Prefrontal excitatory/inhibitory balance in stress and emotional disorders: Evidence for
over-inhibition

Dissertation

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Abstract

Chronic stress-related emotional disorders like anxiety and depression involve imbalances between the excitatory glutamatergic system and the inhibitory γ -aminobutyric acid (GABA) system in the prefrontal cortex (PFC). However, the precise nature and trajectory of excitatory/inhibitory (E/I) imbalances in these conditions is not clear, with the literature reporting glutamatergic and GABAergic findings that are at times contradictory and inconclusive. In this dissertation, I propose and discuss the hypothesis that chronic stress-induced anxiety involves hypoactivity of the PFC due to increased inhibition, mediated in part through increased activity of prefrontal parvalbumin (PV)-expressing inhibitory interneurons. Differing sensitivity of the prefrontal GABAergic system and PV neurons to chronic stress may also underlie sex differences in the prevalence of anxiety disorders, with women presenting with anxiety at higher rates than men. I first present evidence that chronic stress increases the activity of prefrontal PV neurons, and that increased activity of this cell population induces hypoactivity of the PFC. Furthermore, increasing prefrontal PV cell activity using DREADDs (designer receptors exclusively activated by designer drugs) induces anxiety-like behaviors specifically in females (**Chapter 2**). However, acute inhibition of prefrontal PV cells using DREADDs is insufficient to rescue stress-induced anxiety-like behavior, though it does modulate activity and synaptic plasticity in the PFC in a sex-

dependent manner (**Chapter 3**). Furthermore, manipulating the prefrontal GABAergic system during chronic stress can incur vulnerability or resilience to anxiety. Pharmacological enhancement of GABAergic transmission with the GABA positive allosteric modulator lorazepam during chronic stress exposure exacerbates prefrontal inhibition and anxiety-like behaviors in female mice (**Chapter 4**). Lastly, transgenic female mice deficient for the potassium channel Kv3.1, which have reduced activity of fast-spiking neurons like PV cells, show resilience against the anxiogenic effects of chronic stress (**Chapter 5**). Taken together, these results support the hypothesis that increased prefrontal inhibition, mediated at least in part through increased activity of PV interneurons, is causally related to increased anxiety-like behavior in female mice following chronic stress exposure. This hypothesis bears on our understanding of prefrontal E/I imbalances in stress-related conditions, especially with regards to sex differences, and provides insight into new therapeutic approaches for anxiety disorders.

Dedication

To Uncle Gene Klaaren, who always knew I could do it.

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Fields of Study

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Chapter 1. Introduction

Following the publication of two of the most influential books in the field of biology: *On the Origin of Species* (1859) and *The Descent of Man* (1871), Charles Darwin published *The Expression of the Emotions in Man and Animals* (1872). This writing informed many later works about the biological basis of stress and emotion, including Walter Cannon's sympathetic nervous system "fight or flight response" and James Papez's limbic circuitry. Darwin himself writes about the connection between exposure to stress and experiencing "low spirits". He writes: "*After the mind has suffered from an acute paroxysm of grief, and the cause still continues, we fall into a state of low spirits; or we may be utterly cast down and dejected [...] If we expect to suffer, **we are anxious**; if we have no hope of relief, **we despair***" (emphases are mine) (Darwin, 1872).

These words from Darwin are perhaps one of the earliest references to the relationship between chronic stress exposure and affective neuropsychiatric disorders like anxiety and depression. One in four Americans report experiencing some form of extreme stress (Anderson et al., 2016). Over 1 in 4 Americans have also experienced or will develop an affective neuropsychiatric disorder by the end of their life (Kendler et al., 1999). This extremely high prevalence occurs in part because, as Darwin proposed in 1872, chronic stress exposure is a major risk factor for the development of anxiety and depression (Kendler et al., 1999; Duman and Monteggia, 2006; Mineur et al., 2006).

Biological sex impacts this relationship between stress exposure and emotional dysfunction. In comparison to men, women are at an increased risk for developing anxiety or depression and also show higher rates of co-morbidity between these disorders (de Graaf et al., 2002; Wittchen et al., 2011; Kessler et al., 2012; Bale and Epperson, 2015). Understanding the mechanistic connections between stress and affective disorders like anxiety and depression, especially with regards to sex differences, is critical for developing effective treatments for these pervasive disorders.

The research presented in this dissertation focuses on the prefrontal cortex (PFC), a brain region essentially involved in the stress response and emotional regulation. Increasing evidence suggests that the balance between excitatory and inhibitory neurotransmission in the PFC is important for emotional behaviors and is affected by stress. This dissertation focuses primarily on the inhibitory side of excitatory/inhibitory (E/I) balance in the PFC, specifically the inhibitory γ -aminobutyric acid (GABA) system and its sex-specific role in the stress response and emotional behaviors. To introduce this research, I will begin with a discussion of the prefrontal GABAergic system in the context of a balance between excitatory and inhibitory neurotransmission. This will be followed by a review of the current literature about how E/I balance is perturbed by chronic stress exposure, including sex differences. Finally, I will introduce the series of experiments performed in this dissertation to investigate the sex-specific role of the GABAergic system in the stress response and emotional behaviors, concluding with a hypothesis of E/I imbalance and over-inhibition in stress-induced anxiety.

The prefrontal cortex and excitatory/inhibitory balance

The PFC is involved in emotional expression and regulation (Dixon et al., 2017) and cognitive behaviors (Fuster and Bressler, 2015). The PFC is also highly stress-responsive (Arnsten, 2009; McEwen et al., 2016). The rodent medial PFC (mPFC) is subdivided into three subregions: the anterior cingulate cortex (ACC), prelimbic (PrL) cortex, and infralimbic (IL) cortex. In the human brain, these subregions are thought to be homologous to Brodmann areas 24b, 32, and 25, respectively (McKlveen et al., 2015). The PrL and IL cortices in particular show high connectivity with limbic regions of the brain such as the nucleus accumbens and amygdala and therefore play a substantial role in modulating emotional behaviors (Adhikari et al., 2015; Wood et al., 2019). The subregions of the PFC have distinct functional connectivity with other brain regions and thus differing roles in neuropsychiatric disorders (Heidbreder and Groenewegen, 2003).

The functioning of the PFC relies on a balance between excitatory and inhibitory neurotransmission. This excitatory/inhibitory (E/I) balance arises from a complex interplay of heterogeneous glutamatergic and GABAergic neurons interacting within a local circuit. In particular, GABAergic interneurons that express the calcium-binding protein parvalbumin (PV) are a major regulator of E/I balance as they represent more than 50% of the total population of GABAergic interneurons in the frontal cortex and provide strong, fast-spiking inhibitory control near or on the cell bodies of excitatory pyramidal cells (Kubota et al., 1994; Ferguson and Gao, 2018a). Consequently, PV cells play an important role in prefrontal-dependent emotional and cognitive behaviors (Ferguson and Gao, 2018a).

E/I tone is also set by other inhibitory interneurons in the PFC. Somatostatin (SST)-expressing cells provide modulatory inhibitory inputs by targeting the dendrites of pyramidal neurons (Tremblay et al., 2016). SST cells also inhibit PV cells (Pfeffer et al., 2013). Cells expressing the ionotropic serotonin receptor 5HT3a (5HT3aR) also represent a heterogeneous class of inhibitory interneurons, some of which also express vasoactive intestinal peptide (VIP). VIP neurons preferentially inhibit other interneurons, including SST and PV cells (Hensch, 2014; Tremblay et al., 2016). SST and 5HT3aR neurons can co-express other inhibitory interneuron markers like calretinin (CR), cholecystokinin (CCK), and neuropeptide Y (NPY) (Tremblay et al., 2016). Together, PV, SST, and 5HT3aR cells account for nearly all of neocortical GABAergic interneurons (Rudy et al., 2011). Inhibitory interneurons also receive excitatory input locally from pyramidal cells or from other parts of the cortex or subcortical regions (Hattori et al., 2017). The result is a complex logic circuit of excitatory, inhibitory and dis-inhibitory connections that together modulate E/I balance and function in the PFC.

Dysregulations in prefrontal E/I balance have frequently been implicated in neuropsychiatric conditions such as schizophrenia, autism spectrum disorders, and intellectual disabilities. Most of these disorders are thought to involve deficient inhibitory control, largely mediated by deficiencies in PV-expressing GABAergic interneurons. On that subject, readers are referred to several comprehensive review articles about the psychopathology of reduced PV cell activity and over-excitation in prefrontal circuits (Yizhar et al., 2011; Nelson and Valakh, 2015; Ferguson and Gao, 2018a; Selten et al., 2018). The other end of the E/I spectrum – over-inhibition and reduced circuit activity –

has not been as extensively studied. Increasing evidence suggests that the PFC is hypoactive in stress-induced affective disorders like anxiety and depression, and that the GABAergic system and PV cells play an important role in the stress response and emotional dysfunction (Maguire, 2014; McKlveen et al., 2016; Shepard et al., 2016; Shepard and Coutellier, 2018). Given the well-established link between chronic stress exposure and neuropsychiatric disorders like depression and anxiety (Kendler et al., 1999; Duman and Monteggia, 2006; Mineur et al., 2006), understanding the mechanistic connection between stress and emotional dysfunction is critical to developing effective treatments for these disorders.

Development of prefrontal E/I balance

In order to understand E/I imbalances that occur in stress and emotional disorders, it is helpful to first examine how E/I balance in the PFC develops. Prefrontal E/I balance is established largely during adolescence through maturation of the GABAergic system. Some of the mechanisms involved in this process may provide insight into how E/I balance is disrupted in stress-induced affective disorders, and may also provide insight into novel therapeutic approaches for rebalancing glutamatergic and GABAergic transmission in stress-induced affective disorders.

Postnatal life is characterized by critical periods of plasticity in which brain regions show heightened activity-dependent sensitivity to experience with long-lasting effects. Like other young brain regions, the immature PFC is hyper-excitabile and plastic. As development proceeds, the prefrontal GABAergic system matures to introduce more

inhibitory control and stability into the network (Bavelier et al., 2010; Le Magueresse and Monyer, 2013) (Figure 1). The mechanisms for increased network stability are numerous (Takesian and Hensch, 2013), but they include increased glutamatergic inputs onto prefrontal PV interneurons that enables greater inhibitory control over pyramidal cells (Caballero et al., 2016). PV immunoreactivity and mRNA/protein expression increases during juvenile and adolescent development (Caballero et al., 2014a; Ueda et al., 2015; Ueno et al., 2017). In mice, *PV* gene expression in the PFC peaks and subsequently plateaus around postnatal day (PND) 35 (Ueda et al., 2015). CR expression correspondingly decreases during adolescence. Given that CR neurons likely exert inhibitory control over other interneurons like PV cells, adolescent downregulation of CR expression may facilitate dis-inhibition of PV cells (Caballero et al., 2014a). GABAergic maturation also includes increases in perineuronal net (PNN) density (Takesian and Hensch, 2013). PNNs are extracellular matrices of proteins that preferentially surround PV cells and enhance their excitability (Balmer, 2016). In the visual cortex, PNNs increase inhibitory GABAergic currents during the critical period of plasticity (Liu et al., 2013). In the PFC, PNN density peaks around PND 28 (Baker et al., 2017; Ueno et al., 2017). Increased PV cell activity and PNN density is associated with the closing of critical periods of plasticity (Umemori et al., 2015) and the functional maturation of brain regions like the PFC. In adulthood, PV cells continue to play a major role in regulating the inhibitory side of E/I balance and thus in regulating prefrontal-dependent cognitive and emotional functions (Ferguson and Gao, 2018a). Gene expression of other GABAergic system markers, like *GAD1*, *SST*, and *VIP*, do not change significantly

during adolescent development of the PFC (Ueda et al., 2015), but still contribute to functional E/I balance in adulthood.

Though much of the work described above was conducted in male rodents, biological sex is a highly relevant factor for the mechanisms of E/I maturation during adolescence. Ovariectomy prior to puberty onset blocks the increase in inhibitory neurotransmission in the frontal cortex of female mice, and pre-pubertal hormone treatment accelerates GABAergic maturation (Piekarski et al., 2017). The adolescent increase in PV protein expression and cell counts appears to occur earlier in females than in males (Du et al., 2018). At least in the hippocampus, an adolescent increase in PV expression in females is estradiol-dependent (Wu et al., 2014), though it is unknown whether this is also true for the PFC. Adolescent development of E/I balance also involves pruning of excitatory synapses in the PFC (Hoftman and Lewis, 2011; Petanjek et al., 2011). Loss of prefrontal excitatory synapses during adolescence is more pronounced in females according to rodent studies (Koss et al., 2014; Drzewiecki et al., 2016), and greater thinning of the PFC during adolescence correlates with increased emotional regulation abilities in women (Vijayakumar et al., 2014). In addition to synaptic pruning, loss of non-GABAergic cells also occurs in the PFC during adolescence (Markham et al., 2007; Willing and Juraska, 2015). Neuronal loss is more pronounced in females during this developmental period due to the effect of ovarian hormones (Koss et al., 2015).

Prefrontal E/I balance in emotional disorders and chronic stress

Chronic stress in adulthood perturbs mature E/I balance in the PFC and contributes to emotional disturbances like those in anxiety and depression (Wieronska et al., 2011; Lener et al., 2017), though the precise nature and time course of this imbalance is far from clear. As is evident from research on the topic, there are conflicting and occasionally paradoxical results about glutamatergic and GABAergic dysfunction in humans with emotional disorders (Table 1) and animal models of chronic stress and emotional dysfunction (Table 2). It is important to note that the majority of studies reviewed here focus on the PFC as a whole or report similar results across subregions. Subregions of the PFC are thought to play distinct roles in neuropsychiatric disorders and emotional behaviors (Heidbreder and Groenewegen, 2003; Adhikari et al., 2015), but at present there is insufficient evidence to draw conclusions about the nature of prefrontal E/I imbalances in stress and emotional disorders according to subregion.

Model	Sex	Excitation-related Findings	Inhibition-related Findings	Behavioral Phenotype	Reference
Human Studies					
Post-mortem tissue from clinically depressed patients	unspecified	↓ immediate early gene expression	Not tested	N/A	(Covington et al., 2010)
Post-mortem tissue from clinically depressed patients	Men and women (not analyzed by sex)	↓ NR2A, NR2B, and PSD-95 expression	Not tested	N/A	(Feyissa et al., 2009)
Post-mortem tissue from clinically depressed patients	Men and women (not analyzed by sex)	Not tested	↓ GAD67 expression	N/A	(Karolewicz et al., 2010)
Magnetic resonance spectroscopy (MRS) in major depression patients	Men and women (not analyzed by sex)	↓ glutamate/ glutamine levels	↓ GABA levels	Met DSM-IV criteria for current major depressive episode, high score on depression rating scale	(Hasler et al., 2007)
MRS in panic disorder patients	Women	Not tested	↓ GABA levels	High scores on panic disorder severity scale and state anxiety level test	(Long et al., 2013)
MRS in healthy subjects	Men and women	No correlation with trait anxiety	↑ GABA levels correlated with ↑ trait anxiety; no sex differences	Trait anxiety measurements	(Delli Pizzi et al., 2016)
MRS in PTSD patients	Men and women (not analyzed by sex)	Not tested	↑ GABA levels	High score on PTSD measurement scale	(Michels et al., 2014)
Post-mortem tissue from clinically depressed patients	Men and women (not analyzed by sex)	Not tested	↓ SST expression	N/A	(Sibille et al., 2011)
Post-mortem tissue from clinically depressed patients	Men and women (not analyzed by sex)	Not tested	↓ CB cell counts	N/A	(Rajkowska et al., 2007)

Table 1. E/I markers in the adult prefrontal cortex of humans.

MRS: magnetic resonance spectroscopy; NR: NMDA receptor; PSD-95: post-synaptic density; GAD67: glutamate decarboxylase; SST: somatostatin; CB: calbindin; DSM: Diagnostic and Statistical Manual of Mental Disorders; PTSD: post-traumatic stress disorder; N/A: not applicable

Model	Sex	Excitation-related Findings	Inhibition-related Findings	Behavioral Phenotype	Reference
Rodent Studies					
GABA_AR $\gamma 2$(+/-) mice	unknown	↓ glutamate receptor expression ↓ glutamatergic synapses	↓ GABAergic synapses	Anxiety- and depression-like behaviors	(Ren et al., 2016)
Chronic variable stress (14-day) in rats	Males	↑ inhibitory synapses on glutamatergic cells	↑ mIPSC frequency, ↓ glucocorticoid receptor on PV cells	Impaired decision-making	(McKlveen et al., 2016)
Chronic restraint stress (21-day) in rats	Males	↓ dendritic arborization and spine density of pyramidal cells	Not tested	Not tested	(Cook and Wellman, 2004; Radley et al., 2006)
Chronic restraint stress (7-day) in rats	Males and females	Pyramidal cell dendritic branching: males ↓, females ↑	Not tested	Not tested	(Garrett and Wellman, 2009)
Chronic mild stress (9-week) in rats	Males	↑ pyramidal cell excitability	↓ PV, CR, CCK cell counts ↓ mIPSC frequency	Anhedonia, impaired cognition	(Czéh et al., 2018)
Chronic social isolation stress (21-day) in rats	Males	Not tested	↓ PV cell counts	Depression-like behaviors	(Todorović et al., 2019)
Chronic social defeat stress (10-day) in mice	Males	↓ immediate early gene expression	Not tested	Depression-like behaviors, reduced social interaction	(Covington et al., 2010)
Chronic restraint stress (21-day) in mice	Males	Not tested	↑ dendritic arborization of GAD-expressing cells ↓ GAD67-expressing somata ↑ GABA-A $\alpha 1$ expression	Not tested	(Gilabert-Juan et al., 2013)
Chronic unpredictable stress (5-week) in rats	Males	Not tested	↓ GAD67, SST, NPY expression	Not tested	(Banasr et al., 2017)
Social isolation stress (28-day) in mice	Males	↓ immediate early gene and mGluR-1 and -2 expression	Not tested	Anxiety- and depression-like behaviors	(Ieraci et al., 2016)
Social stress (5-day) in rats	Males and females	Females: ↓ pyramidal cell excitability	Not tested	Not tested	(Urban and Valentino, 2017)
Social defeat stress (10-day) in mice	Males	↓ glutamate, glutamine, EAAT2 expression	↓ GABA, GAD1 expression	Depression-like behaviors	(Veeraiah et al., 2014)

Table 2. Prefrontal E/I markers in adult rodent models of emotional dysfunction.

GABA_AR: GABA_A receptor; mGluR-1: metabotropic glutamate receptor; EAAT2: excitatory amino acid transporter; mIPSC: miniature inhibitory post-synaptic current; PV: parvalbumin; CR: calretinin; CCK: cholecystokinin; GAD: glutamate decarboxylase; SST: somatostatin; NPY: neuropeptide Y

The biphasic effects of stress on the glutamatergic system

The literature supports evidence for both increased and decreased glutamatergic transmission in the PFC in the context of chronic stress and affective disorders (Sanacora et al., 2012). Some of the contradictory findings may be attributable to the time course of stress effects (acute vs. chronic). Stress has a biphasic effect on glutamatergic transmission that is mirrored in its effects on dendritic arborization and spine density of pyramidal neurons in the PFC. The effects of stress on glutamatergic transmission in the PFC and on pyramidal cell morphology and excitatory synapse number have been comprehensively reviewed (Popoli et al., 2011; Musazzi et al., 2015). Acute stress increases prefrontal glutamatergic transmission and enhances dendritic complexity and spine density of pyramidal neurons, often accompanied by mild cognitive enhancement (Musazzi et al., 2015). Chronic stress causes dendritic atrophy of these same neurons in the PFC (Cook and Wellman, 2004; Radley et al., 2006). This decrease in dendritic arborization and spine density of prefrontal pyramidal neurons is thought to be one of the mechanisms behind reduced activity and plasticity in the PFC following stress and in affective disorders. Chronic stress reduces activity in the PFC, impairing its function and contributing to emotional dysregulation (Negrón-Oyarzo et al., 2016; Son et al., 2018). PFC tissue from both chronically stressed mice and from clinically depressed human patients display reduced expression of immediate early genes (IEGs), indicative of decreased activity of this brain region (Covington et al., 2010). Magnetic resonance spectroscopy (MRS) studies in depressed patients also reveal decreased glutamate in the PFC (Hasler et al., 2007).

If reduced prefrontal activity is associated with emotional dysfunction, one would expect that increased activity of the PFC would contribute to healthy emotional functioning. Engagement of the PFC is indeed associated with stress resilience and recovery. Increased prefrontal activity during chronic stress appears to promote coping and resilience in human and rodent studies (Vialou et al., 2014; Sinha et al., 2016). Stimulation of the PFC has also been shown to reduce anxiety- and depression-related behaviors in rodents (Covington et al., 2010; Kumar et al., 2013; Fuchikami et al., 2015; Pati et al., 2018; Reznikov et al., 2018). PFC activation is also necessary for the extinction of learned fear (Fucich et al., 2018; Ramanathan et al., 2018) via decreasing activity in the amygdala (Quirk et al., 2003).

The rodent studies cited above were conducted exclusively in male mice, and the human studies did not analyze sex differences. There is increasing evidence to suggest that the PFC of females may respond differently to stress (Wellman et al., 2018). Females show unique, estrogen-dependent patterns of pyramidal cell dendritic remodeling in the PFC and PFC-amygdala connections following chronic stress (Garrett and Wellman, 2009; Shansky et al., 2010). Following chronic stress, males show the above-mentioned dendritic retraction in the PFC, but females do not (or even show dendritic hypertrophy), possibly due to protective effects of estrogen (Wei et al., 2014; Wellman et al., 2018). After a period of rest from stress, dendritic retraction in males recovers but does not change in females. This suggests that in the days following chronic stress, the PFC of females is less plastic than that of males. If this is the case, females may be more susceptible to a “second hit” of a novel stressor: while prior chronic stress exposure

blunts novel acute stress-induced neuronal activation in males, females may have a more exaggerated response to subsequent stressors. Therefore, dendritic retraction may be an adaptive, protective mechanism against initial stress exposure that males employ but females do not, rendering females more susceptible to chronic stress and emotional dysfunction (Moench and Wellman, 2017; Wellman et al., 2018). Supporting the hypothesis that females are more susceptible to a “second hit” of stress, chronically stressed female rats given a rest period show enhanced activity in limbic brain regions following a novel acute stressor compared to their unstressed counterparts. By contrast, male rats showed blunted activity in the PFC and limbic brain regions under the same conditions (Moench et al., 2019). This research implies that plasticity of the PFC following stress is important for resilience and recovery, and that chronic or repeated stress may impair prefrontal plasticity especially in females.

