corticostriatal LTP is enhanced in the DMS, but as a motor skill is automatized, through repeated training, corticostriatal LTD in the DLS is augmented. This highlights the fundamental role that plasticity plays in the myriad actions expressed in our daily lives, and it should come as no surprise that aberrant corticostriatal synaptic plasticity contributes to the compulsive behavior seen in obsessive compulsive disorder (Graybiel & Rauch, 2000) and addiction (Gerdeman et al., 2003).

These corticostriatal plasticity mechanisms do not exist in a glutamate-only vacuum. Corticostriatal LTD is dependent on local dopamine release that activates D2 dopamine receptors on MSNS and cholinergic interneurons (Wang et al., 2006; Lerner & Kreitzer 2012; Augustin et al., 2018). This orchestrated corticostriatal and dopamine signaling mobilizes the release of endocannabinoids that act on presynaptic cannabinoid type-1 (CB1) receptors to reduce synaptic transmission from cortical terminals onto MSNs (Gerdeman & Lovinger, 2000). The complex interplay of acetylcholine, dopamine, endocannabinoid neuromodulatory signaling underlying this glutamatergic corticostriatal plasticity leads to potential LTD perturbation by several abused substances that target these component neurotransmitter systems (e.g., nicotine, stimulants, cannabinoids, and ethanol).

As the corticostriatal synapse is the most abundant synapse type on MSNs and corticostriatal LTD is linked to action learning (Yin et al., 2009), corticostriatal LTD represents a key mechanism by which drugs of abuse expedite the automatization of actions leading to drug reward. Accordingly, manipulation of the underlying neurotransmitter systems with drugs of abuse or experimental manipulations modulates habit formation. For example, chronic cocaine exposure biases the use of habitual action strategies for drug reward in male rats (Schmitzer-Torbert et al., 2015; Zapata et al., 2010). Inactivation of the dopamine neurons that project to the DLS abolishes habitual lever pressing behavior in male rats (Faure et al., 2005). Additionally, Murray et al. (2012) demonstrated that simply inhibiting dopamine receptors in the DLS is sufficient to abolish habitual cocaine-seeking behavior in male rats. Therefore, psychostimulants are positioned to disrupt corticostriatal synaptic transmission to promote habitual drug-seeking and -taking behavior. Chronic drug exposure may have more generalized effects on reward-related behavior, as chronic ethanol exposure (Corbit et al. 2012) and amphetamine exposure (Nelson & Killcross, 2006) both enhance habitual responding for natural rewards in male rats. Indeed, clinical reports indicate that individuals with substance use disorder feel overpowered by habitual actions, including but not limited to habitual drug use (Seo & Sinha 2014).

Provided the critical role of cholinergic neurons in DLS LTD, it is likely that nicotine, an acetylcholine nicotinic receptor agonist, significantly impacts this form of plasticity and, therefore, could have a dramatic impact on habitual behavior. In a recent human study, it was shown that that the degree of nicotine dependence in men and women was positively correlated to habitual responding on a stimulus-response task (Luijten et al., 2019). The authors noted that this effect was accompanied by reductions in goal-directed responding, resulting in a shift toward predominantly habitual responses. In line with these results, preclinical studies of nicotine use demonstrate that, initially, nicotine seeking is goal-directed (Clemens et al., 2014), but with additional training and exposure, responding for nicotine becomes habitual in male rats (Loughlin et al., 2017). As discussed above, this transition from goal-directed behavior to habitual responding involves corticostriatal plasticity and this process is certainly modulated by repeated nicotine exposure. For example, in brain slices, nicotine exposure depresses excitatory transmission onto MSNs, an effect that is dependent on D2 dopamine receptor activation (Licheri et al., 2018). Additionally, Partridge et al. (2002) demonstrated that activation of nicotinic receptors facilitates corticostriatal LTD in the DLS. Collectively, these results suggest that the chronic nicotine exposure may dramatically influence striatal plasticity to underlie habitual behaviors that emerge with repeated drug use.

In addition to drugs that act directly on the transmitter systems underlying corticostriatal LTD, such as nicotine on acetylcholine receptors, chronic ethanol also disrupts striatal LTD by modulating cannabinoid signaling. Chronic ethanol exposure impairs striatal LTD in male rats (Adermark et al 2011) and mice (Depoy et al. 2013), which is recovered by a high dose application of a CB1 receptor agonist (Adermark et al. 2011). Even acute ethanol exposure disrupts endocannabinoid mediated LTD in the dorsal striatum in male rats (Clarke & Adermark 2010). The effect of chronic ethanol exposure on cannabinoid signaling is likely arises from ethanol-induced decreases in CB1 mRNA expression in the striatum (Adermark et al., 2011). Indeed, men with alcohol use disorder exhibit significantly reduced CB1 receptor binding, a proxy for CB1 receptor expression, across multiple brain regions including the dorsal and ventral striatum (Hirvoven et al., 2012). Notably, these authors revealed that the degree of CB1 receptor binding negatively correlates with the duration of alcohol abuse, further supporting the relationship between ethanol exposure and CB1 receptor expression (Hirvoven et al., 2012).

The effects of chronic ethanol exposure on striatal endocannabinoid signaling and corticostriatal plasticity likely impact subsequent ethanol consumption, as systemic administration of a CB1 receptor agonists increases voluntary ethanol consumption in male rats (Colombo et al., 2002), while CB1 receptor antagonists decreases voluntary ethanol consumption (Serra et al., 2001). Subsequent studies demonstrated that delivery of a CB1 receptor antagonist into the nucleus accumbens reduces ethanol self-administration in male rats (Caillé et al., 2007, Alvarez-Jaimes et al. 2009). However, future studies are needed to determine if these effects are replicated in the dorsal striatum. Regardless, these studies collectively demonstrate that multiple drugs of abuse significantly impact DLS synaptic plasticity, which is a key mechanism for action learning. Therefore, dissecting the mechanisms underlying various forms of striatal glutamatergic plasticity and how drugs of abuse hijack them may lead to new treatment avenues.

1.9.4 Thalamus→nucleus accumbens/dorsal striatum

The thalamus is a collection of nuclei supporting a multitude of functions. Some of these nuclei can be characterized as sensory relay nuclei, as they receive primary sensory input from the periphery and relay this information to the cortex. Another set of thalamic nuclei are categorized as association nuclei, which integrate information from a variety of cortical and subcortical sources and output to multiple cortical and subcortical areas (Cover & Mathur 2021; Sherman 2016). The striatum receives glutamatergic projections from a subset of association thalamic nuclei, called the intralaminar nuclei of the thalamus, which is anatomically divided into rostral and caudal regions (**Fig. 1.2**; Parent et al., 1983; Matsumoto et al., 2000). The caudal region of the intralaminar nuclei contains the center median (CM) and parafascicular (Pf) nuclei in primates, while in rodents the caudal region only consists of the Pf nucleus (Groenewegen & Berendse, 1994; Van der Werf et al., 2002). The rostral intralaminar nuclei (rILN) consists of the central medial, the paracentral, and the central lateral nuclei (Cover & Mathur, 2021; Fisher & Reynolds, 2014). Additionally, the midline thalamus, consisting of the paraventricular nucleus (PVN) and paratenial nucleus, sends projections to the dorsal striatum and nucleus accumbens in the rat (**Fig. 1.2**; Vertes & Hoover, 2008). These excitatory afferents synapse onto striatal MSNs and exhibit greater projection density onto direct pathway neurons, as measured by electron microscopy (Sidibé & Smith, 1996). The information traveling through this thalamostriatal circuit depolarizes MSNs to a subthreshold "up state" (Shulz et al., 2009) that likely informs the striatum of salient events that, in conjunction with dopamine signals, reinforces action learning (Fisher & Reynolds, 2014). Notably, there is considerable convergence of thalamostriatal and corticostriatal projections onto striatal MSNs (Hunnicutt et al., 2016) that likely interact to influence striatal function and action expression.

A key driver of drug addiction is the conditioned response to drug-related stimuli that persists despite negative feedback, suggesting aberrant thalamic function in chronic users (Huang et al., 2017). Supporting this hypothesis, chronic drug exposure increases non-specific thalamic activity in response to drug cues in men and women (Zilverstand et al., 2018). FMRI studies demonstrate hyperresponsivity of the thalamus to drug cues in male and female cocaine users (Ray et al., 2015), heroin users (Li et al., 2013), and alcohol-dependent individuals (Feldstein Ewing et al., 2010). Therefore, an understanding of the interaction between thalamic structures and the striatum is required to conceptualize how this aberrant thalamic activity shapes drug-seeking behavior.

Early models of basal ganglia function suggest that the striatum regulates appropriate actions by providing start and stop commands to downstream control circuits (Graybiel, 1998; Jin & Costa, 2015). This hypothesis is supported by electrophysiology recordings from rodents (Barnes at al., 2005; Jin & Costa, 2010) and primates (Desrochers et al., 2015) showing that activity in the striatum aligns with the initiation and termination of trained motor behaviors. Within this model, the striatum is activated by cortical projections from motor cortex that dictate which striatal motor program is executed. However, more recent studies demonstrate that the striatum encodes important kinematic information for the elements that comprise an action sequence, including action speed (Wolf et al.,2019; Roberts et al. 2019).

Recently it was demonstrated that once learned, an action can be executed without the participation of motor cortex, suggesting that a motor program is potentially acquired from the cortex and stored in the dorsal striatum where it is then re-activated by an alternative source (Kawai et al., 2015). Since MSNs require excitatory input to fire action potentials, re-activation of a stored motor program would theoretically require drive from an excitatory structure outside the cortex. A candidate that would meet the necessary requirements to drive MSN activity and, therefore, learned actions that are stored in the dorsal striatum, is the intralaminar thalamus. The intralaminar thalamus provides extensive excitatory projections to the striatum. Supporting the role of intralaminar thalamostriatal circuitry in motor learning, Wolff et al. (2019) demonstrated that initial motor learning requires cortical drive of the DLS, but once a motor skill is learned these actions are evoked by thalamic inputs to the DLS. The authors suggest that the motor cortex transfers relevant motor information to the intralaminar thalamus through convergent activation of MSNs in a manner that requires synaptic plasticity. Testing this hypothesis, the authors demonstrate that inhibiting long-term synaptic plasticity with PKM zeta inhibitor ZIP in the striatum, but not the cortex, disrupts motor skill learning. This work suggests that thalamostriatal circuits play a critical role in the expression of, at least, well-learned actions that support drug-seeking behavior.

Given this role of the intralaminar thalamus, inputs from these nuclei to the striatum unsurprisingly play an important role in drug-seeking behavior: inactivation of the rostral grouping of the intralaminar nuclei (including central lateral, central medial, and paracentral nuclei) projections to the DMS decreases methamphetamine-seeking behavior after 30 days of withdrawal in male rats (Li et al., 2018). As such, these thalamostriatal circuits likely play a key role in the so-called incubation of craving for drug reward during extended withdrawal. Despite the apparent importance of this thalamostriatal circuit in mediating drug-seeking behavior, very little is known about how drugs of abuse modulate thalamostriatal signaling. While a simple assumption would be that both cortical and thalamic excitatory synapses function similarly, slice electrophysiology studies demonstrate that these inputs exhibit different glutamate release probabilities (Ding et al., 2008) and that thalamostriatal inputs, but not cortical inputs, undergo synaptic depression by activation of mu-opioid receptors (Atwood et al., 2014). Importantly, this mu-opioid dependent LTD was lost after acute injection of oxycodone, suggesting that opiate exposure selectively impacts thalamostriatal transmission.

In addition to direct contacts onto striatal MSNs, thalamic terminals arising from intralaminar nuclei also innervate striatal cholinergic interneurons (Lapper & Bolam, 1992; Aosaki et al., 1995). This circuit has received considerable attention since cholinergic signaling plays a central role in striatal-dependent learning (Diaz del Guante et al., 1991). Striatal cholinergic interneurons are tonically active (Wilson et al., 1990; Aosaki et al., 1995; Kawaguchi et al., 1995), but exhibit burst-pause firing behavior in response to salient reward and aversive sensory input in monkeys (Yamada et al., 2004). This burst-pause behavior is abolished by pharmacological inactivation of the monkey CM/Pf (Matsumoto et al., 2000). Additionally, optogenetically activating intralaminar nucleus terminals produces burst firing in cholinergic interneurons (Ding et al., 2010). This burst activity was coupled to a decrease in MSN activity, followed by a selective increase in sensitivity of indirect pathway MSNs to cortical stimulation (Ding et al., 2010). This suggests that by resetting cortical input, this thalamic input facilitates shifting behavior to respond to updated information about a reward contingency. Supporting this idea, in a reversal learning task where an animal learns that a previously rewarding stimulus is no longer rewarding, there is an increase in striatal acetylcholine that arises with Pf activation in rats (Brown et al., 2010). Moreover, inactivating Pf inputs to the striatum produces a learning deficit in rats when action-outcome contingencies have changed (Bradfield et al., 2013). Thus, the Pf-to-cholinergic interneuron circuit may update new and existing information to enable accurate goal-directed actions.

As striatal acetylcholine and dopamine act in concert to mediate behavioral reinforcement and drug-related behavior, it stands to reason that thalamostriatal inputs arising from the intralaminar nuclei may also modulate dopamine release in the striatum. Indeed, Threlfell et al. (2012) showed in the dorsal striatum of mice that activation of Pf inputs elicits dopamine release in a cholinergic interneuron-dependent manner. In addition, the rILN also display this ability: activation of these afferents in the dorsal striatum elicits stable dopamine release (Cover et al., 2019). Thus, intralaminar thalamic afferent activation drives cholinergic interneuron release of acetylcholine, which in turn, activates nicotinic receptors on nigrostriatal terminals to release dopamine (Threlfell et al., 2012, Cover et al., 2019). This suggests a possible mechanism for how nicotine may modulate striatal dopamine and promote reward-driven behaviors. For example, Cover et al. (2019) demonstrated that mice will self-stimulate the rILN→dorsal striatum circuit, which is dependent on D1 dopamine receptor activation. Considering that the rILN are required for incubation of methamphetamine craving in a D1 dopamine receptor-dependent manner in male rats (Li et al., 2018), the rILN are positioned as an emerging target for therapeutic intervention in addiction.

In addition to the intralaminar nuclei, the PVN has also received considerable attention for its role in reinstatement of drug-seeking behavior, which is thought to manifest from associations between drug cues and the resulting drug reward (Huang et al., 2017). Indeed, PVN inactivation studies demonstrate that this structure is necessary for reinstatement of drug-seeking behavior, including cocaine-seeking behavior in male rats (Matzeau et al., 2015; Hamlin et al., 2009). PVN neurons that project to the nucleus accumbens appear to be critical for reinstatement of drug-seeking behavior, as reinstatement of ethanol seeking results in greater activation of striatal-projecting PVN neurons (Hamlin et al., 2009) and selective inhibition of this circuit attenuates the acquisition of cocaine self-administration behaviors in male rats (Neuman et al., 2016). Taken together, these studies suggest that several thalamic nuclei projecting to the striatal complex act in concert to prime behavioral engagement in drug-seeking behavior once these behaviors are learned, and stored, in ensembles of striatal MSNs (Cover & Mathur 2021).

1.9.5 Amygdala→nucleus accumbens/dorsal striatum

The amygdala is a limbic structure that encodes emotional valence of environmental stimuli (Kaada, 1972). The collection of nuclei forming the amygdaloid complex consists of three distinct subregions, the basolateral amygdala (BLA), the medial amygdala, and the central amygdala (CeA), each of which play unique roles in emotional processing (Hrybouski et al., 2016). The striatum receives inputs predominantly from the BLA (Christie et al. 1987; McDonald 1991, 1992; Pan et al., 2010) that specifically arise from glutamatergic pyramidal (class I) BLA neurons but not GABAergic (class II and III) BLA neurons (Christie et al. 1987; McDonald 1992; Zorilla & Koob 2013). These BLA projections primarily innervate the nucleus accumbens (**Fig. 1.2**; Krettek & Price, 1978, Keley et al., 1982), but also more sparsely innervate the dorsal striatum (Pan et al., 2010; Cho et al., 2013).

The BLA is of particular interest for this chapter as it receives robust sensory information from prefrontal cortex and thalamus and relays this information to the striatum to influence motivational learning (Wassum & Izquierdo, 2016). Structurally, Cho et al.

(2013) revealed that the topographical organization in the cortex is maintained throughout the cortico-amygdala-striatal circuit, which the authors suggest promotes the integration of internal emotional states with external social and sensory information to inform emotionally complex behaviors. Zooming in on the BLA projections to the nucleus accumbens, Ambroggi et al., (2008) demonstrated that cue-evoked excitation of accumbens MSNs depends on the BLA and that inactivating the BLA diminishes lever-pressing performance on a discriminative stimulus task in male rats. Additionally, projections from the BLA and the mPFC converge in the nucleus accumbens, suggesting that the BLA may also influence motivated behavior by modulating cortical drive of nucleus accumbens MSNs (McGinty & Grace, 2008). For example, train stimulating of BLA inputs to the nucleus accumbens depresses cortically-evoked spiking of striatal MSNs (McGinty & Grace, 2009). Conversely, Popescu et al. (2007) demonstrated that BLA inputs to the accumbens potentiate corticostriatal LTP through NMDA receptor activation. While the direction of these two forms of modulation are inconsistent, perhaps due to different stimulation conditions of amygdala projection neurons, these two studies clearly demonstrate the ability of the amygdala to modulate cortical drive of the nucleus accumbens. This likely serves an important mechanism by which the amygdala provides emotional incentive to shape motivated behavior that is driven by the cortex.

In addition to the major input to the nucleus accumbens, the BLA also sends projections to the medial and posterior regions of the DMS (Kelley et al., 1982). This circuit may encode emotional significance to guide goal-directed actions to a reward (Corbit et al., 2012). This contribution may additionally require communication with cortical structures, as Fisher et al (2020) recently demonstrated that amygdala projections to the mPFC, but not the DMS, are critical for goal-directed learning in male and female mice. However further investigation of this specific BLA projection is needed to further characterize its functional contributions to motivated behavior.

Drugs of abuse dramatically impact BLA function is it impairs the ability to properly update reward incentive values for environmental cues. This results in maladaptive behavioral responses including the propensity to express habitual actions (Wassum & Izquierdo, 2016). Functional imaging studies demonstrate amygdala dysfunction in nicotine addicted men and women (Mihov & Hurlemann, 2012), where smoking cues elicit increased amygdala activity in abstinent smokers but not in nonsmokers (Due et al., 2002). Additionally, the amygdala response to unpleasant stimuli is decreased in nicotine using men and women, indicating impaired encoding of emotional stimuli (Kobiella et al., 2010). Similarly, alcohol intoxication dysregulates amygdala response to emotional cues in men and women (Gilman et al., 2008). In cocaine-dependent men and women there is elevated activity in the amygdala (Kilts et al., 2001; Bonson et al., 2002) and these effects last for months after termination of drug treatment, which demonstrates the long-lasting effects of drug use on brain function (Schoenbaum & Setlow, 2005). Unfortunately, these functional imaging studies could not specify the exact location of activation within the amygdaloid complex. However, considering the rodent studies that will be discussed in this section, it is likely that BLA dysfunction is integral to substance use disorder. A prevailing concept is that a dysfunctional BLA response to normal emotional cues may predispose one to ignore salient, emotionally laden cues in the environment that would normally shift behavior away from drug seeking.

Another view is supported by Parkes and Balleine (2013) who reported that the BLA encodes outcome values forming an “incentive memory” that influences future actions in male rats. In the context of substance abuse, actions that result in the acquisition of drug are similarly encoded by the BLA to form “drug memories” that guide future behavior toward a known drug reward (Luo et al., 2013). Supporting this, inactivating the BLA prevents the formation of these drug memories including cocaine- (Fuchs et al., 2002) and morphine-induced conditioned place preference in male rodents (Rezayof et al., 2002). Furthermore, inactivating the BLA abolishes cocaine seeking behavior in male rats (Whitelaw et al., 1996).

The process of encoding drug-reward associations likely occurs through dopamine release from VTA dopamine neurons that send extensive projections to the BLA and nucleus accumbens (Loughlin & Fallon, 1982; Haber & Fudge 1997). For example, male rats self-administering cocaine exhibit a dopamine surge in the amygdala and nucleus accumbens while pressing for the cocaine-paired lever but not the unpaired lever (Weiss et al., 2000). Following this result, infusion of a D1 but not D2 dopamine receptor antagonist into the BLA prevents cue-induced reinstatement of cocaine-seeking behavior in male rats (See et al., 2001). Subsequent studies revealed that BLA projections to the nucleus accumbens drive cue-induced reinstatement of cocaine-seeking behavior, as inhibiting this circuit pharmacologically (Di Ciano & Everitt, 2004) or chemogenetically (Puaud et al., 2021) prevents reinstatement of cocaine-seeking behavior in male rats.

The BLA also plays a key role in value encoding for ethanol reward that is critical for ethanol-seeking behavior (Gremel & Cunningham, 2009). Several mechanisms may be at play to underlie this effect. In a mechanically isolated neuron/bouton preparation, acute ethanol exposure increases GABAergic transmission from local interneurons in male rats (Zhu & Lovinger, 2006; Silberman et al., 2009), while chronic ethanol exposure increases glutamatergic signaling onto BLA pyramidal neurons in male and female rats (Läck et al., 2007; Morales et al., 2018). Providing a possible mechanism underlying this effect, it was recently demonstrated that chronic ethanol exposure upregulates acetylcholine release in the BLA that activates presynaptic alpha-7 nicotinic acetylcholine receptors to enhance glutamatergic transmission (Sizer et al., 2021). Additionally, withdrawal from chronic ethanol exposure robustly decreases GABA release from interneurons in the BLA of male rats (Diaz et al., 2011) and increases glutamatergic transmission through increased AMPA receptor expression (Christian et al., 2012). These authors suggested that by reducing inhibitory tone and increasing excitation of the BLA, chronic ethanol exposure produces long-lasting changes of amygdala function that ultimately shape incentive learning. For example, Gass et al. (2011) recorded simultaneous glutamate release in the BLA and nucleus accumbens in chronic ethanol-treated male rats and demonstrated increased glutamate release in both structures during cue-induced reinstatement of ethanol-seeking behavior. This would suggest that the BLA drives nucleus accumbens projection neurons to promote ethanol-seeking behavior. Supporting this idea, pharmacologically inactivating the BLA or the nucleus accumbens reduces Pavlovian responses for ethanol reward in male rats (Chaudhri et al. 2013; Millan et al. 2015). Following these results, Millan et al. (2017) demonstrated that specifically disrupting synaptic transmission from the BLA to the nucleus accumbens using optogenetics reduces conditioned ethanol consumption in rats, supporting the role of this circuit in ethanol reward associative learning (Millan et al. 2017).

Relapse is a significant obstacle in recovering substance abusers that is thought to arise from conditioned responses to drug-cues in the environment that illicit drug-seeking behavior (Weiss 2005). Examining the role of the BLA→nucleus accumbens circuit in ethanol relapse, Keistler et al. (2017) ablated BLA projection to the accumbens in male rats self-administering ethanol and tested cue-induced reinstatement following ethanol extinction. The authors found that ablating the BLA→ nucleus accumbens projection attenuates cue-induced ethanol reinstatement, but also that ethanol reinstatement is completely blocked when inputs from the mPFC→ nucleus accumbens are ablated. Considering the electrophysiology data previously discussed, where inputs from the BLA modulate cortical excitation of accumbens MSNs (McGinty & Grace, 2008), these data collectively demonstrate that the BLA encodes important reward cues from the environment, including drug-associated cues, and relays this information to the nucleus accumbens to guide behavior toward drug acquisition.

As an individual with substance use disorder cycles through episodes of repeated drug abuse and withdrawal, the stress response system adapts by becoming hyperactive. This produces anxiety and reward deficits that promote further drug use in order to alleviate these negative symptoms (Koob et al., 2014; Koob & Volkow 2016). Contributing to this viscous cycle, stress negatively impacts the function of both the amygdala and the nucleus accumbens, which play a key role in the negative affective state seen in addiction. Specifically, the amygdala and nucleus accumbens exhibit aberrant responses to reward and aversive cues, respectively, where the degree of this aberrant response predicts a greater risk of alcohol abuse in young men and women (Nikolova et al. 2016). This dysregulated activity is associated with increased stimulus-response behavior in men

(Vogel et al., 2016), which as we mentioned previously contributes to compulsive drug seeking and consumption in addiction. Notably, administration of an antagonist to the receptor for the stress hormone mineralocorticoid, corrects aberrant amygdala activity and stimulus-response behavior in men (Vogel et al., 2016). The dysregulated activity of the amygdala and nucleus accumbens may arise from a loss of top-down control from the mPFC, as chronic stress decreases activity in the mPFC and increases activity in the BLA in male rats (Piggott et al., 2019).

1.9.6 Hippocampus→nucleus accumbens

The hippocampus, located in the medial temporal lobe of the brain, is one of the most well-studied systems in the brain (Bird & Burgess, 2008). Dating back to early clinical studies of patients with damage to the temporal lobe, we learned that the hippocampus plays a fundamental role in binding memories to form complex representations that are necessary for spatial and contextual memory (Kutlu & Gould, 2016). In the context of drug abuse, the hippocampus promotes maladaptive drug-context associations that enable an individual to navigate toward a known drug source. This likely occurs through excitatory projections from the hippocampus to the nucleus accumbens, which synapse onto and excite accumbal MSNs (**Fig. 1.2**; Groenewegen et al., 1982, 1996; Kelley & Domesick, 1982). Additionally, the hippocampus indirectly influences the dorsal striatum through its connections with the mPFC and OFC and provides important spatial information to help overcome multiple possible actions related to a specific location in order to maintain accurate goal-directed behavior (Goodroe et al., 2018).

Subsequent neuronal tract-tracing studies demonstrate that the hippocampus and mPFC send excitatory projections that converge onto nucleus accumbens MSNs (French & Totterdell, 2002), giving rise to the idea that hippocampal input influences and possibly gates information from the cortex. Provided that MSNs do not fire spontaneously but instead require a significant amount of excitatory input to reach spike threshold (Pennartz et al., 1994), convergent excitatory input may be necessary to elicit MSN spike firing. Supporting this idea, excitation of only hippocampal inputs brings MSNs to a depolarized state (O'Donnell & Grace, 1995), and only in this depolarized state can inputs from the mPFC evoke spike firing (French & Totterdell, 2002). Hippocampal gating of cortical input to the nucleus accumbens is likely the phenomenon underlying the ability to use contextual cues to maintain goal-directed action despite changes in, or in response to, spatial context.

Chronic drug exposure has a dramatic impact on hippocampal function, disrupting hippocampal neurogenesis (Eisch & Harburg, 2006) and synaptic plasticity in rodents (Thompson, Gosnell, & Wagner, 2002) that collectively contribute to maladaptive behavior in addiction. For instance, amphetamines, after their release as a prescription drug in 1935, were marketed for their ability to enhance memory recall (Zeeuws & Soetens, 2007) attention (Johnson et al., 2000) and working memory (Mattay et al., 2000) in men and women. In contrast, chronic amphetamine use produces dramatic deficits in memory, attention and decision making in men and women (Ornstein et al., 2000; Nordahl et al., 2003; Simon et al., 2010). Preclinical studies demonstrate the specific impact of chronic amphetamine use on hippocampal-dependent learning tasks, including deficits in spatial object recognition and spatial navigation on the Morris water maze in male mice (Mandillo et al., 2003). This suggests an enhancement of hippocampal→nucleus accumbens activity, particularly in early stages of addiction, that serves to strengthen contextual memories associated with drug use that are later triggered by environmental stimuli signaling drug availability.

Further supporting this concept, acute cocaine exposure enhances hippocampus→nucleus accumbens synaptic strength in male mice (Britt et al., 2012). This occurs by increasing postsynaptic AMPA receptor expression in MSNs, specifically onto direct pathway MSNs (Pascoli et al., 2014). Following the go/no-go model of striatal function, such hippocampus-to-accumbens cocaine-induced enhancement of direct pathway output would foster drug reinforcement. Indeed, Pascoli et al. (2014) demonstrated that reversing plasticity from these hippocampal inputs abolishes drug-seeking behavior in male and female mice. Similarly, acute ethanol exposure enhances glutamatergic synaptic transmission from hippocampus to direct pathway MSNs and abolishes LTD at these synapses by increasing the insertion of calcium permeable AMPA receptors into the postsynaptic membrane (Kircher et al., 2019). These preclinical studies demonstrate that acute drug exposure facilitates hippocampal drive of nucleus accumbens that likely subserves context-dependent drug reinforcement. However, chronic drug exposure impairs hippocampal function and disrupts hippocampus-dependent learning that likely facilitates continued drug use despite attempts to learn new reward associations related to sobriety (Kutlu & Gould, 2016).

1.10 Inhibitory Striatal Circuits in Motivated Behavior and Addiction

In addition to excitatory projections driving MSN activity, the striatum receives inhibitory signals from extrinsic and intrinsic sources. For example, the dorsal pallidum sends inhibitory projections that terminate in the dorsal striatum (Staines, Atmadja, & Fibiger, 1981; Staines & Fibiger, 1984) and the ventral pallidum sends inhibitory projections to the nucleus accumbens (**Fig. 1.2**; Churchill & Kalivas 1994). These projections provide the largest extrinsic source of GABAergic inhibition to the striatum and synapse onto striatal MSNs, cholinergic interneurons and parvalbumin expressing fast-spiking interneurons (Mallet et al., 2012). The reticular thalamic nucleus, a thin shell of neurons surrounding the dorsal thalamus, provides an additional source of extrinsic GABAergic inhibition to the striatum (**Fig. 1.2**; Minderhound, 1971). These projections synapse onto and inhibit parvalbumin-expressing GABAergic fast-spiking striatal interneurons (Klug et al., 2018). In addition to these early studied sources of extrinsic inhibition, cortical somatostatin-expressing interneurons send inhibitory projections to the striatum that directly inhibit MSNs, thereby providing an addition source of extrinsic inhibitory control (Rock et al., 2016).

Locally, the striatum receives strong inhibitory control from interneurons, which includes the fast-spiking interneurons, the low-threshold spiking interneurons, the calretinin expressing interneurons, and the TH-expressing interneurons (Dubach et al., 1987; Koós & Tepper, 1999; Ibáñez-Sandoval et al., 2010; Tepper et al., 2010). The most well-studied inhibitory interneuron in the striatum is the fast-spiking interneuron (Tepper et al., 2010) that synapses onto proximal MSN dendrites and somata (Kita et al., 1990) and evokes large-amplitude inhibitory postsynaptic potentials (Koós & Tepper, 1999, 2002). Fast-spiking interneurons synapse onto both direct and indirect MSNs as well as other fast-spiking interneurons, but do not synapse onto cholinergic interneurons or low-threshold spiking interneurons (Gittis et al., 2010). In addition to the reciprocal synaptic connections between pairs of fast-spiking interneurons, these neurons form an interconnected network through dendro-dendritic gap junctions (Kita et al., 1990; Koós & Tepper, 1999). Together, fast-spiking interneurons delay or entirely blocking action potentials in many MSNs simultaneously (Koós & Tepper, 1999). Through this network, striatal fast-spiking interneurons provide a strong feedforward inhibition that regulates MSN spike timing to shape the striatal output signal (Koós & Tepper, 1999; Tepper, 2010).

The low-threshold spiking interneurons were characterized, in part, by the presence of a low-threshold calcium spike (Kawaguchi, 1993), and a significant portion of recorded neurons exhibit spontaneous activity (Tepper et al., 2010). The low-threshold spiking interneurons synapse onto distal dendrites of MSNs (DiFiglia & Aronin, 1982; Kubota & Kawaguchi, 2000) and form synaptic contacts with cholinergic interneurons (Vuillet et al., 1989a, b). The inhibitory input from these neurons produces relatively weak GABAergic inhibitory postsynaptic currents in MSNs (Gittis et al., 2010). This may be due to low expression of the GABA synthetic enzyme glutamate decarboxylase expressed in these neurons (Chesselet & Robbins, 1989; Kubota & Kawaguchi, 2000).

