The hemisphere-specific effects of chronic stress on rat prefrontal cortical function and dendritic morphology

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Abstract

Stressful life events have long been viewed as risk factors for neuropsychiatric disorders and while the pathogenesis remains unclear, stress-induced alterations in the function of the prefrontal cortex (PFC) are generally seen as potential targets. The prefrontal cortical regulation of stress responses is lateralized. Therefore, in order to mount an appropriate stress response, the hemispheres must somehow communicate. There is evidence now that prefrontal cortical callosal neurons are responsible for this interhemispheric communication of stress-relevant information. Callosal neurons are pyramidal neurons that send homotopic projections to the contralateral PFC and are responsible for the majority of the PFC glutamate stress response.

Exposure to chronic stress has been widely reported to induce dendritic atrophy of pyramidal neurons in the PFC. Importantly, these morphological alterations appear to be lateralized in nature; chronic stress eliminates the inherent asymmetry in dendritic morphology between the left and the right PFC. Our first experiment tested the hypothesis that prefrontal cortical callosal neurons would have a lateralized pattern of remodelling in response to chronic stress. A retrograde microbead tracer was injected into male rats' PFC 6 days prior to 21 days of restraint stress. After completion of the stress regimen, microbead-labelled callosal neurons in the prelimbic and infralimbic cortices were loaded with the fluorescent dye Lucifer Yellow to visualize their dendritic arbors. We observed a significant reduction in the total dendritic length and branching of callosal neurons in both the right and the left hemispheres. However, the distribution of these stress-induced alterations in dendritic morphology along the dendritic arbor differed between hemispheres: both proximal and distal dendrites underwent stress-induced

changes in dendritic morphology in the right hemisphere, while in the left hemisphere, only distal dendrites were affected.

In a second experiment, we investigated if chronic stress affected the prefrontal cortical glutamate stress response, which is mainly due to the activation of callosal neurons in the opposite hemisphere. After cessation of stress, *in vivo* microdialysis was performed to measure the glutamate stress response to an acute tail pinch stressor. In the left PFC, chronic stress prolonged the glutamate stress response. In contrast, there was no stress-induced change in the right PFC glutamate stress response. These findings imply that the more widespread dendritic atrophy in the right PFC could underlie the enhanced glutamate stress response measured in the left PFC. Such hemisphere-specific alterations in morphology and function of callosal neurons could affect the inter-hemispheric exchange of stress-relevant information, and ultimately contribute to the prefrontal cognitive deficits associated with chronic stress.

Résumé

Le stress chronique a longtemps été considéré comme un facteur de risque pour plusieurs maladies neuropsychiatriques et bien que la pathogenèse reste incertaine, les modifications causées par le stress à la fonction du cortex préfrontal sont généralement considérés comme des cibles potentielles. La régulation des réactions de stress par le cortex préfrontal est latéralisée. Donc, afin de produire une réponse au stress approprié, les hémisphères doivent communiquer entre eux. Nous avançons l'hypothèse que les neurones calleux du cortex préfrontal sont responsables pour cette communication inter-hémisphérique de l'information pertinente au stress. Les neurones calleux sont des cellules pyramidales qui envoient des projections homotypiques au PFC controlatéral. Ils sont responsables pour la majorité de la réponse au stress glutamatergique dans le cortex préfrontal.

Il a été démontré que le stress chronique résulte en une l'atrophie dendritique des cellules pyramidales du cortex préfrontal. De plus, il semble que le remodelage dendritique induit par le stress soit spécifique à chaque hémisphère: c'est-à-dire que le stress affecte principalement les dendrites dans l'hémisphère droit, abolissant l'asymétrie hémisphérique inhérente de la morphologie dendritique. Notre première expérience a testé l'hypothèse selon laquelle les neurones calleux du cortex préfrontal subiraient des changements à la morphologie dendritique induits par le stress dans une manière latéralisée. Six jours après que des rats mâles aient été soumis à 21 jours de contention, un traceur rétrograde microbead a été injecté dans le cortex préfrontal. Après la fin du traitement de stress, les neurones calleux marqués avec des microbeads dans les cortex prélimbique et infralimbic ont été injectés de Lucifer Yellow un colorant fluorescent permettant de visualiser leurs dendrites. Une réduction significative de la longueur et branchement des dendrites des neurones calleux a été observée dans les hémisphères

droit et gauche. Cependant, la distribution de ces altérations morphologiques le long de l'arbre dendritique différait entre les hémisphères: les dendrites proximales et distales ont subi des changements morphologiques induits par le stress dans l'hémisphère droit, tandis que dans l'hémisphère gauche, seules les dendrites distales ont été affectées.

Dans une deuxième expérience, nous avons étudié comment le stress chronique affecte la réponse glutamatergique au stress dans le cortex préfrontal, une réponse qui est principalement attribuable à l'activation des neurones calleux localisés dans l'hémisphère opposé. Suite au régime de stress chronique, la microdialyse *in vivo* a été utilisée pour mesurer la réponse glutamatergique à un stresseur aigu de pincement de la queue. Chez les animaux stressés chroniquement, une réponse glutamatergique prolongée a été observée dans le cortex préfrontal gauche mais non dans l'hémisphère droite. Ces résultats mènent à l'hypothèse que l'atrophie dendritique plus extensive dans le cortex préfrontal droit pourrait avoir contribuée au prolongement de la réponse glutamatergique au stress observée dans le cortex préfrontal gauche. Ces altérations morphologiques et fonctionnelles des neurones calleux pourraient affecter l'échange inter-hémisphérique de l'information pertinente au stress, et finalement peuvent contribuer aux déficits cognitifs préfrontaux associés au stress chronique.

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Contribution of Authors

Dr. Alain Gratton supervised and helped design my research. Luc Moquin made all microdialysis probes used in the experiments. In addition, Luc Moquin performed HPLC on all microdialysis samples. As the main researcher of this project, I executed all experiments described in this thesis. For the dendritic morphology experiment, I performed all tracer injection surgeries, chronic restraint stress, perfusions, Lucifer Yellow iontophoretic injections, immunocytochemistry techniques, morphometric reconstructions and analyses. For the microdialysis experiment, I performed all cannulation surgeries, chronic restraint stress, *in vivo* microdialysis experiments, and chromatogram analyses.

The current thesis was written by me and edited by my supervisor Dr. Alain Gratton.

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List of Abbreviations

3D	Three dimension
5-HT	Serotonin
aBST	Anterior bed nuclei of the stria terminalis
aCSF	Artificial cerebrospinal fluid
ACC	Anterior cingulate cortex
ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
AP	Anteroposterior
BLA	Basolateral amygdala
CORT	Corticosterone
CRH	Corticotropin-releasing hormone
CS	Conditioned stimulus
CSF	Cerebrospinal fluid
DA	Dopamine
DAB	3, 3'-diaminobenzidine
DV	Dorsoventral
EEG	Electroencephalography
EPSC	Excitatory post-synaptic current
fMRI	Functional magnetic resonance imaging

GAD	General anxiety disorder
GC	Glucocorticoid
GR	Glucocorticoid receptor
GLU	Glutamate
HPA	Hypothalamic-pituitary-adrenal
HPLC	High pressure liquid chromatography
IEG	Immediate early gene
IL	Infralimbic cortex
LTP	Long term potentiation
LY	Lucifer Yellow
ML	Mediolateral
mPFC	Medial prefrontal cortex
NMDA	N-methyl-D-aspartate
PB	Phosphate buffer
PFA	Paraformaldehyde
PFC	Prefrontal cortex
PKC	Phosphate kinase C
PL	Prelimbic
PTSD	Post-traumatic stress disorder
RIA	Radioimmunoassay
SEM	Standard error of the mean

TMS Transcranial magnetic stimulation

TTX Tetrodotoxin

VTA Ventral tegmental area

1. General Introduction

1.1. Frame of reference

Our research focuses on the effects of chronic stress on neural morphology and function of the medial prefrontal cortex (mPFC). As will be argued later, chronic exposure to stressors has long been recognized as a risk factor for several psychiatric disorders such as depression, anxiety disorders and drug addiction (McEwen, 2004) and while the mechanisms by which this occurs remain unclear, those involving the PFC are generally seen as potential targets. The PFC is involved in higher order cognitive and emotional processing as well as in regulating several important responses to stressors. A growing body of evidence also shows that there is hemispheric specialization in the PFC-mediated regulation of the behavioural, neuroendocrine, and autonomic responses to stressors (Gratton & Sullivan, 2005). Thus, in order to mount an appropriate response to stress, the right and the left PFC must somehow communicate with each other. Recent evidence has implicated prefrontal cortical callosal neurons in the inter-hemispheric communication of stress-relevant information. This class of glutamate-containing pyramidal neurons send homotopic projections to the contralateral PFC (Ferino et al., 1987). Furthermore, the prefrontal cortical glutamate stress response has been shown to predominantly reflect the activity of callosal neurons whose cell bodies are located in the opposite hemisphere (Lupinsky et al., 2010). From this, it has been hypothesized that disruption of the normal inter-hemispheric exchange of information could lead to disturbances in PFC-mediated stress responses. Such a disruption in prefrontal cortical function could emerge as a result of chronic exposure to stressors.

In addition to callosal neurons, the PFC contains pyramidal neurons that send widespread projections to other brain regions (Sesack et al., 1989). Chronic stress regimens as well as exogenous glucocorticoid (GC) administration are now known to result in long-lasting

morphological changes to the apical dendrites of pyramidal neurons in the PFC (Cook & Wellman, 2004; Radley et al., 2004; Cerqueira et al., 2006). These include decreased dendritic length and branching, and reduced spine density (Cook & Wellman, 2004; Radley et al., 2004; Cerqueira et al., 2006; Radley et al, 2006). Moreover, these morphological alterations appear to be lateralized in nature; chronic stress eliminates the inherent asymmetry in dendritic morphology between the left and the right PFC (Perez-Cruz et al., 2007). Stress-induced morphological changes have been hypothesized to lead to prefrontal cortical dysfunction and to maladaptive stress responsivity (Holmes & Wellman, 2008). Indeed, chronically stressed rats display decreased neuronal excitability and impairments in prefrontal cortical-dependent behavioural tasks (Liston et al., 2006; Liu & Aghajanian, 2008, Wilber et al., 2011; Dias-Ferreira et al., 2009; Hains et al., 2009).