The biphasic effects on glutamatergic transmission in the PFC (an initial increase in stress-evoked glutamate release followed by prefrontal hypoactivity), as well as sex differences inherent in this process, may explain in part the discrepancies in the literature about glutamatergic imbalances in stress and emotional disorders presented in Table 1 and Table 2. Glutamatergic findings will vary depending on factors like whether the stressor is acute or chronic, stress duration, recovery time, and sex of the animal, and genetic vulnerabilities (Figure 2). Collectively, the literature supports the hypothesis that under most chronic stress conditions and emotional dysfunction, activity and plasticity of the PFC is impaired and reduced.

Bidirectional GABAergic dysfunction

The literature on GABAergic dysfunction in stress and affective disorders is inconclusive and at times contradictory, with many studies reporting decreased GABAergic function but some finding evidence for increased inhibition (Table 1 and Table 2). The time course of GABAergic dysfunction has also not yet been comprehensively studied, but similarly to the glutamatergic system, there may be biphasic or even multiphasic effects of stress on the GABAergic system that help explain the bidirectional findings. Many studies report reduced GABA concentrations and markers of the GABAergic system in the stressed and/or anxious and depressed PFC (Ghosal et al., 2017; Fogaça and Duman, 2019). An MRS study of humans with panic disorder found decreased GABA levels in the anterior cingulate cortex and medial PFC (Long et al., 2013), as did an MRS study of major depressive disorder patients (Hasler et al., 2007). In rodent studies, chronic stress also reduces inhibitory transmission in the PFC (Veeraiah et al., 2014; Czéh et al., 2018). Support for the GABAergic deficit hypothesis of stress and affective dysfunction (Lüscher et al., 2011) also comes from studies in mice deficient for the $\gamma 2$ subunit of the GABA_A receptor (GABA_AR), which show anxiety- and depression-like phenotypes along with suppressed GABAergic and glutamatergic system activity (Ren et al., 2016).

By contrast, there are several studies that report findings indicative of increased GABAergic system activity and prefrontal inhibition in conditions of stress and affective dysfunction. Chronic stress increases the frequency of miniature inhibitory postsynaptic currents (IPSCs) in the PFC and increases inhibitory synapses onto glutamatergic cells (McKlveen et al., 2016). Chronic stress also induces dendritic hypertrophy in a

subpopulation of GAD-expressing inhibitory interneurons identified as Martinotti cells, and has bidirectional effects on the expression of certain GABAergic system markers (decreased GAD67 and increased GABA_Aα1 expression) (Gilabert-Juan et al., 2013). These studies were conducted in male rodents. In females, increased prefrontal PV cell activity may be involved in the stress response and emotional behaviors (see the following section, “*Prefrontal parvalbumin cells and chronic stress*”, for further discussion.)

Collectively, the literature about GABAergic dysfunction in chronic stress and affective disorders remains inconclusive. There is strong evidence that the PFC is hypoactive in stress-induced affective disorders like anxiety and depression, and that the GABAergic system plays an important role in the stress response and emotional dysfunction. However, this is alongside numerous reports of GABAergic deficits in human and rodent studies of chronic stress and affective dysfunction. As with the glutamatergic system, GABAergic dysfunction in stress and affective disorders is likely multiphasic and also impacted by external factors such as duration of stress exposure, recovery time, severity, controllability, and predictability (Anisman and Matheson, 2005), as well as internal variables like genetics and biological sex. A critical challenge for stress research in the coming decades will be to map these variables onto the complex landscape of excitatory and inhibitory interactions in the PFC.

Prefrontal parvalbumin cells and chronic stress

A main driver for prefrontal hypoactivity may be increased activity of inhibitory PV interneurons. Chronic stress exposure increases the number of cells that are immunoreactive for PV, as well as PV mRNA expression, predominantly in female mice (Figure 3 and Figure 4) (Shepard et al., 2016; Shepard and Coutellier, 2018). The reported increase in the number of PV-positive cells most likely reflects an activity-dependent increase in PV protein expression levels (Kinney et al., 2006; Filice et al., 2016) that allows for immunohistochemistry detection. Chronic stress also increases the number of PV cells double-labeled with phospho-ERK and increases the number of VGlut1 puncta on PV neurons in females, indicative of enhanced neuronal activity and excitatory input (Shepard and Coutellier, 2018). This immunohistochemical evidence indicates that chronic stress increases PV cell activity in the PFC. This may be a mechanism contributing to prefrontal hypoactivity following chronic stress. A stress-induced elevation in PV cell counts corresponds with a reduced number of PV-negative, cFos-positive cells in the PFC of female, but not male, mice (Shepard et al., 2016) (Figures 3 and 4), suggestive of reduced activity levels in the PFC, given that the majority of total neurons in the PFC are glutamatergic (Granato and De Giorgio, 2014; Tremblay et al., 2016). In male rats, chronic stress increases prefrontal inhibitory currents and decreases glucocorticoid receptor immunoreactivity specifically in PV neurons in the PFC. These findings suggest that chronic stress increases prefrontal inhibition possibly through a loss of a glucocorticoid receptor-mediated brake on PV cell activity (McKlveen et al., 2016). The hypothesis that increased PV cell activity following chronic stress is a

mechanism of prefrontal hypoactivity is supported by electrophysiological studies showing that activation of PV cells increases IPSCs in target pyramidal cells and reduces network excitability (Winkelmann et al., 2014; Safari et al., 2017). At the behavioral level, increased PV expression correlates with increased emotionality behaviors in females but not males (Shepard et al., 2016).

Another study used targeted expression of gain-of-function glycine receptors in PV cells to increase their activity, and found that this decreases network excitability and results in an anxious phenotype (Winkelmann et al., 2014). Increased PV cell activity using the same genetic manipulation also reduces extinction of contextual fear memory (Çaliskan et al., 2016), again suggesting that PV cells represent a cellular substrate for fear and anxiety behaviors. These gain-of-function studies were conducted in male mice and were also not brain-region specific, so more research is needed to parse out the role of PV cells in different brain regions on emotional behaviors in male and female mice.

However, others have reported decreased PV cell counts in the PFC following chronic stress (Czéh et al., 2018; Todorović et al., 2019), countering the hypothesis of increased prefrontal inhibition mediated by prefrontal PV cells. The study by Czéh et al. also reported reduced counts of other GABAergic cells, including CR and CCK, along with increased pyramidal cell excitability. These findings are more in line with hypotheses of GABAergic deficits in stress and affective disorders. When attempting to reconcile these findings, it is important to consider the heterogeneity of stressful experiences. Czéh et al. utilized a 9-week chronic mild stress protocol, much longer than the 2- and 4-week protocols utilized when increased PV cell counts were found (Shepard

et al., 2016; Shepard and Coutellier, 2018). Todorović et al. utilized social isolation, a stressor that varies fundamentally in nature from chronic mild stress protocols. The length and type of stressor may therefore play a role in the effects of chronic stress on prefrontal PV cells (increased vs. decreased). Chemogenetic studies have also reported that acute activation of prefrontal PV cells is anxiolytic (Ferguson and Gao, 2018b), and acute suppression of prefrontal PV cell activity promotes helplessness (Perova et al., 2015). Both studies were conducted in male rodents, again emphasizing the possible role of sex differences. These studies were also acute manipulations, which likely have different behavioral effects than chronic excitation or inhibition. Independent of the nature of the changes to PV neurons after stress, these results altogether show that prefrontal PV cells are highly sensitive to stress and regulate aspects of emotional behaviors. Because changes in prefrontal activity has been reliably implicated in depression and anxiety (Thompson et al., 2015), these findings emphasize the importance of studying in depth the contribution of prefrontal PV neurons to mood disorders.

Evidence for over-inhibition

Part of the inconclusive nature of findings related to GABAergic dysfunction in stress and affective disorders comes from a lack of research into this topic, especially with regards to sex differences. Furthermore, the majority of research is correlational.

GABAergic changes have been observed in human studies of emotional disorders and animal models of stress and affective dysfunction, but rarely have GABA-related findings been causally linked to changes in emotional behaviors. In this dissertation, I examine the sex-specific role of the GABAergic system in the stress response and emotional

behaviors. The experiments presented here manipulate the GABAergic system using chemogenetic, pharmacological, or transgenic methods to causally connect increases or decreases in inhibition with behavioral outcomes. The findings support the hypothesis that chronic stress leads to over-inhibition in the PFC, predominately in females. This over-inhibition is mediated in part by PV cells and is related to increased anxiety-like behaviors following chronic stress exposure.

Chronic stress increases prefrontal PV mRNA and protein expression in the female PFC, indicative of increased activity of these cells (Figure 3) (Shepard et al., 2016). Following up on this research, I used chemogenetics (designer receptors exclusively activated by designer drugs [DREADDs]) to increase prefrontal PV cell activity in the absence of chronic stress and found that chronic excitation of these cells effectively recapitulates sex-specific effects of chronic stress, namely increased anxiety-related behaviors in female mice (**Chapter 2**). I next examined whether decreasing the activity of prefrontal PV cells would rescue anxiety-like behavior in females following chronic stress, again using chemogenetics. Acute inhibition of prefrontal PV cells following chronic stress was found to be insufficient to rescue anxiety-like behaviors but did alter markers of neural activity and synaptic plasticity in a sex- and stress-dependent manner (**Chapter 3**).

Having demonstrated a sex-specific, causal role for prefrontal PV cells in anxiety-related behaviors, I turned to pharmacological and transgenic techniques for manipulating the activity of the GABAergic system during chronic stress exposure in females to induce resilience or vulnerability. First, I administered lorazepam, a positive allosteric modulator

for GABA receptors, during the first 2 weeks of a 4-week chronic stress exposure. Lorazepam administration in concurrence with chronic stress exacerbates anxiety-related behaviors. Chronic lorazepam administration also elevates mRNA expression of *GAD1*, the GABA synthesizing enzyme, which positively correlates with increased anxiety-like behaviors (**Chapter 4**). Second, I exposed female mice deficient for the potassium channel Kv3.1 to chronic stress. Kv3.1 is expressed predominately in PV neurons and facilitates their fast-spiking activity (Rudy and McBain, 2001), so Kv3.1 deficiency reduces the activity of PV cells and may thus incur resilience or vulnerability to chronic stress. Kv3.1 knockout females do not show increased anxiety-like behavior following chronic stress, unlike their wildtype and heterozygous littermates, indicating that reduced PV cell activity is associated with stress resilience (**Chapter 5**).

Together, these experiments provide evidence for over-inhibition as a mechanism connecting chronic stress exposure to emotional dysfunction, specifically increased anxiety-like behavior in females. Over-inhibition involves increases in brakes on activity and plasticity, such as PV cell activity, which are typically associated with the closing of developmental critical periods of plasticity. Therapeutic interventions may involve lifting these brakes and reinstating activity and plasticity in the PFC, restoring E/I balance and healthy emotional functioning. The potential of this therapeutic avenue remains an open and critical question. The mechanisms of sex differences in the sensitivity of the prefrontal GABAergic system and PV cells to chronic stress also requires further investigation. Gaining a deeper understanding of the role of prefrontal PV cells and inhibitory transmission in the stress response and emotional dysfunction will invaluablely

contribute to resolving the complexity of E/I interactions in disorders like anxiety and depression. The long-term hope of this research is to facilitate the clinical quest for relief for those suffering from these debilitating forms of “low spirits” (Darwin, 1872).

Figure 1. Development of excitatory/inhibitory balance.

The adolescent prefrontal cortex is hyper-excitable and plastic. As development proceeds, the GABAergic system matures to introduce more inhibitory control into the network and stabilize/consolidate neuronal circuitry. E: excitation; I: inhibition. Figure modified (Bavelier et al., 2010).

Scale according to:

- Nature of stressor
- Stressor duration
- Recovery time
- Sex
- Genetics

Figure 2. Time course of adult stress effects on glutamatergic transmission and activity in the prefrontal cortex.

Acutely, stress exposure increases glutamatergic transmission and prefrontal activity. In cases of chronic stress exposure, this initial increase eventually results in reduced excitation and prefrontal hypoactivity. The nature of this trajectory is heterogeneous and impacted by multiple different factors including external ones relating to the stress itself, as well as internal ones such as sex and genetic vulnerability. E: excitation; I: inhibition.

Figure 3. Number of PV and cFos+ in the PFC of female mice.

Number of PV-positive cells (**A, B**), and of non-PV cells positive to cFos (**C, D**) in the PrL PFC (**A, C**) and IL PFC (**B, D**) after exposure to the open-field in female mice handled daily (control) or exposed to 4 weeks of UCMS (n = 12 sections from 3 animals in each group). (Top picture) Representative picture of double staining for PV and cFos in the PFC of a control female; (Bottom picture) Representative picture of double staining for PV and cFos in the PrL PFC of a control (top) and a UCMS-exposed (bottom) female. Scale bar = 20 μ m. * p = 0.05. UCMS = unpredictable chronic mild stress (Shepard et al., 2016).

Figure 4. Number of PV and cFos+ in the PFC of male mice.

Number of PV-positive cells (**A, B**), and of non-PV cells positive to cFos (**C, D**) in the PrL PFC (**A, C**) and IL PFC (**B, D**) after exposure to the open-field in male mice handled daily (control) or exposed to 4 weeks of UCMS (n = 12 sections from 3 animals in each group). (Top picture) Representative picture of double staining for PV and cFos in the PFC of a control male; (Bottom picture) Representative picture of double staining for PV and cFos in the PrL PFC of a control (top) and a UCMS-exposed (bottom) male. Scale bar = 20 μ m. * p = 0.05. UCMS = unpredictable chronic mild stress (Shepard et al., 2016).

Chapter 2. Prefrontal parvalbumin cells are sensitive to stress and mediate anxiety-related behaviors in female mice

Introduction

Abnormal functioning of the prefrontal cortex (PFC) is associated with emotional dysregulation as seen in mood disorders like depression or anxiety. Deficits in glutamatergic transmission, and thereby an overall lower level of prefrontal activity, has been related to depressive symptoms and anxious behaviors (Martinot et al., 1990; Shin et al., 2001; Phelps et al., 2004; Thompson et al., 2015). The sources of changes in prefrontal activity and their relationship with behavioral symptoms remain unclear. Understanding the mechanisms underlying this prefrontal hypoactivity in mood disorders must remain a priority to promote the development of efficacious and reliable pharmacological therapies.

The PFC contains a heterogeneous population of excitatory pyramidal and inhibitory GABAergic neurons that directly regulate its activity. Prefrontal GABAergic interneurons are the primary regulators of principal projection neurons' spiking activity. Their dysregulation is likely to affect prefrontal activity and emotional behaviors. In support of this idea, rodent-based studies have demonstrated that modulation of the activity of specific subpopulations of GABAergic interneurons induces changes in frontal activity and alters emotional behaviors. For instance, acutely blocking the activity of somatostatin-positive inhibitory neurons in the PFC of male mice increased their

behavioral emotionality (Soumier and Sibille, 2014). The effects of chronic social stress on depressive- and anxiety-like behaviors of male mice are mediated by cholecystokinin-expressing GABAergic neurons that regulate PFC activity (Vialou et al., 2014).

Recently, our work demonstrated that exposure to chronic mild stress increases prefrontal PV mRNA expression and the number of parvalbumin (PV)-expressing interneurons, which corresponds with heightened anxiety-like behaviors in female, but not male, mice (Shepard et al., 2016; Shepard and Coutellier, 2018). Because PV expression is activity-dependent (Jiang and Swann, 2005; Tropea et al., 2006), the increase in expression we observed could reflect increased activity of PV cells after stress. PV cells provide strong, fast-spiking inhibitory signals to principle projection neurons in the PFC (Tremblay et al., 2016) and contribute to healthy cognitive and emotional functioning (Ferguson and Gao, 2018a). They are integral to balancing activity levels of pyramidal cells in the PFC and therefore, increased PV-dependent GABAergic transmission could be contributing to the hypoactivity of the PFC observed in mood disorders. Surprisingly, prefrontal PV cells have not been thoroughly explored in the context of emotional dysregulations and prefrontal hypoactivity. Some work has highlighted a role of hippocampal PV cells in anxiety (Zou et al., 2016; Murthy et al., 2019), but whether PV cells in the PFC contribute also to mood disorders marked by prefrontal hypoactivity is unclear. Our recent work (Shepard et al., 2016; Shepard and Coutellier, 2018) strongly suggests that plasticity of prefrontal PV neurons contributes to anxiety dysregulations following stress exposure, a known risk factor to mood disorders (Kendler et al., 1999; Duman and Monteggia, 2006; Mineur et al., 2006). Our work also

indicates that the potential role played by PV cells in regulating anxiety could be modulated by biological sex, since we reported a positive correlation between increased anxiety-like behaviors and increased PV expression in the PFC in females but not males (Shepard et al., 2016).

Here, we verify whether prefrontal PV cell activity is increased by stress and whether this causally contributes to emotional dysfunction in a sex-specific manner. We first demonstrate a stress-induced increase in prefrontal PV cell activity as measured by PV/cFos immunolabeling. We then used a chemogenetic approach in male and female mice to increase the activity of prefrontal PV cells in the absence of stress to test whether this intervention was anxiogenic. We injected a Cre-dependent DREADD (designer receptor exclusively active by designer drugs) virus into the PFC of PV:Cre mice and administered the DREADD ligand clozapine-N-oxide (CNO) to induce activation of PV cells in the PFC. Our findings show that chronic DREADD-induced activation of these cells decreases prefrontal activity in both sexes and increases anxiety-related behaviors in female mice. This information provides novel insight into sex-specific neural mechanisms underlying anxiety disorders and identifies a potential new avenue for treatment.

Materials and Methods

Animals

Adult male and female C57Bl/6J mice were ordered from Jackson Laboratory (Maine, US) and were used for chronic stress exposure and evaluation of PV/cFos double-

immunolabeling. They were group-housed per sex (3-5 mice per cage, unless specified otherwise) and allowed to habituate to the colony room for one week before experimental procedures began.

PV:Cre adult male and female mice (B6;129P2-Pvalbtm1(cre)Arbr/J) were used for the DREADD experiments. PV:Cre animals were bred in our colony from parents purchased from Jackson Laboratory. After weaning, mice were maintained group-housed per sex (3-5 mice per cage) and undisturbed (aside from regular cage cleaning) until experimental procedures started.

All mice had access to food and water ad libitum and were maintained on a 12-hour reverse light/dark cycle. All experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee of The Ohio State University and were performed based on the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Unpredictable chronic mild stress (UCMS) and PV/cFos immunohistochemistry

The UCMS paradigm used is the same as we previously described (Shepard et al., 2016) and consisted of single-housing the animals and of daily exposure over 4 weeks to alternating mild stressors presented in a random order according to an unpredictable schedule. Stressors included: absence of nesting material for 24 hours, 20° cage tilt on the vertical axis for 6 hours, absence of bedding in the cage for 8 hours, restraint stress in the dark for 8 minutes, and restraint stress under bright light for 4 minutes. Control animals were handled once daily for 1-2 minutes throughout the UCMS period.

To measure activity of prefrontal PV cells in the PFC after UCMS, we used a double immunofluorescent approach for PV and cFos. Twenty-four hours after the last handling (control group) or stressor (UCMS group), mice were exposed to the open field (OF) test (see “*Behavioral testing*” section for description) to stimulate cFos induction. Ninety minutes after exposure to the open field, animals were perfused with 4% cold paraformaldehyde (PFA). Brains were removed and kept in 4% PFA at 4°C overnight before storage in a sucrose solution (30% sucrose). Brains were frozen on dry ice and sectioned at 50 µm using a cryostat so as to obtain 3 sets of sections containing the PFC. Free-floating staining was performed on 1 set of sections using a rabbit anti-PV antibody (1:100, Abcam, Ab11427), and a goat anti-cFos antibody (1:300, SantaCruz Biotechnology, sc-52G), used as a marker of neuronal activity. Sections were then incubated with Alexa Fluor donkey anti-goat 488 and Alexa Fluor donkey anti-rabbit 555 secondary antibodies (1:500, Thermofisher A11055 and A31572, respectively). Quantitative analysis of PV cells expressing cFos (indicating activity of this specific neuronal population) in the mPFC was achieved using the unbiased stereology method with StereoInvestigator software from MBF Bioscience (Williston, VT) as previously described (Shepard et al., 2016). Cells were counted in every 3 sections. Accuracy of the estimate of the total numbers of cells was assured by verifying that the mean coefficient of error (CE) of Gundersen (Gundersen et al., 1999) was below 0.10.

Stereotaxic viral vector injection and verification of specificity

AAV DREADD vectors were obtained from the University of North Carolina Vector Core Facilities (Chapel Hill, NC). Adult PV:Cre mice were injected bilaterally with 0.5 µl/side of AAV2/hSyn-DIO-hm3D(Gq)-mCherry ('hm3DGq') or with the AAV2/hSyn-DIO-mCherry ('control virus') ($\sim 10^{12}$ vg/ml) into the medial PFC (mPFC – including the central part of the prelimbic (PrL) and the infralimbic (IL) cortex). Coordinates were antero-posterior + 1.7 mm; medio-lateral $\pm$ 0.2 mm; dorso-ventral -2.6 mm. Viruses were injected at a rate of 0.1 µl/minute. The syringe remained in place for 10 minutes before being removed. A period of 21 days was allowed to obtain full viral expression specifically within PV cells.

To verify accuracy of injection site, we collected and processed the brains of all mice after completion of all experiments. One set of PFC sections was stained using a rabbit anti-DsRed antibody (1:1000, Clontech Laboratories Inc) followed by an Alexa Fluor anti-rabbit 555 secondary antibody (1:500, Thermofisher). In addition, we quantified cell-specific expression of the DREADD viral infection to PV cells. Six sets of sections from three mice injected with the control virus and three mice injected with the hm3DGq virus were selected randomly. Sections were incubated with a guinea pig anti-PV antibody (1:500, Synaptic Systems) and a rabbit anti-DsRed antibody (1:1000, Clontech Laboratories Inc). Secondary antibodies were a donkey anti guinea pig CF488A conjugate (1:500) and Alexa Fluor donkey anti-rabbit 555 (1:500). Quantification was achieved using the unbiased stereology method to count the percentage of PV cells expressing DsRed, and percentage of DsRed cells expressing PV.

