UNIVERSITY OF CALIFORNIA, IRVINE

Investigating the Role of *Gilz* in Regulating Cocaine-Induced Cellular Activity and Cocaine-Associated Behaviors

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

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2024

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Acknowledgements

I will be forever grateful to my committee chair, Professor Marcelo Wood, for his unwavering support and continual guidance throughout graduate school. Dr. Wood demonstrated not only a contagious sense of encouragement, but also an acute sense of empathy. He taught me how to look past the technical and into the narrative components of science. He taught me how to remain deeply invested in my research while retaining the ability to set aside its weightier effects. Most importantly, he taught me how to show up for people, even when studying the mouse. I would also like to thank my committee members: Dr. Javier Díaz-Alonso, Dr. Christie Fowler, and Dr. Kim Green for offering thought-provoking questions and advice. Their feedback and support have been critical in helping shape not only my thesis, but also my relationship to science.

To all the past and current members of the Wood lab, thank you for the guidance, support, and comradery. Dr. Dina Matheos walked patiently with me through endless molecular protocols and offered insightful discussion on topics both scientific and otherwise. Dr. Enïko Kramár provided critical additions to my thesis project and was always keen to connect in the lab. Dr. Jessica Childs showed me the meaning of tenacity in the series of experiments she conducted for this thesis. Others in the Wood lab who have shown me the many ways in which science can be rewarding include: Carlene Chinn, Vanessa Alizo, Alyssa Rodriguez, Dr. Ashley Keiser, and Dr. Franklin Garcia. Dr. Rianne Campbell mentored me briefly during our short overlap in the Wood lab, and her incredible technical and management capabilities served to continually inspire me throughout graduate school.

Financial support was provided by the National Institute on Drug Abuse, F31 Grant DA059264.

To my past mentors, Dr. Zuoxin Wang and Christine Meneer. I thank you for investing in me, and for teaching me how to invest in myself.

To my late grandfather, James Rudgers. I thank you for your unending love and support. You always inspired me to reach further.

To my siblings, Hannah, Josh, and Jonah. Hannah taught me the art of rhetoric. Josh taught me the art of discipline. And Jonah taught me the art of heart.

Finally, to my parents. To whom I owe all that I am. Thank you, Mom and Dad, for teaching me to do what is right and to treat others kindly.

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Funding:

Ruth L. Kirschstein National Research Service Award Individual Predoctoral Fellowship in Health-Related Research (F31) 2024
National Institute on Drug Abuse: F31-DA059264
"Investigating the Role of *Gilz*, an X-linked Gene, in Regulating Cocaine-Induced Cellular Activity and Cocaine-Associated Memory"

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UCI Center for Neurobiology of Learning and Memory John W. Haycock Memorial Graduate Student Award	2024
UCI Department of Neurobiology & Behavior Best Poster Award	2023
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Peer-Reviewed Publications

Donovan M, Mackey CS, Platt GN, **Rounds J**, Brown AN, Trickey DJ, Liu Y, Jones KM, Wang Z. (2020). Social isolation alters behavior, the gut-immune-brain axis, and neurochemical circuits in male and female prairie voles. *Neurobiol Stress*.

Rounds, J.S., Wood, M.A., Dudek, S. (Ed.). (2023). Activity-dependent transcription and its role in cocaine and opioid addiction. *Transcriptional Regulation by Neuronal Activity: To the Nucleus and Back 2.0*. Springer Science & Business Media. (Accepted for publication).

Chen, C. C., Han, J., **Rounds, J**., Chinn, C. A., Li, X., Nikan, M., Myszka, M., Tong, L., Bredy, T. W., Wood,M. A., Luptak, A. (2024). Inhibition of Cpeb3 ribozyme elevates CPEB3 protein expression and polyadenylation of its target mRNAs and enhances object location memory*. eLife* 13, e90116.

Keiser AA, Dong T, Kramár EA, Butler CW, Chen S, Matheos DP, Rodriguez A, **Rounds JS**, Beardwood J, Augustynski AS, Al-Shammari A, Alaghband Y, Alizo Vera V, Berchtold NC, Shanur S, Baldi P, Cotman CW, Wood MA. (2024) Specific exercise patterns generate an epigenetic molecular memory window that drives long-term memory formation and identifies ACVR1C as a bidirectional regulator of memory in mice. *Nat Commun* 15, 3836.

Rounds, J., Chinn, C., Childs, J., Kramár, E.A, Alizo Vera, V., Lai, Y., Matheos, D., Wood, M.A. (2024) *Gilz* splice variants regulate nucleus accumbens long-term potentiation and cocaine intravenous self-administration in mice. (in prep).

Chinn, C.A., **Rounds, J**., Kramár, E.A., Tam, S., Matheos, D., Wood, M.A. (2024) The X-linked gene *Gilz* regulates formation and maintenance of fear memory in the basolateral amygdala. (in prep).

Abstract of the Dissertation

Investigating the Role of *Gilz* in Regulating Cocaine-Induced Cellular Activity and Cocaine-Associated Behaviors

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Doctor of Philosophy in Biological Sciences
University of California, Irvine 2024
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Exposure to drugs of abuse, such as cocaine, engages transcriptional machinery to alter gene expression profiles, ultimately giving rise to long-term changes in cellular function, synaptic plasticity, and drug-related behavior. Previous work from our lab has identified *glucocorticoid-induced leucine zipper* (*Gilz*) as an epigenetically regulated gene that may be responsible for orchestrating long-term transcriptional adaptations underlying such behaviors. However, it remains unknown whether the distinct mRNA splice variants encoded by the *Gilz* gene play unique roles in this context. It is also unclear whether *Gilz* splice variants in the brain differentially respond to stress-related events, despite the body of literature demonstrating *Gilz* as a downstream transcriptional regulator of glucocorticoid-signaling. In the present dissertation, we investigated whether *Gilz* splice variants exhibit differential responses to cocaine- or stress-exposure, and we investigated whether *Gilz* is necessary for (1) long-term potentiation (LTP) in the nucleus accumbens (NAc), (2) cocaine-conditioned place preference, and (3) cocaine self-administration behaviors. We demonstrate that *Gilz* acts in the NAc to regulate LTP, in the VTA to regulate CPP, and that *Gilz* is necessary for reinstatement of cocaine-seeking behavior in male mice. We also demonstrate that *Gilz* splice variants differentially respond to a variety of stress-related stimuli. Overall, this work illustrates how cocaine or stress may

engage *Gilz* in distinct brain regions to regulate cellular and behavioral responses to maladaptive experiences.

Introduction

A. Substance use disorder & stress

Substance Use Disorder (SUD) is a progressive neuropsychiatric disorder characterized by loss of control over drug intake, preoccupation, and continued use despite negative consequences[3]. SUDs are often chronic, relapsing disorders which have profound effects on the health of individuals and societies. It is estimated that the United States spends more than $400B annually to address the effects of substance abuse, and that annual drug-involved overdose deaths have increased by 75% in the past five years[4,5]. In addition to the rampant ongoing opioid epidemic, recent data show that psychostimulants (*e.g*. cocaine, methamphetamine) are among the leading contributors to overdose deaths, with a 36% increase from 2020-2021[6]. While current treatment methods have varying efficacies, roughly half of individuals diagnosed with SUDs are reported to relapse following abstinence[7]. For these reasons it is imperative that research efforts are focused on investigating how illicit drugs lead to such persistent changes in brain function and behavior.

Bidirectional effects of drug abuse and stress

A variety of factors underly addiction vulnerability as well as behavioral progression through patterns of drug abuse. Of these factors, environmental stress exerts a notably powerful influence on the behavior of individuals with SUDs. Stress can broadly be defined as a state of mental or emotional strain resulting from adverse circumstances[8], and the unpredictable and unavoidable nature of stress can further potentiate the harmful consequences of drug-related behavior. This influence is reflected in epidemiological data showing high rates of comorbidity between stress-related conditions and substance

abuse. For instance, prior trauma is associated with increased frequency and severity of SUDs, and roughly 80% of patients diagnosed with post-traumatic stress disorder (PTSD) go on to develop comorbid SUD symptoms[9-11]. Moreover, drug users report intensified cravings during stressful periods, and stress-induced increases in blood cortisol levels are positively correlated with drug intake in cocaine abusers[12,13]. Animal models have also provided evidence that stressful experiences can increase susceptibility to substance abuse. Exposure to a variety of stressors (e.g. electric foot shock, social isolation, forced swim) not only facilitates acquisition and escalation of drug self-administration, but also potentiates reinstatement of extinguished drug-seeking[14-18]. These findings represent only part of an overwhelming body of evidence implicating the role of stress in substance abuse.

Conversely, repeated exposure to DOAs can have adverse effects on stress-responsive systems. Individuals with drug use disorders are nearly twice as likely to develop anxiety disorders[19], and withdrawal from various DOAs has anxiogenic effects in abstinent SUD patients[20]. Prior experience with drugs leads to enhanced stress responses, and individuals with SUD are at increased risk of developing PTSD[21,22]. In addition to the drug itself, presentation of a drug-associated cue alone is sufficient to increase anxiety ratings in cocaine-dependent individuals[23]. Acute cocaine exposure has been associated with dose-dependent increases in glucocorticoid levels (i.e. corticosterone in rodents and cortisol in humans)[24-26]. Thus, not only does stress affect individuals' drug-seeking behavior, but repeated exposure to DOAs significantly alters stress-related conditions. Taken together, the above findings suggest a bidirectional

relationship between drug use and stress, highlighting the need to understand interactions between their underlying biological mechanisms.

Sex differences in drug abuse and stress-reactivity

There are sex differences in rates of SUDs and stress-related disorders, as well as the efficacy of treatment approaches. Men display higher prevalence of drug use and abuse across virtually all classes of illicit drugs, while women are more likely to experience stress-related psychiatric disorders (e.g. PTSD, depression, generalized anxiety disorder) as a result of substance abuse[27-30]. Nicotine-replacement options are more effective at treating men with tobacco use disorder, but females respond better to a variety of cocaine-replacement therapies[31,32]. Despite a higher prevalence in SUD among males, females tend to exhibit a "telescoping effect," progressing from initial use to dependence more quickly[33,34]. This effect is further exacerbated by exposure to stress[35-37]. In addition to the numerous sociopolitical factors affecting these differences, there is a clear need to consider biological sex when investigating the underlying mechanisms of both stress and drug use.

Rodent studies have helped elucidate some of the mechanisms contributing to sex differences in reward processing and drug use. For instance, ovarian hormones play a role in the propensity of female rats to develop a cocaine-conditioned place preference (CPP) at lower doses and to display enhanced CPP reinstatement[38-40]. Furthermore, exogenous estradiol treatment following gonadectomy facilitates acquisition of cocaine self-administration in females but not males[41]. The role of androgen hormones is less clear, with mixed results on cocaine-induced behavioral responses[42]. In addition to endocrine actions, cocaine dependence is associated with a 4:1 monozygotic:dizygotic

twin concordance ratio, and heritability is roughly 21% higher in males[43-45]. These findings suggest a sex-dependent polygenic risk associated with cocaine abuse and highlight the importance of designing experiments to understand the role of both genetic and endocrine factors in drug use.

Sex differences in stress reactivity to DOAs are also widely published, arising from divergent hypothalamic-pituitary adrenal (HPA)-axis as well as neurobiological responses to stress. HPA-axis activation in response to cocaine is higher in female rats compared to males[46]. Cues associated with stress lead to increased drug craving in both males and females, but the degree of response in females depends on estrus phase[47]. In rats, exposure to social stress potentiates reinstatement of cocaine-seeking behavior for both sexes, but females show a greater degree of stress-induced reinstatement that is also dependent upon estrous[48,49]. The above stated differences in behavioral and chemical responses to stress and DOAs arise from signal interactions at both the intracellular and circuit levels.

B. Signaling mechanisms in drug reward and stress

Dopaminergic signaling

Cocaine acts in the brain by blocking monoamine transporters, primarily the dopamine transporter (DAT), to inhibit reuptake of monoamines into presynaptic terminals[50]. The resulting increase in extracellular dopamine (DA) concentrations, specifically in the mesolimbic pathway from the ventral tegmental area (VTA) to the nucleus accumbens (NAc), is thought to represent the common initial target for DOAs[51]. This mesolimbic pathway plays a role in both associative learning and reward processing.

The NAc has two subregions (i.e. the core and shell) with distinct roles for encoding reward information in the context of cocaine exposure. The NAc core is critical for processing reward prediction errors (RPEs) that facilitate associative learning[53]. This associative learning arises from neural adaptations that allow environmental contexts and cues to elicit novel behavioral responses following repeated drug exposure[20]. In short, midbrain DA-ergic neurons terminating in the NAc core exhibit strong phasic responses upon receipt of unexpected rewards following predictive cues, reflecting a similar model of firing patterns thought to underlie learning[54]. The resulting effects of this altered midbrain firing are what drive a novel association between a conditioned stimulus (i.e. cue) and an unconditioned stimulus (i.e. psychostimulant effects of cocaine). Numerous studies in rodent models have shown that, in addition to cocaine-induced phasic increases in NAc DA, the same neurochemical effects can result from presentation of a cocaine-associated cue[55]. Furthermore, presentation of either drug-related or stress-related cues is sufficient to reinstate previously extinguished patterns of cocaine seeking behavior in drug self-administration models[56,57]. These findings support data from human subjects with active cocaine use disorder, where PET images show decreased binding availability of type-II dopamine receptors (D2Rs) in the striatum following presentation of cocaine-associated cues. Notably, the presentation of these cues was also associated with increased drug craving in the same subjects[58]. The presentation of stress-related cues also evoke cravings in subjects with a history of cocaine abuse[59]. Alternatively, the NAc shell is implicated in the processing of unconditioned reward stimuli. Lesion studies demonstrate a role for the NAc shell in potentiating unconditioned effects of cocaine, and extracellular DA increases following non-contingent cocaine administration are greater in

the NAc shell compared to the NAc core[60-62]. While neurotransmission in this mesolimbic pathway is thus a key substrate of reward processing, cocaine is also responsible for altering DA-ergic signaling in a number of other brain regions implicated in decision-making, pleasure, and associative learning (i.e. medial prefrontal cortex, amygdala, hippocampus)[52]. The resulting changes in these regions have lasting effects on how individuals respond to their environment.

To understand how cocaine exposure can induce neuroadaptations, it is necessary to outline the cellular mechanisms of DA signaling and their downstream effects on synaptic plasticity. Extracellular DA binds to either type-I (D1R) or type-II (D2R) receptors, both of which are expressed in reward- and memory-related circuitry (i.e. mPFC, hippocampus, amygdala)[Error! Reference source not found.,63]. D1Rs are $G_{\alpha s}$-coupled receptors while D2Rs are $G_{\alpha i/o}$-coupled receptors that stimulate or inhibit adenylyl cyclase-mediated increases in intracellular levels of cyclic-AMP (cAMP), respectively[63]. Extracellular signal-related kinase (ERK), a member of the mitogen-activated protein kinase (MAPK) family, is an important downstream target of this DA-mediated signaling cascade[64]. Acute and chronic injections of cocaine lead to increased phosphorylation of ERK and subsequent activation of the transcription factor CREB (cAMP response element-binding protein) as well as immediate early genes *c-Fos* and *Zif268* in the NAc, amygdala, and mPFC[65,66]. Phosphorylation of ERK in the NAc is necessary for consolidation of cocaine-conditioned associations during CPP training as well as expression of conditioned responses during CPP testing[67,68]. In addition, both D1Rs and D2Rs regulate nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB, a transcription factor with roles in inflammation, plasticity, and memory) through various mechanisms[69-71]. Chronic cocaine induces NF-

κB-dependent transcription of many genes known to maintain cell structure in the NAc of mice, and NF-κB inhibition blocks formation of a cocaine-CPP[72].

Glucocorticoid signaling

The HPA axis is a key component of the physiological stress response. Following a stressful stimulus, the neuropeptide corticotropin-releasing factor (CRF) is released from the paraventricular nucleus of the hypothalamus (PVN). CRF then binds G_s-coupled receptors (CRFR1s) to stimulate release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland into the bloodstream. ACTH signals reach the adrenal cortex and promote secretion of glucocorticoids (GCs, corticosterone in rodents and cortisol in humans). Circulating GCs readily cross the blood brain barrier and bind to glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs)[73]. GR-dependent actions are the primary downstream-effectors of the HPA axis, and despite their effects being typically adaptive, GR mechanisms are also responsible for pathologies arising from abnormal HPA axis activation[12,74-76]. This section will thus focus on GC-GR signaling, but it should be noted that MR signaling helps maintain basal activity of the HPA axis as well as neuronal metabolism[77,78].

GRs are ubiquitously expressed throughout the brain and in the periphery[79]. Upon diffusion of GCs through the cell membrane, bound GRs undergo a conformational change resulting in their dissociation from a multiprotein complex and subsequent translocation into the nucleus[80-82]. Nuclear GR homodimers bind to glucocorticoid response elements (GREs), DNA motifs found in the promoter regions of GC-responsive genes, to regulate gene expression by interacting with a variety of transcription factors (TFs), chromatin remodeling machinery, and RNA-polymerase II[84-86]. Notably, GRs also

mediate gene expression through direct protein-protein interactions with TFs NF-κB and activator protein 1 (AP-1, a heterodimer of c-Fos and c-Jun)[87-89]. The wide-reaching genomic effects of GC-GR signaling regulate a variety of cellular processes and represent key substrates for the interfaced responses to stress and DOAs.

Like stress, cocaine self-administration leads to elevated GC levels in humans and rats[90,91]. Moreover, GC secretion is necessary for a number of cocaine-induced behavioral adaptations. Repeated exposure to stress leads to GC-dependent increases in cocaine self-administration in rats, while selective GR deletion in NAc DA cells attenuates cocaine self-administration, locomotor sensitization, and CPP[17,92,93]. GR activation following forced swim has been shown to alter excitability of DA cells in the VTA[94]. Similarly, acute stress induces GR-dependent changes in NAc glutamatergic synaptic plasticity and DA efflux in the mPFC[95,96]. While the precise mechanisms underlying GR-mediated signaling in cocaine-induced neuroplasticity remain unclear, numerous studies point to the transcriptional regulation of immediate early genes as well as genes related to glutamatergic transmission and dendritic spine morphology[97,98]. Thus, transient neurochemical alterations in both DA and GCs may give rise to behavioral adaptations by engaging transcriptional machinery to modulate the structure and function of drug- and stress-responsive cells.

C. Transcriptional & epigenetic regulation of cocaine-induced plasticity

Cocaine-induced transcription

Transcription is critical for long-term synaptic plasticity, and cocaine exposure in both humans and rodents has been shown to promote robust transcriptional changes in

several of the reward-related brain regions described above[99-101]. Early studies examined cocaine-responsive changes in the activation of immediate early genes, and recent work has identified transcriptional responses to cocaine that durably alter processes related to synaptic function, metabolism, cytoskeletal structure, and GC-response.[102-105].

Both acute and chronic exposure to cocaine leads to upregulation of immediate early genes (IEGs) previously implicated in learning-induced plasticity. IEGs are a class of genes rapidly and transiently activated following a variety of extracellular stimuli[106]. In addition to encoding cytoskeletal proteins and DNA-binding proteins, neuronal IEGs also encode TFs which go on to orchestrate genomic functions underlying synaptic plasticity[106-108]. Numerous studies have demonstrated that IEGs (e.g. *FosB*, *Nr4a2*, *Npas4*) are transcribed in a paradigm- and region-specific manner following cocaine exposure, and these IEGs induce downstream transcriptomic changes associated with the establishment of persistent neuronal and behavioral adaptations underlying drug addiction[109-111]. For instance, cocaine-induced transcription of *ΔFosB* (a degradation-resistant splice variant of FosB) in the NAc regulates transcriptional programs affecting structural and synaptic plasticity to promote locomotor sensitization and cocaine-CPP[102,112,113]. Apart from *ΔFosB*, however, IEG activation is transient and thus stable cellular adaptations are carried out via a second wave of activity-dependent transcription.

Beyond the rapid effects of cocaine, lasting changes to synaptic plasticity are carried out by TFs such as CREB and NF-κB. Cocaine-induced CREB activation occurs via increased phosphorylation of MAPK/ERK in the mPFC, NAc, and amygdala[65,66]. CREB was one of the earliest identified TFs implicated in the formation of long-term memory, and it has also emerged as a negative regulator of the behavioral effects of

cocaine[114,115]. In the NAc, CREB activation mediates cocaine induction of the GluN2B subunit of glutamatergic NMDARs as well as corresponding alterations in dendritic spine morphology and intrinsic excitability[116,118]. More recent studies have shown cocaine-dependent enhancement of CREB-regulated gene expression throughout brain reward circuitry[99].

In contrast to CREB, the mechanism of cocaine-induced NF-κB transcription is not well understood. NF-κB, a TF known for its role in inflammation and immune responses, has more recently emerged as a regulator of synaptic plasticity underlying memory processes[70,117]. Russo *et al* found that chronic cocaine-induced NF-κB transcription in the NAc is necessary for regulating the rewarding responses to the drug[72]. Furthermore, downstream activation of several NF-κB-responsive genes (known for their roles in cell growth and survival) leads to increased dendritic spine density in NAc MSNs. Thus, rather than affecting synaptic transmission via receptor regulation, cocaine-induced transcription of NF-κB is thought to mediate downstream structural adaptations to the drug.

Another key TF implicated in cocaine action is the glucocorticoid receptor. Extended access to cocaine leads to increased GR expression throughout brain reward circuitry, and GR-mediated transcriptional activity is thought to be a potential mechanism underlying GC-dependent cocaine-induced neuroplasticity[92-94]. In short, current models posit that cocaine exposure elevates GC levels, which stimulate increased GR expression and leave stress- and drug-responsive cells primed for transcriptional adaptations to future cocaine use[119]. This model is thought to underly the “stage setting” effects that leave individuals vulnerable to stress- or cocaine-primed relapse. Selective GR deletion

in NAc dopaminoceptive neurons attenuates acquisition of both cocaine self-administration and cocaine-CPP in mice, suggesting that DA may also be an upstream regulator of GR-mediated transcription[92,127]. While many studies have characterized GR-dependent transcriptomic responses to stressful stimuli, it remains unclear which downstream targets of GRs contribute specifically to cocaine-induced plasticity underlying drug-associated behaviors.

Taken together, these findings demonstrate that exposure to cocaine induces transcription of various plasticity-related genes implicated in memory formation, cell growth, and stress responses. To understand how these transcriptional profiles can be sustained as persistent alterations in cell function and drug-related behavior, it is important to consider epigenetic mechanisms responsible for modifying and remodeling the chromatin landscape.

Epigenetics in cocaine-associated behavior

In the field of neurobiology, epigenetics is used as a term to describe the set of processes regulating gene expression without altering the nucleotide sequence of DNA itself. While this section will focus on epigenetic regulation of chromatin structure, RNA modifications and non-coding RNAs have been reviewed elsewhere with respect to their role in drug addiction[120]. Chromatin is composed of nucleosome units where DNA is wrapped around histone octamers with two copies each of H2A, H2B, H3 and H4. These subunits harbor amino-terminal histone tails which undergo a variety of post-translational modifications (PTMs, i.e. acetylation, methylation, phosphorylation, ubiquitination, etc.) that affect their structure and DNA-binding affinity. Among these, histone acetylation and

methylation are the most widely studied in the fields of learning, memory, and drug addiction[120,121].

Histone PTMs are controlled by enzyme families and represent epigenetic marks that affect gene transcription through both permissive and instructive mechanisms. For instance, histone acetylation generally promotes transcriptional activation by (A) neutralizing the positive charge of lysine residues to relax DNA-histone binding, and (B) actively recruiting enzymes known as “readers” – transcriptional regulators that recognize and bind to histone modifications. Acetyl groups are added and removed by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively, and the actions of these enzymes have been implicated in numerous mechanisms of activity-dependent transcription[120]. In response to acute or chronic cocaine exposure, acetylation levels are increased both globally and at the promoter regions of plasticity-related TFs (e.g. CREB, NF-κB)[72]. The resulting upregulation of these plasticity-related genes has been shown to dramatically alter mesolimbic circuitry, resulting in persistent behavioral responses to DOAs such as cocaine[Error! Reference source not found.]. Conversely, experimental manipulations of HDACs and HATs have been widely used to change behavioral responses to cocaine in a context-dependent manner.

Of particular interest to the context of this proposal, GC signaling has been shown to enhance memory consolidation via increased histone acetylation, and HDAC inhibition results in similar enhancements[122]. Our lab has demonstrated that cocaine-induced acetylation at the promoters of IEGs *c-Fos* and *Nr4a2* can be enhanced by focal deletion of HDAC3 in the NAc, and that HDAC3 negatively regulates cocaine-induced CPP

acquisition[123]. This finding, however, represents only a limited view of the functional role of HDAC3 in the context of memory.

HDAC3 is the most highly expressed HDAC of its class in the brain and is a well-established negative regulator of both long-term memory and synaptic plasticity. HDAC3 inhibition in the hippocampus ameliorates impairments in LTP and long-term object location memory in mice expressing an inactive form of CREB-binding protein (CBP, a well-studied HAT)[124]. Furthermore, HDAC3 inhibition in the NAc promotes extinction of cocaine-CPP in a manner resistant to reinstatement by enhancing mechanisms of memory consolidation[125]. It has recently been shown that cocaine-induced changes in HDAC3 activity occur in a cell type-specific manner, such that chronic cocaine increases *Hdac3* expression in D1 MSNs but not D2 MSNs[126]. Collectively these findings demonstrate that, among the many epigenetic regulators, HDAC3 is a key target for understanding neurobiological processes underlying plasticity, memory, and cocaine-induced behavioral adaptations. In fact, recent data from our lab have revealed that HDAC3 may regulate transcription of an intriguing substrate known as *GILZ*.

D. Glucocorticoid-induced leucine zipper (*Gilz*)

Of the many genes regulated by GR-mediated transcription, one of the most rapidly and invariably induced is *glucocorticoid-induced leucine zipper* (*Gilz*, a.k.a *Tsc22d3*). *Gilz* was first identified in 1997 as a dexamethasone-induced protein responsible for protecting T cells from apoptosis in lymphatic tissues, and many subsequent studies have focused on the role of GILZ in producing anti-inflammatory effects in various cell types[128,129]. Similar to its murine ortholog, human *Gilz* is ubiquitously

upregulated by GCs in both lymphoid and non-lymphoid tissues, including the brain[130-132]. Once translated, GILZ proteins then go on to interact with several transcription factors and cellular signaling pathways mentioned above (*i.e*. AP-1, NF-κB, MAPK/ERK), suggesting that its role in the cell may extend beyond inflammation[133-135]. To establish an understanding of how GILZ may mediate interactions between stress and drug use, it is first necessary to outline the mechanisms regulating *Gilz* transcription as well as its downstream molecular effects.

The Gilz gene and its protein isoforms

Gilz is an X-linked gene with five exons that can produce three different transcription splice variants (see **Fig. 1.1**)[136]. Numerous glucocorticoid response elements (GREs) present in each of *Gilz*'s two promoter regions are responsible for its role as a primary GR-target gene[137]. Consistent with this model of GC-dependent transcription, stress-induced upregulation of *Gilz* mRNA in both mPFC and hippocampus is abolished by adrenalectomy in mice[132]. In addition to the role of GRs, transcription of *Gilz* is positively associated with H3Ac and H3K4me3 levels and negatively associated with H3K9me3 levels in *Gilz* promoter regions[138]. Furthermore, the upstream promoter harbors a CREB-response element that is sensitive to estrogen-dependent transcriptional regulation[139]. These findings suggest that stress-associated signaling may work in concert with other signaling mechanisms to regulate cellular levels of GILZ protein.

The canonical protein isoform (GILZ-1) exhibits high homology between its murine and human form (137 and 134 aa, respectively)[129]. GILZ-3 is retained as a long non-coding RNA, and the remaining two protein isoforms (GILZ-2 and GILZ-5) differ only in their N-terminal domains. All protein isoforms share a TGF-β stimulated clone (TSC) box

domain, a leucine zipper (LZ) domain, and a proline/glutamic-acid rich (PER) domain at their C-terminals[140]. These functional domains are essential for direct protein-protein binding interactions and thus are largely responsible for determining the molecular actions of GILZ within cells.