Whether the dendritic morphology of callosal neurons is affected by chronic stress has not been investigated. Thus, the question of interest: Are callosal neurons among the mPFC pyramidal neurons that undergo morphological remodelling during chronic stress? The resulting functional alterations to callosal neurons would be expected to disrupt normal inter-hemispheric communication and, consequently, lead to an imbalance in left versus right prefrontal cortical function. The two objectives of the present research are to document chronic stress-induced changes in 1) the dendritic morphology of identified prefrontal cortical callosal neurons and 2) the left versus right prefrontal cortical glutamate responses to an acute stressor.

1.2. The role of the PFC in stress response regulation

The PFC mediates higher order executive functions that select and process information so that behaviour can be suitably planned, directed, and controlled for the prevailing environmental conditions (Holmes & Wellman, 2008). Numerous studies demonstrate that the PFC plays a major role in coordinating the physiological and behavioural responses to stress. The induction of immediate early genes (IEG) is proposed to correlate with widespread neural activity. The expression of the IEG c-Fos increases in the rodent PFC after an exposure to a stressor, demonstrating that neurons in the PFC are activated during stress (Singewald, 2007). The mPFC, in particular, has an important role in the modulation of the autonomic and endocrine stress responses. The prelimbic (PL) and infralimbic (IL) cortices have opposing roles in coordinating autonomic stress responses, with output from the IL and PL PFC serving to activate and inhibit, respectively, brain stem autonomic control nuclei (Ulrich-Lai & Herman, 2009). The mPFC plays an inhibitory role in the feedback regulation of the hypothalamic-pituitary-adrenal (HPA) axis stress response. Figueiredo et al. (2003) showed that bilateral mPFC lesioned rats have increased c-Fos levels in the hypothalamic paraventricular nucleus and have potentiated adrenocorticotropic hormone (ACTH) secretion after restraint stress compared to sham-operated animals. The negative feedback control of the HPA axis is thought to occur through the activation glucocorticoid receptors (GR) in the mPFC. In support of this hypothesis, inserting corticosterone (CORT) implants in the rat PFC decreases the ACTH response to restraint stress (Akana et al., 2001).

1.3. The mPFC-mediated stress response is lateralized

Numerous animal studies have shown that the PFC-mediated regulation of the behavioural, neuroendocrine, and autonomic responses to stressors is inherently lateralized. Neveu and Moya (1997) classified mice as left-pawed, ambidextrous, or right-pawed using a paw preference test in a food reaching task. They found that elevated CORT levels after a one hour period of restraint stress were significantly associated with a left paw preference, suggesting that, to some degree, right brain dominance is associated with increased HPA responsivity. Indeed, right side excitotoxic lesions of infralimbic cortex suppress the restraint stress-induced CORT response to the same extent as bilateral lesions in rats (Sullivan & Gratton, 1999). In contrast, the CORT responses of rats with left side lesions do not differ from sham-lesioned rats, indicating that an intact right PFC is necessary to mount a robust HPA stress response. When animals are subjected to a longer cold-restraint stress period, right IL lesions dramatically reduce the formation of gastric ulcers (Gratton & Sullivan, 1999), a measure of autonomically-mediated stress pathology. In addition to these right PFC-mediated physiological functions, excitotoxic lesions of the right, but not left IL PFC, cause rats to spend more time in the open arms of an elevated plus maze (Sullivan & Gratton, 2002). Therefore, the right ventral mPFC integrates stress-related sensory inputs with the proper neuroendocrine and autonomic outputs and also plays an important role in in the optimization of cautious and adaptive behaviour in potentially threatening situations.

1.4. The PFC dopamine stress response

Under normal circumstances, it is thought that the predominantly inhibitory mesocortical dopaminergic inputs to the PFC have an adaptive role in protecting against the pathological effects of stress. Indeed, the selective destruction of right mPFC dopaminergic neurons with 6-hydroxydopamine (6-OHDA) significantly increases stress ulcer formation (Sullivan & Szechtman, 1995). High dopamine (DA) concentrations in the right mPFC are significantly correlated with successful escape performance following a prolonged, uncontrollable foot shock stressor, suggesting that increased dopaminergic activity in this region facilitates behavioural coping strategies (Carlson et al., 1993). Rodents placed in a brightly lit, stressful novel environment are shown to have increased dopamine metabolism in the right PFC (Berridge et al., 1999). Interestingly, when these animals are able to chew an inedible object, a displacement behaviour which reduces HPA activity (Hennesy & Foy, 1987), the lateralized increase in dopamine metabolism diminishes. This result indicates that when the stressfulness of a situation is reduced, the intrinsic activity in the right PFC and its need for dopaminergic modulation are also decreased. The above findings demonstrate that the right mPFC is necessary for the optimal activation of physiological stress responses, but disinhibition or excessive activity in this region is maladaptive.

Stressors activate numerous neurochemical systems in the PFC, but one of the most comprehensively studied is the dopamine stress response. The PFC receives dopaminergic input from the mesocortical dopamine pathway that arises from the ventral tegmental area (VTA) (Seamans & Yang, 2004). When animals are physically stressed or exposed to a species-typical threat, extracellular levels of dopamine markedly increase in the PFC (Abercrombie et al., 1989; Stevenson et al., 2003). However, there are strong hemispheric biases in the PFC dopamine

stress responses which depend on the duration, controllability, and severity of the stressor (Berridge et al., 1999; Sullivan & Szechtman, 1995; Carlson et al., 1991; Carlson et al., 1993). Non-physical, psychological stressors, such as predator odour, predominantly activate dopamine transmission in the right PFC, while dopamine transmission in the left PFC is, at least initially, activated by physical stressors like tail-pinch (Sullivan & Gratton, 1998). Importantly, the activation of prefrontal cortical dopaminergic transmission by physical stressors shifts from an initial left hemisphere bias to a right hemisphere bias in response to more intense physical stressors, such as restraint or foot shock, or when the exposure to stress is prolonged or perceived as uncontrollable (Carlson et al., 1991). It is hypothesized that, when the left PFC is ineffective in mediating the response to a prolonged or inescapable stressor, the right PFC begins to predominate. Under these circumstances, it is thought that the increase in dopaminergic transmission in the right PFC could function to prevent the deleterious consequences of prolonged right PFC activity. Indeed, as previously mentioned, dopamine-depleting lesions in the right PFC are associated with maladaptive neuroendocrine and autonomic stress responses (Sullivan & Szechtman, 1995).

Together, these findings suggest that the effects of different stressors, and the coping behaviours they initiate, are executed by distinct prefrontal cortical mechanisms that are inherently lateralized and modulated by dopamine. Furthermore, they imply that when faced with a stressful situation, the right and the left PFC must somehow communicate in order to produce a suitable stress response. The exact mechanism underlying this interhemispheric exchange of stress-relevant information remains to be elucidated; however, glutamate-containing callosal neurons have been hypothesized to be probable candidates.

1.5. Interhemispheric communication in the PFC

1.5.1. A role for callosal neurons?

Callosal projection neurons are glutamatergic pyramidal neurons that are primarily located in layers II, III, and V of the mPFC (Ferino et al., 1987). Axons of these neurons enter the ipsilateral forceps minor, cross to the contralateral hemisphere through the genu of the corpus callosum, and pass from the contralateral forceps minor to terminate homotopically in both superficial and deep layers of the opposite PFC (Ferino et al., 1987). In the contralateral PFC, projections from callosal neurons synapse on pyramidal neurons and GABAergic interneurons (Carr & Sesack, 1998). The activity of callosal neurons is regulated by dopamine, GABA, and glutamate (Lupinsky et al., 2010; Kruse et al., 2009). Based on the widespread distribution of callosal neurons in the mPFC, it is thought that their activity is regulated by the main glutamatergic systems projecting to the mPFC which originate from the basolateral amygdala, dorsomedial thalamus, the hippocampus, as well as the contralateral mPFC (Bacon et al., 1996; Pirot et al., 1994; Gigg et al., 1994; Sesack et al., 1989). Using in situ hybridization, Gaspar et al. (1995) showed that pyramidal neurons which were retrogradely labelled from the contralateral PFC express mRNA for D1 and D2 dopamine receptors (Gaspar et al., 1995). Given this finding, it is not surprising that dopaminergic terminals synapse on the distal dendrites and spines of callosal neurons in the mPFC (Carr & Sesack, 2000). Prefrontal cortical callosal neurons also receive inputs from nerve terminals forming asymmetric synapses, which are typically excitatory (Carr & Sesack, 2000). This indicates that dopaminergic and, presumably, glutamatergic axons converge synaptically on callosal neurons in the PFC. Therefore, dopamine can alter the activity of callosal neurons through direct synaptic actions as well as by regulating the inputs to these cells.