Pharmacogenetic activation of prefrontal PV cells

To achieve specific activation of prefrontal PV cells, we injected mice intraperitoneally with clozapine-N-oxide (CNO) prepared in 0.9% saline. Chronic activation of prefrontal PV cells was achieved through a 21-day period of daily CNO injection (0.5mg/kg/day). Acute activation of these cells was achieved through one single injection of the same dose of CNO 30 minutes prior to the open field test. The CNO dose (0.5mg/kg) was chosen based on previous work showing that it is sufficient to induce changes in firing activity of PV cells (Soumier and Sibille, 2014; Zou et al., 2016) and to avoid chronic desensitization in our chronic activation group.

To confirm these previous reports, we used a small cohort of mice infused with the hm3DGq or the control virus in the mPFC. After 21 days, two control virus mice and three hm3DGq mice were treated acutely with a single injection of CNO (0.5mg/kg), while three other hm3DGq-mice were treated chronically (once daily for 21 days) with CNO (0.5mg/kg). To verify that our CNO injection leads to activation of PV cells, brains were collected 90 minutes after the acute or the last CNO injection. Sections from the PFC were double-stained for DsRed (rabbit anti-DsRed antibody; 1:1000, Clontech Laboratories Inc) and cFos (goat anti-cFos; 1:300, Santa Cruz). Double-labeled DsRed/cFos cells and single-labeled cFos cells were counted using the unbiased stereology method.

Behavioral testing

All behavioral assays were conducted during the dark phase. Mice were habituated to experimenter handling for the 3 days prior to the start of behavioral testing for 1 minute per day; on the days of testing, mice were allowed a one-hour habituation period in the testing room prior to the beginning of testing. Mice were tested in no more than one test per day with an inter-test period of 24 hours, with the exception of the forced swim test which was conducted 3 days after novelty suppressed feeding to allow mice to recover from the food restriction associated with this test. Mice chronically injected with CNO were tested 4 days after the last CNO injection. The 4-day recovery period allowed us to avoid acute effects of CNO on our behavioral endpoints. We used $N = 8-9$ mice per virus group, per sex. However, two videos of the OF and one of the FST were corrupted, leading to $N = 7$ for the control male and female groups in OF, and $N = 7$ for the control male group in the FST. Both hm3DGq- and control virus-expressing mice were chronically injected with CNO. To control for a potential effect of chronic exposure to CNO on behaviors, a group of adult C57Bl6/J mice was injected with CNO or vehicle once daily over a 21-day period and then behaviorally tested ($N = 6$ per treatment per sex). Acutely-injected mice with CNO were tested in the open field test 30 minutes after the CNO injection. We used $N = 8-9$ mice per virus group, per sex.

Open field test (OF). The OF was conducted as previously described (Shepard et al., 2016). Briefly, each mouse was placed in an unfamiliar square arena (40x40cm) and were allowed to explore for a period of 10 minutes under bright light conditions. Trials were video-recorded, and videos analyzed using the Ethovision software XT 11.5 (Noldus).

Total distance traveled was used as indicator as general activity level; time spent, and distance travelled in the center of the OF vs. near the walls were used as measures of anxiety-like behaviors.

Object recognition test (ORT). Animals were tested for their long-term memory abilities in the ORT. During the learning phase, mice were placed in the OF arena with two identical unfamiliar objects that they were allowed to explore for 10 minutes. Twenty-four hours later, mice were placed back in the arena with an object from the learning phase ('familiar object') and a never-before met object ('novel object'). They were again allowed to freely explore the objects for a 10-minute period. Each phase was video-recorded and video scored off-line by an experimenter blind to the group of the animals. The time spent sniffing each object during the learning and testing phase was recorded; a mouse was considered sniffing an object when its nose contacted the object or was directed at the object within 1 cm. A discrimination ratio was calculated as: (time sniffing novel object – time sniffing familiar object)/total time sniffing.

Novelty-suppressed feeding (NSF). The NSF was adapted from Brachman et al (2016). Mice were food restricted for a period of 24 hours (no food present in home cage). Testing took place in an unfamiliar arena (30cm x 50cm) under bright light conditions. The floor of the arena was covered with clean bedding. At the time of testing, a single pellet of regular chow was placed in the center of the arena on a slightly elevated platform. Each mouse was placed in a corner of the arena. The latency to feed was recorded and used as a measure of anxiety-like behavior; a maximum of 10 minutes was allowed. Mice were then returned in their home cage with the food pellet for a period of 5

minutes. The total amount of food eaten within the home cage was recorded as an indicator of motivation to feed.

Forced swim test (FST). The FST was conducted as previously described (Shepard et al., 2016). Mice were placed in a glass cylinder (height: 30cm; diameter: 15cm) filled with water (24-25°C) for a period of 6 minutes. Each trial was recorded via a side-camera to allow for offline analysis by an experimenter blind to the group of the animal. The total time spent immobile (defined as the complete absence of movement, aside the ones required for breathing) over the 6-minute period of the test was scored as an indicator of depressive-like behaviors.

Statistical Analysis

Data were analyzed using the software Prism 5.01 (GraphPad Software Inc., CA, USA). Due to previous findings showing sex-specific emotional behaviors in rodents (e.g. female rodents being less anxious and more active in the open field test than males) (Domonkos et al., 2017; Kokras et al., 2018), statistical analyses were conducted separately in males and females. This approach is used frequently to answer questions about differentiated patterns of response to stress in male and female subjects (Li et al., 2016; Marrocco et al., 2017; Rapanelli et al., 2017). Furthermore, since the current work is a follow-up to our previous study (Shepard et al., 2016), this statistical approach allows us to directly compare our findings. A one-way analysis of variance (ANOVA) with type of virus (control vs hM3DGq) as the independent variable and sex as a covariate was run using SPSS for the analysis of the percentage of PV cells expressing the DREADD virus

and the percentage of DsRed cells expressing PV. PV/cFos data were analyzed using the Mann-Whitney test to compare unstressed vs. stressed animals. Behavioral data were analyzed using Student's t-tests to compare the control virus group to the hm3DGq virus group. The number of DsRed expressing cFos after acute or chronic injection of CNO (vs. control), as well as the number of single-labeled cFos cells, were analyzed using one-way ANOVA followed by Tukey's tests. Significance was set at $p \leq 0.05$. Data are presented as mean $\pm$ standard error of the mean (SEM).

Results

Chronic stress exposure increases prefrontal PV cell activity

We previously observed that chronic stress increases PV mRNA expression as well as the number of PV cells in the PFC of female mice (Shepard et al., 2016). We sought to confirm whether these observations indicated increased activity of this cell population by exposing male and female mice to 4 weeks of UCMS and measuring prefrontal PV activity using PV/cFos double-labeling. Chronic stress significantly increased the number of PV/cFos double-labeled cells in the mPFC of females ($p = 0.05$) and males ($p = 0.05$) (Figure 5).

Selective activation of mPFC PV cells and hypofrontality

In our chemogenetic experiments, we first verified that the DREADD virus expression is specific to PV cells. Using the unbiased stereological method in six mice chosen randomly (3 mice injected with the control virus and 3 with the hm3DGq virus), we

verified that $92.23 \pm 1.31\%$ of mPFC PV cells expressed the virus, and that $96.85 \pm 0.70\%$ of DsRed cells co-express PV (Figure 6A-E). We also verified that there were no significant differences in viral expression between males and females (percentage of PV cells expressing DsRed: $p = 0.121$; percentage of DsRed cells expressing PV: $p = 0.567$; data not shown).

We then verified that 0.5mg/kg of CNO is sufficient to induce activation of PV cells after acute or chronic treatment. Acute or chronic CNO injections lead to a similar level of activity of cells expressing the hm3DGq virus: $79.23 \pm 4.46\%$ and $86.92 \pm 2.64\%$ of DsRed⁺ cells express cFos, respectively. This is significantly more than what we observed after CNO injection in mice expressing the control virus ($27.68 \pm 3.89\%$; $F_{2,5} = 61.18$; $p = 0.0003$) (Figure 6F). We finally verified that chemogenetic activation of prefrontal PV cells is sufficient to induce an overall reduction of activity of the PFC, using cFos as a neuronal marker of activity. We observed that both acute and chronic activation of PV cells reduced cFos expression in the PFC 90 minutes after the acute or last CNO injection, which is significantly lower than what was observed in mice expressing the control virus ($F_{2,5} = 17.44$; $p = 0.0056$) (Figure 6G). This provides indication of overall reduced activity of the PFC after activation of PV cells.

Chronic activation of prefrontal PV cells is sufficient to induce an anxiety-like phenotype in female mice

Based on the observation that chronic stress increases the activity of prefrontal PV cells (Figure 5), we hypothesized that exposure to daily stressors over the chronic stress period induces daily activation of PV cells. To test this idea, mice injected with the hm3DGq or

control virus were treated daily with CNO over a 21-day period. Chronic activation of prefrontal PV cells induced an increase in anxiety-like behaviors in females. The time spent in the center of the OF arena was significantly lower in hm3DGq-mice when compared to control mice ($t_{14} = 2.528$; $p = 0.024$; Figure 7B). Also, the distance traveled near the walls of the arena in the OF was higher in hm3DGq mice ($t_{14} = 2.158$; $p = 0.049$; Figure 7C). Finally, their latency to eat the food in the NSF test was longer ($t_{14} = 2.044$; $p = 0.060$; Figure 7D), without significant difference in the amount of food eaten back in their home cage demonstrating similar motivation to eat food ($t_{14} = 0.959$; $p = 0.354$; data not shown). On the other hand, locomotor activity (Figure 7E), despair-like behaviors (Figure 7F) and long-term memory (Figure 7G) were not affected by chronic activation of PV cells. In males, no significant changes in behaviors were observed after chronic activation of PV cells in the mPFC (Figure 7H-M).

We verified that none of the effects reported could have been confounded by the chronic exposure to CNO, rather than by the chronic activation of prefrontal PV cells. Our data showed that C57Bl6/J male and female mice injected once daily with CNO for 21 days did not display differences in body weight, locomotor activity, cognitive functions and emotional behaviors when compared to vehicle injected-mice (Figure 8).

Acute activation of prefrontal PV cells does not induce changes in anxiety-like behaviors in females

After observing that chronic excitation of prefrontal PV induces anxiety-like behavior only in females, we next determined whether acute activation was sufficient to elicit an anxiogenic effect. For this experiment, we only tested animals in the OF 30 minutes after

CNO injection to avoid repeated PV cell activation over multiple days of behavioral testing causing chronic activation. In both females and males, we did not observe any significant change in anxiety-like behavior as measured in the OF after acute activation of prefrontal PV cells (Figure 9).

Discussion

Our findings demonstrate that chronic stress increases the activity of prefrontal PV cells. Using DREADDs to drive activity of this cell population reduces overall activity of the PFC both acutely and chronically. The effects of increased activity of prefrontal PV cells on emotional behaviors is modulated by sex. Chronic activation of PV cells in the PFC increased anxiety-like behaviors in females only. This finding mimics the effects of chronic stress on mice anxiety-like behaviors we previously reported (Shepard et al., 2016), which corresponded in female mice with increased expression of PV mRNA and number of PV cells in the PFC. Together, our results establish for the first time a causal link between increased chronic activity of PFC PV cells and elevated anxiety-related behaviors in females. Further studying changes in the activity of this specific interneuron population could be a novel avenue of research to gain a better understanding of stress-induced anxiety, especially considering that females are at an increased risk for developing an anxiety disorder in comparison to males (Kessler et al., 2012).

Using cFos as a marker of neuronal activity, we confirmed that the previously reported chronic stress-induced increase in total PV mRNA in the PFC corresponds to increased activity of PV cells. Importantly, we noticed that in control animals, males

display higher number of PV/cFos cells than females, and that the increase in the number of PV/cFos after chronic stress is more pronounced in females than in males (98% increase vs. 47%, respectively). This sex difference in baseline values and in stress effects could explain why no anxiety-like phenotype was observed in males after chronic stress (Shepard et al., 2016; Shepard and Coutellier, 2018). Previous work has also shown that male mice need a longer duration stress exposure to elicit an anxiety-like phenotype (Monteiro et al., 2015). It is therefore possible that a longer duration of PV cell activation would be necessary to induce anxiety-like behaviors in males. Alternatively, sex-specific circuits within the mPFC may also mediate anxiety-related behaviors. For example, Li et al. (2016) identified circuitry in the mPFC that is anxiolytic in males but not females. We can speculate on a similar mechanism here, whereby a subpopulation of PV cells in the female, but not male, PFC regulates anxiety-like phenotype. Increasing the length of stress exposure will help address these ideas.

We have previously reported that chronic stress induced a sex-specific increase in anxiety-like behaviors (Shepard et al., 2016; Shepard and Coutellier, 2018). Here, we found that while acute PV cell excitation is insufficient to induce anxiety-related behaviors in either sex, chronic activation of PV cells using DREADDs reproduces the female-specific anxiogenic-like effects of chronic stress. Manipulation of PFC PV cells in female mice does not lead to changes in depressive-like behaviors as measured by the forced swim test. While both anxiety and depressive disorders are characterized by reduced activity of the PFC (Martinot et al., 1990; Shin et al., 2001; Phelps et al., 2004; Thompson et al., 2015), our data suggest that different mechanisms within the PFC might

drive prefrontal hypoactivity in each of these pathologies, or that activity levels in different local prefrontal microcircuits contribute specifically to anxiety- or depressive-like phenotypes. It is also possible that chronic PV cell activation affects facets of depressive-like behaviors not tested for here, such as anhedonia. These data also reveal that whether hypoactivity of the PFC occurs in an acute or chronic manner can play a major role in the appearance of an abnormal phenotype. Here, we verified that increasing the activity of PV cells in the PFC leads to reduced overall prefrontal activity, as previously shown by electrophysiological recordings (Winkelmann et al., 2014; Safari et al., 2017). However, while both acute and chronic activation of PV cells reduce prefrontal activity, only the chronic regimen leads to increased anxiety-like behaviors in females.

The present research points toward a potential novel mechanism underlying anxiety, whereby PV cell over-activity reduces the overall level of activity in the PFC and increases anxiety-like behaviors. These findings are in line with those suggesting that GABA antagonism can be anxiolytic. For example, quercetin, a negative allosteric modulator for GABA_A receptors (GABA_ARs), reduces GABAergic transmission in the PFC (Fan et al., 2018) and has stress-protective and anxiolytic effects (Bhutada et al., 2010; Mehta et al., 2017; Samad et al., 2018). Another negative allosteric modulator for $\alpha 5$ -containing GABA_ARs, MRK-016, also exhibits antidepressant actions (Zanos et al., 2017). However, it is not known whether these compounds act on prefrontal PV cells or on PV synapses with pyramidal cells. Further investigation is also needed to characterize precisely the sex-specific roles played by prefrontal PV cells on emotional behaviors.

To conclude, the present research identifies increased prefrontal PV cell activity as a mechanism of prefrontal hypoactivity as well as a sex-specific modulator of anxiety-like behaviors. Future research is necessary to test the therapeutic potential of targeting prefrontal PV cells as an intervention for stress-induced anxiety disorders. Further investigation is also necessary to elucidate the mechanisms by which chronic stress increases the activity of PV cells in the PFC, as well as the mechanisms behind sex differences in the behavioral effects of chronic PV cell activation. Overall, the findings presented here open a new avenue of research into a cell-specific mechanism potentially underlying sex differences in stress susceptibility and the prevalence of anxiety.

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Figure 5. Chronic stress increases activity of prefrontal PV neurons in both sexes.

(A) Representative image showing PV (red) and cFos (green) double-labeling (white arrow). Scale bar: 10 μm. **(B)** Chronic stress increases the number of PV/cFos double-labeled cells in the PFC of females and **(C)** males. In all figures, summary data are represented as mean ± SEM. * $p = 0.05$.

(A) Experimental schematic of injection site of the DREADD virus in the ventral part of the PFC (the coronal illustration at the level of the PFC comes from Paxinos and Franklin's The Mouse Brain in Stereotaxic Coordinates (Paxinos and Franklin, 2012)). (B) Representative coronal image shows bilateral expression of mCherry localized to the infralimbic cortex (IL) and part of the prelimbic cortex (PrL). Scale bar: 50µm. (C) Representative images showing selective expression of the hM3DGq virus (red) in parvalbumin (PV – green) interneurons. Scale bar: 20µm. (D) Stereological analysis indicated that $92.23 \pm 1.31\%$ of mPFC PV neurons expressed the control or hM3DGq virus, and (E) $96.85 \pm 0.70\%$ DsRed⁺ cells are PV. (F) Verification of hM3DGq virus response to acute and chronic (once per day for 21 days) CNO injection using stereological analysis of the number of DsRed cells expressing cFos: both acute and chronic injection of CNO in the PFC of mice expressing the hM3DGq virus leads to high expression of cFos in DsRed⁺ cells when compared to cells expressing the control virus. Representative image of DsRed⁺ cells expressing cFos (white arrow) vs. DsRed⁺ cells not expressing cFos (white arrowhead). Scale bar: 20µm. (G) Verification that acute and chronic chemogenetic activation of prefrontal PV cells lead to hypofunction of the PFC: both regimens of CNO injection reduce significantly the amount of cFos⁺ cells, showing overall hypoactivity of the PFC. Representative images of cFos expression in the PFC of mice expressing the control or the hM3DGq virus. Scale bar: 20µm. ** $p < 0.001$; *** $p < 0.0001$.

Figure 7. Chemogenetic chronic activation of PV neurons in the PFC of PV:Cre male and female mice increases anxiety-like behaviors in a sex-specific manner.

(A) Experimental schematic of the timeline followed. Mice injected with the control (AAV-DIO-mCherry) or the AAV-DIO-hM3DGq virus received a daily injection of CNO over the course of 21 days. Four days after the last injection, mice were tested once daily in a behavioral test to assess various sub-domains of emotional behaviors and cognitive functions. (B-G) Behavioral endpoints in control or hM3DGq female mice. Female mice that experienced chronic activation of PFC PV neurons displayed increased anxiety-like behaviors as shown by less time spent in the center of the open field (B), more distance travelled near the walls of the open field arena (C) and increased latency to eat the food in the novelty suppressed feeding test (D). Analysis of the total distance traveled in the open field (E), the time spent immobile in the forced swim test (F), and the discrimination ratio in the object recognition test (G) indicated no significant effect of the chronic activation of PFC PV neurons on these behaviors. (H-M) Behavioral endpoints in control or hM3DGq male mice. Chronic activation of PFC PV neurons did not impact emotional and cognitive behaviors in males. In all figures, summary data are represented as mean $\pm$ SEM. τ : $p = 0.06$. $*p \leq 0.05$. $N = 7-9$ per group per sex. CNO: clozapine-N-oxide; OF: open field; ORT: object recognition test; NSF: novelty-suppressed feeding test; FST: forced swim test.

Figure 8. Chronic injection of CNO in male and female C57Bl/6J mice does not affect general health or behaviors.

(**A**) Experimental scheme of the timeline followed. Mice were injected daily with 0.5mg/kg of CNO over the course of 21 days. Body weight was recorded throughout the injection period, and behaviors were tested four days after the last injection. (**B-G**) In females, chronic exposure to CNO did not affect body weight (**B**), general activity (**C**), anxiety-like behaviors tested in the open field test (**D**) and novelty-suppressed feeding tests (**E**), cognitive functions tested in the object recognition test (**F**), or depressive-like behaviors tested in the forced swim test (**G**), or. (**H-M**). Similar in males, CNO did not impact any behaviors. In all figures, summary data are represented as mean $\pm$ SEM. $N = 6$ per group per sex. CNO: clozapine-N-oxide; OF: open field; ORT: object recognition test; NSF: novelty-suppressed feeding test; FST: forced swim test.

Figure 9. Chemogenetic acute activation of PV neurons in the PFC of PV:Cre male and female mice does not impact anxiety-like behavior.

(A) Experimental schematic of the timeline followed. Mice injected with the control (AAV-DIO-mCherry) or the AAV-DIO-hM3DGq virus received one injection of CNO 30 minutes prior to the OF. (B; C; D) Acute activation of PFC PV neurons in female mice did not impact time spent in the center, locomotion near the walls or total locomotion in the novel arena. (E; F; G) The same was true in males. $N = 8-9$ per group per sex. OF: open field.

Chapter 3. Acute chemogenetic inhibition of prefrontal parvalbumin cells does not rescue stress-induced anxiety-like behaviors

Introduction

Chronic stress increases prefrontal parvalbumin (PV) expression, which is associated with hypoactivity in the prefrontal cortex (PFC) and behavioral emotionality in female mice (Shepard et al., 2016). An increase in prefrontal PV cell activity is causally related to increased anxiety-like behavior in females (Chapter 2). This raises the possibility that inhibiting prefrontal PV cells following chronic stress may rescue stress-induced increases in anxiety-like behaviors in these mice. More broadly, reducing inhibition in PFC may be a therapeutic intervention for anxiety disorders.

The present study utilizes designer receptors exclusively activated by designer drugs (DREADDs) to acutely inhibit PV cells in the PFC after chronic stress exposure and examines the effects on emotional behaviors. Very few studies have been conducted using DREADDs in the context of stress and PV cell activity. One experiment demonstrated that activation of PV cells in the dentate gyrus using DREADDs is anxiolytic and facilitates fear extinction (Zou et al., 2016). There is also evidence demonstrating that DREADDs are effectual interventions after chronic stress exposure: chemogenetic excitation of pyramidal cells in the PFC successfully reduces activity in the basolateral amygdala (BLA) and rescues stress-induced increases in emotional behaviors, as does chemogenetic inhibition of excitatory cells in the BLA itself (Wei et al., 2017).

Additionally, it is possible to elicit rapid antidepressant responses in mice using ketamine or optogenetics to stimulate excitatory neurons in the PFC, both acute manipulations (Li et al., 2011; Fuchikami et al., 2015). Acute ketamine treatment also reduces PV and GAD67 expression in the PFC (Zhou et al., 2015), supporting the hypothesis that acutely reducing prefrontal inhibition (by suppressing PV cell activity) may rescue stress-induced emotional dysfunction.