The TSC box domain shared by all isoforms is important for binding Ras, a key protein in the MAPK signal transduction pathway underlying cell growth[135]. Notably, the N-terminal unique to GILZ-1 has been shown to interact with Raf-1, another member of the MAPK pathway[141]. Consistent with these findings, mouse KO models have demonstrated that *Gilz* deficiency results in male sterility via hyperactivation of Ras signaling[142].

Several studies have investigated the functional role of the LZ domain. LZ motifs are constituted by leucine residues at every seventh amino acid in a short α-helix, resulting in formation of stable coiled-coil quaternary structures[143]. This motif allows for homo- and heterodimerization and is shared with numerous transcription factors implicated in cocaine-induced plasticity (*e.g*. Fos, Jun, CREB)[144]. However, it should be noted that although the LZ domain is necessary for GILZ homodimerization, there have been no studies showing LZ-dependent heterodimerization or direct DNA binding, likely due to the absence of a neighboring basic-rich region[145,146]. Interestingly, *in-vitro* binding assays demonstrated the N-terminal domain, and not the LZ domain, is necessary for GILZ binding c-Jun and c-Fos, components of the AP-1 transcription factor[133]. Thus, while GILZ shares some characteristics typical of transcription factors, its precise role in regulating transcription remains unclear.

The PER domain near the C-terminus of all isoforms has been widely studied with respect to GILZ:NF-κB interaction. Upon homodimerization, GILZ binds the p65 subunit of NF-κB and prevents nuclear translocation[134]. This GILZ-dependent inhibitory activity prevents transcription of NF-κB target genes[145].

These findings collectively demonstrate that (1) *Gilz* expression is influenced by epigenetic modifications at the promoter region, (2) endocrine signaling (*i.e*. GCs, estrogen) plays a critical role in *Gilz* transcription, and (3) GILZ protein isoforms interact with a number of effector proteins implicated in cocaine-induced plasticity.

The role of Gilz in plasticity and drug abuse

To date, most studies of *Gilz* have focused on its anti-inflammatory properties, and only limited studies have investigated its role in plasticity[129]. A recent publication from our lab identified *Gilz* as an HDAC3-regulated gene whose transcriptional activation is associated with long-term memory formation[2]. *Gilz* was among a small set of activity-dependent genes for which focal deletion of HDAC3 in the hippocampus rescued age-related dysregulation. Notably, this manipulation also rescued performance on an object location memory (OLM) task, suggesting hippocampal *Gilz* may contribute to cellular mechanisms underlying memory consolidation.

Similarly, the effects of drug exposure on *Gilz* expression have not been widely examined. One study demonstrates *Gilz* expression is reduced in the VTA of alcohol-preferring rats following binge-like alcohol drinking[151]. In the mouse striatum, however, *Gilz* mRNA is upregulated in response to acute ethanol as well as morphine and heroin[152]. Furthermore, *Gilz* is also induced in the striatum following morphine-CPP training sessions, but not after a CPP testing session. Immunohistochemical staining shows that

morphine-induced transcription of *Gilz* is localized to neuronal cells, and shRNA-mediated knockdown of GILZ in cultured neurons resulted in changes to dendritic spine shape. Results from this study indicate that GILZ may be recruited in neurons to induce structural changes during consolidation of a drug-associated memory. It should be noted, however, that opioids and cocaine may induce distinct cellular adaptations to achieve similar behavioral outcomes. Recent RNA-sequencing data from our lab shows paradigm-specific changes in VTA *Gilz* expression following cocaine exposure[105]. However, there are currently no studies investigating a causal role of *Gilz* in regulating cellular adaptations underlying cocaine-induced plasticity. **The experiments described here provide the first evidence for splice variant-specific roles of *Gilz* in synaptic plasticity and cocaine-associated behaviors.**

Chapter 1: *Gilz* expression in the brain.

Rationale:

Transcription of activity-dependent genes is critical for orchestrating cellular adaptations underlying long-term changes in behavior. A prior study from our lab identified *Gilz* as a possible HDAC3-regulated target gene involved in the consolidation of long-term memory formation[2]. RNAseq analysis from Kwapis *et al.* (2018) identified *Gilz* as a novel target on a short list of genes with well-established functions governing both synaptic plasticity and behavioral (*i.e.*, *Egr-1*, *Nr4a1*, *Per1*). These four genes were upregulated in the hippocampus of young adult mice during memory consolidation, failed to be expressed in the aging hippocampus, and the age-related failure to express was ameliorated by HDAC3 focal deletion in aging mice[2]. Our findings in the Kwapis *et al.* (2018) study suggested that *Gilz* may have a role in learning and memory related functions. While the expression, distribution, and functions of *Gilz* have been investigated in peripheral tissues within the body, there is a paucity of knowledge focused on *Gilz* in the brain[163].

The limited studies that do exist point towards *Gilz* regulation by glucocorticoid signaling and stress-induced expression. For instance, Cari *et al*. (2015) outline a strategy for using RT-qPCR to quantify *Gilz* in various tissues (including the brain) following administration of dexamethasone, a synthetic glucocorticoid known to induce *Gilz* transcription. With regard to stress, Yachi *et al.* (2007) demonstrate restraint stress-induced *Gilz* upregulation in the prefrontal cortex and hippocampus of mice[130,132]. Furthermore, *Gilz* upregulation in the striatum has been found in response to various

drugs of abuse[152]. Importantly, however, these findings make no mention of which *Gilz* splice variant(s) are being examined and reported.

Alternative splicing events are an important factor contributing to transcriptional regulation not only in the context of memory, but also in the context of long-term synaptic and behavioral adaptations to cocaine[164,165]. For this reason, we sought to examine the expression of each verifiable *Gilz* splice variant in brain tissue. The goal of these experiments was to identify which *Gilz* variants correspond to validated transcript IDs in trusted databases (*i.e.* Ensembl, MGI, NCBI) and design RT-qPCR primers to reliably distinguish between these variants when gathering information regarding *Gilz* expression patterns in the brain. It is necessary to understand which transcripts are expressed, when are they expressed, and where are they expressed in order to begin to study the function of GILZ in the brain.

Materials & Methods:

Mice:

Male and female C57BL/6J mice (Jackson Laboratories, n = 80 total), were all single-housed and within 2-3 months old during behavioral testing. All animals had *ad libitum* access to food and water unless otherwise specified. Experiments were performed during the light phase of a 12-hr light/dark cycle. All experiments were conducted in accordance with the National Institutes of Health *Guideline for Animal Care and Use* and were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

Drugs:

Cocaine-HCl was purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and dissolved in saline (0.9% NaCl). Cocaine-HCl is expressed as the weight of the salt. For cocaine- experiments, cocaine-HCl was dissolved to a final concentration at 2.0 mg/mL and administered in a volume of 10 ml/kg body weight, resulting in a final dose of 20 mg/kg. Cocaine-HCl and saline were administered intraperitoneally (i.p).

Quantitative RT-qPCR:

RT-qPCR was performed as described previously[2,149]. Two half-millimeter punches were collected from PFC, NAc, BLA, DHC, VHC, and VTA in two consecutive 250 um slices of tissue. RNA was isolated from punches using an RNeasy Minikit (QIAGEN) and cDNA was created using the Transcriptor First Strand cDNA Synthesis kit (Roche Applied Science). The following primers were used, designed using the Integrated DNA Technology PrimerQuest tool:

Variant ID	Forward primer	Reverse primer	Ensembl ID
Gilz-1	ATGGAGGTGGCGGTCTAT	GGAGGCACTGTTATCCAGTTT	ENSMUST00000055738.12
Gilz-2	CAACATAATGCGCCAGGATTC	TCTGGTCTATGTTGCGGTTG	ENSMUST00000112996.9
Gilz-5	GCGTACATCAGGTGGTTCTT	ACATTTCAGCCTCCTTATTCCC	ENSMUST00000123898.2
Hprt1	TGCTCGAGATGTCATGAAGG	CTTTTATGTCCCCCGTTGAC	ENST00000298556.8

Gilz probes were conjugated to FAM/Zen/3IABkFQ while *Hprt1* probes were conjugated to HEX/Zen/3IABkFQ to allow for multiplexing in the Roche LightCycle 480 II machine (Roche Applied Sciences). All values were normalized to *Hprt1* expression levels. For baseline expression (Figure 1.1), each group was compared relative to the lowest group average. Analyses and statistics were performed using the Roche proprietary algorithms and REST 2009 software based on the Pfaffl method (Pfaffl, 2001, 2002).

Chromatin Immunoprecipitation:

ChIP was performed as described previously[149], based on the protocol from the Millipore ChIP kit. Tissue was cross-linked with 1% formaldehyde (Sigma), lysed and sonicated, and chromatin was immunoprecipitated overnight with 5 µL of anti- HDAC3 (Millipore) or 5 µL of anti-mouse IgG (negative control, Millipore). The immunoprecipitate was collected using magnetic protein A beads (Millipore). After washing, chromatin was eluted from the beads and reverse cross-linked in the presence of proteinase K before column purification of DNA. *Gilz* promoter enrichment in ChIP samples was measured by quantitative real-time PCR using the Roche 480 LightCycler and SYBR green. Primer sequences for the promoter, designed by the Primer 3 program are F: TTCTCTGTTCCGCTCATGACGT, and R: CTTCTCAGTTGCTAGCTGCAATCG. Five µL of input, anti-HDAC3 IgG, or anti- mouse IgG immunoprecipitate from 4 separate mice in each condition were examined in duplicate. To normalize ChIP-qPCR data, we used the percent input method. The input sample was adjusted to 100% and both the IP and IgG samples were calculated as a percent of this input using the formula: 100*AE^(adjusted input – Ct (IP)). An in-plate standard curve determined amplification efficiency (AE).

Cocaine-conditioned place preference:

A modified version of our unbiased conditioned place preference (CPP) was performed as described in previous studies[123]. Briefly, all mice were handled for 2 minutes for 3 consecutive days prior to the experiment (days 1-3). Baseline preferences for three compartments in the CPP apparatus were assessed by placing the animals in the center compartment of the apparatus with free access to three distinct compartments for 15 min (day 4). Time spent in each compartment was recorded. Following this pretest, mice in experiment 2 (Fig. 1.2) received a single conditioning session (30-min) with paired with an i.p. injection of either cocaine-HCl (10 mg/kg) or 0.9% saline. Mice in experiment 3 (Fig. 1.3) were conditioned over two or four consecutive days, receiving either cocaine-HCl (10 mg/kg, IP; Sigma) or 0.9% saline. 24 hours following the last conditioning session, post-conditioning preference was tested in animals while they were in a drug-free state. Animals were allowed to freely explore all compartments of the CPP apparatus to assess preference, established as the difference between time spent in the cocaine-paired chamber and the saline-paired chamber, in seconds. Time spent was tracked automatically from MPEG videos using EthoVision 3.1 software (Noldus Technology).

Statistical analysis:

Graphpad Prism 9 was used. All data are expressed as mean ± SEM. For baseline RT-qPCR, two-way analyses of variance were run, followed by Bonferroni's multiple comparisons to compare groups to the brain region with lowest relative abundance. For RT-qPCR following CPP, data were normalized to *Hprt* then to saline-exposed controls for each brain region and each sex before running two-way ANOVAs followed by Bonferroni's multiple comparisons. CPP behavioral data were analyzed with two-way

ANOVAs followed by Dunnett's multiple comparisons to compare each group to saline-treated controls. One-way ANOVAs were run in ChIP pulse-chase experiments to compare different time points to untreated controls. Significance was set at $p < 0.05$ for all tests.

Results:

***Gilz* splice variants are highly expressed in reward regions.**

We first examined comparative expression of *Gilz* mRNA splice variants by extending our scope to include not only the dorsal hippocampus, where we first detected *Gilz*, but also reward-related brain regions, where we have previously demonstrated overlapping functions for hippocampal and accumbal plasticity-related genes[2,123-126]. Tissue was harvested from the prefrontal cortex (PFC), nucleus accumbens (NAc), basolateral amygdala (BLA), dorsal hippocampus (DHC), ventral hippocampus (VHC), and ventral tegmental area (VTA) of adult male and female mice to be examined via RT-qPCR. For these studies we chose to focus on previously validated consensus sequences when assigning nomenclature for *Gilz* splice variants. Specifically, "*Gilz-1*" denotes the splice variant encoding the most widely examined 137-aa GILZ isoform, "*Gilz-2*" denotes the variant encoding a 201-aa isoform, and we chose "*Gilz-5*" to denote the variant encoding the 113-aa isoform due to a lack of consensus in the literature on the identity of "*Gilz-4*"[140]. We were unable to identify the splice variant denoted as *Gilz-4* in one study, which does not align to the mouse genome[136]. *Gilz-3* may be a long non-coding RNA, which we also did not attempt to pursue at this time.

Our splice variant expression analyses thus focused on *Gilz*-1, -2, and -5. RT-qPCR data were normalized to the housekeeping gene *Hprt1* and are expressed relative to the lowest group average for each splice variant. Primer assays designed to target unique sequences within Gilz-1, -2, and -5 protein-coding regions were validated by TA cloning (Fig. 1.1A). We found basal *Gilz-1* expression levels were lowest in the PFC and were significantly higher in the NAc (Fig. 1.1B $t_{24} = 3.13$, $p = 0.022$) and the VTA (Fig. 1.1B $t_{24} = 14.0$, $p < 0.0001$). *Gilz-2* expression levels were also relatively low in the PFC and were significantly higher in the DHC (Fig. 1.1C $t_{22} = 3.10$, $p = 0.025$), the VHC (Fig. 1.1C $t_{22} = 7.24$, $p < 0.0001$) and the VTA (Fig. 1.1C $t_{22} = 5.98$, $p < 0.0001$). *Gilz-5* expression was relatively lower in the PFC than nearly all other regions (Fig. 1.1D NAc: $t_{24} = 3.90$, $p = 0.003$, BLA: $t_{24} = 3.11$, $p = 0.024$. DHC: $t_{24} = 2.39$, $p = 0.033$, VTA: $t_{24} = 14.75$, $p < 0.0001$). Despite its position on the X chromosome, no sex differences were observed for any *Gilz* splice variant in all brain regions. These findings suggest *Gilz* functions not only in the dorsal hippocampus, as previously observed, but also in reward-related brain regions.

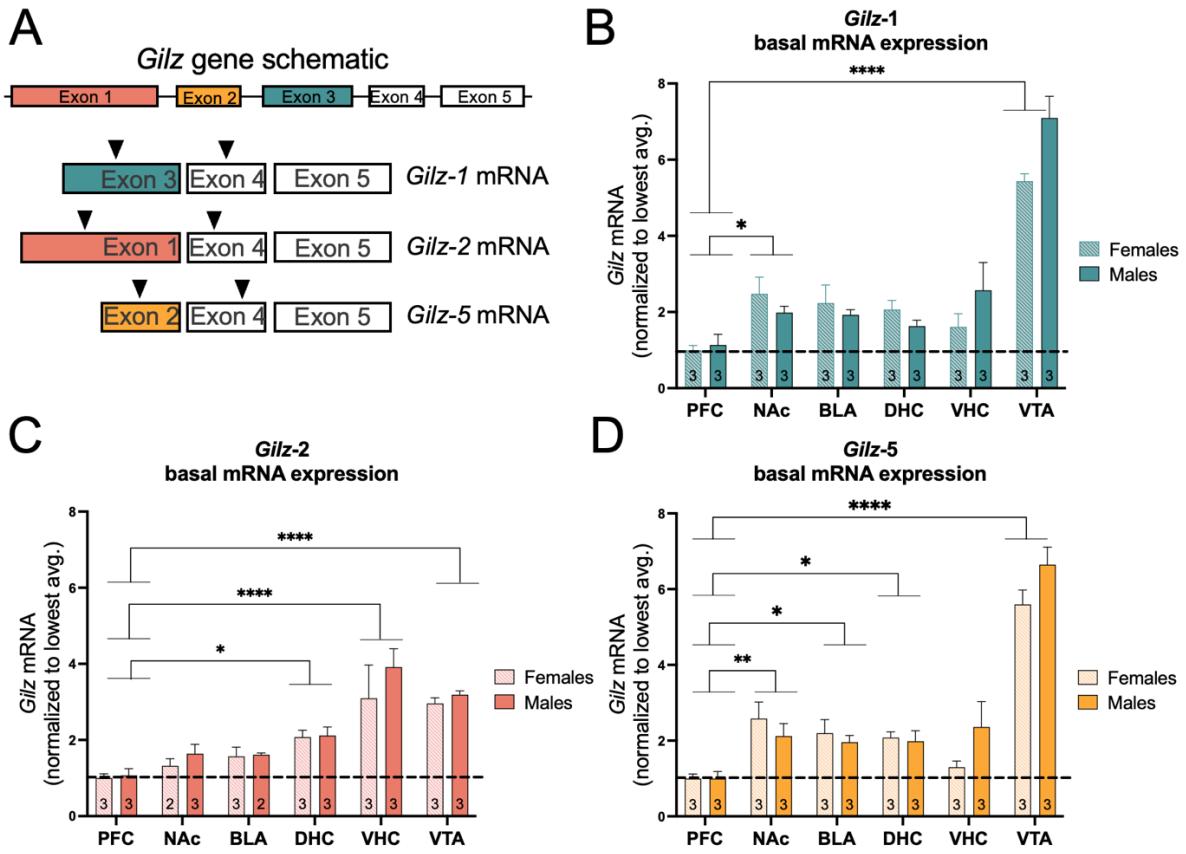

Figure 1.1: Validating sequence-specific detection, and mapping *Gilz* in males and females. (**A**) Schematic showing exon splicing patterns of *Gilz*-1, *Gilz*-2, and *Gilz*-5 mRNA. Exons 1-3 represent unique regions used to design sequence-specific RT-qPCR primer assays. Primer locations denoted by arrows. (**B**) *Gilz-1* mRNA expression under home cage conditions in males and females. 2-way ANOVA: main effect of brain region: $F_{5,24}$ = 49.43, p < 0.0001, no main effect of sex: $F_{1,24}$ = 1.386, p = 0.2506. Bonferroni's multiple comparisons: PFC vs NAc t_{24} = 3.13, p = 0.022, PFC vs VTA t_{24} = 14.0, p < 0.0001. (**C**) *Gilz-2* mRNA expression under home cage conditions in males and females. 2-way ANOVA: main effect of brain region: $F_{5,22}$ = 15.58, p < 0.0001, no main effect of sex: $F_{1,22}$ = 1.524, p = 0.2300. Bonferroni's multiple comparisons: PFC vs DHC t_{22} = 3.10, p = 0.025, PFC vs VHC t_{22} = 7.24, p < 0.0001, PFC vs VTA t_{22} = 5.98, p < 0.0001. (**D**) *Gilz-5* mRNA expression under home cage conditions in males and females. 2-way ANOVA: main effect of brain region: $F_{5,24}$ = 53.79, p < 0.0001, no main effect of sex: $F_{1,24}$ = 1.192, p = 0.2857. Bonferroni's multiple comparisons: PFC vs NAc t_{24} = 3.90, p = 0.003, PFC vs BLA: t_{24} = 3.11, p = 0.024. PFC vs DHC: t_{24} = 2.39, p = 0.033, PFC vs VTA: t_{24} = 14.75, p < 0.0001. All Cps were normalized to Hprt then normalized to the lowest group average (dashed lines) for relative quantification. Data are represented as mean + SEM. *p < 0.05, **p < 0.01, *** p <0.001.

Gilz splice variant expression in the NAc or VTA is not altered during consolidation of cocaine-associated memory.

We next examined whether *Gilz* splice variants are differentially expressed during consolidation of cocaine-associated memory formation. RT-qPCR was performed on NAc and VTA tissue harvested from both males and females 1-hr after a conditioning session in a cocaine-CPP apparatus. This time period corresponds to induction of activity-dependent genes necessary for memory consolidation and represents a reward-associated analog to our initial findings identifying hippocampal *Gilz* upregulation during consolidation of object location memory[2-123]. Surprisingly, we observed no cocaine-induced effects on *Gilz* splice variant expression in the NAc or VTA of either sex during consolidation (Fig. 1.2B-G, Males: *Gilz*-1 $F_{1,16} = 0.6563$, $p = 0.4298$; *Gilz*-2 $F_{1,17} = 0.1053$, $p = 0.7495$; *Gilz*-5 $F_{1,18} = 0.1546$, $p = 0.6988$. Females: *Gilz*-1 $F_{1,19} = 1.024$, $p = 0.3242$; *Gilz*-2 $F_{1,20} = 0.5592$, $p = 0.4633$; *Gilz*-5 $F_{1,19} = 0.1619$, $p = 0.6919$). This suggests that while *Gilz* expression is readily observable in these regions, further increases in expression do not appear to be occurring during the consolidation phase of cocaine-associated memory formation as measured using cocaine-induced conditioned place preference.

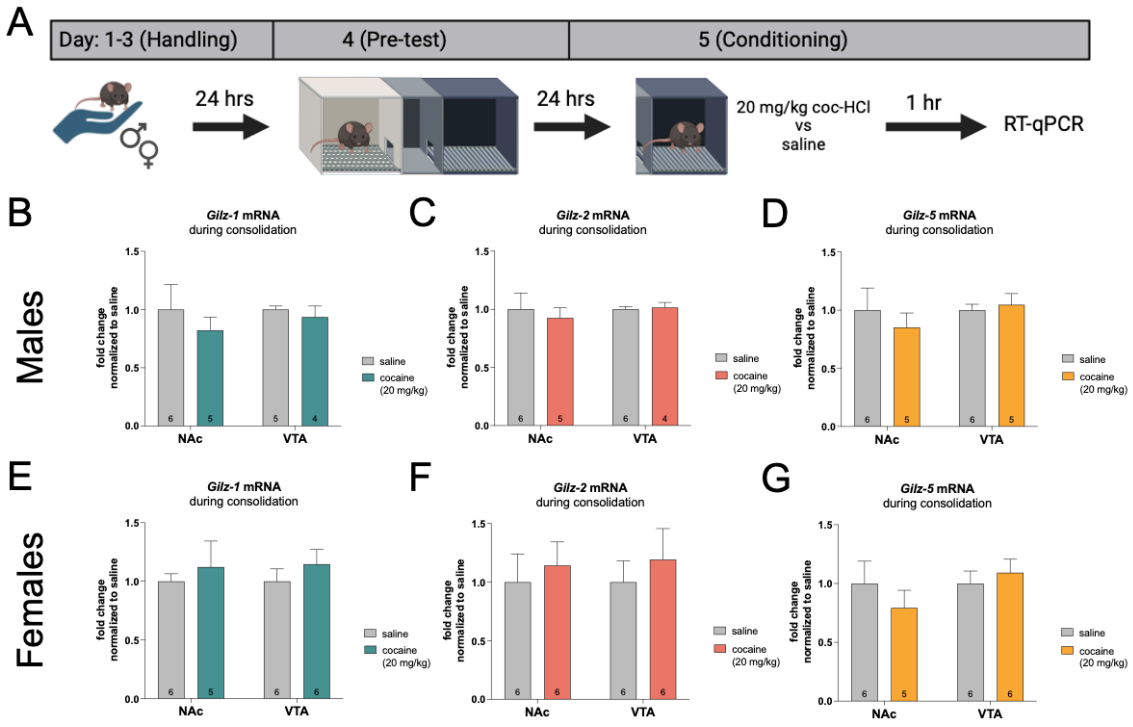

Figure 1.1: *Gilz* expression is not affected during consolidation of reward-associated memory in males or females. (**A**) Male and female mice were given a CPP pre-test followed by a single 30-min session of conditioning with cocaine (20 mg/kg) or saline, i.p. then sacrificed one hour later for RT-qPCR. (**B**) Male *Gilz-1* mRNA following a single conditioning session. 2-way ANOVA: no effect of cocaine $F_{1,16}$ = 0.6563, p = 0.4298. (**C**) Male *Gilz-2* mRNA following a single conditioning session. 2-way ANOVA: no effect of cocaine $F_{1,17}$ = 0.1053, p = 0.7495. (**D**) Male *Gilz-5* mRNA following a single conditioning session. 2-way ANOVA: no effect of cocaine $F_{1,18}$ = 0.1546, p = 0.6988. (**E**) Female *Gilz-1* mRNA following a single conditioning session. 2-way ANOVA: no effect of cocaine $F_{1,19}$ = 1.024, p = 0.3242. (**F**) Female *Gilz-2* mRNA following a single conditioning session. 2-way ANOVA: no effect of cocaine $F_{1,20}$ = 0.5592, p = 0.4633. (**G**) Female *Gilz-5* mRNA following a single conditioning session. 2-way ANOVA: no effect of cocaine $F_{1,19}$ = 0.1619, p = 0.6919. Data were normalized to saline controls and are represented as mean + SEM.

Gilz splice variant expression in the NAc or VTA is not altered during retrieval/reconsolidation of cocaine-associated memory.

We next sought to examine whether *Gilz* is differentially regulated during retrieval of cocaine-associated memory, as the transcriptional mechanisms engaged during this process are somewhat distinct from those engaged during consolidation[67]. Here, male and female mice underwent a modified version of our lab's CPP protocol (Fig 1.3A), where one group was only conditioned with saline and the other two groups received 1 or 2 pairings of cocaine (20 mg/kg, i.p.), respectively. Post-conditioning tests were

administered to engage retrieval of the previously consolidated associative memories. We observed CPP acquisition in both sexes, such that animals that received 2 pairings of cocaine exhibited more robust CPP than those which received only one pairing. (Fig 1.3B, Males: pairing x conditioning interaction $F_{2,30}$ = 8.377, p = 0.0013. Dunnett's post-hoc: "saline vs. 1-pairing" q_{30} = 3.05, p = 0.009, and "saline vs. 2-pairing" q_{30}=4.881, p < 0.0001. Fig 1.3G, Females: pairing x conditioning interaction $F_{2,26}$ = 10.23, p = 0.0005. Dunnett's post-hoc "saline vs. 1-pairing" q_{52} = 2.284, p = 0.0485 and "saline vs. 2-pairing" q_{52} = 4.769, p < 0.0001). However, these behavioral findings were not associated with significant changes in *Gilz* splice variant post-test expression in the NAc or VTA of either sex (Fig. 1.3C-E, G-I).