The calretinin-expressing interneurons are much less studied, and our knowledge of this cell type depends on immunostaining, as these cells have not been knowingly recorded electrophysiologically (Tepper et al., 2010). These morphological studies indicate that calretinin-expressing interneurons extend aspiny dendrites and can be categorized into three distinct subtypes in the mouse striatum (Tepper et al., 2010). Lastly the TH-expressing interneurons are comprised of a heterogenous class that has been characterized into four types based on intrinsic electrophysiological properties and morphology (Ibáñez-Sandoval et al., 2010). Type I, II, and III neurons innervate striatal MSNs and elicit large amplitude inhibitory post-synaptic potentials that are strong enough to delay MSN action potential firing (Ibáñez-Sandoval et al., 2010). Thus, these TH-positive interneurons provide an additional source of inhibitory control of MSNs.

Although less studied than glutamatergic transmission, there is significant evidence demonstrating that drugs of abuse modulate striatal GABAergic transmission, which likely contributes to maladaptive behaviors seen in substance use disorder. For example, acute cocaine and amphetamine exposure depresses GABAergic inhibitory postsynaptic currents on striatal MSNs, which is mediated by activation of the D2 dopamine receptor in male rats (Centonze et al., 2002). Additionally, opiates, including morphine and heroin, elevate VTA dopaminergic neuron activity by inhibiting presynaptic GABAergic interneurons (Xi & Stein, 2002). For example, acute opiate administration decreases the firing rate of interneurons while increasing the firing rate of VTA dopamine neurons in male rats (Kelley et al., 1980). Additionally, administering gamma-vinyl GABA in to the VTA, an irreversible GABA-transaminase inhibitor, completely blocks heroin-induced dopamine release in the nucleus accumbens and heroin self-administration in male rats (Xi & Stein 2000). This opiate-induced dopamine release is mediated by $GABA_B$ receptors in the VTA, as administering a $GABA_B$ agonist blocks heroin self-administration and nucleus accumbens dopamine release in male rats (Xi & Stein 1998,1999). Further supporting for the involvement of the $GABA_B$ receptor in opiate reward, Tsuji et al. (1996) demonstrated that administering a $GABA_B$ agonist also inhibits morphine conditioned place preference in male rats.

Arguably the most well studied drug-mediated change of GABAergic transmission in the brain, including in the striatum, is by ethanol (Davis & Wu, 2001). While ethanol directly interacts with $GABA_A$ receptors to enhance GABA responses in dorsal root ganglion preparations (Lobo & Harris, 2009), ethanol actions on GABAergic synapses in the striatum is more complicated. In the nucleus accumbens, ethanol does not alter baseline GABAergic synaptic transmission (Patton et al., 2019). Instead, a TrkB-dependent form of long-term synaptic depression of inhibitory inputs to the nucleus accumbens is enhanced by ethanol exposure in male and female mice, which may serve as a physiological substrate for ethanol modulation of nucleus accumbens-mediated reward encoding (Patton et al., 2019). In the dorsal striatum, chronic ethanol exposure selectively reduces the frequency and amplitude of spontaneous GABAergic transmission onto MSNs in the putamen of male cynomolgus monkeys (Cuzon Carlson et al., 2011) and decreases the frequency of inhibitory transmission onto dorsal striatum MSNs in male mice (Wilcox et al., 2014). Notably, this effect was more prominent in the DLS compared to the DMS, and this difference may be due to differential effects of chronic alcohol exposure on D1 versus D2 MSNs, as Cheng et al. (2017) demonstrated that chronic ethanol consumption strengthens inhibitory transmission onto D2 neurons in the DMS of male mice. However, a more detailed investigation using transgenic mouse lines is needed to determine whether inhibitory transmission is altered by alcohol exposure specifically onto D1 or D2 MSNs in both subregions of the dorsal striatum. Interestingly, acute ethanol exposure does not directly impact MSN physiology, but instead indirectly influences MSN activity through its actions on striatal interneurons. For example, acute alcohol exposure increases intrinsic excitability of FSIs in male and female rats by suppressing potassium currents (Blomeley et al., 2011). Furthermore, acute ethanol exposure reduces inhibitory transmission from striatal fast-spiking interneurons onto MSNs by activating presynaptic delta-opioid receptors in male and female mice (Patton et al., 2016). Notably, these striatal fast-spiking interneurons are critical for habit formation (O'Hare et al., 2017) and support the formation of habitual action strategies that drive compulsive ethanol consumption (Patton et al., 2020). Continued investigation into the effects of abused substances on GABAergic transmission in the striatum will benefit from technologies that allow circuit-specific manipulations to reveal the specific sources of GABAergic transmission that are impacted by acute and chronic exposure to various drugs of abuse

1.11 Sex Differences in Substance Use Disorder

It is important to note that many, if not most, of the studies examining the effects of abused substances on striatal function were conducted in either men, or male rodent models. Therefore, much of our current understanding of how drugs of abuse impact the striatum are viewed from the perspective of the male brain. This historical trend of male-centric research presents a significant obstacle for the development of effective treatment for women with substance use disorder, as drug use and relapse behavior manifests differently in women than in men (Becker & Hu 2008). Historically, men are two-to-three times more likely to develop a substance use disorder (Becker & Hu 2008), drink larger amounts of alcohol, and exhibit more frequent binge drinking episodes than women (Becker & Koob 2016). This difference may lie in biological differences as well as cultural influences.

As our cultural perspective of gender stereotypes evolve and the opportunities for women increase, the gap in alcohol consumption between the sexes has begun to narrow. Women born between 1912-1932 only reported a 2% prevalence of binge drinking, whereas women born between 1968-1984 reported a 16% prevalence of binge drinking

(Keyes et al., 2008). Further, the 2019 National Survey on Drug Use and Health (NSDUH) found that 29.7% of men and 22.2% of women ages 18 and older reported that they engaged in binge drinking in the past month.

In contrast to the higher alcohol consumption rates seen in men, women are more likely than men: to begin cocaine use at an earlier age (Chen & Kandel 2002); to consume greater amounts of cocaine; and to exhibit a longer duration of cocaine use (Kosten et al. 1993). Provided these differences in drug consumption between men and women, it is important to understand the biological mechanisms underlying these sex differences in order to develop more personalized therapies for substance use disorder.

While cultural influences significantly impact the likelihood and the onset of initial drug exposure, how an individual responds to and continues using a drug is, in part, influenced by sex hormones (Anker & Carroll 2010). In a clinical study, the subjective effects of acute amphetamine were self-reported in women across menstrual cycle; revealing that the positive sensation of amphetamine increased during the follicular phase of the menstrual cycle, where estradiol levels slowly rise, and progesterone levels decrease (Becker & Hu 2008). Further, administration of estradiol increases the positive sensation of acute amphetamine treatment in women (Justice & de Wit 2000). These studies suggest that ovarian hormones positively modulate the subjective effects of stimulants, which likely contributes to the higher stimulant abuse rates seen in women compared to men. In line with this hypothesis, female rats exhibit a greater locomotor response to amphetamine (Becker et al. 1982) and cocaine compared to male rats (Segarra et al. 2010). Additionally, cocaine-induced CPP is mediated by estradiol in female rats (Segarra et al. 2014) and mice (Satta et al. 2018).

One mechanism by which estradiol enhances the rewarding effects of stimulant use is through the modulation of dopamine signaling in the striatum. Acute administration of cocaine produces higher levels of electrically-evoked dopamine release events in the DMS in female rats compared to male rats (Walker, Ray, & Kuhn 2006) and this effect is attenuated in ovariectomized female rats but reinstated by a physiological dose of estrogen (Becker et al. 1999). Additionally, amphetamine-induced CPP is accompanied by a greater increase in dopamine release in the nucleus accumbens of female rats compared to male rats (Robinson & Becker 1982). Not surprisingly then, the rewarding effects of stimulant use are due, in part, to activation of estrogen receptors in the mesolimbic system. Indeed, knockdown of the estrogen beta receptor prevents cocaine-induced CPP in female mice (Satta et al. 2018).

As we previously mentioned, men historically drink alcohol more than women. However, women are more likely to develop medical problems from excessive drinking (Erol & Karpyak 2015). As such, examining sex differences in animal models of alcohol drinking is critical. In nonhuman primates, male (cynomolgus) monkeys drink more than females (Vivian et al. 2001). However, in rodents female mice (Eriksson & Pikkarainen, 1968; Sneddon et al. 2019) and rats (Li & Lumeng, 1984) drink more ethanol than males. Similar to cocaine use, sex differences in ethanol consumption is linked to ovarian hormones, as Almeida et al. (1998) demonstrated that ethanol consumption in neonatally estrogenized female rats, which confers a masculinized adult brain, is reduced to levels similar to male rats. Potentially underlying this difference are fluctuations in sex hormone during critical developmental periods and across estrous cycle that modulates the nucleus accumbens to promote drug consumption (Tonn Eisinger et al. 2018). For example, adult female rats drink more than males and exhibit greater ethanol-induced dopamine release into the nucleus accumbens (Blanchard et al. 1993). However, adolescent male rats drink more ethanol compared to adolescent females (Vetter O'Hagen et al. 2009). Collectively, these studies demonstrate the profound impact that sex hormones, specifically estradiol, exert on drug consumption. It is therefore imperative that we continue exploring at a more detailed circuit level the biological mechanisms underlying sex differences in drug consumption, withdrawal, and relapse to inform sex-specific therapies for substance use disorder.

1.12 Introduction Summary

The striatal complex plays a critical role in the expression of reward-driven actions. As we explore the environment, cues predicting rewards are encoded in the nucleus accumbens. As this occurs, internal emotional information from the amygdala and external contextual information from the hippocampus is integrated in the nucleus accumbens to motivate behavior toward further reward acquisition. As reward acquisition continues, the actions that achieve rewards are encoded in the DMS. During this stage, information about the reward outcome is continuously updated through projections from the medial prefrontal cortex and orbitofrontal cortex to the DMS, and if an action no longer results in a reward, behavior flexibly adapts to this feedback. If an action continues to be reinforced through reward acquisition, an automatized action becomes encoded in the DLS by incoming information from somatosensory and motor cortices. Drugs of abuse modulate each step of this dynamic and parallel processing to facilitate enhanced motivation to perform highly automatized, compulsive drug-seeking behavior.

Drugs of abuse exert these effects on the striatal complex largely by interacting with the rich neurotransmitter systems that orchestrate synaptic plasticity events. Drug-induced neural circuit-specific synaptic plasticity ultimately affects what information is stored in the striatal complex, which includes pathologically strong motivation to perform habitual actions geared toward compulsive drug-seeking and -taking behaviors. These stored action sequences, consisting of ensembles of striatal interneurons and projection neurons alike, are engaged by thalamic structures to elicit stimulus-response strategies that underlie compulsive drug seeking and consumption, a key feature of addiction that thwarts efforts to limit drug use. Clearly, the circuit-specific mechanisms underlying this transition to compulsive drug use is intimidatingly multidimensional. While the task to devise novel addiction treatments appears daunting, the complexity of the problem also presents a plethora of mechanisms to target for therapeutic strategy development that should be considered through a sex-specific lens.

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1.14 Initial Hypotheses and Predictions

Overarching Hypothesis: Chronic ethanol exposure dysregulates dorsal striatum fast-spiking interneurons to promote compulsive drinking

Hypothesis 1 (Chapter 2): Dorsal striatum fast-spiking interneurons are necessary for compulsive ethanol consumption

> **Prediction 1.1**: viral ablation of dorsal striatum fast-spiking interneurons will prevent the expression of compulsive ethanol consumption in mice

Hypothesis 2 (chapter 3): Chronic ethanol exposure dysregulates dorsal striatum fast-spiking interneuron physiology

> **Prediction 2.1**: Chronic ethanol exposure modulates either GABAergic or Glutamatergic synaptic transmission onto dorsal striatum fast-spiking interneurons

Hypothesis 3 (chapter 4): Acute bath application of ethanol modulates GABAergic transmission onto dorsal striatum fast-spiking interneurons

> **Prediction 3.1**: Acute bath application of ethanol enhances GABAergic transmission onto dorsal striatum fast-spiking interneurons

Chapter 2: Compulsive Alcohol Consumption is Regulated by Dorsal Striatum Fast-Spiking Interneurons

2.1 Introduction

Repeated exposure to drugs of abuse, like alcohol, facilitates habit learning and thwarts efforts to abstain from further consumption (Graybiel 2005; Everitt et al.,2001). Chronic alcohol users increase habitual responding on stimulus-response task and this correlates with the duration of alcohol dependence (Sjoerds et al., 2013; Everitt & Robbins 2016). In rodents, 4-6 weeks of voluntary ethanol exposure promotes habitual ethanol consumption (Hopf et al., 2010; Lesscher et al. 2010) and accelerates habitual responding for sucrose reward (Corbit et al., 2012). Collectively, this suggests that chronic ethanol exposure targets neuronal substrates governing habit formation. Determining these substrates is the first step toward devising strategies toward treating compulsive alcohol drinking, which is notoriously treatment resistant (Burch et al., 2019; Garbusow et al., 2014; Kranzler & Soyka 2018; Maisto et al., 2018; Stock 2017).

Habitual behavior requires a dynamic interaction between the cortex and basal ganglia (Ashby et al., 2010; Balleine et al., 2007; Gremel & Costa 2013; Knowlton et al., 1996; Yin et al., 2009). Lesion studies demonstrate the necessity of the dorsolateral striatum, which is innervated by sensorimotor cortices, in the acquisition and expression of habitual behavior (Dickinson et al., 1995; Yin et al., 2004; Yin et al., 2005; Yin et al., 2006). The dorsolateral striatum is characterized by an enrichment of the parvalbumin (PV) expressing fast-spiking interneurons (FSIs) that provide powerful inhibitory control over striatal medium spiny projection neurons (Gerfen & Surmeier 2011; Kawaguchi 1993; Schlösser et al., 1999; Luk & Sadikot 2001; Tepper et al., 2004; Gittis et al., 2010). FSIs are implicated in habitual responding for a sucrose reward (O'Hare et al., 2017). Whether FSIs are a key ethanol target giving rise to compulsive ethanol consumption is not known.

Ethanol alters FSI intrinsic excitability (Blomeley et al., 2011) and disrupts FSI synaptic transmission onto medium spiny projection neurons (Patton et al., 2016). This supports the possibility that ethanol targets FSIs to promote compulsive consumption. Therefore, in the following study, we selectively ablated dorsal striatal FSIs in both male and female C57BL/6J mice undergoing a voluntary chronic intermittent ethanol consumption paradigm, drinking-in-the-dark (DID). We analyzed lick sequence behavior across the drinking paradigm to determine if striatal FSIs regulate the adaptations in drinking behavior exhibited by compulsive drinking rats (Darevsky et al., 2018). We show that FSI ablation reduces ethanol consumption and alters ethanol lick sequence behavior. Additionally, when ethanol is adulterated with the bitterant quinine, FSI ablation attenuates ethanol consumption. Collectively these results provide evidence for a causal role of dorsal striatum FSIs in compulsive ethanol consumption.

2.2 Methods

2.2.1Animals

All experiments were performed in accordance with NIH guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Maryland Baltimore and the National Institute on Alcohol Abuse and Alcoholism. Mice were housed with littermates (2-5 per cage) under a reverse 12-hour light/dark cycle (lights off at 0900 hours, on at 2100 hours) with *ad libitum* access to food and water.

2.2.2 FSI ablation

To selectively ablate PV-containing FSIs in the dorsal striatum, $\geq$2-month old PV-*cre* transgenic mice on a C57BL/6J background (31,32) were injected bilaterally with AAV5-flex-taCasp3-TEVp (UNC, Chapel Hill Vector Core) or AAV5-dio-EYFP (Addgene) at a volume of 500nl/side and a rate of 20nl/min at the following coordinates relative to bregma: anterior-posterior + 0.6 mm; medial-lateral $\pm$ 2.25 mm; dorsal-ventral -3.4 mm.

Mice were initially anesthetized with 4% vaporized isoflurane (VetEquip) and maintained at 1-2.5% isoflurane for the duration of the surgery based on toe-pinch response. A topical analgesic (2% lidocaine) was applied to the incision site following surgery. For additional pain management, mice received carprofen (5mg/kg, i.p.) every 24h for three days following surgery and had a three-week recovery period before behavioral testing.

2.2.3 Drinking-in-the-dark (DID)

The DID protocol was adapted from Rhodes *et. al* (2005). Mice (5 males, 5 females per group) were randomly assigned to the water, sucrose, or ethanol drinking groups. Two-three hours into the dark cycle (1100) mice were placed into modified home cages (18x18x23cm) with *ad libitum* access to food that was weighed at the end of each week. In order to achieve a clean lickometer signal, we elevated the drinking spout to disallow climbing and paw grabbing, which created false signals on the lickometer. Through a pilot test, we found that our animals would not willingly drink 20% ethanol from this spout height but would instead learn to drink from an elevated sipper providing a sucrose solution. We therefore added a 4-day sucrose fading session to train our mice to drink from the elevated sipper prior to the 4-week DID procedure (i.e. week 0). During the 4-week DID procedure, mice were given two hours of access to the drinking bottle in the first three days, and on day four mice were given four hours of access. Following four days of drinking, mice underwent three days of forced abstinence. On week 4, blood samples were collected from tail nicks immediately following a two-hour session to determine the blood ethanol concentration. Blood serum values were measured spectrophotometrically using an enzymatic assay (Sigma MAK076). On weeks 0 and 4, mice were given a two-bottle choice test of water and ethanol (two-hour duration) to determine drinking preference. On days two through four of week 5, the bitterant quinine (0.3mM) was added to the bottles to assess compulsive drinking.

2.2.4 Lickometer

A grated steel platform was placed on the floor of the chamber below the metal sipper of the drinking bottle and was connected to the ground of an analog-to-digital (AD) converter (Digidata, Molecular Devices). The input signal was connected to the metal sipper that created a circuit that closed when the mouse licked the sipper while standing on the steel platform. Lick events were recorded using Clampex software (Molecular Devices). Data were analyzed offline with Matlab (Mathworks R2018a). Events of 1-2 licks were defined as unorganized licks, while events of $\geq$3 licks, each lick occurring within 2s or less, were classified as organized drinking bouts (Wilcox et al., 2014).

2.2.5 Immunohistochemistry and ablation quantification

Mice (4 males, 3 females per group) were anesthetized with isoflurane and then intracardially perfused with saline followed by ice-cold paraformaldehyde (4%). Fixed brains were extracted and 100µm sections were collected using a vibratome (Leica). Slices were incubated in a mouse anti-PV antibody (1:1,000; clone PARV-19, Sigma-Aldrich) overnight at 23°C. The following day, slices were washed with a solution of phosphate buffered saline with Triton-X100 (0.2%) and incubated in Cy3 conjugated secondary antibody for four hours at 23°C (1:2,000; Jackson ImmunoResearch). Fluorescent images were visualized on a Nikon Eclipse Ti2 fluorescent microscope and captured with a Nikon DS-Qi2 camera. Cell counts were blindly collected from a region of interest encompassing the dorsolateral striatum. Automated cell counts were performed on single sections with Nikon Instruments Elements Advanced Research software (5.11.01). Briefly, fluorescent signal was restricted to a three-point circle threshold, and objects were excluded based on object circularity and diameter.

2.2.6 Acute slice preparation

Mice (3 males, 2 females per group) were deeply anesthetized with isoflurane then perfused with a carbogen-bubbled N-Methyl-D-Glutamine (NMDG) artificial cerebral spinal fluid (aCSF) solution (92 mM NMDG, 2.5 mM KCL, 1.25 mM NaH_2PO_4,30 mM $NaHCO_3$, 20 mM HEPES, 25 mM glucose, 2 mM thiourea, 5 mM Na-ascorbate, 3 mM NA-pyruvate, 0.5 mM $CaCl_2$, and 10 mM $MgCl_2$) before decapitation and brain removal. 250 µm coronal sections were collected in ice cold NMDG aCSF. Sections were incubated at 32°C for 12 minutes and then transferred to HEPES holding solution (92mM NaCl, 2.5mM KCL, 1.25, 1.25mM NaH_2PO_4, 30mM $NaHCO_3$, 20mM HEPES, 25mM glucose, 2mM thiourea, 5mM

Na-ascorbate, 3mM NA-pyruvate, 2mM $CaCl_2$, and 2mM $MgCl_2$) and stored at room temperature until recording.

2.2.7 Whole-cell voltage-clamp electrophysiology

Slices were hemisected, placed into a recording chamber, and perfused with temperature controlled aCSF (29-31°C) containing 50 µM DL-AP5 and 5 µM NBQX. Dorsolateral striatum medium spiny neurons were visualized using infrared differential interference contrast light microscopy and Q-capture camera and associated Pro 7 software. Striatal medium spiny projection neurons (MSNs) were voltage clamped at -60 mV using a MultiClamp 700B Amplifier (Molecular Devices). Spontaneous inhibitory postsynaptic current (IPSC) events were recorded using a borosilicate glass pipette (3-5 MO resistance) filled with CsCl-based internal solution (150 mM CsCl, 10 mM HEPES, 2 mM $MgCl_2$, 0.3 mM Na-GTP, 5 mM QX-314, 3 mM Mg-ATP, and 0.2 mM BAPTA). Signals were filtered at 2 kHz, digitized at 10 kHz and acquired using Clampex 10.4.1.4 software (Molecular Devices). Data were analyzed offline using Mini Analysis Program (Synaptosoft).

2.2.8 Behavior in the open field

Mice (5 males, 5 females per group) were habituated to a plexiglass open-field arena (30x30x25 cm) affixed with a ceiling camera to register mouse locomotor behavior via Ethovision (Noldus). The following day, mice received an intraperitoneal (i.p.) injection of ethanol (2 mg/kg) that was previously shown to produce blood ethanol concentrations (BECs) comparable to that achieved by DID (Rhodes et al., 2010). 30 minutes after injection, mice were placed in the open-field arena and locomotor behavior was recorded for 15 minutes. The following day an additional open-field trial was conducted to collect sober locomotor activity. All treatment groups were counterbalanced for ethanol and baseline trials.

2.2.9 Statistical analysis

All statistical analyses were performed in GraphPad Prism 8.0. Data are represented as mean $\pm$ SEM. Fluid consumption and the number of drinking bouts are derived from the weekly averages collected from the 2h drinking sessions. Bout length, bout length distribution, and coefficient of variation are derived from bouts expressed throughout the entire 4-week DID paradigm. Comparisons between two groups were analyzed with a Student's t-test while comparisons of three or more groups were analyzed with two-way ANOVA. Multiple comparisons were corrected with Sidak's test or Dunnett's test when comparing multiple time points to a single baseline. Effect sizes are reported by partial eta squared. Horizontal brackets indicate statistical significance within a treatment group, while vertical brackets indicate statistical significance between treatment groups. Simple linear regression was performed for correlation analysis and all $p < 0.05$ designations indicate a slope that is significantly non-zero. For all data, N is defined as an individual animal. For patch clamp data, N consists of an average recording from two to three cells for each animal.

2.3 Results

FSI ablation was achieved using injections of control fluorophore (control)- or genetically engineered caspase3 (taCasp3)- expressing viruses into the dorsal striatum (**Figure 2.1A**).

Viral-mediated FSI ablation resulted in a significant decrease in the number of PV-immunoreactive cells in the dorsolateral striatum (control = 186.4 $\pm$ 29.4 cells, taCasp3 = 34.43 $\pm$ 8.0 cells; $t_{12} = 4.987$, $p < 0.005$, $\eta^2 = 0.67$, Student's t-test, **Figure 2.1B,C**). FSI ablation was further characterized by recording spontaneous IPSC events from MSNs in the dorsolateral striatum using whole-cell voltage clamp electrophysiology. FSI ablation significantly decreased the amplitude of sIPSC events (control = 59.78 $\pm$ 2.41 pA, FSI ablation = 44.83 $\pm$ 3.9 pA; $t_{(8)} = 3.217$, $p < 0.05$, $\eta^2 = 0.56$, Student's t-test, **Figure 2.1D, E**) but had no effect on sIPSC event frequency ($t_{(8)} = 0.13$, $p = 0.90$, Student's t-test, **Figure 2.1F**).

To determine if FSI ablation affected gross motor function under normal or inebriated conditions, we analyzed locomotor behavior in an open field arena. We found no main effect of FSI ablation on total distance traveled under baseline conditions or following an acute injection of ethanol (2mg/kg, i.p.) ($F_{(1,35)} = 0.342$, $p = 0.56$, two-way ANOVA, **Figure 2.1G, H**). No main effect of FSI ablation was observed for mean velocity in the control condition or following acute injection of ethanol (2mg/kg, i.p.) ($F_{(1,35)} = 0.032$, $p = 0.86$, two-way ANOVA, **Figure 2.1I**). Additionally, we found no main effect of FSI ablation on the number of movement initiations in the control condition or following acute ethanol administration ($F_{(1,36)} = 1.03$, $p = 0.32$, two-way ANOVA, Figure **2.1J**).

To examine whether food consummatory behavior was disrupted by FSI ablation, which may indirectly affect ethanol consumption through differences in daily caloric intake, we measured weekly food pellet consumption ingested during DID. We found no main effect FSI ablation on food consumption in water drinking animals ($F_{(1,18)} = 0.16$, $p = 0.70$; two-way ANOVA, **Figure 2.1K**) or in ethanol drinking animals ($F_{(1,36)} = 0.26$, p =

0.61, two-way ANOVA). Additionally we found no effect of time on consumption in either water ($F_{(3,46)}$ = 2.7, p = 0.065, two-way ANOVA) or ethanol drinking animals ($F_{(2,41)}$ = 1.31, p = 0.28, two-way ANOVA).

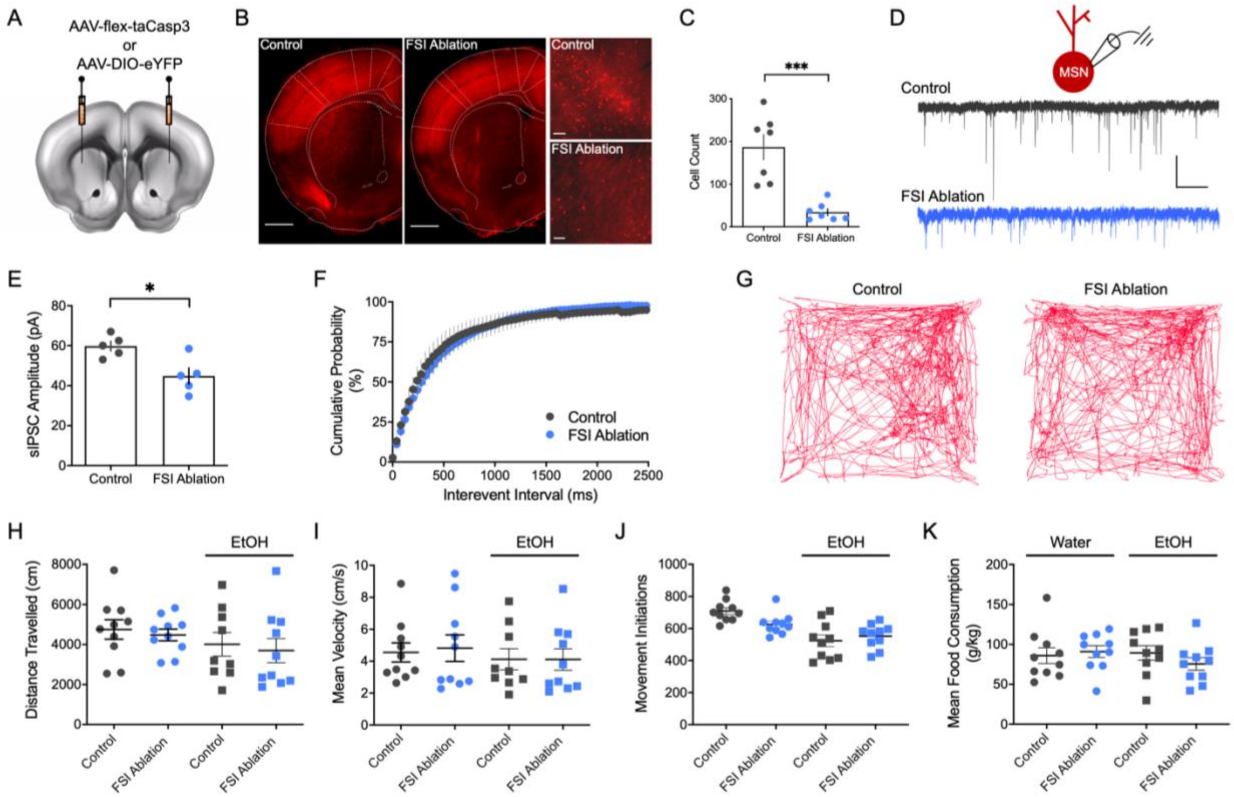

Figure 2.1 Viral ablation of striatal fast spiking interneurons (FSIs). A. A schematic depicting bilateral injection sites of genetically engineered caspase 3 (taCasp3)-expressing virus for dorsal striatal FSI ablations. **B.** Immunostain for parvalbumin from control (left) and taCasp3-ablated (middle) animals. Scale bar 1000μm. 20x magnification of parvalbumin expression from control (top right) and taCasp3 ablated (bottom right) animals. Scale bar 100μm. **C.** taCasp3-mediated FSI ablation significantly reduced the number of parvalbumin-positive neurons in the dorsal striatum (N = 7 animals). **D.** A schematic of whole-cell voltage clamp recording of a medium spiny neuron (MSN) (top). Representative traces of spontaneous inhibitory postsynaptic current (sIPSC) events recorded from MSNs from control (dark grey) and FSI-ablated (blue) animals (bottom). Scale bars: vertical, 50 pA; horizontal, 5s. **E.** taCasp3-mediated FSI ablation reduced sIPSC event amplitude (N = 5 animals). **F.** A cumulative frequency distribution of sIPSC event frequency showing that frequency was not affected by FSI ablation (N = 5 animals). **G.** Representative ambulatory traces from control and FSI-ablated animals exploring an open-field arena. FSI ablation does not affect total distance traveled (**H**), mean velocity (**I**), or the number of movement initiations (**J**) in either a sober state or following acute injection of ethanol (EtOH, 2mg/kg i.p.). **K.** FSI ablation does not reduce food consumption across the 4-week DID paradigm. *$p < 0.05$, *** $p < 0.005$. Data represented as mean $\pm$ SEM.

During the four-week DID paradigm fluid consumption was tracked using a custom lickometer, and the data for the two-hour sessions were analyzed as they represented the large majority of all drinking sessions (**Figure 2.2A-B**). We found that licks positively correlated with ethanol consumption (g/kg) for both control ($R^2 = 0.30$, $p < 0.001$) and FSI ablated animals (R2 = 0.34, $p < 0.001$). We found no main effect of FSI ablation on water consumption ($F_{(1,18)} = 0.384$, $p = 0.543$, two-way ANOVA, **Figure 2.2C**) or sucrose consumption ($F_{(1,18)} = 0.0282$, $p = 0.8684$, two-way ANOVA, **Figure 2.2D**). However, we found a main effect of FSI ablation on ethanol consumption that was significantly different by week 4 (control = 71.25 $\pm$ 6.52 licks, FSI ablation 49.28 $\pm$ 3.76 licks, $F_{(1,18)} = 5.73$, $p < 0.05$, $\eta^2 = 0.14$, two-way ANOVA; Sidak's multiple comparisons test, **Figure 2.2E**). To determine if this reduction in licks in the ablated group was driven by changes in ethanol preference, we subjected mice to a two-bottle choice task at the beginning and end of the DID procedure. Both control (baseline = 51.04 $\pm$ 2.70 %, post-DID = 72.27 $\pm$ 4.00 %, $t_{(18)} = 5.44$, $p < 0.001$, $\eta^2 = 0.60$, Student's t-test) and FSI-ablated animals exhibited a significant preference for ethanol over water consumption (baseline = 51.43 $\pm$ 3.56 %, post-DID = 68.29 $\pm$ 3.98 %; $t_{(18)} = 3.197$, $p < 0.01$, $\eta^2 = 0.38$, Student's t-test, **Figure 2.2F**).