Chronic stress also impairs prefrontal activity and glutamate-dependent plasticity (Kuipers et al., 2003; Jett et al., 2017). We further hypothesize that suppressing PV cell activity will rescue stress-induced deficits in activity and plasticity of the PFC. In order to test this hypothesis, we examined stress- and DREADD-induced changes to markers of prefrontal activity and excitatory synaptic plasticity. We predict that chronic stress will reduce expression of markers of activity and plasticity, and that this reduction may be rescued by PV cell inhibition. Prefrontal activity and plasticity were measured by quantifying expression of activity-related cytoskeleton-associated protein (Arc; also known as Arg3.1). Arc is an immediate early gene (IEG) that is rapidly transcribed in an activity-dependent manner and mediates glutamate-dependent plasticity, including synaptic remodeling (Shepherd et al., 2006). To further examine excitatory synaptic plasticity, we also quantified expression of post-synaptic density (PSD)-95. PSD-95 is a marker of excitatory synapses and is reduced by chronic stress. Increased PSD-95 expression by fast-acting antidepressants could be indicative of a rescue of stress-induced synapse loss in the PFC (Li et al., 2011).

Chronic stress has been shown to impair excitatory synaptic plasticity (Kuipers et al., 2003), but it conversely may enhance inhibitory synaptic plasticity. A recent study found that chronic stress increases expression of markers associated with plasticity of interneurons in the PFC (Pesarico et al., 2019). We hypothesize that chronic stress enhances inhibitory synaptic plasticity, and that this too may be rescued by PV cell inhibition. To test the hypothesis that chronic stress increases interneuron plasticity, we measured expression of neuronal pentraxin 2 (Nptx2, also known as NARP). Nptx2 is synthesized in excitatory neurons. When translated, Nptx2 is secreted into the extracellular space where it accumulates at excitatory synapses of nearby PV cells and facilitates the insertion of GluR4-containing AMPA receptors into their membrane (Chang et al., 2010). In this way, Nptx2 increases excitatory drive onto PV cells and increases inhibition (Doyle et al., 2010).

The findings of this study show that chronic stress elicits anxiety-like behaviors in both male and female mice, which is not rescued by acute prefrontal PV cell inhibition in either sex. Chronic stress and DREADD manipulation of prefrontal PV cells impact markers of activity and plasticity in a sex-dependent manner. Specifically, *Arc* mRNA expression in females was increased by PV cell inhibition in control mice but not in stressed mice. Chronic stress decreased mRNA expression of *Arc* and *Nptx2* in males. Together, these findings suggest that acute PV cell inhibition can increase prefrontal activity and glutamate-dependent plasticity in female mice, but that this acute intervention is insufficient to overcome stress-induced increases in anxiety-like behavior and reductions in prefrontal activity and plasticity.

Materials and Methods

Animals

PV:Cre adult male and female mice (B6;129P2-Pvalbtm1(cre)Arbr/J) were used.

Experimental animals were bred in the colony from parents purchased from Jackson Laboratory (ME, USA). At postnatal day (PND) 21, litters were weaned, and mice were maintained group-housed per sex (3-5 mice per cage) and undisturbed (aside from regular cage cleaning) until experimental procedures started.

All mice had access to food and water ad libitum and were maintained on a 12-hour reverse light/dark cycle. All experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee of The Ohio State University and were performed based on the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Stereotaxic viral injection

The DREADD virus, pAAV-hSyn-DIO-hM4D(Gi)-mCherry ('hM4DGi'), was purchased from Addgene (Addgene plasmid #44362-AAV2). The control virus, pAAV-hSyn-DIO-mCherry ('control virus'), was also purchased from Addgene (Addgene plasmid #50459-AAV2). PV:Cre mice were injected bilaterally with 0.7 μ l/side of hM4DGi or control virus ($\sim 10^{12}$ vg/ml) into the most ventral part of the medial PFC (mPFC – including the central part of the prelimbic (PrL) and infralimbic (IL) cortex). Coordinates were antero-posterior + 1.7 mm; medio-lateral $\pm$ 0.2 mm; dorso-ventral -2.6 mm. Viruses were

injected at a rate of 0.1 μ l/minute. The syringe remained in place for 10 minutes before being removed at a rate of 1mm/minute.

Unpredictable chronic mild stress (UCMS)

The UCMS paradigm used is the same as the one previously utilized in the lab (Shepard et al., 2016; Page and Coutellier, 2018; Shepard and Coutellier, 2018) and consisted of single-housing the animals and of daily exposure to alternating mild stressors presented in a random order according to an unpredictable schedule for 28 days. Stressors included: absence of nesting material for 24 hours, 20° cage tilt on the vertical axis for 6 hours, absence of bedding in the cage for 8 hours, restraint stress in the dark for 8 minutes, and restraint stress under bright light for 4 minutes. Control animals were handled once daily for 1-2 minutes throughout the UCMS period. UCMS (or daily handling) began 3 days after stereotaxic viral injection surgery.

Behavioral testing

Mice were handled once daily for 1-2 minutes over the 3 days preceding testing to reduce any anxiety caused by interacting with the experimenter. Behavioral testing was performed during the dark phase of the mice's light cycle. Mice were acclimated to the testing room in the dark for at least one hour prior to testing. Dim white lights (~50 lux) were turned on during the testing period. Mice were exposed to the elevated plus maze (EPM) on one day, followed by the open field (OF) and the forced swim test (FST) on separate days.

The EPM test consisted of a raised (74-cm) arena with four arms (each 34 cm long). The two closed arms were surrounded by 22-cm-high black walls. Mice were allowed to explore the maze for 5 min, after which they were returned to their home cage and the arena was cleaned with 70% ethanol prior to testing the next mouse. The OF test used a 40 x 40 cm gray square arena with opaque white walls. Mice were allowed to explore the arena for 10 minutes and were returned to their home cage and the arena cleaned with 70% ethanol. The OF test was recorded with an overhead camera for offline analysis. Automated, unbiased analyses were conducted using the EthoVision XT software from Noldus Information Technology (Wageningen, Netherlands). The duration of time spent in the open arms of the EPM and the center of the OF were measured as indicators of anxiety-like behaviors. As total locomotor activity can bias the interpretation of these behaviors, the ratio of distance traveled in the open arms to total distance traveled was calculated for the EPM and distance traveled in the center relative to total distance traveled was calculated for the OF (Guilloux et al., 2011; Delprato et al., 2017).

On the final day, mice were tested in the forced swim test (FST): they were placed in a glass cylinder (height: 30 cm; diameter: 15 cm) filled with water (24-25°C) for 6 minutes, after which they were placed back in their home cage on a heated pad until dry. The FST was also video-recorded for offline analysis. Latency to first immobility and total time spent immobile in the forced swim tested were scored manually by a blinded experimenter.

Brain collection and processing

Ninety minutes after the completion of the FST, mice were anaesthetized with isoflurane and transcardially perfused with 25 mL of ice-cold 0.1 M phosphate-buffered saline (PBS). The brains were then removed and cut along the midline to separate the left and right hemisphere. One hemisphere was flash frozen on dry ice and the other hemisphere was placed in fresh 4% paraformaldehyde (PFA) for 2 days at 4°C, after which they were placed in 30% sucrose until they sank. The fixed hemispheres were then frozen on dry ice and sectioned at 50 µm with a cryostat. After sectioning, the thin slices were stored in cryoprotectant at -20°C until immunostaining.

To verify accuracy of injection site, the PFC of each mouse was stained using a rabbit anti-DsRed antibody (1:1000, Clontech Laboratories Inc, #632496) and a goat anti-PV antibody (1:2500, Swant, PVG-213) followed by an Alexa Fluor anti-rabbit 555 secondary antibody (1:500, Thermofisher A31572) and an Alexa Fluor anti-goat 488 secondary antibody (1:250, Thermofisher A11055). The percentage of PV cells expressing the virus (DsRed), as well as the percentage of DsRed cells expressing PV, was quantified using the unbiased stereology method with StereoInvestigator software from MBF Bioscience (Williston, VT) on 3 sets of PFC sections from 3 mice.

The flash frozen hemispheres were stored at -80°C until dissection. Dissection of the PFC was performed in a cold room on dry ice according to the *Mouse Brain in Stereotaxic Coordinates* (Paxinos and Franklin, 2012). Target mRNA were quantified using real-time polymerase chain reaction (RT-PCR). Brain tissues were processed as previously described (Coutellier et al., 2015). The target cDNA and the reference target

glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were amplified simultaneously in the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The following primer sequences were used:

Arc: Fwd: TACCGTTAGCCCCTATGCCATC,

Rev: TGATATTGCTGAGCCTCAACTG (Spiegel et al., 2014);

PSD-95: Fwd: GCCCTGTTTGATTACGACAA;

Rev: CTCATAGCTCAGAACCGAGT (Sun et al., 2009);

Nptx2: Fwd: AGAGGGTGACTGAGCTGGAG,

Rev: CTTCTTGATCTTGCCATACAGGT (Spiegel et al., 2014);

GAPDH: Fwd: CATGGCCTTCCGTGTTTCCT,

Rev: TGATGTCATCATACTTGGCAGGTT (Page et al., 2018).

Statistical analysis

Data were analyzed using the software Prism 5.01 (GraphPad Software Inc., CA, USA).

Due to previous findings showing sex-specific emotional behaviors in rodents (e.g. female rodents being less anxious and more active in the open field test than males) (Domonkos et al., 2017; Kokras et al., 2018), statistical analyses were conducted separately in males and females. This approach is used frequently to answer questions about differentiated patterns of response to stress in male and female subjects (Li et al., 2016; Marrocco et al., 2017; Rapanelli et al., 2017). Furthermore, since the current work is a follow-up to our previous study (Shepard et al., 2016), this statistical approach allowed us to directly compare our findings. Two-way analysis of variance (ANOVA)

was used to analyze the data with condition (no stress vs. stress) and virus (control vs. hM4DGi) as the independent variables. Data are presented as mean $\pm$ standard error of the mean (SEM). For post-hoc tests, Tukey's multiple comparisons adjustment was used.

Results

Efficacy and selectivity of DREADD viral expression

In the PFC, $87.79 \pm 2.472\%$ of PV cells co-expressed DsRed, and $81.96 \pm 4.33\%$ of DsRed cells co-expressed PV (Figure 10C-D).

Chronic stress increases anxiety-like behavior in the open field test in male and female mice, which is not rescued by acute prefrontal PV cell inhibition

For females, there were no significant differences in time spent in the open arms of the elevated plus maze (stress effect: $F_{1,30} = 1.266$, $p = 0.269$; virus effect: $F_{1,30} = 0.334$, $p = 0.568$; Figure 11A). There were also no significant differences in the distance ratio in the EPM (stress effect: $F_{1,30} = 0.607$, $p = 0.442$; virus effect: $F_{1,30} = 0.044$, $p = 0.836$; Figure 11B). In the open field, stressed females spent significantly less time in the center than unstressed females ($F_{1,30} = 30.35$, $p < 0.0001$), with no effect of virus ($F_{1,30} = 0.961$, $p = 0.335$). There was a trending interaction effect such that acute prefrontal PV cell inhibition increased time spent in the center of the OF in control animals ($F_{1,30} = 3.954$, $p = 0.056$; Figure 11C). Stressed females also had significantly reduced distance traveled in the center relative to total distance traveled ($F_{1,30} = 17.37$, $p = 0.0002$), again with no effect of virus ($F_{1,30} = 2.735$, $p = 0.109$). There was again a trend towards a significant

interaction effect such that acute prefrontal PV cell inhibition increased center distance ratio ($F_{1,30} = 3.736, p = 0.063$; Figure 11D).

The findings for males were similar. There were no significant differences in time spent in the open arms of the EPM (stress effect: $F_{1,26} = 0.0005, p = 0.982$; virus effect: $F_{1,26} = 1.801, p = 0.191$; Figure 12A), nor in distance ratio in the EPM (stress effect: $F_{1,26} = 3.176, p = 0.086$; virus effect: $F_{1,26} = 0.144, p = 0.708$; Figure 12B). In the open field, stressed males spent significantly less time in the center than unstressed males ($F_{1,26} = 23.52, p < 0.0001$) with no effect of virus ($F_{1,26} = 0.204, p = 0.655$; Figure 12C). There was no effect of stress on relative center distance ($F_{1,26} = 0.295, p = 0.592$); however, hM4DGi-treated males had significantly higher center distance traveled relative to total distance ($F_{1,26} = 5.551, p = 0.026$; Figure 12D). There were no significant interaction effects.

No changes in depression-like behaviors following chronic stress or acute prefrontal PV cell inhibition

For females, there was no effect of chronic stress ($F_{1,29} = 0.108, p = 0.745$) or DREADD virus ($F_{1,29} = 0.140, p = 0.711$) on the latency to immobility in the forced swim test (Figure 11E). For total time spent immobile, there was also no effect of chronic stress ($F_{1,29} = 0.022, p = 0.884$) or DREADD virus ($F_{1,29} = 1.746, p = 0.197$; Figure 11F). There were no significant interaction effects.

For males, there was also no effect of chronic stress ($F_{1,26} = 0.756, p = 0.393$) or virus ($F_{1,26} = 2.526, p = 0.124$) on latency to immobility in the forced swim test (Figure 12E). There were also no significant differences in total time spent immobile (stress

effect: $F_{1,26} = 1.752, p = 0.197$; virus effect: $F_{1,26} = 0.518, p = 0.478$; Figure 12F). There was a near-significant interaction effect ($F_{1,26} = 3.948, p = 0.058$) but no significant multiple comparisons.

Chronic stress and acute prefrontal PV cell inhibition have sex-specific effects on prefrontal Arc and Nptx2 mRNA expression

In females, there was a significant interaction between stress and virus ($F_{1,15} = 5.562, p = 0.0324$) on prefrontal *Arc* mRNA expression (Figure 13A). There were no significant multiple comparisons. There were also no significant differences in prefrontal *PSD-95* mRNA expression (stress effect: $F_{1,15} = 0.427, p = 0.523$; virus effect: $F_{1,15} = 0.106, p = 0.749$; Figure 13B). There were also no significant differences in *Nptx2* mRNA expression (stress effect: $F_{1,15} = 1.229, p = 0.285$; virus effect: $F_{1,15} = 1.517, p = 0.237$; Figure 13C).

In males, chronic stress reduces *Arc* mRNA expression in the PFC ($F_{1,15} = 9.831, p = 0.0068$; Figure 14A). There were no significant differences in *PSD-95* expression (stress effect: $F_{1,15} = 0.071, p = 0.793$; virus effect: $F_{1,15} = 0.259, p = 0.618$; Figure 14B). Chronic stress reduces prefrontal *Nptx2* expression ($F_{1,15} = 6.370, p = 0.023$), with no effect of acute PV cell inhibition ($F_{1,15} = 0.00205, p = 0.965$; Figure 14C).

Discussion

The main conclusion from this experiment is that acute prefrontal PV cell inhibition is insufficient to rescue stress-induced anxiety-like behaviors in both males and females. Chronic stress and the DREADD manipulation also impact markers of prefrontal activity

and synaptic plasticity in a sex-specific manner. Acute prefrontal PV cell inhibition increases *Arc* in female mice, but only in the absence of chronic stress. Chronic stress, by contrast, blunts *Arc* expression in both sexes. Chronic stress also reduces expression of *Nptx2* in males.

In this study, acute PV cell inhibition was insufficient to induce changes in emotional behaviors. The one exception was that acute PV cell inhibition increased center distance ratio in the OF in male mice. However, this was in the absence of any effect of chronic stress in this particular measurement in males. There was also a trend towards reduced anxiety-like behavior following acute PV cell inhibition in control females, hinting at a possible anxiolytic effect of this intervention. There were no stress effects in the EPM and FST in this study, emphasizing the need for more robust and reliable indicators of emotional behaviors as these tests can be highly variable (Hogg, 1996; Suvrathan et al., 2010; Bogdanova et al., 2013; Albani et al., 2015). It is also possible that the behavioral effects of the hM4DGi DREADD virus were limited by relatively lower and non-specific expression compared to the hM3DGq virus utilized in Chapter 2. Further refining the expression and specificity of the DREADD virus, perhaps by adjusting the titer of virus or volume injected, would facilitate the efficacy of future studies about the effects of prefrontal PV cell activity on emotional behaviors.

We previously reported that the UCMS paradigm used here increased anxiety-like behavior in females but not males (Shepard et al., 2016). However, in this study, we observe an anxiogenic effect of chronic stress in males as well. This is possibly attributable to chronic stress followed by a novel acute stress (restraint and i.p. injection

of CNO) 30 minutes prior to behavioral testing that elicited anxiety-like behaviors in stressed males in this case, though further sex-specific replication of the anxiogenic effects of this UCMS paradigm is warranted.

Previous studies have shown that it is possible to elicit rapid changes in emotional behaviors (anxiolytic and antidepressant-like effects) in mice using the acute interventions of ketamine or optogenetics to activate excitatory neurons in the PFC (Li et al., 2011; Fuchikami et al., 2015). However, acute inhibition of prefrontal PV cells does not elicit similar rapid effects. It is likely that chronic PV cell inhibition would be necessary to alleviate the anxiogenic effects of chronic stress. This is further supported by the fact that no changes in anxiety-like behaviors were observed after acute excitation of prefrontal PV cells (Chapter 2). As discussed in Chapter 2, even though both acute and chronic PV cell excitation cause hypoactivity of the PFC, only chronic hypofrontality is associated with increased anxiety in females. Similarly, acute PV cell inhibition may increase prefrontal activity, but only chronic increases in prefrontal activity levels may rescue stress-induced anxiety. A follow-up study should investigate the therapeutic potential of chronically inhibiting prefrontal PV cells after chronic stress.

Even though acute PV cell inhibition did not rescue anxiety-like behaviors, prefrontal PV cells may still represent a therapeutic target for anxiety. Antidepressants like selective serotonin reuptake inhibitors (SSRIs), which are frequently used to treat anxiety (Feighner, 1999; Davidson, 2004), reduce the expression of PV in the PFC (Ohira et al., 2013). The fast-acting antidepressant ketamine also has anxiolytic effects (Li et al., 2010; Fuchikami et al., 2015) and reduces PV expression (Zhou et al., 2015). However,

other studies suggest that the reduction in PV expression may not be involved in the efficaciousness of these treatments. Mice lacking N-Methyl-d-aspartic (NMDA) receptors, the target of ketamine, specifically in PV cells still show an antidepressant-like response to ketamine; anxiety-like behavior was not evaluated (Pozzi et al., 2014). Scopolamine, an antagonist of M1-type muscarinic acetylcholine receptors and fast-acting antidepressant, has anxiolytic effects that do not depend on prefrontal PV cells (Wohleb et al., 2016). It is possible that a reduction in prefrontal PV cell activity is a downstream, but not direct, effect of treatments like SSRIs, ketamine, and scopolamine. Overall, the responsibility of PV cells in modulating anxiolytic responses is still being unraveled and further studies are warranted.

Acute prefrontal PV cell inhibition modulates prefrontal activity and glutamate-dependent plasticity, as measured by expression of the IEG *Arc*, in females but not males. In unstressed females, *Arc* expression was elevated by the DREADD manipulation, which could represent an increase in activity in the PFC due to PV cell inhibition. This observation also supports the idea presented in Chapter 2 that PV cells in the PFC regulate molecular mechanisms underlying anxiety-like behavior and the stress response in females. Previous work has shown that *Arc* expression is reduced in male mice susceptible to chronic stress but remains high in resilient mice (Covington et al., 2010). There is currently little information on *Arc* in females and further experiments are necessary to understand the relationship between prefrontal PV cells and *Arc* expression.

In females, *Arc* expression was reduced when the DREADD manipulation was combined with chronic stress. This observation suggests that chronic stress blunts activity

in the PFC, which is in line with the previous reports of prefrontal hypoactivity in emotional disorders (Martinot et al., 1990; Shin et al., 2001; Phelps et al., 2004; Thompson et al., 2015). The hypothesis of prefrontal hypoactivity is supported by the fact that *Arc* expression is also reduced in chronically stressed males; prefrontal PV cell inhibition did not rescue this decrease in prefrontal activity. Previous research has shown that *Arc* expression in the PFC is reduced by chronic stress in male rodents (Covington et al., 2010; Ons et al., 2010). *Arc* expression is also reduced in humans with major depression (Covington et al., 2010; Li et al., 2015) and is elevated by chronic treatment with antidepressants and by acute ketamine administration (Pei et al., 2003; Molteni et al., 2008; Li et al., 2010). Overall, the present findings support these previous reports of reduced *Arc* expression in cases of chronic stress but demonstrate that acute PV cell inhibition is unable to rescue this deficit.

There were no changes in *PSD-95* mRNA expression in the PFC in male or female mice. Chronic stress has previously been shown to reduce PSD-95 protein expression, which is rescued by fast-acting antidepressant treatment (Li et al., 2011). It may have been insufficient to observe mRNA in the present study; only protein expression may reveal stress-induced changes to PSD-95. Furthermore, prefrontal PV cell activity does not appear to alter *PSD-95* gene expression, at least acutely.

Chronic stress, but not the DREADD manipulation, also impacted inhibitory plasticity as measured by *Nptx2* expression in a sex-dependent manner. Specifically, chronic stress reduced prefrontal *Nptx2* expression in males but not females. *Nptx2* expression facilitates the activation of PV neurons (Chang et al., 2010). Our previous

work has shown that chronic stress increases PV cell activity in female mice but not males (Shepard et al., 2016; Shepard and Coutellier, 2018). It is possible that downregulation of *Nptx2* expression by chronic stress in males helps to prevent an increase in PV cell activity in the face of chronic stress. However, males still displayed stress-induced anxiety-like behavior in the present study, indicating that reduced *Nptx2* expression does not protect against anxiety. *Nptx2* in the hippocampus alleviates anxiety (Chang et al., 2018), but at present it is unknown how prefrontal *Nptx2* modulates emotional behaviors.

In conclusion, acute prefrontal PV cell inhibition is insufficient to rescue stress-induced anxiety-like behaviors in male and female mice. Prefrontal PV cells may still represent a therapeutic target for anxiety, but chronic inhibition of this cell population is likely necessary. This research also reveals that prefrontal PV cells in female mice regulate activity and glutamate-dependent plasticity, which is worthy of future investigation as a possible mechanism underlying anxiety-like behaviors and the stress response in females.

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Figure 10. Experimental timeline and AAV-DIO-hM4DG viral injection.

(A) Schematic of experimental timeline. **(B)** Experimental schematic of injection site of the DREADD virus in the ventral part of the medial prefrontal cortex (mPFC). The coronal illustration at the level of the PFC comes from Paxinos and Franklin's *The Mouse Brain in Stereotaxic Coordinates* (Paxinos and Franklin, 2012). **(C)** Percentage of PV cells expressing the DREADD virus. **(D)** Percentage of DsRed-positive, viral-infected cells expressing PV. **(E)** Representative coronal images shows expression of DsRed localized to the mPFC at 5x (left). Double-labeled DsRed and PV cell at 63x (right). Scale bar = 10 μ m. UCMS: unpredictable chronic mild stress. EPM: elevated plus maze. OF: open field. FST: forced swim test. CNO: clozapine-N-oxide. PV: parvalbumin.