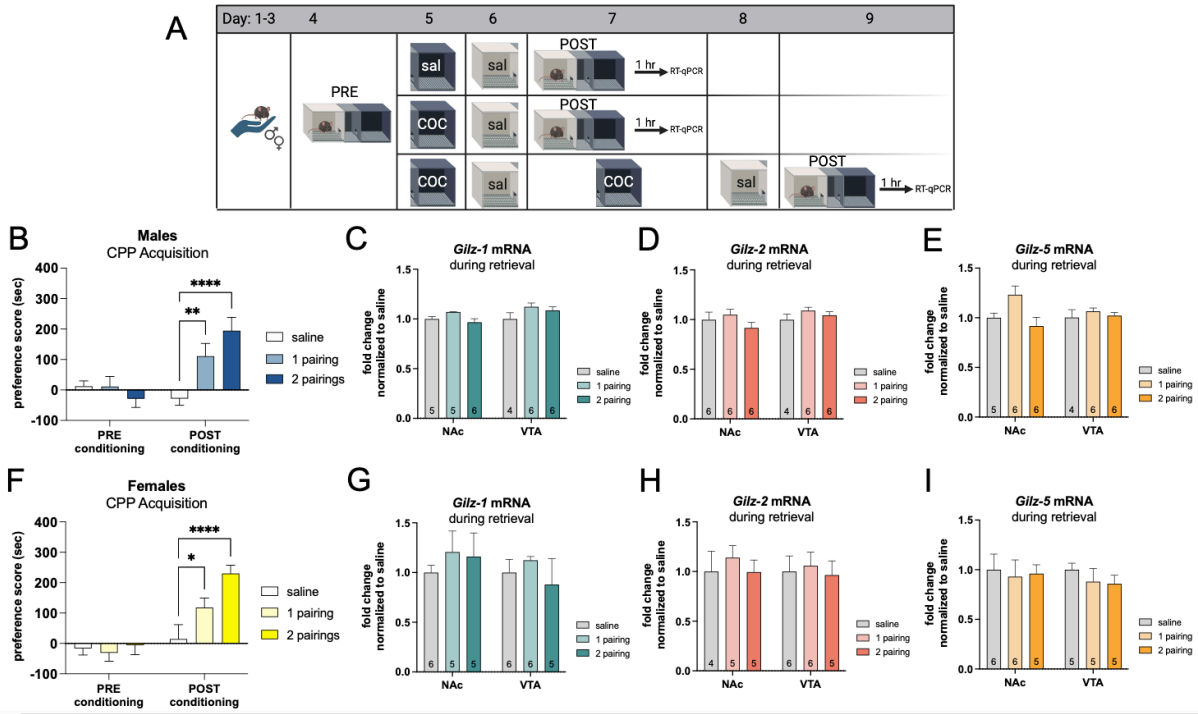

Figure 1.3: *Gilz* expression is not affected during retrieval while expressing CPP. (**A**) Male and female mice were given a CPP pre-test followed by a modified conditioning protocol with either saline only, or alterations of cocaine (20 mg/kg) and saline, i.p then sacrificed one hour later for RT-qPCR. (**B**) Male CPP behavior. 2-way ANOVA: pairing x conditioning interaction $F_{2,30}$ = 8.377, p = 0.0013. Dunnett's post-hoc: "saline vs. 1-pairing" q_{30} = 3.05, p = 0.009, and "saline vs. 2-pairing" q_{30}=4.881, p < 0.0001. (**C**) Male *Gilz-1* mRNA following CPP. 2-way ANOVA: no effect of cocaine $F_{2,27}$ = 0.6563, p = 0.4298. (**D**) Male *Gilz-2*

mRNA following CPP. 2-way ANOVA: no effect of cocaine $F_{2,28} = 1.561$, $p = 0.2277$. (**E**) Male *Gilz-5* mRNA following CPP. 2-way ANOVA: no effect of cocaine $F_{2,27} = 0.7992$, $p = 0.4301$. (**F**) Female CPP behavior pairing x conditioning interaction $F_{2,26} = 10.23$, $p = 0.0005$. Dunnett's post-hoc "saline vs. 1-pairing" $q_{52} = 2.284$, $p = 0.0485$ and "saline vs. 2-pairing" $q_{52} = 4.769$, $p < 0.0001$. (**G**) Female *Gilz-1* mRNA following CPP. 2-way ANOVA: no effect of cocaine $F_{2,27} = 0.5890$, $p = 0.5619$. (**H**) Female *Gilz-2* mRNA following CPP. 2-way ANOVA: no effect of cocaine $F_{2,25} = 0.3880$, $p = 0.6824$. (**I**) Female *Gilz-5* mRNA following CPP. 2-way ANOVA: no effect of cocaine $F_{2,26} = 0.3473$, $p = 0.7099$. RT-qPCR data were normalized to saline controls and are represented as mean + SEM. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

HDAC3 occupies the *Gilz* promoter region, and its removal is associated with splice variant-specific *Gilz* induction.

As mentioned above, the Kwapis *et al*. (2018) study suggested that *Gilz* may be regulated by HDAC3. Thus, ChIP-qPCR was used to assess HDAC3 occupancy at the promoter region of *Gilz* in various tissues. We observed significantly higher HDAC3 occupancy relative to IgG control samples in HT22 cells (Fig. 1.4A, $t_8 = 2.67$, $p = 0.029$) as well as DHC and NAc tissue from adult males (Fig. 1.4B-C, DHC: $t_8 = 2.35$, $p = 0.047$. NAc: $t_8 = 2.68$, $p = 0.028$). Furthermore, when HT22 cells were treated with KCl to simulate an activation event, HDAC3 occupancy was reduced over time (Fig. 1.4D, 30-min vs none: $q_8 = 6.48$, $p = 0.0005$. 60-min vs none: $q_8 = 5.52$, $p = 0.0015$). Finally, this reduced occupancy was associated with induction of *Gilz*-1 (Fig. 1.4E, 90-min vs none: $q_{13} = 12.64$, $p < 0.0001$) and reduction of *Gilz*-5 (Fig. 1.4F, 360-min vs none: $q_{13} = 5.68$, $p = 0.019$). These results suggest that not only is HDAC3 a possible regulator of *Gilz* transcription, but that its role may differentially contribute to the transcription of specific splice variants.

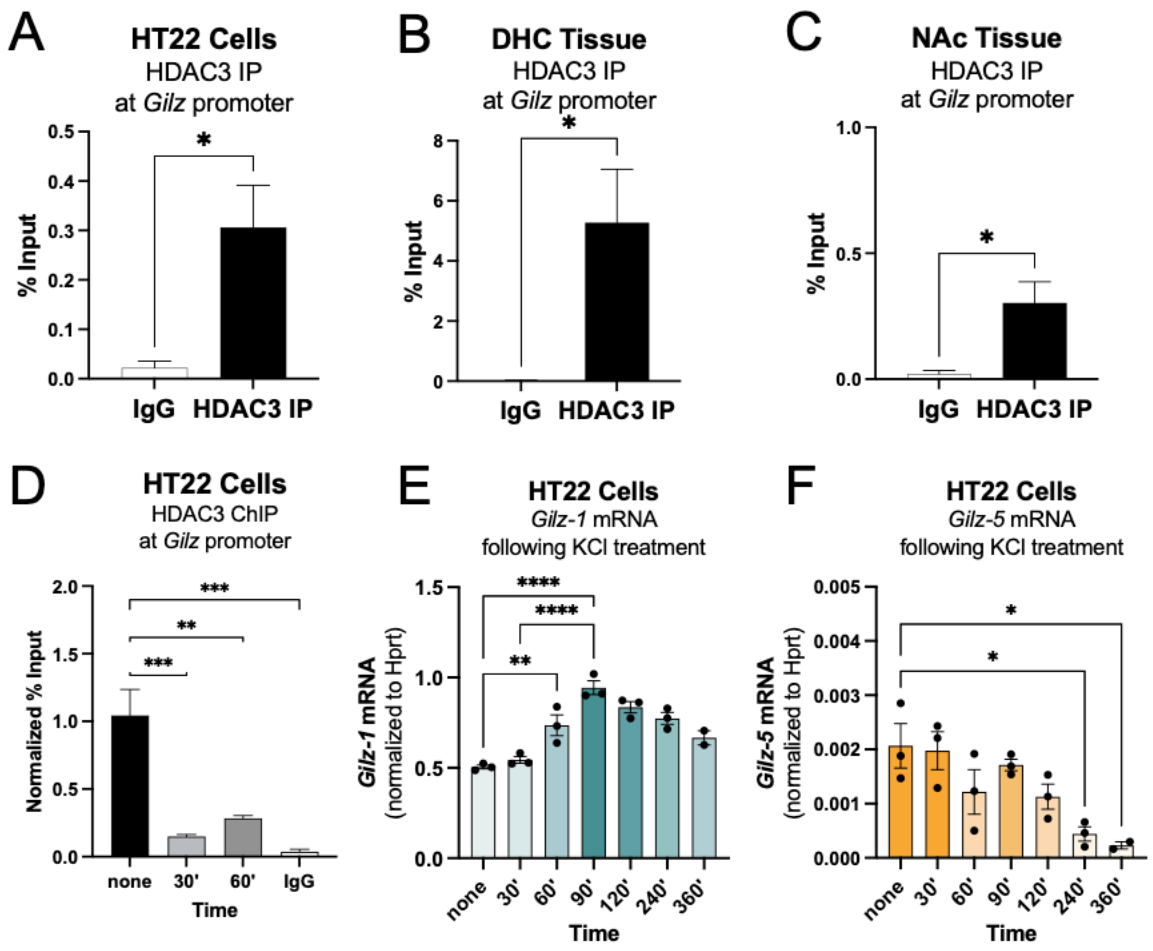

Figure 1.4: HDAC occupies the *Gilz* promoter *in vitro* and in brain tissue, and stimulation with KCl leads to HDAC3 dissociation and variant-specific *Gilz* induction. (**A**) HDAC3 chromatin immunoprecipitation at the *Gilz* promoter region in HT22 cells. HDAC3 IP is greater than IgG, $t_8 = 2.67$, $p = 0.029$. (**B**) HDAC3 chromatin immunoprecipitation at the *Gilz* promoter region in DHC tissue. HDAC3 IP is greater than IgG, $t_8 = 2.35$, $p = 0.047$. (**C**) HDAC3 chromatin immunoprecipitation at the *Gilz* promoter region in NAc tissue. HDAC3 IP is greater than IgG, $t_8 = 2.68$, $p = 0.028$). (**D**) KCl treatment leads to reduced HDAC3 occupancy at the *Gilz* promoter in HT22 cells. 30-min vs none: $q_8 = 6.48$, $p = 0.0005$. 60-min vs none: $q_8 = 5.52$, $p = 0.0015$. (**E**) *Gilz*-1 mRNA is upregulated in HT22 cells following KCl treatment. Dunnett's multiple comparisons, 90-min vs none: $q_{13} = 12.64$, $p < 0.0001$. (**F**) *Gilz*-5 mRNA is downregulated in HT22 cells following KCl treatment. Dunnett's multiple comparisons, 360-min vs none: $q_{13} = 5.68$, $p = 0.019$). Data are represented as mean + SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.00,1$****$p < 0.0001$.

Discussion:

This study provides evidence of the feasibility of detecting unique *Gilz* splice variants in brain tissue. In addition, we find that *Gilz* splice variant expression is not significantly altered in the NAc or VTA of either sex during consolidation or retrieval of

reward-associated memory using a CPP paradigm. However, our chromatin-immunoprecipitation data confirm that HDAC3 is a likely regulator of *Gilz* expression *in vivo*. Together, these findings suggest that while *Gilz* in the hippocampus may be an HDAC3-target gene implicated in memory consolidation, its role in the brain reward system may serve a unique function[2].

The method of variant-specific *Gilz* detection outlined here provides a strategy for assessing the possible unique roles of alternately spliced *Gilz* variants in the brain that has remained previously unreported. While *Gilz* splice variants have been assessed in peripheral tissue, the limited set of studies probing *Gilz* function in the brain have not been variant-specific[132,136,152]. However, numerous studies have demonstrated the value of considering splice variants with unique functional domains when assessing gene expression[170]. Here, TA cloning results validate our ability to reliably amplify unique sequences within *Gilz* splice variants. We observed no sex differences in the expression of *Gilz*-1, *Gilz*-2, or *Gilz*-5 across various brain regions implicated in memory and reward. While *Gilz* is an X-linked gene, it has been shown to be subject to normal mosaic patterns of X chromosome-inactivation (XCI) in peripheral tissue[170]. Thus, XCI is a likely mechanism contributing to similar patterns of *Gilz* expression between males and females in the brain regions we assessed.

We also observed consistent patterns of relatively high *Gilz*-1, *Gilz*-2, and *Gilz*-5 expression in the VTA under home cage conditions. This observation expanded upon our previously reported findings to suggest that *Gilz* may function not only to regulate hippocampal plasticity, but also to regulate plasticity associated with reward, as the VTA (and its projections to the NAc) is highly implicated in drug-induced neuroadaptations. We

thus employed a set of previously validated conditioned place preference (CPP) protocols to examine whether *Gilz* splice variant expression was affected in the NAc and VTA in response to cocaine-associated memory processes.

We observed no significant changes in *Gilz* splice variant expression in the NAc or VTA of either sex during consolidation of a cocaine-associated memory. Furthermore, when males and females were subjected to either 1 or 2 pairings of cocaine during CPP conditioning, we observed escalations in their degree of preference during CPP post-tests, but these behavioral effects were not associated with significant changes in *Gilz* expression during retrieval of cocaine-associated memory. Thus, a possible role for *Gilz* in regulating reward-associated plasticity may not involve activity-dependent induction of new *Gilz* transcripts, but rather activity-dependent alterations in the functions of existing *Gilz* transcripts within the cell. For instance, GILZ protein isoforms may differentially engage with their known binding partners (*i.e.*, NF-kB, AP-1, Ras) to regulate transcription in the context of cocaine exposure. This would represent a divergence from the anticipated role of hippocampal *Gilz*, and we thus sought to examine whether *Gilz* is indeed an HDAC3 target gene both within and outside the hippocampus.

Our chromatin-immunoprecipitation findings from HT22 cells demonstrate HDAC3 occupancy at the *Gilz* promoter region. We also observed HDAC3 occupancy at the *Gilz* promoter in hippocampal and NAc tissue samples. Notably, this occupancy was considerably higher in the hippocampal tissue, which may indicate that HDAC3 exhibits a more robust effect on the transcriptional regulation of *Gilz* in this region compared to the NAc. Thus, while HDAC3 has been shown to regulate the expression of key plasticity-related genes in both regions, these ChIP findings may offer a partial explanation of why

Gilz appears to be associated with consolidation in the hippocampus but not the NAc[2,126]. Furthermore, results from HT22 cells support a more direct form of evidence implicating HDAC3 in regulating *Gilz* transcription, such that KCl-induced reductions in HDAC3 occupancy were associated with the consequent upregulation of *Gilz*-1 and downregulation of *Gilz*-5. This bidirectional effect not only serves to exemplify the value of considering splice variant-specificity, but also establishes *Gilz*-1 as a possible variant of interest in these studies.

Overall, these findings suggest brain-region specific roles for *Gilz*, offer a more direct validation of our initial HDAC3 target gene hypothesis, and support the need for splice variant-specific detection when studying *Gilz* in the brain. While we found no cocaine-induced changes to *Gilz* expression, it should be emphasized that expression is not equal to function. Similar studies into the function of HDAC3 itself (or other HDAC3 targets, *i.e.*, CREST) have demonstrated that cocaine does not affect mRNA or protein expression levels, but rather affects the phosphorylation state to engage stable downstream neuroadaptations[125,149]. Thus, while the effects of *Gilz* induction may be more reliably inferred following stress rather than drug exposure (see Yachi *et al.*, 2007), high *Gilz* expression in reward regions does still suggest a functional role in the context of reward.

Chapter 2: *Gilz* in NAc LTP.

Rationale:

Numerous studies from our lab and others have demonstrated the value of using long-term potentiation (LTP) studies to examine the function of genes implicated in synaptic plasticity[2,126,147]. In particular, HDAC3-regulated genes have been implicated in contributing to transcription-dependent processes engaged by LTP associated with long-term behavioral adaptations[126,166]. The glutamatergic pathway from the PFC to NAc, which is engaged to drive such behavioral changes in the context of cocaine, represents a valuable target for probing the function of *Gilz* in the reward system, as *Gilz* has been shown to be induced by stress or drug exposure in these regions respectively[132,152,167,168].

Cocaine exposure results in pronounced transcriptomic adaptations within distinct cell populations (including cell populations within the NAc and PFC)[99]. While our prior findings suggest *de novo* transcription of *Gilz* splice variants in these regions is not a consequence of cocaine-associated memory formation, it is possible cocaine drives neuroadaptations in these regions by engaging or altering the function of GILZ in the cell by perturbing the balance of its homeostatic interaction dynamics. In particular, the GILZ-1 isoform harbors functional domains that allow it to interact not only with NF-kB (as is shared with other isoforms) but also with AP-1 and Raf-1 (part of the MAPK/ERK signaling pathway)[129]. The collection of these binding partners positions the *Gilz-1* splice variant as an intriguing candidate that may orchestrate a variety of the transcriptomic neuroadaptations observed in the reward system. In the studies described below, we aimed to assess the role of *Gilz* in NAc LTP using siRNA or genetically modified mice in males and females prior to electrophysiological recordings.

Materials & Methods:

Mice:

Male and female C57BL/6J mice (Jackson Laboratories) were all single-housed and within 2-3 months old during behavioral testing. *Gilz*^{KO} males, *Gilz*^{HET} females, and *Gilz*^{WT} littermates were bred on a C57BL/6J background and maintained on the same conditions. All animals had *ad libitum* access to food and water unless otherwise specified. Experiments were performed during the light phase of a 12-hr light/dark cycle. All experiments were conducted in accordance with the National Institutes of Health *Guideline for Animal Care and Use* and were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

Quantitative RT-qPCR:

RT-qPCR was performed as described previously[2,149]. Two half-millimeter punches were collected from the NAc in two consecutive 250 um slices of tissue. RNA was isolated from punches using an RNeasy Minikit (QIAGEN) and cDNA was created using the Transcriptor First Strand cDNA Synthesis kit (Roche Applied Science). The following primers were used, designed using the Integrated DNA Technology PrimerQuest tool:

Variant ID	Forward primer	Reverse primer	Ensembl ID
Gilz-1	ATGGAGGTGGCGGTCTAT	GGAGGCACTGTTATCCAGTTT	ENSMUST00000055738.12

Gilz-2	CAACATAATGCGCC AGGATTC	TCTGGTCTATGTT GCGGTTG	ENSMUST00000112996 .9
Gilz-5	GCGTACATCAGGTG GTTCTT	ACATTTCAGCCTC CTTATTCCC	ENSMUST00000123898 .2
Hprt1	TGCTCGAGATGTCA TGAAGG	CTTTTATGTCCCC CGTTGAC	ENST00000298556.8

Gilz probes were conjugated to FAM/Zen/3IABkFQ while *Hprt1* probes were conjugated to HEX/Zen/3IABkFQ to allow for multiplexing in the Roche LightCycle 480 II machine (Roche Applied Sciences). All values were normalized to *Hprt1* expression levels prior to normalization to control group averages. Analyses and statistics were performed using the Roche proprietary algorithms and REST 2009 software based on the Pfaffl method (Pfaffl, 2001, 2002).

AAV Production:

Wild-type *Gilz*-1 was amplified from mouse accumbal cDNA and cloned into a modified pAAV-IRES-V5 plasmid, under control of the CMV promoter and β-globin intron. For the Empty Vector control, the *Gilz*-1 coding sequence was not present, but all other elements remain. Adeno-associated virus (AAV) was made by the Center for Neural Circuit Mapping (University of California Irvine) from the above described plasmids and was serotyped with AAV1. The final titer of AAV-*Gilz*-1 was 3.23×10^{12} GC/mL and the final titer of AAV-EV was 7.88×10^{13} GC/mL.

siRNA:

A SMARTpool consisting of 4 individual siRNAs was designed and purchased from Horizon Discovery. These siRNAs were targeted against unique regions in the 3'-UTR that were shared by all *Gilz* splice variants. The Accell SMARTpool formulation is reconstituted and delivered for transfection at 100uM in water solvent. A non-targeting control siRNA was also purchased from Horizon Discovery and delivered under the same conditions.

Surgery:

Mice were induced to anesthesia with 4% isoflurane in oxygen and maintained at 1.5-2% for the duration of surgery. Animals were injected with either a-*Gilz* siRNA, non-targeting siRNA, AAV-*Gilz*-1-V5 or AAV-EV-V5. 0.5 µl of solution was infused bilaterally into the NAc (AP): +1.3 mm; (ML): ±1.1 mm; (DV): −4.5 mm relative to bregma. Solutions were infused at a rate of 6 µl /hr by using a 30 gauge Neuros Hamilton syringe (product #65459-01) mounted to either a Harvard Apparatus Nanomite Syringe Pump (product #MA1 70-2217) or Leica Biosystems Nanoinjector Motorized f/Stereotaxics (product #39462901). All infusions used the Leica Microsystems Angle Two Stereotaxic System. For siRNA experiments, animals were allowed to recover for 24-48 hrs. For viral experiments, animals were allowed to recover for three weeks to ensure viral expression.

Elevated Plus Maze:

The plus-maze was conducted by an experimenter blind to the experimental groups. The maze consists of two open arms (30 × 5 cm) and two closed arms (30 × 5 × 15 cm), that are connected by a central platform (5 × 5 cm). The maze was elevated 40 cm above the floor. During the test, mice were recorded for 5 min on the apparatus, with initially placing each mouse onto the central platform facing one of the open arms.

Between subjects, the maze was cleaned with 70% ethanol. The percentage of time spent in the closed and open arms was scored using ANY-maze software.

Slice Preparation and Recording:

Parasagittal slices containing the NAc core were prepared from siRNA-infused mice, or *Gilz*-mutated mice with or without viral infusions (2-3 months of age). Following isoflurane anesthesia, mice were decapitated and the brain was quickly removed and submerged in ice-cold, oxygenated dissection medium containing the following: 124 mM NaCl, 3 mM KCl, 1.25 mM KH_2PO_4, 5 mM $MgSO_4$, 2.5 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose. Following removal of the cerebellum and lateral aspects of both hemispheres, parasagittal slices (320 mm) were cut from the blocked brain using a FHC vibrating tissue slicer (Model:OTS-5000). The tissue was then transferred to an interface recording chamber containing preheated artificial cerebrospinal fluid (aCSF) of the following composition: 124 mM NaCl, 3 mM KCl, 1.25 mM KH_2PO_4, 1.5 mM $MgSO_4$, 2.5 mM $CaCl_2$, 26 mM NaHCO3, 10 mM glucose, and 10 mM picrotoxin to reduce feedforward inhibition. Slices were continuously perfused with this solution at a rate of 1.0–1.5 $mlmin^{-1}$, while the surface of the slices were exposed to warm, humidified 95% O_2/5% CO_2 at 31 $\pm$ 1°C. Recordings began following at least 1.5 h of incubation.

Stimulation of glutamatergic afferent fibres within the NAc was achieved by placing a bipolar stainless steel stimulation electrode (25 mm in diameter, FHC) just below the anterior commissure. Activation of field (f)EPSPs were recorded using a glass pipette (2–3 MΩ) positioned caudal or caudal–ventral to the stimulation electrode. Thus, correct placement of electrodes within the NAc was confirmed by visual inspection of the slice and comparison with mouse brain atlas (Paxinos and Watson; 0.84–1.08 lateral to

midline). Two parasagittal slices/hemisphere containing a large portion of the NAc core were obtained for each animal. Pulses were administered at 0.05 Hz using a current that elicited a 30–40% maximal response. Measurements of fEPSP slope (measured at 10–90% fall of the slope) were recorded during a minimum 20- min stable baseline period at which time long-term potentiation (LTP) was induced by delivering three to five trains (intertrain interval of 1 min), each train containing three “theta” bursts, with each burst consisting of four pulses at 100 Hz and the bursts themselves separated by 200 ms (TBS). The stimulation intensity was not increased during the delivery of TBS. Data were collected and digitized by NAC 2.0 Neurodata Acquisition System (Theta Burst Corp.) and stored on a disk.

Statistical analysis:

Graphpad Prism 9 was used. All data are expressed as mean ± SEM. For RT-qPCR assessment of *Gilz* knockdown (via siRNA or genomic manipulation), data for each splice variant were normalized to *Hprt* then to control group averages before being analyzed on a splice variant-specific basis with unpaired two-tailed t-tests. Unpaired two-tailed t-tests were used to analyze mean LTP potentiation and distance traveled in open field tests. One-way ANOVA followed by Tukey’s multiple comparisons was used for mean LTP potentiation in the *Gilz*-1 viral study. Two-way repeated measures ANOVAs followed by Sidak’s or Dunnett’s multiple comparisons were used to analyze I/O curves, PPF, and habituation data. Two-way ANOVAs followed by Sidak’s multiple comparisons were used for EPM, open field tests, and RT-qPCR validation of *Gilz*-1 overexpression. Significance was set at $p < 0.05$ for all tests.

Results:

Intra-NAc siRNA infusions lead to acute reduction of *Gilz* splice variants.

As *Gilz* has been shown to interact with key binding partners (*i.e.* NF-κB, AP-1) known to regulate reward-associated transcription, we next sought to test the hypothesis that *Gilz* is necessary for synaptic plasticity in glutamatergic projections from the PFC to the NAc[72,169]. To examine the role of *Gilz* in NAc LTP, we first confirmed our siRNA approach. For this, a siRNA SMARTpool was designed to deliver a mixture of sequences targeted at the 3'-UTRs of all *Gilz* splice variants. Male and female mice received bilateral infusions of α-*Gilz* siRNA or nontargeting control siRNA into the NAc core (Fig. 2.1A). Animals were sacrificed 48 hours post-surgery and tissue samples were collected from the NAc core for RT-qPCR analysis to examine *Gilz* knockdown. Anti-*Gilz* siRNA significantly blunted *Gilz* expression in both sexes, thus demonstrating feasibility for an unbiased approach to site-specific reduction of all *Gilz* splice variants (Fig. 2.1B-C siRNA main effects: Males, $F_{1,24} = 22.7$, $p < 0.0001$; Females, $F_{1,18} = 10.9$, $p = 0.004$).

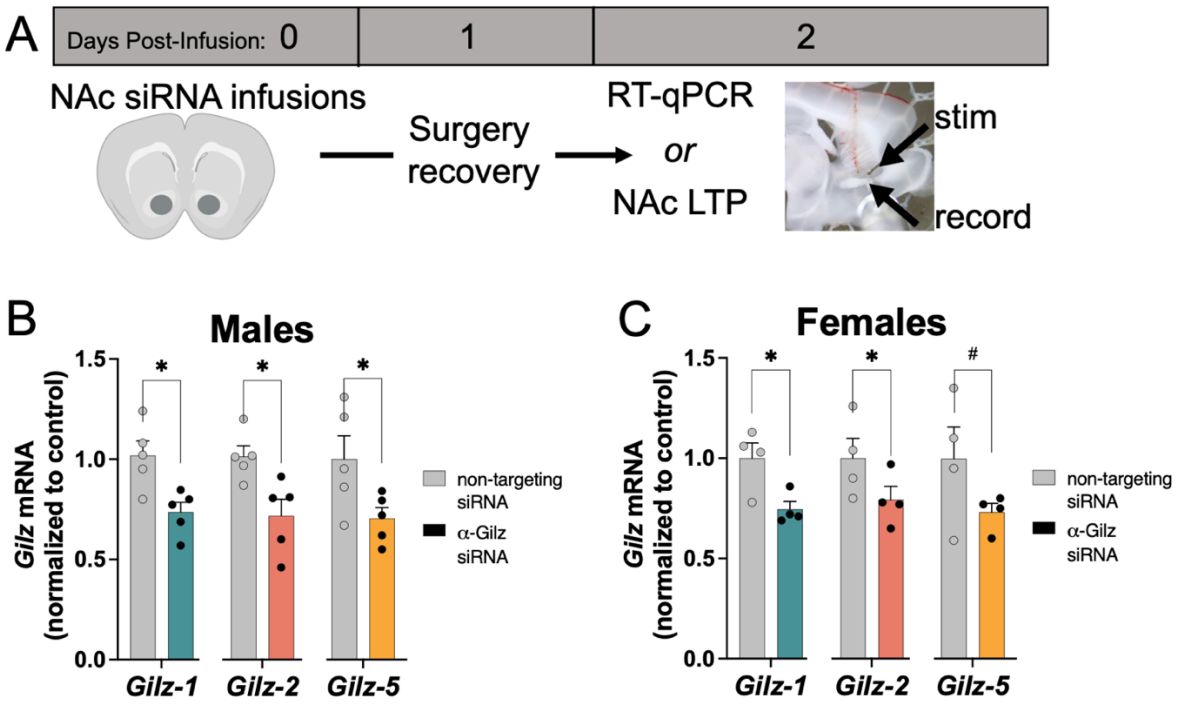

Figure 2.1: SMARTpool siRNA blunts expression of all *Gilz* splice variants in the NAc of males and females. (**A**) Male and female mice received bilateral NAc infusions of either *α-Gilz* siRNA or non-targeting control siRNA 48 hours prior to tissue collection for RT-qPCR or LTP. (**B**) Male qPCR data showing *α-Gilz* siRNA blunted expression of all splice variants ($F_{(1,24)}$ = 22.7, p < 0.0001). (**C**) Female qPCR data showing *α-Gilz* siRNA blunted expression of all splice variants ($F_{(1,18)}$ = 10.9, p = 0.0004).Data are represented as mean + SEM. $^{*}p < 0.05$ $^{\#}p < 0.1$.

Intra-NAc siRNA impairs LTP in males but not females.

A separate cohort of male and female mice received the same infusions of either *α-Gilz* siRNA or non-targeting control siRNA into the NAc 48 hours prior to euthanasia. Slices were then prepared for extracellular field potential recordings from PFC glutamatergic afferents to the NAc (Fig. 2.2). After establishing baseline fEPSP slopes, theta burst stimulation delivered to males and females that received non-targeting siRNA resulted in stable levels of potentiation consistent with other reports in our laboratory[126,149]. In males, *α-Gilz* siRNA significantly blunted potentiation during the 50-60 min post-TBS period (Fig. 2.2C, t_{12} = 6.97, p < 0.0001). In females, *α-Gilz* siRNA had no significant effect on potentiation during this same post-TBS period (Fig. 2.2D, t_{14} =

0.40, p = 0.693). These data suggest *Gilz* is necessary for maintenance of late-phase potentiation at glutamatergic synapses in the NAc core of males but not females.