To assess BEC, blood was collected during week 4 of the DID paradigm. Control animals exhibited higher BECs compared to FSI ablated littermates (control = 75.2 $\pm$ 11.5 mg/dl, FSI ablation = 30.35 $\pm$ 8.40 mg/dl, $t_{(18)} = 3.15$, $p <0.01$, $\eta^2 = 0.36$ Student's t-test, **Figure 2.2G**). The significant reduction in BEC in the face of a modest reduction in total ethanol consumption during week 4 spurred us to analyze which group drank more at end of the session, when blood was collected, relative to total consumption on the day of blood collection. We found that control animals exhibited relatively more licks in the last 30 mins than FSI ablated animals (control = 34.47 $\pm$ 3.93 %, FSI ablated = 20.62 $\pm$ 3.82 %, $t_{(16)} = 2.528$, $p < 0.05$, $\eta^2 = 0.28$ Student's t-test).

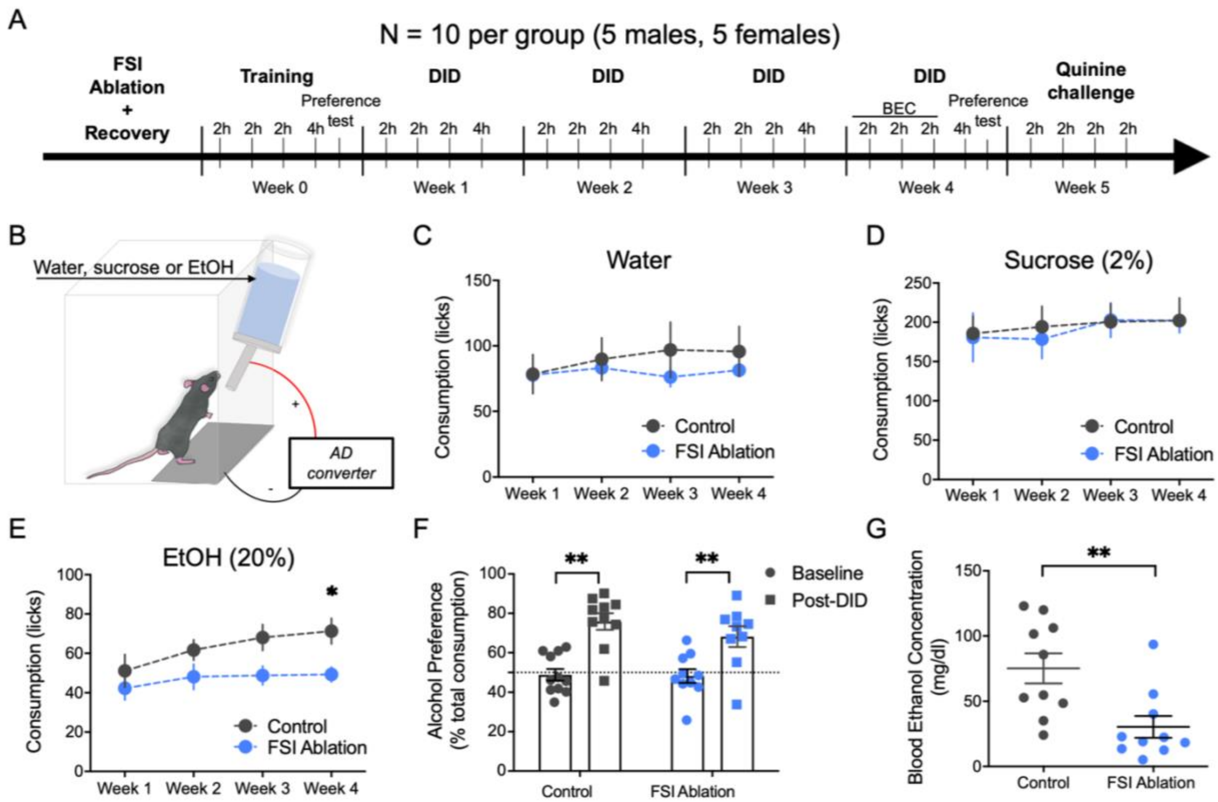

Figure 2.2 Striatal FSI ablation reduced voluntary ethanol consumption. A. Experimental timeline of FSI ablation, drinking-in-the-dark (DID), EtOH preference tests, and quinine challenge. **B.** Schematic of the DID lickometer apparatus including the analog-to-digital (AD) converter. **C-E.** Average weekly fluid consumption (number of licks) from control (black) and FSI ablated animals (blue) collected from two hour (2h) DID sessions across the four-week drinking paradigm. FSI ablation did not affect water consumption (**C**) or sucrose consumption (**D**), but reduced alcohol consumption in week 4 only (**E**). **F.** Both control and FSI-ablated animals exhibited an EtOH preference following the four-weeks of EtOH consumption. **G.** Blood ethanol concentration from animals during week 4 of DID were lower in FSI-ablated animals. * $p < 0.05$, ** $p < 0.01$. Data represented in mean $\pm$ SEM.

Since the dorsal striatum is implicated in motor sequence learning (Sneddon et al., 2019; Botvineck et al. 2009; Dezfouli et al., 2014; Jin & Costa 2010), and lick bouts are implicated in compulsive ethanol consumption in rats (Darevsky et al., 2018), we examined whether striatal FSIs influence ethanol lick sequence behavior (**Figure 2.3**). We found no main effect of FSI ablation on the number of water licking bouts ($F_{(1,18)} = 0.16$, $p = 0.69$, two-way ANOVA, Figure **2.3A, D**), or sucrose licking bouts ($F_{(1,18)} = 0.013$, $p = 0.91$, two-way ANOVA, **Figure 2.3B, E**), but found that FSI ablation reduced the number of ethanol licking bouts (control = 15.28 $\pm$ 1.01 licks, FSI ablation = 9.55 $\pm$ 0.27 licks, $F_{(1,18)} = 11.75$, $p < 0.01$, $\eta^2 = 0.26$, two-way ANOVA, **Figure 2.3C, F**). Additionally, we found a main effect of time in the ethanol drinking animals, where the control mice exhibited an increase in the number of ethanol drinking bouts by week 3, which was not observed in FSI-ablated animals (week 1 control: 11.02 $\pm$ 1.9, week 4 control = 16.63 $\pm$ 2.01, $F_{(3,54)} = 3.12$, $p < 0.05$, $\eta^2 = 0.06$, Dunnett's multiple comparisons test, **Figure 2.3F**). However, we found no main effect of FSI ablation on unorganized licks from mice consuming water ($F_{(1,71)} = 0.04$, $p = 0.84$,two-way ANOVA, **Figure 2.3G**), sucrose ($F_{(1,71)} = 0.02$, $p = 0.89$, two-way ANOVA, **Figure 2.3H)** or ethanol ($F_{(1,71)} = 2.45$, $p = 0.12$,two-way ANOVA, **Figure 2.3I**).

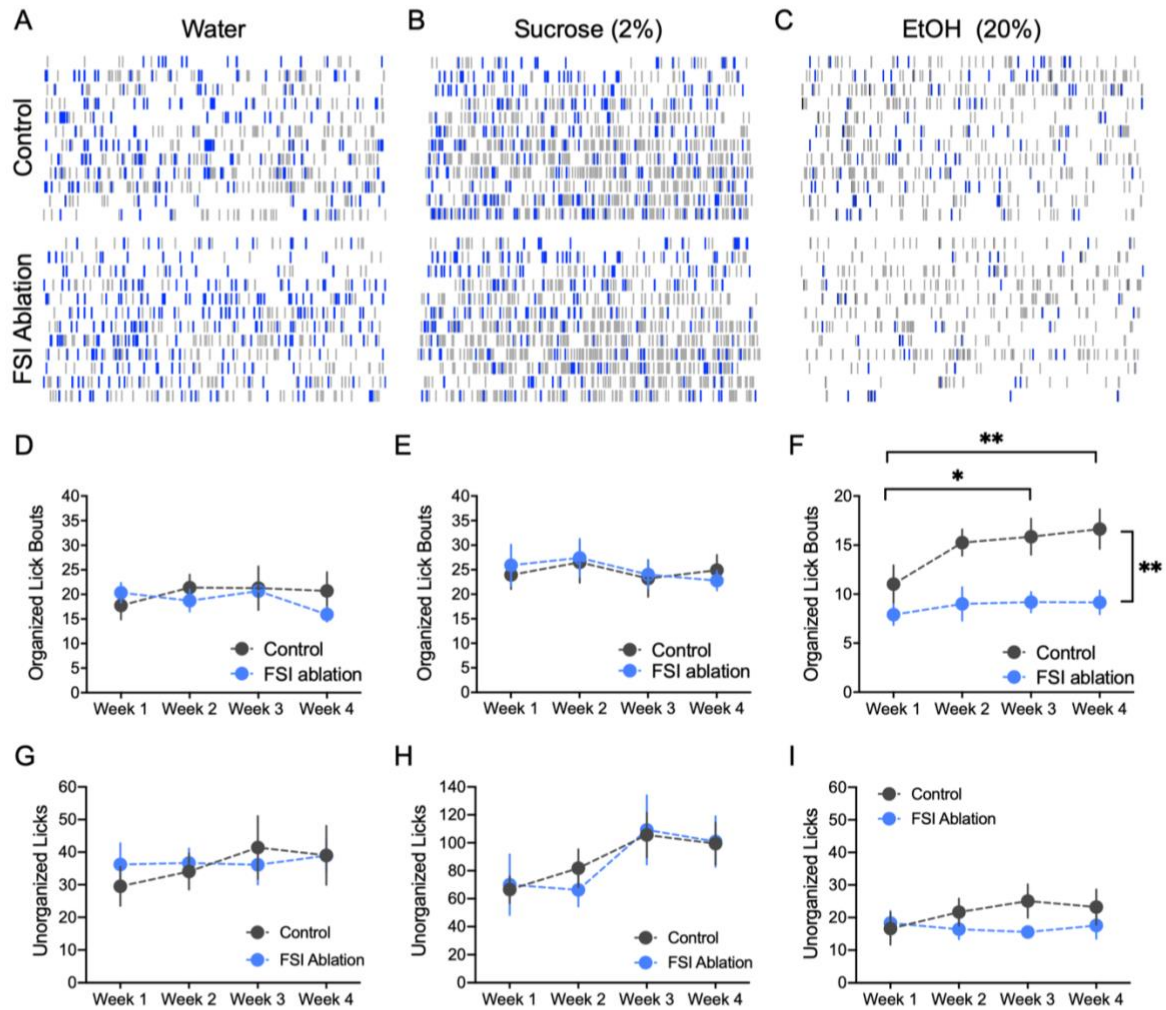

Figure 2.3 Striatal FSI ablation selectively reduced EtOH drinking bouts. A-C. Representative raster plots depicting individual licks (gray) and drinking bouts (blue) collected from control (top row) and FSI-ablated animals (bottom row) during the 2hr session across the 4-week DID paradigm. FSI ablation had no effect on the number of water drinking bouts (**D**) or sucrose drinking bouts (**E**). **F.** Control animals escalated the number of EtOH drinking bouts during the 4-week DID paradigm, exhibiting a significant increase in weeks 3 and 4 versus week 1, while FSI-ablated animals exhibited significantly fewer EtOH drinking bouts compared to controls from weeks 2-4. FSI ablation had no effect on unorganized licking from animals drinking water (G), sucrose (H), or ethanol (I). * $p < 0.05$, ** $p < 0.01$. Data represented as mean $\pm$ SEM.

We next examined if FSI ablation altered bout licking microstructure (**Figure 2.4**). We found that FSI ablation had no effect on the average bout length of water drinking animals ($t_{(18)} = 0.06$, $p = 0.95$, Student's t-test, **Figure 2.4A, D**) or sucrose drinking animals ($t_{(18)} = 1.09$, $p = 0.29$, Student's t-test, **Figure 2.4B, E**). However, FSI ablation increased the average bout length in ethanol drinking animals (control = 4.40 $\pm$ 0.20 licks, FSI ablation = 5.53 $\pm$ 0.41 licks, $t_{(18)} = 2.49$, $p < 0.05$, $\eta^2 = 0.26$, Student's t-test, **Figure 2.4C, F**). We then performed a frequency distribution analysis of bout lengths to determine the source of this mean difference. As expected, we found no effect of FSI ablation on bout length distribution from water ($F_{(1,72)} = 0.038$, $p = 0.85$, two-way ANOVA, **Figure 2.4G**) or sucrose drinking animals ($F_{(1,72)} = 0.01$, $p = 0.99$, two-way ANOVA, **Figure 2.4H**). However, there was a significant size x ablation interaction in the ethanol drinking condition ($F_{(3,72)} = 10.17$, $p < 0.001$, $\eta^2 = 0.11$) where FSI-ablated animals exhibited a significant decrease in the proportion of small ethanol bouts (three to four licks per bout) in FSI-ablated animals (control = 50.45 $\pm$ 2.53 %, FSI ablation = 33.75 $\pm$ 4.17 %) and an increase in the proportion of large ethanol bouts (nine or more licks per bout) (control = 7.61 $\pm$ 1.85 %, FSI ablation = 18.64 $\pm$ 4.12 %, Sidak's multiple comparisons test, **Figure 2.4I)**. We noticed that FSI ablated animals exhibited a wider distribution of bout lengths from our frequency distribution. We therefore calculated the coefficient of variance for the bout length exhibited by each animal, and compared mean variance across treatment group for each drinking condition. We found that FSI ablation had no effect on water ($t_{(18)} = 0.95$, $p = 0.35$, Student's t-test, **Figure 2.4J**) or sucrose bout length variance ($t_{(18)} = 0.36$, $p = 0.72$, **Figure 2.4K**). However, consistent with our frequency distribution data, we found that ethanol-drinking FSI-ablated animals exhibited more bout length variance compared to controls (control 40. 26 $\pm$ 2.61 %, FSI ablation 55.66 $\pm$ 5.34 %, $t_{(18)} = 2.59$, $p < 0.05$, $\eta^2 = 0.27$, Student's t-test, **Figure 2.4L**).

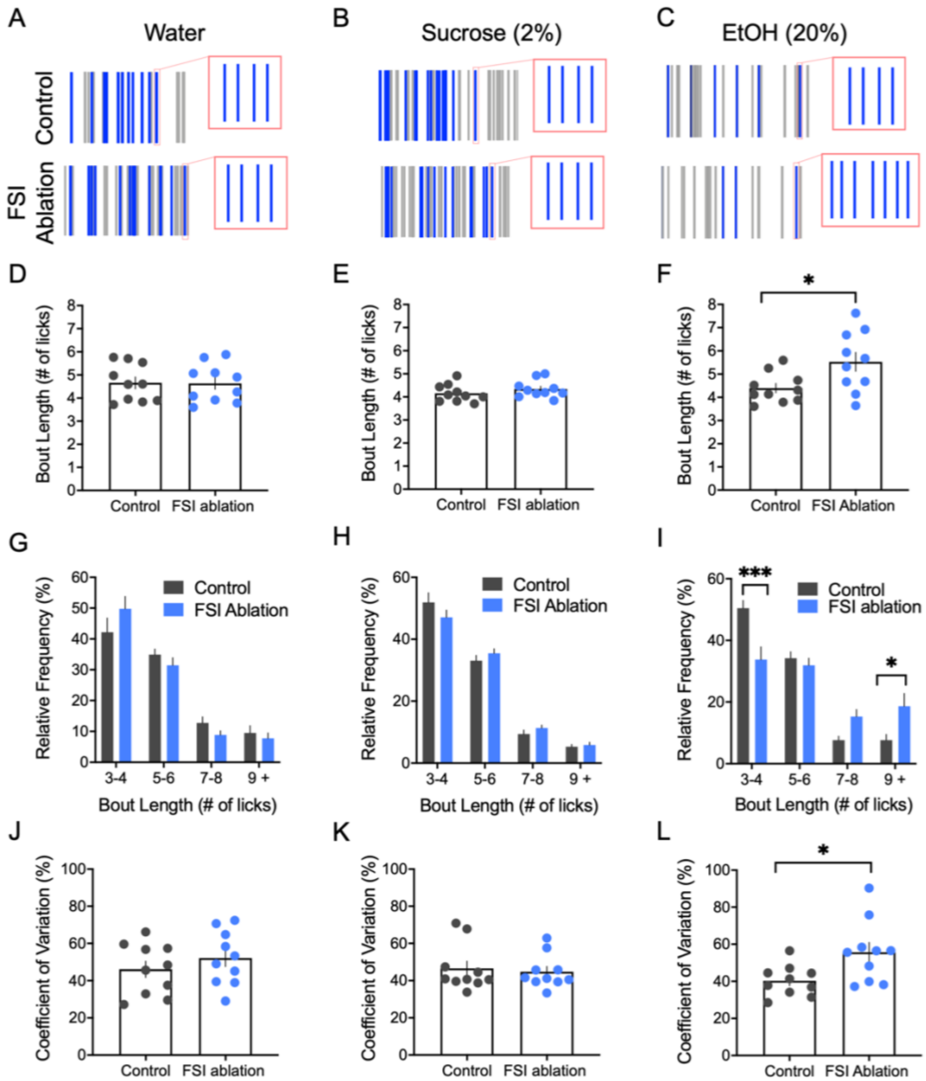

Figure 2.4 Striatal FSI ablation selectively disorganized EtOH drinking bout microstructure. A-C. Representative raster plot of a 30 min window from a 2 hr EtOH drinking session depicting individual licks (gray) and drinking bouts (blue) from control (top row) and FSI-ablated animals (bottom row). Inserts: magnification of an individual drinking bout illustrating the difference in bout length between control and FSI-ablated animals. **D-F.** Mean bout length from control and FSI-ablated animals across drinking condition. FSI ablation had no effect on water (**D**) or sucrose (**E**) bout length, but increased mean bout length from EtOH-drinking animals (**F**). **G-I.** A frequency distribution of drinking bouts binned by number of licks across drinking condition. FSI ablation had no effect on water (**G**) or sucrose (**H**) bout length distribution. **I.** Control animals exhibited a greater proportion of small drinking bouts (three to four licks), while FSI ablated animals exhibited a greater proportion of larger drinking bouts ($\geq$ 9 licks). **J-L.** Drinking bout length variance across drinking condition. FSI ablation had no effect on bout length variance from water (**J**) and sucrose (**K**) drinking animals. **L.** FSI ablation resulted in greater variability in the length of ethanol drinking bouts compared to control animals. * $p < 0.05$. Data represented as mean $\pm$ SEM.

Striatal FSIs may encode learning dependent changes in action sequence expression (Dezfouli et al., 2014; Jin & Costa 2010). In addition, compulsive drinking rats adjust their lick frequency within drinking bouts (Darevsky et al., 2018). Therefore, we next assessed lick frequency within drinking bouts across time (**Figure 2.5A-I**). To do this, we categorized bouts into <3 Hz, 3-4 Hz, and >4 Hz lick frequency ranges, and expressed the data as relative frequency. We observed no main effect of FSI ablation on water bout microstructure frequency (<3 Hz: $F_{(1,19)} = 0.02$, $p = 0.88$; 3-4 Hz: $F_{(1,19)} = 0.20$, $p = 0.66$; >4 Hz: $F_{(1,19)} = 0.14$, $p = 0.72$, two-way ANOVA, **Figure 2.5A,D,G**) or sucrose bout microstructure (<3 Hz: $F_{(1,18)} = 1.76$, $p = 0.20$; 3-4 Hz: $F_{(1,18)} = 0.42$, $p = 0.53$; >4 Hz: $F_{(1,18)} = 0.59$, $p = 0.45$, two-way ANOVA, **Figure 2.5B,E,H**). Additionally, we saw no main effect of time on bout microstructure across the four-week paradigm in either water drinking animals (<3 Hz: $F_{(2,39)} = 0.54$, $p = 0.59$; 3-4 Hz: $F_{(2.3, 45.4)} = 0.45$, $p = 0.68$; >4 Hz: $F_{(2.5, 47.9)} = 0.36$, $p = 0.75$, two-way ANOVA, **Figure 2.5A,D,G**) or sucrose drinking animals (<3 Hz: $F_{(2.4,42.8)} = 0.90$, $p = 0.43$; 3-4 Hz: $F_{(1.8, 33.5)} = 1.59$, $p = 0.22$; > 4 Hz: $F_{(2.1, 37.1)} = 1.30$, $p = 0.29$, two-way ANOVA, **Figure 2.5B,E,H**). However, we found a time x ablation interaction from the ethanol drinking animals in the < 3 Hz lick frequency ($F_{(3,54)} = 3.83$, $p < 0.05$, $\eta^2 = 0.10$) and a main effect of FSI ablation ($F_{(1,18)} = 12.5$, $p < 0.01$, $\eta^2 = 0.20$, **Figure 2.5C).** We additionally observed that control animals exhibited an increase in <3 Hz bout licks at week 4 compared to week 1 that was not observed in FSI-ablated animals (week 1 control = 5.32 $\pm$ 1.15 %, week 4 control = 9.97 $\pm$ 1.49 %, $p < 0.05$, Dunnett's multiple comparisons test, **Figure 2.5C**). Further, we found no main effects in 3-4 Hz events ($F_{(1,18)} = 0.81$, $p = 0.38$, two-way ANOVA; **Figure 2.5F**), but found a main effect of FSI-ablation in > 4 Hz events (control = 64.63 $\pm$ 1.62 %, FSI ablation = 76.13 $\pm$ 1.08 %, $F_{(1,18)} = 5.96$, $p < 0.05$, $\eta^2 = 0.16$, two-way ANOVA, **Figure 2.5I**).

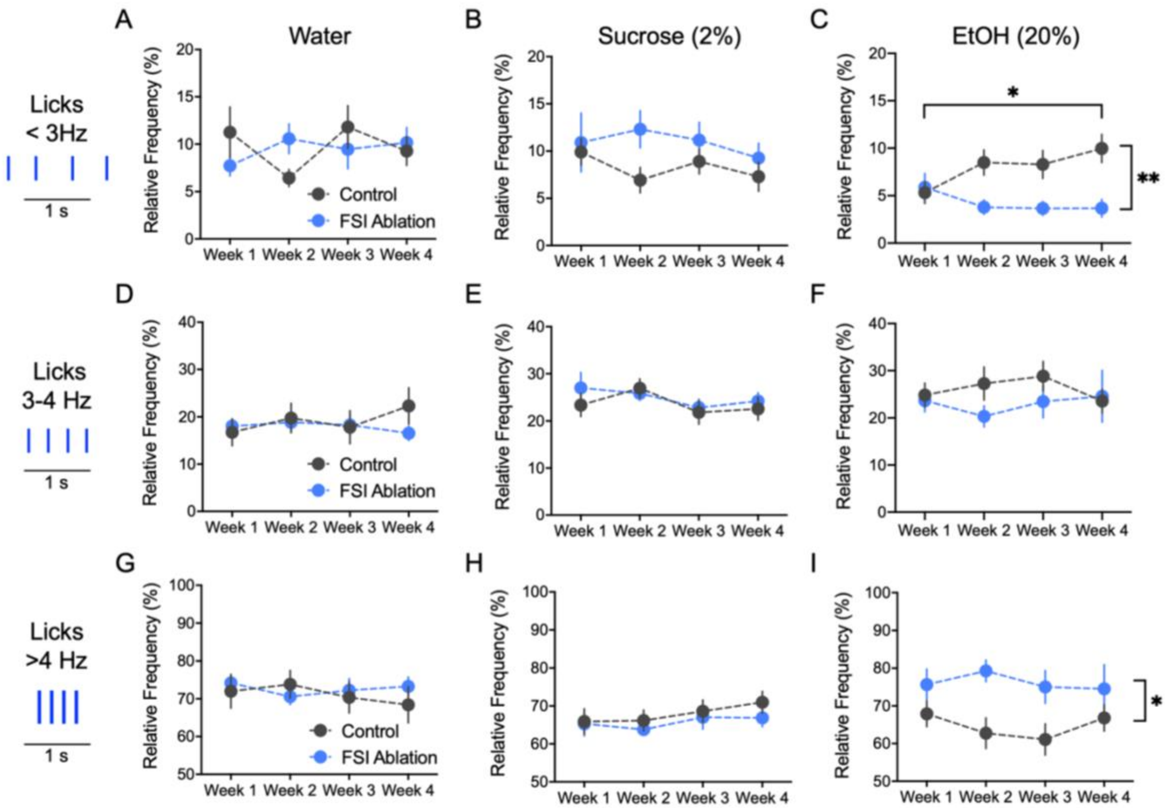

Figure 2.5 Striatal FSI ablation disorganized EtOH lick microstructure speed. A-G) Relative lick frequency collected from bouts during the 4-week drinking paradigm was binned into <3 Hz, 3-4 Hz, and >4 Hz ranges. FSI ablation had no effect on any lick frequency in water (**A,D,G**) or sucrose (**B,E,H**) drinking animals. However, control EtOH drinking animals exhibited a time-dependent increase in <3 Hz frequency licks that reached significance comparing week 1 versus week 4, which was not observed in FSI-ablated animals (**C**). Additionally, FSI-ablated animals exhibited fewer <3 Hz frequency licks than control animals at weeks 2-4. (**C**). **I.** An increase in >4 Hz frequency licks was observed in FSI-ablated animals as compared to control animals at week 2. * $p < 0.05$, ** $p < 0.01$. Data represented as mean $\pm$ SEM.

Finally, we examined the effect of FSI ablation on the expression of compulsive ethanol consumption (**Figure 2.6**). Following the addition of the adulterant quinine into their respective drinking solutions we found that both water ($F_{(2.5, 33.5)} = 56.93$, $p < 0.001$, $\eta^2 = 0.67$, two-way ANOVA, Dunnett's multiple comparison test, **Figure 2.6B**) and sucrose drinkers reduced consumption relative to baseline ($F_{(2.8, 49.5)} = 39.7$, $p <0.001$, $\eta^2 = 0.67$, two-way ANOVA, Dunnett's multiple comparison test, **Figure 2.6C**), but observed no main effect of FSI ablation in either group (water: $F_{(1,18)} = 0.58$, $p = 0.45$; sucrose: $F_{(1,18)} = 0.57$, $p = 0.46$, two-way ANOVA). However, we found a main effect of FSI ablation in the ethanol drinking mice ($F_{(1,68)} = 20$, $p < 0.001$, $\eta^2 = 0.18$, two-way ANOVA), where control animals exhibited greater quinine consumption compared to FSI-ablated animals (Dunnett's multiple comparisons, **Figure 2.6D**). Additionally, we found a main effect of time ($F_{(3,68)} = 5.0$, $p < 0.01$, $\eta^2 = 0.14$, two-way ANOVA), and found that control animals failed to reduce ethanol consumption with the addition of quinine (Dunnett's multiple comparisons, **Figure 2.6D**).

We next analyzed bout behavior from ethanol drinking animals during the three-day quinine challenge to assess whether FSI ablation affected quinine-adulterated lick microstructure. We found that control animals exhibited more ethanol drinking bouts (control = 9.93 $\pm$ 1.05 bouts, FSI ablation = 5.68 $\pm$ 0.81 bouts, $t_{(18)} = 03.143$, $p <0.01$, $\eta^2 = 0.37$ Student's t-test, **Figure 2.6E**) and more unorganized licks than FSI ablated animals (control = 20.8 $\pm$ 3.59 licks, FSI ablation = 10.17 $\pm$ 1.80 licks, $t_{(18)} = 2.65$, $p < 0.05$, $\eta^2 = 0.28$, Student's t-test, **Figure 2.6F**). We additionally found that control mice exhibit a greater relative frequency of < 3Hz licks (control = 10.85 $\pm$ 6.5%, FSI ablation = 5.01 $\pm$ 3.6%, $t_{(18)} = 2.6$, $p < 0.05$, $\eta^2 = 0.27$, Student's t-test). No differences were observed in bout length ($t_{(18)}$ = 0.5, p = 0.63, Student's t-test, **Figure 2.6G**) relative frequency ($F_{(1,64)}$ = 0.08, p = 0.77, **Figure 2.6H**) or bout variance ($t_{(18)}$ = 0.21, p = 0.84, Student's t-test, **Figure 2.6I**).

Since FSI-ablated ethanol-drinking animals exhibited reduced BEC levels compared to intact controls, we examined if BEC levels correlated with compulsive consumption. To do this, we averaged the three-day quinine consumption data to create a compulsive consumption score used for subsequent correlation analysis. We found no correlation between BEC level and compulsive consumption score in either the control group (R^2 = 0.096, p =0.38), or FSI ablated group (R^2 = 0.10, p = 0.37). Additionally, we found no correlation between amount of ethanol consumed during the last week of DID and compulsive consumption in either control animals (R^2 = 0.17, p = 0.23) or FSI ablated animals (R^2 = 0.05, p = 0.96, **Figure 2.6J**). We found a trend in the correlation between the number of ethanol bouts exhibited during the 4-week DID and compulsive consumption score (control: R^2=0.35, p = 0.07). This trend was not present in FSI-ablated animals (R^2= 0.05, p = 0.54, **Figure 2.6K**). Additionally, we found that the degree of variance in bout length exhibited during the 4-week DID paradigm negatively correlated with compulsive consumption score in control animals (R^2 = 0.45, $p < 0.05$) but not in FSI ablated animals (R^2= 0.23, p = 0.16, **Figure 2.6L**).