Figure 11. Chronic stress increases anxiety-like behaviors in females, which is not rescued by acute prefrontal parvalbumin cell inhibition.

(A) No significant differences in time spent in the open arms of the EPM, nor in **(B)** distance traveled in the open arms relative to total distance traveled. **(C)** Chronic stress significantly reduces time spent in the center of the OF, as well as **(D)** distance traveled in the center relative to total distance traveled. **(E)** No significant differences in latency to immobility in the FST nor in **(F)** total immobility. $N = 7-10$ per group. In all figures, data are presented as mean $\pm$ SEM. #: interaction effect. *** $p \leq 0.001$. EPM: elevated plus maze; OF: open field test; FST: forced swim test.

Figure 12. Chronic stress increases anxiety-like behaviors in males, which is not rescued by acute prefrontal parvalbumin cell inhibition.

(A) No significant differences in time spent in the open arms of the EPM, nor in **(B)** distance traveled in the open arms relative to total distance traveled. **(C)** Chronic stress significantly reduces time spent in the center of the OF. **(D)** Acute prefrontal PV cell inhibition increases distance traveled in the center relative to total distance traveled. **(E)** No significant differences in latency to immobility in the FST nor in **(F)** total immobility. $N = 7-8$ per group. In all figures, data are presented as mean $\pm$ SEM. #: interaction effect. $*p \leq 0.05$; $***p \leq 0.001$. EPM: elevated plus maze; OF: open field test; FST: forced swim test.

Figure 13. Prefrontal expression of *Arc* is impacted by chronic stress and acute prefrontal PV cell inhibition in females.

(A) Chronic stress and acute prefrontal PV cell inhibition interact to affect *Arc* expression in the PFC. (B) No significant differences in *PSD-95* or (C) *Nptx2* expression. $N = 4-5$ per group. In all figures, data are presented as mean $\pm$ SEM. #: significant interaction effect ($p \leq 0.05$).

Figure 14. Chronic stress reduces prefrontal expression of *Arc* and *Nptx2* in males.

(A) Chronic stress significantly reduces *Arc* mRNA expression. **(B)** No significant differences in *PSD-95* expression. **(C)** Chronic stress significantly reduces *Nptx2* mRNA expression. $N = 4-5$ per group. In all figures, data are presented as mean $\pm$ SEM. $*p \leq 0.05$; $**p \leq 0.01$.

Chapter 4: Chronic lorazepam administration increases prefrontal inhibition and exacerbates stress-induced anxiety in female mice

Introduction

The prefrontal cortex (PFC) relies on a balance between excitatory and inhibitory neurotransmission for a range of higher-order functions, such as working memory, planning, decision-making, error monitoring, and emotional regulation (Fuster and Bressler, 2015; Dixon et al., 2017). Chronic stress perturbs prefrontal excitatory/inhibitory (E/I) balance and leads to emotional dysregulations, as seen in disorders like anxiety (Wieronska et al., 2011; Page and Coutellier, 2019). As previously discussed (Chapter 1), the precise nature of E/I imbalances in stress and affective disorders is still being unraveled.

Stress appears to have biphasic effects on the prefrontal glutamatergic system. Acutely, stress enhances excitatory transmission in the PFC. Chronically, stress impedes glutamatergic transmission and causes prefrontal hypoactivity, along with emotional dysfunction (Musazzi et al., 2015; Son et al., 2018) (Figure 2). There are numerous reports of GABAergic deficits in stress and emotional disorders (Lüscher et al., 2011; Ghosal et al., 2017; Fogaça and Duman, 2019) alongside contradictory evidence for increased prefrontal inhibition (McKlveen et al., 2016; Shepard et al., 2016). Chapter 2 supports the hypothesis that increased prefrontal inhibition decreases prefrontal activity and drives anxiety in female mice.

A major open question for this line of research is the nature of the interactions between the excitatory and inhibitory systems in the PFC in stress and emotional disorders. Excitation and inhibition are inextricably intertwined with each other. The excitatory and inhibitory systems work to keep prefrontal and associated networks in balance in a homeostatic manner. If a network is hyper-excitabile beyond a set point, mechanisms of inhibitory control engage in order to keep excitation in check. For example, elevated activity levels can cause compensatory increases in the amplitude and/or frequency of inhibitory post-synaptic currents (IPSCs) (Wenner, 2011). Reduced inhibition can cause compensatory decreases in excitation (Seybold et al., 2012). Following this line of thinking, it has been proposed that reduced GABAergic functioning could be a causative factor in impaired glutamatergic transmission resulting from chronic stress (Ghosal et al., 2017). The reverse is also possible, that elevated excitation causes compensatory increases in inhibitory control. However, these ideas have not yet been tested.

The present study builds on previous findings that stress initially causes an increase in excitatory transmission but chronically leads to prefrontal hypoactivity, (Musazzi et al., 2015) (Figure 2). The present study also continues to investigate the hypothesis that anxiety disorders in females involve increased prefrontal inhibition (Shepard et al., 2016; Chapter 2). Lorazepam (LZ), a positive allosteric modulator for GABA_A receptors (Saari et al., 2011), was administered during the first 2 weeks of a 4-week chronic stress protocol in adult females to test whether this intervention would help or hurt stress-induced anxiety-related behaviors. If anxiety disorders indeed involve

increased prefrontal inhibition, further increasing GABAergic transmission would be expected to exacerbate the detrimental effects of chronic stress. However, it is also possible that increased inhibitory control during the initial phases of chronic stress exposure could be protective against its anxiogenic effects. Stress initially increases excitation but then leads to prefrontal hypoactivity (Musazzi et al., 2015). The “tipping point” into prefrontal hypoactivity may arise from the inhibitory system over-compensating for increased excitation (Wenner, 2011). If this is the case, enhancing GABAergic transmission during the initial phases of stress exposure may help counter-balance excitation and keep the network in stable balance. Either outcome contributes to understanding the nature of the relationship between excitation and inhibition in chronic stress and anxiety disorders.

The results from the experiment support the first outcome proposed above: enhancing inhibition in the PFC during the first 2 weeks of a 4-week chronic stress exposure exacerbates stress-induced anxiety-like behaviors in females. Furthermore, increased pre-synaptic inhibition positively correlates with increased anxiety-like behaviors. These findings further support the hypothesis of prefrontal over-inhibition as a mechanism for stress-induced anxiety.

Materials and Methods

Animals

Adult (8-week old) female C57Bl/6J mice were ordered from Jackson Laboratory (Maine, US). Mice were group-housed (4 mice per cage, unless specified otherwise) and

maintained on a 12-hour reverse light/dark cycle with access to food and water *ad libitum*. Animals were given one week to habituate to the facility prior to the start of the experiment. After habituation, the animals were divided into 4 groups ($N = 8$ per group): control vehicle, control lorazepam (LZ), stress vehicle, stress LZ. Only females were used in this experiment as a follow-up to previous research showing that females are more susceptible to stress-induced anxiety (Shepard et al., 2016), and that increased prefrontal inhibition induces anxiety-like behaviors in female mice only (Chapter 2).

All procedures related to animal maintenance and experimentation were approved by The Ohio State University Office of Responsible Research Practices and conformed to the U.S. National Institute of Health Guide for the Care and Use of Laboratory animals. Animals were cared for according to regulations set forth by the Institutional Animal Care and Use Committee (IACUC) to minimize pain and discomfort and reduce the number of animals used.

Unpredictable chronic mild stress procedure

The unpredictable chronic mild stress (UCMS) procedure is the same as we used previously (Shepard et al., 2016; Shepard and Coutellier, 2018). It consisted of daily exposure to alternating mild stressors for 4 weeks. The following stressors were presented in random order: absence of nesting material for 24 hours, 20° cage tilt on the vertical axis for 6 hours, absence of bedding in the cage for 8 hours, restraint stress in the dark for 8 minutes, and restraint stress under bright light for 4 minutes. Stressors were applied according to an unpredictable schedule. Stressed mice were single-housed on the

first day of stress exposure until euthanasia. Control mice remained group-housed and were handled once daily for 1-2 minutes for 4 weeks. Body weights were collected daily during the UCMS/handling procedure, and at the time of euthanasia.

Lorazepam treatment

LZ was prepared at 100 µg/kg in distilled water. The corresponding vehicle control solution was 0.9% NaCl. This dose and vehicle were chosen based on previous research (Ramirez et al., 2016). LZ or vehicle was administered via intraperitoneal (i.p.) injection at a volume of 0.01 ml/g body weight (i.e., for a mouse that weighs 30 g the volume injected was 0.3 ml). Treatment was administered 30 minutes prior (Bourin et al., 1992) to each variate stress session (or handling) for the first 2 weeks of the UCMS procedure. For the last 2 weeks of the UCMS or handling, no injection was received. Two weeks of LZ treatment was chosen based on our previous study that used the same UCMS paradigm for 2 or 4 weeks (Shepard et al., 2016). In this study, 2 weeks slightly elevated anxiety-like behaviors as well as prefrontal parvalbumin mRNA expression, while 4 weeks produced more substantial elevations in these measurements. This led us to hypothesize that the first 2 weeks of a 4-week UCMS procedure may be a window of opportunity for intervention in the GABAergic system.

Behavioral tests

Behavioral testing was performed during the dark phase of the mice's light cycle. Mice were acclimated to the testing room in the dark for at least one hour prior to testing. Mice

were exposed to the elevated plus maze (EPM) on one day and the open field (OF) test on the following day. Only anxiety measurements were used in this experiment as increased prefrontal inhibition appears to specifically modulate anxiety-like behaviors in females (Chapter 2).

The EPM was conducted under red light and consisted of a raised (74 cm) arena with four arms (each 34 cm long). The two closed arms were surrounded by 22 cm-high black walls. Mice were allowed to explore the maze for 5 minutes, after which they were returned to their home cage and the arena was cleaned with 70% ethanol prior to testing the next mouse. The OF test used a 40 x 40 cm gray square arena with opaque white walls under dim white lights (~50 lux). Mice were allowed to explore the arena for 10 minutes and were again returned to their home cage and the arena cleaned with 70% ethanol.

Both the EPM and OF tests were recorded with an overhead camera for offline analysis. Automated, unbiased analyses were conducted using the EthoVision XT software from Noldus Information Technology (Wageningen, Netherlands). As total locomotor activity can bias the interpretation of these behaviors, we also calculated open arm entry ratio/total entries for EPM, and thigmotaxis ratio (distance traveled near walls/total distance) for the OF (Guilloux et al., 2011; Delprato et al., 2017).

Following the completion of behavioral testing, mice were anaesthetized with isoflurane and rapidly decapitated; the brains were flash frozen on dry ice for molecular analyses.

mRNA expression

The flash frozen brains from the 4-week and 2-week cohorts were stored at -80°C until dissection. Dissection of the PFC was performed in a cold room on dry ice according to the *Mouse Brain in Stereotaxic Coordinates* (Paxinos and Franklin, 2012). Target mRNA were quantified using real-time polymerase chain reaction (RT-PCR). Brain tissues were processed as previously described (Coutellier et al., 2015). The target cDNA and the reference target glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were amplified simultaneously in the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The following primer sequences were used:

GAD1: Fwd: CCTTTCTATGCCGCTGAGTT,

Rev: CACGACTGTTTACGGAGCAT (Page et al., 2018);

GABA_Aα1: Fwd: CAAGAGCAGAAGTTGTCTATGAGT

Rev: GCACGGCAGATATGTTTGAATAAC (Shepard et al., 2017)

Egr1: Fwd: TCCTCTCCATCACATGCCTG,

Rev: CACTCTGACACATGCTCCAG (Page et al., 2018);

GAPDH: Fwd: CATGGCCTTCCGTGTTTCCT,

Rev: TGATGTCATCATACTTGGCAGGTT (Page et al., 2018).

Statistical analysis

Data were analyzed using 2-way analysis of variance (ANOVA) using the software Prism 5.01 (GraphPad Software Inc., CA, USA) with stress (control vs. UCMS) and drug (vehicle vs. LZ) as the independent variables. For multiple comparisons, Tukey's

multiple comparisons adjustment was used. Data are presented as mean $\pm$ standard error of the mean (SEM).

For the behavioral data, a Z-score of anxiety-like behavior was calculated (Guilloux et al., 2011; Page and Coutellier, 2018). Repeated behavioral testing in mice can produce biases and interpretation of data is often affected by individual variability; this method helps overcome these issues by allowing dimensionless comparison across the different parameters and behavioral tests. It consists of first measuring a Z-score for each parameter in each test using the following equation:

$$Z = \frac{X - \mu}{\sigma}$$

X represents an individual data point for the observed parameter, and μ and σ represent the mean and standard deviation for a control reference group, respectively. The unstressed vehicle group was used as the reference group. The direction of measurements for each parameter was adjusted so that higher Z-scores represented increased anxiety. The Z-scores for each parameter within a test were averaged together to obtain an overall Z-score for the test, as follows:

$$Z_{EPM} = \frac{Z_{(time\ in\ open\ arms)} + Z_{(time\ in\ closed\ arms)} + Z_{(open\ arm\ entries)} + Z_{(open\ entry\ ratio)}}{4}$$

$$Z_{OF} = \frac{Z_{(time\ in\ center)} + Z_{(time\ in\ walls)} + Z_{(center\ entries)} + Z_{(thigmotaxis\ ratio)}}{4}$$

Lastly, the Z-scores for each test were averaged together to achieve an overall Z-score of anxiety:

$$Z_{anxiety} = \frac{Z_{EPM} + Z_{OF}}{2}$$

Z-score calculations were performed in R (The R Foundation, Vienna, Austria). Correlations between behavioral outputs and mRNA expression levels were conducted using two-tailed Pearson's correlation analyses in GraphPad Prism 5.01 and graphed in R.

Results

Anxiety-like behaviors are increased by stress and exacerbated by lorazepam

Chronic stress reduced the time spent in the open arms of the elevated plus maze ($F_{1,28} = 3.65$, $p = 0.066$; Figure 15A) and significantly increased the time spent in the closed arms of the elevated plus maze ($F_{1,28} = 10.48$, $p = 0.0031$; Figure 15B). The stress + LZ group spent the most time in the closed arms of the elevated plus maze (compared to control + vehicle: $p = 0.054$; compared to control LZ: $p = 0.065$). Chronic stress also reduced the number of entries in to the open arms ($F_{1,28} = 8.74$, $p = 0.0063$; Figure 15C). Stress + LZ animals again showed the highest anxiety (lowest number of entries into the open arms; compared to control + vehicle: $p = 0.033$; compared to control + LZ: $p = 0.037$). We also calculated the ratio of open arm entries to total zone entries to control for any possible changes in locomotor activity in the EPM. Chronic stress also significantly reduced the EPM entry ratio (open arm entry ratio/total entries) ($F_{1,28} = 4.83$, $p = 0.036$; Figure 15D). Again, the stress + LZ group showed the highest anxiety-like behavior (lowest entry ratio; compared to control + vehicle: $p = 0.037$).

Chronic stress also reduced time spent in the center of the open field ($F_{1,28} = 5.80$, $p = 0.023$; Figure 15E) and increased time spent near the walls ($F_{1,28} = 5.80$, $p = 0.023$; Figure 15F). Specifically, LZ administration reduced anxiety-related behavior in the

control group, but not in the stressed group. For time spent in the center or the walls of the open field, the control + LZ had the lowest anxiety (most time spent in center and least time spent near walls; compared to stress + vehicle: $p = 0.0277$). Stress or LZ administration did not have any effect on the number of entries into the center of the OF (Figure 15G). To control for possible changes in locomotor activity in the OF, we also calculated thigmotaxis ratio (distance traveled near walls/total distance traveled): chronic stress increased thigmotaxis ratio ($F_{1,28} = 5.77$, $p = 0.023$; Figure 15H) with no effect of LZ.

Chronic stress increased Z-scores in the EPM ($F_{1,28} = 8.621$, $p = 0.0066$; Figure 16A) and the OF ($F_{1,28} = 4.875$, $p = 0.036$; Figure 16B). For the Z-EPM score, stress + LZ mice had the highest score, which was significantly different compared to control + vehicle ($p = 0.027$). Chronic stress significantly increased Z-anxiety score (indicating higher anxiety-like behaviors) ($F_{1,28} = 12.3$, $p = 0.0015$; Figure 16C). Again, the stress + LZ group had the highest Z-anxiety score (compared to control + LZ: $p = 0.013$; compared to vehicle + control: $p = 0.056$).

Body weight gain decreases after 2 weeks of chronic stress and is normalized by 4 weeks, with no effect of lorazepam

Body weights were tracked daily and analyzed after 2 and 4 weeks of chronic stress exposure. The gain in body weight from the start of the experiment was analyzed at 2 and 4 weeks. Two weeks of chronic stress exposure significantly reduced body weight gain ($F_{1,28} = 5.28$, $p = 0.029$; Figure 17A) with no effect of LZ. There were no differences in

body weight gain after 4 weeks of UCMS (Figure 17B). There were also no significant differences in absolute body weight after 4 weeks of UCMS (Figure 17C).

Lorazepam administration increases prefrontal pre-synaptic inhibition

LZ treatment increased prefrontal *GAD1* mRNA expression ($F_{1,11} = 6.416, p = 0.028$; Figure 18A). There was no significant effect of stress alone ($F_{1,11} = 2.264, p = 0.161$); however, stress + LZ animals had the highest expression levels of *GAD1* compared to the other groups. LZ treatment also reduced prefrontal *GABA-A α 1* expression ($F_{1,11} = 6.004, p = 0.032$; Figure 18B), with no significant effect of stress ($F_{1,11} = 2.358, p = 0.153$). Stress + vehicle animals had the highest expression of *GABA-A α 1* in the PFC. Chronic stress trended towards reducing prefrontal *Egr1* mRNA expression levels (stress effect: $F_{1,11} = 4.342, p = 0.0613$) with no effect of LZ ($F_{1,11} = 0.0419, p = 0.842$; Figure 18C).

Increased GAD1 expression correlates with increased anxiety in the elevated plus maze

Increased prefrontal expression of *GAD1* was correlated with less time spent in the open arms of the EPM ($r = -0.505, p = 0.055$) and a reduced entry ratio in the EPM ($r = -0.547, p = 0.035$). Increased *GAD1* expression also trended towards correlating with a higher Z-score on the EPM ($r = 0.478, p = 0.072$). There were no significant or trending correlations between *GAD1* expression and behavior in the OF, nor between *GABA-A α 1* or *Egr1* expression and any anxiety measurements (Table 3; Figure 19).

Discussion

The findings from this study show that 4 weeks of chronic stress combined with lorazepam treatment during the first 2 weeks increases anxiety-like behaviors in adult female mice. This increase in anxiety was correlated with an increase in prefrontal inhibition as measured by *GAD1* mRNA expression. These results support the hypothesis that chronic stress-induced anxiety disorders involve over-inhibition in the PFC. As acute lorazepam is commonly used to treat anxiety and panic, chronic use should be approached with caution, especially under conditions of stress exposure. Interventions that reduce inhibition in the PFC may instead be therapeutic in the long term for anxiety disorders.

These conclusions could be considered surprising or counterintuitive given the classically anxiolytic effects of lorazepam. Lorazepam and other benzodiazepines are frequently used to treat anxiety as they act as positive allosteric modulators of GABA_A receptors to enhance GABAergic transmission (Saari et al., 2011), and are thought to act at the hypothalamus to suppress the hypothalamic-pituitary-adrenal (HPA) axis stress response (Arvat et al., 2002; Ramirez et al., 2016). Benzodiazepine treatment has also been shown to have protective effects against stress. Two days of pre-treatment with lorazepam or clonazepam prior to a 6-day repeated social defeat stress prevented stress-induced anxiety (Ramirez et al., 2016), and administration of diazepam prior to each daily stress exposure over 4 weeks prevented depression-like behavior (Zhao et al., 2012). Importantly, Ramirez et al. did not administer benzodiazepines chronically. Zhao et al. did use chronic administration; however, they did not test for anxiety-like behavior

and used much higher doses (0.5, 1, and 2 mg/kg). Moreover, both these studies used male mice. Chronic stress in conjunction with diazepam treatment has been shown to prevent anxiety-like behaviors as measured by the EPM and light/dark box, but again, this study used only male mice (Wang et al., 2014). To our knowledge, this is the first study to examine the effects of chronic stress in conjunction with benzodiazepine treatment in females.

There also have been few studies examining the effects of benzodiazepine administration on the PFC. Chronic diazepam treatment over 21 days has been found to reduce spine density in prefrontal pyramidal neurons of male mice (Curto et al., 2016). This is surprisingly reminiscent of the effects of chronic stress (Moench and Wellman, 2015), supporting the notion that chronic benzodiazepine treatment has detrimental effects on excitation in the PFC and may contribute to prefrontal hypoactivity. Pretreatment with diazepam prior to repeated stress exposure may alter glutamate dynamics and E/I balance in the PFC, preventing the PFC from adapting its response to multiple stressors, according to a study in male rats (Bagley and Moghaddam, 1997). The male PFC may be better able to adapt to the effects of repeated stress exposure compared to the female PFC (Moench and Wellman, 2017; Wellman et al., 2018; Moench et al., 2019). Plasticity of the PFC following stress is important for resilience and recovery, and chronic stress may impair prefrontal plasticity especially in females. Lorazepam treatment in conjunction with chronic stress may further impair the ability of the PFC to adapt to repeated stress exposure, exacerbating anxiety-like behavior and prefrontal hypoactivity.

Understanding biphasic or multiphasic effects of chronic or repeated stress on E/I balance and anxiety-related behavior is of critical importance. Glutamate and GABA dynamics in the PFC may be key determinants of the stress response (Bagley and Moghaddam, 1997). In the present study, if lorazepam treatment had been shorter or earlier (i.e., one pretreatment prior to stress exposure, or 1 week of treatment), it may have been able to stabilize E/I balance and prevent the development of anxiety-like behavior. Conversely, if treatment had been longer (3 or 4 weeks), it may have further exacerbated anxiety-like behavior and prefrontal hypoactivity. We elected to administer lorazepam for 2 weeks based on our previous research demonstrating a slight elevation in anxiety-like behavior and parvalbumin mRNA in the PFC following 2 weeks of chronic stress, compared to a stronger elevation after 4 weeks of chronic stress (Shepard et al., 2016). This suggested that the first 2 weeks of chronic stress exposure may be a window of opportunity for manipulating the stress response. It would be worthwhile for future studies to conduct time-series experiments that manipulate the prefrontal GABAergic system at different time points and for longer or shorter durations to examine the effects on E/I balance and emotional behaviors. Such experiments would also help scale the axes in Figure 2 and provide a clearer picture of E/I dynamics under chronic stress conditions, revealing windows of opportunities for therapeutic intervention.