The effects of α-*Gilz* siRNA on baseline transmission in the NAc were next assessed using an input/output (I/O) curve to detect changes in excitability and paired-pulse facilitation (PPF) to measure frequency facilitation. In males, both I/O and PPF were similar across groups (Fig. 2.2E,G, I/O no main effect of siRNA: $F_{1,70}$ = 0.6901, p = 0.4089; PPF no main effect of siRNA: $F_{1,4}$ = 0.1262, p = 0.7404). In females, PPF was similar across groups, however α-*Gilz* siRNA had an effect on the I/O curve, such that slices from α-*Gilz* siRNA-treated females exhibited increased membrane excitability (Fig. 2.2F,H, I/O main effect of siRNA: $F_{1,98}$ = 12.28, p = 0.0007; PPF no main effect of siRNA: $F_{1,7}$ = 0.6765, p = 0.4379; Fig. 2I, K). These collective data suggest that in males, blunting *Gilz* expression impairs transcription-dependent LTP in the NAc, while in females, the absence of siRNA-mediated LTP impairments may be due to compensatory alterations in membrane excitability.

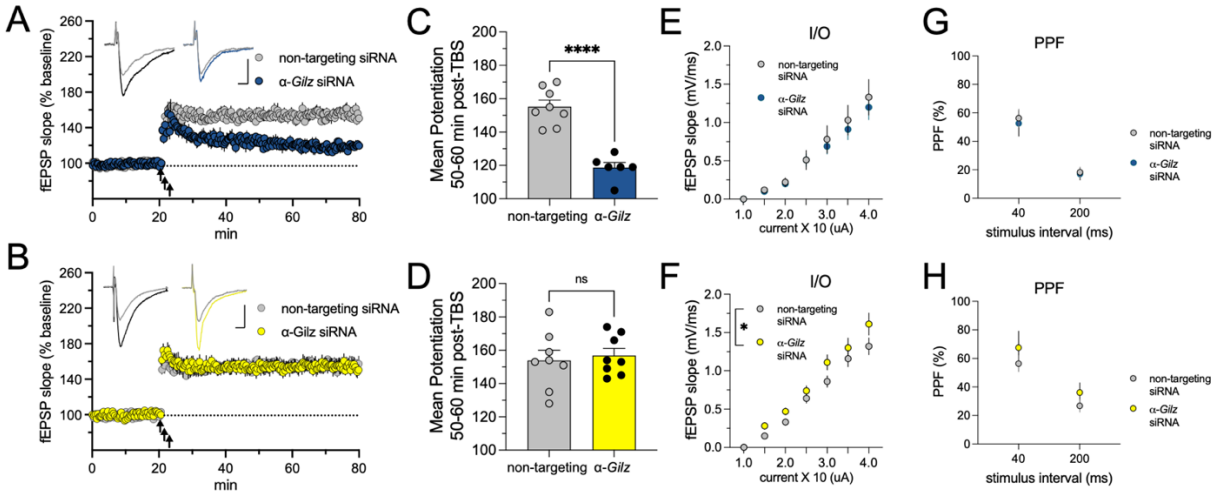

Figure 2.2: SMARTpool siRNA impairs NAc LTP in males but not females. (**A**) fEPSP slope before and after theta-burst stimulation showing effects of siRNA infusions on NAc long-term potentiation in males. (**B**)

fEPSP slope before and after theta-burst stimulation showing effects of siRNA infusions on NAc long-term potentiation in females. Insets are representative traces. Scale bars represent 5ms x 1mV. (**C**) Summary of mean potentiation during the 50-60 min post-stimulation interval demonstrates an siRNA-mediated impairment in males: $t_{12} = 6.97$, $p < 0.0001$. (**D**) Summary of mean potentiation during the 50-60 min post-stimulation interval show no effect of siRNA in females: $t_{14} = 0.40$, $p = 0.693$. (**E**) I/O curve results suggest no effect of siRNA in males $F_{1,70} = 0.6901$, $p = 0.4089$. (**F**) I/O curve results show a main effect of siRNA in females $F_{1,98} = 12.28$, $p = 0.0007$. (**G**) Paired-pulse facilitation results in males suggest no effect of siRNA $F_{1,4} = 0.1262$, $p = 0.7404$. (**H**) Paired-pulse facilitation results in females suggest no effect of siRNA $F_{1,7} = 0.6765$, $p = 0.4379$. Data are represented as mean + SEM. $^{*}p < 0.05$ $^{****}p < 0.0001$.

Exon 4 deletion from *Gilz* gene leads to blunted global expression but does not affect baseline behavioral measures.

To further investigate the role of *Gilz* in plasticity, we generated a line of mice on the C57BL/6J background lacking a 310 base pair sequence of genomic DNA surrounding exon 4 of the *Gilz* gene on the X chromosome (Fig. 2.3A). This exon encodes amino acids 42-58 of *GILZ*-1 and is shared by all protein-coding *Gilz* mRNA splice variants. As GILZ is necessary for spermatogenesis, hemizygous $Gilz^{KO}$ males are rendered sterile and cannot be crossed with heterozygous females ($Gilz^{HET}$), thus preventing us from generating homozygous $Gilz^{KO}$ females[142]. Consequently, no female-derived results conducted with this mouse line are directly compared to male-derived results, and we instead focus on intra-sex group comparisons.

To assess the effects of exon 4 deletion on expression of individual *Gilz* splice variants, we harvested NAc tissue from adult male $Gilz^{KO}$ and female $Gilz^{HET}$ mice as well as their $Gilz^{WT}$ littermates. In males, hemizygous *Gilz* deletion yields a total knockout of all mRNA splice variants (Fig. 2.3C, Main effect of genotype: $F_{(1,29)} = 393.3$, $p < 0.0001$). In females, heterozygous *Gilz* deletion yields a 50% reduction in mRNA splice variant expression (Fig. 2.3D, Main effect of genotype: $F_{(1,28)} = 59.95$, $p < 0.0001$).

We next tested separate cohorts of naïve males and females on a series of behavioral tests to examine whether deletion of *Gilz* may affect performance such as

ambulation, exploratory behaviors, anxiety-like behavior, and general learning in the form of habituation. In both males and females, a 5-minute session on an elevated plus maze yielded no significant effects of genotype on the percent of time mice spent in the open arms of the apparatus (Fig. 2.3E, Males: no main effect of genotype, $F_{(1,26)} = 0.5941$, $p = 0.4478$; open arm: $t_{26} = 2.097$, $p = 0.0896$; Fig. 2.3F, Females: no main effect of genotype, $F_{(1,30)} = 0.5904$, $p = 0.4483$; open arm: $t_{30} = 1.394$, $p = 0.9428$). Similarly, after a 10-minute session in an open field apparatus, there were no effects of genotype on time spent in the inner zone of the apparatus (Fig. 2.3G, Males inner zone: $t_{26} = 0.7179$, $p = 0.7288$; Fig. 2.3H, Females inner zone: $t_{30} = 0.2959$, $p = 0.9468$). Together, these findings suggest homozygous *Gilz* deletion in males and heterozygous *Gilz* deletion in females does not affect performance on the EPM.

In the open field test, both males and females exhibited normal levels of general ambulation. There were no effects of genotype on total distance traveled during the 10-minute session for either sex, indicating hemizygous *Gilz* deletion in males and heterozygous *Gilz* deletion in females does not affect baseline processes associated with general ambulatory behavior (Fig. 2.3I-J, Males: $t_{13} = 1.01$, $p = 0.3309$; Females: $t_{15} = 1.449$, $p = 0.1678$).

When mice were exposed to daily habituation sessions in an open chamber, there were no effects of genotype on distance traveled (Fig. 2.3K-L, Males: $F_{(1,13)} = 0.4400$, $p = 0.5187$; Females: $F_{(1,15)} = 0.4289$, $p = 0.5225$). Furthermore, we observed main effects of Test Session that were not specific to genotype for each sex (Fig. 2.3K-L, Males: $F_{(2.141,27.83)} = 10.48$, $p = 0.0003$; Females: $F_{(3.098, 43.37)} = 33.27$, $p < 0.0001$). These results indicate hemizygous or heterozygous *Gilz* deletion does not affect animals' ability to learn

from repeated exposure to a context. Collectively, these tests serve to demonstrate the viability of using this genetically-modified mouse line for further study.

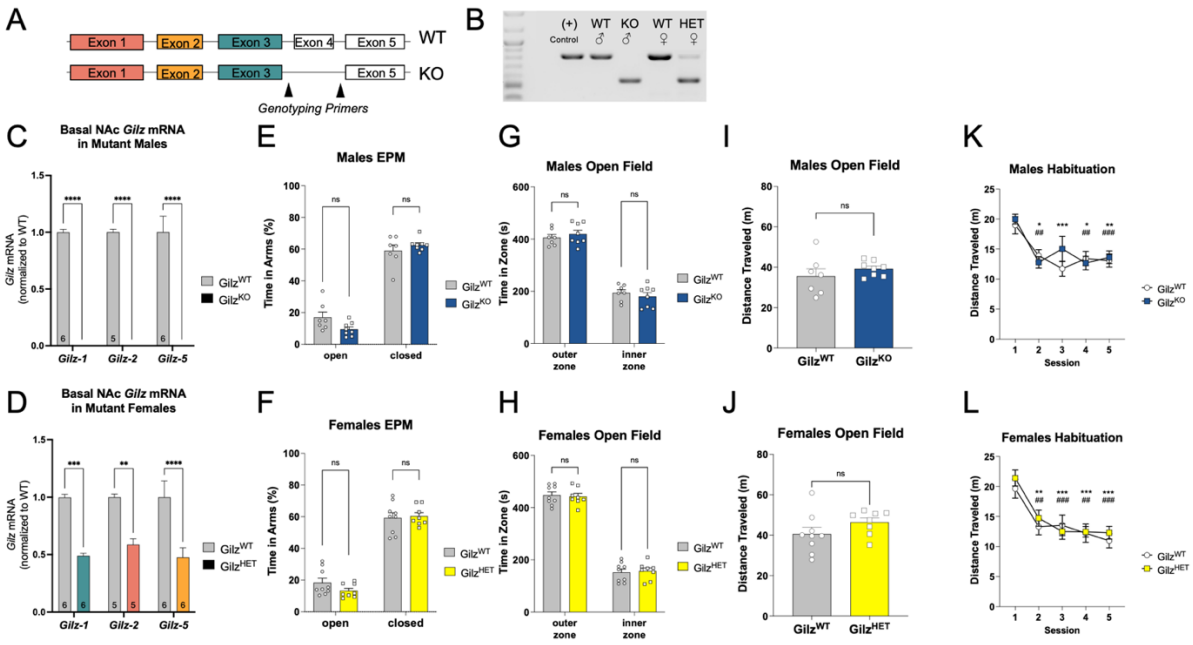

Figure 2.3: Validation of *Gilz*^{KO} males and *Gilz*^{HET} females with no phenotypic abnormalities. (**A**) Schematic showing exon 4 deletion and genotyping primers. (**B**), Representative gel comparison of 815bp WT bands and 505bp mutated-*Gilz* bands. (**C**) *Gilz*^{KO} in males leads to a complete knockout of all *Gilz* splice variants in NAc tissue, Main effect of genotype: $F_{(1,29)}$ = 393.3, p < 0.0001. (**D**) *Gilz*^{HET} in females leads to a ~50% reduction of all splice variants in NAc tissue from females. Main effect of genotype: $F_{(1,28)}$ = 59.95, p < 0.0001. RT-qPCR data were obtained using primer assays from Figure 1.1 and were normalized to *Hprt1* and to WT littermate controls. (**E,F**) *Gilz* mutation does not affect time spent in open or closed arms of an EPM apparatus for males or females (Males: no main effect of genotype, $F_{(1,26)}$ = 0.5941, p = 0.4478; open arm: t_{26} = 2.097, p = 0.0896; Females: no main effect of genotype, $F_{(1,30)}$ = 0.5904, p = 0.4483; open arm: t_{30} = 1.394, p = 0.9428). (**G,H**) *Gilz* mutation does not affect time spent in either the inner or outer zone of an open field apparatus for males or females (Males inner zone: t_{26} = 0.7179, p = 0.7288; Females inner zone: t_{30} = 0.2959, p = 0.9468). (**I,J**) *Gilz* mutation does not affect distance traveled throughout the duration of an open field test in males or females (Males: t_{13} = 1.01, p = 0.3309; Females: t_{15} = 1.449, p = 0.1678). (**K,L**) *Gilz* mutation does not affect habituation during repeated exposure to an open chamber. In both males and females, all groups exhibit a reduction in distance traveled over the course of repeated test sessions Males: $F_{(1,13)}$ = 0.4400, p = 0.5187; Females: $F_{(1,15)}$ = 0.4289, p = 0.5225. Habituation test results are compared to session 1 and are denoted with * for WT mice and with # for *Gilz*-mutated mice. Data are presented as mean + SEM.**p < 0.01, *** p <0.001, ****p < 0.0001.

NAc LTP is impaired in *Gilz*^{KO} males but not *Gilz*^{HET} females.

Compared to our modest siRNA knockdown protocol, genomic deletion of *Gilz* (in either a hemizygous or heterozygous fashion) yields a more robust ablation of all *Gilz* mRNA transcripts. We sought to examine whether brain sections taken from *Gilz*^{KO} males

or *Gilz*^{HET} females exhibited patterns of cellular responses to theta burst-induced stimulation similar to our prior findings following modest reduction via siRNA. We hypothesized that global knockout of *Gilz* in males would yield similar impairments in NAc LTP to those observed following acute, local *Gilz* knockdown via siRNA. Furthermore, we hypothesized that a more robust knockdown in *Gilz*^{HET} females would result in NAc LTP impairments that were not seen following modest siRNA-mediated knockdown.

Adult male *Gilz*^{KO} and female *Gilz*^{HET} were euthanized alongside *Gilz*^{WT} littermates and brain sections were prepared for electrophysiological recordings from the NAc. After establishing baseline fEPSP slopes, theta burst stimulation delivered to *Gilz*^{WT} animals of both sexes resulted in stable levels of potentiation consistent with other reports in our laboratory[126,149]. In females, heterozygous *Gilz*^{KO} had no significant effect on potentiation during the 50-60 min post-TBS period (Fig. 2.4A-B, $t_{14} = 1.09$, $p = 0.294$). In males, hemizygous *Gilz*^{KO} resulted in a significant impairment in potentiation during this same post-TBS period (Fig. 2.4C-D, $t_{22} = 5.315$, $p < 0.0001$).

The effects of genomic *Gilz* manipulation on baseline transmission in the NAc were assessed as above, using an input/output (I/O) curve and paired-pulse facilitation (PPF). In females, both I/O and PPF were similar across groups (Fig. 2.4E-F, I/O no main effect of genotype: $F_{(1,20)} = 0.6290$, $p = 0.4270$; PPF no main effect of genotype: $F_{(1,12)} = 0.4220$, $p = 0.5282$). Likewise in males, both I/O and PPF were similar across groups (Fig. 2.4G-H, I/O no main effect of genotype: $F_{(1,22)} = 0.9005$, $p = 0.3530$; PPF no main effect of genotype: $F_{(1,12)} = 0.6904$, $p = 0.4150$). Collectively, these data support our hypothesis that *Gilz*-reductions impair NAc LTP in males but not females, further supporting the

notion GILZ is necessary for late-phase potentiation at glutamatergic synapses in the NAc core of males while suggesting the role of GILZ in this process may not extend to females.

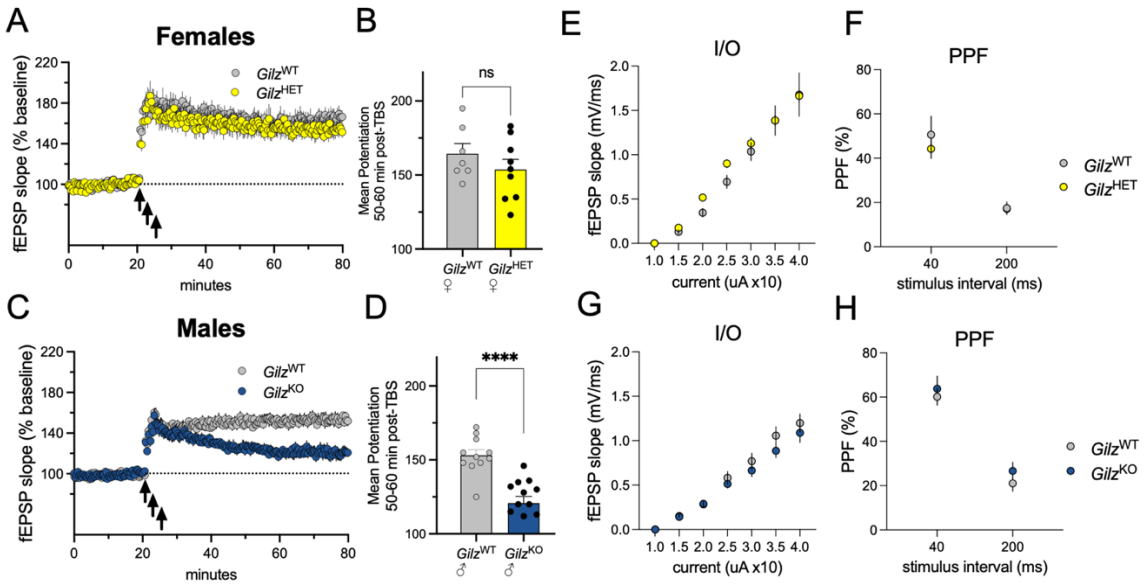

Figure 2.4: *Gilz*^{KO} males exhibit NAc LTP impairments while *Gilz*^{HET} females do not. (**A**) fEPSP slope before and after theta-burst stimulation showing no effects of *Gilz*^{HET} on NAc long-term potentiation in females. (**B**) Summary of mean potentiation during the 50-60 min post-stimulation interval show no effect of *Gilz*^{HET} in females: $t_{14} = 1.09$, $p = 0.294$. (**C**) fEPSP slope before and after theta-burst stimulation showing effects of *Gilz*^{KO} on NAc long-term potentiation in males. (**D**) Summary of mean potentiation during the 50-60 min post-stimulation interval show LTP impairments in males: $t_{22} = 5.315$, $p < 0.0001$. (**E**) I/O curve results suggest no effect of *Gilz*^{HET} in females $F_{1,20} = 0.6290$, $p = 0.4270$. (**F**) Paired-pulse facilitation results in females suggest no effect of *Gilz*^{HET} $F_{1,12} = 0.4220$, $p = 0.5282$. (**G**) I/O curve results suggest no effect of *Gilz*^{KO} in males $F_{1,22} = 0.9005$, $p = 0.3530$. (**H**) Paired-pulse facilitation results in males suggest no effect of *Gilz*^{KO} $F_{1,12} = 0.6904$, $p = 0.4150$. Data are represented as mean + SEM. ****$p < 0.0001$.

Viral delivery of WT *Gilz-1* leads to selective overexpression with splice variant-specificity *in vivo.*

Because the canonical *Gilz*-1 variant has been implicated as a regulator of dendrite morphology in the striatum and hippocampus[152], and because its corresponding GILZ-1 isoform is the most potent transcriptional regulator via interactions with NF-κB[145], we next sought to examine whether impairments in NAc LTP can be rescued by selective overexpression of *Gilz-1*. Additionally, the design of this rescue experiment allows us to

further assess whether the impairments observed in *Gilz*^{KO} mice were indeed due to *Gilz*-specific (as opposed to compensatory) mechanisms. WT *Gilz-1* with a 3' V5 tag was cloned into a plasmid under the CMV promoter and virally packaged in AAV1 (Fig. 2.5A). A cohort of adult mice received bilateral intra-NAc viral infusions to selectively overexpress *Gilz*-1 in *Gilz*^{KO} males. *Gilz*^{WT} and *Gilz*^{KO} males were infused with empty vector control virus, and a separate group of *Gilz*^{KO} males received AAV-*Gilz*-1 infusions. Mice were sacrificed 3 weeks after surgery and tissue was collected from the NAc. RT-qPCR analysis from this tissue demonstrates the viability of not only rescuing natural levels of *Gilz-1* in *Gilz*^{KO} mice lacking other variants, but also of enhancing *Gilz-1* expression past steady-state levels observed in *Gilz*^{WT} controls (Fig. 2.5B, Splice variant × virus: $F_{(4,27)} = 3.90$, $p = 0.0126$; WT+e.v. *vs* KO+*Gilz*-1: $t_{27} = 4.088$, $p = 0.0031$; KO+e.v. vs KO+*Gilz-1*: $t_{27} = 4.191$, $p = 0.0024$). Furthermore, *in vitro* transfection shows viral enhancements in GILZ-1 expression concurrent with V5 tagging. Compared to EV-treated controls, cells transfected with our *Gilz-1* construct exhibit robust upregulation at both 24- and 48-hours post-transfection, as measured by α-V5 *and* α-GILZ antibodies (Fig. 2.5C).

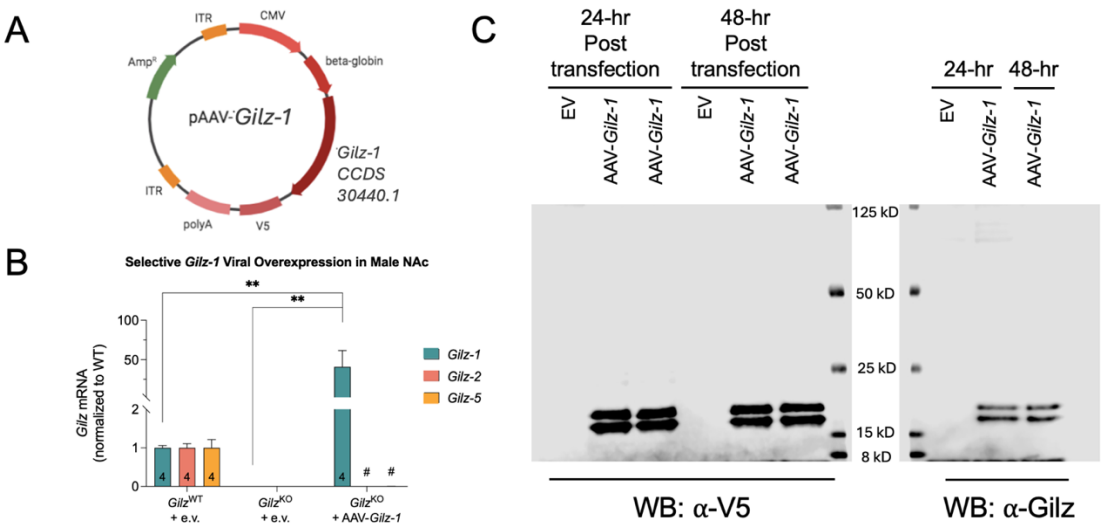

Figure 2.5: Design and validation of AAV construct to selectively overexpress *Gilz*-1 variant. (**A**) Plasmid construct map showing CMV promoter, beta-globin intron, WT *Gilz-1* CCDS sequence, and V5 epitope. (**B**) RT-qPCR data from NAc tissue showing selective overexpression of *Gilz*-1 in *Gilz*^{KO} males that received AAV-Gilz-1 viral infusions. Splice variant × virus: $F_{(4,27)} = 3.90$, $p = 0.0126$; WT+e.v. *vs* KO+*Gilz*-1: $t_{27} = 4.088$, $p = 0.0031$; KO+e.v. vs KO+*Gilz-1*: $t_{27} = 4.191$, $p = 0.0024$. (**C**) Western blot images showing AAV-Gilz-1-mediated upregulation of GILZ protein and V5 tag. Images show clear bands near the molecular weight of GILZ-1 (17 kD) at both 24- and 48-hours post-transfection compared to empty vector-treated controls.Data are represented as mean + SEM. $^{**}p < 0.01$, $^{\#}p < 0.05$ compared to *Gilz*-1 from the same treatment group.

Gilz-1 overexpression rescues NAc LTP impairments in *Gilz*^{KO} males.

A separate cohort of males received the same infusions under the same experimental design, but here tissue collected after viral expression was prepared for NAc LTP as stated above (Fig. 2.6). The data acquired in this experiment replicated our prior siRNA-mediated findings such that in mice expressing empty vector, hemizygous *Gilz*^{KO} males exhibited a significant impairment in potentiation during the 50-60 min post-TBS period compared to *Gilz*^{WT} counterparts (Fig. 2.6A-B, WT+e.v. vs KO+e.v., $q_{27} = 9.672$, $p < 0.0001$). Furthermore, viral overexpression of *Gilz-1* was sufficient to rescue this deficit in NAc LTP, as *Gilz*^{KO} mice expressing *Gilz-1* exhibited potentiation levels that were higher than their empty vector counterparts and comparable to *Gilz*^{WT} controls (Fig. 2.6B, KO+*Gilz-1* vs KO+e.v., $q_{27} = 12.52$, $p < 0.0001$; KO+*Gilz-1* vs WT+e.v., $q_{27} = 2.285$, $p =$

0.2565). These findings provide a twofold advantage. First, they reproduce our observations of NAc LTP impairments in *Gilz*^{KO} males. Second, they add rigor to the overall work by suggesting the function of *Gilz* in synaptic plasticity may be splice variant-specific.

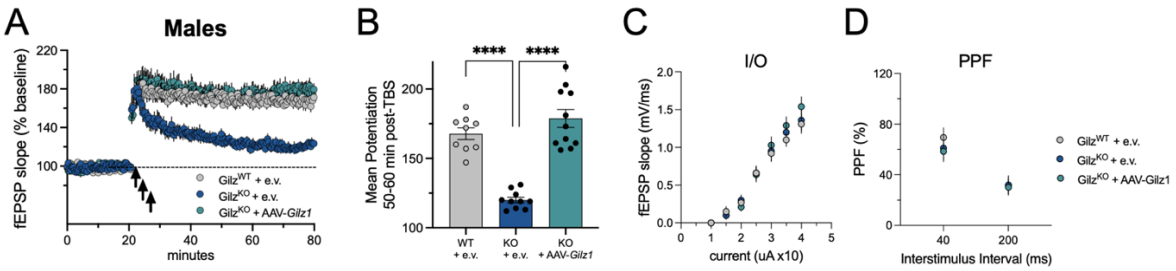

Figure 2.6: Selective overexpression of *Gilz-1* variant rescues NAc LTP impairments in *Gilz*^{KO} males. (**A**) fEPSP slope before and after theta-burst stimulation showing the effects of AAV-*Gilz*-1 (vs empty vector) in *Gilz*^{KO} compared to WT controls on NAc long-term potentiation in males. (**B**) Summary of mean potentiation during the 50-60 min post-stimulation interval show an impairment in *Gilz*^{KO} males that is rescued by AAV-*Gilz*-1 WT+e.v. vs KO+e.v., $q_{27} = 9.672$, $p < 0.0001$; KO+*Gilz-1* vs KO+e.v., $q_{27} = 12.52$, $p < 0.0001$; KO+*Gilz-1* vs WT+e.v., $q_{27} = 2.285$, $p = 0.2565$. (**C**) I/O curve results suggest no effect of *Gilz*^{KO} or AAV-*Gilz*-1 in males $F_{2,18} = 0.0188$, $p = 0.9813$. (**D**) Paired-pulse facilitation results suggest no effect of *Gilz*^{KO} or AAV-*Gilz*-1 in males $F_{2,28} = 0.2285$, $p = 0.7972$. Data are represented as mean + SEM. ****$p < 0.0001$.

Discussion

In this set of experiments, we show that *Gilz* splice variants can be site-specifically reduced by siRNA or globally reduced by genomic manipulation, and that such reductions lead to impairments in LTP in the NAc of males but not females. Additionally, we show that the *Gilz*-1 variant can be selectively overexpressed in *Gilz*^{KO} males, and that this is sufficient to rescue NAc LTP impairments. We also present data that begin to demonstrate the feasibility of continuing to use *Gilz*^{KO} males and *Gilz*^{HET} females for future studies.