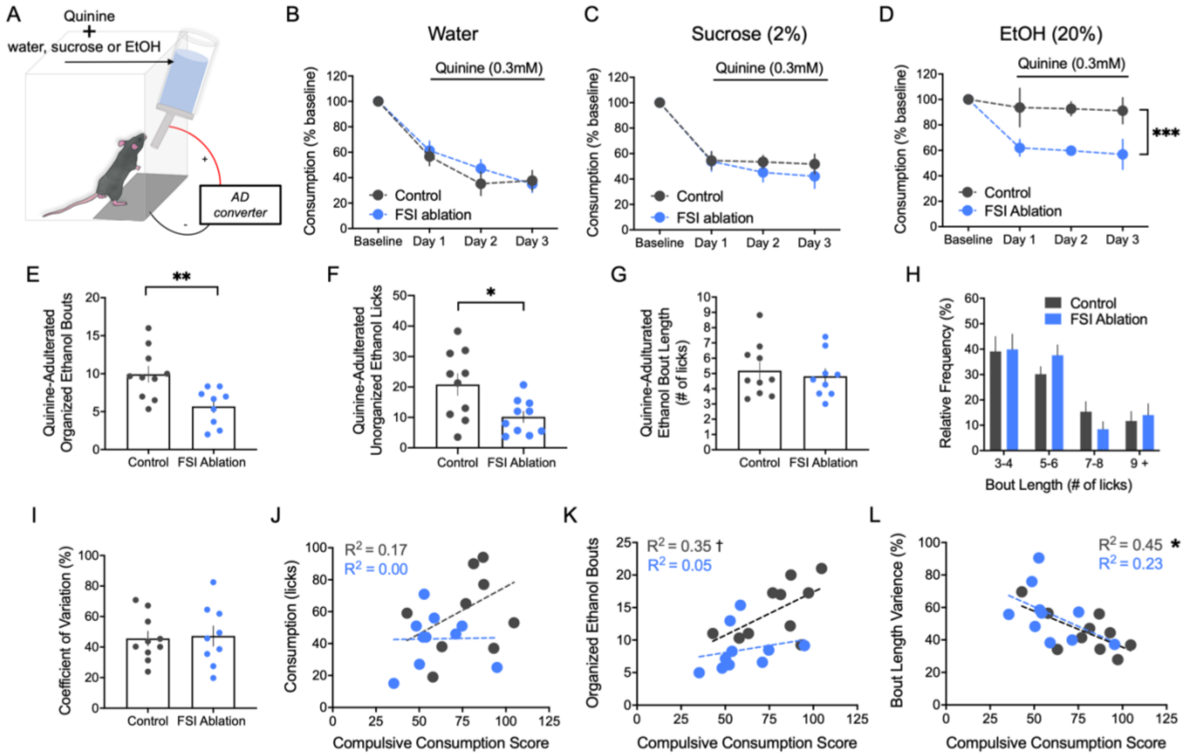

Figure 2.6 Striatal FSI ablation attenuated compulsive EtOH consumption. A) A schematic of the DID lickometer apparatus depicting for the quinine challenge that quinine is added to water, sucrose, or EtOH for water, sucrose, and EtOH drinkers, respectively, following the 4-week DID paradigm. **B-D.** Fluid consumption from the three-day quinine challenge. With the addition of the bitterant quinine (0.3mM), control and FSI-ablated animals exhibited reduced water (**B**) and sucrose consumption (**C**). **D.** Control mice exhibited quinine-insensitive EtOH consumption, which was attenuated by FSI ablation across all three days. FSI ablation reduced the number of EtOH drinking bouts (E) and unorganized ethanol licks (F) during the quinine challenge. However, FSI ablation did not affect the mean bout length (**G**) or bout length distribution (**H**) or variance (**I**). **J.** A correlation of the mean quantity of EtOH consumed during week 4 of DID with the degree of compulsive drinking during the 3-day quinine challenge. **K.** A correlation of the mean number of EtOH drinking bouts during the 4-week DID paradigm with the degree of compulsive EtOH consumption in control and FSI ablated animals. **L.** A correlation of EtOH drinking bout length variance from the 4-week DID paradigm with the degree of compulsive EtOH consumption. † $p = 0.07$, * $p < 0.05$, ** $p < 0.01$. Data represented as mean ± SEM.

2.4 Discussion

We found that FSI ablation did not impact any index of water or sucrose consumption, but reduced voluntary ethanol consumption and disrupted organized ethanol drinking microstructure by reducing the number of ethanol drinking bouts and increasing the number of licks within a bout. Further, FSI ablation significantly attenuated compulsive ethanol consumption, despite having no effect on ethanol (unadulterated) preference. Collectively, these data causally implicate striatal FSIs in the pathophysiology of compulsive alcohol drinking.

Repeated training of sequentially executed motor actions, like lever pressing, results in the organization and expression of action sequences that are optimally-timed for reward acquisition (Botvineck et al., 2009;Dezfouli et al., 2014). Consistent with this, we found that control mice adapted their lick behavior over the four-week DID paradigm by selectively increasing the expression of ethanol drinking bouts, but not unorganized licks, which coincided with higher ethanol intake. Supporting the findings that the dorsal striatum is engaged during action sequence behavior (Jin & Costa 2010; Jin et al., 2014; Aldridge & Berridge 1998; Martiros et al., 2018), selective ablation of striatal FSIs disallowed the increase in the number of ethanol bouts and prevented the increase in relative expression of low frequency (<3 Hz) bout licks exhibited by control mice. These data therefore highlight a role for FSIs in organizing action sequences.

How FSIs organize action sequences remains an open question. Providing some clues, extant data demonstrates that FSIs are active during action sequence behavior (Jin & Costa 2010; Martiros et al., 2018), and encode kinematic properties of action (Kim et al., 2019; Roberts et al., 2019) as ensembles (Roberts et al., 2019) that regulate the concerted activity of striatal MSNs (Kim et al., 2019, Bakhurin et al., 2016; Duhne et al., 2020; Gritton et al., 2019; Klaus et al., 2011; Owen et al., 2018). Here we found that animals with intact FSIs exhibited distinct bout sequences centered around four licks per bout, while FSI-ablated animals exhibited greater variability in bout length and, at times, exhibited unconstrained bouts of 20 or more licks. These data suggest that FSIs contribute to a learning process for habitual action sequence constraint, which is mediated by the dorsolateral striatum (Rueda-Orozco & Robbe 2015).

Chronic ethanol exposure facilitates habit learning (Sjoerds et al., 2013; Everitt & Robbins 2016; Hopf et al., 2010; Lesscher et al., 2010; Corbit et al., 2012). In line with this evidence, we found that four weeks of DID produced compulsive ethanol consumption. FSI ablation abolished this compulsive ethanol consumption but also reduced overall ethanol consumption during the DID paradigm. It is possible that FSI-ablated animals were incapable of achieving a compulsive drinking phenotype simply due to not preferring ethanol or insufficient total ethanol exposure over the course of the DID paradigm. However, FSI ablation did not affect ethanol preference. In addition, the degree of compulsive consumption and the amount of ethanol consumed (total licks) were not significantly correlated. This might suggest that the reduction in ethanol consumption in FSI-ablated animals during the four-week DID paradigm may instead be due to the removal of the organized, compulsive drinking component from overall consumption. Supporting this, control and FSI-ablated mice began the DID paradigm drinking an equivalent amount, but, over time, FSI-ablated animals failed to increase the number of drinking bouts compared to control animals.

Finally, we found that two elements of drinking behavior corresponded with compulsive ethanol consumption. Organized drinking, expressed as the number of drinking bouts an animal exhibited during the four-week DID paradigm, positively correlated with the compulsive drinking score, while the variance in number of licks within a bout negatively correlated with the compulsive drinking score. We found that bout length and bout variance were normalized in the FSI-ablated mice during the quinine challenge, suggesting that the DLS was no longer recruited. It is possible that in this state, the DMS was re-engaged to elicit the goal-directed response resulting in normalized bout parameters. Supporting this idea, we found that FSI-ablation had no effect on water or sucrose bout parameters, and that these mice similarly failed to compulsively consume these solutions. Collectively, these data support previous work illustrating organization of lick microstructure in compulsive drinking rats (Darevsky et al., 2018) and identifies a cellular substrate contributing to this effect.

These data may suggest that alcohol targets FSIs to fuel the repeated execution of an organized action sequence, which ultimately leads to the compulsive behavior expression that is mediated, at least in part, by the dorsolateral striatum. Recent work from Walker et al. (2020) reveals differential expression of G protein-coupled receptors in the putamen of AUD patients and demonstrates that M4 muscarinic receptor activity, expressed on D1 MSNS, regulates alcohol consumption in rats. Considering these results with our own data, this suggest that alcohol regulates striatal FSIs through a microcircuit feedback mechanism mediated by the M4 muscarinic receptor. However, the involvement of the M4 receptor requires future research. Given that the data herein support that elimination of less than 1% of all striatal neurons attenuates ethanol drinking and abrogates compulsive ethanol consumption, FSIs represent a compelling target for pharmacological intervention in alcohol use disorder.

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Chapter 3: Chronic Ethanol Exposure Remodels Perineuronal Nets and Inhibitory Synapses on Striatal Fast-Spiking Interneurons

3.1 Introduction

Continued drinking in the face of negative consequences, or compulsive drinking, is a prominent feature of alcohol use disorder that is not adequately addressed by current pharmacotherapies (Swift & Aston 2015). The dorsolateral striatum, or putamen in humans and non-human primates, encodes habitual actions that are thought to underlie compulsive drinking in alcohol use disorder (Gerdemen et al., 2003; Graybiel 2008; Everitt & Robbins, 2005; Koob & Volkow 2010, 2016). There is clear evidence from both human and preclinical rodent models that chronic ethanol exposure promotes the hyperactivation of the dorsolateral striatum and subsequently biases behavior from goal-directed to habitual action strategies on instrumental tasks (Everitt et al., 2008; Everitt & Robbins 2016; Lüscher et al., 2020). For example, the duration of alcohol dependence in men and women correlates with intensity of alcohol-cue-induced responses in the putamen (Sjoerds et al., 2014). These participants additionally exhibited an overreliance on habitual responses during a computer-based instrumental learning task (Sjoerds et al., 2014). In preclinical studies, 4 weeks of voluntary ethanol consumption accelerates habitual control of natural reward seeking (Corbit et al., 2012), whereas extended ethanol exposure (3-4 months intermittent access) leads to compulsive drinking in rats (Hopf et al. 2010).

Parvalbumin expressing fast-spiking interneurons (FSIs) are a subpopulation of inhibitory interneurons that comprise approximately 1% of the neuron population in the striatum (Kawaguchi et al., 1995). They are enriched in the dorsolateral striatum (i.e., putamen) and provide the strongest form of feedforward inhibition onto striatal projection neurons (Tepper et al., 2010; Kita et al., 1990; Koós & Tepper, 1999, 2002). Notably, dorsal striatum FSIs are necessary for the expression of habitual actions for natural rewards (O'Hare et al., 2017), and we recently demonstrated that dorsal striatum FSIs encode the development of binge-like ethanol consumption and are necessary for the expression of compulsive-like drinking in C57BL6J mice (Patton et al., 2020).

Surrounding the majority of fast-spiking interneurons is a unique sub compartment of the extracellular matrix called perineuronal nets. These nets consist of chondroitin sulfate proteoglycans (e.g., aggrecan, brevican, neurocan), link proteins (e.g., HAPLN1, tenacin-R) and hyaluronan that condense around the soma and proximal neurites of fast-spiking interneurons to regulate synaptic transmission and neuronal excitability (Balmer 2016; Giamanco & Matthews 2012; Fawcett et al. 2019; Burket et al., 2021). Notably, HAPLN1 and aggrecan are expressed by PNN containing neurons in rodents, while other PNN components are expressed by both neurons and glia (Matthews et al., 2002; Dino et al., 2006; Galtrev et al., 2008).

While initial studies on perineuronal nets focused on their contribution to synaptic development (Pizzorusso et al., 2002), more recent work demonstrates that perineuronal nets continue to regulate neuronal function into adulthood (Lee et al., 2012; Carstens et al. 2016; Fawcett et al., 2019). For example, perineuronal nets regulate hippocampal synaptic plasticity (Frischknecht et al., 2009) and are necessary for a hippocampal- dependent memory task (Hylin et al. 2013). Importantly, perineuronal nets are dynamically expressed and can be modified by several factors including FSI activity (Dityatev et al., 2007), circadian rhythm (Pantazopoulos et al., 2020), stress (Laham & Gould 2022), and environmental enrichment (Stamenkovic et al., 2017; O'Connor et al. 2019). Additionally, more recent evidence indicates that drugs of abuse, including alcohol, modify perineuronal net expression to possibly subserve long-lasting changes in drug-related behavior (Chen et al., 2015; Slaker et al., 2016; Lasek et al., 2017; Chen & Lasek 2020; Brown & Sorg 2022; Dannenhoffer et al., 2022).

Provided these recent observations, and the necessity of dorsal striatum FSIs for compulsive-like ethanol consumption, we hypothesize that chronic alcohol exposure modifies perineuronal net expression in the dorsal striatum to regulate FSI physiology. In the following work, we demonstrate that 5 weeks of vapor ethanol exposure specifically reduces GABAergic synaptic transmission, but not glutamatergic transmission onto dorsal striatum FSIs. This reduction in GABAergic transmission was mediated by a decrease in the number of GABAergic synapses, primarily on somata and proximal dendrites of FSIs where perineuronal nets are expressed. Following this observation, we discovered that 5-week ethanol vapor exposure reduced the number of perineuronal net positive FSIs and reduced perineuronal net protein expression in the dorsal striatum. Notably, enzymatic reduction of perineuronal nets reduces GABAergic transmission onto dorsolateral striatum FSIs similarly to 5-week ethanol vapor exposure, suggesting that repeated alcohol exposure reduces GABAergic synaptic transmission by modifying perineuronal nets.

While previous literature demonstrates 6-week voluntary ethanol consumption increases cortical perineuronal net expression (Chen et al., 2015), here we show 5-week ethanol vapor exposure also degrades perineuronal nets in the striatum, illustrating brain region specific regulation of extracellular matrix in response to repeated ethanol exposure. The subsequent remodeling of GABAergic synapses onto dorsolateral striatum fast-spiking interneurons may significantly impact striatum-mediated behaviors, as fast-spiking interneurons are known to regulate habits (O'Hare et al., 2017) and compulsive-like ethanol consumption (Patton et al., 2020). Taken together, this study provides novel insight into the pathophysiology of chronic ethanol exposure and suggests that future therapies targeting perineuronal nets are a viable strategy to treat substance use disorder.

3.2 Methods

3.2.1 Animals

All experiments were performed in accordance with NIH guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Maryland Baltimore and the National Institute on Alcohol Abuse and Alcoholism. All C57BL/6J mice were housed with littermates (2-5 per cage) under a normal 12-hour light/dark cycle (lights on at 0900 hours, off at 2100 hours) with *ad libitum* access to food and water.

3.2.2 Surgical procedures

All stereotaxic injections were performed under isoflurane anesthesia (5% induction; 2%–3% maintenance) and viruses were injected at a rate of 30nl/min with a 25G syringe (Hamilton Company). For pain management, a topical lidocaine ointment (2.5% lidocaine, 2.5% prilocaine; Akorn) was applied to the incision site and systemic carprofen injections (5 mg/kg SQ) were administered for a minimum of 3 days following surgery. To isolate GABAergic projections from the globus pallidus or the reticular thalamic nucleus to the dorsolateral striatum, parvalbumin cre x tdTomato (PV-tdT) expressing mice on a C57BL/6J background (Tanahira et al., 2009; Mathur et al., 2013) were stereotaxically injected with a virus expressing channelrhodopsin (ChR2; AAV5-hSyn-ChR2-eYFP) in the globus pallidus (GP:AP −0.4 mm, ML ± 2.0 mm, DV −3.7 mm; 200 nl/side) or the reticular thalamic nucleus (RTN: AP −0.58 mm, ML ± 1.25 mm, DV −3.5 mm; 200 nl/side). To degrade perineuronal nets, PV-tdT mice were stereotaxically injected with chondroitinase ABC (ChABC: 15 units/mL in PBS w 1% BSA; Sigma) or vehicle unilaterally into the dorsolateral striatum (AP +0.60 mm, ML ± 2.25 mm, DV −3.0 mm; 500 nl/side). To label postsynaptic GABAergic density within PV cells, PV-tdT mice were stereotaxically injected with a fibronectin intrabody against Gephyrin (AAV5-EF1A-DIO-Gephyrin.FingR-eGFP; Benssussen et al., 2020) in the dorsolateral striatum (AP +0.60 mm, ML ± 2.25 mm, DV −3.0 mm; 500 nl/side).

3.2.3 Chronic Intermittent Ethanol Exposure (CIE)

Randomly sampled male and female mice were placed in Plexiglass inhalation chambers (60x36x60 cm) and exposed to ethanol vapor or air 16 hours/day, four days a week for a total of 5 weeks. Ethanol was volatilized by passing air through a tube submerged in 100% ethanol. After four days in the inhalation chambers, mice underwent a 72-hour forced abstinence period from ethanol. Control vapor chambers delivered only air without ethanol vapor. Vapor chamber ethanol concentrations were monitored daily and air flow was adjusted to produce ethanol concentrations within 1.8-2.0 % ethanol content measured by a digital alcohol breath tester (FFtopu). These conditions produce stable blood ethanol concentrations in C57BL/6J mice ranging from 150-200mg/dl (Lopez and Becker 2003). The alcohol dehydrogenase inhibitor Pyrazole (1 mmol/kg, i.p.) was injected to both control and ethanol vapor chamber mice on weeks 4-5 to stabilize blood ethanol concentrations.

3.2.4 Immunohistochemistry

Mice were transcardially perfused with room-temperature 0.1 M Phosphate Buffered Saline (PBS), pH 7.3, followed by room-temperature 4% (w/v) paraformaldehyde in PBS. Brains were extracted and post-fixed with 4% paraformaldehyde in PBS at 4°C for 24-48h. Coronal sections (50 µm) were cut using a Leica VT1000S vibrating microtome. For perineuronal net analysis, serial sections were stained with Wisteria floribunda agglutinin (WFA) conjugated to fluorescein (1:1,000; ThermoFisher L32481). Fast-spiking interneurons were fluorescently labeled with an anti-parvalbumin antibody (mouse; 1:1,1000; Sigma P3088). Presynaptic GABA terminals were fluorescently labeled with an anti-vesicular GABA transporter (VGAT) antibody (rabbit; 1:1,000; Synaptic Systems 131002). Slices were incubated in secondary antibodies conjugated to Alexa 594 (Donkey; 1:1000) at room temp for 4h.

3.2.5 Acute slice preparation

Randomly sampled male and female mice were deeply anesthetized with isoflurane before rapid decapitation and brain removal. 250 µm coronal sections were collected in ice cold modified artificial cerebral spinal fluid (aCSF: 194 mM sucrose, 30 mM NaCl, 4.5 mM KCl, 1 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, and 10 mM D-glucose bubbled with 95% oxygen, 5% carbon dioxide) using a Leica VT1200 vibrating microtome. Brain sections were then transferred to regular aCSF (124 mM NaCl, 4.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, and 10 mM D-glucose, bubbled with

95% oxygen, 5% carbon dioxide) and incubated at 32°C for 30 min for recovery, followed by room temperature until recording.

3.2.6 3D neuronal reconstruction

Striatal fast-spiking interneurons were filled with biocytin (5%; Tocris 3349) with a borosilicate glass pipette (3-5 MΩ resistance) under 40x immersion objective. Slices (250 µm) were fixed in 4% paraformaldehyde overnight at 4°C. The next day, slices were washed in 0.1 M PBS (3 x 20 min) and blocked with 1% bovine serum albumin (BSA) in PBS + 0.3% Triton X-100 (PBS-T) for 2 h at room temperature. Slices were incubated with Alexa Fluor 594-streptavidin (Invitrogen, #S32356) (1:1000, 1% BSA in 0.3% PBS-T) overnight at 4°C. The next day, slices were washed in PBS (3 x 20 min), mounted on slides, and coverslipped using ProLong Diamond Antifade (Invitrogen, #P36965). Images were acquired with a Nikon W1 microscope (see confocal methods) and neurons were reconstructed in Neurolucida 360 (version 2020.1.1) using semi-automated reconstruction with a directional kernels algorithm.

3.2.7 Microscopy

For 3D neuronal reconstruction, confocal images were acquired with a Nikon W1 microscope equipped with a 561 nm laser. Neurons were imaged using a 40x oil (1.30 NA) objective with a lateral resolution of 0.160 µm per pixel and a 0.200 µm z-step. For perineuronal net analysis and gephyrin-VGAT colocalization, confocal images were acquired with a Nikon A1 microscope equipped with a 488 and 561 nm lasers. Neurons were imaged using a 40x oil (1.40 NA) objective with a lateral resolution of 0.2 µm per pixel and a 0.20 µm z-step. Images were denoised with Nikon NIS-ElementsDenoise.ai software. Surface models and filament models were generated using Imaris Cell Imaging Software (Oxford Instruments). Colocalized gephyrin-vGAT puncta were quantified using the dot-model function with masking application in Imaris. Fluorescent images for population perineuronal net analysis were visualized on a Nikon Eclipse Ti2 fluorescent microscope and captured with a Nikon DS-Qi2 camera. Perineuronal nets surrounding fast-spiking interneurons were analyzed by a fluorescent intensity profile threshold, where enriched interneurons contained a signal of $\geq$ 1000 intensity units from background and undetected nets were classified by a signal intensity < 500 intensity units from background.

3.2.8 Whole-cell Patch-clamp electrophysiology

Slices were hemisected, placed into a recording chamber, and perfused with temperature controlled aCSF (29-31°C) during the recording procedure (aCSF: 124 mM NaCL, 4.5 mM KCl, 1 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 10 mM D-glucose, 2 mM $CaCl_2$, bubbled with 95% oxygen, 5% carbon dioxide). Post synaptic currents were electrically evoked every 20 s using a constant current isolated stimulator (Digitimer Ltd model DS3) and a concentric bipolar stimulating electrode (World Precision Instruments) located approximately 150 µm from the recording electrode. Excitatory post synaptic currents (EPSCs) were pharmacologically isolated by adding picrotoxin (50 µM) to the recording aCSF and inhibitory post synaptic currents (IPSCs) was isolated with DL-AP5 (50 µM) and CNQX (5 µM).

Optogenetically-evoked currents were generated from an LED driver (Thorlabs, LEDD1B T-Cube) and a 470 nm LED delivering blue light (2 - 4ms pulse duration) through an optical fiber located approximately 150µm from the recording electrode. For optically-evoked asynchronous currents, calcium was replaced with strontium (2 mM) in the recording aCSF. To photo-uncage GABA, a 2 ms pulse of blue light (470 nm) was delivered to an acute slice preparation bath perfused with 250 µM ruthenium-bipyridine-triphenylphosphine caged GABA (RuBi-GABA) (Tocris 4709; Rial Verde et al., 2008). Spontaneous inhibitory currents (sIPSCs) were pharmacologically isolated by adding DL-AP5 (50 µM) and CNQX (5 µM) to the recording aCSF.

Dorsolateral striatum FSIs were fluorescently visualized using Eclipse FN1 Nikon microscope and Intenslight C-HGFIE (Nikon). Images were digitally rendered using a Hamamatsu ORCA-ER digital camera and associated HC image live 4.8.0 software. Striatal fast-spiking interneurons were voltage clamped at -60 mV using a MultiClamp 700B Amplifier (Molecular Devices).

Electrically-evoked currents excitatory currents were recorded using a borosilicate glass pipette (3-5 MΩ resistance) filled with Cs-Meth-based internal solution (120 mM $CsMeSO_3$, 10 mM HEPES, 5 mM NaCl, 10 mM TEA-Cl, 1.1 mM EGTA, 0.3 mM Na-GTP, 5 mM QX-314, 4 mM Mg-ATP).Optogenetically and electrically-evoked inhibitory currents were recording using a borosilicate glass pipette (3-5 MΩ resistance) filled with Cs-Cl-based internal solution (150 mM CsCl, 10 mM HEPES, 2 mM $MgCl_2$, 0.3 mM Na-GTP, 5 mM QX-314, 3 mM Mg-ATP, and 0.2 mM BAPTA). Signals were filtered at 2k Hz, digitized at 10 kHz and acquired using Clampex 10.4.1.4 software (Molecular Devices). Data were analyzed offline using Clampfit software (Molecular Devices) and Mini Analysis Program (Synaptosoft).

3.2.9 Western blot

Following 5 weeks of ethanol exposure, mice were sacrificed by rapid decapitation and brains were immediately removed for tissue collection. The dorsal striatum (AP: +1.3 - 0.3 mm from bregma) was dissected from a 1 mm coronal section cut from a mouse brain mold. Left and right hemispheres were pooled and flash frozen in crushed dry ice. Tissue was stored at -80°C until further processing. Tissue lysates were prepared in RIPA lysis buffer (Milllipore 20-188) containing protease (Promega G6521) and phosphatase inhibitors (Sigma 4906845001). Protein content was normalized via BCA assay and 10µg of protein was loaded onto NuPAGE Novex Tris-Acetate mini protein gels (Novex EA0375) and run with Tris-Acetate buffer (Novex LA0041). Gels were electroblotted onto nitrocellulose membrane (0.45 µ pore size) using XCell II Blot Module (ThermoFisher). Membranes were blocked in Intercept blocking buffer (1:2 in TBS; Li-Cor) for 2h at room temperature before incubation overnight 4 °C in primary antibody: HAPLN1 (Goat; 1:1,000; R&D Systems AF2608) or Aggrecan (Rabbit; 1:1,000; Millipore AB1031). Membranes were washed three times for 10 min in TBS-T (0.1% Tween 20) before incubation in secondary antibodies: Donkey anti-rabbit (IRDye 926; 1:10,000; Li-Cor or Donkey anti-goat (IRDye 800; 1:10,000; Li-Cor). Images were captured using an Odyssey CLX (Li-Cor). Protein signal was normalized with a total protein stain (Revert total protein stain; Li-cor) and data are represented as % control.

3.2.10 Statistical analysis

All statistical analyses were performed in GraphPad Prism 9.0. Data are represented as mean $\pm$ SEM. Comparisons between two groups were analyzed with a Student's t-test while comparisons of three or more groups were analyzed with two-way ANOVA. Multiple comparisons were corrected with Sidak's test or Dunnett's test when comparing multiple time points to a single baseline. For the Sholl analysis, a mixed-effect model was performed (Wilson et al., 2017). All N are specifically defined in the legend of each figure. For data derived from chronic ethanol exposed mice, N is defined as an individual animal. This N is derived from an average recording from 2-3 cells per animal or an average fluorescent quantification of 5-10 cells per animal. For ChABC treatment, N is defined as an individual animal derived from an average recording from 2-3 cells.

3.3 Results

We first investigated whether chronic ethanol exposure influenced the cellular morphology of dorsolateral striatum fast-spiking interneurons, as this would likely result in profound changes to FSI function. Dorsolateral striatum FSIs were dye filled and 3D reconstructed (**Figure 3.1A,B**) and the resulting Sholl analysis revealed no statistically significant differences in dendritic complexity between control and chronic ethanol treated mice ($F_{(1,18)} = 0.037$, $p = 0.85$; Mixed-effect model; Wilson et al. 2017; **Figure 3.1C**). We then performed whole-cell patch clamp recordings from dorsolateral striatum FSIs to determine if chronic ethanol exposure modulated synaptic strength onto FSIs. We found no difference in the amplitude of electrically-evoked excitatory synaptic currents across increasing stimulus intensities ($F_{(1,10)} = 0.058$, $p = 0.82$; two-way repeated measures ANOVA; **Figure 3.1D**). However, chronic ethanol exposure dramatically reduced electrically-evoked inhibitory transmission onto dorsolateral striatum FSIs ($F_{(1,10)} = 19.59$, $p = 0.001$; two-way repeated measures ANOVA; **Figure 3.1E**). Following these results, we recorded spontaneous inhibitory release events onto dorsolateral striatum FSIs to determine if chronic ethanol exposure modulated a pre or post synaptic mechanism to regulate GABAergic transmission. FSIs from chronic ethanol exposed mice received less frequent spontaneous inhibitory currents (sIPSCs) compared to FSIs from control mice ($t_{(18)} = 3.12$, $p < 0.001$; Student's t-test; **Figure 3.1F,G**) but there was no change in the amplitude of the sIPSCs ($t_{(18)} = 1.47$, $p = 0.16$; Student's t-test; **Figure 3.1H).**

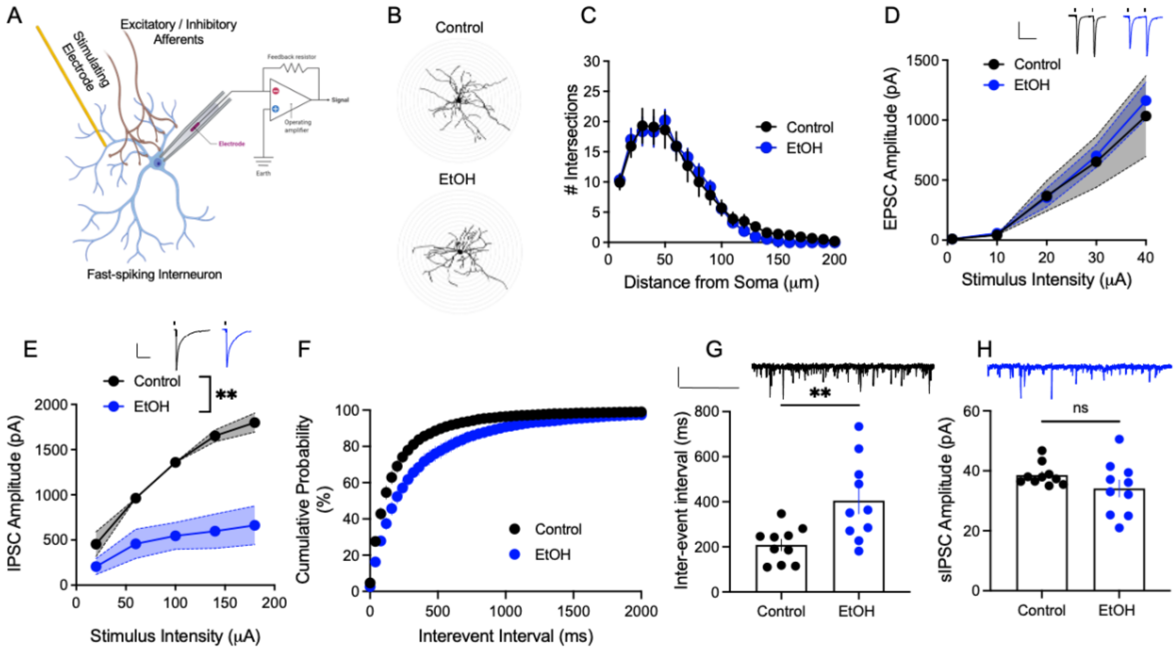


Figure 3.1 Chronic ethanol exposure specifically reduces inhibitory synaptic transmission onto dorsal striatum fast-spiking interneurons (FSIs). A) schematic of whole-cell patch clamp recording from a dorsal striatum FSI. **B**) Representative dye-filled cells from control (top) and chronic ethanol (bottom) treated mice (sholl ring radius 10µm increments). **C**) Sholl analysis reveals no impact of chronic ethanol exposure on FSI dendrite branch complexity (N=10 cells, 5 mice per group, 3M 2F). **D**) Chronic ethanol exposure does not impact electrically-evoked excitatory synaptic transmission onto dorsal striatum FSIs (N=6 mice per group, 3M 3F). **E**) However, chronic ethanol exposure dramatically reduces inhibitory synaptic transmission onto FSIs (N=6 mice per group, 3M 3F). Inlet: representative electrically evoked synaptic currents from control (black) and chronic ethanol (blue) treated mice (scale bars 500pA, 100ms). **F-H**) Spontaneous inhibitory synaptic transmission onto dorsal striatum FSIs (N=10 mice per group, 5M 5F). Inlet: representative traces from control (black) and chronic ethanol (blue) treated mice (scale bar 100pA, 2s). Chronic ethanol exposure reduces the frequency of spontaneous inhibitory events (**F,G**) but does not impact event amplitude (**H**). All data represented as mean $\pm$ SEM. ** $P < .01$.

Two major extrinsic sources of GABA to the dorsal striatum, the globus pallidus (GP) and the reticular thalamic nucleus (RTN), synapse onto and inhibit striatal fast spiking interneurons (Mallet et al. 2012; Klug et al. 2018). To investigate which GABAergic projections were specifically impacted by chronic ethanol exposure, we virally expressed ChR2 into the globus pallidus or the reticular thalamic nucleus and recorded optically-evoked inhibitory post synaptic currents onto FSIs. GABAergic transmission from the GP was dramatically reduced by chronic ethanol exposure ($F_{(1,70)} = 42.45$, $p < 0.0001$; two-way repeated measures ANOVA; **Figure 3.2A**), but there was no change in the paired-pulse ratio (PPR) from the GP ($t_{(14)} = 1.07$, $p = 0.30$, Student's t-test; **Figure 3.2B**). Since we found a decrease in the frequency of sIPSCs (**Figure 3.1F, G**) but saw no difference in PPR (**Figure 3.2B**), we decided to specifically isolate spontaneous release events from the GP by analyzing optically-evoked asynchronous events enabled by replacing calcium with strontium in the external recording solution. Chronic ethanol exposure reduced the frequency of asynchronous release events from the GP ($t_{(10)} = 4.14$, $p < 0.005$; Student's t-test; **Figure 3.2C**) but had no impact on event amplitude ($t_{(10)} = 0.09$, $p = 0.93$; Student's t-test; **Figure 3.2D**).