1.5.2. Regulation of the PFC glutamate stress response

Stressors cause glutamate levels to increase in the mPFC (Moghaddam, 1993). Lupinsky et al. (2010) used *in vivo* microdialysis and local drug application to investigate if the glutamate stress response reflects increased interhemispheric communication by callosal projection neurons (see Figure 1 for schematic summarizing these findings). They found that tail pinch stress elicited similar increases in both the left and right PFC. The prefrontal cortical glutamate stress responses were significantly attenuated by sodium channel blockade with tetrotodoxin (TTX) and when perfusate calcium was omitted; indicating that the stress-induced increases in extracellular PFC glutamate reflects neurotransmitter release from an impulse- and calcium-dependent neuronal pool. The glutamate stress response does not originate from glial cells, since cysteine-glutamate transporter blockade did not affect the stress response. Unilateral excitotoxic lesions with ibotenic acid profoundly diminished the glutamate stress response in the opposite hemisphere. Furthermore, unilateral PFC mGlu2/3 receptor activation with LY379268 blocked the opposite PFC glutamate stress response. Activation of presynaptic Glu2/3 receptors inhibited glutamate release (Cartmell & Schoepp, 2000), which explains the decreased activation of callosal neurons projecting to the contralateral PFC. These results suggest that glutamatergic callosal neurons are the main contributors to the glutamate stress response and that this stress response is itself regulated by glutamate. Lupinsky et al. (2010) also found that the glutamate stress response was facilitated by contralateral $GABA_B$ receptor activation with baclofen. This finding is thought to be due to an interaction between GABA- and dopamine-sensitive mechanisms. The direct action of GABA on pyramidal neurons is mediated predominantly by $GABA_A$ receptors (Pirot et al., 1992); therefore, it is thought that the enhancement of the glutamate stress response is caused through indirect mechanisms: $GABA_B$ receptor activation

inhibits dopamine release and potentiates the glutamate stress response in the opposite hemisphere. Indeed, local D1 receptor blockade with SCH23390 in the left PFC potently enhanced the glutamate stress response in the right PFC. Interestingly, the administration of SCH23390 in the right PFC produced a comparatively weaker increase of the left PFC glutamate stress response. To explain these results, Lupinsky et al. (2010) hypothesized that SCH23390 reduces the activity of GABAergic interneurons expressing D1 receptors that, in the presence of dopamine, exert an inhibitory influence on callosal neurons. Taken together, these results indicate that a significant component of the prefrontal cortical glutamate stress response is due to increased interhemispheric communication by pyramidal callosal neurons. Furthermore, dopamine exerts an indirect D1 receptor-mediated inhibitory influence on this communication. However, this control appears to be asymmetrical in nature: dopaminergic input to the left PFC exerts a relatively stronger inhibitory influence on glutamatergic transmission in the right PFC than the right PFC does over left PFC glutamatergic transmission.

The left and right PFC play important, yet contrasting, roles in regulating the responses to different stressors. A large body of evidence indicates that an imbalance between left and right PFC-mediated function is involved in maladaptive stress responses and various pathopsychological symptoms (Gratton & Sullivan, 2005). Recently, numerous studies have shown that exposure to chronic stress leads to changes in the dendritic morphology of mPFC pyramidal neurons as well as deficits in PFC-mediated tasks (Holmes & Wellman, 2009). A subpopulation of the mPFC pyramidal neurons that undergo morphological remodelling during chronic stress could include callosal neurons that are involved in the inter-hemispheric exchange of stress relevant information. As such, functional alterations to this subset of neurons could

negatively affect normal inter-hemispheric communication, leading to imbalances in left versus right prefrontal cortical function.

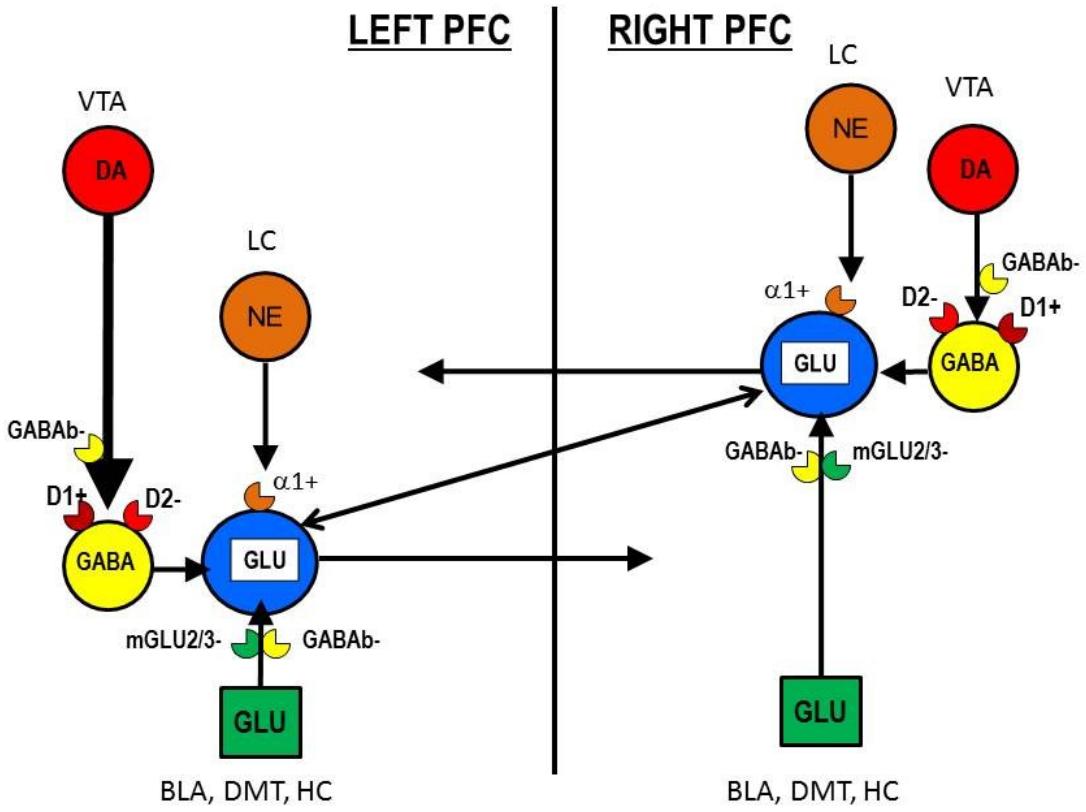

Figure 1. Schematic summarizing the regulation of the PFC GLU stress response.

1.6. Effects of chronic stress on the PFC

1.6.1. Stress-induced dendritic remodelling in the mPFC

In rats, repeated exposure to stressors has a profound impact on neuronal plasticity, most notably, in the hippocampal formation, the mPFC, and the amygdala (Czeh et al., 2008). These structures are extensively interconnected and regulate the HPA axis (Herman et al., 2003). Magarinos and McEwen (1995a) were the first to show that chronic stress induces dendritic atrophy of pyramidal neurons in the hippocampus. However, a rapidly growing number of studies have demonstrated that prolonged restraint stress leads to changes in the dendritic arborisation of pyramidal neurons in layers II, III and V of the mPFC (Cook & Wellman, 2004; Radley et al., 2004; Liston et al., 2006; Perez-Cruz et al., 2007; Liu & Aghajanian, 2008). The main findings from these studies are that chronic stress significantly reduces the total apical dendritic length and branching by 20 to 30%. Dendritic spines are also affected by stress: twenty-one days of restraint stress caused a 16% reduction in spine density on the distal apical dendrites of layer II/III pyramidal cells in the mPFC (Radley et al., 2006). In addition to spine loss, chronic stress causes morphological changes in the distal apical dendritic spines. Bloss et al. (2011) found that the spines on distal apical dendrites of chronically stressed animals have a larger head diameter compared to unstressed controls. Chronic stress has no effect on the length, branching, and spine density of basilar dendrites of pyramidal neurons in the mPFC (Cook & Wellman, 2004; Perez-Cruz et al., 2007; Radley et al., 2006; but see Perez-Cruz et al., 2009). Surprisingly, even daily injections of vehicle, a presumably mild stressor, cause dendritic remodelling of pyramidal neurons in the mPFC (Brown et al., 2005). However, it seems as though these morphological changes are reversible: Radley et al. (2005) showed that there was no difference in the apical dendritic length and branch number of layer II/III mPFC neurons between

chronically stressed rats allowed to recover for 3 weeks and control rats. In contrast, Goldwater et al. (2009) found that the chronic stress-induced apical dendritic retraction and spine loss of distal dendrites was not reversed when rats were allowed to recover for 3 weeks. Instead, recovery resulted in an over extension of proximal dendritic arbours and in spine growth. Differences in dendritic recovery between the two studies could be due to the fact that different neuronal subpopulations were sampled: Radley et al. studied layer II/III mPFC neurons, while Goldwater et al. investigated IL PFC neurons in layer V. It is thought that some of the chronic stress-induced effects are likely to be mediated by the activation of GRs, since the administration of the GR-selective agonist dexamethasone and of the natural adrenosteroid, CORT, resulted in a reduction in the length of apical dendrites of mPFC pyramidal neurons, similar to those seen following chronic stress exposure (Cerqueira et al., 2007). However, unlike chronic restraint stress, these treatments did not affect the number of dendritic branches. Unfortunately, most of these studies did not differentiate between subregions of the mPFC or between the hemispheres.

1.6.2. Hemisphere-specific effects of chronic stress on dendritic morphology in the mPFC

Perez-Cruz et al. (2007) performed a detailed analysis of the inherent differences and stress-induced changes in dendritic morphology between mPFC subareas and also between hemispheres. Interestingly, they found that pyramidal neurons in the right PL and IL cortices had longer apical dendrites compared to cells in the left mPFC, but no hemispheric asymmetry was observed in ACC pyramidal neurons. Chronic restraint stress mainly affected the right mPFC, abolishing the inherent hemispheric asymmetry in dendritic morphology. Moreover, the effect of chronic stress on dendritic branching and length was different in the IL, PL, and anterior cingulate cortex (ACC). Of particular interest is the finding that chronic stress robustly affected dendritic length in the IL cortex, where stress-induced increases in GLU transmission occur

(Lupinsky et al., 2010; Hascup et al. unpublished results, 2011). Perez-Cruz et al. did not investigate inherent hemispheric asymmetries in spine density or type in the mPFC.

1.6.3. Chronic stress-induced dendritic remodelling: Why and how does it occur?

The functional significance of stress-induced dendritic remodelling and the mechanism by which it occurs are unknown. It is well-established that exposure to stressors increases glutamatergic neurotransmission in the mPFC (Moghaddam, 1993). One explanation for dendritic remodelling is that neurons reduce their surface area to protect themselves against the excitotoxic effects of increased glutamate transmission during extended periods of stress (Czeh et al., 2008). Decreasing the total surface area by dendritic atrophy would reduce the amount of potentially excitotoxic synaptic inputs received by mPFC pyramidal neurons. GC administration induces changes in dendritic morphology similar to those produced by chronic stress (Cerqueira et al., 2007), which suggests that disregulation of the HPA axis could contribute to stress-induced dendritic remodelling. Czeh et al. (2008) hypothesized that brief exposures to stress initiate homeostatic neuronal changes that are beneficial for the survival of the animal. However, when stress becomes chronic and overwhelming, the system deteriorates, which increases cellular vulnerability. Indeed, inhibition of either GRs or N-methyl-D-aspartic acid (NMDA) receptors prevents chronic restraint stress-induced dendritic retraction (Liu & Aghajanian, 2008; Magarinos & McEwen, 1995b). Interestingly, repeated injection of a low dose of amphetamine in juvenile rats increases dendritic length and branching in mPFC pyramidal neurons (Diaz Heijtz et al., 2003). Amphetamine administration causes dopamine and glutamate concentrations to increase in the PFC in a manner similar to stress (Berridge et al., 2006; Reid et al., 1997). It is unclear why repeated amphetamine administration should increase dendritic length and branching when chronic stress induces dendritic retraction and reduce branching? Our hypothesis

posits that the remodelling of pyramidal neurons reflects functional imbalances in the inputs to these cells. Thus, depending on the stimulus and on its magnitude, neurotransmitter and hormone levels are differentially altered and dendrites are remodelled accordingly.