Because *Gilz* was initially detected in association with memory consolidation, and because GILZ is known to interact with a key set of transcription factors necessary for synaptic plasticity, we sought to examine whether *Gilz* expression contributes to the transcription-dependent maintenance of LTP[2,129]. Our first studies in this effort show that nonselective site-specific reduction of the *Gilz*-1, *Gilz*-2, and *Gilz*-5 variants in the NAc is achievable by *in vivo* siRNA infusions. Thus, while *Gilz* splice variants harbor unique upstream sequences (corresponding to unique N-terminal domains in their associated protein isoforms), targeting siRNA at the consensus 3'-UTRs of these variants is sufficient for unbiased, acute reduction of *Gilz* expression.

We show that siRNA-mediated blunting of *Gilz* expression in the NAc significantly impairs theta burst-induced LTP in males but not females. Thus, in males, *Gilz* may regulate transcription-dependent synaptic adaptions in reward-associated circuitry. Numerous studies from our lab and others have demonstrated the value in investigating the role of drug-responsive genes in PFC afferents to NAc, as this pathway directly regulates behavioral adaptations to cocaine, such as locomotor sensitization and cued reinstatement of cocaine seeking[126,149,167,172]. Notably, we detected no sex-differences in siRNA-blunted *Gilz* expression in the NAc, yet females exhibited no siRNA-mediated LTP impairments. This suggests a possible divergence in the cellular role for *Gilz* between males and females.

To further probe the importance of *Gilz* in reward-related plasticity, we next sought to investigate the effects of more robust *Gilz* reduction on NAc LTP. For this, a mutant mouse line lacking exon 4 for a functional knockout was first generated and validated. We observed that hemizygous $Gilz^{KO}$ males indeed expressed neither *Gilz*-1, *Gilz*-2, or

Gilz-5 splice variants in the NAc under basal conditions. Notably, *Gilz* is necessary for spermatogenesis and *Gilz*^{KO} males are consequently rendered sterile, thus restricting our capabilities to generating only *Gilz*^{HET} females using this genomic approach[142]. While this does represent a notable limitation to our experimental design, we observed a ~50% reduction of all *Gilz* splice variants in the NAc of *Gilz*^{HET} females, which offered a manipulation that was more robust than the siRNA-mediated approach above but less complete than a global conventional knockout. Nonetheless, we refrain from making definitive claims with regard to sex-specific mechanisms in these experiments. It will be important for future studies to examine a complete knockout of *Gilz* in females.

Both males and females from this mutant line were subjected to behavioral tests to assess whether *Gilz*^{KO} or *Gilz*^{HET} affected general measures of ambulatory, anxiety-like, or learning-related behaviors. Our results indicate that reduced levels of global *Gilz* expression do not lead to any baseline differences in these measures, which is an important validation in light of prior findings demonstrating a sex-specific role for *Gilz* in response to lifetime accumulation of anxiogenic events[173]. Collectively, the findings from these behavioral tests served to validate the feasibility of our mouse line for use in the interpretation of experiment-specific behavioral phenotypes.

In *Gilz*^{KO} males, we observed an impairment in NAc LTP consistent with our siRNA-mediated findings. This suggests a mechanism that is not circumvented by compensatory biological adaptations to *Gilz* deficiency, and indicates that perhaps *de novo* transcription of specific *Gilz* splice variants is required for this form of synaptic plasticity. Thus, we turned to a variant-specific approach of replacing *Gilz* in our knockout mice.

The canonical *Gilz*-1 variant has been implicated as a regulator of dendrite morphology in the striatum and hippocampus, and its corresponding GILZ-1 isoform is the most potent transcriptional regulator via interactions with NF-κB[136,145,152]. Furthermore, GILZ-1 expresses a unique N-terminal domain that has been shown to regulate transcription by interacting with AP-1 and Raf-1 (part of the MAPK/ERK pathway)[133]. AP-1 directly regulates cocaine-induced transcriptional adaptations underlying neuritic outgrowth in the NAc, and the MAPK/ERK indirectly regulates cocaine-induced transcriptional adaptations in the NAc and PFC by induction of Fos and CREB[64-66,174]. These combined functional domains may thus underlie a possible variant-specific role for *Gilz*-1.

Here, we demonstrated how *Gilz*-1 can be selectively overexpressed in the NAc of *Gilz*^{KO} males via viral infusion. Importantly, this selective replacement is sufficient to rescue LTP impairments in these mice, further supporting the notion that *Gilz* is a key contributor to synaptic plasticity. However, the precise subcellular function of *Gilz*-1 in this context remains to be fully understood, as this viral approach does not specifically rescue expression in the glutamatergic afferents that are stimulated by the theta-burst protocol used here. Of the many heterogenous cellular subtypes in the NAc, glutamatergic afferents from PFC innervate both D1- and D2-subtype MSNs, whose unique contributions to NAc plasticity suggest future studies may benefit from considering cell-type as a variable when assessing *Gilz* function[1].

Collectively, these experiments demonstrate a role for *Gilz* in regulating transcription-dependent mechanisms of synaptic plasticity in the NAc of males but not females. Thus, while *Gilz* is not induced in reward circuitry following cocaine exposure

(see Chapter 1), our hypothesis moving forward is that GILZ does function within these cell populations to indirectly regulate transcription underlying cocaine-induced behavioral adaptations.

Chapter 3: *Gilz* in cocaine-CPP.

Rationale:

In reward circuitry, genes engaged by NAc LTP often contribute to cellular adaptations necessary for the acquisition and expression of cocaine-conditioned place preference (CPP)[126,149,175,176]. The associative memory processes involved in cocaine-CPP share some similarities with those involved in object location memory (including the DHC, where *Gilz* was originally implicated) but also engage a unique set of genes in nonoverlapping regions with the brain's reward circuitry, including the NAc, VTA, and PFC[2,123,125,177]. Not only have we observed high expression of all *Gilz* splice variants in the NAc and VTA, as well as notable expression in the PFC, but also our LTP-related findings have positioned *Gilz* as a gene that may function in this reward-related circuitry to govern CPP-related behaviors.

While there is a paucity of knowledge regarding the precise function of *Gilz* in the brain, limited studies do offer further insight into potential links between *Gilz* expression and drug-related behaviors. Piechota *et al.* (2010) detected an association between expression levels of striatal *Gilz* and the acquisition of morphine-CPP, although no direct role for *Gilz* was assessed[152]. RNA-seq data from Walker *et al*. (2018) suggest a positive correlation between *Gilz* levels in the ventral striatum and a composite measure of various behavioral measures in cocaine self-administration classified as an "addiction index.[99]" To date, however, no studies have identified a direct link between *Gilz* and cocaine-CPP, but our lab and others have demonstrated the value in probing whether genes implicated in NAc LTP (as *Gilz* has been) also contribute to cocaine-CPP[149,178]. The goal of this study

was to examine whether site-specific or global *Gilz* reduction affects the acquisition of cocaine-CPP in males or females.

Materials & Methods:

Mice:

Male and female C57BL/6J mice (Jackson Laboratories) were all single-housed and within 2-3 months old during behavioral testing. *Gilz*^{KO} males, *Gilz*^{HET} females, and *Gilz*^{WT} littermates were bred on a C57BL/6J background and maintained on the same conditions. All animals had *ad libitum* access to food and water unless otherwise specified. Experiments were performed during the light phase of a 12-hr light/dark cycle. All experiments were conducted in accordance with the National Institutes of Health *Guideline for Animal Care and Use* and were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

AAV Production:

Wild-type *Gilz*-1 was amplified from mouse accumbal cDNA and cloned into a modified pAAV-IRES-V5 plasmid, under control of the CMV promoter and β-globin intron. For the Empty Vector control, the *Gilz*-1 coding sequence was not present, but all other elements remain. Adeno-associated virus (AAV) was made by the Center for Neural Circuit Mapping (University of California Irvine) from the above described plasmids and was serotyped with AAV1. The final titer of AAV-*Gilz*-1 was 3.23×10^{12} GC/mL and the final titer of AAV-EV was 7.88×10^{13} GC/mL.

siRNA:

A SMARTpool consisting of 4 individual siRNAs was designed and purchased from Horizon Discovery. These siRNAs were targeted against unique regions in the 3'-UTR that were shared by all *Gilz* splice variants. The Accell SMARTpool formulation is reconstituted and delivered for transfection at 100uM in water solvent. A non-targeting control siRNA was also purchased from Horizon Discovery and delivered under the same conditions.

Surgery:

Mice were induced to anesthesia with 4% isoflurane in oxygen and maintained at 1.5-2% for the duration of surgery. Animals were injected with either a-*Gilz* siRNA, non-targeting siRNA, AAV-*Gilz*-1-V5 or AAV-EV-V5. 0.5 µl of solution was infused bilaterally into the NAc (AP): +1.3 mm; (ML): ±1.1 mm; (DV): −4.5 mm relative to bregma. Solutions were infused at a rate of 6 µl /hr by using a 30 gauge Neuros Hamilton syringe (product #65459-01) mounted to either a Harvard Apparatus Nanomite Syringe Pump (product #MA1 70-2217) or Leica Biosystems Nanoinjector Motorized f/Stereotaxics (product #39462901). All infusions used the Leica Microsystems Angle Two Stereotaxic System. For siRNA experiments, animals were allowed to recover for 24-48 hrs. For viral experiments, animals were allowed to recover for three weeks to ensure viral expression.

Cocaine-conditioned place preference:

Unbiased conditioned place preference (CPP) was performed as described in previous studies[149]. Briefly, all mice were handled for 2 minutes for 3 consecutive days prior to the experiment (days 1-3). Baseline preferences for three compartments in the CPP apparatus were assessed by placing the animals in the center compartment of the apparatus with free access to three distinct compartments for 15 min (day 4). Time spent

in each compartment was recorded. Following this pretest, mice received intra-NAc or intra-VTA infusions of siRNA or viral constructs. For siRNA studies, conditioning began 48 hours post-surgery. For viral studies, conditioning began 21 days post-surgery. Mice were conditioned over four consecutive days, receiving either cocaine-HCl (5 mg/kg or 10 mg/kg, IP; Sigma) or 0.9% saline. 24 hours following the last conditioning session, post-conditioning preference was tested in animals while they were in a drug-free state. Animals were allowed to freely explore all compartments of the CPP apparatus to assess preference, established as the difference between time spent in the cocaine-paired chamber and the saline-paired chamber, in seconds. For viral studies, this test was administered once daily for CPP extinction. Extinction was defined as 2 consecutive days where individual group scores were not significantly different from zero (one-tailed t-tests). For reinstatement, mice were given a single 5 mg/kg i.p. injection of cocaine immediately prior to a final CPP test. Time spent was tracked automatically from MPEG videos using EthoVision 3.1 software (Noldus Technology).

Cocaine-Induced Locomotion:

This test examines the locomotor activating effects of cocaine in animals following experimenter-administered cocaine injections. Mice were handled for 2 min for 3 days (day 1-3) and were habituated to the activity apparatus (Plexiglas open field with sawdust bedding; base 16 cm × 32 cm) for 30 min per day for 2 consecutive days (days 4-5). Following habituation, locomotor activity was recorded for 30 minutes after an intraperitoneal injection of 10 mg/kg Cocaine-HCl for 5 days (day 6-10). Locomotor activity (total distance traveled) was monitored and tracked automatically from MPEG videos using EthoVision 3.1 software (Noldus Technology, Leesburg, VA).

Results:

Acute *Gilz* reduction does not affect cocaine-CPP acquisition at a 5 mg/kg dose.

Our previous findings implicating *Gilz* in NAc LTP as well as our findings linking *Gilz* to memory consolidation led us to first test the role of NAc *Gilz* in the acquisition of cocaine-CPP, which is a consolidation-dependent behavioral measure[2]. Independent cohorts of male and female mice were subjected to our cocaine-CPP acquisition protocol after receiving α-*Gilz* or non-targeting control siRNA infusions into the NAc (Fig 3.1A). Mice were then conditioned with 5 mg/kg cocaine and saline before being administered a post-test to probe for CPP acquisition. In both males and females, we observed a main effect of conditioning (Fig 3.1B-C, Males: $F_{1,14} = 21.95$, $p = 0.0004$. Females: $F_{1,16} = 27.46$, $p < 0.0001$). However, Šidák's multiple comparisons revealed no effect of α-*Gilz* siRNA on post-test scores, suggesting that partial knockdown of *Gilz* splice variants under these conditions does not affect CPP acquisition (Fig 3.1B-C, Males: $t_{28} = 0.301$, $p = 0.945$. Females: $t_{32} = 0.379$, $p = 0.914$).

We next sought to examine whether VTA *Gilz* contributes to cocaine-CPP acquisition. Not only do dopaminergic projections from VTA to NAc represent a primary target for drugs of abuse including cocaine[51], but also we have observed high expression levels of all *Gilz* splice variants in the VTA (see Chapter 1). Thus, independent cohorts of male and female mice were exposed to the same experimental protocol described above, but instead received intra-VTA siRNA infusions prior to conditioning. In both males and females, we again observed a main effect of conditioning (Fig 3.1D-E, Males: $F_{1,16} = 17.82$, $p = 0.0006$. Females: $F_{1,17} = 19.37$, $p = 0.0004$). Similar to our NAc findings,

however, Šidák's multiple comparisons again revealed no effect of α-*Gilz* siRNA on post-test scores, suggesting that partial knockdown of *Gilz* splice variants under these conditions does not affect CPP acquisition (Fig 3.1D-E, Males: t_{32} = 0.001, p > 0.999. Females: t_{34} = 0.526, p = 0.841).

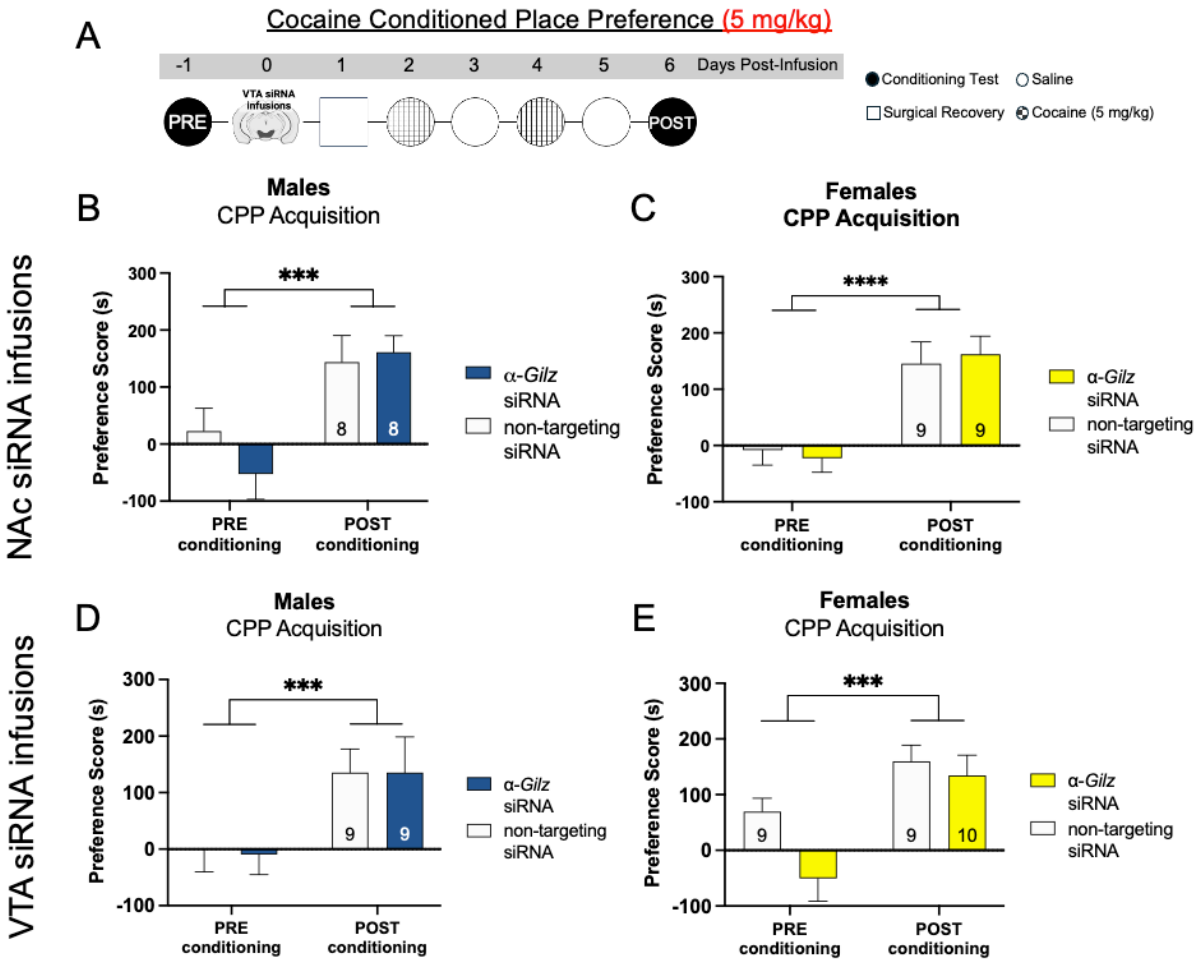

Figure 3.1: α-*Gilz* siRNA does not affect 5 mg/kg cocaine-CPP acquisition. (**A**) Males and females were administered a CPP pre-test after 3 days of handling. The following day, mice received bilateral infusions of either α-*Gilz* siRNA or non-targeting control siRNA into either the NAc or VTA. 48 hours later, mice began a regimen of once-daily alternating conditioning sessions with 5 mg/kg cocaine and saline, followed by a CPP post-test. (**B**) intra-NAc α-*Gilz* siRNA did not affect 5 mg/kg CPP acquisition in males (main effect of conditioning: $F_{1,14}$ = 21.95, p = 0.0004, Šidák's multiple comparisons at post-test: t_{28} = 0.301, p = 0.945). (**C**) intra-NAc α-*Gilz* siRNA did not affect 5 mg/kg CPP acquisition in females (main effect of conditioning: $F_{1,16}$ = 27.46, p < 0.0001. Šidák's multiple comparisons at post-test: t_{32} = 0.379, p = 0.914). (**D**) intra-VTA α-*Gilz* siRNA did not affect 5 mg/kg CPP acquisition in males (main effect of conditioning: $F_{1,16}$ = 17.82, p = 0.0006. Šidák's multiple comparisons at post-test: t_{32} = 0.001, p > 0.999). (**E**) intra-VTA α-*Gilz* siRNA did not affect 5 mg/kg CPP acquisition in females (main effect of conditioning: $F_{1,17}$ = 19.37, p

= 0.0004. Šidák's multiple comparisons at post-test: t_{34} = 0.526, p = 0.841). Data are presented as mean + SEM. ***p < 0.001, ****p < 0.0001.

VTA *Gilz* reduction impairs cocaine-CPP acquisition in males at a 10 mg/kg dose.

It has been well established that different doses of cocaine not only lead to different behavioral performance in CPP, but also that these doses lead to differential regulation of gene expression profiles in reward-related brain regions[179,181]. As such, we sought to test the effects of acute *Gilz* knockdown at a higher dose of cocaine-conditioning. Here, males and females underwent the same manipulations described above but with 10 mg/kg cocaine conditioning (Fig. 3.2A). As with the lower dose, this dose has been validated in our lab to engage key mechanisms under investigation[123.] Here, following NAc siRNA infusions, we again observed a main effect of conditioning in both males and females (Fig. 3.2B-C, Males: $F_{1,14}$ = 135.9, p < 0.0001. Females: $F_{1,13}$ = 28.23, p < 0.0001). Šidák's multiple comparisons again revealed no effect of α-*Gilz* siRNA on post-test scores (Fig. 3.2B-C, Males: t_{31} = 0.458, p = 0.878. Females: t_{29} = 0.144, p = 0.912).

Notably, when siRNA was infused into the VTA prior to conditioning with 10 mg/kg cocaine, we observed a significant impairment in CPP acquisition in males that received α-*Gilz* siRNA (Fig. 3.2D, t_{34} = 2.703, p = 0.0212, Šidák's multiple comparisons). This effect was specific to males, as females exhibited CPP independent of siRNA-group (Fig. 3.2E, Main effect of conditioning: $F_{1,15}$ = 69.18, p < 0.0001. Šidák's multiple comparisons at post-test: t_{30} = 1.751, p = 0.172). These results suggest *Gilz* acts within the VTA of males to regulate processes underlying the acquisition or expression of cocaine-CPP.

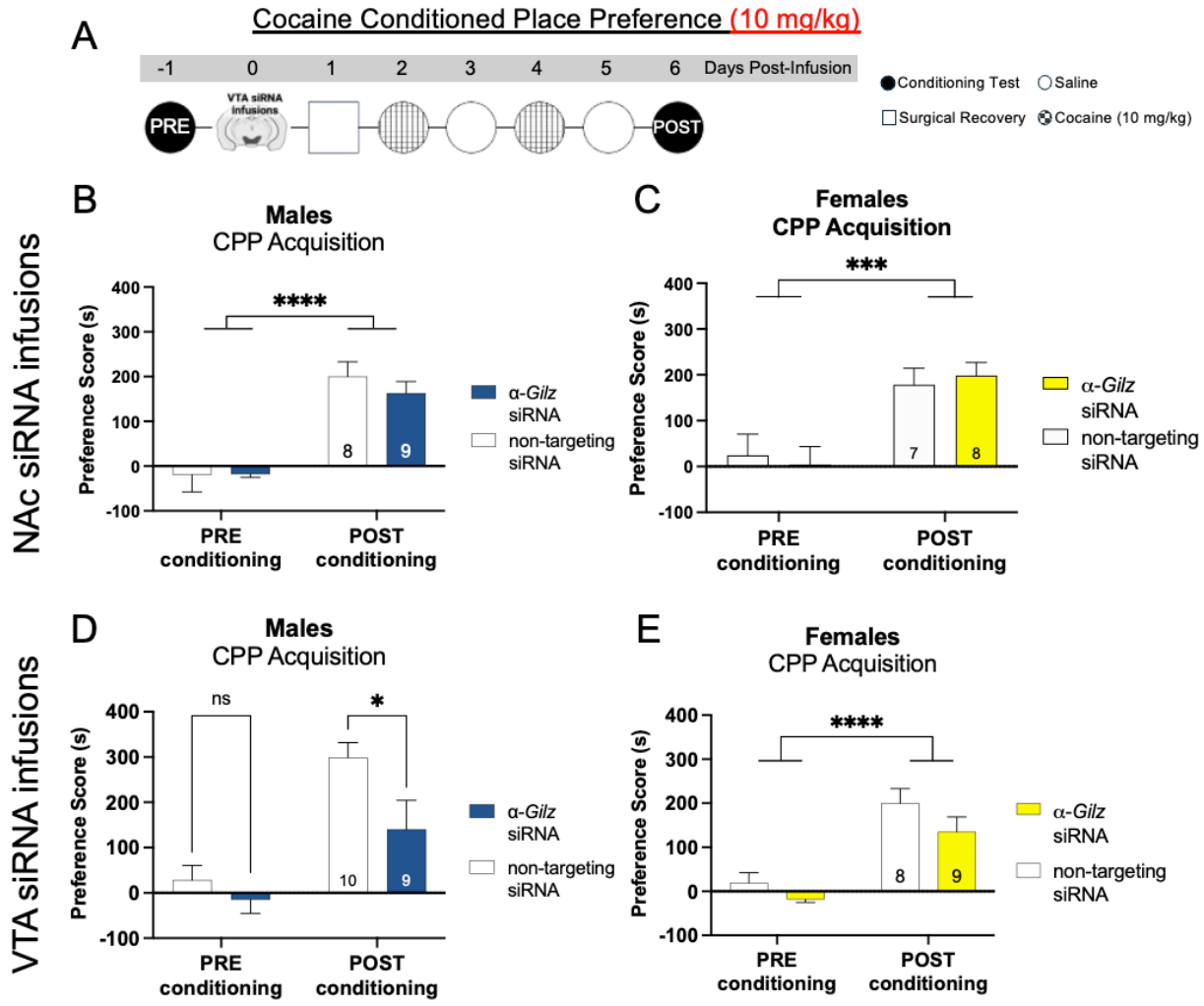

Figure 3.2: α-*Gilz* siRNA in VTA blunts 10 mg/kg CPP acquisition in males but not females. (**A**) Males and females were administered a CPP pre-test after 3 days of handling. The following day, mice received bilateral infusions of either α-*Gilz* siRNA or non-targeting control siRNA into either the NAc or VTA. 48 hours later, mice began a regimen of once-daily alternating conditioning sessions with 10 mg/kg cocaine and saline, followed by a CPP post-test. (**B**) intra-NAc α-*Gilz* siRNA did not affect 10 mg/kg CPP acquisition in males (main effect of conditioning: $F_{1,14} = 135.9$, $p < 0.0001$, Šidák's multiple comparisons at post-test $t_{31} = 0.458$, $p = 0.878$). (**C**) intra-NAc α-*Gilz* siRNA did not affect 10 mg/kg CPP acquisition in females (main effect of conditioning: $F_{1,13} = 28.23$, $p < 0.0001$. Šidák's multiple comparisons at post-test $t_{29} = 0.144$, $p = 0.912$). (**D**) intra-VTA α-*Gilz* siRNA impaired 10 mg/kg CPP acquisition in males (Šidák's multiple comparisons at post-test: $t_{34} = 2.703$, $p = 0.0212$). (**E**) intra-VTA α-*Gilz* siRNA did not affect 10 mg/kg CPP acquisition in females (main effect of conditioning: $F_{1,15} = 69.18$, $p < 0.0001$. Šidák's multiple comparisons at post-test: $t_{30} = 1.751$, $p = 0.172$). Data are presented as mean + SEM. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$.

Gilz^{KO} males and *Gilz*^{HET} females exhibit similar cocaine-CPP acquisition to WT controls.

The results outlined in our LTP studies (see Chapter 2) suggest NAc LTP impairments observed following siRNA treatment were conserved in *Gilz*^{KO} males. We sought to examine whether this conserved role for *Gilz* also translated to our behavioral results observed in CPP. For this, adult *Gilz*^{KO} males, *Gilz*^{HET} females and WT littermates were subjected to our unmodified CPP protocol and conditioned with 10 mg/kg cocaine. Here, we observed main effects of conditioning for both sexes (Fig. 3.3A-B, Males: $F_{1,17}$ = 89.85, p < 0.0001. Females: $F_{1,13}$ = 71.35, p < 0.0001). Surprisingly, Šidák's multiple comparisons revealed no interactions between genotype during the post-test (Fig. 3.3A-B, Males: : t_{34} = 0.4366, p = 0.8879. Females: t_{26} = 0.3786, p = 0.914). In males, this suggests the CPP impairment we observed following VTA siRNA infusions may depend on the acute, site-specific effects of *Gilz* reduction in contrast to the effects of global knockout.

Gilz^{KO} males and _Gilz_^{HET} females exhibit similar cocaine sensitization to WT controls.

We next sought to examine whether genomic *Gilz* manipulations affected mechanisms associated with the inherent rewarding properties of cocaine (Fig. 3.3C). If the rewarding properties of cocaine are indeed affected, that may cloud our interpretations derived from CPP findings. For this, we employed a cocaine psychomotor sensitization protocol. *Gilz*^{KO} and *Gilz*^{HET} mice were used alongside WT littermates due to our inability to use siRNA for prolonged *Gilz* reduction throughout the sensitization protocol. In both males and females, we observed no effect of genotype (Fig. 3.3D-E, Males: $F_{1,14}$ = 1.244, p = 0.283. Females: $F_{1,14}$ = 0.295, p = 0.595). Indeed, both sexes

exhibited the anticipated increases in locomotor activity on day 5 compared to day 1 (Fig. 3.3D-E, Šidák's multiple comparisons, Males: t_{83} = 6.055, p < 0.0001. Females: t_{84} = 3.178, p = 0.0124). These combined data suggest that while *Gilz* may function acutely to regulate processes underlying cocaine-CPP, genomic *Gilz* reduction does not affect psychomotor sensitization to cocaine.