Next, we explored optically-evoked inhibitory transmission from the RTN. Similar to GP inputs, chronic ethanol exposure reduced the amplitude of optically-evoked inhibitory synaptic currents from the RTN onto dorsolateral striatum FSIs ($F_{(1,14)} = 10.36$, $p < 0.01$; two-way repeated measures ANOVA; **Figure 3.2E**). Additionally, chronic ethanol exposure did not impact PPR from the RTN ($t_{(14)} = 0.58$, $p = 0.57$; Student's t-test; **Figure 3.2F**). Chronic ethanol exposure reduced the frequency of optically-evoked strontium-enabled asynchronous inhibitory synaptic release events from the RTN ($t_{(10)} =$

6.20, $p < 0.001$; Student's t-test; **Figure 3.2G**) but had no impact on event amplitude ($t_{(10)} = 0.41$, $p = 0.69$; Student's t-test; **Figure 3.2H**).

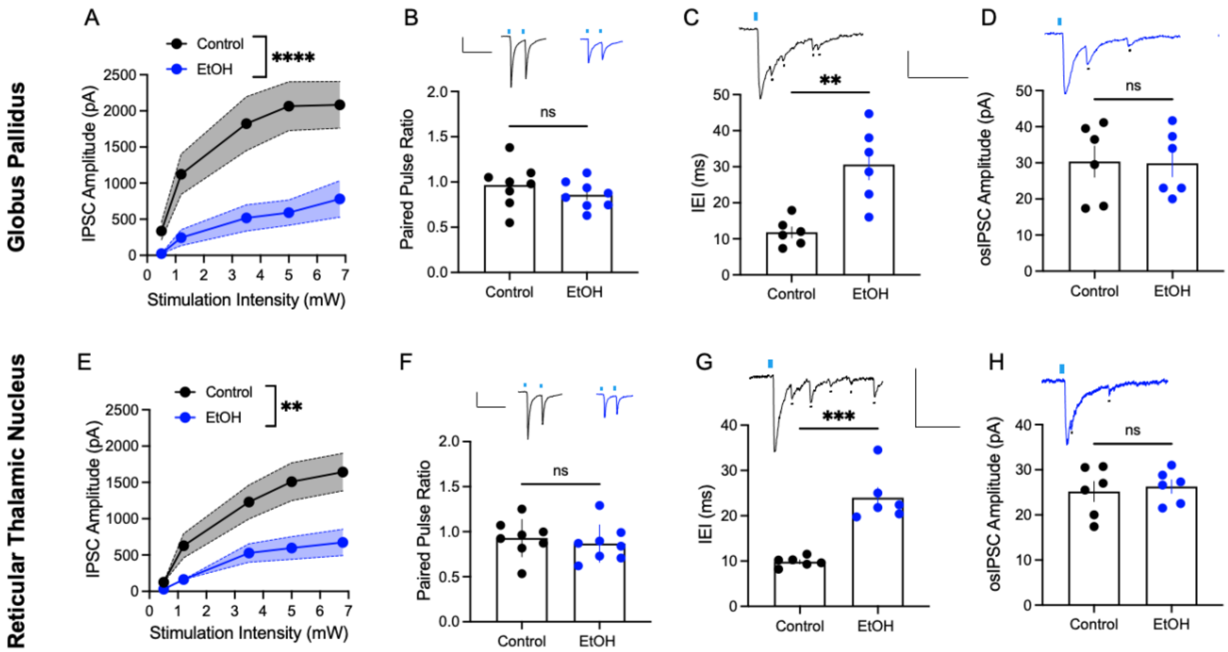


Figure 3.2 Chronic ethanol exposure reduces inhibitory synaptic transmission from the globus pallidus (GP) and the reticular thalamic nucleus (RTN). A) Chronic ethanol exposure reduces optically-evoked inhibitory currents from the GP but (**B**) has no impact on release probability (N=8 mice per group, 4M 4F) Inlet: representative traces from control (black) and chronic ethanol (blue) treated mice (scale bar 500pa, 100ms). **C,D**) Optically-evoked strontium asynchronous inhibitory post synaptic currents from the GP. Chronic ethanol exposure reduces the (**C**) frequency but not the (**D**) amplitude of asynchronous inhibitory currents from the GP. Inlet: representative traces from control (black) and chronic ethanol (blue) treated mice (scale bar 200pA, 20ms). **E**) Chronic ethanol exposure reduces optically-evoked inhibitory currents from the RTN but (**F**) has no impact on release probability (N=8 mice per group, 4M 4F; scale bar 500pa, 100ms). **G**) Chronic ethanol exposure reduces the frequency of asynchronous inhibitory currents but (**H**) does not impact asynchronous current amplitude (N= 6 mice per group, 3M 3F; scale bar 200pA, 20ms). All data represented as mean $\pm$ SEM. ** P < .01, *** p<.001, **** p< .0001.

The reduction in the frequency of sIPSCs arising from the GP and RTN suggested that the reduction in evoked synaptic transmission is regulated by a pre-synaptic mechanism. However, we found that the PPR of RTN- and GP-derived synaptic currents were unchanged following chronic ethanol exposure (**Figure 3.2B,F**). A possible explanation is that chronic ethanol exposure reduces the number of active GABAergic synapses. To test this hypothesis, we virally expressed a GFP intrabody against the postsynaptic GABAergic synapse marker, gephyrin, into striatal FSIs and immunostained for the pre-synaptic GABAergic marker VGAT. Using Imaris imaging software, we quantified colocalized VGAT- and gephyrin-positive puncta to identify putative GABAergic synapses (**Figure 3.3A**). Chronic ethanol exposed mice exhibited fewer GABAergic synapses onto dorsolateral striatum FSIs compared to control mice (**Figure 3.3B-D**). Notably, the loss of synapses was more pronounced on the somata ($t_{(14)} = 4.491$, $p < 0.001$; Student's t-test; **Figure 3.3B**) and proximal branches of FSIs ($t_{(14)} = 6.58$, $p < 0.0001$; Student's t-test; **Figure 3.3C**), but still present on distal processes ($t_{(14)} = 2.16$, $p < 0.05$; Student's t-test; **Figure 3.3D**).

To further investigate whether chronic ethanol exposure resulted in a postsynaptic silencing of inhibitory synapses or merely a loss of presynaptic elements that leave postsynaptic sites intact (and unsilenced), we photo-uncaged RuBI-GABA in a slice preparation and recorded from FSIs using whole-cell patch clamp electrophysiology in voltage clamp mode. We found that uncaging IPSCs (uIPSCs) onto FSIs from control and chronic ethanol exposed mice were not significantly different, suggesting that postsynaptic inhibitory synapses remain unsilenced ($F_{(1,14)} = 0.19$, $p = 0.67$; **Figure 3.3E,F**).

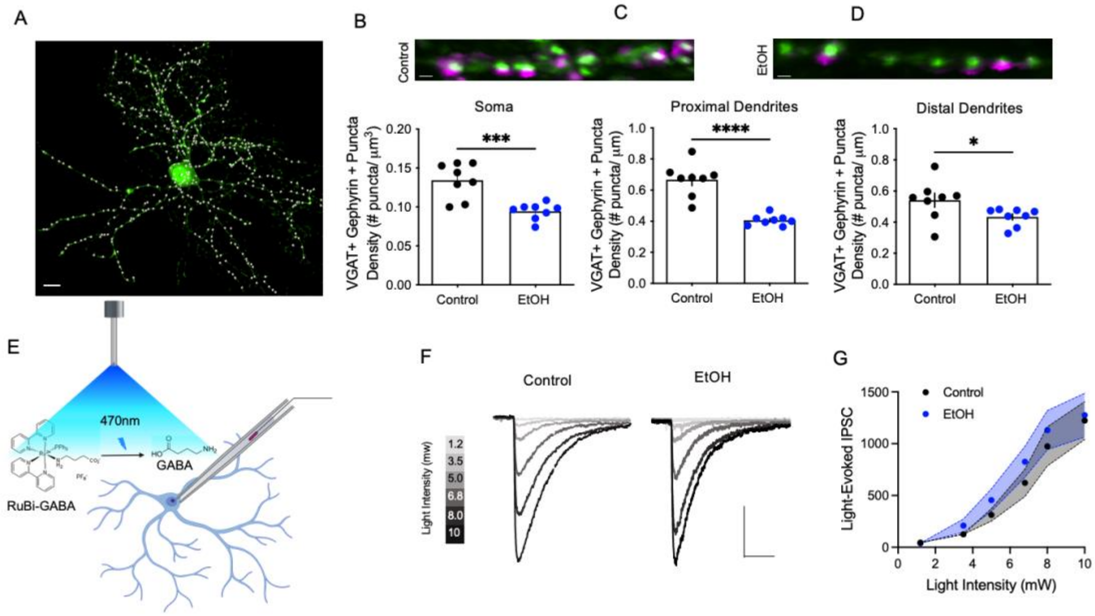


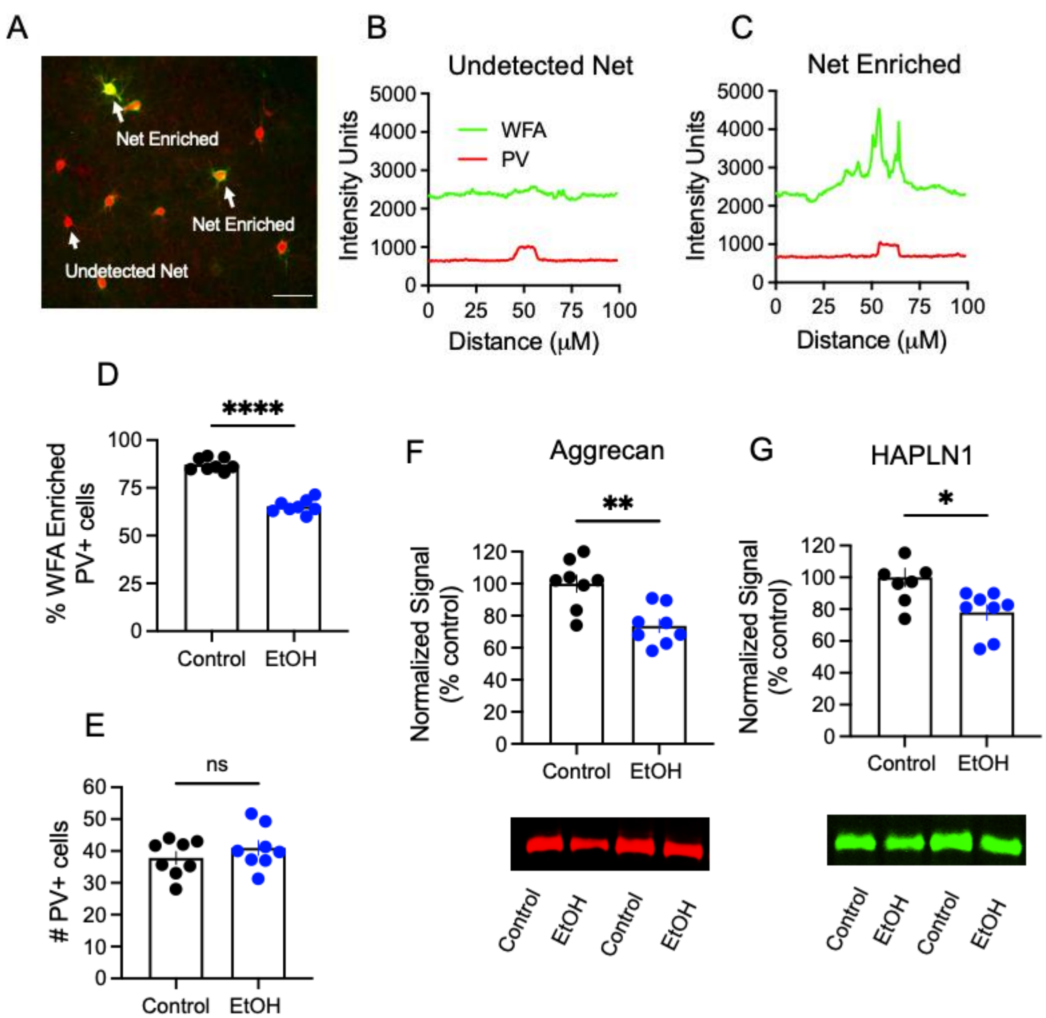
Figure 3.3 Chronic ethanol exposure reduces GABAergic synaptic contacts onto dorsal striatum FSIs. A) Representative dorsal striatum FSI expressing a cre-dependent gephyrin-GFP intrabody (green) with an immunofluorescent marker for vesicular GABA transporter (VGAT) puncta. VGAT positive puncta were masked onto the GFP signal and colocalized signals (white dots) were labeled with a dot function model to quantify GABAergic synapses (scale bar 10µm). **B-D**) quantification of GABAergic synapses onto cellular subdivisions of dorsal striatum FSIs. Top: representative fluorescent image of proximal neuronal branch from control (left) and chronic ethanol (right) treated mice depicting colocalized VGAT (purple) puncta onto gephyrin (green) presynaptic boutons (scale bar 1µm). **B**) Chronic ethanol exposure dramatically reduces the density of colocalized VGAT puncta onto the somas and **C**) proximal dendrites ($\leq$ 20µm from soma) of dorsal striatum FSIs. **D**) Additionally, chronic ethanol exposure reduces the density of colocalized VGAT puncta onto distal dendrites (> 40µm from soma). N=8 mice per group, 4M 4F. **E**) schematic of whole-cell patch clamp recording from fast-spiking interneuron during photo-uncaging of RuBi-GABA-II. **F**) Representative traces of light-evoked GABA currents from fast spiking interneurons of control (left) and chronic ethanol (right) treated mice at increasing stimulus intensity (scale bar = 200pA, 100ms). **G**) Group quantification of light-evoked inhibitory currents from control (black) and chronic ethanol (blue) treated mice at increasing stimulus intensity (n=8 mice per group). All data represented as mean $\pm$ SEM. * P < .05, *** p<.001, **** p< .0001

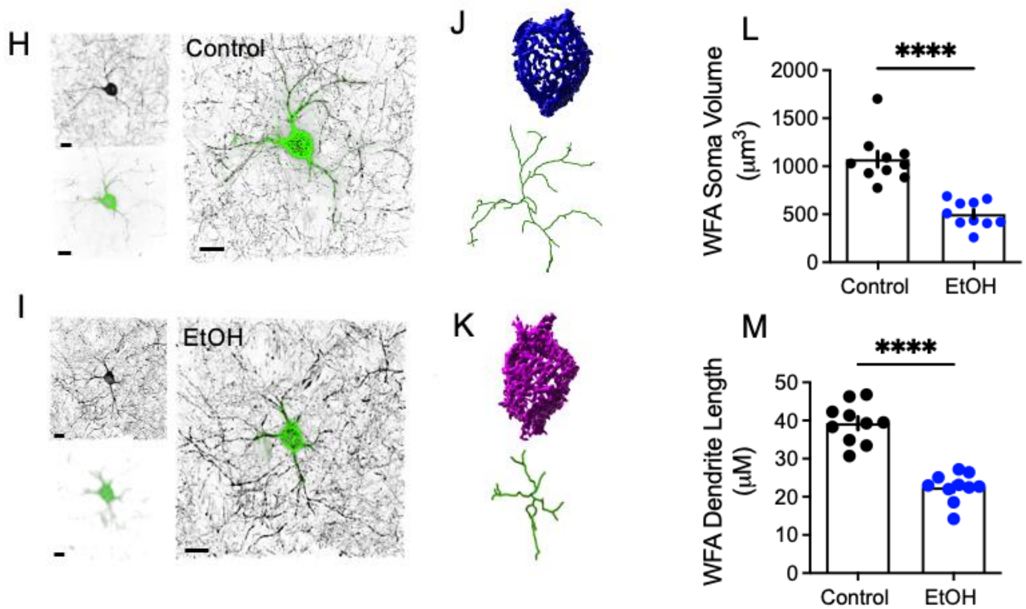
As the reduction in putative GABAergic synapses was more prominent around the somata and proximal processes of FSIs, we looked for a perisomatic mechanism that would account for region specific synapse loss. Perineuronal nets, a sub compartment of the extracellular matrix, condense around the somata and proximal branches of FSIs and are known to regulate synaptic transmission onto FSIs (Wang & Fawcett 2012; Carstens et al. 2016; Fawcett et al. 2019). We therefore investigated whether chronic ethanol exposure modulated perineuronal net expression. We first confirmed that perineuronal nets are indeed present in the dorsal striatum of adult C57BL/6J mice and surround PV+ FSIs (**Figure 3.4A**) consistent with previous literature (Lee, Leamey, & Sawatari 2012). We noticed that not every FSI was enriched with perineuronal net protein, but that a subpopulation could be categorized as being "undetected" for perineuronal nets based on a fluorescent signal intensity threshold (**Figure 3.4A-C**).

We next examined the effect of chronic ethanol exposure on perineuronal net presence surrounding FSIs. Five weeks of ethanol vapor chamber exposure reduced the number of detected perineuronal net-enriched FSIs ($t_{(14)} = 12.83$, $p < 0.0001$; Student's t-test; **Figure 3.4D**), but had no impact on the total number of PV+ FSIs ($t_{(14)} = 1.01$, $p = 0.33$; Student's t-test; **Figure 3.4E**). The reduction in perineuronal net-positive (PNN+) FSIs was not evident after 2-weeks of ethanol exposure (mean control = 64% PNN+, mean EtOH = 68% PNN+; $t_{(10)} = 0.85$, $p = 0.41$; Student's t-test). We followed these results by blotting for two proteins that are associated with perineuronal nets, the proteoglycan aggrecan and the link protein hyaluronan and proteogclycan link protein 1 (HAPLN1) (Yi et al., 2012; Fawcett et al., 2019). Consistent with our population analysis, aggrecan expression was reduced in dorsolateral striatum punches from chronic ethanol exposed mice ($t_{(14)} = 3.87$, $p < 0.01$; Student's t-test; **Figure 3.4F**) as was HAPLN1 expression compared to control animal striatal punches ($t_{(14)} = 2.91$, $p < 0.05$; Student's t-test; **Figure 3.4G**).

To determine whether specific cellular sub-compartments were impacted by chronic ethanol exposure, we confocally imaged perineuronal net signal from control (**Figure 3.4H**) and chronic ethanol treated mice using WFA (**Figure 3.4I**) and generated 3D surface expression models of perineuronal net signal surrounding FSI somata as well as branch models of perineuronal net signal extending into proximal branches (**Figure 3.4J,K**). Analysis of these models revealed a significant reduction in perineuronal net WFA+ signal surrounding FSI somata ($t_{(18)} = 6.24$, $p < 0.0001$; Student's t-test; **Figure 3.4L**) and a reduction in the length of perineuronal net WFA+ signal extending along FSI branches ($t_{(18)} = 8.23$, $p < 0.0001$; Student's t-test; **Figure 3.4M**). However, there was no difference in the branch length of the PV+ neurons analyzed for this experiment (mean control = 49.22 µm, mean EtOH = 49.3 µm; $t_{(18)} = 0.03$, $p = 0.98$; Student's t-test).

Figure 3.4 Chronic ethanol exposure reduces perineuronal net protein expression in the dorsal striatum. **A**) Immunofluorescent image of parvalbumin+ FSIs (red) and WFA+ perineuronal nets (green) depicting net enriched and undetected net cells (scale bar 100µm). **B,C**) Fluorescent intensity profile of an undetected net cell and a net enriched cell. **D**) Chronic ethanol exposure reduces the % of net enriched FSIs in the dorsal striatum but (**E**) does not reduce the number PV+ FSIs (N=10 mice per group, 5M 5F). Chronic ethanol exposure reduces the expression of perineuronal net proteins (**F**) aggrecan and (**G**) HAPLN1 in the dorsal striatum (N=8 mice per group, 4M 4F). **H,I**) Representative confocal fluorescent images of PV+ FSI (black) and WFA+ perineuronal net (green) from a control (**H**) and chronic ethanol (**I**) treated mice (scale bar 15µm). **J,K**) Top: 3D volume reconstruction of WFA perineuronal net fluorescent signal from control (**J**) and chronic ethanol (**K**) treated mice. Bottom: Filament model of WFA perineuronal net fluorescent signal. **L**) Chronic ethanol exposure reduces the volume of WFA fluorescent signal surrounding PV+ FSI somas. **M**) Chronic ethanol exposure reduces the average branch length covered by WFA perineuronal net fluorescent signal (N=10 mice per group, 5M 5F). All data represented as mean $\pm$ SEM. *P < 0.05. ** P < 0.01, **** P < 0.0001.





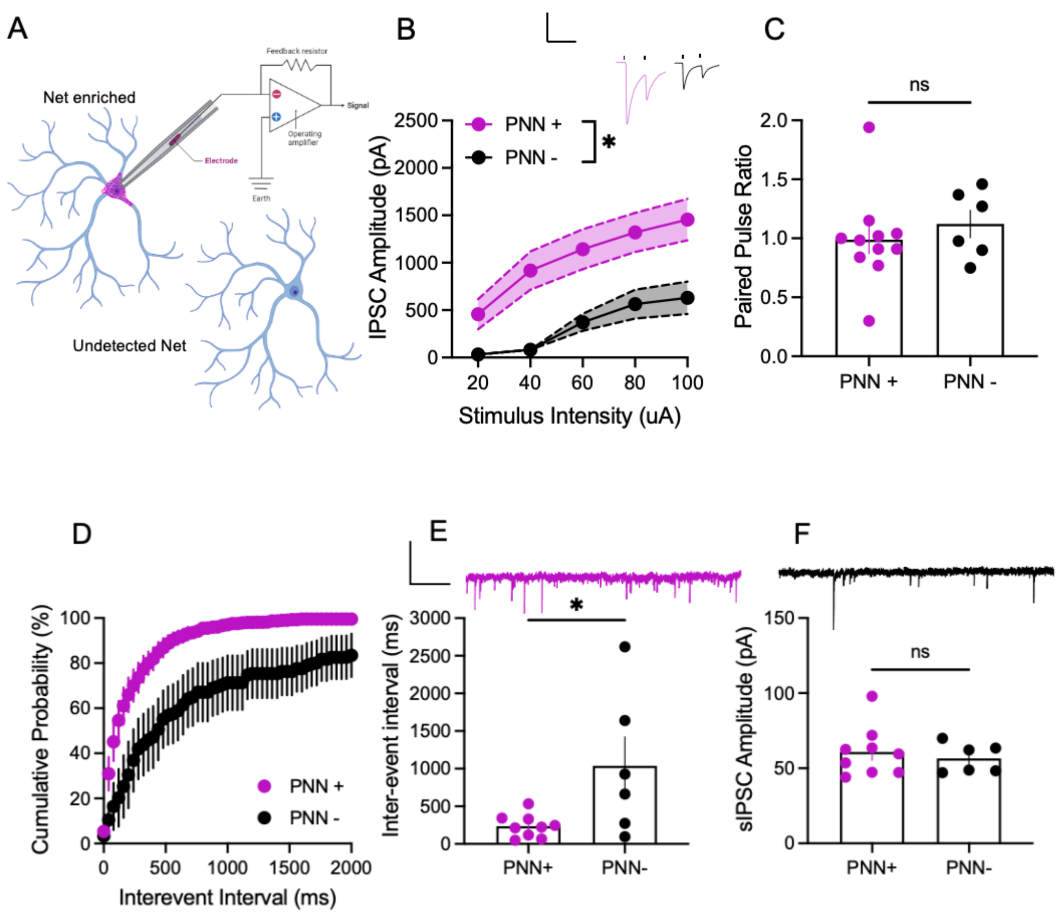
H
Control
I
EtOH
J
K
L
****
WFA Soma Volume (μm^3)
2000
1500
1000
500
0
Control
EtOH
M
****
WFA Dendrite Length (μM)
50
40
30
20
10
0
Control
EtOH

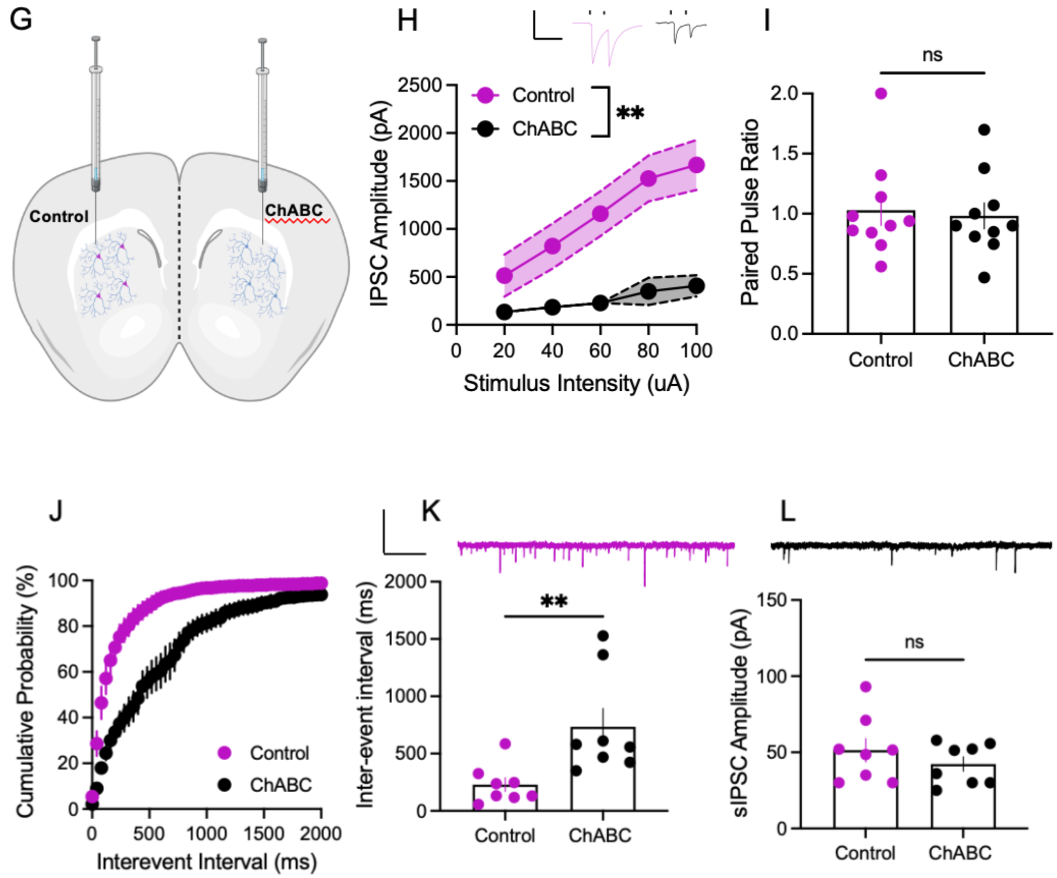
During brain development, perineuronal nets regulate the formation of synaptic connections (Pizzorusso et al., 2002). We therefore investigated whether perineuronal nets regulated GABAergic transmission onto dorsolateral striatum fast-spiking interneurons. Since dorsolateral striatum FSIs contain both net enriched and undetected net populations, we recorded electrically-evoked GABAergic transmission onto these two FSI sub-populations (**Figure 3.5A**). PNN+ FSIs exhibited larger electically-evoked IPSC amplitudes compared to PNN- FSIs ($F_{(1,15)} = 8.10$, $p < 0.05$; two-way repeated measures ANOVA; **Figure 3.5B**). This difference was not due to a change in presynaptic release probability, as PNN+ and PNN- FSIs exhibited no mean difference in the PPR ($t_{(15)} = 0.74$, $p = 0.47$; Student's t-test; **Figure 3.5C**). To further explore differences in GABAergic transmission between these FSI sub-populations, we recorded sIPSCs onto PNN+ and PNN- FSIs. PNN+ FSIs exhibited greater frequency of sIPSC events compared to PNN- FSIs ($t_{(13)} = 2.53$, $p < 0.05$; Student's t-test; **Figure 3.5D,E**), but there was no change in sIPSC amplitude ($t_{(13)} = 0.56$, $p = 0.58$; Student's t-test; **Figure 3.5F**).

Since chronic ethanol exposure degrades perineuronal nets (**Figure 3.4**) and dorsolateral striatum PNN+ FSIs exhibited stronger GABAergic input (**Figure 3.5A-F**), we next examined the consequence of enzymatically degrading PNNs on GABAergic transmission onto dorsolateral striatum FSIs. Enzymatic degradation of PNNs with Chondroitinase ABC (ChABC) dramatically reduced the number of PNN+ FSIs at 3-4 days following stereotaxic microinjection into the dorsolateral striatum (mean control = 70% PNN+, mean ChABC = 26% PNN+; $t_{(6)} = 5.15$, $p < 0.01$; Student's t-test). This effect was still evident at 14 days post injection (mean control = 65% PNN+, mean ChABC = 40% PNN+; $t_{(14)} = 4.5$, $p < 0.0001$; Student's t-test). Notably degrading PNNs had no impact on the number of PV+ FSIs (mean control 147 PV+ cells, mean ChABC = 149 PV+ cells; $t_{(14)} = 0.30$, $p = 0.77$; Student's t-test of day 14 samples).

Next, we recorded dorsolateral striatum FSIs and compared GABAergic transmission from control and ChABC treated tissue (**Figure 3.5G**). Enzymatically degrading PNNs dramatically reduced electrically-evoked IPSCs onto dorsolateral striatum FSIs ($F_{(1, 18)} = 14.89$, $p < 0.01$; two-way repeated measures ANOVA; **Figure 3.5H**). This reduction in GABAergic transmission was not due to a change in presynaptic release probability as ChABC treatment did not affect PPR ($t_{(18)} = 0.27$, $p = 0.79$; Student's t-test; **Figure 3.5I**). Again, we recorded sIPSCs onto dorsolateral striatum FSIs from control and ChABC treated mice. Degrading PNNs reduced the frequency of sIPSC events ($t_{(14)} = 3.0$, $p < 0.01$; Student's t-test; **Figure 3.5JK**), but had no impact on sIPSC event amplitude ($t_{(14)} = 1.0$, $p = 0.33$, Student's t-test; **Figure 3.5L**).

Figure 3.5 Perineuronal nets (PNNs) stabilize inhibitory synaptic transmission onto dorsal striatum FSIs. **A**) schematic of whole-cell patch recording from net enriched (PNN+) or undetected net (PNN-) FSI. **B**) PNN+ FSIs exhibit larger evoked inhibitory synaptic currents compared to PNN- FSIs (N=11 PNN+ cells, 6 PNN- cells; 5M 6F, 3M 3F). Inlet: representative traces from PNN+ (pink) and PNN- (blue) FSIs (scale bar 1nA, 100ms). **C**) The presence of an enriched perineuronal net does not impact inhibitory synaptic release probability. **D-F**) spontaneous inhibitory post synaptic currents recorded from dorsal striatum FSIs (N=11 PNN+ cells, 6 PNN- cells; 5M 6F, 3M 3F). Inlet: representative traces from PNN+ (pink) and PNN- (black) FSIs (scale bar 100pA, 1s). **D,E**) PNN+ FSIs exhibit greater frequency spontaneous inhibitory currents but (**F**) no change in current amplitude. **G**) schematic of unilateral microinjection of chondroitinase ABC (ChABC) into the dorsal striatum to enzymatically degrade PNNs. **H**) Degrading PNNS with ChABC dramatically reduces electrically-evoked inhibitory synaptic currents onto dorsal striatum FSIs but (**I**) does not impact synaptic release probability (N=10 mice, 5M 5F; scale bar 1nA, 100ms). **J,K**) Degrading PNNS with ChABC reduces the frequency of spontaneous inhibitory currents onto dorsal striatum FSIs but (**L**) does not impact event amplitude (N=8 mice, 4M 4F; scale bar 100pA, 1s). All data represented as mean $\pm$ SEM. *P < 0.05. ** P < 0.01.