In addition to neuronal structural changes, it is becoming increasingly clear that stress affects the serotonergic modulation of mPFC neurons. The majority of the excitatory postsynaptic currents (EPSCs) induced by serotonin (5-HT) are generated by the apical tuft of layer V pyramidal neurons in the mPFC (Liu & Aghajanian, 2008). Chronic stress selectively induces a decrease in the complexity and spine density of the apical dendrites of these neurons (Radley et al., 2006; Liu & Aghajanian, 2008). Taken together, these results led Liu and Aghajanian (2008) to speculate that chronic stress would significantly decrease the 5-HT-induced EPSCs in layer V pyramidal neurons in the mPFC. Accordingly, electrophysiological recordings revealed that chronic stress reduced the 5-HT-induced EPSC frequency by 26%. Liu & Aghajanian (2008) further hypothesized that stress-induced elevation in CORT concentrations could be a possible mechanism for stress-induced morphological changes and the altered serotonergic modulation in the mPFC. Indeed, the layer V pyramidal neurons of rats that were administered CORT showed apical dendritic atrophy, decreased spine density as well as a 37% reduction in 5-HT-induced EPSCs. Moreover, treatment with a GR antagonist prior to stress exposure prevented both the stress-induced morphological and electrophysiological alterations from occurring. This study suggests that chronic stress alters both the apical dendritic morphology and the serotonergic excitation of layer V pyramidal neurons in the mPFC and that these effects could be caused by stress-induced over-activation of the HPA-axis.

Chronic stress also causes disregulation of dopamine transmission in the mPFC (Di Chiara et al., 1999; Mizoguchi et al., 2000). Chronically stressed rats have increased dopamine

transmission in the mPFC in response to aversive (tail pinch) and rewarding (food pellet) stimuli (Di Chiara et al., 1999). Mizoguchi et al. (2000) showed that chronically stressed rats were impaired in a spatial working memory task. In addition, under basal conditions, dopamine concentration in the mPFC was reduced by 80% in chronically stressed animals; however, prefrontal cortical D1 receptor density was increased in stressed animals. Indeed, working spatial memory impairment was rescued by administration of a D1 receptor agonist (Mizoguchi et al., 2000). Taken together, these results indicate that stress-induced deficiencies in working memory are at least partly mediated by changes to a D1 receptor-sensitive mechanism in the PFC. Evidence consistent with this idea comes from a study by Goldwater et al. (2009) who studied how chronic stress affects the D1 receptor-mediated modulation of long term potentiation (LTP) induction and maintenance in layer V PFC (IL) cortical pyramidal neurons. Surprisingly, measures of LTP did not differ in chronically stressed and control rats. However, in the presence of the D1 receptor agonist SKF38393, the neurons of stressed animals had impaired LTP. Moreover, the IL PFC of stressed animals had decreased D1 receptor ligand binding density, indicating that chronic stress led to D1 receptor down-regulation in IL cortical pyramidal neurons. It is likely that all of the above mentioned changes in neurotransmitter signalling contribute to the functional consequences associated with chronic exposure to stressors.

1.6.4. Effect of stress-induced morphological changes on prefrontal cortical function

Countless studies have investigated the behavioural effects of chronic exposure to stressors; however, only a few have examined how chronic stress-induced behavioural deficits are functionally related to the morphological remodelling of PFC pyramidal neurons. Liston et al. (2006) demonstrated that chronically restrained rats had impairments in attentional set-shifting, a mPFC mediated function. In stressed rats but not controls, decreased dendritic

arborisation in the mPFC predicted impaired attentional set-shifting performance. Wilber et al. (2011) investigated the firing of IL and PL PFC neurons in stressed and control animals during the acquisition and extinction of a fear conditioning task. Chronically stressed rats had increased freezing behaviour and altered neuronal firing in both the IL and PL cortices during the acquisition phase. There were no differences in neuronal firing or freezing behaviour between stressed and control animals during the extinction phase, which consisted of presenting the conditioned stimulus (CS) alone. However, when the CS was presented the following day (extinction retrieval), stressed rats displayed increased freezing behaviour and reduced neuronal firing in the PL and IL cortices compared to control rats, indicating deficits in extinction retrieval. Moreover, the reduction in firing in the IL cortex was significantly correlated with poor extinction retrieval. During stressful events, there is a large increase in catecholamine release in the mPFC, which leads to the activation of protein kinase C (PKC) intracellular signalling (Hains & Arnsten, 2008). PKC signalling reduces prefrontal neuronal firing and impairs working memory (Birnbaum et al., 2004). In the hippocampus, excessive PKC signalling *in vitro* induces spine retraction through disruption of the actin cytoskeleton (Calbrese & Halpain, 2005). Therefore, Hains et al. (2009) hypothesized that increased PKC activity during chronic stress could be contributing to stress-induced morphological and cognitive changes. To test this hypothesis, they investigated if inhibition of PKC with chelerythine before daily chronic variable stress would rescue working memory impairments and reverse distal apical remodelling in pyramidal neurons in the PL cortex. Chronically stressed rats performed worse than controls on a spatial delayed alternation task in a T maze, a task that assesses working memory; however, stressed rats treated with chelerythine performed at a level that was not statistically different than controls. Although chelerythine treatment did not prevent apical dendritic atrophy, this treatment

did reverse apical dendritic spine loss. Additionally, greater apical spine density significantly correlated with better performance in the working memory task. This study suggests that PKC could be involved in the intracellular signalling cascade responsible for stress-induced morphological and cognitive alterations. Furthermore, these findings imply that spines located on distal apical dendrites are important for working memory and that preventing stress-induced spine loss may rescue the cognitive deficits associated with stress. In all, these studies provide direct evidence that dendritic remodelling in the mPFC may underlie the functional deficits observed in chronically stressed animals.

1.6.5. Effect of chronic stress on individual neuronal circuits in the PFC

Recent work has focused on characterizing the stress-induced remodelling of individual prefrontal cortical neuronal circuits. In these studies, retrograde tracers are utilized to identify specific populations of neurons. Shansky et al. (2009) investigated the effect of 10 days of immobilization stress on the dendritic morphology of neurons projecting from the IL cortex to the basolateral nucleus of the amygdala (BLA) and the entorhinal cortex (EC). Similar to what had been observed in previous studies, unlabelled, randomly selected prefrontal cortical neurons showed stress-induced apical dendritic retraction. EC-projecting neurons in the IL cortex also displayed reduced apical dendritic length and branching. In contrast, BLA-projecting neurons exhibited no stress-induced dendritic remodelling or spine loss. These results indicate that the prefrontal cortical projections to the BLA are particularly resilient against the effects of chronic stress and that stress-induced changes in dendritic morphology in the mPFC are circuit-specific. Radley et al. (2013) studied the effect of 14 days of chronic variable stress on PL cortical neurons that project to the anterior bed nuclei of the stria terminalis (aBST). During stressful situations, a subpopulation of GABAergic neurons in the aBST integrates and relay inhibitory

signals to HPA axis-effector neurons (Radley et al., 2009). These inhibitory aBST neurons receive inputs from mPFC pyramidal neurons. Thus, Radley et al. (2013) studied the effect of stress on retrogradely labelled aBST-projecting neurons in the PL cortex. They found that aBST-projecting PL cortical neurons underwent apical dendritic retraction and loss of branching. Furthermore, these neurons displayed a selective loss of mature mushroom-shaped spines. To further investigate the effect of stress on this neuronal circuit, Radley et al. (2013) compared the CORT response and the IEG Fos activation in aBST-projecting PL cortical neurons of chronically stressed and control animals. When subjected to 30 minutes of restraint stress, chronically stressed animals had dramatically higher CORT levels compared to unstressed controls. Chronically stressed animals also had decreased Fos activation in aBST-projecting PL cortical neurons. These results indicate that stress-induced remodelling in the PL cortex to aBST pathway can reduce the ability of GABAergic aBST neurons to relay inhibitory signals to HPA axis-effector neurons. Future studies should investigate the stress-induced dendritic alterations of individual prefrontal cortical circuits because this will allow for a better understanding of how stress affects their underlying function.

2. Experiment 1:

The effect of chronic stress on the dendritic morphology of callosal neurons

2.1. Rationale

In Experiment 1, we investigated if a history of chronic stress altered the dendritic morphology of prefrontal cortical callosal neurons in a hemisphere-specific manner. Chronic restraint stress results in long-lasting regressive structural alterations to the dendrites of prefrontal cortical pyramidal neurons (Cook & Wellman, 2004; Radley et al., 2004). The dendritic morphology of pyramidal neurons in the PFC is inherently asymmetric. Importantly, chronic stress-induced dendritic remodelling occurs in a hemisphere-dependent manner: stress mainly affects dendrites in the right PFC, which abolishes this inherent asymmetry in dendritic morphology. We also know that prefrontal cortical callosal neurons mediate a portion of the interhemispheric exchange of stress-relevant information. Our hypothesis posits that a subpopulation of the mPFC pyramidal neurons that undergo morphological remodelling during chronic stress includes callosal neurons. However, certain neural circuits do not undergo stress-induced morphological changes (Shanksy et al., 2009), indicating that the effects of stress on dendrites are neural circuit-specific. Thus, the objective of the present experiment was to confirm whether or not chronic stress alters the dendritic morphology of identified left versus right PFC callosal neurons.