Two weeks of lorazepam treatment out of a 4-week chronic stress exposure was also chosen to try to avoid benzodiazepine withdrawal symptoms. Chronic use of benzodiazepines can cause dependence and cessation of use can lead to withdrawal. One of the most common symptoms of withdrawal is rebound anxiety (Pétursson, 1994).

Therefore, it is possible that the elevated anxiety observed in chronic stress + lorazepam-treated mice is part of a discontinuation syndrome. If this were the case, we would also expect to see elevated anxiety in the unstressed lorazepam treated mice, which we do not. A previous study found that withdrawal from 2 mg/kg of chronic diazepam treatment over 14 days did not increase anxiety-like behavior in male rats (Kovačević et al., 2014). However, we cannot entirely rule out the possibility of rebound anxiety in this study. A low dose of lorazepam (100 µg/kg) was also chosen to alleviate this possibility.

Selective serotonin reuptake inhibitors (SSRIs) are thought to be more effective than benzodiazepines for the treatment of generalized anxiety (Davidson, 2004). One possible explanation is that SSRIs reduce markers of inhibitory transmission in the PFC (Sung et al., 2008; Ohira et al., 2013; Guirado et al., 2014; Lee et al., 2018). These interventions would elevate activity levels in the PFC, which has been shown to be therapeutic for anxiety and depression (Covington et al., 2010; Kumar et al., 2013; Fuchikami et al., 2015; Reznikov et al., 2018). By contrast, chronic benzodiazepine treatment may be exacerbating prefrontal hypoactivity, as shown by elevations in *GAD1* expression that correlated with increased anxiety-like behavior.

One finding from the present study is that the increase in prefrontal inhibition by chronic stress and lorazepam treatment appears to be pre-synaptic, as we observed elevated pre-synaptic *GAD1* mRNA but reduced post-synaptic *GABA-A α 1* expression by lorazepam treatment. An early study also demonstrated decreased cortical *GABA_A* receptor subunit concentrations ($\alpha 1$ and $\gamma 2$) following 14 days of lorazepam administration (Kang and Miller, 1991). This is likely a compensatory response to

increased GABAergic transmission. We also observed an inclination towards a stress-induced increase in *GABA-A α 1* expression in vehicle-treated animals that was abolished by lorazepam treatment. This replicates previous findings that stress increases cortical $\alpha 1$ and $\gamma 2$ mRNA expression (Kang et al., 1991). It is important to note that other studies have shown decreased *GAD1* in the frontal cortex by chronic stress, which was associated with depression-like behaviors (Orsetti et al., 2008; Veeraiah et al., 2014). These studies used male rodents. These contradictory findings still highlight the fact that GABAergic changes (deficits vs. over-inhibition) in stress-induced emotional disorders remain inconclusive, especially with regards to sex differences.

Chronic stress trended toward reducing prefrontal activity in the PFC as measured by expression of *Egr1*, an immediate early gene. This observation supports the hypothesis of prefrontal hypoactivity under conditions of stress and emotional dysfunction (Musazzi et al., 2015; Son et al., 2018). Lorazepam administration did not further reduce *Egr1* levels, which might be expected if GABA agonism is exacerbating stress-induced prefrontal hypoactivity. Levels of *Egr1* also did not correlate with any measurements of anxiety-like behavior. Absolute estimates of prefrontal activity may not be the best indicator of emotional behaviors, however. As we saw in Chapter 2, both acute and chronic activation of inhibitory parvalbumin (PV) cells induced hypoactivity of the PFC as measured by cFos expression, though only chronic PV cell activation increased anxiety-like behavior. The acute reduction in prefrontal activity was not associated with anxiety. Therefore, measurements of activity in the PFC do not always correspond directly with changes in emotional behaviors.

Overall, the results from the present study support the hypothesis that over-inhibition in the PFC is part of the pathogenesis of anxiety-like behavior in females. Pharmaceutical interventions that decrease, rather than increase, GABAergic transmission in the PFC may be therapeutic for anxiety disorders. Further research is needed to continue parsing the dynamics of prefrontal E/I balance in stress and emotional disorders like anxiety.

Figure 15. Lorazepam treatment exacerbates chronic stress-induced anxiety-like behaviors.

(A-D) Chronic stress increased anxiety-like behaviors in the elevated plus maze (EPM). Stress + lorazepam-treated animals had the highest anxiety-like behaviors in the EPM. **(E-H)** Chronic stress increased anxiety-like behaviors in the open field (OF). Lorazepam treatment in control animals increased time spent in the center of the OF and reduced time spent near the walls, but this anxiolytic effect was abolished in chronically stressed mice. In all figures, summary data are represented as mean $\pm$ SEM. $N = 8$ per group. $*p \leq 0.05$. $**p \leq 0.01$. a,b: different letters are significantly different from one another. LZ: lorazepam.

Figure 16. Z-scores for anxiety tests show lorazepam exacerbates stress-induced anxiety-like behaviors.

Chronic stress increases Z-scores in elevated plus maze (EPM) (A), open field (OF) (B), and overall anxiety (C). For the Z-EPM and Z-anxiety scores, stress + lorazepam-treated animals had the highest measures of anxiety-like behavior. In all figures, summary data are represented as mean $\pm$ SEM. $N = 8$ per group. $*p \leq 0.05$. $**p \leq 0.01$. a,b: different letters are significantly different from one another. LZ: lorazepam.

Figure 17. Body weight gain decreases after 2 weeks of chronic stress and is normalized after 4 weeks.

(A) Chronically stressed females had reduced body weight gain compared to controls after 2 weeks of chronic stress. (B) There were no significant differences in body weight gain after 4 weeks of chronic stress, nor any differences in (C) absolute body weights. In all figures, summary data are represented as mean $\pm$ SEM. $N = 8$ per group. $*p \leq 0.05$. LZ: lorazepam.

Figure 18. LZ administration and chronic stress increase prefrontal pre-synaptic inhibition.

(A) Lorazepam treatment increases prefrontal *GAD1* expression and (B) decreases *GABA-A α 1* expression. (C) Chronic stress trends towards decreasing *Egr1* expression. In all figures, summary data are represented as mean $\pm$ SEM. $N = 3-4$ per group. * $p \leq 0.05$. LZ: lorazepam.

Figure 19. Increased *GAD1* expression correlates with increased measurements of anxiety-like behavior.

Increased *GAD1* expression in the prefrontal cortex is correlated with **(A)** decreased time spent in the open arms of the elevated plus maze (EPM), **(B)** reduced entry ratio in the elevated plus maze, and **(C)** increased Z-EPM score, all indicative of increased anxiety-like behaviors. Gray shading represents the 95% confidence interval around the blue line of best fit.

	<i>GAD1</i>	<i>GABA-Aα1</i>	<i>Egr1</i>
EPM			
Open arm duration	$r = -0.505$ $p = 0.055$ ^t	$r = -0.116$ $p = 0.682$	$r = 0.331$ $p = 0.229$
Closed arm duration	$r = 0.359$ $p = 0.189$	$r = 0.080$ $p = 0.776$	$r = -0.171$ $p = 0.541$
Open arm entries	$r = -0.449$ $p = 0.093$	$r = -0.245$ $p = 0.378$	$r = 0.329$ $p = 0.232$
Entry ratio	$r = -0.547$ $p = 0.035$ *	$r = -0.217$ $p = 0.437$	$r = 0.327$ $p = 0.234$
OF			
Center duration	$r = 0.246$ $p = 0.377$	$r = -0.154$ $p = 0.583$	$r = -0.117$ $p = 0.679$
Walls duration	$r = -0.246$ $p = 0.377$	$r = 0.154$ $p = 0.583$	$r = 0.117$ $p = 0.679$
Center entries	$r = -0.073$ $p = 0.797$	$r = 0.239$ $p = 0.392$	$r = -0.314$ $p = 0.254$
Thigmotaxis ratio	$r = -0.102$ $p = 0.717$	$r = 0.173$ $p = 0.538$	$r = 0.119$ $p = 0.674$
Z-scores			
Z-EPM	$r = 0.478$ $p = 0.072$ ^t	$r = 0.162$ $p = 0.564$	$r = -0.292$ $p = 0.292$
Z-OF	$r = -0.169$ $p = 0.548$	$r = 0.096$ $p = 0.733$	$r = 0.169$ $p = 0.545$
Z-anxiety	$r = 0.289$ $p = 0.297$	$r = 0.169$ $p = 0.548$	$r = -0.146$ $p = 0.604$

Table 3. Correlations between behavioral data and expression of inhibitory and activity markers in the prefrontal cortex.

EPM: elevated plus maze; OF: open field. t: $p \leq 0.08$; * $p \leq 0.05$.

Chapter 5: Kv3.1 deletion incurs resilience against chronic stress-induced anxiety in females

Introduction

Anxiety is a stress-related mood disorder that is associated with reduced activity of the prefrontal cortex (PFC) (Motzkin et al., 2015; Wang et al., 2015; Park et al., 2016). Increased prefrontal inhibition may be a mechanism contributing to prefrontal hypoactivity in stress-induced mood disorders like anxiety (Shepard et al., 2016), with particular relevance to the increased prevalence of this disorder in women (Kessler et al., 2012). Increased prefrontal parvalbumin (PV) cell excitation reduces activity in the PFC and increases anxiety-like behaviors in female mice (Chapter 2). Furthermore, increasing prefrontal inhibition during chronic stress exposure exacerbates stress-induced anxiety-like behaviors in females (Chapter 4). Therefore, reducing prefrontal inhibition during chronic stress exposure, thus increasing activity in the PFC, may incur resilience against stress-induced anxiety.

PV cells are a key target for manipulating levels of inhibition because these cells provide strong, fast-spiking inhibitory control on or near the cell bodies of excitatory pyramidal cells (Tremblay et al., 2016). This makes PV cells one of the dominant regulators of neuronal activity and excitatory/inhibitory (E/I) balance within the PFC and other brain regions (Ferguson and Gao, 2018a). Voltage-gated potassium (K⁺) ion channels (Kv channels) are highly expressed in PV neurons and help endow these cells

with their fast-spiking properties (Rudy and McBain, 2001). Kv channels regulate neuronal excitability by controlling K⁺ efflux and thus the dynamics of action potential firing. They can rapidly open and close, quickly resetting neuronal membrane potential to allow for high frequency firing (Macica et al., 2003; Labro et al., 2015; Kaczmarek and Zhang, 2017). In particular, the Kv3.1 isoform is highly expressed in PV-expressing interneurons (Chow et al., 1999; McDonald and Mascagni, 2006).

Transgenic mice deficient for the *Kv3.1* gene have altered fast-spiking neuron dynamics and show behavioral abnormalities (Ho et al., 1997). Kv3.1-null male mice display hyperlocomotion and decreased anxiety- and depression-like behaviors compared to their wildtype counterparts (Parekh et al., 2018). Interestingly, antidepressants like fluoxetine and paroxetine, which are frequently used to treat anxiety disorders (Feighner, 1999; Davidson, 2004), block the Kv3.1 channel (Sung et al., 2008; Lee et al., 2018). Together, these observations lend support to the hypothesis that increased PV cell activity is part of the pathogenesis of anxiety and that reducing PV cell activity via manipulation of the Kv3.1 channel may be therapeutic or preventative. However, the behavioral effects of Kv3.1 deficiency in female mice, as well as the effects of Kv3.1 deficiency in combination with chronic stress exposure, have not been examined.

The present study utilizes transgenic Kv3.1 wildtype (WT), heterozygous (HET), and knock-out (KO) female mice exposed to chronic stress to examine their susceptibility or resilience to the anxiogenic effects of stress. The study is ongoing, but present results indicate that KO females do not display the anxiogenic effects of chronic stress. Chronic stress induced anxiety-like behaviors in WT and HET mice, but not KO mice, suggesting

that global removal of the Kv3.1 channel incurs resilience against chronic stress, presumably through reduced PV cell excitability and increased activity in the PFC, though the precise mechanisms have yet to be examined.

Materials and Methods

Animals

Female Kv3.1 WT, HET, and KO mice were used to determine the role of PV cell activity in stress vulnerability. Original breeder pairs were obtained from the laboratory of Dr. Leonard Kaczmarek at the Yale School of Medicine (New Haven, CT). Mice were then bred in-house using HET x HET breeder pairs. Litters were weaned at postnatal day (PND) 21 and group-housed by sex with 2 to 5 mice per cage, unless specified otherwise.

Only female mice were used as a follow-up to our previous research showing that females are more susceptible to stress-induced anxiety and increased prefrontal PV cell activity (Shepard et al., 2016), and that increased prefrontal inhibition induces anxiety-like behaviors in female mice only (Chapter 2).

All mice had access to food and water ad libitum and were maintained on a 12-hour reverse light/dark cycle. All procedures related to animal maintenance and experimentation were approved by The Ohio State University Office of Responsible Research Practices and conformed to the U.S. National Institute of Health Guide for the Care and Use of Laboratory animals. Animals were cared for according to regulations set forth by the Institutional Animal Care and Use Committee (IACUC) to minimize pain and discomfort and reduce the number of animals used.

Unpredictable chronic mild stress procedure

The unpredictable chronic mild stress (UCMS) procedure is the same as we used previously (Shepard et al., 2016; Shepard and Coutellier, 2018). It consisted of daily exposure to alternating mild stressors for 4 weeks. The following stressors were presented in random order: absence of nesting material for 24 hours, 20° cage tilt on the vertical axis for 6 hours, absence of bedding in the cage for 8 hours, restraint stress in the dark for 8 minutes, and restraint stress under bright light for 4 minutes. Stressors were applied according to an unpredictable schedule. Stressed mice were single-housed on the first day of stress exposure until euthanasia. Control mice remained group-housed and were handled once daily for 1-2 minutes for 4 weeks.

Behavioral testing

Behavioral testing was performed during the dark phase of the mice's light cycle. Mice were acclimated to the testing room in the dark for at least one hour prior to testing. Mice were exposed to three anxiety tests in the following order, with one test per day: elevated plus maze (EPM), open field (OF), and marble bury (MB). Only anxiety measurements were used in this experiment as increased prefrontal inhibition appears to specifically modulate anxiety-like behaviors in females (Chapter 2).

The EPM was conducted under red light and consisted of a raised (74 cm) arena with four arms (each 34 cm long). The two closed arms were surrounded by 22 cm-high black walls. Mice were allowed to explore the maze for 5 minutes, after which they were returned to their home cage and the arena was cleaned with 70% ethanol prior to testing

the next mouse. The OF test used a 40 x 40 cm gray square arena with opaque white walls under dim white lights (~50 lux). Mice were allowed to explore the arena for 10 minutes and were again returned to their home cage and the arena cleaned with 70% ethanol.

Both the EPM and OF tests were recorded with an overhead camera for offline analysis. Automated, unbiased analyses were conducted using the EthoVision XT software from Noldus Information Technology (Wageningen, Netherlands). As total locomotor activity can bias the interpretation of these behaviors, we also calculated distance ratios: open arm entry ratio/total entries for EPM, and thigmotaxis ratio (distance traveled near walls/total distance) for the OF (Guilloux et al., 2011; Delprato et al., 2017).

For the MB test, 20 marbles were evenly placed in a 5 x 4 grid on top of approximately 10 cm of clean corncob bedding in a standard size clean mouse cage. The mouse was taken from its home cage and placed in the test cage under dim white lighting. The mouse was allowed to explore the cage for 30 minutes. After 30 minutes the mouse was removed from the test cage and placed back in its home cage. The buried marbles were then counted. Any marble more than half covered with bedding was considered buried. Marbles were cleaned after each test with 70% EtOH and a new cage was used for each mouse. More buried marbles is indicative of increased anxiety-like behavior as indicated by neophobia and/or compulsivity (Kedia and Chattarji, 2014).

Ninety minutes after the MB test, half of the mice were anaesthetized with isoflurane and rapidly decapitated; the brains were flash frozen on dry ice. The other half

of the mice were perfused with cold 4% paraformaldehyde (PFA). Brains were removed and kept in 4% PFA at 4°C overnight before storage in a sucrose solution (30% sucrose). The flash frozen and perfused brains are being stored for future molecular analyses.

Additional behavioral analyses are ongoing to achieve full group sizes. The data presented here include the following *N*s: 7 WT control; 12 HET control; 7 KO control; 5 WT stress; 10 HET stress; 6 KO stress. One HET stress animal was found dead after completing the EPM and OF test, leaving *N* = 9 for the HET stress group in the MB and the Z-anxiety analysis (see below).

Statistical analysis

Data were analyzed using 2-way analysis of variance (ANOVA) using the software Prism 5.01 (GraphPad Software Inc., CA, USA) with stress (control vs. UCMS) and genotype (WT vs. HET vs. KO) as the independent variables. For multiple comparisons, Tukey's multiple comparisons adjustment was used. Data are presented as mean ± standard error of the mean (SEM).

A Z-score of anxiety-like behavior was calculated (Guilloux et al., 2011; Page and Coutellier, 2018). Repeated behavioral testing in mice can produce biases and interpretation of data is often affected by individual variability; this method helps overcome these issues by allowing dimensionless comparison across the different parameters and behavioral tests. It consists of first measuring a Z-score for each parameter in each test using the following equation:

$$Z = \frac{X - \mu}{\sigma}$$

X represents an individual data point for the observed parameter, and μ and σ represent the mean and standard deviation for a control reference group, respectively. The unstressed WT group was used as the reference group. The direction of measurements for each parameter was adjusted so that higher Z-scores represented increased anxiety. The Z-scores for each parameter within a test were averaged together to obtain an overall Z-score for the test, as follows (MB Z-score was not averaged as there was only one measurement in this behavioral test):

$$Z_{EPM} = \frac{Z_{(time\ in\ open\ arms)} + Z_{(time\ in\ closed\ arms)} + Z_{(open\ arm\ entries)} + Z_{(open\ entry\ ratio)}}{4}$$

$$Z_{OF} = \frac{Z_{(time\ in\ center)} + Z_{(time\ in\ walls)} + Z_{(center\ entries)} + Z_{(thigmotaxis\ ratio)}}{4}$$

Lastly, the Z-scores for each test were averaged together to achieve an overall Z-score of anxiety:

$$Z_{anxiety} = \frac{Z_{EPM} + Z_{OF} + Z_{MB}}{3}$$

Z-score calculations were performed in R (The R Foundation, Vienna, Austria).

Results

Kv3.1 KO females do not show anxiety-like behavior following chronic stress

In the EPM, there was a significant effect of genotype on time spent in the open arms of the maze, with KO animals spending the most time in the open arms ($F_{2,41} = 6.733$, $p = 0.003$). The effect of stress was not significant ($F_{1,41} = 2.068$, $p = 0.158$). There was a trending effect of stress for the time spent in the closed arms ($F_{1,41} = 3.313$, $p = 0.076$). There was a significant effect of stress on the number of open arm entries ($F_{1,41} = 4.428$,

$p = 0.042$), as well as the entry ratio ($F_{1,41} = 5.969, p = 0.019$). For the entry ratio, there was a non-significant effect of genotype such that KO animals had the highest entry ratio ($F_{2,41} = 2.59, p = 0.087$) (Figure 20).

In the OF, there was a significant interaction effect for time spent in the center ($F_{2,41} = 3.333, p = 0.046$) such that chronic stress reduced center duration in WT and HET mice but increased it in KO animals. There was also a significant anxiogenic effect of stress ($F_{1,41} = 4.052, p = 0.05$). KO animals had significantly more entries into the center than WTs and HETs (genotype effect: $F_{2,41} = 3.61, p = 0.036$). Conversely, stressed WT and HET animals spent more time near the walls while stressed KO animals spent less time near the walls (interaction effect: $F_{2,41} = 3.382, p = 0.044$; stress effect: $F_{1,41} = 4.126, p = 0.05$). Thigmotaxis ratio was increased by stress in WT and HET animals but not in KOs (stress effect: $F_{1,41} = 4.183, p = 0.047$) (Figure 21).

In the MB test, there was a significant effect of genotype ($F_{2,40} = 7.093, p = 0.0023$) in which KO animals buried the least marbles. There was no significant effect of stress ($F_{1,40} = 0.687, p = 0.412$) (Figure 22).

The Z-score analyses support these findings. There was a significant anxiogenic effect of stress in the Z-EPM score ($F_{1,41} = 4.682, p = 0.036$). There was also a significant effect of genotype ($F_{2,41} = 3.942, p = 0.027$) with KO animals showing the lowest Z-EPM score (lowest anxiety-like behavior). For the Z-OF, there was a significant effect of stress ($F_{1,41} = 4.342, p = 0.044$), where stress increased anxiety-like behavior in WT and HET animals but not in KOs. For the Z-MB, there was a significant genotype effect ($F_{2,40} = 7.093, p = 0.0023$) such that KO animals had the lowest anxiety-like behavior. There was

also a significant genotype effect in the overall measurement of anxiety ($F_{2,40} = 8.599$, $p = 0.0008$), again with KO animals displaying the lowest Z-score (lowest anxiety-like behavior) (Figure 23).

Discussion

The main findings from this study show that KO females are resilient to the anxiogenic effects of chronic stress. In the EPM, stressed WT females showed increased anxiety-like behavior while control and stressed KOs showed comparable lower levels of anxiety-like behavior. Both control and stressed HET animals appear to have slightly increased anxiety-like behavior relative to WT controls, with no apparent stress differences, possibly reflecting a “ceiling effect” of increased anxiety. In the OF, WT and HET animals showed a similar pattern, with stressed animals showing increased anxiety-like behavior relative to controls. KO animals, however, showed an opposite pattern. Stressed KOs displayed decreased anxiety-like behavior relative to their control counterparts. In the MB test, KOs showed the lowest level of anxiety-like behavior; however, there were no significant stress effects. According to the overall Z-score of anxiety, the anxiogenic effect of stress was strongest in WTs, smaller in HETs, and non-existent in KOs, suggesting a “dose-dependent” effect of reduced Kv3.1 expression.