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3.4 Discussion

We found that 5-week ethanol vapor exposure specifically reduced GABAergic transmission onto dorsolateral striatum FSIs but had no impact on glutamatergic transmission. This effect was evident in two major GABAergic projections to the dorsal striatum, the globus pallidus and the reticular thalamic nucleus. Chronic ethanol vapor exposure reduced the number of GABAergic synaptic contacts onto dorsal striatum FSIs that were predominately localized to the somata and proximal dendrites where perineuronal nets reside. Notably, chronic ethanol vapor exposure degraded perineuronal net protein expression and enzymatically degrading PNNs reduced GABAergic transmission in the dorsolateral striatum. Collectively, these data suggest that PNNs are a viable therapeutic target for future interventions against alcohol use disorder.

The dorsolateral striatum encodes the habitual actions that underlie compulsive behavior seen in substance use disorder (Graybiel 2008; Everitt & Robbins, 2005). Recently we demonstrated that dorsolateral striatum fast-spiking interneurons are necessary for the expression of compulsive-like ethanol consumption (Patton et al., 2022). Following these results, here we demonstrate that chronic ethanol vapor exposure specifically reduces GABAergic, but not glutamatergic input onto dorsolateral striatum FSIs. This effect was evident in two major GABAergic projections the dorsolateral striatum, the globus pallidus, and the reticular thalamic nucleus (Mallet et al. 2012; Klug et al. 2018), suggesting a cell-wide modification of GABAergic inputs. However, future investigation into the FSI-FSI synapse is warranted. Notably, a reduction in GABAergic input, with no change in glutamatergic input, would dramatically enhance FSI firing. Compounding this effect, acute ethanol exposure increases FSI intrinsic excitability

(Blomeley et al., 2011) and reduces FSI transmission onto spiny projection neurons (Patton et al., 2015). Provided the critical role of striatal FSIs for neuronal ensemble formation (Duhne et al., 2020), chronic ethanol exposure may dramatically influence the formation of striatal ensembles, including those recruited for drug acquisition (McKeon et al., 2022; Roberts et al., 2019; Klaus et al., 2017).

Chronic ethanol vapor exposure reduced the number of putative GABAergic synapses onto dorsolateral striatum FSIs. Multiple cellular mechanisms, including synaptic pruning could possibly underly this phenomenon (Wiedemann 2008). Indeed previous work indicates that 10 days of voluntary ethanol consumption induces synaptic pruning in the prefrontal cortex (Socodato et al., 2020). However, in the dorsolateral striatum, synapse loss was predominately localized to the soma and proximal processes of FSIs, indicating a perisomatic mechanism that is susceptible to ethanol exposure.

PNNs condense around the soma and proximal processes of PV-expressing fast-spiking interneurons to regulate neuronal excitability and synaptic transmission (Balmer 2016; Giamanco & Matthews 2012; Fawcett et al. 2019; Burket et al., 2021). Notably, drugs of abuse, including alcohol, modulate perineuronal net expression through multiple proposed mechanisms (Slaker et al., 2016; Brown & Sorg 2022; Dannenhoffer et al., 2022; Chen et al., 2015; Lasek et al., 2017; Chen & Lasek 2020). Drug-induced modifications to PNNs are thought to re-open the critical period of synaptic plasticity to enable the formation of drug memories and drug-associated behaviors (Lasek et al., 2017; Slaker et al., 2016). In line with this work, we demonstrate that chronic ethanol vapor exposure modulates perineuronal net protein expression in the dorsolateral striatum. Specifcally, the reduction in PNN expression we observed alings with the PNN loss reported in the retrosplenial cortex of ethanol exposed mice (Lewin et al., 2018). However, in the insular (Chen et al., 2015) and prefrontal cortex (Coleman et al., 2014), repeated ethanol exposure increases PNN expression. This difference is also evident following cocaine exposure (see table 2 in Brown & Sorg 2022), suggesting region specific sensitivity to drugs of abuse, where local microenviroments differentially respond to drugs resulting in either upregulation of PNN proteins or conversely enhanced degradation of PNNs.

PNNs are dynamically expressed to maintain a homeostatic neuronal enviroment, where activity FSIs promote PNN sythesis (Dityatev et al., 2007) and signals from both FSIs and neighboring cells, including micgroglia, secrete enzymes to degrade PNNS (Fawcett et al. 2019). While the exact mechanism underlying ethanol-induced degredation of PNNs in the dorsal striatum has yet to be determined, substantial evidence points to regulation of PNN degrading enzymes, specifically matrix metalloproeinase-9 (MMP-9). In human studies, serum levels of MMP-9 are elevated during alcohol intoxication (Zdanowicz et al., 2022) and in individuals with a history of alcohol abuse (Sillanaukee et al., 2002). In rats, MMP-9 expression increased in the nuclues accumbens of alcohol dependent rats (Go et al., 2018), possibly by increasing oxidative stress (Koken et al., 2010). Notably, intracerebroventricular administration of a broad spectrum MMP inhibitor attenuates the escalation of ethanol self-administration in rats (Smith et al., 2011). However due to the brain-wide delivery of this treatment, the specific contribution of dorsolateral striatum MMP-9 activity to ethanol consumption is yet to be determined.

Enzymatically degrading PNNs resulted in a dramatic decease in GABAergic transmission onto dorsolateral striatum FSIs. Similar to the chronic ethanol condition, we observed a reduction in the frequency of spontaneous inhibitory synaptic transmission, but no change in the event amplitude, suggesting that destabilizing PNNs reduces the number of GABAergic contacts onto FSIs. In agreement with this notion, genetic deletion of PNN protein reduces the frequency of spontaneous inhibitory transmission onto FSIs in the hippocampus (Favuzzi et al., 2017). Additionally, enzymatically degrading PNNs or genetically eliminating PNN proteins leads to a reduction in inhibitory puncta in the preisomatic region of FSIs (Carcellar et al., 2020; Gottschling et al., 2019).

To the best of our knowledge, this is the first study demonstrating drug-induced PNN modifications and synapse elimination in the dorsolateral striatum. By degrading PNNs, we believe ethanol may be opening a critical period in the striatum for habit learning that facilitates escelated ethanol consumption and the expression of compuslive-like drinking. Therefore future therapeutic interventions targeted toward PNN remodeling may serve as a viable treatment strategy to reduce ethanol consumption in substance use disorder.

3.5 References

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Chapter 4: Alcohol Potentiates Multiple GABAergic Inputs to Dorsal Striatum Fast-Spiking Interneurons

4.1 Introduction

Plastic control of synaptic strength enables complex adaptations to an ever-changing environment required for reward acquisition and survival (Citri & Malenka 2007). However, synaptic plasticity is also targeted by drugs of abuse, including alcohol, to promote maladaptive, compulsive drug (i.e., reward) seeking that defines substance use disorder (Koob & Volkow 2009; 2016). Therefore, investigating how alcohol modifies synaptic plasticity in areas of the brain responsible for generating compulsive actions, such as the dorsal striatum, stands to reveal mechanisms underlying substance use disorder and how they may be targeted therapeutically.

The dorsolateral striatum, or putamen in humans and non-human primates, integrates multisensory information with motor commands to reinforce and refine motor actions that result in the acquisition of a reward (Haber 2016). 4-8 weeks of alcohol consumption drives habitual ethanol seeking in rats (Corbit et al., 2012). As such, alcohol exposure is positioned to pathologically modulate dorsolateral striatum synapses to promote actions that result in alcohol acquisition (Gerdeman et al., 2003). Within the dorsolateral striatum, parvalbumin expressing fast-spiking interneurons (FSIs) are a subpopulation of inhibitory interneurons that comprise approximately 1% of the neuron population in the striatum (Kawaguchi et al., 1995). They are enriched in the dorsolateral striatum (i.e., putamen) and provide powerful feedforward inhibition onto striatal projection neurons (Tepper et al., 2010; Kita et al., 1990; Koós & Tepper, 1999, 2002). FSIs are necessary for compulsive ethanol consumption in C57BL6J mice (Patton et al., 2020). What remains to be determined is how ethanol exposure modulates dorsolateral synaptic strength and, in particular, synaptic strength related to FSIs.

Acute ethanol exposure significantly impacts GABAergic transmission in the dorsolateral striatum, resulting in depression of GABAergic transmission from spiny projection neurons and FSIs onto spiny projection neurons in the dorsolateral striatum (Patton et al., 2016). This reflects the finding that 6 weeks of voluntary ethanol consumption suppresses GABAergic transmission onto striatal projection neurons (Wilcox et al 2014; Carlson et al 2018). This has led to the notion that ethanol disinhibits dorsolateral striatal projection neurons to facilitate ethanol habits (Chen et al., 2011; Blomeley et al., 2011; Wilcox et al., 2014; Patton et al., 2016). Given that striatal GABAergic synapses are sensitive to ethanol, a significant gap in knowledge is whether GABAergic control of FSIs is impacted as well. A finding of ethanol-induced depression of these synapses would challenge the dorsolateral striatum disinhibition model of ethanol habit formation, while potentiation of these synapses by ethanol would support the disinhibition model. Testing this, we found that acute bath application of ethanol at a concentration reached during episodes of binge drinking (i.e., 50mM) potentiated the two primary GABAergic synapses onto FSIs arising from the globus pallidus and the reticular thalamic nucleus. Collectively, these data add to our understanding of how ethanol interacts with the dorsolateral striatum, a critical hub of habit learning in the brain.

4.2 Methods

4.2.1 Animals

All experiments were performed in accordance with NIH guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Maryland Baltimore and the National Institute on Alcohol Abuse and Alcoholism. All C57BL/6J mice were housed with littermates (2-5 per cage) under a normal 12-hour light/dark cycle (lights on at 0900 hours, off at 2100 hours) with *ad libitum* access to food and water.

4.2.2 Surgical procedures

All stereotaxic injections were performed under isoflurane anesthesia (5% induction; 2%–3% maintenance) and viruses were injected at a rate of 30 nL/min with a 25 G syringe (Hamilton Company). For pain management, a topical lidocaine ointment (2.5% lidocaine, 2.5% prilocaine; Akorn) was applied to the incision site and systemic carprofen injections (5 mg/kg S.C.) were administered for a minimum of three days following surgery. To isolate GABAergic projections from the globus pallidus or the reticular thalamic nucleus to the dorsal striatum, parvalbumin-cre x tdTomato expressing mice were stereotaxically injected with an adeno-associated virus expressing channelrhodopsin (AAV5-hSyn-ChR2-eYFP) in the globus pallidus (AP −0.4mm, ML ± 2.0mm, DV −3.7mm; 200 nL/side) or the reticular thalamic nucleus (AP −0.58mm, ML ± 1.25mm, DV −3.5mm; 200 nL/side).

4.2.3 Fluorescent microscopy

Mice were transcardially perfused with room-temperature 0.1 M phosphate buffered saline (PBS), pH 7.3, followed by room-temperature 4% (w/v) paraformaldehyde in PBS. Brains were extracted and post-fixed with 4% paraformaldehyde in PBS at 4°C for 24-48h. Coronal sections (50 μm) were cut using a Leica VT1000S vibrating microtome. Fluorescent images were visualized on a Nikon Eclipse Ti2 fluorescent microscope using a 4X objective (0.20 NA) and captured with a Nikon DS-Qi2 camera.

4.2.4 Acute slice preparation

Mice were deeply anesthetized with isoflurane before rapid decapitation and brain removal. 250 µm coronal sections were collected in ice-cold modified artificial cerebral spinal fluid (aCSF: 95% oxygen, 5% carbon dioxide, 194 mM sucrose, 30 mM NaCl, 4.5 mM KCl, 1 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, and 10 mM D-glucose) using a Leica VT1200 vibrating microtome. Brain sections were then transferred to regular aCSF (95% oxygen, 5% carbon dioxide, 124 mM NaCl, 4.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, and 10 mM D-glucose) and incubated at 32°C for 30 min for recovery. Slices were then removed from the incubator and stored at room temperature until recording.

4.2.5 Whole cell electrophysiology

Slices were hemisected, placed into a recording chamber, and perfused with temperature controlled aCSF (29-31°C) during the recording procedure (aCSF: 124 mM NaCL, 4.5 mM KCl, 1 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 10 mM D-glucose, 2 mM $CaCl_2$). Optogenetically evoked currents were generated from an LED driver (Thorlabs, LEDD1B T-Cube) and an 470nm LED delivering blue light (2-4 ms) through an optical fiber located approximately 150 µm from the recording electrode. GABAergic transmission was pharmacologically isolated by adding DL-AP5 (50μM) and NBQX (5μM) to the recording aCSF. Dorsolateral striatum FSIs were fluorescently visualized using Eclipse FN1 Nikon microscope and Intenslight C-HGFIE (Nikon). Images were digitally rendered using Hamamatsu ORCA-ER digital camera and associated HC image live 4.8.0. FSIs were voltage clamped at -60 mV using a MultiClamp 700B Amplifier (Molecular Devices). Optogenetically evoked currents were recording using a borosilicate glass pipette (3-5 MΩ resistance) filled with an internal solution (120mM $CsMeSO_3$, 10mM HEPES, 5mM NaCl, 10mM TEA-Cl, 1.1mM EGTA, 0.3mM Na-GTP, 5mM QX-314, 4mM Mg-ATP). For some recordings, 10 mM BAPTA was added to the internal solution. Signals were filtered at 2 kHz, digitized at 10 kHz and acquired using Clampex 10.4.1.4 software (Molecular Devices). Data were analyzed offline using Clampfit software (Molecular Devices) and Mini Analysis Program software (Synaptosoft).

4.2.6 Chronic intermittent ethanol exposure

mice were placed in Plexiglass inhalation chambers (81x56x37 cm) and exposed to chronic intermittent ethanol (CIE) vapor or air 16 hours/day, 4 days a week for a total of 5 weeks. Ethanol was volatilized by passing compressed air (10-20 psi) through a tube submerged in 100% ethanol. After four days in the inhalation chambers, mice underwent a 72-hour forced abstinence period. Control vapor chambers delivered only air without ethanol vapor. Vapor chamber ethanol concentrations were monitored daily and air flow was adjusted to produce ethanol concentrations within 1.8-2.0% ethanol content measured by a digital alcohol breath tester (FFtopu). These conditions produce stable blood ethanol concentrations in C57BL/6J mice ranging from 150-200 mg/dl (Lopez and Becker 2003).

On weeks 4-5, the alcohol dehydrogenase inhibitor pyrazole (1 mmol/kg, i.p.) was injected 30min prior to each session to stabilize blood ethanol concentrations.

4.2.7 Statistical analysis

All statistical analyses were performed in GraphPad Prism 9.0. Data are represented as mean $\pm$ standard error of the mean (SEM). Comparisons between three groups were analyzed with a two-way ANOVA. Multiple comparisons were corrected with a Dunnett's test when comparing multiple time points to a single baseline. For the acute ethanol exposure experiments, N is defined as an individual neuron. For the chronic ethanol exposure experiments, N is defined as an individual animal derived from an average recording of 2-3 cells. Baseline synaptic transmission values were determined by the average amplitude from 4min of the baseline recording period. Ethanol exposure values were determined by the average amplitude from min 8-12 of the ethanol application period, while ethanol removal values were determined by the last 20-25 min of the recording.

4.3 Results

We first recorded GABAergic transmission from the globus pallidus to FSIs (GP→FSI) using optogenetics (**Figure 4.1A,B**). We found that acute bath application of 17 mM ethanol, the legal driving limit in most of the United States (Wallner, Hanchar, & Olsen 2014), had no impact on GABAergic transmission at the GP→FSI synapse ($F_{(1.51, 9.05)} = 0.15$, $p = 0.53$; repeated measures ANOVA; **Figure 4.1C**). However, at a binge level of ethanol (50mM), GABAergic transmission at the GP→FSI synapse was potentiated ($F_{(1.5, 10.5)} = 10.0$, Dunnett's adjusted $p < 0.01$; repeated measures ANOVA; **Figure 4.1D**). This potentiation was sustained after ethanol removal (Dunnett's adjusted $p < 0.05$). We found a transient change in release probability during ethanol application ($F_{(1.36,\ 9.52)} = 9.9$, Dunnett's adjusted $p < 0.01$; repeated measures ANOVA; **Figure 4.1E**) that did not persist after the ethanol removal (Dunnett's adjusted $p = 0.22$). These results suggest that a post-synaptic phenomenon was likely driving the sustained enhancement of GABAergic transmission. Since many forms of post-synaptic GABAergic long-term potentiation involve an increase in intracellular calcium (Caillard, Ben-Ari, & Gaiarsa 1999; Nugent & Kauer 2008), we added the calcium chelator BAPTA (10mM) to the intracellular recording solution to sequester any fluxes in calcium within FSIs. We found that the addition of BATPA (10 mM) prevented the ability of acute ethanol (50 mM) to potentiate GABAergic transmission at the GP→FSI synapse ($F_{(1.5,\ 8.98)} = 4.8$, Dunnett's adjusted $p = 0.83$; repeated measures ANOVA; **Figure 4.1F**).

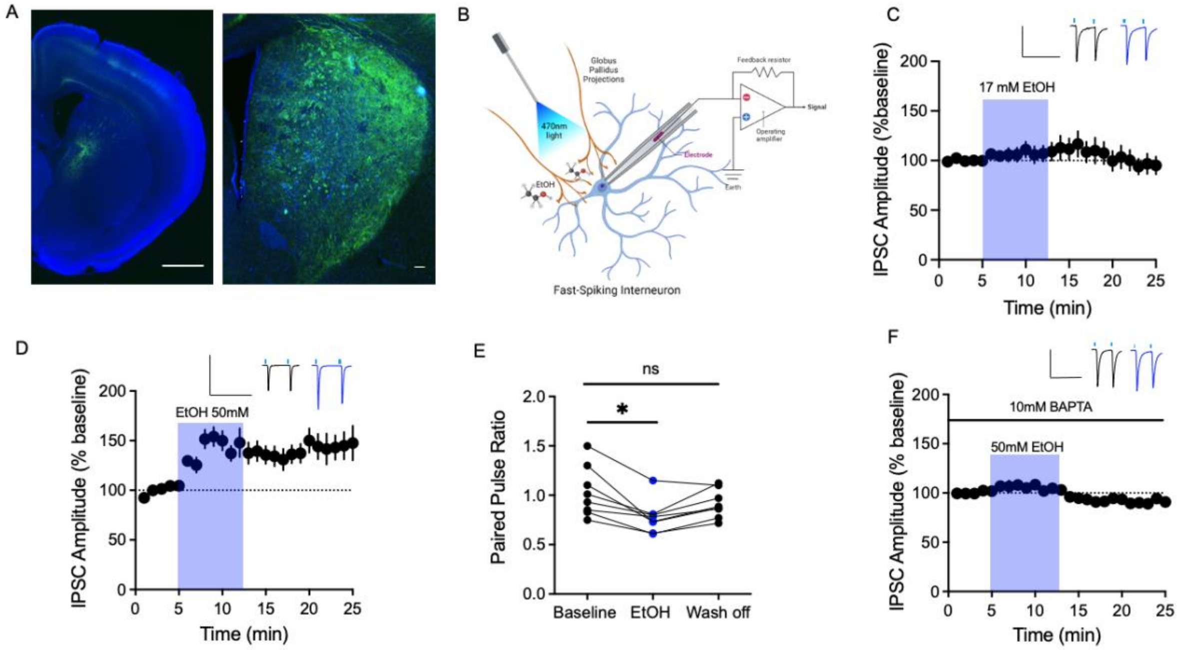

Figure 4.1 Acute ethanol exposure enhances inhibitory synaptic transmission from the globus pallidus (GP). **A**) Representative fluorescent image of stereotaxic injection site into the GP (left scale bar = 500µm; right = 25µm). **B**) Schematic of whole-cell patch configuration. **C**) Acute bath application of 17mM ethanol does not potentiate inhibitory synaptic transmission at the GP→FSI synapse (N=8 cells, 4M 4F). **D**) However, bath application of 50mM ethanol induces a sustained potentiation of inhibitory transmission at the GP→FSI synapse (N = 8 cells, 4M 4F). **E**) Bath application of 50mM ethanol induces a transient increase in the paired-pulse ratio that does not sustain after ethanol removal. **F**) The addition of the calcium chelator BAPTA (10mM) to the patch pipette prevents acute-ethanol induced potentiation of inhibitory transmission at the GP→FSI synapse (N=8 cells, 4M 4F). Inlet: representative traces from control (black) and chronic ethanol (blue) treated mice (scale bar 400pa, 100ms). All data represented as mean ± SEM. *P < 0.05.

Following the results from the GP→ FSI synapse, we next recorded synaptic transmission from RTN→ FSI to determine if ethanol modification to GABAergic input was circuit specific (**Figure 4.2A,B**). We found that acute bath application of 17 mM potentiated GABAergic transmission at the RTN→FSI synapse ($F_{(1.52,\ 10.61)} = 2.75$, Dunnett's adjusted $p < 0.05$; repeated measures ANOVA; **Figure 4.2C**), but this effect did not sustain following ethanol removal (Dunnett's adjusted $p = 0.20$). However, bath application of 50 mM ethanol resulted in a significantly potentiated GABAergic transmission at the RTN→ FSI synapse ($F_{(1.39,\ 9.70)} = 8.79$, Dunnett's adjusted $p < 0.05$; repeated measures ANOVA; **Figure 4.2D**) that sustained after the ethanol removal (Dunnett's adjusted $p < 0.05$). Additionally, we found no change in the paired-pulse ratio following acute ethanol (50 mM) exposure at the RTN→ FSI synapse ($F_{(1.57,\ 10.96)} = 0.58$, $p = 0.53$; repeated measures ANOVA; **Figure 4.2E**). We then tested whether sequestration of calcium fluxes with the calcium chelator BAPTA (10mM) would also prevent ethanol augmentation at the RTN→FSI synapse. Indeed, similar to what we observed at the GP→FSI synapse, the addition of BAPTA to the internal solution prevented ethanol potentiation at the RTN→FSI synapse ($F_{(1.06,\ 7.43)} = 1.79$, Dunnett's adjust $p = 0.815$; repeated measures ANOVA; **Figure 4.2F**).

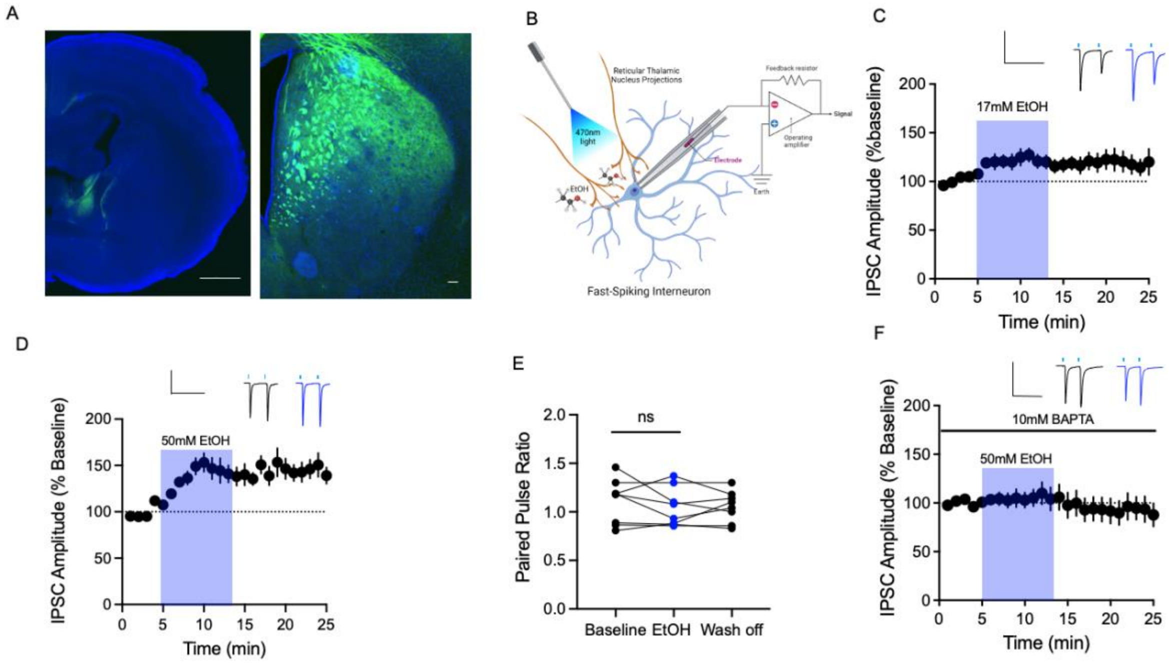

Figure 4.2 Acute ethanol exposure enhances inhibitory synaptic transmission from the reticular thalamic nucleus (RTN). **A**) Representative fluorescent image of stereotaxic injection site into the RTN (left scale bar =500µm; right = 25µm). **B**) Schematic of whole-cell patch configuration. **C**) Acute bath application of 17mM ethanol transiently potentiates inhibitory transmission at the RTN→FSI synapse (N=8 cells, 4M 4F). **D**) Bath application of 50 mM ethanol induces a sustained potentiation of inhibitory transmission at the RTN→FSI synapse (N = 8 cells, 4M 4F). **E**) Bath application of 50mM ethanol does not effect paired-pulse ratio at RTN→FSI synapse. **F**) The addition of the calcium chelator BAPTA (10mM) to the patch pipette prevents acute-ethanol induced potentiation of inhibitory transmission a the RTN→FSI synapse (N=8 cells, 4M 4F). Inlet: representative traces from control (black) and chronic ethanol (blue) treated mice (scale bar 200pa, 100ms).

We next investigated whether repeated ethanol exposure induces synaptic modifications to GABAergic transmission. We therefore recorded optically-evoked synaptic currents from mice that underwent 5 weeks of intermittent ethanol exposure (**Figure 4.3A**; see CIE methods). In control animals chronically exposed to air vapor, we confirmed that acute ethanol exposure (50mM) augments GABAergic transmission at the GP→FSI synapse ($F_{(1.99, 7.94)} = 10.86$, Dunnett's adjusted $p < 0.05$; **Figure 4.3B**) and at the RTN→FSI synapse ($F_{(1.30, 6.50)}=13.33$, Dunnett's adjusted $p < 0.01$; **Figure 4.3C**). We next recorded from mice chronically exposed to CIE and found that chronic ethanol exposure prevented acute ethanol-induced potentiation of GABAergic transmission at the GP→FSI synapse ($F_{(1.78, 8.25)} = 1.94$, Dunnett's adjusted $p = 0.37$; repeated measures ANOVA; **Figure 4.3D**). Additionally, we found that chronic ethanol exposure attenuated acute ethanol-induced potentiation of GABAergic transmission at the RTN→ FSI synapse ($F_{(1.50, 10.21)} = 3.64$, Dunnett's adjusted $p = 0.11$; repeated measures ANOVA; **Figure 4.3E**).

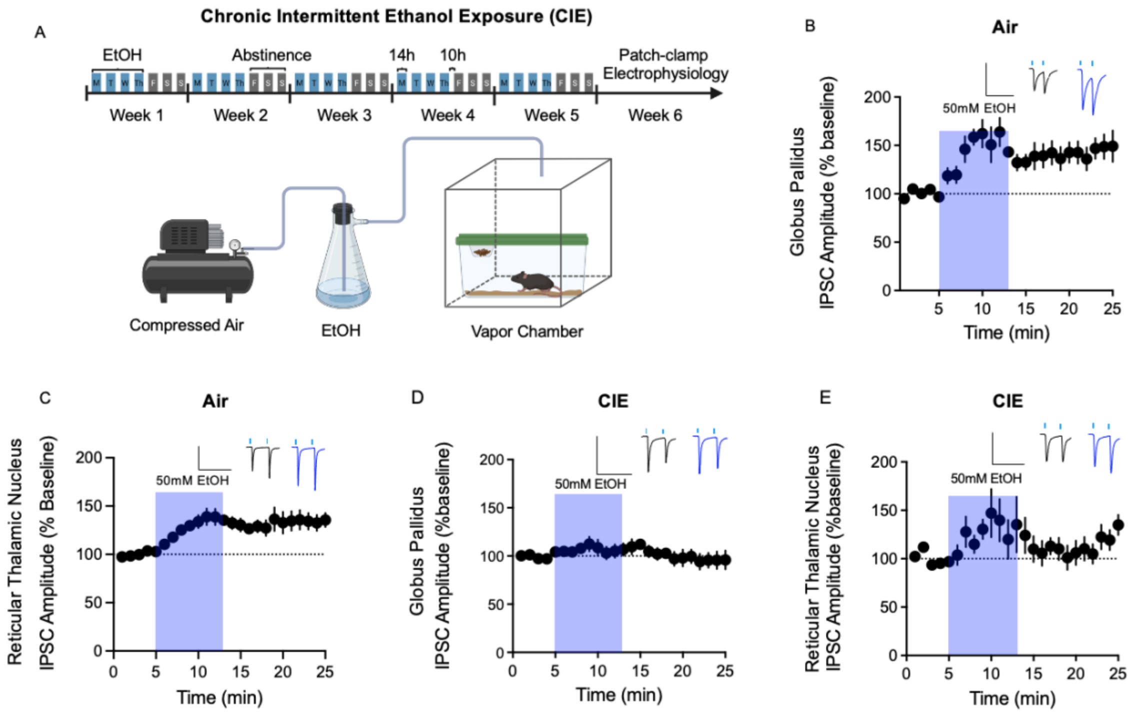

Figure 4.3 Chronic ethanol exposure attenuates acute-ethanol potentiation of inhibitory transmission at the GP→FSI and RTN→FSI synapses. A) Experimental timeline and schematic of chronic intermittent ethanol vapor chamber paradigm. Control mice exposed to air vapor chamber still exhibit acute-ethanol potentiation of inhibitory transmission at the GP→FSI (B) and RTN→FSI (C) synapses (N=6 animals, 3M 3F). Chronic ethanol exposure attenuates the ability of acute ethanol (50mM) to potentiate inhibitory transmission at the GP→FSI (D) and RTN→FSI (E) synapses (N = 8 animals, 4M 4F). Inlet: representative traces from baseline (black) and acute ethanol exposure (blue; scale bar 200pa, 100ms).

4.4 Discussion

We found that 50 mM acute ethanol application *ex vivo* induced a sustained potentiation of GABAergic transmission onto dorsal striatum FSIs from two extrinsic projections, the GP and RTN. This GABAergic potentiation is mediated by postsynaptic intracellular calcium, as the addition of the calcium chelator BAPTA to the intracellular recording solution prevented the ethanol-induced potentiation at these two synapses. Finally, we demonstrated that chronic ethanol exposure attenuated the ability of acute ethanol to potentiate GABAergic transmission from both the GP and RTN. The acute ethanol-induced potentiation of these synapses is consistent with the notion that ethanol disinhibits the dorsolateral striatum.

As motor actions are learned and mastered, striatal neurons organize into functional ensembles consisting of MSNs and interneurons (Jin & Costa 2010; Martiros et al., 2018; Roberts et al., 2019; Bakhurin et al., 2016; Gritton et al., 2019) to facilitate the expression of a refined motor sequence (Jin & Costa 2010; Aldridge & Berridge 1998; Martiros et al., 2018). FSIs significantly contribute to striatal ensemble organization which likely underlies their ability to regulate motor sequences expressed as habits (O'Hare et al., 2017) and compulsive-like drinking (Patton et al. 2021). Photo-stimulation of FSIs activates and refines striatal MSN ensembles (Duhne et al., 2020) and facilitates the expression of motor actions (Gritton et al., 2019). Recently, McKeon et al. (2022) demonstrated that FSI ensembles arises from concerted cortical input. However, the effect of ethanol exposure on GABAergic regulation of FSIs may also affect ensemble formation. Specifically, chronic ethanol vapor exposed mice are insensitive to ethanol augmentation of GABAergic control of FSIs which would promote FSI activity to engage ensembles and habits.