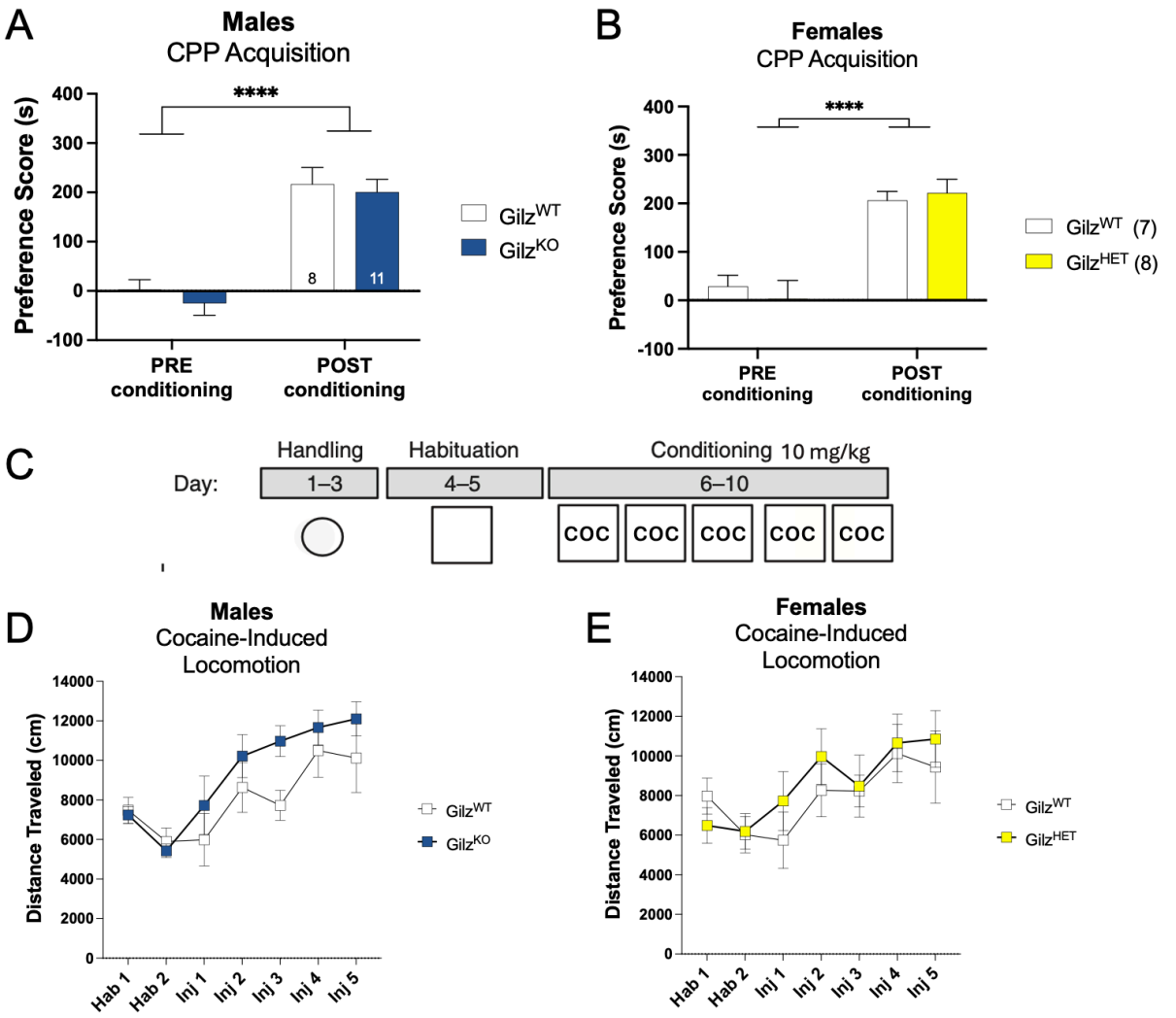

Figure 3.3: *Gilz*^{KO} in males and *Gilz*^{HET} in females does not affect CPP acquisition or locomotor sensitization. (**A**) *Gilz*^{KO} males exhibit 10 mg/kg cocaine-CPP acquisition comparable to WT controls (main effect of conditioning: $F_{1,17}$ = 89.85, p < 0.0001, Šidák's multiple comparisons at post-test: t_{34} = 0.4366, p = 0.8879). (**B**) *Gilz*^{HET} females exhibit 10 mg/kg cocaine-CPP acquisition comparable to WT controls (main effect of conditioning: $F_{1,13}$ = 71.35, p < 0.0001, Šidák's multiple comparisons at post-test: t_{26} = 0.3786, p = 0.914). (**C**) Cocaine locomotor sensitization protocol. After 3 days of handling, males and females from independent cohorts were habituated to open chambers for 2 daily sessions. Mice received 10 mg/kg cocaine i.p. injections daily followed immediately by daily re-exposure to the testing chambers. Locomotion

was tracked each day. (**D**) Males exhibit cocaine-induced locomotor sensitization over successive days regardless of genotype (Šidák's multiple comparisons: day 5 vs day 1 $t_{83} = 6.055$, $p < 0.0001$. No main effect of genotype: $F_{1,14} = 1.244$, $p = 0.283$). (**E**) Females exhibit cocaine-induced locomotor sensitization over successive days regardless of genotype (Šidák's multiple comparisons: day 5 vs day 1 $t_{84} = 3.178$, $p = 0.0124$. No main effect of genotype: $F_{1,14} = 0.295$, $p = 0.595$). Data are presented as mean + SEM. ****$p < 0.0001$.

Gilz-1 overexpression in VTA does not affect acquisition, extinction, or reinstatement of cocaine-CPP.

Our prior findings have suggested a splice variant-specific role for *Gilz*-1 in reward-related processes. First, we detected notably high levels of *Gilz*-1 expression in the VTA of both sexes (see Chapter 1). Then, we demonstrated that *Gilz*-1 overexpression rescues NAc LTP impairments in *Gilz*^{KO} males (see Chapter 2). These findings position *Gilz*-1 as a candidate underlying the CPP impairments we observed following VTA *Gilz* knockdown. Thus, we sought to utilize our previously validated AAV-*Gilz*-1-V5 construct to test the behavioral effects of overexpression. Here, males received viral infusions in the VTA after CPP pre-tests (Fig. 3.4A). Following a 3-week period for viral expression, mice were then conditioned with 10 mg/kg cocaine. We observed no effects of viral group, as both groups exhibited comparable levels of CPP acquisition (Fig. 3.4B, Main effect of conditioning: $F_{1,13} = 47.84$, $p < 0.0001$, Šidák's comparison at post-test: $t_{26} = 0.293$, $p = 0.948$).

In addition to regulating acquisition of cocaine-CPP, the VTA is also engaged by reinstatement of previously extinguished CPP[182]. However, the subcellular populations within VTA that contribute to these processes are not identical. For instance, GABA-ergic projections from VTA to NAc are disinhibited during reinstatement, but not acquisition, of cocaine-CPP[180]. Thus, we sought to examine whether VTA *Gilz* may also function to

regulate CPP reinstatement. The same cohort described immediately above was subjected to daily CPP extinction tests before undergoing a cocaine-primed (5 mg/kg) reinstatement test. Both groups exhibited normal extinction behavior (Fig. 3.4B, Šidák's comparison "post" vs "E6": t_{14} = 3.973, p = 0.0097). Both groups also exhibited CPP reinstatement (Fig. 3.4B, Šidák's comparison "E6" vs "RI": t_{14} = 3.17, p = 0.0467). We observed no effect of virus on reinstatement (Fig. 3.4B, Šidák's comparison at RI: $t_{12.58}$ = 0.361, p > 0.9999). These results suggest that *Gilz*-1 overexpression in the VTA does not singularly affect the associative memory processes engaged by acquisition, extinction, or reinstatement of cocaine-CPP under these conditions.

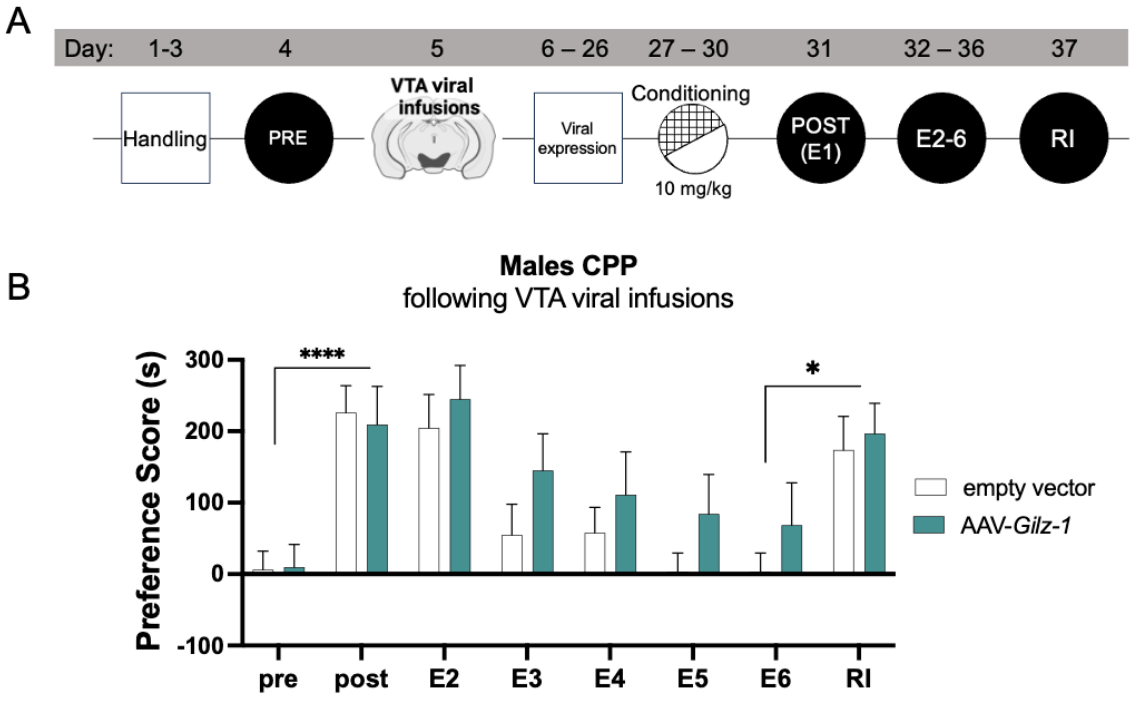

Figure 3.4: *Gilz*-1 overexpression in VTA does not affect acquisition, extinction, or reinstatement of cocaine-CPP in males. (**A**) After a CPP pre-test, WT males received intra-VTA infusions of either AAV-*Gilz*-1 or empty vector. After a period allowed for viral expression, mice were conditioned with 10 mg/kg cocaine and saline i.p. injections. A CPP post test was administered to assess acquisition. As this test doubles as the first extinction session, a series of successive extinction sessions followed. Mice received a priming dose of cocaine (5 mg/kg) immediately before reintroduction to the CPP chambers for the reinstatement test. (**B**) AAV-*Gilz*-1 does not affect CPP behaviors. Main effect of conditioning on acquisition: $F_{1,13}$ = 47.84, p < 0.0001, Šidák's multiple comparisons at post-test t_{26} = 0.293, p = 0.948). Šidák's multiple comparisons between post-test and E6: t_{14} = 3.973, p = 0.0097 collapsed across viral groups. Šidák's multiple comparisons between E6 and RI: t_{14} = 3.17, p = 0.0467 collapsed across viral groups. Šidák's

multiple comparisons between viral groups during RI: $t_{12.58} = 0.361$, $p > 0.9999$. Data are presented as mean + SEM. *$p < 0.05$, ****$p < 0.0001$.

Discussion:

We found that VTA *Gilz* is necessary for acquisition of cocaine-CPP in males but not females. Interestingly, while VTA afferents to NAc are a key substrate of cocaine-induced neuroadaptations, and while we previously observed a reliable role for NAc *Gilz* in synaptic plasticity, our findings here also show that NAc *Gilz* does not appear to regulate cocaine-CPP[51]. Nonetheless, the studies described above represent the first direct evidence for *Gilz*-dependent regulation of drug-associated behaviors.

At a lower dose (5 mg/kg) siRNA-mediated reduction of *Gilz* splice variants had no significant effects on CPP acquisition. The impairment we observed in males was specific to our 10 mg/kg conditioning protocol. Even slight perturbations in drug administration protocols can lead to distinct outcomes, and different doses of cocaine have been shown to differentially induce expression profiles of immediate early genes (*i.e., Fos, Egr-1*) which go on to orchestrate intricate transcriptomic responses[179]. Furthermore, higher doses of cocaine lead to distinct pharmacokinetic environments in cells expressing dopamine transporters (DATs), which in turn differentially affect the downstream signaling cascades (MAPK/ERK) and transcription factors (AP-1) known to interact with *Gilz*[129,183,184]. Thus, the mechanism by which *Gilz* regulates CPP acquisition may be not only region-specific, but also dose-specific.

In addition to the baseline differences in *Gilz* splice variant expression between the NAc and VTA, cocaine has been shown to activate distinct subcellular populations within these regions. For instance, while much research has focused on excitatory and dopaminergic transmission in the context of reward, cocaine exposure has been shown

to evoke potentiation of GABA release from specifically from D1-type NAc afferents to the VTA[185]. Furthermore, cocaine differentially acts within D1- vs D2-type MSNs of the NAc to regulate other HDAC3 target genes as well as behavioral adaptations[126]. In our lab, efforts to manipulate HDAC3 in the NAc yield distinct outcomes depending on the level of cell type-specificity in our experimental approaches[123,126]. Finally, recent efforts have been made to better understand the distinct transcriptomic profiles induced by drugs of abuse in the highly heterogenous cellular subtypes of both the NAc and VTA[186-188]. Thus, *Gilz* may only be acting in particular subpopulations of the VTA during CPP acquisition. Indeed, *Gilz* may also play a role in NAc subpopulations that is being occluded by our unbiased knockdown approaches.

While future work may refine the set of cells in which *Gilz* functions are probed, the collective findings from these studies begin to link the role for *Gilz* in synaptic plasticity with its involvement in functional, behavioral consequences to cocaine exposure.

Chapter 4: *Gilz* in cocaine-IVSA.

Rationale:

Relapse to drug-seeking represents a key manifestation of the long-term adaptations underlying substance use disorders. Even after prolonged periods of abstinence or withdrawal, cues and contexts associated with the rewarding effects of cocaine retain an enduring ability to reinstate drug-seeking behaviors in humans and rodents[189]. For instance, following a 10-week period of abstinence, cocaine-dependent patients who experience relapse exhibit increased brain activation in sensory, motor, and cognitive-emotional processing areas when presented with cocaine-related cues[190]. Many rodent studies have demonstrated how either diffuse and multimodal "context" cues or "discrete" cues that have acquired incentive salience can robustly reinstate cocaine-seeking behaviors after various schedules of abstinence[191]. The vulnerability to relapse is thus a critical avenue for investigation in the pursuit of substance abuse treatments.

Intravenous drug self-administration (IVSA) is a gold standard method for modelling addiction-like behaviors in animal models. In IVSA models, rodents are trained to perform an operant response (*e.g.*, lever pressing) in order to receive a dose of a drug, typically delivered alongside the presentation of a discrete cue. Generally, mice and rats gradually acquire increasing patterns of responding over repeated training sessions, indicating both an ability to learn the task and a motivation for the rewarding effects of a drug. Importantly, one of the key components that distinguishes IVSA from CPP is the volitional nature with which the drug is administered. Mice will expend considerable effort to receive cocaine infusions, as is indicated by behavioral escalations in progressive ratio schedules[192].

Similar to the effects observed in CPP, removing the drug-paired consequences of lever pressing leads to a gradual extinction in previously established behaviors. There are, however, various ways to model cessation of cocaine use in the IVSA model[191]. A period of forced abstinence (where animals are returned to their home cage for an extended duration in the absence of cocaine) can lead to increases in cue-induced cocaine seeking during a re-exposure session[193]. This "incubation of craving" is thought to arise from a multitude of neural mechanisms, but much work has suggested that overlapping stress- and reward-related circuitry is engaged during both abstinence and reinstatement[12]. (While incubation of craving represents a distinct avenue of study in the field of addiction, it is not a phenomenon under direct study in the experiments outlined below but is rather used as a precursor step in our examination of reinstatement behavior.) In particular, glucocorticoid (GC)-dependent mechanisms engaged in the PFC, NAc, and VTA contribute to the induction of reinstatement-related plasticity. Blocking GCs in these regions has modest effects on both acquisition and extinction cocaine-IVSA while completely abolishing subsequent reinstatement[119]. This establishes a need for investigating GC-related pathways in the context of reinstatement.

Gilz is a primary target of GC-dependent regulation, and its downstream interactions with signaling pathways (*i.e.*, MAPK/ERK) and transcription factors (*i.e.*, AP-1 and NF-kB) suggest *Gilz* may orchestrate the transcriptional adaptations that give rise to reinstatement[137,141,145]. Indeed, a recent study from our lab illustrated the diverse transcriptomic responses to distinct forms of cocaine exposure and found that cocaine-IVSA engages gene networks associated with GC-receptor signaling pathways[105]. This study highlighted the paradigm-specific effects of cocaine exposure, thus demonstrating

how a particular gene may play differential roles in cocaine-CPP vs cocaine-IVSA. Thus, we hypothesized that *Gilz* may instead be engaged by the stress-related components of abstinence and reinstatement and have a role in reinstatement-related behavior.

Materials & Methods:

Mice:

Gilz^{KO} males, and *Gilz*^{WT} littermates were bred on a C57BL/6J background and were group-housed with *ad libitum* access to food and water until 1 week prior to experimental onset. Mice were within 2-6 months old during behavioral testing and were all single-housed one week prior to experimental onset. Experiments were performed during the light phase of a 12-hr light/dark cycle. All experiments were conducted in accordance with the National Institutes of Health *Guideline for Animal Care and Use* and were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

Quantitative RT-qPCR:

RT-qPCR was performed as described previously[2]. Two half-millimeter punches were collected from the PFC, NAc and DHC in two consecutive 250 um slices of tissue. RNA was isolated from punches using an RNeasy Minikit (QIAGEN) and cDNA was created using the Transcriptor First Strand cDNA Synthesis kit (Roche Applied Science).

AAV Production:

Wild-type *Gilz*-1 was amplified from mouse accumbal cDNA and cloned into a modified pAAV-IRES-V5 plasmid, under control of the CMV promoter and β-globin intron.

For the Empty Vector control, the *Gilz*-1 coding sequence was not present, but all other elements remain. Adeno-associated virus (AAV) was made by the Center for Neural Circuit Mapping (University of California Irvine) from the above described plasmids and was serotyped with AAV1. The final titer of AAV-*Gilz*-1 was 3.23×10^{12} GC/mL and the final titer of AAV-EV was 7.88×10^{13} GC/mL.

Surgery:

Mice were induced to anesthesia with 4% isoflurane in oxygen and maintained at 1.5-2% for the duration of surgery. Animals were injected with either a-*Gilz* siRNA, non-targeting siRNA, AAV-*Gilz*-1-V5 or AAV-EV-V5. 0.5 µl of solution was infused bilaterally into the NAc (AP): +1.3 mm; (ML): ±1.1 mm; (DV): −4.5 mm relative to bregma. Solutions were infused at a rate of 6 µl /hr by using a 30 gauge Neuros Hamilton syringe (product #65459-01) mounted to either a Harvard Apparatus Nanomite Syringe Pump (product #MA1 70-2217) or Leica Biosystems Nanoinjector Motorized f/Stereotaxics (product #39462901). All infusions used the Leica Microsystems Angle Two Stereotaxic System. For siRNA experiments, animals were allowed to recover for 24-48 hrs. For viral experiments, animals were allowed to recover for three weeks to ensure viral expression.

Jugular vein catheterization

One week before behavior, animals were implanted with an indwelling back-mounted jugular vein catheter for intravenous cocaine self-administration as previously described[194]. During 5–7 days of recovery, catheters were flushed daily (heparinized saline, 100USP/ml in 0.9% saline and enrofloxacin) to maintain catheter patency, which were verified before and after the self-administration period by observing a 5–10 s sedation after infusing the fast-acting anesthetic propofol (propofol sodium, Patterson

Vet). After recovery, animals were food restricted to 90% of presurgical weight over 3–4 days before the start of behavior.

Cocaine intravenous self-administration:

Mice were allowed to self-administer cocaine in operant conditioning chambers (MedAssociates) in 12 daily 1-h sessions. Most animals acquired self-administration within the first session; otherwise, on the second day (and third if needed) levers were baited with a drop of condensed milk. Failing to acquire self-administration or having a malfunctioning catheter were exclusion criteria. Animals advanced to fixed-ratio 2 (FR2) schedule after four days of FR1. During self-administration, active lever presses elicited a cocaine reward in cocaine-SA mice (8.5mg/kg/infusion) and saline in yoked mice, along with a cue presentation (light/tone). There were no programmed consequences for inactive lever presses. Following self-administration, mice received a 30-day home cage withdrawal followed by a 5-h extinction session in which presses on the previously active lever were not rewarded or cued. Extinction in Figure 4.3 is detailed in the results section. Immediately after extinction, a 70 min cued reinstatement was induced with cue priming during the first 10 min. During reinstatement, presses on the previously active lever resulted in cue presentation but no reward. Brains were flash-frozen in isopentane 1-h after termination of the reinstatement test. Self-administration and extinction were analyzed using a two-way repeated measures ANOVA (Prism 9, GraphPad Software Inc.). Reinstatement data were analyzed using a one-way ANOVA. p values < 0.05 were considered significant.

Results:

***Gilz*^{KO} males exhibit impairments in cue-primed reinstatement of cocaine-IVSA.**

Male *Gilz*^{KO} and *Gilz*^{WT} littermates were trained to self-administer cocaine, which was followed by a 30-day withdrawal used to drive craving and reinstatement of extinguished cocaine seeking (Fig. 4.1A). During cocaine self-administration, presses on the active lever exceeded presses on the inactive lever in both groups (Fig. 4.1B, WT active vs inactive; $F_{1,10}$ = 20.15, p = 0.0012; KO active vs inactive: $F_{1,10}$ = 18.09, p = 0.0017). During self-administration, there were no differences between groups in responses to the active lever ($F_{1,10}$ = 4.38, p = 0.0628) or in the amount of cocaine consumed (WT vs KO cocaine infusions; $F_{1,10}$ = 4.689, p = 0.0556; WT average = 26.64 rewards per session, KO average = 38.72 rewards per session). After 30 days of home cage withdrawal there were no differences between groups in extinction of presses on the lever previously designated as active (Fig. 4.1C, WT vs KO "active"; $F_{1,10}$ = 0.3609, p = 0.5614). At the end of extinction (Hr 5), all animals received 10 min of cue priming to induce reinstatement, which was followed by a 1-h cued reinstatement session. Behaviorally experienced *Gilz*^{WT} mice showed high reinstatement of "active" lever-pressing compared to behaviorally experienced *Gilz*^{KO} mice (Fig. 4.1D, Tukey's multiple comparisons: WT vs KO "active": q_{20}=5.289, p = 0.0065), demonstrating that *Gilz* contributes to relapse-like behavior.

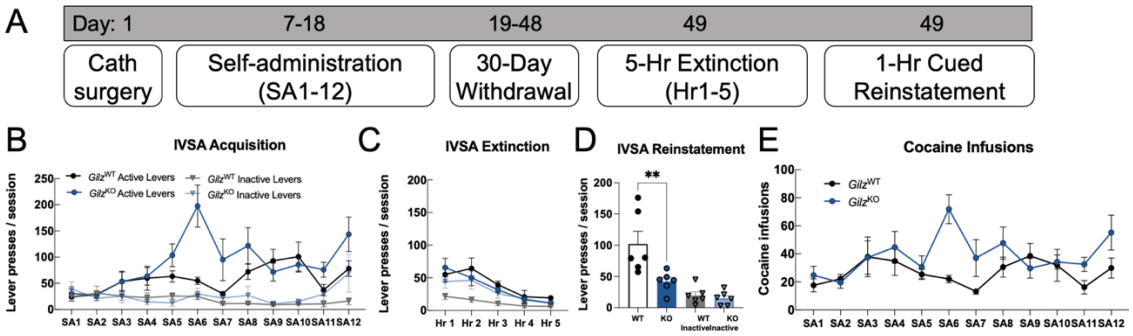

Figure 4.1: *Gilz*^{KO} leads to impairments in cue-primed reinstatement of cocaine self-administration. (**A**) Schematic of experimental timeline. (**B**) *Gilz*^{KO} had no effect on discrimination between active and inactive levers during acquisition of cocaine self-administration, (2-way ANOVA: no main effect of genotype: $F_{1,10} = 4.38$, $p = 0.0628$). (**C**) After 30 days of home cage withdrawal, *Gilz*^{KO} had no effect on extinction (WT vs KO "active"; $F_{1,10} = 0.3609$, $p = 0.5614$. (**D**) While WT mice exhibited reinstatement of lever pressing behavior following a 10-min period of cue-priming, *Gilz*^{KO} mice did not (Tukey's multiple comparisons: WT vs KO "active": $q_{20}=5.289$, $p = 0.0065$). (**E**) *Gilz*^{KO} did not affect cocaine intake across the same acquisition sessions plotted in panel B, although *Gilz*^{KO} mice infused more cocaine on SA6 (2-way ANOVA, no main effect of genotype: $F_{1,10} = 4.689$, $p = 0.0556$). Data are presented as mean + SEM. **p < 0.01.

Cue-primed reinstatement of cocaine-IVSA in WT males leads to a trend in *Gilz*-1 induction in the NAc.

We next sought to delineate the effects of *Gilz*^{KO} on interactions with accumulated cocaine experience vs interactions with the general behavioral protocol in the absence of cocaine by replicating our self-administration experiment with the inclusion of saline-yoked control mice. Here, adult male *Gilz*^{KO} and *Gilz*^{WT} mice were again subjected to our cocaine self-administration protocol, but a subset of each group were assigned to receive only passive saline infusions consistent with those of a cocaine self-administering (coc-SA) counterpart. Thus, for a saline-yoked mouse, presses on the "active" lever were recorded but did not directly correlate with saline infusion schedules. In coc-SA mice, presses on the active lever again exceeded presses on the inactive lever in both groups (Fig. 4.2A, WT active vs inactive; $F_{1,24} = 61.01$, $p < 0.0001$; KO active vs inactive: $F_{1,20} = 17.67$, $p = 0.0004$), and there were no differences between groups in responses to the active lever ($F_{1,22} = 2.566$, $p = 0.1234$) or in the amount of cocaine consumed (WT vs KO cocaine infusions; $F_{1,22} = 1.494$, $p = 0.2345$; WT average = 33.40 rewards per session,

KO average = 39.39 rewards per session). After 30 days of home cage withdrawal from cocaine-SA we again observed no effect of genotype on extinction behavior (Fig. 4.2C, WT vs KO "active"; $F_{1,22} = 0.0331$, $p = 0.8572$). Notably, *Gilz*^{WT} mice again showed high reinstatement compared to *Gilz*^{KO} mice (Fig. 4.2E, $q_{44} = 4.158$, $p = 0.0258$), which serves as a robust replication of our earlier findings (Fig. 4.1D).

Furthermore, in both *Gilz*^{WT} and *Gilz*^{KO} mice, active lever presses for cocaine exceeded active lever presses for saline (Fig. 4.2A-B, WT coc-SA vs saline: $F_{1,16} = 31.01$, $p < 0.0001$; KO coc-SA vs saline: $F_{1,14} = 11.49$, $p = 0.0044$), and cocaine infusions outnumbered saline infusions (Fig. 4.2G-H, WT cocaine vs saline infusions: $F_{1,16} = 32.77$, $p < 0.0001$; KO cocaine vs saline infusions: $F_{1,14} = 24.03$, $p = 0.0002$).

To begin examining whether the effects we observed in reinstatement behavior are due to the function of specific *Gilz* splice variants, we randomly selected a subset of *Gilz*^{WT} mice from both the coc-SA and saline-yoked groups to assess *Gilz* expression following the reinstatement test. Animals were sacrificed 1-hour after the termination of the test, and NAc tissue was processed for analysis via RT-qPCR (Fig. 4.2I). The PFC and DHC were again included in this analysis to determine if reinstatement-associated effects were region specific. Although we found no significant effects of cocaine-SA on *Gilz* expression, there was a trend toward induction of *Gilz*-1 in the NAc of cocaine-SA animals (Fig. 4.2J, $t_7 = 2.212$, $p = 0.0626$). Together, these data suggest *Gilz*-1 in the NAc may contribute not only to the cellular mechanisms engaged during LTP (see Chapter 2) but also to the functions governing reinstatement of cocaine-seeking behavior.