In order to determine if chronic stress affects the dendritic morphology of prefrontal cortical callosal neurons, a retrograde microbead tracer was injected into male rats' PFC 6 days prior to the start of the restraint stress or non-stress regimens. The chronic stress regimen consisted of 6 hour episodes of restraint stress for 21 consecutive days. Control rats were handled daily. During the chronic stress regimen, we investigated the effects of chronic stress on weight gain and plasma CORT levels. To do this, weight was recorded daily and blood samples were collected on the 1st, 10th, and 21st day of restraint stress. After completion of the stress regimen,

microbead-labelled callosal neurons in the PL and IL PFC were loaded with the fluorescent dye Lucifer Yellow to visualize their dendritic arbors. Next, morphometric analyses were performed to compare the dendritic morphology of callosal neurons in the left versus right PFC from chronically stressed and control animals.

2.2. Materials and Methods

2.2.1. Animals

Adult male Long-Evans rats (Charles River) were singly housed and acclimated to the animal colony for 5-7 days before the experiment. Food and water was available *ad libitum*. Animals were maintained in a temperature-controlled room (22-24˚C) on a 12 hour light/dark cycle (lights on at 09:00am). At the beginning of the experiment, animals weighed between 300 and 400 grams.

2.2.2. Chronic restraint stress regimen

Animals were randomly assigned to the experimental (chronic stress) and control groups. During the habituation phase of the experiment, all rats were handled and weighed daily for three days to become familiarized with human contact. During the second experimental phase, lasting 21 days, animals in the stress group were subjected to daily restraint stress for six hours (10:00am – 04:00pm). The chronic restraint protocol was executed according to a well-recognized paradigm (Magariños & McEwen, 1995a). Stressed rats were placed in a plastic rat restrainer in their home cages and did not have access to food and water. Additionally, stressed rats were weighed daily. Control rats were not subjected to any form of stress but were handled and weighed daily.

2.2.3. Corticosterone radioimmunoassay

Blood samples were collected from a subset of animals in the chronically stressed group using the tail snip procedure. Samples were taken on the first, tenth, and twenty-first days of the chronic stress regimen four times per day: when the rats were first restrained (0 hours), and 1, 3,

and 6 hours subsequently. Plasma was analysed for concentrations of CORT using a rat-specific radioimmunoassay (RIA) kit from MP Biomedicals (Orangeburg, NY). The limit of detection and the total binding percentage for the CORT assay were 0.3125 μg/dl and 49.7%, respectively.

2.2.4. Retrograde labelling surgery

Animals were anesthetized using isoflurane by inhalation. Each rat was secured in a stereotaxic apparatus, the skull was exposed, and a hole was drilled above the mPFC (anteroposterior (AP) +3 mm, mediolateral (ML) ±0.5 mm, dorsoventral (DV) -4.2 mm). A solution of retrograde fluorescent microbead tracer (FluoSpheres carboxylate-modified, 0.04 μm, 580/605; Molecular Probes) was deposited through pressure injection using a 0.5 μl Hamilton Neuros Syringe (Reno, NV) held in place by a stereotaxic attachment. Two hundred nl of the tracer was injected at a rate of ~50 nl/min, and the apparatus was left undisturbed for 5 minutes before syringe removal, to allow for diffusion. The bore hole was sealed with bone wax. The incision was closed with monofilament sutures and antibiotic gel (BNP Ointment; bacitracin zinc: 400 IU, neomycin sulfate: 5 mg, polymyxin B sulfate: 5000 IU) was applied to the wound. Carprofen (5 mg/kg, s.c.) was injected subcutaneously as a postoperative analgesic. Animals were allowed to recover for 3 days before beginning the habituation phase of the chronic stress regime and, subsequently, the 21 day chronic stress or non-stress regimen.

2.2.5. Sacrifice and tissue preparation

Twenty-four hours after cessation of the final stressor, animals were deeply anesthetized with rodent cocktail (ketamine 25 mg/ml, acepromazine 1 mg/ml, xylazine 5 mg/ml in 0.9% saline i.p. at a dose of 1 ml/kg body weight) and perfused transcardially with a vascular rinse, followed by 4% PFA in 0.1 M phosphate buffer (PB), pH 7.4. After perfusion, the brains were

carefully removed and postfixed for 24 hours in 4% paraformaldehyde (PFA) in 0.1 M PB. After postfixation, the brains were rinsed twice with and stored in 0.1 м PB. The brains were sub-blocked to contain the PFC and the hemisphere that was injected with tracer was marked with a small knick. Two hundred and fifty μm sections were collected using a vibrating blade microtome (VT1200 S, Leica, Concord, ON) and stored in wells filled with 0.01% sodium azide in 0.1 м PB.

2.2.6. Intracellular LY dye injection

Coronal sections of 250 μm thickness containing the mPFC were placed in an iontophoretic chamber filled with 0.1M PB. Sections were viewed under fluorescence using a fixed-stage microscope. Microbead tracer placement in the opposite PL and IL mPFC was verified for each animal. A sharp microelectrode (~130 MΩ) was used to inject Lucifer Yellow dye (LY; dilithium salt, Sigma-Aldrich, St. Louis, MO, dissolved at 1% in standard internal electrode solution) into microbead-labelled neurons in layer 2/3 of the PL and IL cortices. Injections were made by iontophoresis using a direct current of 1-2 nA for 8-10 minutes, or until the tips of the distal dendrites appeared bright when visualized with the fluorescent microscope. The technique for intracellular injection involved observing the diffusion of LY from the microelectrode tip under 40X magnification; LY diffusion becomes restricted intracellularly when the microelectrode hits a cell body or dendritic process. Five to ten labelled neurons were injected per mPFC section and 3-4 sections were used per animal.

2.2.7. Immunocytochemical staining of LY

We employed the immunocytochemical protocol for staining LY-loaded neurons previously described by Bories *et al.* (2013). Following the intracellular LY injections, the

cortical slices were washed in 0.1 M PB three times for 10 minutes and were incubated overnight in a solution of 0.1 M PB, 0.1% Triton, and 5% sucrose. The next day, three 10 minute washes in 0.1% Triton and 0.1 M PB were performed. Afterward, slices were incubated in a quenching solution (0.1 M PB, 0.1% Triton, and 0.3% H_2O_2) for 2 hours at room temperature. Slices were incubated for two hours at room temperature in a preblocking solution [0.1 M PB, 0.1% Triton, 10% normal goat serum (NGS; Vector Laboratories, Burlingame, CA)] before they were incubated overnight at 4°C with a rabbit anti-LY primary antibody (1:10,000; Molecular Probes, Invitrogen, Eugene, OR) in 0.1 M PB, 0.1% Triton, and 5% NGS. On day three, slices were washed three times in 0.1 M PB for 10 minutes. After the washes, slices were incubated in a biotinylated goat anti-rabbit IgG antibody (1:500, Vector Laboratories, Burlingame, CA) in 0.1 M PB for 2 hours at room temperature. Slices were rinsed again in 0.1 M PB three times for 10 minutes. The reaction solution was made from an ABC kit (1 drop reagent A, 1 drop reagent B in 5 ml 0.1 M PB; Vectastain ABC Elite kit, Vector Laboratories, Burlingame, CA) and was prepared 30 minutes before use. This solution was applied to slices for 90 minutes at room temperature. Once again, sections were washed in 0.1 M PB for 10 minutes three times. Sections were then allowed to react with the 3, 3'-diaminobenzidine (DAB) substrate (2 drops H_2O_2, 2 drops buffer, 2 drops nickel solution, and 4 drops DAB in 5 ml distilled H_2O; DAB Peroxidase Substrate Kit, Vector Laboratories, Burlingame, CA) until the desired staining was obtained, approximately 2 minutes. Finally, sections were washed in 0.1 M PB three times for 10 minutes, mounted on gelatin subbed slides and allowed to dry overnight. To visualize the cell layers, mounted sections underwent a light Nissl stain using cresyl violet dye. Subsequently, the sections were dehydrated in ascending alcohols and xylene and were cover slipped with Permount (Fischer Scientific, Fair Lawn, NJ).

2.2.8. Morphometric analyses

We performed the dendritic and neuronal morphometric reconstructions unaware of the treatment condition of each rat. A total of 140 callosal neurons were reconstructed and analysed in this study. For each animal, an average of six LY-filled callosal neurons with cell bodies in layer 2/3 of the PL or IL cortices was analysed. To be considered for analysis, neurons were required to have complete filling of dendritic projections, as evidenced by well-defined dendritic endings. A strict criteria described by Radley et al. (2013) was employed for the inclusion of callosal neurons apical dendrites for morphometric analysis. The fact that the primary shaft of the apical dendrite typically ran parallel from the top surface of the section enhanced the probability for retaining the complete apical dendritic tree. However, because LY microinjections were performed on sections with a width of 250 μm, it was effectively impossible to obtain six neurons per animal with entirely intact apical dendritic projections with no truncations. Therefore, apical dendrites that were included for analysis had intact secondary and tertiary branches, and truncations were permissible only in collateral branches that appeared to be close to the point of termination and were not likely to make any additional branchings.

Callosal neurons were traced under a X 100 (NA 1.40) oil immersion objective (Olympus BX51 light microscope) and their processes were analysed in three dimension (3D) within single sections using Neurolucida software (Neurolucida v. 8.10.2; MBF Bioscience, Williston, VT). Cell bodies were analysed in two-dimensions (area at its largest cross-sectional diameter) because of software limitations associated with measuring and tracing DAB-stained somas in three-dimensions.