We observed a transient decrease in synaptic release probability during ethanol application at the GP→FSI synapse, suggesting that a pre-synaptic mechanism underlies acute ethanol augmentation of GABAergic transmission. Similarly, Varodayan et al. (2017a) demonstrated that acute ethanol acts on presynaptic P/Q-type calcium channels in the central amygdala to promote GABA release. However, the presently observed decrease in synaptic release probability did not sustain after ethanol removal, pointing to a post-synaptic mechanism underlying the sustained augmentation of GABAergic transmission. Many forms of post-synaptic GABAergic potentiation involve a rise in intracellular calcium (Caillard, Ben-Ari, & Gaiarsa 1999; Nugent & Kauer 2008). Supporting this notion, In the present study we observed that sequestering calcium flux with BAPTA prevented acute ethanol-induced augmentation of GABAergic transmission from both the GP and RTN.

Numerous *in vitro* studies demonstrate that acute ethanol increases intracellular calcium levels through release from internal stores, raising the possibility that IP3 signaling may underly GABAergic potentiation in FSIs (Daneill & Harris, 1989; Mironov & Hermann, 1996; Xiao et al., 2005; Kelm et al., 2007). However, in the central amygdala, acute ethanol exposure increases activity of L-type voltage-gated calcium channels providing an alternative source of calcium (Hell et al., 1993; Varodayan et al., 2017b). While future studies are warranted to investigate the precise mechanism underlying the presently studied GABAergic potentiation, these collective works highlight the importance of calcium signaling in ethanol modulation of GABAergic synaptic strength.

This body of work makes considerable steps toward uncovering a novel mechanism by which acute ethanol modulates GABAergic transmission in the dorsal striatum. We know this mechanism is not circuit specific since both GP→FSI and RTN→FSI synapses are potentiated by acute ethanol administration, but instead requires a post-synaptic calcium flux. Additionally we demonstrate that chronic ethanol vapor exposure attenuates the ability of acute ethanol to potentiate GABAergic transmission onto FSIs from both circuits. This could arise from multiple mechanisms including $GABA_A$ receptor desensitization (Dopico & Lovinger 2009), activity-dependent dysregulation of calcium signaling (Kawamoto et al., 2012), or homeostatic plasticity (Carpenter-Hyland & Chandler 2006; Spencer et al., 2016). Collectively this work highlights the impact of ethanol exposure on GABAergic signaling in the dorsal striatum and suggests that modulation of inhibitory control of fast-spiking interneurons contributes to the development of habitual actions geared toward drug acquisition including compulsive drinking.

4.5 References

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Chapter 5: Discussion

5.1 The role of striatal FSIs in ensemble formation and compulsive drinking

While FSIs represent a small portion of neurons in the striatum (approximately 1%), they play a large role in shaping the activity of striatal projections neurons and therefore the motor output signal relayed through the basal ganglia system. How exactly FSIs regulate striatal microcircuitry is not entirely understood. However, *in vitro* calcium studies and slice electrophysiology recordings shed light on how this small subset of neurons regulates striatal function. Recall that as actions are learned and mastered, MSN activity organizes into functional neuron ensembles consisting of D1 and D2 MSNs (Jin & Costa 2010; Martiros et al., 2018; Bakhurin et al., 2016; Gritton et al., 2019). These functional ensembles promote task-oriented actions (i.e., direct pathway activation) while simultaneously inhibiting unwanted actions (i.e., indirect pathway activation) that collectively facilitate the expression of a refined motor sequence (Jin & Costa 2010; Aldridge & Berridge 1998; Martiros et al., 2018). Once a motor sequence is mastered and repeatedly enforced, these actions are expressed as habits and, in extreme cases, compulsive actions (Graybiel 2005; Everitt et al., 2001, 2016).

FSIs play a significant role in the formation of MSN ensembles (Duhne et al., 2020), which likely underlies their ability to regulate the expression of habitual behavior (O'Hare et al., 2017). Notably, FSIs preferentially inhibit D1 MSNs over D2 MSNs (Gittis et al., 2010; Bahuguna et al., 2015), which likely refines the recruited population of D1 MSNs resulting in a more selective MSN response. Although FSIs represent only 1% of the neuron population, they account for a larger fraction (5-11%) of striatal ensembles (Duhne et al., 2020). Since MSNs receive input from 2-4 FSIs (Tepper & Koós 2017), this collectively suggests that inhibitory control is a major constituent of striatal microcircuits (**Figure 5.1**). Provided that FSIs send inhibitory projections to MSN somata (Tepper et al., 2004), one would hypothesize that this strong feed-forward inhibition gaits MSN activity to influence ensemble formation. Indeed, there is evidence to support this notion, where photo-stimulating FSIs reconfigures existing striatal MSN ensembles (Duhne et al., 2020). However, these authors also demonstrated that in resting microcircuits, photo-activation of FSIs turns on MSN ensembles (Duhne et al., 2020), adding confusion to the role of FSIs in gating MSN activity. However, the authors remind us that FSIs send collateral projections to other FSIs, where activation of one FSI would in turn inhibit the activity of a neighboring FSI, presumably within the same neuronal ensemble. This lateral inhibition would result in the disinhibition of select MSNs that would activate upon cortical excitation.

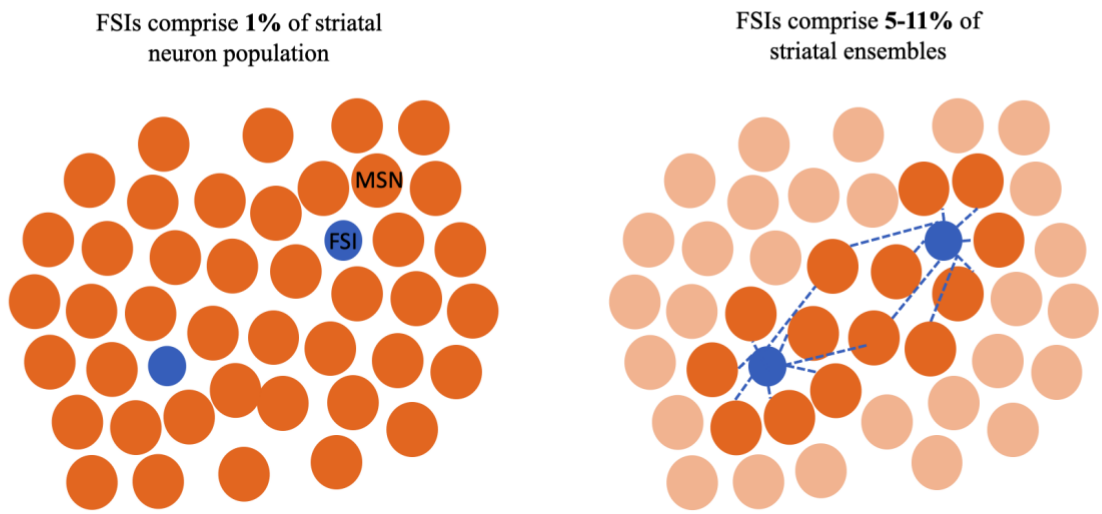


Figure 5.1 FSIs make up a small portion of the striatal neuronal population (1%) but represent a larger fraction of striatal ensembles (5-11%). Left: cartoon depicting a population of striatal neurons consisting of MSNs (orange) and FSIs (blue). Right: cartoon depicting a striatal neuron ensemble consisting of MSNs (bright orange) and FSIs (blue).

In addition to this lateral inhibition hypothesis, it is important to consider the state in which a population of MSNs receives a GABAergic signal from an FSI (i.e., the chloride driving force). MSNs exhibit biphasic resting potentials, existing in either an up-state (-60mV) or a down-state (-80mV) (Wilson & Kawaguchi 1996). Notably this down-state resting potential is well below the chloride reversal potential (-55mV). Therefore, if a collection of MSNs resting in their down state receive GABAergic input from an FSI, this signal would depolarize MSNs and facilitate cortical drive of MSN ensembles. This could provide an additional explanation as to why photo-activation of FSIs turns on MSN ensembles.

Subsequent *in vivo* studies further support for role of FSIs in striatal ensemble formation while additionally providing a link between striatal microcircuits and action sequence learning. In awake behaving animals, FSIs themselves form functional ensembles to encode action speed (Roberts et al., 2019). Notably, photo-activation of FSIs facilitates motor action, likely by refining task-relevant MSN ensembles (Gritton et al., 2019). Further supporting the role of striatal FSIs in motor skill learning, I demonstrated that ablating FSIs disrupts the expression of organized lick sequence behavior in mice drinking ethanol (**Figure 2.3**). This result was particularly striking, since ablating FSIs in mice drinking either sucrose or water had no effect on lick sequence behavior, suggesting that innate drinking behavior is predominantly driven by an alternative brain region that does not require modulation by the dorsal striatum (**Figure 2.3**). This default drinking behavior is likely conserved, by evolutionary pressure, across species to facilitate species survival. Indeed, innate behaviors including feeding and reproduction do not require significant motor refinement by the striatum, but instead are driven by more evolutionarily conserved hindbrain structures (Bout 1998). Specifically, subregions of the pons and medulla provide critical motor signals to the spinal cord to execute a specific motor command (Arber & Costa 2018, 2022). These motor signals are conveyed along command lines that innervate distinct body regions to regulate specific movements. Orofacial movements such as licking, for example, are controlled by hypoglossal neurons in the medulla that innervate the muscles of the tongue (Moore et al., 2014; Rossi et al., 2016). It is therefore likely that in the sucrose and water drinking condition, command lines from the medulla predominantly control licking behavior, which is why ablating striatal FSIs had no consequence on the expression of this motor command.

Ablating FSIs in the ethanol drinking mice did however generate an effect on lick sequence behavior, suggesting that ethanol drinking somehow uniquely engaged dorsal striatum activity, and once engaged the absence of functional FSIs manifested aberrant licking behavior. While hindbrain structures control basic motor commands, the basal ganglia system, via inhibitory afferents from the substantia nigra pars reticulata, regulates motor command lines in the hind brain to select a relevant action and to indicate how vigorously that action should be performed (Arber & Costa 2018). It is possible that the bitter taste of ethanol requires a learning phase where mice overcome the aversive obstacle to obtain the rewarding effects of ethanol. This would initially require cognitive control to engage a goal-direct behavior to acquire ethanol reward. However, following repeated execution and reinforcement of this behavior, the DLS would then engage to elicit stimulus-response behavior. Supporting this idea, I found that the effect of FSI ablation on lick sequence behavior manifested by week-3 of the drinking paradigm (**Figure 2.3**), but was not evident at earlier time points, suggesting a time-dependent adaptation in motor behavior. Specifically, I found the mice preferentially express lick sequences of 3-4 licks, but that over time, ethanol exposed mice express these organized lick sequences more frequently (**Figure 2.3**). Notably, water and sucrose drinking mice express the same 3-4 lick sequence length, suggesting that this is the default motor command for fluid consumption in mice. Once the striatum transmits the vigor signal for this lick command, the action sequence is then expressed more frequently. This data agrees with previous FSI circuit manipulations, demonstrating that photo-stimulation of FSIs engages motor actions and activates MSN ensembles (Gritton et al., 2019; Duhne et al., 2020). One could postulate that cortical activation of FSIs promotes a lick ensemble to facilitate escalated ethanol consumption. Notably, I found that only mice with intact FSIs exhibited escalated ethanol consumption, likely through the coinciding expression of organized lick sequences.

Interestingly, ethanol drinking mice with ablated FSIs were unable to constrain their organized lick sequences to this "default" lick length, but instead exhibited more variability in lick sequence length, where at times they exhibited "super bouts" of 15> licks (**Figure 2.4**). Following the observations from Duhne et al. (2020), this would suggest that FSIs simultaneously activate and refine an MSN ensemble to a definable motor action sequence, in this case a lick sequence of 3-4 licks. Therefore, without intact FSIs, the ability to refine the MSN ensemble is lost, resulting in variable lick sequence length, and no escalating expression of the sequence, despite presumable reward feedback from ethanol intake. Further support for this hypothesis comes from two preliminary studies in which I chemogenetically manipulated FSIs in mice undergoing the Drinking in the Dark paradigm. First, I found that chemogenetically inhibiting FSIs reduced the expression of organized lick sequences (unpublished data), suggesting that like ablating FSIs, reducing inhibitory control over MSNs prevents escalated expression of a motor sequence. I subsequently found that chemogenetically exciting FSIs produced no change in the frequency of organized lick sequences, but instead augmented the length of the lick action sequence (unpublished data). One might think that enhancing FSI transmission onto MSNs would further refine a motor ensemble, however these mice instead exhibited augmented motor sequence length. It is possible that exciting FSIs recruits a larger population of MSNs through lateral inhibition of FSIs, that in turn disinhibit connected MSNs resulting in a larger striatal ensemble and longer action sequence. Additionally, by raising the resting membrane potential of FSIs, excitatory DREADDS could scramble the refined motor command from the cortex/thalamus resulting unintended FSI activation resulting in a larger ensemble recruitment. Collectively these data support the notion that FSI transmission onto MSNs facilitates the activation and refinement of an MSN ensembles that enable the proper execution of a desired motor sequence.

Once mastered, these organized action sequences encoded by the dorsolateral striatum can be elicited almost automatically by sensory input from the environment, resulting in "stimulus-response" or habitual behavior. The development and expression of habits is critical to our understanding of substance use disorder, as many drugs of abuse, including alcohol, facilitate the formation of habitual actions resulting in continued drug use despite efforts to abstain (Graybiel 2005; Everitt & Robbins 2005, 2016). Provided the critical function of dorsal striatum FSIs in ensemble formation and the expression of motor sequences, it is no surprise that FSIs also play a role in the expression of habits (O'Hare et al., 2017). Extending upon this work I demonstrated that chronic ethanol exposure recruits dorsal striatum FSIs for the expression of compulsive drinking, a core feature of alcohol use disorder (Figure 2.6). This result provides a novel substrate for future cell-specific therapies targeted to compulsive drinking in alcohol use disorder.

5.2 The effect of chronic ethanol exposure on striatal FSI microcircuitry

In addition to the classical engagement of the striatum in reward-based motor learning, ethanol exposure additionally influences dorsal striatum activity through its pharmacological action on both resident neurons and afferent projections. Acute ethanol exposure modulates the activity of midbrain dopamine neurons that project to both dorsal and ventral striatum resulting in profound modifications to basal ganglia function to shape motor learning (Deehan et al., 2016; Abrahao et al., 2017). Additionally, acute ethanol exposure modulates the two dominant forms of corticostriatal plasticity that are critical for instrumental learning. Specifically, ethanol exposure reversing NMDA dependent LTP to long term depression in the dorsomedial striatum (Yin et al., 2007) and impairs endocannabinoid-mediated LTD in the dorsolateral striatum (Adermark et al., 2011; Depoy et al., 2013). The consequence of this polypharmacological modulation is preferential activity of the dorsolateral striatum, as evidenced by human imaging studies of chronic alcohol abusers (Sjoerds et al., 2014). While these studies illustrate the significant impact of ethanol exposure on MSN function, what remains to be understood is how repeated ethanol exposure influences striatal FSIs to facilitate habitual actions geared toward drug acquisition, such as compulsive drinking.

To address this question, I recorded electrically-evoked synaptic transmission onto dorsal striatum FSIs from chronic ethanol exposed mice. While 5-weeks voluntary ethanol consumption increases the frequency of spontaneous excitatory transmission onto MSNs

(Rangel-Barajas et al., 2021), I surprisingly found no impact of 5-week ethanol vapor exposure on basal excitatory transmission onto FSIs (**Figure 3.1**). Collectively this suggests that ethanol exposure modifies release probability at excitatory terminals specifically synapsing onto MSNs but not FSIs. This specificity could arise from unique G-protein receptor expression at MSN terminals, or unique expression of an ethanol sensitive isoform of adenylyl cyclase at glutamatergic terminals synapsing on MSNs.

In addition to the ethanol-induced increase in basal excitatory transmission onto MSNs, Muñoz et al. (2018) demonstrated that acute ethanol exposure blocks mu-opioid LTD specifically onto projection neurons in the DLS, which is critical for the automatization of a motor skill (Yin et al. 2009; Hawes et al., 2015). It is therefore still plausible that while 5-week ethanol vapor exposure does not impact basal excitatory transmission onto dorsal striatum FSIs, it may still modulate an unknown form of excitatory synaptic plasticity. Future experiments are warranted to determine whether high frequency stimulation of excitatory inputs to the DLS similarly induce LTD in FSIs or whether this form of plasticity is specific to striatal projection neurons. These results would provide important insight into how cortical excitation during motor learning shapes striatal ensemble formation.

Despite no observed change in basal excitatory transmission onto FSIs, I did however observe a dramatic reduction in inhibitory transmission arising from two extrinsic GABA nuclei, the globus pallidus and the reticular thalamic nucleus (**Figure 3.2**). Further investigation revealed a prominent loss of inhibitory terminals predominantly expressed on the soma and proximal neurites of FSI. Notably, the ethanol-induced deficit in GABAergic transmission was no longer observed when GABA input was controlled by a photo-caged

GABA compound, providing further support for inhibitory synapse loss (**Figure 3.3**). While neuronal/synaptic atrophy is typically associated with neurodegenerative disorders, like Parkinson's and Alzheimer's disease, post-mortem studies depict neuronal loss in chronic alcohol abusers, specifically in the cerebellum and prefrontal cortex (Brooks 2000; de la Monte & Kril 2013). This includes both gray and white matter volume reduction, where white matter loss primarily stems from glia dysregulation and degeneration of synaptic terminals (de la Monte & Kril 2013). For example, Socodato et al. (2020) demonstrated ethanol-induced synaptic pruning in the prefrontal cortex due to aberrant microglia phagocytosis.

Contributing to this line of work, I demonstrated that 5-week ethanol vapor exposure induces synapse elimination of GABAergic terminals within the striatum, which, to the best of my knowledge, is the first reported incident of ethanol-induced striatal synapse loss. What remains to be determined is this precise status of this synapse loss. In other words, are the inhibitory terminals still intact and merely retracted from the post-synaptic zone or is there complete irreversible synapse elimination. Understanding the precise state of this synapse loss will determine whether the system will resolve with time and therefore provide a critical time window for potential relapse and recovery.

Since this is the first reported incident of striatal inhibitory synapse loss from chronic ethanol exposure, the exact mechanism underlying this phenomenon is somewhat of a mystery. However, uniquely surrounding the somas and proximal neurites of FSIs, the same subcellular location where I observed predominant synapse loss, is a sub-compartment of the extracellular matrix called perineuronal nets. Notably, perineuronal nets regulate synapse formation and synaptic transmission (Giamanco & Matthews 2012;

Fawcett et al. 2019). Therefore, I hypothesized that chronic ethanol exposure modifies perineuronal net expression, which has been demonstrated in neighboring cortical structures (Chen et al., 2015; Brown & Sorg 2022; Dannenhoffer et al., 2022). Supporting this hypothesis, I observed that 5-week ethanol vapor exposure degrades perineuronal net protein expression in the dorsal striatum resulting in a reduction in the number of net enriched FSIs specifically in the dorsolateral striatum (**Figure 3.4**). While I observed net degradation in the striatum, it has previously been demonstrated that 6-week voluntary ethanol consumption enhances net protein expression in the insular cortex (Chen et al., 2015). Collectively these data demonstrate that the effect of chronic ethanol exposure on perineuronal net expression is not brain-wide, but instead varies across brain regions, suggesting that local microenvironments differentially respond to ethanol exposure resulting in either upregulation of net proteins or enhanced net degradation. Further exploration into the exact mechanisms regulating perineuronal net expression will likely reveal how these different microenvironments respond to repeated ethanol exposure and may yield novel therapeutic substrates for substance use disorder.

Since perineuronal nets are known to regulate synapse formation, I next investigated whether the degradation of perineuronal nets and the paralleled loss of inhibitory inputs onto FSIs were, in fact, somehow connected. One possibility is that by altering inhibitory input onto FSIs, ethanol modifies FSI firing properties resulting in activity-dependent modification to perineuronal net protein, as it is well known that perineuronal net expression is modified by neuronal activity (Dityatev et al., 2007; Fawcett et al. 2019). Alternatively, ethanol may initially target perineuronal nets resulting in synaptic modifications to inputs residing within the perineuronal net domain. Indeed, serum enzyme levels of net degrading proteases (i.e., MMP-9) are elevated in humans during alcohol intoxication (Zdanowicz et al., 2022), providing a possible mechanism by which ethanol exposure may degrade perineuronal nets. Supporting this perineuronal net-targeted hypothesis, I demonstrated that enzymatically degrading perineuronal nets results in a dramatic reduction in GABAergic transmission onto FSIs, similar to what I observed in 5-week ethanol vapor exposed mice.

A third possibility that connects perineuronal net degradation with synapse elimination involves the recruitment of local immune cells. Ethanol targets microglia, the brain resident macrophage, to induce neuroinflammation and enhance phagocytosis (Fernandez-Lizarbe et al., 2009; Henriques et al., 2018). For example, 10 days of ethanol consumption induces microglia-mediated aberrant synaptic pruning of excitatory synapses in the prefrontal cortex (Socodato et al., 2020). Additionally, administration of lipopolysaccharide treatment or poly polyinosinic-polycytidylic acid (Poly I:C), which are known to increase peripheral cytokine expression, promotes microglia phagocytosis of perineuronal net proteins (Crasper et al., 2020; Venturina et al., 2021; Wegryzn et al., 2021). What remains to be determined is whether ethanol exposure signals striatal microglia to degrade perineuronal nets and phagocytose inhibitory terminals. However, in support of this hypothesis, I observed that acute ethanol exposure (2g/kg i.p.) transitions the morphological profile of microglia in the dorsolateral striatum (**Figure 5.2**). Further transcriptional and proteomic signatures are needed to validate whether microglia are responding to ethanol exposure to phagocytose perineuronal nets and/or inhibitory synaptic terminals.

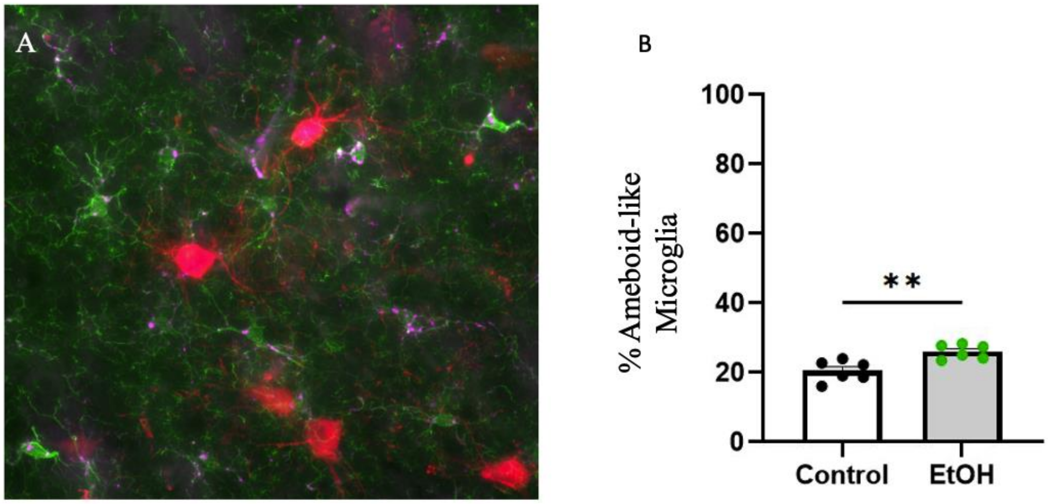


Figure 5.2 Acute ethanol vapor exposure induces a morphological transition in dorsal striatum microglia. **A**) Immunofluorescent image of Iba1 positive microglia (green) co-labeled with lysosomal maker CD68 (magenta) surrounding PV positive interneurons (red). **B**) acute ethanol vapor exposure (30min) increases the number of ameboid-like microglia in the dorsolateral striatum (Schwartz et al. 2012).

Despite not knowing the exact cause-effect relationship of inhibitory synapse loss and perineuronal net degradation, one can still speculate how these observations would influence the expression of compulsive drinking through the modulation of striatal ensembles. First, a reduction in inhibitory gaiting of FSIs, with no change in excitatory control, would disinhibit striatal FSIs allowing stronger feedforward inhibition onto MSNs and lateral inhibition onto neighboring FSIs. Since Dunhe et al. (2020) revealed that photo stimulating FSIs activates MSN ensembles, one would hypothesize that disinhibition of FSIs similarly facilitates striatal ensemble expression (**Figure 5.3**). Interestingly, not only does ethanol exposure promote habitual actions aimed toward the drug itself, but this phenomenon also transfers to natural rewards (Corbit et al., 2012), providing insight to clinical reports from alcohol abusers who feel trapped in a cycle of habitual actions including but not limited to habitual drinking (Seo & Sinha 2014).

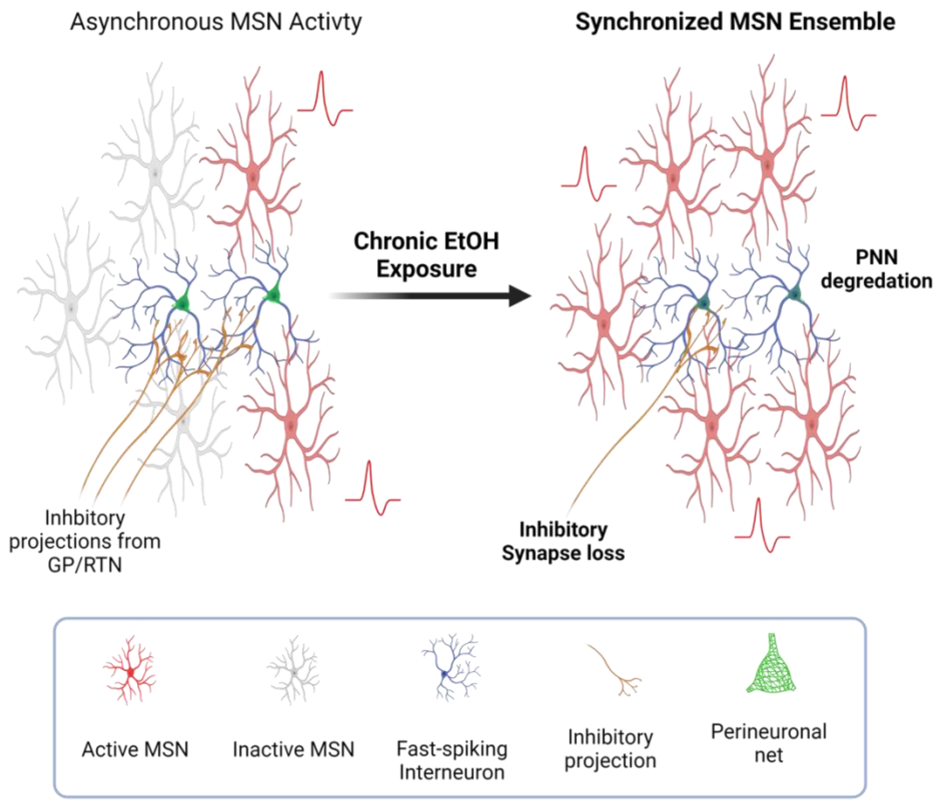


Figure 5.3 Chronic ethanol exposure facilitates striatal ensemble formation. Chronic ethanol exposure reduces inhibitory synaptic transmission onto striatal FSIs by reducing the number of inhibitory synaptic contacts. In parallel, chronic ethanol exposure degrades perineuronal nets surround striatal FSIs. The resulting disinhibition promotes FSI activity to engage a synchronous MSN ensemble. Additional decrease in GABAergic transmission from FSIs→MSNs following chronic ethanol exposure further promotes a refined MSN ensemble that promotes actions geared toward the acquisition of ethanol reward (i.e., lick sequences).

5.3 The effect of acute ethanol exposure on FSI microcircuitry

While I previously demonstrated the effect of chronic ethanol vapor exposure on synaptic transmission to dorsal striatum FSIs, this merely provides a single snapshot of the brain after 5 weeks of ethanol exposure. I subsequently investigated how acute ethanol exposure modulates FSI physiology *ex vivo*. Blomely et al. (2011) demonstrated that acute ethanol exposure increases FSI excitability by modulating potassium channel function (Blomely et al., 2011). Additionally, acute ethanol exposure depresses synaptic transmission from FSIs on to dorsal striatum MSNs through a presynaptic mechanism (Patton et al., 2016). Adding to this body of work, I investigated the effect of acute ethanol exposure on GABAergic transmission from the two extrinsic inhibitory projections that I previously demonstrated are sensitive to chronic ethanol vapor exposure, the GP and RTN.

The effects of acute ethanol exposure on GABAergic transmission are widely studied, as many of the behavioral responses to ethanol administration, including anxiolysis and sedative effects are due, in part, to ethanol actions on the $GABA_A$ receptor (Boehm et al., 2006; Kumar et al., 2009). Indeed, many of ethanol's behavioral actions parallel what is observed following administration of a $GABA_A$ receptor agonist (Grobin et al., 1998), and notably administration of a $GABA_A$ antagonist decreases ethanol behavioral actions, including self-administration (Hyytiä & Koob 1995). The $GABA_A$ receptor exists as a pentameric structure of several subunits [alpha $_{1\text{-}6.}$, beta $_{1\text{-}3}$, gamma $_{1\text{-}3}$ delta, epsilon, theta, pi, rho $_{1\text{-}3}$] that combine and give rise to multiple receptor configurations (Olsen & Sieghart 2008). These receptors are located both synaptically and extra-synaptically, where synaptic receptors are primarily identified by the presence of an alpha$_1$ subunit and extra-synaptic receptors are identified by the expression of an alpha$_4$ subunit (Mody 2001). Synaptic

$GABA_A$ receptors mediate transient inhibition, while extra-synaptic GABA receptors regulate tonic inhibition from "spillover" of GABA (100 nM – 1 uM) during synaptic events (Belelli et al., 2009; Kumar et al., 2009). Numerous studies demonstrate that acute ethanol enhances $GABA_A$ transmission through multiple proposed mechanisms including direct receptor modulation (Nestoros 1980; Henriksen & Wiesner 1988). However, where ethanol interacts with the $GABA_A$ receptor is not entirely clear (Ticku 1990) as it does not displace GABA or benzodiazepine binding (Ticku 1988; Greenberg et al., 1984). Interestingly, administration of an inverse agonist at the benzodiazepine site partially blocks ethanol-mediated enhancement of GABA-activated chloride flux (Harris et al., 1998) and attenuates ethanol self-administration (June et al., 1994), suggesting that ethanol may interact near this allosteric site.

However, not all of ethanol's behavioral effects are blocked by benzodiazepine inverse agonist treatment, and the effects of this treatment are specific to low dose ethanol administration (<30 mM) (Wallner & Olsen 2008). Adding further confusion, ethanol enhancement of GABAergic transmission is not consistently demonstrated throughout the brain (Siggins et al., 1987), suggesting a diversity of $GABA_A$ receptor subunit composition with selective sensitivity to ethanol. Indeed, several studies have demonstrated that low concentrations of ethanol (10-17mM) target alpha-4 receptors found extra-synaptically (Wallner et al., 2014), where administration of an antagonist to this receptor, Ro15-4513, attenuates voluntary ethanol consumption in rats (June et al., 1994). However, at higher concentrations (50-100mM), ethanol affects both synaptic and extrasynaptic $GABA_A$ receptors (Jia et al., 2008).