The behavioral analyses for this experiment are ongoing to achieve full group sizes and upcoming results may strengthen or weaken these findings. The main conclusion currently is that KO females have the lowest anxiety-like behavior and appear resilient to the anxiogenic effects of chronic stress. Low anxiety in Kv3.1 KOs has

previously been reported in males (Parekh et al., 2018); these results replicate this observation in females. This study is the first to combine Kv3.1 deficiency with chronic stress. The evidence for stress resilience in Kv3.1 KO mice supports the hypothesis of over-inhibition in stress-induced anxiety. Kv3.1 KO animals have decreased activity of fast-spiking interneurons like PV cells and therefore reduced inhibitory signaling (Parekh et al., 2018). We hypothesize, therefore, that this genetic reduction in inhibitory neurotransmission prevents stress-induced over-inhibition and anxiety-like behavior.

One limitation of this study is that transgenic Kv3.1 deficiency or deletion is global and not limited to the PFC. This makes the interpretation of the results more difficult, as the mechanisms behind stress resilience in Kv3.1 KO mice could be due to altered inhibitory transmission in other brain regions. For instance, Kv3.1 channels are also found in the amygdala and hippocampus (Kaczmarek and Zhang, 2017), other brain regions highly involved in the regulation of emotional behaviors and the stress response. Future molecular analyses will measure expression of glutamatergic and GABAergic markers in the PFC to determine whether Kv3.1 deficiency in combination with chronic stress impacts E/I balance specifically in this brain region. For instance, it was proposed in Chapter 3 that increased expression of the immediate early gene (IEG) *Arc* may be a marker of resilience associated with decreased prefrontal PV cell activity. If this is indeed the case, we would expect that in female Kv3.1 KO mice, which do not show an anxiety-like phenotype following chronic stress, *Arc* expression would remain high.

Kv3.1 channels are also present in subsets of somatostatin- and calbindin-expressing interneurons (Chow et al., 1999; McDonald and Mascagni, 2006), so a

thorough cell type-specific analysis of the effects of Kv3.1 deletion is also warranted. It would be important in future cellular analyses to examine the number of prefrontal PV cells expressing cFos, a marker of neuronal activity, to ensure that activity of this neuronal population in the PFC is indeed reduced in the Kv3.1 transgenic line. A similar analysis with somatostatin and calbindin interneurons in the PFC would further elucidate the possible contribution of other cell types to the behavioral phenotypes observed here. Based on previous findings showing increased activity of prefrontal PV cells following chronic stress associated with an anxiety-like phenotype (Shepard et al., 2016; Chapter 2), we would further expect that chronic stress increases PV cell activity in the PFC of WT and HET females but not in the KOs.

Other potassium channels also modulate cortical PV cell activity (Du et al., 1998) and could be involved in prefrontal E/I balance and the stress response. In male rats, chronic stress increases frontal cortex expression of Kv2.1, which is rescued by fluoxetine treatment (there was a non-statistically significant increase in expression of Kv3.1 in the frontal cortex by chronic stress) (Chen et al., 2015). It would be informative to examine prefrontal expression of Kv2.1 and Kv3.1 in female wild-type animals following chronic stress exposure, with the hypothesis that chronic stress would similarly elevate expression of these channels.

In conclusion, the present findings support the hypothesis that stress-induced anxiety in females involves increased prefrontal inhibition mediated by PV cells. Reduced PV cell activity via transgenic knockout of Kv3.1 expression incurs resilience to chronic stress-induced anxiety in female mice; behavioral results are ongoing to achieve

full group sizes and confirm these findings. Future molecular and cellular analyses will also be conducted to verify the behavioral results are related to activity in the PFC. The observation of stress resilience in Kv3.1 KO mice is also in line with previous work showing that increased activity of the PFC is associated with anxiolytic behavior (Fuchikami et al., 2015; Pati et al., 2018; Reznikov et al., 2018). Reducing prefrontal inhibition may therefore be a therapeutic strategy for increasing activity in the PFC and preventing or treating anxiety disorders.

Figure 20. Kv3.1 KO mice do not show stress-induced anxiety in the elevated plus maze.

(A) KOs spent the most time in the open arms of the elevated plus maze. **(B)** There was a non-significant effect of stress in time spent in the closed arms of the EPM. **(C)** There was a significant effect of stress in the number of open arm entries; this effect did not apply to KOs. **(D)** There was a significant effect of stress on entry ratio in the EPM, again with KO animals showing the highest entry ratio. There was a trending effect of genotype. **(E)** Representative heat maps of activity in the EPM. $N = 7$ WT control; 12 HET control; 7 KO control; 5 WT stress; 10 HET stress; 6 KO stress. $*p \leq 0.05$; $**p \leq 0.01$. EPM: elevated plus maze. WT: wild-type; HET: heterozygous; KO: knockout.

Figure 21. Chronic stress increases anxiety-like behavior in the open field in WT and HET females but not KOs.

(A) Chronic stress decreases time spent in the center of the open field in WT and HET females, but not in KOs. **(B)** KO animals have the highest number of center entries in the open field. **(C)** Chronic stress increases time spent near the walls in WT and HET mice but not in KOs. **(D)** Chronic stress increases thigmotaxis ratio in WT and HETs but not in KOs. **(E)** Representative heat maps of activity in the open field. $N = 7$ WT control; 12 HET control; 7 KO control; 5 WT stress; 10 HET stress; 6 KO stress. #: significant interaction effect ($p \leq 0.05$). *: $p \leq 0.05$. WT: wild-type; HET: heterozygous; KO: knockout.

Figure 22. KO females show the lowest anxiety-like behavior in the MB test.

(A) KO females bury the least number of marbles compared to WT and HETs. **(B)** Representative images of marbles buried. $N = 7$ WT control; 12 HET control; 7 KO control; 5 WT stress; 9 HET stress; 6 KO stress. $**p \leq 0.01$. WT: wild-type; HET: heterozygous; KO: knockout.

Figure 23. Z-scores of behavioral tests indicate reduced anxiety-like behavior in KO females.

(A) Stress increases anxiety-like behavior in the EPM in WT and HET animals, but not KOs. There is also a significant effect of genotype, with KOs showing the lowest anxiety-like behavior. (B) Chronic stress increases Z-OF score in WT and HET animals but not KOs. (C) KO animals have the lowest anxiety-like behavior in the MB test, as well as in (D) the overall Z-score of anxiety. $N = 7$ WT control; 12 HET control; 7 KO control; 5 WT stress; 10 HET stress (9 for MB and Z-anxiety); 6 KO stress. $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$. WT: wild-type; HET: heterozygous; KO: knockout; EPM: elevated plus maze; OF: open field; MB: marble bury.

Chapter 6: Conclusions

Summary: Evidence for over-inhibition

Excitatory/inhibitory (E/I) balance in the prefrontal cortex plays an integral role in emotional behaviors and is affected by chronic stress (Wieronska et al., 2011; Lener et al., 2017; McKlveen et al., 2019). Both the excitatory, glutamatergic side and the inhibitory, GABAergic side of E/I balance are perturbed by stress, and aberrations in both systems have been associated with emotional pathology (Sanacora et al., 2012; Musazzi et al., 2015; Fogaça and Duman, 2019). Most studies support the conclusion that glutamatergic activity in the PFC is reduced by chronic stress (Popoli et al., 2011; Musazzi et al., 2015). On the inhibitory side, the GABAergic deficit hypothesis (Lüscher et al., 2011; Lüscher and Möhler, 2019) is prevalent but has been challenged by others suggesting increased inhibitory neurotransmission in the PFC following chronic stress (McKlveen et al., 2016). Our work also challenged the GABAergic deficit hypothesis of stress and emotional dysfunction when we discovered that chronic stress increased the number of parvalbumin (PV) interneurons in the PFC, suggesting increased activity of this inhibitory cell population (Shepard et al., 2016; Shepard and Coutellier, 2018). In the series of experiments performed in this dissertation, I and my colleagues used chemogenetic, pharmacological, and transgenic techniques to manipulate the activity of the inhibitory GABAergic system and examine the effects on the stress response and

emotional behaviors. In these experiments, we also examined sex differences as increased PV expression in the PFC was observed predominately in females and correlated with increased emotionality in females only. We hypothesized that differing sensitivity of the prefrontal GABAergic system to chronic stress could be a mechanism behind the increased prevalence of emotional disorders in women compared to men (de Graaf et al., 2002; Wittchen et al., 2011; Kessler et al., 2012; Bale and Epperson, 2015).

The findings presented in this dissertation collectively support the hypothesis that chronic stress leads to over-inhibition in the PFC in both male and female mice, and this over-inhibition is causally related to increased anxiety-like behavior specifically in females. Furthermore, over-inhibition is mediated at least in part by increased activity of prefrontal PV interneurons. Chronic stress increases the activity of prefrontal PV cells in both male and female mice, as measured by the number of PV cells expressing the neuronal activity marker cFos. Using designer receptors exclusively activated by designer drugs (DREADDs) to drive activity of this cell population in the absence of chronic stress both acutely and chronically reduced the number of active (cFos-expressing) cells in the PFC, indicative of hypoactivity. Only the chronic DREADD-induced excitation of prefrontal PV cells increased anxiety-like behavior in female mice. These findings revealed that increased activity of prefrontal PV cells is indeed a mechanism of prefrontal hypoactivity, and this hypoactivity is causally connected to anxiety-like behaviors in females (**Chapter 2**).

Given that increased activity of prefrontal PV cells recapitulates the sex-specific effects of chronic stress, namely increased anxiety-like behavior in females (Shepard et

al., 2016, Chapter 2), we hypothesized that inhibiting this cell population following chronic stress exposure may be therapeutic for stress-induced anxiety. We used DREADDs to acutely inhibit prefrontal PV cells following 4 weeks of unpredictable chronic mild stress (UCMS) but found this was insufficient to rescue increased anxiety-like behaviors. We also observed DREADD- and stress-dependent changes in expression of markers of excitatory activity and plasticity (*Arc*) as well as inhibitory plasticity (*Nptx2*). Acute prefrontal PV cell inhibition increases *Arc* expression in unstressed females, while *Arc* expression in the PFC is reduced in stressed females and males. This finding reveals a role for prefrontal PV cells in regulating activity and glutamate-dependent plasticity that is worthy of future investigation as a possible mechanism of stress resilience or vulnerability. Furthermore, chronic stress decreases *Nptx2* expression in male mice, which may also be indicative of sex differences in stress effects on inhibitory synaptic plasticity (**Chapter 3**).

Chronic stress has biphasic effects on glutamatergic transmission. Acute stress increases activity in the PFC while chronic stress causes hypoactivity (Musazzi et al., 2015; Son et al., 2018). This “tipping point” from hyper- to hypoactivity may arise from inhibitory over-compensation for the initial increase in excitation. We hypothesized that pharmacologically enhancing GABAergic transmission during the early phases of chronic stress would alleviate the stress response by maintaining E/I balance. Administration of the GABA positive allosteric modulator lorazepam during the first 2 weeks of a 4-week UCMS procedure in female mice instead exacerbated anxiety-like behavior, which correlated with an increase in prefrontal inhibition as measured by

GAD1 expression. These observations further support the hypothesis of over-inhibition as a mechanism of anxiety (**Chapter 4**).

Building on the previous experiment, we lastly examined whether decreasing inhibitory neurotransmission mediated by fast-spiking interneurons like PV cells during chronic stress would incur resilience to stress-induced anxiety. The potassium channel Kv3.1 endows PV cells with their fast-spiking properties, so we used Kv3.1 transgenic females which have reduced activity of this neuronal population. When exposed to chronic stress, Kv3.1 knockout females do not display stress-induced anxiety-like behaviors, unlike their wildtype and heterozygous littermates. Ongoing molecular analyses will explore the possible mechanisms related to this resilience, but the current findings further support the hypothesis of over-inhibition in stress and anxiety disorders in females (**Chapter 5**).

Prefrontal over-inhibition, mediated at least in part by PV cells, is a novel mechanism for stress-induced anxiety in females and opens a new avenue of research into a possible sex-specific therapeutic target for anxiety disorders. However, several unanswered questions remain. The following sections will discuss some of the future directions and implications of this research and the hypothesis of over-inhibition.

Untangling anxiety and depression

This dissertation presents the hypothesis that chronic stress increases inhibition in the PFC, mediated in part by increased activity of prefrontal PV cells, and this over-inhibition elevates anxiety-like behaviors in females. We did not find evidence that over-

inhibition increases depression-like behaviors; however, we cannot rule out this possibility. Depression and anxiety are highly comorbid with each other in humans and rodent models (de Graaf et al., 2002; Hu et al., 2017). Furthermore, both anxiety and depression are characterized by reduced activity of the PFC (Martinot et al., 1990; Shin et al., 2001; Phelps et al., 2004; Thompson et al., 2015). Various antidepressant drugs like ketamine or fluoxetine also reduce PV cell activity and elevate prefrontal activity levels to rescue behavioral phenotypes (see “*Inhibition as a therapeutic target*” section in this chapter for more information). These antidepressants often also have anxiolytic effects, but we cannot exclude their relevance to depression. It would likely be an oversight to say that prefrontal PV cells regulate anxiety-like but not depression-like behaviors, especially considering we only used the forced swim test to measure depression-like behaviors in the various experiments presented here. Emotional behaviors are nuanced and multifaceted. For instance, depression can involve despair-related behaviors (measured by tests such as tail suspension and forced swim in rodents), anhedonia (measured by the sucrose preference test), and/or a lack of personal care/grooming (as indicated by the splash test and coat condition) (Hu et al., 2017). More recent behavioral paradigms are also starting to address the cognitive bias towards stimuli with negative valence that can be part of depression (Stuart et al., 2013). Controversy over the forced swim test suggests that it does not measure depression-like or despair-related behaviors at all, but rather is an indicator of stress coping strategies and cannot be used as a readout of an animal model of depression (Molendijk and de Kloet, 2019). Therefore, based on the forced swim test alone, it is not possible to conclude whether prefrontal PV cells mediate

depression-like behaviors in rodents. Further studies should employ behavioral tests with more construct validity for depression in order to investigate the role of prefrontal PV cells in facets of depression-like behaviors. If prefrontal PV cells do indeed regulate aspects of both depression and anxiety, this would highlight a novel mechanism of comorbidity that could be targeted as a therapeutic intervention for both stress-related conditions.

It is still possible that prefrontal PV cells regulate anxiety-like but not depression-like behaviors. As mentioned in the discussion for Chapter 2, this would suggest that the prefrontal hypoactivity found in anxiety and depressive disorders has distinct underlying mechanisms depending on the condition. For instance, increased PV cell activity may cause over-inhibition that is unique to an anxiety phenotype, whereas other cell populations and/or circuits may be involved in hypoactivity that leads to depression-like behaviors. Depression may be more related to prefrontal SST-expressing interneurons while anxiety is regulated by circuits containing PV cells. In human PFC tissue from depressed patients, SST expression is downregulated with no changes in PV (Sibille et al., 2011). Thinking beyond the PFC, depression likely involves alterations in prefrontal-nucleus accumbens circuitry while anxiety depends on prefrontal-amygdala interactions (Adhikari et al., 2015; Chang et al., 2015; Janak and Tye, 2015), though it is currently unknown how prefrontal PV cells play into these pathways. If prefrontal PV cells are not involved in depression-like behaviors, this would be a highly informative insight into distinct mechanisms underlying depression vs. anxiety, helping untangle their comorbidity. Overall, either possibility (emotional comorbidity vs. differentiation)

provides important information about how prefrontal PV cells regulate the emotional landscape that is disrupted by chronic stress. As changes in the prefrontal GABAergic system more generally have been implicated in both anxiety and depression (Lüscher et al., 2011; Wieronska et al., 2011; Lener et al., 2017), the following sections that discuss these more general GABAergic changes will refer to both conditions. When prefrontal PV cells specifically are discussed, their relevance will be limited to anxiety as their role in depression is currently inconclusive.

Integrating over-inhibition with GABAergic deficits

The hypothesis of over-inhibition raises the question: How is it possible to reconcile inconsistent or contradictory findings about glutamatergic and GABAergic dysfunction in stress and affective disorders? More specifically, how does hypoactivity in the PFC, and the hypothesis of over-inhibition, fit with reports of GABAergic deficits? In both human and rodent studies (Table 1 and Table 2), the most consistent findings suggest suppression of both glutamatergic and GABAergic systems in stress and emotional dysregulation, with some exceptions. The time course of stress may provide some insight. Stress is a highly heterogeneous and variable experience, depending on factors like duration of exposure, time since exposure, severity, controllability, and predictability (Anisman and Matheson, 2005). The stress paradigm used, as well as when during that stress paradigm data are collected, will impact the direction of glutamatergic and GABAergic findings due to the biphasic and bidirectional effects of stress on these systems. A critical challenge for stress research in the coming decades will be to map the

variable effects of stress onto the complex landscape of excitatory and inhibitory interactions in the PFC and other brain regions. Such a map would help provide a clear scale for the axes in Figure 2, which as of now are undefined and hypothetical.

Another core concept that may help reconcile glutamatergic and GABAergic findings in stress and affective dysfunction is homeostatic plasticity. Homeostatic plasticity mechanisms help keep excitation and inhibition in balance. If a network is hyper-excitable beyond a set point, mechanisms of inhibitory control engage in order to keep excitation in check. For example, elevated activity levels can cause compensatory increases in the amplitude and/or frequency of IPSCs (Wenner, 2011). And vice-versa – if excitation is suppressed, levels of inhibition subsequently decrease in order to maintain a certain level of activity. In this way, the glutamatergic and GABAergic systems can concordantly be suppressed or elevated depending on changes in network activity. GABAergic deficits in stress-induced emotional dysfunction, therefore, may still be compatible with the hypothesis of prefrontal hypoactivity and over-inhibition. The glutamatergic and GABAergic systems may both be suppressed, but inhibition can still outweigh excitation resulting in a net effect of prefrontal hypoactivity (Figure 24). Such a phenomenon could arise if GABAergic deficits are over-compensated for by an increase in inhibition, which could be explained by the heterogeneity of GABAergic interneuron subtypes that differentially regulate the activity of pyramidal cells. For instance, a stress-induced loss of local, dendritic inhibition to pyramidal cells mediated by somatostatin (SST)-expressing interneurons (Lin and Sibille, 2015) could result in over-compensation by PV-mediated soma-targeting inhibition, thus reducing overall cortical excitation

(Ghosal et al., 2017) (Figure 24B). Figure 24 represents this possible explanation for how the hypothesis of over-inhibition in the PFC fits with reports of GABAergic deficits in stress and affective dysfunction, though this remains to be tested. It has been suggested that rapid-acting antidepressants like ketamine re-engage homeostatic plasticity to elevate both glutamatergic and GABAergic systems again and restore proper E/I balance (Raab-Graham et al., 2016; Workman et al., 2018). In support of this idea, mice deficient for the $\gamma 2$ subunit of the GABA_AR, which show emotional dysfunction, show suppressed glutamatergic and GABAergic system activity, both of which are restored by ketamine treatment (Ren et al., 2016).

A cell type-specific approach to understanding GABAergic imbalances in stress may also be key to untangling inconclusive results. The PFC contains a highly heterogeneous population of GABAergic interneurons with different inputs, spiking properties, and cellular targets (Tremblay et al., 2016; McKlveen et al., 2019). Some interneuron subtypes may be underactive (GABAergic deficits) while others are over-active (over-inhibition). As previously mentioned, a cell type-specific approach may also help untangle the mechanisms behind depression vs. anxiety. This dissertation focuses primarily on PV cells as a cell-specific mechanism of prefrontal over-inhibition and anxiety-like behavior specifically, and the following sections expand upon how PV cells may become over-active in cases of stress and anxiety.

Possible mechanisms of increased prefrontal parvalbumin cell activity

An important question for the future of the research presented here is to investigate the mechanisms by which chronic stress increases the activity of prefrontal PV cells. Stress may induce structural changes to PV cells, and/or alter their microcircuit and macrocircuit inputs that influence their excitability, thus altering activity in the PFC and anxiety-like behavior.

Structural changes that may influence function

As previously discussed in the introduction (Chapter 1), PV cells are surrounded by perineuronal nets (PNNs) that enhance their excitability (Balmer, 2016). A recent study found that chronic stress increases PNN density in the PFC of adult male rats (Pesarico et al., 2019). A corresponding analysis has not been conducted in females, but it is possible that chronic stress increases PNN coverage of PV cells, increasing their activity and contributing to over-inhibition in the PFC. In support of this hypothesis, chronic antidepressant treatment, commonly used to treat anxiety, reduces PV expression and PNNs in the PFC (Ohira et al., 2013).

A hallmark of stress-induced changes to the PFC include reduced dendritic arborization and spine density of pyramidal cells, a likely mechanism contributing to prefrontal hypoactivity (Cook and Wellman, 2004; Radley et al., 2006; Moench and Wellman, 2017). It would be interesting to examine whether chronic stress also alters the dendritic morphology of PV cells in the PFC. In this case, the hypothesis would be that chronic stress would increase dendritic arborization and spine density of prefrontal PV

cells, thereby increasing excitatory synaptic drive onto these cells. Chronic stress does increase excitatory VGlut1 puncta on PV cells (Shepard and Coutellier, 2018) as well as inhibitory appositions onto excitatory cells (McKlveen et al., 2016) but it is unknown whether these indicators of synaptic changes also corresponds with morphological changes to PV cells. Chronic stress has also been shown to cause dendritic hypertrophy of Martinotti cells, a subtype of somatostatin SST-expressing interneuron (Gilabert-Juan et al., 2013).

Glucocorticoids (the “stress hormone”; cortisol in humans and corticosterone in rodents) may also play a role in PV-mediated over-inhibition. Glucocorticoid receptors (GRs) in the PFC help turn down activity in the hypothalamic-pituitary-adrenal (HPA) axis, the stress response that mediates the release of glucocorticoids (Hill et al., 2011; Heck and Handa, 2019; McKlveen et al., 2019). This negative feedback action of glucocorticoids in the PFC may be mediated by inhibitory neurons, according to studies conducted in male rodents. Application of corticosterone to prefrontal cortical slices suppresses GABA release onto excitatory neurons to increase the activity of the PFC (Hill et al., 2011). Furthermore, GRs are present on about 50% of PV interneurons in the infralimbic subregion of the PFC (McKlveen et al., 2016). Chronic stress decreases the number of GRs present on PV neurons in the PFC of male rodents, which corresponds with increased inhibitory tone. Therefore, it seems likely that under basal conditions, GRs on PV cells decrease PV cell activity to increase prefrontal excitation and regulation of the HPA axis, but under conditions of chronic stress, a loss of GRs on PV cells contributes to over-inhibition and a loss of prefrontal-mediated control over the stress

response (McKlveen et al., 2016). Similar studies have not been conducted in female rodents, but it is possible that a stress-induced loss of GRs on PV cells in the PFC is more pronounced in females, or that females have less GRs on PV cells at baseline, weakening negative feedback of the HPA axis and exacerbating the stress response and prefrontal over-inhibition (Figure 25).