2.2.9. Data format and statistical analysis

Group data for the morphometric analyses were obtained by taking the average values from each animal (average of six neurons, six animals per group) in retrogradely-labelled callosal neurons from the right or the left PL and IL of unstressed controls and chronically stressed animals. Inclusion for analysis required proper placement of tracer injection within the opposite mPFC. Several dendritic morphological features were assessed. To assess overall changes in callosal neuron morphology, cell body size and the total length, total volume, and number of branches of apical dendrites were compared across groups using *t* tests. Apical terminal and noding branch length were also compared between groups using *t* tests. A 3D version of the Sholl analysis (Sholl, 1956) was utilized to further investigate the spatial distribution of apical dendritic projections. This analysis employs a series of 10 μm concentric circles around the soma allowing for the quantification of the distribution of each parameter within a certain radius from the cell body. These data obtained were then summed over 20 μm intervals. The resulting data was compared using two-way repeated-measures analysis of variance (ANOVA) (group x radial distance from the soma) followed by appropriate planned comparisons. Furthermore, the total number and length for each branch order were compared across groups using two-way repeated-measures ANOVA (group x branch order) followed by appropriate planned comparisons. For all statistical analyses, planned comparisons were done using *F* tests performed within the context of the overall ANOVA, comparing groups at each radius or branch order. All measurements are expressed as mean ± standard error of the mean (SEM) and $p \leq 0.05$ was considered to be significant in all statistical tests.

2.3. Results

2.3.1. Effect of chronic stress on weight gain

Chronically stressed rats gained less weight than control animals over the course of the 21 day restraint stress regimen. Figure 2 is a comparison of the body weight gain, expressed as a percentage of the initial weight, in stressed (n = 28) and control (n = 24) rats. Throughout the chronic stress regimen, stressed rats gained significantly less weight than control animals (Figure 2 A; two-way repeated measures ANOVA, stress x time interaction, $F_{(20, 1000)} = 49.59$, $p < 0.0001$). On the final day of the restraint stress regimen, chronically stressed rats had gained 46% less weight than control animals over the course of the chronic stress regimen (Figure 2 B; controls versus stressed for % weight gain, $t_{(50)} = 8.587$, $p < 0.00001$).

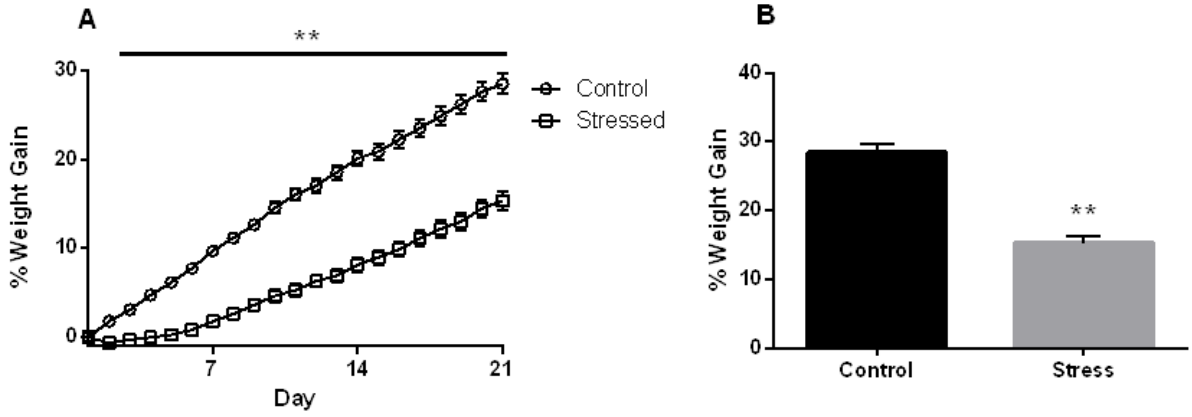

Figure 2. Twenty-one days of restraint stress caused decreased weight gain. A) Changes in body weight over the course of the study in chronically stressed and control rats. Chronically stressed rats gained significantly less weight over the course of the chronic stress regimen. B) Percent weight gain on the final day of chronic stress or non-stress regimen. Stressed animals had gained significantly less weight than control animals. $^{**}p < 0.0001$ versus weight gain of control group.

2.3.2. Effect of chronic stress on plasma CORT levels

Figure 3 shows the acute plasma CORT responses to the 1st, 10th, and 21st once daily exposures to a 6 hour episode of restraint stress. Two findings emerged from this analysis. First, restraint stress caused CORT secretion to increase from basal levels (hour 0) on days 1, 10, and 21 of the stress regimen (Figure 3 A, B, & C; 2 two-way repeated measures ANOVA, effect of time, $F_{(3, 27)} = 49.59$, $p < 0.0001$). On day 1, *post hoc* analysis confirmed that CORT levels remained significantly elevated throughout the 6 hour restraint period (Figure 3 A, Table 1; Bonferroni correction, $p < 0.05$). However, on days 10 and 21, the CORT levels were not consistently elevated from basal levels during the restraint period (Figure 3 B & C; Table 1). Chronic restraint stress did not significantly alter basal CORT levels measured before restraint stress (hour 0) over the course of the chronic stress regimen (Figure 3 D; Bonferroni correction, $p > 0.05$). Second, the CORT response to restraint was attenuated at day 10 of the chronic stress regimen. The CORT response was significantly more robust on day 1 compared to days 10 and 21 (Figure 3 D; two-way repeated measures ANOVA, day x time interaction, $F_{(6, 54)} = 49.59$, $p < 0.001$).

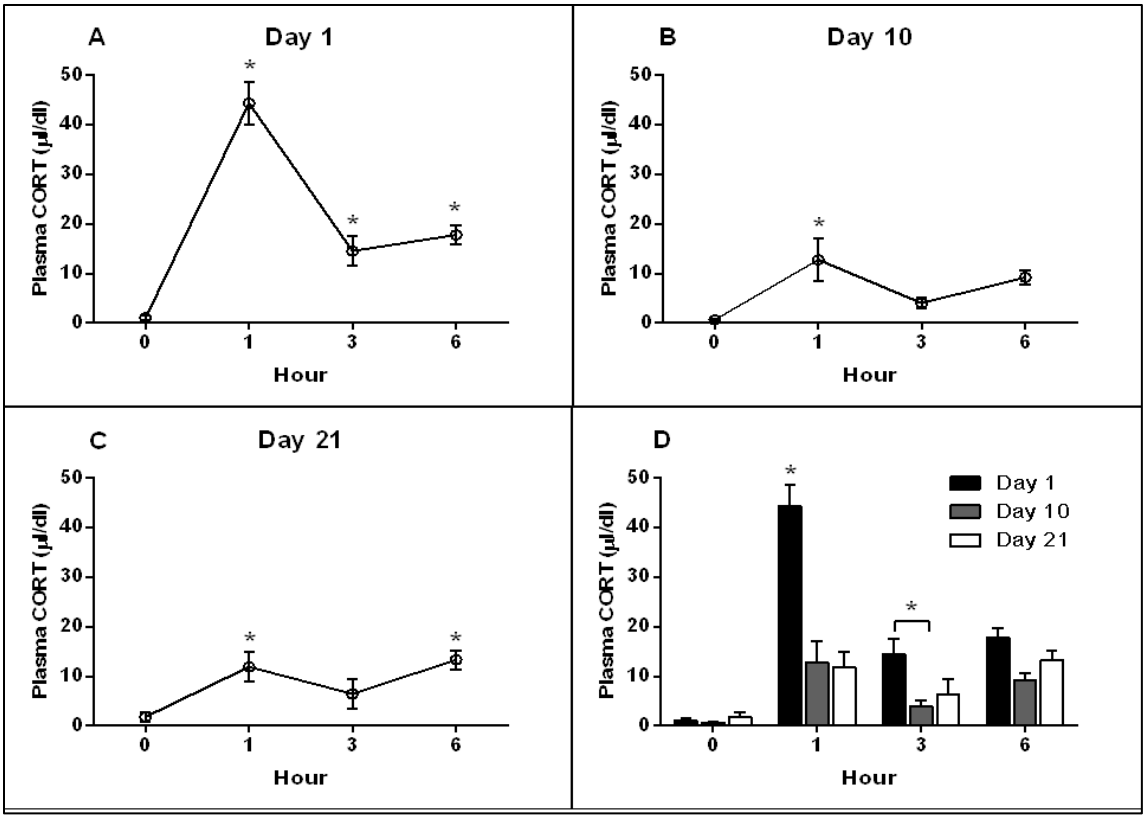

Figure 3. Plasma CORT levels during 21 days of restraint stress. A, B, & C) Restraint stress caused CORT concentration to increase from basal levels on days 1, 10, and 21 of the chronic stress regimen. *$p < 0.05$ versus basal levels at hour 0. d) CORT levels over the course of the chronic stress regimen. By day 10, the CORT response to restraint stress was attenuated. *$p <$ 0.05 versus days 10 and 21 of restraint stress.

Table 1. Plasma corticosterone (CORT) levels (μg/dl) during 21 days of restraint stress.

	Day 1	**Day 10**	**Day 21**
Hour 0	1.06 ± 0.37	0.64 ± 0.20	1.79 ± 0.88
Hour 1	44.30 ± 4.24*	12.78 ± 4.34*	11.89 ± 2.91*
Hour 3	14.53 ± 3.00*	4.00 ± 1.11	6.46 ± 3.00
Hour 6	17.78 ± 1.90*	9.22 ± 1.44	13.30 ± 1.93*

Values represent the mean ± SEM. Asterisks represent significant differences from the CORT level at hour 0 for each day; *$P < 0.05$; two-way repeated measures ANOVA, Bonferroni correction *post hoc* test.

2.3.3. Tracer placements and retrograde labelling patterns

Numerous lines of evidence support a role for prefrontal cortical callosal neurons in the interhemispheric exchange of stress relevant information. Callosal neurons are glutamate-containing pyramidal neurons that send projections to the contralateral PFC. To examine the effect of chronic restraint stress on the structural plasticity of callosal neurons, tracer injections of fluorescent microbeads were made in the contralateral mPFC 6 days before stress exposure. Tracer injections were centered in the PL and IL cortices, with tracer fluorescence extending dorsally into the ACC (Figure 4 A & B). Following tracer injection into the mPFC, numerous callosal neurons were labelled within homotopic (PL and IL) regions of the contralateral PFC. Retrogradely labelled callosal neurons were distributed throughout layers II to V of the PL and IL cortices (Figure 4 C). The observed retrograde labelling pattern is consistent with previous studies using conventional and viral retrograde tracers (Ferino et al., 1987; Carr & Sesack, 2000).