In agreement with previous literature, I found that low dose ethanol exposure (17mM) had little to no effect on inhibitory synaptic transmission at the GP→FSI and RTN→FSI synapses. However, high dose ethanol exposure (50mM) enhanced GABAergic transmission from both input regions (**Figure 4.1,4.2**). Interestingly, this effect sustained after ethanol wash-off, suggesting that ethanol is not acting as a positive allosteric modulator at this receptor, but instead induces sustained GABAergic plasticity. In neonatal rat hippocampus, repetitive neuronal depolarization induces long-term potentiation of $GABA_A$ post-synaptic potentials that is blocked by intracellular injection of the calcium chelator BAPTA (Caillard et al., 1999). In the visual cortex, long term $GABA_A$ potentiation is mediated by IP3 receptor-dependent calcium flux (Komatsu 1996). In line with these observations, I found that ethanol-induced GABA LTP was mediated by a post-synaptic phenomenon, since I observed no sustained change in presynaptic release probability (**Figure 4.1,4.2**). I additionally found that sequestering calcium fluxes postsynaptically with the chelator BAPTA (20mM) prevented ethanol-induced GABA LTP (**Figure 4.1,4.2**). The exact source of this calcium flux remains to be determined.

Several studies demonstrate the effects of ethanol exposure on voltage-gated calcium channels, including the L-type channels that are primarily expressed on neuronal somata and dendrites (Gandhi & Ross 1989; Varodayan et al., 2017; Walter & Messing 1999). Alternatively, in cultured neurons ethanol modulates calcium release from intracellular stores (Kim et al., 2021). Despite not know the precise mechanism of calcium flux, these studies collectively highlight the importance of calcium signaling in ethanol's acute effects on the central nervous system. Notably, this likely subserves numerous behavior effects of acute ethanol administration, as systemically administering a calcium chelator blocks voluntary ethanol consumption and the sedative effects of acute ethanol administration (Baliño et al., 2012).

It is interesting to speculate how enhanced inhibitory transmission onto dorsal striatum FSIs would impact striatal function and corresponding behavior. Enhanced inhibition of FSIs would disinhibit striatal projection neurons. Indeed Wilcox et al. (2014) has observed that acute ethanol exposure depresses spontaneous inhibitory synaptic transmission onto striatal MSNs from an unknown inhibitory source. Provided my observations, it is likely that FSIs contribute to this disinhibition. Indeed acute ethanol exposure depresses GABAergic synaptic transmission at the FSI→ MSN synapse as well as collateral inhibition at MSN→MSN synapses (Patton et al., 2015).

Notably, enhanced inhibitory control of FSIs would facilitate the activation of the DLS thereby biasing the dorsal striatum for habitual actions. However, it is important to note that in freely behaving mice, manipulations to the DLS were not observed until 3-weeks of voluntary ethanol consumption, suggesting that while acute ethanol exposure may begin to promote DLS engagement, repeated ethanol exposure is required to ultimately engage DLS output. This effect likely arises from both disinhibition of the DLS and simultaneous inhibition of cortical control over goal-directed DMS output (Lüscher et al., 2020; Perry et al., 2011; Kähkönen et al., 2003; Yang et al., 2016) (**Figure 5.4**).

Additionally, Patton et al. (2016) demonstrated that acute ethanol exposure reduces GABAergic transmission from FSIs to MSNs through a presynaptic mechanism. In a neuronal circuit, presynaptic LTD serves as a high-pass filter (Bidoret et al., 2009), where only high-frequency signals are propagated to the post synaptic neuron. This high-pass filter would refine the motor command initially driven by cortical/thalamic input onto FSIs, likely resulting in the execution of a selective action sequence geared toward ethanol reward (i.e., lick bouts). Importantly, these collective effects of ethanol on striatal function likely arise from modification of synaptic GABAergic transmission that, as I mentioned previously, requires high concentrations of ethanol (50-100mM). Therefore, it is unlikely that low-dose ethanol exposure would produce the same deleterious effects on striatal function, which reinforces the notion that alcohol misuse, especially repeated binge-drinking, promotes maladaptive changes to the brain while moderate consumption is likely benign.

After repeated exposure, the effects of ethanol on inhibitory transmission to striatal FSIs shifts, where acute ethanol potentiates inhibitory transmission, but chronic exposure dramatically reduces inhibitory transmission. This transition likely enables the expression of habitual action sequences geared toward ethanol acquisition (Patton et al., 2021). To further investigate this transition, I recorded the effect of an additional acute ethanol challenge in 5-week ethanol vapor exposed mice and observed that the additional acute challenge no longer potentiated inhibitory transmission, suggesting that the system has undergone a homeostatic adaptation to repeated ethanol exposure. In other words, while acute administration augments inhibitory transmission, repeated potentiation results in a down regulation of inhibitory transmission to re-establish a set point of inhibitory homeostasis. Notably, not all inhibitory inputs are lost following 5-week ethanol vapor exposure, and those that remain appear to be insensitive to the additional acute ethanol challenge. It is possible that only the ethanol sensitive synapses undergo this adaptive change to chronic ethanol exposure, leaving the ethanol insensitive synapses unchanged. This would require local regulation at the post synaptic zone, since I demonstrated the acute potentiation of inhibitory transmission was due to a post-synaptic calcium flux. It is possible that there is synapse specific expression of voltage-gated calcium channels that are selectively sensitive to ethanol exposure. In response to acute ethanol exposure, these ethanol sensitive synapses undergo GABA LTP. However, following repeated exposure these same synapses could undergo perineuronal net modification to enable synaptic retraction to re-establish homeostatic inhibitory transmission. As a result, the remaining inhibitory synapses after chronic ethanol exposure are insensitive to acute ethanol exposure, which is why the additional acute challenge no longer potentiates GABAergic transmission. As I mentioned previously, at this new set point, disinhibition of FSIs likely promotes the recruitment and refinement of MSN ensembles to facilitate ethanol consumption.

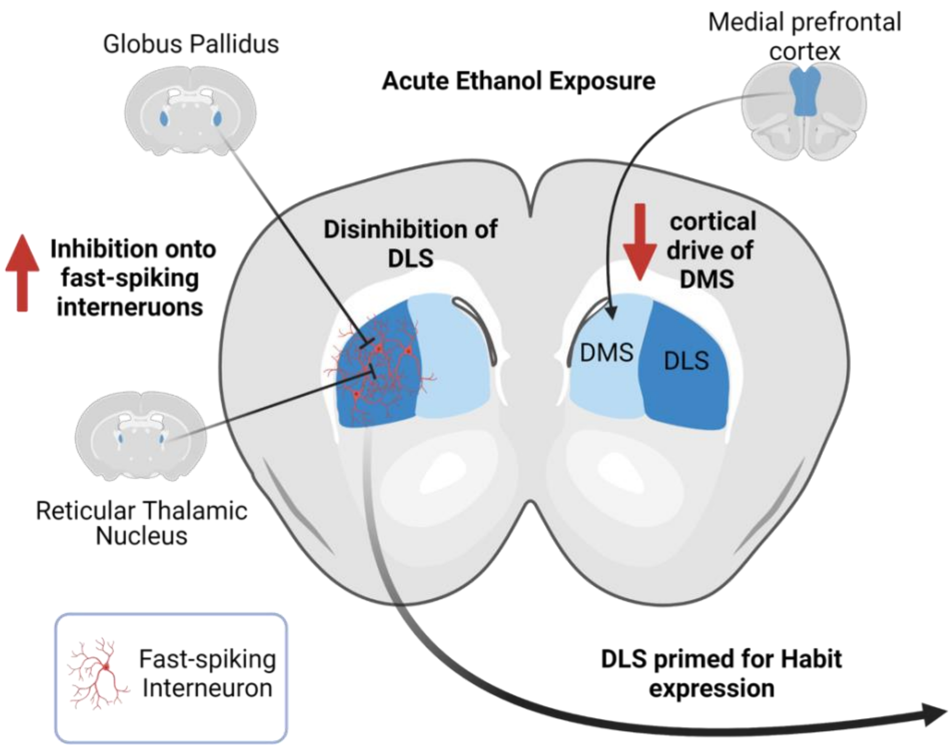


Figure 5.4 Acute ethanol exposure primes the dorsolateral striatum for habitual behavior. Acute ethanol exposure enhances GABAergic transmission from the GP and RTN onto dorsal striatum FSIs. Inhibition of FSIs disinhibits striatal MSNs thereby promoting activity in the DLS. Concomitantly, acute ethanol exposure reduces cortical control over the DMS (Kähkönen et al., 2003; Yin et al., 2007; Glass, 2011) thereby shifting the balance of dorsal striatum activity toward the DLS. Collectively acute ethanol exposure primes the dorsal striatum for DLS driven habitual actions geared toward ethanol reward.

5.4 Future directions

Provided all the evidence I have presented thus far demonstrating the effects of both acute and chronic ethanol exposure on dorsal striatum FSIs, one would ideally be able to lever the synaptic mechanisms gleaned from these studies to pharmacologically counteract ethanol's detrimental effects on the striatum. One such possibility could involve modulating the transition in inhibitory transmission that occurs from acute to chronic ethanol exposure. Specifically, if one could somehow prevent acute ethanol exposure from augmenting GABAergic input onto FSIs, presumably this would prevent ethanol-mediated recruitment of the DLS and subsequently stop the homeostatic adaptation that ultimately results in the retraction/elimination of ethanol-sensitive GABAergic inputs onto FSIs. We know that acute ethanol exposure enhances GABAergic input onto FSIs through an unknown post-synaptic mechanism that involves calcium flux.

As I mentioned previously, there are two potential mechanisms mediating calcium flux, through voltage-gated calcium channel activation and/or release from intracellular stores, and both mechanisms are affected by acute ethanol exposure (Walter & Messing 1999; Varodayan et al., 2017; Kim et al., 2021). However, from my observations, not all synapses were lost following 5-week ethanol vapor exposure, and those that do remain were insensitive to acute ethanol exposure, suggesting that a global mechanism, like release from intracellular stores or microdomains, is unlikely. What is more likely is that selective synapses specifically express an ethanol-sensitive voltage-gated calcium channel.

One possible calcium channel is the L-type voltage-gated channel, of which there are two subtypes Cav1.2 and Cav1.3 expressed somatodendritically in the central nervous system (Hell et al., 1993). Supporting this hypothesis, ethanol discrimination is blocked by the L-type calcium channel blocker isradipine (Colombo et al., 1994; Green & Grant 1999) and administration of two different L-type channel antagonists, verapamil and isradipine, decreases ethanol consumption in alcohol preferring rats (Rezvani & Janowsky 1990; Fadda et al., 1992). Unfortunately, in clinical trials, L-type calcium channel blockers failed to blunt the behavioral effects of acute ethanol administration and the drug combination significantly lowered blood pressure (Perez-Reyes & Hicks 1992; Zacny & Yajnik 1993; Rush & Passaglia 1998). However, it is important to note that these clinical studies did not investigate the ability of L-type channel blockers to attenuate ethanol consumption, but instead focused on the subjective and motor effects of alcohol intoxication. Future slice electrophysiology experiments should explore whether L-type channels modulate acute ethanol augmentation of GABAergic input onto striatal FSIs and whether blocking this effect would prevent inhibitory synapse remodeling.

In addition to blocking ethanol potentiation of GABAergic input onto FSIs, one could alternatively block the synaptic remodeling that occurs after 5-week ethanol vapor exposure. As I mentioned previously, ethanol may initially target perineuronal nets to enable remodeling of inhibitory synaptic inputs. Provided the elevated levels of the extracellular matrix degrading enzyme MMP-9 in human serum during alcohol intoxication (Zdanowicz et al., 2022), it is possible that inhibiting MMP-9 activity in the striatum would restore perineuronal net protein expression and subsequently correct inhibitory synapse remodeling. This, in theory, would reduce the activation of MSN ensembles geared toward ethanol acquisition and therefore prevent the escelation of ethanol consumption and further compulsive drinking. Supporting the role of MMP-9 activity in ethanol-induced striatal dysfunction, intracerebroventricular infusion of a broad spectrum MMP inhibitor (FN-439) attenuates escalation of ethanol self-administration in rats (Smith et al., 2011). While this compound was delivered througout the brain, and therefore it is imppossible to discern where in the brain this enzyme was primarily acting, it is possible that FN-439 prevented striatal perineuronal net degredation to attenuate ethanol consumption.

Conversely, increasing the expression of PNN proteins, specifically in dorsal striatum FSIs, could counteract ethanol-induced degradation of PNNs. Notably, the two PNN components that are reduced by chronic ethanol expsoure, Aggrecan and HAPLN1, are expressed by PNN-containing neurons in rodents, while other PNN constituents are expressed by both neurons and glia (Matthews et al., 2002; Dino et al., 2006; Galtrey et al., 2008). Therefore, designing an adeno-associated virus containing a cre-dependent plasmid expressing both of these proteins could theoretically restore PNN expression specifically around FSIs. However, maintaining a biologically optimal protein expression level with a viral plasmid may present technical problems, where over expression may yield deleterious effects. Future studies exploring this virus based expression approach could provide a novel cell-type specific tool to manipulate PNN expression in the striatum to determine its causal role in compuslive ethanol consumption.

An alternative approach to regulating perineuronal net and inhibitory synapse remodeling is through migroglia phagocytosis, which, as I mentioned previously, is known to regulate both of these processes in the brain (Socodato et al., 2020; Crasper et al., 2020; Venturina et al., 2021; Wegryzn et al., 2021). Minocycline, a second-generation tetracycline antibiotic, is commonly used to inhibit microglia activation (e.g., Kobayashi et al., 2013) and notably, minocycline treatment attenuates voluntary ethanol consumption in mice (Agrawal et al., 2011). Currently, there is a double-blind, placebo-controlled clinical trial investigating minocycline's effects on alcohol response in humans (NCT02187211). Future studies that locally administer minocycline to the striatum may reveal whether local microglia activation promotes escalated ethanol consumption and compulsive drinking in mice.

In addition to correcting ethanol's effects on inhibitory transmission, one could also directly target dorsal striatum FSIs to attenuate ethanol consumption. In a small pilot study, I chemogenetically inhibited FSIs in mice voluntarily drinking ethanol for 4-weeks and found that initially the inhibitory DREADDs trended to decrease voluntary consumption (**Figure 5.5**). However, by the fourth week their drinking rebounded back to the level of controls. It is possible that repeated DREADDs activation induced a homeostatic adaptation in FSIs, that if true, provides important insight for future FSI-targeted therapies administered chronically.

In my initial study of FSI recruitment in voluntary ethanol consumption, the effect of FSI ablation predominantly occurred during the third week of the drinking paradigm, suggesting that inhibiting FSIs at this timepoint would be more effective and could possibly prevent a rebound effect during the remainder of the study. Additionally, inhibiting FSIs during the quinine challenge on the fifth week of the drinking paradigm, the same time point where I observed the dramatic reduction in inhibitory transmission and synapse loss, has a greater potential to attenuate compulsive drinking. Future studies chemogenetically inhibiting FSIs at these specific timepoints are needed.

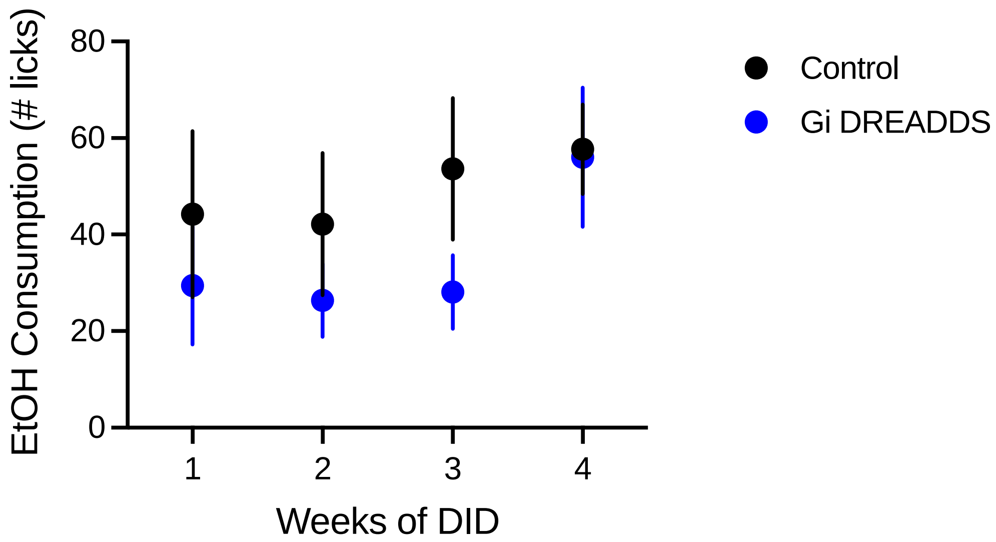


Figure 5.4 Chemogenetic inhibition of striatal FSIs transiently trends to reduce voluntary ethanol consumption. Average weekly ethanol consumption (# licks) across the 4-week drinking paradigm from control fluorophore expressing mice (black) and mice expressing the hM4Di. Administration of the DREADD receptor agonist DCZ (10ug/kg, i.p.) 30min prior to each drinking session trended to induce a transient decrease in voluntary ethanol consumption by week 3 that recovered by week 4 (n = 5 mice per group). Data represented as mean ± SEM.

If the results of the proposed experiments are promising, one could then identify a novel receptor target uniquely expressed on FSIs for cell-type specific pharmacological manipulation. Working toward this goal, in collaboration with the University of Maryland Institute for Genome Science, I collected striatal samples from 5-week ethanol vapor exposed mice that were processed for single nuclear genome sequencing (**Figure 5.6**). The transcriptional information from this dataset could provide novel pharmacological targets to restore inhibition onto FSIs.

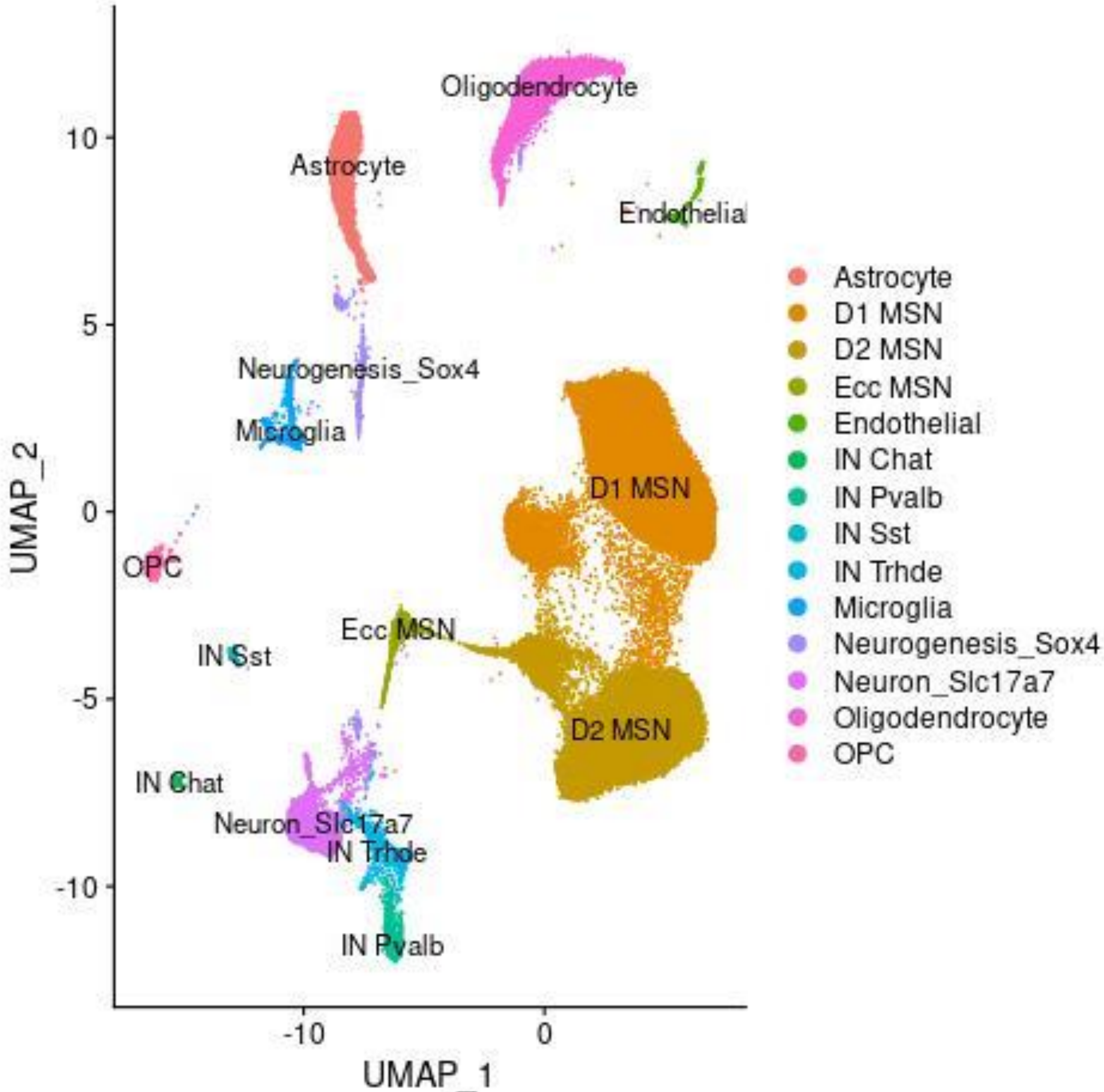
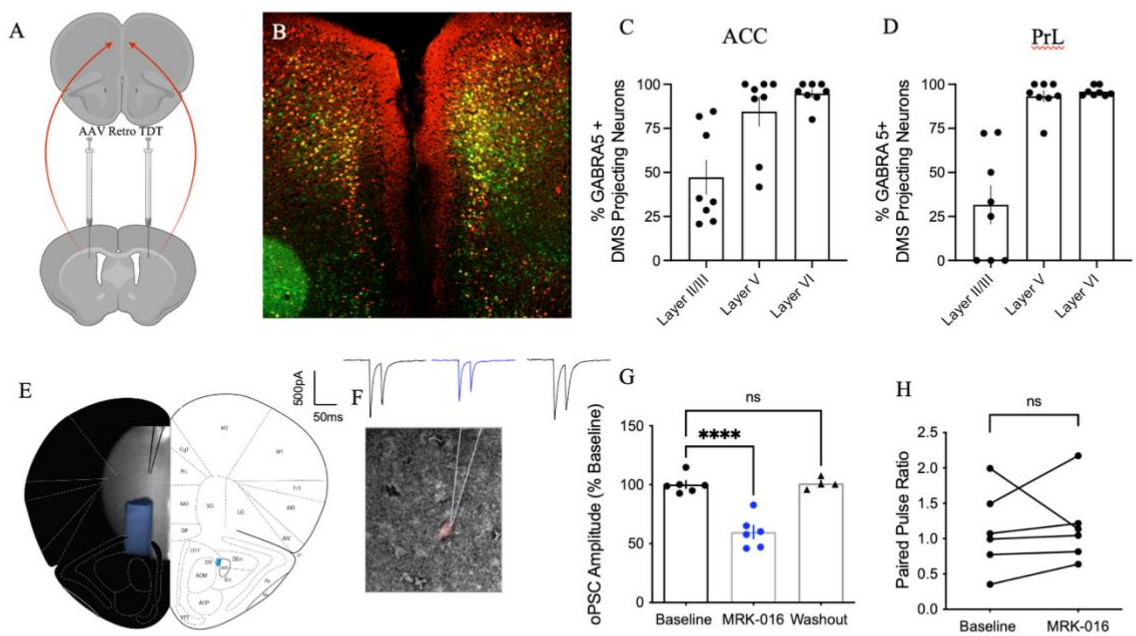


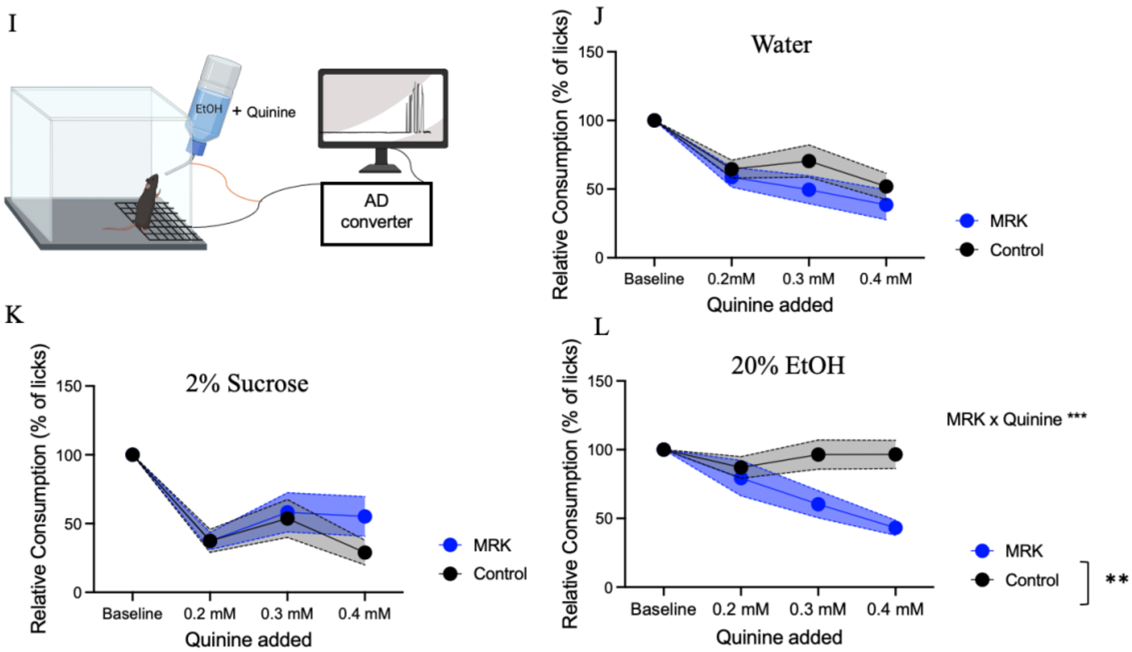
Figure 5.6 Cluster analysis of single nuclear sequencing data from striatal samples of control and chronic ethanol exposed mice. Dimension reduction of the single nuclear expression data identified 14 distinct cell populations within the striatal samples, including parvalbumin expressing FSIs. Subsequent expression analysis will reveal cell-specific transcripts differentially regulated by chronic ethanol exposure.

Considerable attention of this thesis has focused on the pathological recruitment of the DLS following chronic ethanol exposure. However, it is important to consider that the DLS circuit does not work in isolation, but instead works in balance with the DMS to promote appropriate behavioral responses based on an everchanging environment. While I have focused on ethanol-induced modifications to the DLS to attenuate compulsive ethanol consumption, it is equally likely that manipulating the DMS would attenuate compulsive drinking. In other words, one could pharmacologically re-activate the DMS to reinstate goal-directed behavior, thus reducing habitual ethanol consumption.

One promising therapeutic that aims to restore goal-directed behavior is MRK-016 that acts as a selective negative allosteric modulator at alpha-5 containing $GABA_A$ receptors (Atack et al., 2009; Jacob 2019). Notably, alpha-5 receptors are predominately expressed in the hippocampus and the medial prefrontal cortex suggesting few off target side-effects of systemic treatment (Wisden et al., 1992; Sur et al.,1999). In the prefrontal cortex, I demonstrated that approximately 85% of layer 5 pyramidal neurons in the prelimbic cortex projecting to the dorsomedial striatum express the alpha-5 containing receptors (**Figure 5.7**). Importantly, these DMS projecting neurons are functionally responsive to MRK-016 and become disinhibited following acute bath-application *ex vivo* (**Figure 5.7**), suggesting that administration of this compound could reinstate cognitive control over DMS mediated behavior, including ethanol consumption. Indeed, when administered to chronic ethanol exposed mice, MRK-016 attenuates compulsive drinking (**Figure 5.7**). Future studies will determine if the effects of MRK-016 are due to re-activation of DMS or through activation of a different brain circuit.

Figure 5.7 MRK-016 attenuates compulsive-like ethanol consumption in mice. **A**) schematic of viral injection of retrograde AAV expressing TDT to label DMS projecting pyramidal neurons in the prefrontal cortex. **B**) RNA scope fluorescent image of TDT positive DMS projecting cortical neurons (red) co-labeled with RNA for the alpha-5 subunit of the GABAA receptor (GABRA5; green). **C-D**) Quantification of double positive (GABRA5 + / TDT +) neurons in the anterior cingulate cortex (ACC) and the prelimbic prefrontal cortex (PrL). **E**) Schematic of whole-cell patch clamp recording from DMS projecting cortical neurons in the PrL. **F**) Brightfield image of TDT + cortical neuron in the PrL. Insert: optically evoked inhibitory transmission at the FSI→TDT+ pyramidal neuron synapse in the PRL from control (black) and MRK-016 bath application (blue) and MRK removal (black). **G**) MRK-016 reduced inhibitory synaptic transmission at the FSI→ TDT+ pyramidal cell synapse. **H**) MRK-016 does not impact synaptic release probability. **I**) Schematic of Drinking in the Dark box. **S**ystemic administration of MRK-016 30mins prior to each drinking session does not impact quinine-adulterated water consumption (**J**) or sucrose consumption (**K**), but significantly attenuates quinine-adulterated ethanol consumption (**L**). ** $p < 0.01$, *** $p < 0.001$. Data represented as mean $\pm$ SEM.




I
EtOH
+ Quinine
AD converter
J
Water
Relative Consumption (% of licks)
150
100
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Baseline
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0.4 mM
Quinine added
MRK
Control
K
2% Sucrose
Relative Consumption (% of licks)
150
100
50
0
Baseline
0.2 mM
0.3 mM
0.4 mM
Quinine added
MRK
Control
L
20% EtOH
Relative Consumption (% of licks)
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Baseline
0.2 mM
0.3 mM
0.4 mM
Quinine added
MRK x Quinine ***
MRK
Control
**

Therapeutic strategies that re-engage prefrontal cortical control have historically been the focus for the treatment of substance use disorder (Stock 2017). One such therapeutic strategy, cognitive behavioral therapy, is empirically effective for treating alcohol and substance use disorder (Dutra et al., 2008; Magil & Ray 2009). However, the treatment effect is often small (g=0.154) and diminishes after a 12-month follow-up (Magil & Ray 2009). Collectively, this suggests that combined cognitive and pharmacological therapies may be a more efficacious treatment strategy for substance use disorder, specifically those that aim to simultaneously engage goal-directed behavior and suppress habitual DLS-mediated behavior.

During my literature search into possible solutions to the prevalent alcohol abuse that currently and historically have afflicted society, I came across an interesting idea put forth by Wallner and colleagues (2014 ,2008). By studying the pharmacological actions of ethanol on the central nervous system, they propose that one day perhaps society could generate an alcohol mimetic that would still produce the same positive effects of responsible alcohol consumption, without the health consequences associated with ethanol and its toxic metabolites (Zhang et al., 2004; Wallner & Olsen 2008). This dream drug would have a controlled time-course of action that could be regulated by administering a synthetic enzyme or selective antagonist. Imagine being able to go out with friends, become intoxicated on this synthetic compound, and then block its effects when you need to drive home.

While this dream drug may exist one day in the distant future, it seems unlikely that society will produce an alcohol mimetic drug with our current available tools and knowledge of the central nervous system. Indeed, provided what I have demonstrated here, ethanol has numerous effects on the brain, targeting many receptor proteins as well as interacting with the membrane bilayer itself (Li et al., 1994). In fact, it is a marvelous feat of biology that human beings co-evolved with a microorganismal metabolic byproduct that penetrates the blood brain barrier to induce numerous modifications to CNS function that both benefit and harm society.

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