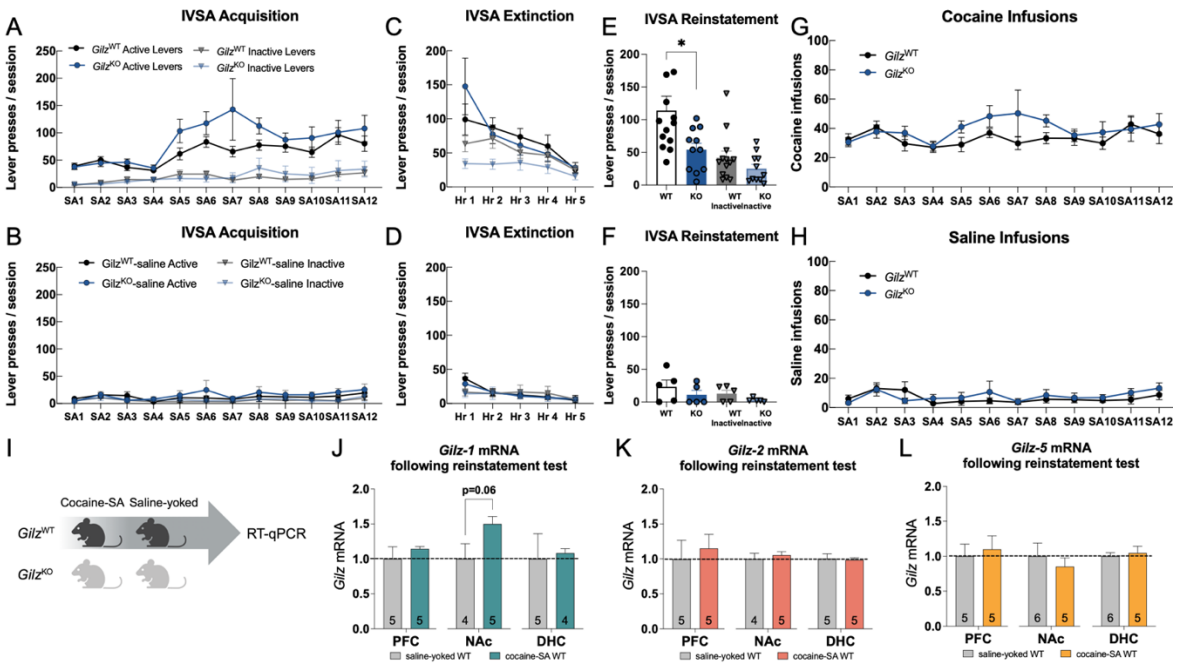

Figure 4.2: *Gilz*^{KO}-related impairments are specific to cocaine reinstatement and Gilz-1 exhibits a trend toward induction in the NAc following reinstatement. (**A,B**) *Gilz*^{KO} had no effect on discrimination between active and inactive levers during acquisition of cocaine self-administration, (RM 2-way ANOVA: no main effect of genotype: $F_{1,22}$ = 2.566, p = 0.1234). And in both *Gilz*^{WT} and *Gilz*^{KO} mice, active lever presses for cocaine exceeded active lever presses for saline (RM 2-way ANOVA, Tukey's multiple comparisons: WT coc-SA vs saline: $F_{1,16}$ = 31.01, p < 0.0001; KO coc-SA vs saline: $F_{1,14}$ = 11.49, p = 0.0044 (**C,D**) After 30 days of home cage withdrawal, *Gilz*^{KO} had no effect on extinction (RM 2-way ANOVA, Tukey's multiple comparisons: WT vs KO "active"; $F_{1,22}$ = 0.0331, p = 0.8572. (**E,F**) While WT coc-SA mice exhibited reinstatement of lever pressing behavior following a 10-min period of cue-priming, *Gilz*^{KO} mice did not (2-way ANOVA, Tukey's multiple comparisons: WT vs KO "active": q_{44} = 4.158, p = 0.0258; WT "active" vs WT "inactive": q_{44} = 5.236, p = 0.0032). (**G,H**) *Gilz*^{KO} did not affect cocaine intake across the same acquisition sessions plotted in panels A-B, (2-way ANOVA, no main effect of genotype WT vs KO cocaine infusions; $F_{1,22}$ = 1.494, p = 0.2345). **(I**) Following reinstatement testing, a subset of WT mice from both the coc-SA and saline-yoked groups were sacrificed for RT-qPCR on NAc tissue. (**J**) A trend toward *Gilz*-1 mRNA induction was observed in the NAc of coc-SA mice compared to saline-yoked controls, t_7 = 2.212, p = 0.0626. (**K,L**) No significant effects of coc-SA were observed on *Gilz-2* or *Gilz*-5 expression in the PFC, NAc, or DHC. Data are presented as mean + SEM. *p < 0.05.

Gilz-1 overexpression in the NAc of *Gilz*^{KO} males leads to enhanced cocaine-IVSA acquisition.

Our RT-qPCR findings further highlight a potential splice variant-specific role for *Gilz*-1 in regulating not only synaptic (see Chapter 2), but also behavioral responses to cocaine. This result led us to test whether the reinstatement impairment we observed in *Gilz*^{KO} males could be rescued in a similar manner to what we observed when

investigating NAc LTP. Here, *Gilz*^{KO} and *Gilz*^{WT} males received intra-NAc infusions of either AAV-*Gilz*-1-V5 or empty vector (Fig. 4.3A). IVSA training was initiated 3 weeks after viral infusions. Saline-yoked controls were omitted from this experiment, and all animals received cocaine-HCl infusions. For visual clarity, inactive lever presses are presented on separate panels. During acquisition, active lever presses exceeded inactive lever presses for all groups (Fig. 4.3B-C, WT+e.v.: $F_{1,26} = 35.04$, $p < 0.0001$. WT+G1: $F_{1,26} = 23.56$, $p < 0.0001$. KO+e.v.: $F_{1,25} = 17.28$, $p = 0.0003$. KO+G1: $F_{1,30} = 32.6$, $p < 0.0001$). Mixed-effects analysis suggests a main group effect during acquisition ($F_{3,54} = 2.926$, $p = 0.0419$), and on SA5, Tukey's multiple comparisons revealed significantly higher responding in *Gilz*^{KO}+AAV-*Gilz*-1 compared to both *Gilz*^{WT} groups (Fig. 4.3B, KO+G1 vs WT+e.v.: adjusted $p = 0.0124$, KO+G1 vs WT+G1: adjusted $p = 0.0093$). These results suggest that viral overexpression of *Gilz*-1 in the NAc of *Gilz*^{KO} males leads to enhanced acquisition of cocaine-IVSA behavior.

Due to high lever pressing behavior observed during acquisition, we employed a modified version of our extinction protocol to ensure more robust extinction following the 30-day abstinence period. Here, mice were extinguished to a predetermined criterion (defined as < 30% of their average responding over the prior three sessions). To account for differences in the amount of time required for mice to reach this criterion, a second 2-hr fixed extinction session was administered the next day. This second session was meant to equilibrate the experience mice had immediately prior to reinstatement.

We observed a main effect of time during extinction day 1 (Fig. 4.3D, $F_{7,298} = 8.692$, $p < 0.0001$), and Dunnett's multiple comparisons revealed significant differences between Hr 1and Hr 8 ($q_{16} = 4.67$, $p = 0.0016$). These results suggest mice extinguished lever-

pressing behavior over the course of day 1, and that *Gilz*-1 viral infusions had no effect in either genotype.

During day 2 of extinction and reinstatement, we observed a main effect of group (Fig. 4.3F, $F_{3,53} = 4.125$, $p = 0.0106$). Across all groups, Dunnett's multiple comparisons revealed a significant decrease between Hr 1 and Hr 2 ($q_{56} = 4.563$, $p < 0.0001$). This was followed by a subsequent increase across groups between Hr 2 and reinstatement ($q_{56} = 6.692$, $p < 0.0001$). These results suggest that while *Gilz*-1 viral expression in *Gilz*^{KO} is associated with higher lever pressing, it does not disrupt patterns of extinction followed by cued-reinstatement.

Collectively, the findings from these studies demonstrate (1) *Gilz*^{KO}-related impairments in reinstatement behavior, (2) marginal *Gilz*-1 induction in the NAc following cocaine-IVSA experience, and (3) enhanced cocaine-IVSA acquisition in *Gilz*^{KO} males with selective *Gilz*-1 overexpression in the NAc.

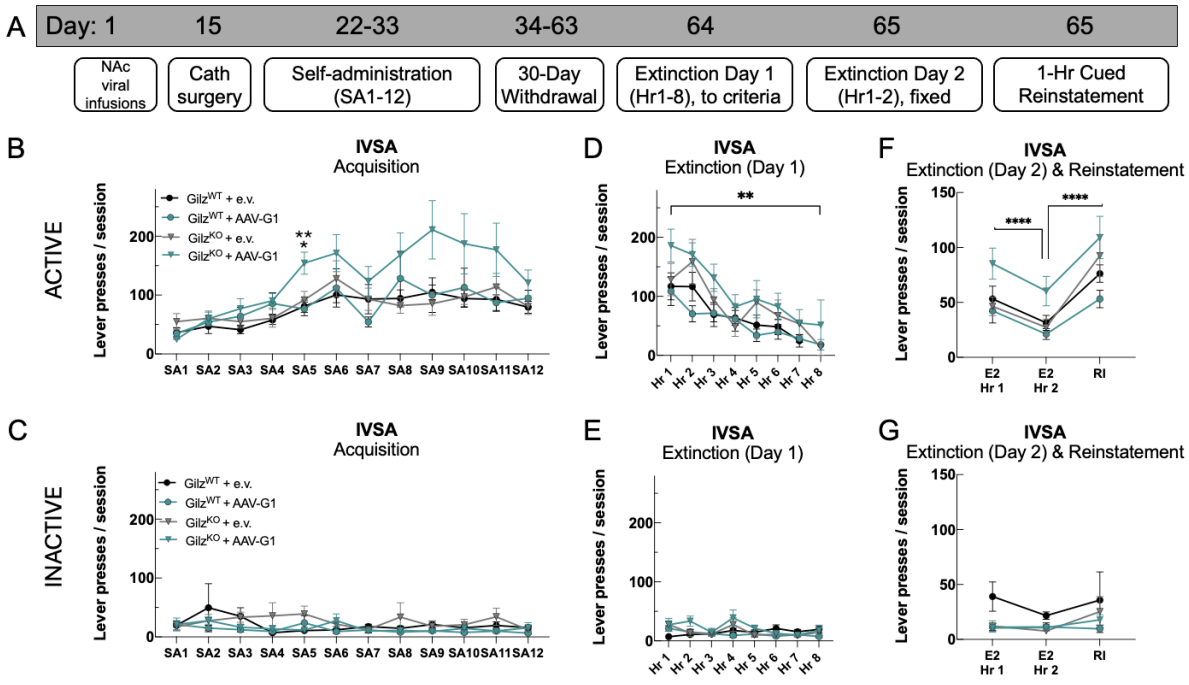

Figure 4.3: *Gilz-1* overexpression in the NAc of *Gilz*^{KO} mice leads to enhanced acquisition of cocaine self-administration behavior. (**A**) Mice received intra-NAc viral infusions of either AAV-*Gilz*-1 or empty vector control prior to IVSA training. Following 12 acquisition sessions and a 30- day home cage withdrawal period, mice underwent Day 1 of extinction to criteria, then Day 2 of extinction for a fixed amount of time. Cue-primed reinstatement tests began immediately after day 2 extinction, in the same session. (**B,C**) *Gilz*-1 overexpression in the NAc resulted in enhanced active-lever pressing behavior during acquisition in *Gilz*^{KO} males only (Mixed effects 2-way ANOVA, main group effect: $F_{3,54} = 2.926$, $p = 0.0419$. Tukey's multiple comparisons on SA5 revealed significantly higher responding in KO males with "G1" overexpression: (*KO+G1 vs WT+e.v.: adjusted $p = 0.0124$, **KO+G1 vs WT+G1: adjusted $p = 0.0093$). (**D,E**) After 30 days of home cage withdrawal, we observed a main effect of time on extinction behavior (RM 2-way ANOVA: $F_{7,298} = 8.692$, $p < 0.0001$), and Dunnett's multiple comparisons revealed significant differences between Hr1 and Hr 8 ($q_{16} = 4.67$, $p = 0.0016$). (**F,G**) During day 2 of extinction and reinstatement, we observed a main effect of group (RM 2-way ANOVA: $F_{3,53} = 4.125$, $p = 0.0106$). Across all groups, Dunnett's multiple comparisons revealed a significant decrease between Hr 1 and Hr 2 ($q_{56} = 4.563$, $p < 0.0001$). This was followed by a subsequent increase across groups between Hr 2 and reinstatement ($q_{56} = 6.692$, $p < 0.0001$). Data are presented as mean + SEM. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

Discussion:

Here, we present direct evidence implicating *Gilz* in cocaine self-administration behaviors. To date, only Walker *et al*. (2018) have published a dataset that suggests *Gilz* correlates with a composite of IVSA behaviors denoted as an "addiction index." Our findings suggest *Gilz* is necessary for cue-primed reinstatement of cocaine-IVSA in males. Furthermore, we observed a trend toward *Gilz*-1-specific induction in the NAc following reinstatement. Finally, we observed enhancements in cocaine-IVSA acquisition following *Gilz*-1 overexpression in the NAc.

Our first experiment illustrated how *Gilz*^{KO} males exhibit impairments in reinstatement behavior. Importantly, these *Gilz*^{KO} mice acquired cocaine-IVSA at similar rates to WT controls. This suggests that, under these conditions, *Gilz* may not be necessary for the mechanisms that *establish* cocaine-seeking behavior, but rather for the subsequent *reengagement* of those mechanisms. It remains to be seen whether *Gilz* functions during the 30-day period of forced abstinence or during the cue-primed reinstatement session. While future studies may utilize our siRNA approach for more precise manipulations, much evidence in the literature would suggest *Gilz* responds to

the stressful components of forced abstinence. Numerous studies have observed adaptations that occur to stress circuitry during cocaine abstinence. For instance, forced abstinence produces an anxiogenic-like effect on an elevated plus maze, which can be abolished by nAChR-blockade in the VTA[195]. Furthermore, forced abstinence alters GC-signaling and CRF expression in the NAc[196]. As *Gilz* is a key regulator of stress-related adaptations, we hypothesize that forced abstinence may recruit GILZ to orchestrate the "stage-setting" effects of incubation through interaction with its previously described binding partners[119]. This will be an important future direction to better understand the role of GILZ in these behaviors.

Our second experiment served not only as a replication of our initial findings (thereby enhancing the rigor of this study) but also demonstrated that our observations were specific to the rewarding effects of cocaine (as opposed to saline) self-administration. Our findings suggest *Gilz*^{KO}-related impairments in operant responding do not manifest in the absence of drug reward. Additionally, the inclusion of saline-yoked controls allowed us to compare gene expression following reinstatement of cocaine- vs saline-SA. We observed a trend toward increases specifically in *Gilz*-1 expression in the NAc of cocaine-SA mice. We also probed *Gilz* expression in the PFC due to our findings implicating *Gilz* in LTP from PFC afferents to NAc. These RT-qPCR results suggest the role for *Gilz* in reinstatement is not only brain region-specific, but also splice variant-specific.

Finally, we overexpressed *Gilz*-1 in the NAc of both *Gilz*^{KO} and WT mice to examine whether this manipulation rescued behavioral deficits in a similar manner to our LTP-rescue experiment. Here, mice were given viral infusions prior to acquisition to avoid

surgery-related disruption of mechanisms that may contribute to forced abstinence. This design however resulted in *Gilz*-1-dependent increases in acquisition of cocaine-IVSA in *Gilz*^{KO} mice only. While this behavioral effect may have engaged neuroadaptations to occlude interpretation of our later reinstatement test, this result on acquisition represents a key finding. Notably, *Gilz*-1 overexpression did not lead to similar acquisition enhancements in WT mice, suggesting that conventional *Gilz*^{KO} may lead to the accumulation of compensatory adaptations that render *Gilz*^{KO} mice sensitive to the effects of *Gilz* replacement.

Indeed, a large body of literature has explored the role of endocrine signaling pathways (including GC-dependent transcription) in not only the initial *organization* of neural circuitry, but also the later *activation* of this circuitry[197,198]. As such, if *Gilz* plays a role in the organization of overlapping stress- and drug-related pathways, then *Gilz*^{KO} mice may harbor alterations in this circuitry that respond differently to later activation, whether it be in the form of stress or drug exposure. Thus, while the collective findings from these studies illuminate a role for *Gilz* in cocaine-IVSA behaviors, further work is needed to understand whether this is due to the engagement of stress-related mechanisms.

Future work may also address some of the limitations to our current experimental approach. First, we chose to study only males for IVSA-related experiments. While this decision was made in light of our male-specific findings related to the role of *Gilz* in NAc LTP and cocaine-CPP (see Chapters 2 and 3), the processes that govern IVSA are distinct, and sex should be considered as a biological variable in future studies. Furthermore, overexpression of WT *Gilz-1* in the NAc was administered in the absence

of a cell type-specific approach, despite a body of literature showing distinct roles for cellular subpopulations in regulating IVSA[126]. Regarding our current viral approach, it may be that high levels of overexpression driven by the CMV promoter in the AAV construct may drive levels of GILZ that are beyond physiologically normal levels, leading to altered behavior.

Chapter 5: *Gilz* in response to stress.

Rationale:

While the primary focus of our studies to date have examined the role of *Gilz* in cocaine-related processes, *Gilz* was originally characterized as a stress-responsive gene. *Gilz* was first identified as a dexamethasone-induced gene with considerable expression in lymphatic tissues, and numerous studies have reported *Gilz* as one of the genes most rapidly and invariably induced by glucocorticoid (GC) signaling[128,131,161]. The *Gilz* promoter harbors 3 GC response elements which, along with other signaling cascades, promote transcriptional activation of all *Gilz* splice variants[136,137]. Although there is a large number of studies examining *Gilz* in peripheral tissues related to inflammation there is paucity of literature examining the effects of GC activation on *Gilz* expression specifically in brain tissue. One of few studies is by Yachi *et al.* (2007) who observed significant increases in *Gilz* mRNA and protein in both the mPFC and hippocampus of male mice exposed to a water-immersion restraint stress[132]. Importantly, adrenalectomized mice failed to exhibit this stress-induced *Gilz* response, thus highlighting the role of GCs in regulating *Gilz*. Furthermore, Yachi *et al*. (2007) found that rapid *Gilz* induction was transient, as expression levels returned to baseline 4 hours post-induction. These findings suggest *Gilz* may act on acute timescales to regulate cellular responses to stress in discrete brain regions.

Over longer periods, Lebow *et al*. (2019) posit that *Gilz* "quantifies" exposure to stressors experienced from late gestation into adulthood, and that low levels of *Gilz* predispose individuals to PTSD in males only[173]. Human tissue samples collected for the Grady Trauma Project displayed correlative relationships between *Gilz* mRNA levels and

the number of traumatic events experienced throughout the lifespan. In the same study by Lebow *et al*. (2019), male mice exposed to prenatal stress went on to exhibit PTSD-like behaviors, and this increased susceptibility was associated with similar downregulations in *Gilz* mRNA in the basolateral amygdala (BLA) that were not detected in females. Thus, not only do *Gilz* mRNA expression levels in the brain correlate with traumatic events in humans (and "PTSD-like" behaviors in rodents), but also there appears to be a possible sex difference in the role of *Gilz* in the brain in response to adverse events.

Importantly, the findings outlined above do not examine specific *Gilz* splice variants and do not specify which variant is being reported. Thus, similar to our studies in the context of cocaine, we sought to examine the responses of distinct *Gilz* splice variants in the brain following exposure to different stimuli known to engage GC signaling. The goal of these studies was to quantify *Gilz*-1, *Gilz*-2, and *Gilz*-5 expression in specific brain regions of males and female mice following acute foot shock, repeated multimodal stress (RMS), and repeated foot shock. In order for future studies to determine the functional role of *Gilz*, we first need to understand which isoforms are expressed in specific brain regions under specific conditions.

Materials & Methods:

Mice:

Male and female C57BL/6J mice (Jackson Laboratories) were all single-housed and within 2-3 months old during behavioral testing. $Gilz^{KO}$ males, $Gilz^{HET}$ females, and $Gilz^{WT}$ littermates were bred on a C57BL/6J background and maintained on the same

conditions. All animals had *ad libitum* access to food and water unless otherwise specified. Experiments were performed during the light phase of a 12-hr light/dark cycle. All experiments were conducted in accordance with the National Institutes of Health *Guideline for Animal Care and Use* and were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

Fear conditioning:

Fear conditioning was conducted in a set of four identical Noldus PhenoTyper chambers. The floor was composed of stainless steel rods (0.9 cm apart) through which foot shocks were delivered. Each chamber is equipped with a lid containing a matrix of infrared LED lights, a tone generator, and an infrared CCD camera with a high-pass filter to block visible light. Chambers were cleaned with 70% ethanol between animals. For conditioning sessions, mice were exposed to the context for 2 minutes followed by a 30-sec tone (80 dB, 2,400 Hz) that co-terminated with a 2-sec (0.75mA) shock. Mice remained in the context for an additional 30s before being removed and transported back to their home cages. Twenty-four hours after training, mice were returned to the conditioning chamber for 5 minutes. Mice were sacrificed one hour later.

Repeated multimodal stress (RMS):

At age P30 to P35, mice were randomly assigned to experimental groups: repeated multiple concurrent stressors (RMS) or stress-free control. This RMS protocol was carried out by the Lur Lab as previously described[203]. For RMS, animals were placed in a well vented restrainer fashioned from 50 ml conical tubes, and five to eight mice were then placed in a clean cage. A high-frequency speaker was placed in the cage to deliver loud noise stimulus. The noise was generated by an Arduino Uno driving an amplifier and

consisted of 0.5–1-s-long beeps randomly selected from a frequency range of 15–30 kHz at 0.5–3 s random intervals. The cage was placed on top of a laboratory rocker under a bright light. Animals were rocked for 1 h/d for 10 consecutive days. A subset of control and stressed mice were weighed every day before the onset of RMS. On day 10 or 11, mice were euthanized for RT-qPCR.

Cocaine-conditioned place preference:

Unbiased conditioned place preference (CPP) was performed as described in previous studies, with modifications[149]. Briefly, mice were given 3 daily 15-min sessions to habituate to the CPP chambers after repeated foot shock exposure. Time spent in each chamber was recorded in a similar fashion to our CPP scoring protocol. Following this habituation, mice were given a 46-day home cage rest period. Mice were then given a final session designated as the pre-test. We observed a general aversion to white chambers (as opposed to checkered chambers), so we chose to counter-condition mice based on their final preference score reflected in the pre-test. Mice were counter-conditioned over four consecutive days, receiving either cocaine-HCl (5 mg/kg, IP; Sigma) or 0.9% saline. 24 hours following the last conditioning session, post-conditioning preference was tested in animals while they were in a drug-free state. Animals were allowed to freely explore all compartments of the CPP apparatus to assess preference, established as the difference between time spent in the cocaine-paired chamber and the saline-paired chamber, in seconds. Time spent was tracked automatically from MPEG videos using EthoVision 3.1 software (Noldus Technology).

Results:

Single foot shock leads to differential regulation of *Gilz* splice variants in the BLA of males and females.

We first sought to examine the effects of a single foot shock on *Gilz* splice variant expression in the BLA of males and females, given the significant body of literature from our lab and others that has highlighted the role of the BLA in regulating behavioral responses to stress-activated GC signaling[122,199,200]. Males and females were handled for 3 days before undergoing a fear conditioning (FC) training session as previously described (Fig. 5.1A)[199]. The training session involved a single acute shock within a 5-min FC training session. We found that this FC training session resulted in splice variant-specific *Gilz* regulation in the BLA of both males and females. *Gilz*-1 was significantly upregulated in comparison to context-exposed controls (Fig. 5.1B-C. Šidák's multiple comparisons, Males: t_{23} = 2.775, p = 0.0319. Females: t_{12} = 5.306, p = 0.0006). *Gilz*-2 expression was unaffected in either sex (Fig. 5.1B-C. Šidák's multiple comparisons, Males: t_{23} = 0.2002, p = 0.9961. Females: t_{12} = 0.5823, p = 0.9211). *Gilz*-5 was reduced in females only (Fig. 5.1B-C. Šidák's multiple comparisons, Males: t_{23} = 1.171, p = 0.5843. Females: t_{12} = 3.398, p = 0.0158). These data suggest a single foot shock leads to variant-specific changes to *Gilz* expression in the BLA of males and females.

Fear conditioning behavior is unaffected in *Gilz*^{KO} males or *Gilz*^{HET} females.

Because *Gilz* is differentially-regulated in the BLA during FC training, we sought to examine whether *Gilz*^{KO} males or *Gilz*^{HET} females exhibited impaired long-term memory for a conditioned context using a fear conditioning test. Separate cohorts of *Gilz*^{KO} males and *Gilz*^{HET} females were exposed to a full FC protocol (with the same single foot shock training), as previously described by our lab (Fig. 5.1D)[199]. During the training session,

we observed no effect of genotype on shock reactivity, an indirect measure of the subjective response to foot shock (Fig. 5.1E-F, Males: t_{18} = 0.4956, p = 0.6262, Females: t_{14} = 0.6049, p = 0.5549). We also observed no effect of genotype on freezing behavior during the FC re-exposure test the next day (Fig. 5.1G-H, Males: t_{17} = 0.1963, p = 0.8467, Females: t_{14} = 0.7498, p = 0.4658). Together, these results indicate that while *Gilz* splice variants are differentially regulated in the BLA by a single foot shock, *Gilz* may not be necessary for the processes underlying the formation of a long-term memory for a context that was associated with a single shock.