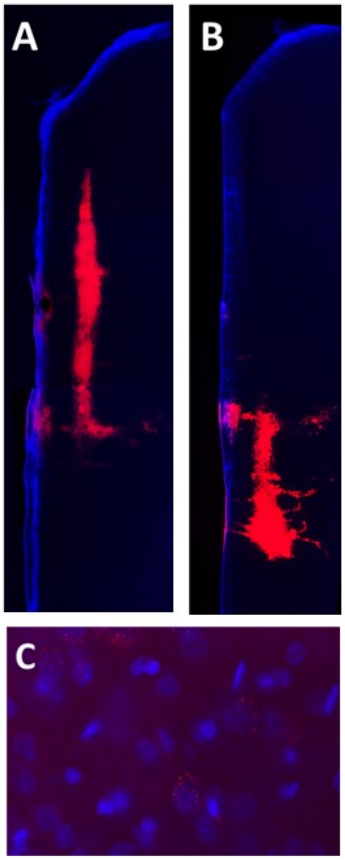

Figure 4. Examples of retrograde tracer injection site and labelling of callosal neurons. A & B) Example of microbead tracer injection in mPFC (red). Section A is anterior to section B and tracer injection continues between slices. C) Examples of retrogradely labelled callosal neuron cell bodies in mPFC contralateral to injection site (red). Nuclei are stained with DAPI (blue).

2.3.4. Overall morphometric analyses

Callosal pyramidal neurons in the PL and IL cortices were labeled after retrograde tracer injection into the opposite PFC. Following tracer injection, rats were exposed to 21 days of chronic restraint stress or similar handling with the absence of stress for controls. Dendritic morphological analyses were performed on the apical dendrites of neurons that were retrogradely labeled and filled with LY in the left and right PFC of chronically stressed and control animals.

In all treatment groups, complete filling of callosal neurons was evident and layer II/III was easily identifiable (Figure 5).

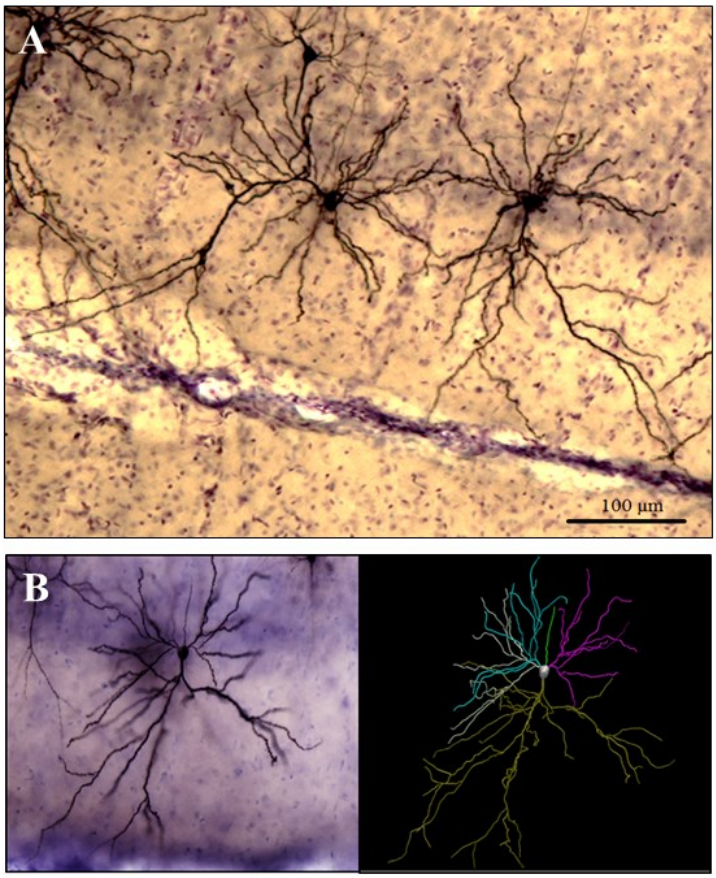

Figure 5. Digital images of LY-filled callosal neurons. A) Stacked digital light micrographs of LY-filled callosal neurons in layer II/III of the medial prefrontal cortex. Scale bar = 100 µm. B) Example neuron in layer II/III of PL that was iontophoretically filled with LY (left) and the 3D digital reconstruction of its dendritic tree (right) using computer-assisted morphometry. The apical dendritic tree (yellow) points downward toward the midline, whereas the axon and basal dendrites project from the cell body in the opposite direction. Only apical dendritic morphology was compared across groups.

In order to determine if there are inherent hemispheric asymmetries in the morphology of prefrontal cortical callosal neurons, the cell body size and the total length, number of branches, and total volume of apical dendrites of unstressed animals were compared. There was no inherent hemispheric asymmetry in cell body size (Figure 6; controls right PFC versus left PFC, $t_{(9)} = -0.031$; $p = 0.98$). Total dendritic length, number of branches, and total volume of callosal neurons in unstressed animals did not differ between the hemispheres (Figures 7, 8, & 9; controls right PFC versus left PFC for length, $t_{(9)} = 0.707$, $p = 0.5$; for branches, $t_{(9)} = 1.058$, $p = 0.32$; for volume, $t_{(9)} = -0.222$, $p = 0.83$). Thus, there was no significant inherent hemispheric asymmetry in the gross morphology of prefrontal cortical callosal neurons.

To assess stress-induced changes in dendritic morphology, the cell body size and the total length, number of branches, and total volume of apical dendrites were compared between chronically stressed and control animals. Chronic stress did not affect the cell body size of callosal neurons in either the right or the left PFC (Figure 6; left PFC, $t_{(11)} = 0.992$, $p = 0.34$; right PFC, $t_{(9)} = -0.952$, $p = 0.37$). However, chronic stress affected apical dendritic length, branching and volume in both hemispheres. Chronic restraint stress significantly decreased the total apical dendritic length by 23 and 24 % in the left and the right PFC, respectively (Figure 7; left PFC, $t_{(11)} = 3.4$, $p = 0.006$; right PFC, $t_{(9)} = 3.873$, $p = 0.004$). Figure 8 demonstrates that the number of apical branches was reduced by chronic stress by 19 % in the left ($t_{(11)} = 2.529$, $p = 0.02801$) and 27 % in the right PFC ($t_{(9)} = 3.314$, $p = 0.009$). Furthermore, chronic stress significantly reduced the total apical dendritic volume by 29 and 24 % in the left and the right PFC, respectively (Figure 9; left PFC, $t_{(11)} = 4.001$, $p = 0.00208$; right PFC, $t_{(9)} = 2.725$, $p = 0.02342$). Therefore, chronic restraint stress significantly reduced the total length, branching, and volume of callosal neurons in both the left and the right PFC.

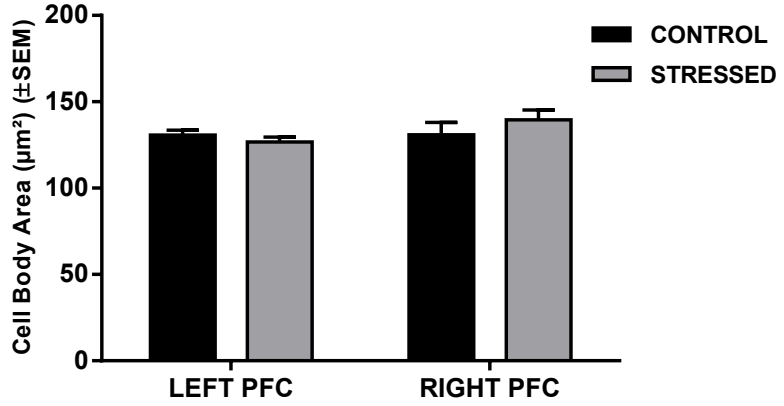

Figure 6. Mean cell body size for control (left PFC n = 6; right PFC n = 5) and chronically stressed rats (left PFC = 7; right PFC = 6). Cell body size did not vary across groups.

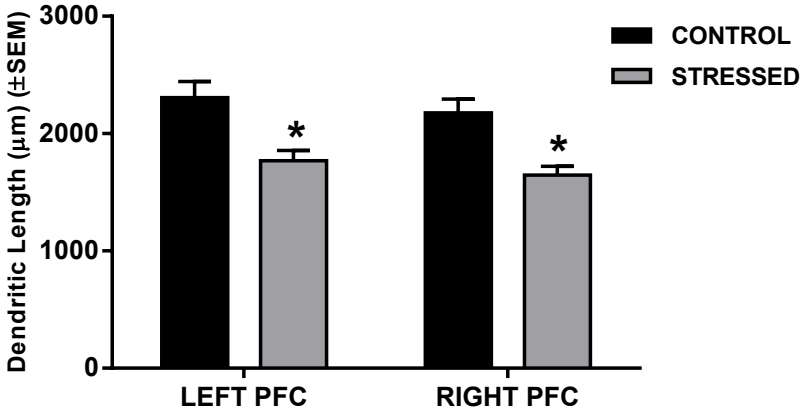

Figure 7. Total apical dendritic length for control (left PFC n = 6; right PFC n = 5) and chronically stressed rats (left PFC = 7; right PFC = 6). The total apical dendritic length of callosal neurons was significantly reduced by chronic stress in both hemispheres. *$p < 0.05$, statistically significant differences from unstressed controls.

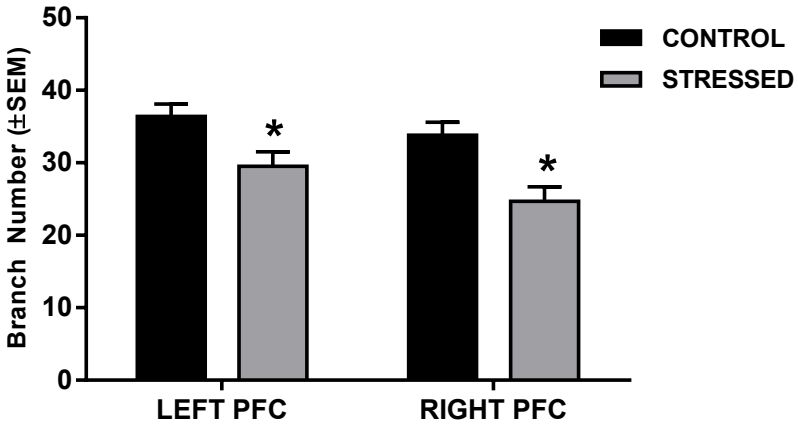

Figure 8. Total number of apical branches for control (left PFC n = 6; right PFC n = 5) and chronically stressed rats (left PFC = 7; right PFC = 6). The total number of apical branches of callosal neurons was significantly reduced by chronic stress in both hemispheres. *$p < 0.05$, statistically significant differences from unstressed controls.