In the context of GABAergic microcircuitry

Other GABAergic cell types also contribute to prefrontal E/I imbalances. Both human and animal studies have identified SST-expressing inhibitory interneurons as a major culprit in emotional dysfunction. SST cells inhibit the dendrites of excitatory pyramidal cells (Tremblay et al., 2016). Mice lacking the gene for the inhibitory neuropeptide SST itself show increased anxiety and depression-like behaviors, and chronic stress decreases expression of SST (Lin and Sibille, 2015; Banasr et al., 2017). SST cells are also thought to modulate some of the sex-specific effects of chronic stress and sex biases in the prevalence of co-morbid anxiety and mood disorders (Seney and Sibille, 2014). SST cells inhibit PV cells (Pfeffer et al., 2013), proposing the hypothesis that impaired functioning of SST cells results in disinhibition of PV cells. In support of this idea, increased activity of SST cells has antidepressant and anti-anxiety benefits (Fuchs et al., 2016). It has not yet been tested whether PV cells mediate the connection between SST cells and anxiety, nor if chronic stress decreases SST-mediated inhibitory inputs to PV cells. If this is the case, this microcircuit would contribute to resolving the previously-discussed paradox in the literature of prefrontal hypoactivity and reduced GABAergic transmission. These

observations are not mutually exclusive if reduced GABAergic transmission is observed due in part to impaired SST cell functioning, while hypoactivity is observed due to the subsequent increase in PV cell activity. This hypothesis has been posited but not yet directly tested (Ghosal et al., 2017) (Figure 25).

Macroircuit inputs possibly driving GABAergic changes in the PFC

PV cells in the PFC receive inputs from subcortical structures that drive their activity and thus drive feedforward inhibition in the PFC. For example, monosynaptic stimulation of the basolateral amygdala (BLA) activates prefrontal PV cells, increasing prefrontal inhibition (Gabbott et al., 2006; Dilgen et al., 2013). BLA-to-PFC inputs are strongest on PV and SST interneurons over pyramidal cells, and this feedforward inhibition predominately contacts corticoamygdala neurons that provide reciprocal connections back to the BLA, highlighting a complex modulatory circuit that is likely involved in prefrontal-dependent control over emotional behaviors (McGarry and Carter, 2016). Some, but not all, chronic stress paradigms increase activity in the BLA (Shepard et al., 2016; Lowery-Gionta et al., 2018; Zhang et al., 2019), enhancing excitatory output at BLA-to-PFC synapses and inducing an anxiety phenotype. Whether a stress-induced increase in excitatory BLA projections to the PFC is driving the activity of prefrontal PV cells remains to be determined. Chronic stress also increases spine density and glutamatergic signaling in BLA projections to the ventral hippocampus, increasing activity of this region (Zhang et al., 2019). The ventral hippocampus directly innervates the PFC and excites PV interneurons (Gabbott et al., 2002; Caballero et al., 2014b).

Again, it is unknown whether stress increases activity of prefrontal PV neurons via increased connectivity from the BLA to the ventral hippocampus to the PFC.

The mediodorsal thalamus (MDT) also helps regulate prefrontal E/I balance (Ferguson and Gao, 2018b). Past research has shown that excitatory projections from MDT neurons synapse directly on PV cells in the PFC, driving feedforward inhibition (Rotaru et al., 2005; Delevich et al., 2015). Increased activity of the MDT would hypothetically increase excitation onto prefrontal PV neurons and increase anxiety-related behaviors. The effect of chronic stress on activity in the MDT, and the relation of the MDT to anxiety disorders via connectivity with prefrontal PV cells, has not yet been studied. The sex-specific nature of macrocircuit alterations in stress and anxiety disorders involving the BLA, ventral hippocampus, MDT, and others, also remains to be studied (Figure 25).

Inhibition as a therapeutic target

If chronic stress and anxiety disorders involve reduced prefrontal activity, in part due to increased activity of prefrontal PV cells, reducing activity of the prefrontal GABAergic system may be an effective therapeutic strategy. For example, reducing PV-cell mediated inhibition may restore levels of activity in the PFC, lifting brakes on plasticity and allowing for therapeutic rewiring of local and long-range circuits. This hypothesis has yet to be directly tested, but there is some supporting evidence from pharmaceuticals and from other brain regions and behavioral paradigms.

GABA agonism or antagonism?

GABA agonism is classically anxiolytic and many common anti-anxiety medications, such as benzodiazepines, exert their therapeutic effects by enhancing GABAergic transmission (Lydiard, 2003). A recent study even reported fast-acting antidepressant effects of novel GABA_AR agonists in both males and females (McMurray et al., 2018). The neurosteroid brexanolone allosterically enhances GABA_A receptor function and has antidepressant and anxiolytic effects (Lüscher and Möhler, 2019). However, there is also recent evidence to suggest that GABA antagonism can be therapeutic for emotional disorders, in support of the hypothesis presented here that these disorders involve over-inhibition in the PFC. For example, quercetin, a negative allosteric modulator for GABA_ARs, reduces GABAergic transmission in the PFC (Fan et al., 2018) and has stress-protective and anxiolytic effects at least in male rodents (females were either not tested or the sex was unspecified) (Bhutada et al., 2010; Mehta et al., 2017; Samad et al., 2018). Other negative allosteric modulators for α 5-containing GABA_ARs, MRK-016 and L-655,708, also exhibit antidepressant actions in males (females were not tested) (Fischell et al., 2015; Zanos et al., 2017). In humans, GABA content in the ventral PFC is positively correlated with trait anxiety with no sex differences observed (Delli Pizzi et al., 2016), suggesting that reducing prefrontal GABA may alleviate anxiety.

The contradictory findings about GABA agonism vs. antagonism may be explainable by differences in GABAergic dysfunction across brain regions in stress and emotional disorders. For instance, positive GABA modulation by benzodiazepines may exert its therapeutic effects within the amygdala (Nuss, 2015). Due to the heterogeneity

of GABAergic interneurons in the PFC, a cell-type specific approach to GABA agonism or antagonism may also be warranted to maximize therapeutic efficacy of intervening in the GABAergic system in this brain region. For example, certain reports indicate that facilitation of SST cell activity is anxiolytic while PV cell excitation is anxiogenic (Fuchs et al., 2016; Chapter 2). The anxiolytic effects of both GABA agonism and antagonism may also relate to the complex and bidirectional effects of chronic stress on GABAergic transmission.

Antidepressants and parvalbumin cells

Antidepressants are frequently used for the treatment of comorbid depression and anxiety or for anxiety alone (Feighner, 1999; Zohar and Westenberg, 2000). Both fluoxetine and ketamine reduce PV expression in the PFC (Ohira et al., 2013; Zhou et al., 2015). A previous study found that the ketamine-induced loss of PV expression involves activation of NADPH oxidase (Behrens et al., 2007). The NADPH oxidase inhibitor apocynin prevents ketamine-induced loss of PV expression in the PFC and also prevents ketamine's antidepressant effects (Zhou et al., 2015), strongly suggesting that at least part of the therapeutic efficacy of antidepressants comes from reducing the activity of prefrontal PV cells. In further support of this, fluoxetine also reduces PNNs around PV cells (Guirado et al., 2014). Venlafaxine reduces PNN integrity in the hippocampus by upregulating matrix metalloproteinase (MMP)-9 (Alaiyed et al., 2019). These studies were conducted in male rodents (or pooled male and female groups). Whether

antidepressants reduce the activity of PV interneurons in females remains to be determined.

The activation of PV cells is driven in part by the potassium channel Kv3.1, which allows PV cells to send inhibitory signals at high frequencies to excitatory pyramidal cells (Rudy and McBain, 2001). Antidepressants like fluoxetine and paroxetine also block the Kv3.1 channel (Sung et al., 2008; Lee et al., 2018). These findings also lend support to the hypothesis that increased PV cell activity is part of the pathogenesis of stress-induced anxiety and that reducing PV cell activity, or prefrontal GABAergic transmission in general, may be therapeutic or preventative (Chapter 5). Kv3.1 currents, and thus neural fast-spiking properties, are suppressed by administration of the element zinc (Kaczmarek and Zhang, 2017). Interestingly, zinc has antidepressant and anxiolytic effects (Partyka et al., 2011; Młyniec et al., 2017) and can be therapeutic following chronic stress (Ding et al., 2016; Omar and Tash, 2017). Zinc deficiency, by contrast, has anxiogenic effects and decreased serum zinc concentrations are present in patients with generalized anxiety disorder (Islam et al., 2013; Młyniec et al., 2017). These findings raise the possibility that zinc exerts its anti-anxiety benefits by reducing ion flow through Kv3.1 channels, thus suppressing PV cell fast-spiking activity to reduce inhibition in the PFC. This idea remains to be tested, though it has been proposed that zinc helps maintain or restore excitatory/inhibitory balance (Młyniec et al., 2017).

Evidence from other brain regions

This dissertation presents the hypothesis that chronic stress increases inhibition in the PFC. This over-inhibition may involve an increase in brakes on plasticity, such as PV cells and PNNs, which are typically associated with the closing of developmental critical periods of plasticity. Therapeutic interventions may involve lifting these brakes and reinstating activity and plasticity in the PFC. Further support for this hypothesis comes from research in other regions of the brain and other behaviorally-relevant networks: the visual cortex and ocular dominance plasticity, the amygdala and fear learning, and the hippocampus and anxiety and other behaviors.

Like the PFC, the visual cortex of early postnatal rodents is highly plastic. One of the first (and one of most well-documented) forms of critical period plasticity relates to sensory experience and occurs in the visual cortex in early postnatal life. During this time, mice display ocular dominance plasticity: if one eye is sutured shut, circuits in primary visual cortex will rewire to reduce inputs from that eye and the cortical territory will be taken over by inputs from the other, open eye. This rewiring can be corrected if the closed eye is re-opened during the critical period. If the closed eye is re-opened during adulthood, the rewiring remains permanent and cannot be reversed without the aid of interventions (Castrén and Hen, 2013). Adult animals are thus unable to express this ocular dominance plasticity; however, it can be reinstated by chronic fluoxetine administration or degradation of PNNs, such as for the treatment of amblyopia (Pizzorusso et al., 2002; Maya-Vetencourt et al., 2008; Lensjø et al., 2017).

In the amygdala, a similar concept has been applied to fear circuitry with therapeutic implications for post-traumatic stress disorder (PTSD) (Nabel et al., 2013). Fear associations in infant animals are rapidly forgotten and easily extinguished, while adults have long-lasting fear memories that are highly prone to reinstatement after extinction. Infant animals subjected to chronic stress in early life also show “adult-like” long-lasting and relapse-prone fear memories (Callaghan et al., 2013). In adult mice, simple extinction does not erase the fear memory. However, two landmark *Science* papers demonstrated that extinction training combined with degradation of PNNs in the amygdala or fluoxetine treatment does result in fear erasure (Gogolla et al., 2009; Karpova et al., 2011).

In the hippocampus, early life stress leads to a premature increase in PV cells (Bath et al., 2016). Conversely, chronic fluoxetine administration leads to a “dematuration” of the dentate gyrus, noted by reduced expression of the mature granule cell marker calbindin, increased granule cell excitability, and reduced connectivity between the dentate gyrus and CA3. These molecular changes were associated with behavioral destabilization, including fluctuating levels of activity and anxiety (Kobayashi et al., 2010, 2011). Electroconvulsive therapy, used for treating depression, also causes a rapid dematuration of hippocampal granule cells (Imoto et al., 2017). Chronic stress has also been shown to increase PNN coverage of PV cells in CA1 (Riga et al., 2017).

A common theme in these brain networks is that during particular developmental periods, a certain level of plasticity exists that allows for circuit rewiring in response to environmental experience due to the hyper-excitable state of the network. As

development progresses into adulthood, these critical periods of plasticity close due to GABAergic maturation and increased inhibitory control, and less experience-dependent rewiring is possible. Chronic stress during early life or adulthood appears to accelerate or strengthen developmental brakes on plasticity, such as PV cell activity or PNN coverage of PV cells. Antidepressant treatments like fluoxetine, ketamine, electroconvulsive therapy, or others assist in lifting brakes on plasticity like PV cells and PNNs and restore activity levels and plasticity in brain networks to again allow for a healthy level of experience-dependent rewiring (Figure 26). Treatment does not appear to re-induce a true “critical period”, which is a positive finding, as critical periods of plasticity are associated with their own vulnerabilities to stress (Hoftman and Lewis, 2011; Chaby et al., 2013). A recent study in the visual cortex supports the trajectory presented in Figure 26: fluoxetine treatment does not induce expression of molecular markers indicative of a “juvenile-like” state, but rather increased expression of markers associated with mature and functional E/I balance, specifically mature glutamate and GABA receptor subunits (Beshara et al., 2015).

The effects of chronic stress during early life and adulthood described here are not generalizable across the lifespan. Chronic stress appears to have an entirely different effect on the prefrontal GABAergic system when it occurs prenatally or during the adolescent critical periods. Here, chronic stress disrupts GABAergic development and leaves prefrontal networks in an immature state, with decreased PV and PNN expression and cognitive deficits like those indicative of schizophrenia phenotypes (Enwright et al., 2016; de Araújo Costa Folha et al., 2017). Stress during adolescence also disrupts

emotional maturation (Page and Coutellier, 2018). In adolescence, enhancing PV cell activity is therapeutic and protective against stress (Chen et al., 2018; Ng et al., 2018). These differences between developmental periods are beyond the scope of this dissertation but are an important consideration.

Sex differences in parvalbumin-mediated over-inhibition

The findings that chronic stress reduces prefrontal activity, in part through increased activity of prefrontal PV cells, does not appear to be sex-specific. Many of the cited studies that support the hypothesis of prefrontal hypoactivity were conducted in males, and chronic stress increases PV/cFos double-labeling in both males and females (Chapter 2). Furthermore, the studies cited in previous sections, which highlight the therapeutic potential of using antidepressants and other interventions to reduce inhibition, are also largely conducted in males. However, there are sex-specific consequences to prefrontal hypoactivity and over-inhibition. Specifically, over-inhibition mediated by prefrontal PV cells increases anxiety-like behaviors in females only, at least at the time points evaluated. It is likely that the effects of stress on prefrontal activity and emotional behaviors scale according to biological sex (Figure 2). Nevertheless, evidence supports that females are more susceptible to the anxiogenic effects of chronic stress and of chronic PV cell activation (Shepard et al., 2016; Chapter 2). The reasons for this are unknown.

Sex hormones and their receptors are likely culprits for differences between males and females. In the hippocampus, estrogen has been shown to regulate prefrontal E/I

balance in females as there is estrous cycle variability in the density of dendritic spines on principal CA1 neurons, as well as cyclic variation in the threshold for seizure activity (McEwen and Milner, 2017). In the PFC, estrogen increases stress sensitivity in females (Shansky et al., 2004, 2010). The relationship between estrogen and E/I balance in the PFC has not been studied, though it is likely that estrogen also impacts prefrontal E/I balance. There is extensive colocalization with PV cells and estrogen receptors (ERs) (Blurton-Jones and Tuszynski, 2002). In the arcuate nucleus and cortex, estrogen treatment increases PV immunoreactivity and expression levels (Ross and Porter, 2002; Sotonyi et al., 2010; Filice et al., 2018). Estrogen treatment also increases the dendritic arborization of cortical PV neurons in male neonatal rats (Ross and Porter, 2002). The relationship between estrogen, stress, and PV cells has not been explored, but it is possible that estrogen and ERs incur sex-specific vulnerability of prefrontal PV cells to chronic stress in a way that increases their excitability. This hypothesis could be tested by examining prefrontal PV cell activity following ovariectomy with or without estrogen replacement in females or estrogen treatment in males.

Sex hormones may also pattern sex differences in E/I balance during critical periods of development, known as the organizational effect of hormones (Schulz et al., 2009; Seney and Sibille, 2014). It was previously discussed in the introduction (Chapter 1) that adolescent maturation of the GABAergic system in brain regions like the frontal cortex and hippocampus is dependent on ovarian hormones in female mice (Wu et al., 2014; Piekarski et al., 2017). It is possible that sex differences in inhibitory tone set during adolescence determine the stress response in adulthood and the nature of E/I

imbalances. Manipulating exposure to sex hormones like estrogen and testosterone during adolescent development in males and females would help determine if the adolescent programming of E/I balance in the PFC is a critical determinant of sex differences in PV-mediated over-inhibition following adult stress exposure.

As previously discussed, SST interneurons inhibit PV cells and may be part of a prefrontal microcircuit that modulates E/I balance and inhibitory tone. Decreased SST expression has been observed in humans with major depression and reduced SST cell functioning could contribute to increased PV cell activity (Ghosal et al., 2017). In humans with major depression, this reduction in SST is more pronounced in women (Seney et al., 2013). This leads to the hypothesis that reduced SST cell functioning may be an upstream mechanism of increased PV cell activity and prefrontal over-inhibition that is more pronounced in females relative to males (Chapter 2). The role of SST in human anxiety specifically has not been explored, though comorbidity between depression and anxiety is common, especially in women (de Graaf et al., 2002).

It is also important to recognize the baseline differences between males and females in markers of prefrontal activity. In Chapter 2, we found that in unstressed animals, males have almost twice as many active PV cells in the PFC (doublelabeled PV/cFos cells). Chronic stress in females increases the number of PV/cFos cells to “male-like” control levels. It is possible that the male PFC is more adapted to increased PV cell activity, whereas increases in PV cell activity in the female PFC represents a larger perturbation to E/I balance. In support of this hypothesis, the level of prefrontal activity-dependent *Arc* mRNA in males was nearly twice that of females. This could suggest that

relative to females, males at baseline have high inhibition (indicated by increased PV cell activity) but also high excitation (indicated by increased *Arc* expression), thus maintaining E/I balance. Females may display lower prefrontal activity at baseline and thus be closer to the threshold for hypoactivity and emotional dysfunction. The baseline differences in glutamatergic and GABAergic system activity within the male and female PFC have not been examined but should be considered when studying sex differences in the stress response.

Concluding remarks

The evidence synthesized in this dissertation supports the hypothesis that chronic stress and affective disorders involve E/I imbalances in the PFC, specifically over-inhibition and prefrontal hypoactivity. Some of the mechanisms behind over-inhibition may be the same as those that increase inhibition during the developmental transition from adolescence to adulthood. Specifically, during adolescent prefrontal maturation, the GABAergic system matures in part through increased activity of PV cells and increased PNN coverage of PV cells. Recent evidence suggests that these brakes on activity and plasticity may be increased by chronic stress and in conditions of anxiety and/or depression. Therapeutic interventions for these disorders involve reducing the activity of PV cells and/or reducing their coverage by PNNs, for instance. In this way, treatment involves restoring activity and plasticity in the PFC to healthy levels and reinstating E/I balance necessary for proper emotional functioning (Figure 26). As many reviews and experiments on the topic of E/I imbalances in stress and affective disorders have focused

on the GABAergic deficit hypothesis of these conditions, it is important to consider the evidence for over-inhibition. The time course of stress, differing magnitudes of glutamatergic vs. GABAergic deficits, mechanisms of homeostatic plasticity, and cell type-specific analyses are all critical factors to consider that may help reconcile inconclusive findings pertaining to E/I balance. Acknowledging the evidence for over-inhibition in the PFC and integrating it into our current understanding of stress and emotional disorders will be invaluable for developing effective interventions. Sex differences in this research are also an essential consideration. The glutamatergic and GABAergic systems of males and females respond differently to stress, which may be important to understanding sex differences in the prevalence of anxiety and depression and developing sex-specific treatments. Future studies must continue to take sex differences into account, as well as continue to investigate the complex interplay between excitatory and inhibitory transmission both in and out of balance.

Figure 24. Hypothesized explanation for reconciling GABAergic deficits in chronic stress and affective disorders with evidence for increased prefrontal inhibition.

(A) The glutamatergic systems and GABAergic systems may both be suppressed in conditions of chronic stress-induced depression and anxiety, but increased inhibition relative to excitation can still result in a net effect of prefrontal hypoactivity. The activity of both systems may be elevated by treatment. **(B)** The phenomenon hypothesized in (A) could arise if (1) decreased local dendritic inhibition by SST cells causes (2) over-compensation by PV cells which provide global, soma-targeting inhibition, (3) thus reducing the activity of pyramidal neurons and contributing to prefrontal hypoactivity. SST: somatostatin; PV: parvalbumin; Pyr: pyramidal cell.

Figure 25. Micro- and macro-circuit interactions with parvalbumin cells in the prefrontal cortex.

Increased PNN coverage of PV cells, as well as dendritic hypertrophy of PV cells, may increase their excitability. Decreased GR expression on PV cells may also increase their activity. PV cells are inhibited by local SST cells. A decrease in SST functionality as seen in stress and affective disorders may result in disinhibition of prefrontal PV cells and thus increased inhibition of target pyramidal neurons. At the macrocircuit level, inputs from the MDT, BLA, and vHPC may also drive activation of PV cells in the PFC. PV: parvalbumin; SST: somatostatin; PNN: perineuronal net; GR: glucocorticoid receptor; Pyr: pyramidal cell; PFC: prefrontal cortex; MDT: medial dorsal thalamus; BLA: basolateral amygdala; vHPC: ventral hippocampus.

Figure 26. Hypothesized changes in E/I balance due to development and chronic stress/emotional dysfunction.

In adolescence, the PFC is more hyper-excitable and plastic. As development progresses into adulthood, increased PV cell activity and PNN coverage of PV cells enhances inhibition to stabilize the network, resulting in mature E/I balance and healthy prefrontal-dependent emotional functioning. Chronic stress and affective disorders like anxiety and depression may involve increases in the same molecular mechanisms that are involved in GABAergic maturation, such as PV cells and PNNs, inducing hypoactivity. Treatment involves restoring levels of activity, potentially in part through reducing brakes on excitation such as PV cells and PNNs to restore a mature, functional E/I balance. E: excitation; I: inhibition; PV: parvalbumin; PNN: perineuronal net.

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