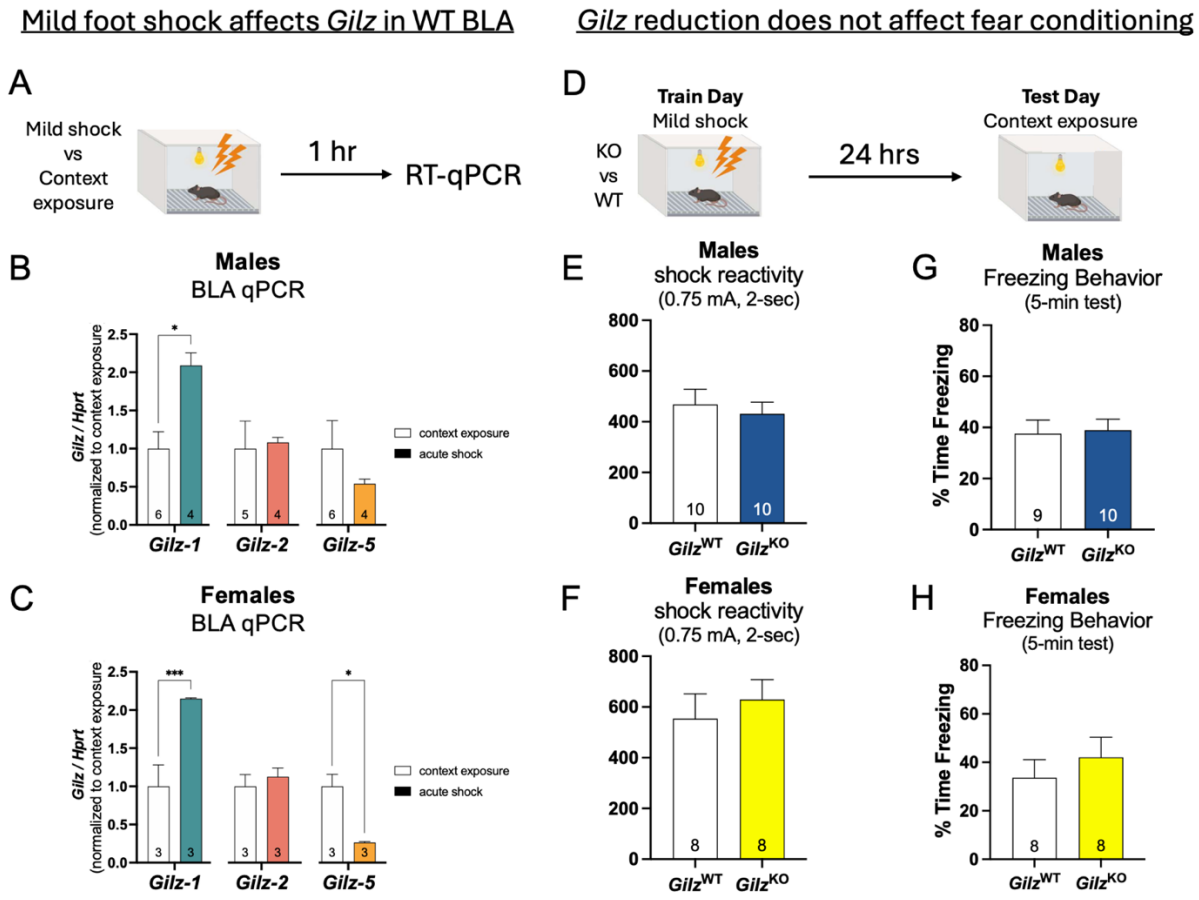

Figure 5.1: Single foot shock leads to differential *Gilz* regulation in BLA, but genomic *Gilz* manipulation does not affect fear conditioning behavior. (**A**) Mice received either a single foot shock or a context exposure without foot shock as a control. Brain were collected 1 hour after the end of the session for RT-qPCR analysis. (**B,C**) Compared to context exposed controls, males and females exposed to a single foot shock exhibited Gilz-1 upregulation in the BLA (Šidák's multiple comparisons, Males: t_{23} = 2.775, p = 0.0319. Females: t_{12} = 5.306, p = 0.0006). *Gilz*-2 expression was unaffected in either sex (Šidák's

multiple comparisons, Males: t_{23} = 0.2002, p = 0.9961. Females: t_{12} = 0.5823, p = 0.9211). *Gilz*-5 was reduced in females only (Šidák's multiple comparisons, Males: t_{23} = 1.171, p = 0.5843. Females: t_{12} = 3.398, p = 0.0158). (**D**) $Gilz^{KO}$ males, $Gilz^{HET}$ females, and WT littermates were exposed to a single foot shock for fear conditioning followed by a test session to measure freezing behavior 24 hours later. (**E,F**) During the training session, we observed no effect of genotype on shock reactivity, an indirect measure of the subjective response to foot shock (Males: t_{18} = 0.4956, p = 0.6262, Females: t_{14} = 0.6049, p = 0.5549). (**G,H**) We observed no effect of genotype on freezing behavior during the FC re-exposure test the next day (Males: t_{17} = 0.1963, p = 0.8467, Females: t_{14} = 0.7498, p = 0.4658). Data are presented as mean + SEM. *p < 0.05, ***p < 0.001.

Repeated multimodal stress (RMS) results in transient *Gilz* induction.

Even more than acute stressors, repeated or chronic exposure to stress constitutes a major risk factor for the development of substance use disorders[201,202]. Furthermore, acute stimuli sometimes considered mild stressors (such as the single foot shock protocol used above) sometimes lack etiological relevance to the complex stressors endured outside of the laboratory setting. In an effort to circumvent these caveats, we collaborated with the laboratory of Dr. Gyuri Lur to utilize a repeated multimodal stressor (RMS) protocol wherein mice are exposed to a variety of concurrent stimuli (*i.e.*, social crowding, physical restraint, loud noise, visual stimuli) for ten days[203]. This protocol leads to robust increases in blood corticosterone, alterations in synaptic connectivity, and short- and long-term memory deficits[205-207]. The effects of RMS are generally more widespread and robust than those produced by comparable unimodal stressors, making RMS a valuable model for beginning to study a possible role for *Gilz* in comorbid stress and addiction[206,208].

Male and female mice were exposed to the RMS protocol by members of the Lur Lab and sacrificed 1-hour after the termination of the final RMS session to assess *Gilz* expression compared to age-matched controls. Here, we examined tissue from the PFC, BLA, and DHC, as these regions are critical for the integration of GC- and drug-related

plasticity. Furthermore, we examined tissue from the posterior parietal cortex, a brain region largely responsible for integration of sensory data that reliably undergoes altered connectivity following RMS[207]. In females, we found RMS-induced upregulation of *Gilz*-1 in all four brain regions (Fig. 5.2A-D, PFC: $t_8 = 8.354$, $p < 0.0001$. BLA: $t_8 = 10.49$, $p < 0.0001$. DHC: $t_7 = 5.205$, $p = 0.0012$. PPC: $t_8 = 4.679$, $p = 0.0016$). *Gilz*-2 upregulation was observed in the PFC, BLA, and DHC of females (Fig. 5.2A-D, PFC: $t_8 = 4.004$, $p = 0.0037$. BLA: $t_8 = 3.755$, $p = 0.0056$. DHC: $t_7 = 3.973$, $p = 0.0054$).

In males, we also observed RMS-induced upregulation of *Gilz*-1 in all four brain regions (Fig. 5.2E-H, PFC: $t_8 = 14.94$, $p < 0.0001$. BLA: $t_8 = 14.24$, $p < 0.0001$. DHC: $t_8 = 9.446$, $p < 0.0001$. PPC: $t_9 = 2.660$, $p = 0.0260$). *Gilz*-2 upregulation patterns in males were also similar to females (Fig. 5.2E-H, PFC: $t_8 = 2.609$, $p = 0.0312$. BLA: $t_8 = 9.185$, $p < 0.0001$. DHC: $t_8 = 5.332$, $p = 0.0007$). Interestingly, *Gilz*-5 was downregulated in the PFC in response to RMS (Fig. 5.2E, $t_9 = 2.571$, $p = 0.0302$). These results suggest that *Gilz* splice variants undergo similar patterns of transcriptional regulation in GC- and reward-related brain regions in both sexes.

Prior studies have reported conflicting findings related to the time course of GC-induced *Gilz* regulation in the brain, with Yachi *et al*. (2007) observing a return to baseline 4 hours after water restraint stress and Lebow *et al*. (2019) observing long term changes concurrent with decreased methylation at the *Gilz* promoter[132,173]. Because RMS leads to long term changes in both gene expression and behavior, we next sought to examine whether RMS-induced changes to *Gilz* are maintained at 24-hours post-manipulation[207,208] A separate cohort of RMS-exposed males were sacrificed one day after the termination of RMS and compared to age-matched controls. We observed no

differences in *Gilz*-1, -2, or -5 expression in all brain regions (Fig. 5.2I-L). These results indicate that RMS-induced changes in *Gilz* expression are transient and subside within a day of removal from RMS.

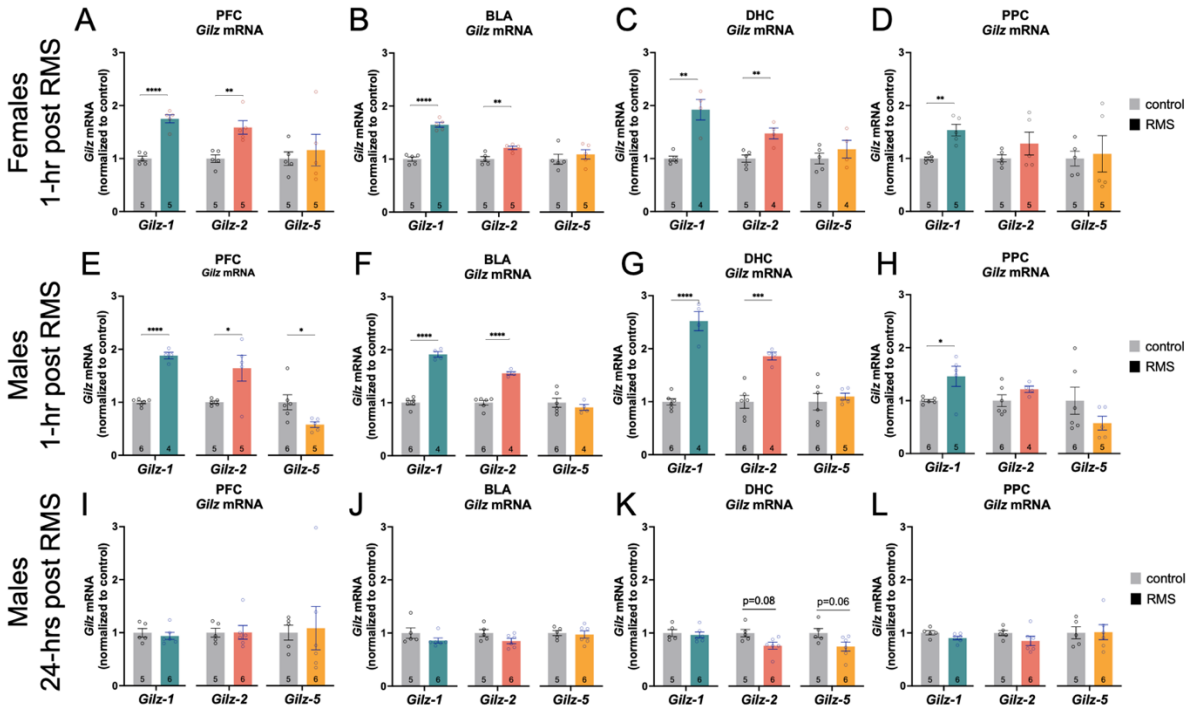

Figure 5.2: Repeated multimodal stress (RMS) leads to differential *Gilz* expression in brain regions associated with stress, reward, and sensory integration. (**A-D**) In female brains collected 1-hr after the final RMS session, we found RMS-induced upregulation of *Gilz*-1 in all four brain regions (PFC: $t_8 = 8.354$, $p < 0.0001$. BLA: $t_8 = 10.49$, $p < 0.0001$. DHC: $t_7 = 5.205$, $p = 0.0012$. PPC: $t_8 = 4.679$, $p = 0.0016$). *Gilz*-2 upregulation was observed in the PFC, BLA, and DHC (PFC: $t_8 = 4.004$, $p = 0.0037$. BLA: $t_8 = 3.755$, $p = 0.0056$. DHC: $t_7 = 3.973$, $p = 0.0054$). (**E-H**) In male brain collected 1-hr after the final RMS session, we also observed RMS-induced upregulation of *Gilz*-1 in all four brain regions (PFC: $t_8 = 14.94$, $p < 0.0001$. BLA: $t_8 = 14.24$, $p < 0.0001$. DHC: $t_8 = 9.446$, $p < 0.0001$. PPC: $t_9 = 2.660$, $p = 0.0260$). *Gilz*-2 upregulation patterns in males were also similar to females (PFC: $t_8 = 2.609$, $p = 0.0312$. BLA: $t_8 = 9.185$, $p < 0.0001$. DHC: $t_8 = 5.332$, $p = 0.0007$). *Gilz*-5 was downregulated in the PFC in response to RMS ($t_9 = 2.571$, $p = 0.0302$). (**I-L**) In male brains collected 1-day after the final RMS session, we observed no significant effects of RMS on *Gilz* expression in any brain region. Data are presented as mean + SEM. $^*p < 0.05$, $^{***}p < 0.001$.

Ten repeated foot shocks result in differential regulation of *Gilz* splice variants in the BLA.

Thus far, we have detected variant-specific transcriptional regulation of *Gilz* following both a single foot shock and repeated multimodal stress. The findings in Lebow *et al*. (2019) described above led us to examine *Gilz* expression patterns in response to repeated foot shocks as a third set of stimuli[173]. This session of repeated foot shocks, developed by Rau & Faneslow (2009), leads to enhanced, maladaptive fear responses and has been used to model aspects of PTSD[209]. Notably, these foot shocks are used in combination with later foot shocks in the stress-enhanced fear learning (SEFL) model of PTSD, but it is used alone in the present study as a means of understanding the acute effects of repeated foot shocks on *Gilz* expression. Because the effects of chronic (RMS) stress on *Gilz* expression were transient, we chose to examine brains at 1-hr and 3-hrs post-shock session (Figure 5.3A).

In both males and females, we observed repeated foot shock-induced *Gilz*-1 expression that persisted 3 hours (Fig. 5.3B-C, Males: main effect of repeated foot shock: $F_{2,9} = 12.17$, $p = 0.0028$. Dunnett's multiple comparisons: context vs 1-hr, $q_9 = 4.552$, $p = 0.0026$, context vs 3-hr, $q_9 = 3.739$, $p = 0.0086$. Females: main effect of repeated foot shock: $F_{2,9} = 16.12$, $p = 0.0011$. Dunnett's multiple comparisons: context vs 1-hr, $q_9 = 4.559$, $p = 0.0026$, context vs 3-hr, $q_9 = 5.179$, $p = 0.0011$). In females, repeated foot shock-induced *Gilz*-2 expression also persisted 3 hours (Fig. 5.3C, Main effect of repeated foot shock: $F_{2,9} = 8.717$, $p = 0.0078$. Dunnett's multiple comparisons: context vs 1-hr, $q_9 = 2.784$, $p = 0.0387$, context vs 3-hr, $q_9 = 4.029$, $p = 0.0056$). Interestingly, in males, we observed repeated foot shock-induced downregulation of *Gilz*-5 that persisted 3 hours (Fig. 5.3B, Main effect of repeated foot shock: $F_{2,9} = 9.943$, $p = 0.0053$. Dunnett's multiple comparisons: context vs 1-hr, $q_9 = 4.17$, $p = 0.0045$, context vs 3-hr, $q_9 = 3.413$,

p = 0.0141). In addition to being another form of foot shock-induced *Gilz* regulation, these results represent another context in which *Gilz*-1 upregulation is accompanied by concurrent *Gilz*-5 downregulation, further highlighting the importance of considering splice variant-specificity.

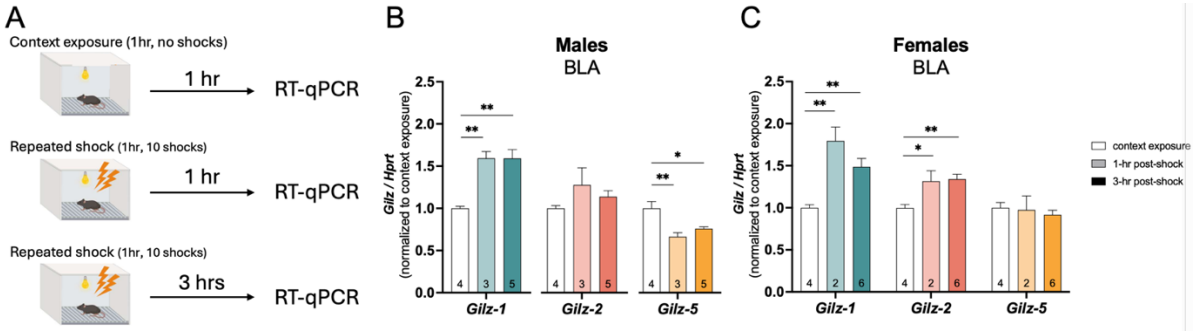

Figure 5.3: Repeated foot shocks lead to differential *Gilz* regulation in BLA. (**A**) Mice received either a context-exposure as control, or 10 repeated unpredictable foot shocks over the span of an hour. Brains were collected either 1-hr or 3-hrs after the end of the session for RT-qPCR. (**B**) In males, we observed repeated foot shock-induced *Gilz*-1 expression that persisted 3 hours (Males: main effect of repeated foot shock: $F_{2,9}$ = 12.17, p = 0.0028. Dunnett's multiple comparisons: context vs 1-hr, q_9 = 4.552, p = 0.0026, context vs 3-hr, q_9 = 3.739, p = 0.0086). We observed repeated foot shock-induced downregulation of *Gilz*-5 that persisted 3 hours (Main effect of repeated foot shock: $F_{2,9}$ = 9.943, p = 0.0053. Dunnett's multiple comparisons: context vs 1-hr, q_9 = 4.17, p = 0.0045, context vs 3-hr, q_9 = 3.413, p = 0.0141). (**C**) In females, we observed repeated foot shock-induced *Gilz*-1 expression that persisted 3 hours (Females: main effect of repeated foot shock: $F_{2,9}$ = 16.12, p = 0.0011. Dunnett's multiple comparisons: context vs 1-hr, q_9 = 4.559, p = 0.0026, context vs 3-hr, q_9 = 5.179, p = 0.0011). Repeated foot shock-induced *Gilz*-2 expression also persisted 3 hours (Main effect of repeated foot shock: $F_{2,9}$ = 8.717, p = 0.0078. Dunnett's multiple comparisons: context vs 1-hr, q_9 = 2.784, p = 0.0387, context vs 3-hr, q_9 = 4.029, p = 0.0056). Data are presented as mean + SEM. *p < 0.05, ***p < 0.001.

Gilz^{KO} males do not exhibit repeated foot shock-related disruptions in cocaine-CPP acquisition.

Because prior exposure to severe stressors is associated with increased frequency and severity of SUDs, and roughly 80% of patients diagnosed with post-traumatic stress disorder (PTSD) go on to develop comorbid SUD symptoms, we next sought to examine whether repeated foot shock-induced transcriptional regulation of *Gilz* contributes to adaptations in cocaine-associated behaviors[9-11]. As before, while we refrain from classifying this manipulation as trauma, many studies have demonstrated the validity in

using repeated foot shocks to model aspects of trauma[209]. Naïve *Gilz*^{KO} males and WT littermates were placed in our fear conditioning chambers with either the presence or absence of our repeated foot shock protocol before undergoing a modified version of cocaine-CPP (Figure 5.4A). To account for heightened anxiety following repeated foot shocks, all mice were habituated to the CPP chambers before an eventual pre-test (Fig. 5.4B). Following 4 days of counter-conditioning with 5 mg/kg cocaine, mice were administered a post-test to assess CPP acquisition. We observed a main effect of cocaine-conditioning (Fig.5.4C, $F_{1,28}$ = 66.94, p < 0.0001). We observed a significant difference in post-test scores between WT mice that received repeated foot shocks vs context exposure (Fig.5.4C, Tukey's multiple comparisons: q_{56} = 3.937, p = 0.0357). This difference was not present when comparing post-test scores of *Gilz*^{KO} mice that received repeated foot shocks vs context exposure (Fig.5.4C, Tukey's multiple comparisons: q_{56} = 2.669, p = 0.2452). These results suggest that WT mice subject to repeated foot shocks exhibit altered CPP behavior as compared to *Gilz*^{KO} mice.

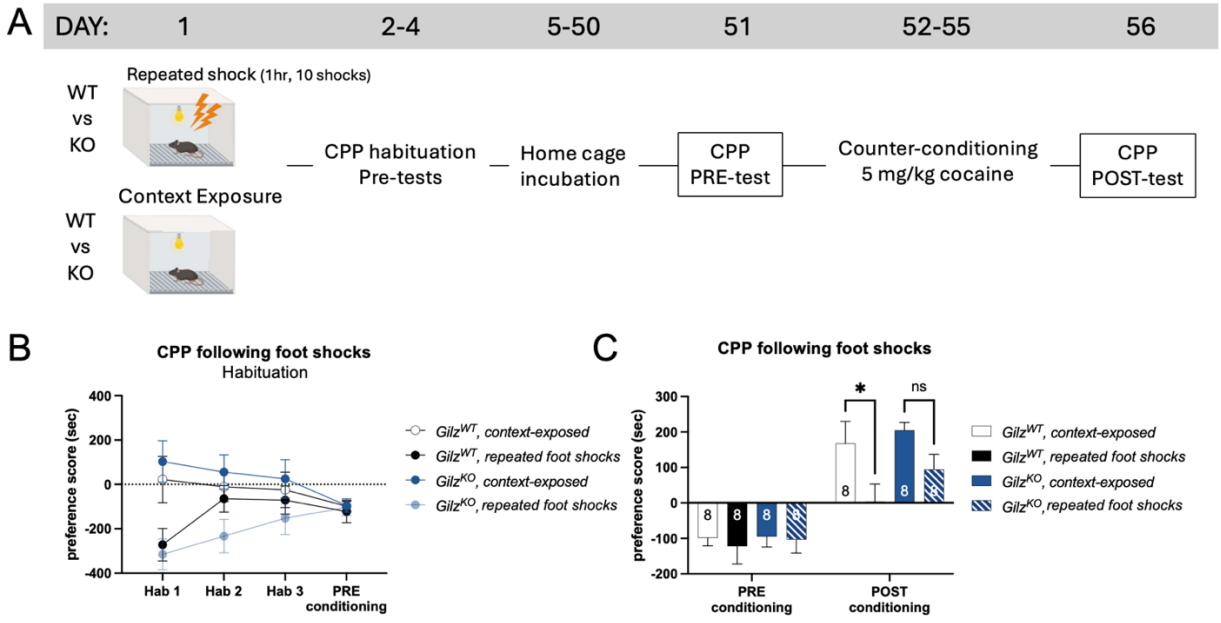

Figure 5.4: Repeated foot shocks blunt CPP acquisition in *Gilz*^{WT} but not *Gilz*^{KO} males. (**A**) *Gilz*^{KO} males and WT littermates received either a context-exposure as control, or 10 repeated unpredictable foot shocks over the span of an hour. Mice were then habituated to the CPP apparatus over the course of 3 once-daily sessions. Following a home cage period, mice were administered a final pre-test before receiving 4 days of counter-conditioning with cocaine (5 mg/kg) or saline i.p. injections and a final post-test. (**B**) Habituation behavior displayed across days illustrates a convergence between groups. Final pre-test scores are similar across groups (1-way ANOVA, $F_{3,28} = 0.1119$, $p = 0.9524$). (**C**) CPP scores. We observed a main effect of cocaine-conditioning ($F_{1,28} = 66.94$, $p < 0.0001$). We observed a significant difference in post-test scores between WT mice that received repeated foot shocks vs context exposure (Tukey's multiple comparisons: $q_{56} = 3.937$, $p = 0.0357$). This difference was not present when comparing post-test scores of *Gilz*^{KO} mice that received repeated foot shocks vs context exposure (Tukey's multiple comparisons: $q_{56} = 2.669$, $p = 0.2452$). Data are presented as mean + SEM. *$p < 0.05$, ***$p < 0.001$.

Discussion:

Here we present initial evidence that a single foot shock, repeated multimodal stress, and repeated foot shocks differentially regulate *Gilz* splice variants in distinct brain regions. Furthermore, we show how disrupting GC-associated regulation of *Gilz* in the brain may affect the downstream mechanisms engaged by cocaine in the development of maladaptive behaviors. Specifically, we show that a single foot shock, repeated multimodal stress, and repeated foot shocks all engage *Gilz* in the BLA of males and females. We also further demonstrate the value of considering splice variant-specificity when studying genetic responses to environmental stressors or drugs, as *Gilz* splice variants are differentially regulated by the various stimuli used in these studies.

Consistent with our earlier findings showing divergent transcriptional regulation of *Gilz*-1 vs *Gilz*-5 following KCl treatment (see Chapter 1), here we also observed that *Gilz*-1 upregulation by these various stimuli was often associated with concurrent *Gilz*-5 downregulation. As such, these bidirectional effects may suggest that the same HDAC3-related mechanisms of *Gilz* regulation observed *in vitro* are also engaged *in vivo* by the stimuli administered in these studies. Furthermore, our bidirectional findings suggest opposing roles for GILZ-1 and GILZ-5 in these contexts. Both isoforms contain the leucine zipper functional domain required for homo- or hetero-dimerization, while GILZ-5 lacks

the N-terminal domain specific to GILZ-1:AP-1 interactions[140,145]. Thus, GILZ-5 may act via heterodimerization to inhibit the activity of GILZ-1, and *Gilz*-5 downregulation in response to stress may reflect a form of transcriptional adaptation that potentiates the effects of *Gilz*-1 upregulation. However, as the current studies are limited to examining changes in *Gilz* mRNA, future work may probe the downstream functional effects associated with these changes.

Both a single and repeated foot shocks lead to robust *Gilz*-1 induction in the BLA, and while we observed no significant effects of $Gilz^{KO}$ on fear conditioning behavior, our later CPP results indicate that *Gilz* may be necessary for orchestrating the effects of repeated foot shocks on CPP acquisition. However, an important caveat to these CPP findings should be noted: we initially hypothesized that prior exposure to repeated foot shocks would result in potentiated, rather than disrupted, CPP acquisition. This hypothesis was derived from literature demonstrating how stress activates reward circuitry to result in enhanced acquisition of cocaine-CPP[211,212]. Nonetheless, while much existing literature utilizes either acute or repeated forms of stress in this context, we have employed a relatively understudied model, which engages both overlapping and nonoverlapping mechanisms in the brain[209]. Since we observe that $Gilz^{KO}$ mice do not exhibit the same foot shock-disrupted CPP acquisition, it may be the case that *Gilz* (and *Gilz*-1 in the BLA particularly) represents a neural substrate for carrying out the effects of repeated foot shock that are later re-engaged during exposure to cocaine.

Taken together, these studies demonstrate that the effects of various forms of environmental stressors on *Gilz* expression in the brain are both context- and variant-

specific, thus highlighting the need for future studies to consider the distinct roles of GILZ isoforms in the context of exposure to stress and/or drugs of abuse.

Conclusions:

Drugs of abuse induce long-term, stable changes in cellular functions underlying maladaptive behavioral consequences. These changes leave individuals vulnerable to cue- or stress-induced relapse in drug use, even after prolonged cessation. Drugs of abuse, such as cocaine, have been shown to orchestrate these changes in cell function by alterations in the epigenetic landscape. In particular, the histone deacetylase enzyme HDAC3 is a powerful regulator of transcriptional adaptations to cocaine. Among the list of key HDAC3-target genes with well-established roles in orchestrating plasticity (i.e., *Egr-1, Nr4a1, Per1*) *Gilz* represents an understudied gene that may act at the interface of glucocorticoid- and dopamine-signaling. The work in this dissertation presents evidence that distinct *Gilz* mRNA splice variants in the brain respond to stress-related events and that *Gilz*'s role in regulating synaptic plasticity in the NAc may underlie its cellular contributions to reinstatement of cocaine-seeking behavior in males.

Throughout this dissertation we present key data to support this conceptual framework implicating *Gilz* in both stress- and drug-responses. In **Chapter 1**, we demonstrate that *Gilz* splice variants in both males and females are expressed in key brain regions known to regulate the effects of glucocorticoids and cocaine. These findings, along with data showing HDAC3 at the *Gilz* promoter differentially regulates *Gilz*-1 vs *Gilz*-5, led us to perform a series of studies to assess the role of *Gilz* splice variants in responses to cocaine or stress exposure. In **Chapter 2**, we demonstrate how acute knockdown of *Gilz* in the NAc of males, but not females, leads to impairments in NAc long-term potentiation. Not only was this impairment replicated in $Gilz^{KO}$ males, but viral overexpression of the *Gilz-1* variant rescued this phenotype. This led to the hypothesis

that *Gilz* may act in reward-related brain regions to regulate plasticity underlying behavioral adaptations to cocaine. In **Chapter 3**, we demonstrate that *Gilz* in the VTA of males is necessary for the acquisition of cocaine-CPP. Interestingly, *Gilz* is not necessary in the NAc under these experimental conditions, leading us to examine whether the PFC-NAc pathway (as examined in our LTP experiments) recruits *Gilz* during reinstatement of cocaine-seeking behavior, as this pathway has been implicated in related studies. In **Chapter 4**, we demonstrate that *Gilz* is necessary for reinstatement of cocaine-IVSA behavior and that *Gilz-1* overexpression leads to enhanced acquisition. Finally, in **Chapter 5**, we provide initial evidence that *Gilz* operates in a splice variant-specific manner to establish stress-related neuroadaptations that are later recruited following exposure to cocaine.

What remains unclear at this time is whether *Gilz* acts within distinct brain regions and/or distinct cell types to regulate reinstatement of cocaine-seeking, and whether GILZ protein isoforms differentially regulate transcriptional profiles necessary for long-term behavioral adaptations. Furthermore, whether these findings are indeed unique to males remains to be seen in light of our present limitations. Additional studies should and will be conducted to further elucidate the molecular targets of GILZ isoforms. However, we speculate that GILZ-1 is a robust transcriptional regulator that interacts with its known binding partners (*i.e.*, NF-kB and AP-1) to regulate their cocaine-responsive activity, while GILZ-5 exhibits inhibitory effects on this GILZ-1 activity by heterodimerization.

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