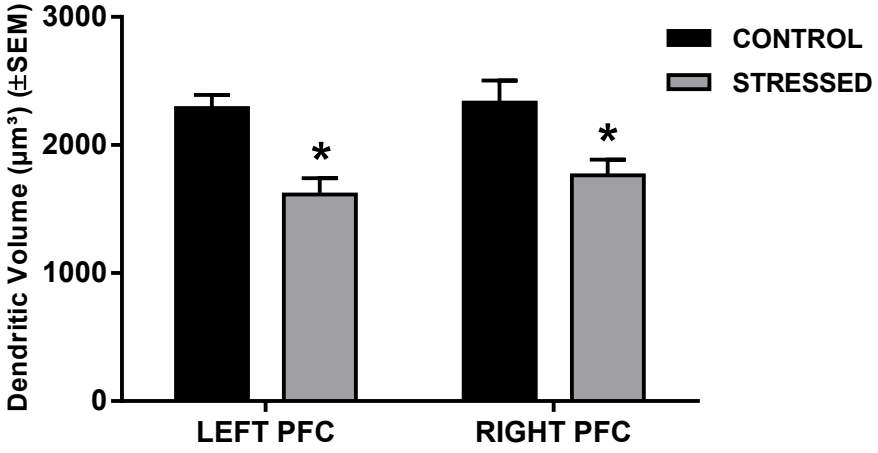

Figure 9. Total apical dendritic volume for control (left PFC n = 6; right PFC n = 5) and chronically stressed rats (left PFC = 7; right PFC = 6). Chronic restraint stress produced significant decreases of total apical dendritic volume in both right and left hemisphere callosal neurons. *$p < 0.05$, statistically significant differences from unstressed controls.

Terminal dendritic branches of prefrontal cortical pyramidal neurons are sensitive to the effects of stress and are thought to be more plastic than other regions of the dendritic arbour (Cook & Wellman, 2004). Thus, the length of terminal apical dendritic branches of callosal neurons was also compared across groups. Figure 10 shows the effect of chronic stress on the total length of terminal apical branches. Chronic restraint stress significantly reduced the total length of terminal apical branches by 26 and 30 % in the left and the right PFC, respectively (Figure 10; left PFC, $t_{(11)} = 3.174$, $p = 0.00886$; right PFC, $t_{(9)} = 4.070$, $p = 0.0028$).

Differences in noding branch length of prefrontal cortical callosal neurons from control and chronically stressed animals were also investigated. Figure 11 displays the effect of chronic stress on the total noding branch length of callosal neurons in the right and the left hemispheres. In the left PFC, chronic restraint stress significantly decreased noding branch length by 17% (Figure 11; $t_{(11)} = 2.605$, $p = 0.02448$). In contrast, there was no significant change in noding branch length in the right PFC of chronically stressed animals (Figure 11; $t_{(9)} = 1.521$, $p = 0.16263$). Thus, chronic stress affected the length of both terminal branches and noding branches of callosal neurons in the left PFC but only altered terminal branches in right prefrontal cortical callosal neurons. These findings demonstrate that there is a hemispheric asymmetry in the sensitivity of the noding branches of prefrontal cortical callosal neurons to stress.

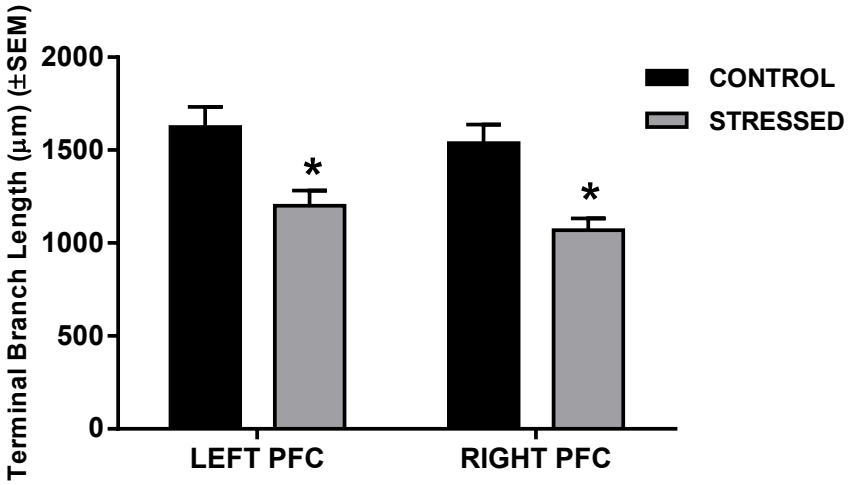

Figure 10. Mean length of apical terminal branches for control (left PFC n = 6; right PFC n = 5) and chronically stressed rats (left PFC = 7; right PFC = 6). The total terminal branch length of callosal neurons was significantly reduced by chronic stress in both the right and the left hemispheres. *$p < 0.05$, statistically significant differences from unstressed controls.

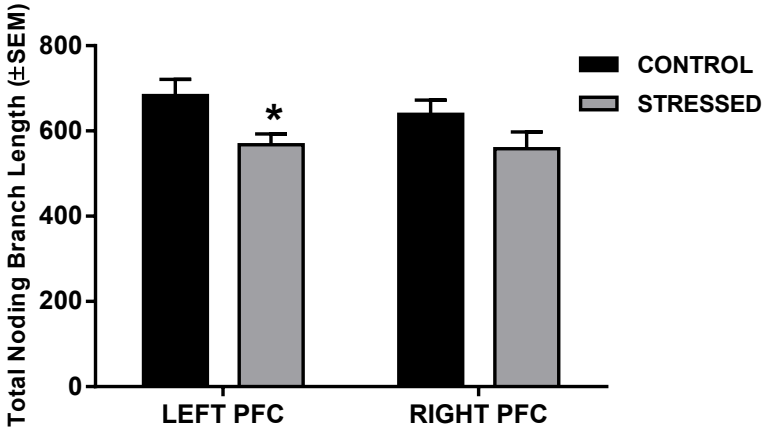

Figure 11. Mean length of apical noding branch length for control (left PFC n = 6; right PFC n = 5) and chronically stressed rats (left PFC = 7; right PFC = 6). Chronic restraint stress produced a significant decrease of total apical noding branch length in the left mPFC callosal neurons, however, callosal neurons in the right mPFC were not affected. *$p < 0.05$, statistically significant differences from unstressed controls.

2.3.5. Sholl analyses

3D Sholl analyses were performed in order to more closely compare the distribution of apical dendrites of callosal neurons from the left versus the right PFC of unstressed control rats. Figure 12 is a comparison of the dendritic length of left and right PFC callosal neurons as a function of radial distance from the soma. There were no inherent hemispheric differences in the overall apical dendritic arbors of callosal neurons in the left compared to the right PFC (for main effect of hemisphere, $F_{(1, 9)} = 0.6792$, $p = 0.4312$). Furthermore, there were no differences in the distribution of dendritic material as a function of radial distance from the soma (for interaction of stress and distance from the soma, $F_{(22, 198)} = 0.5051$, $p = 0.9694$). Thus, there was no inherent hemispheric difference in the dendritic length of prefrontal cortical callosal neurons at any distance from the soma.

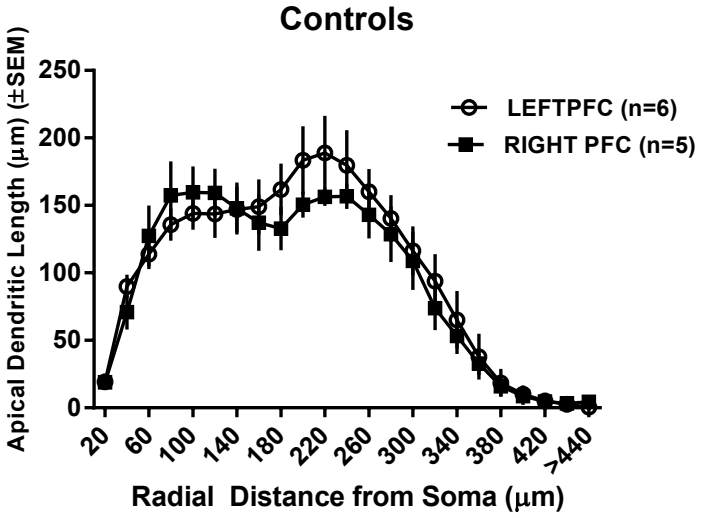

Figure 12. Sholl analysis for summed dendritic length as a function of radial distance from the soma in the left and right PFC of unstressed rats. Data have been summed in 20 µm bins. There was no significant inherent change in dendritic length as a function of radial distance between hemispheres.

Chronic stress significantly reduced the total apical dendritic length and branching of prefrontal cortical callosal neurons; therefore, Sholl analyses were executed to determine the location on the dendritic arbor where these stress-induced morphological rearrangements occurred. Figure 13 displays the effect of chronic stress on apical dendritic length in the right and the left PFC as a function of radial distance from the soma. In the left PFC, chronic stress significantly altered both the overall dendritic arbor (Figure 13 A; for main effect of stress, $F_{(1, 11)} = 11.58$, $p = 0.0059$) and the distribution of dendritic material (for interaction of stress and distance from soma, $F_{(22, 242)} = 1.831$, $p = 0.015$). Planned comparisons revealed that chronic stress significantly reduced the length of distal apical dendrites by 35 to 38 % at radial distances of 220 to 280 μm from the soma (for 220 μm, $F_{(1,11)} = 2.257$, $p = 0.0453$; for 240 μm, $F_{(1, 11)} = 2.565$, $p = 0.0263$; for 260 μm, $F_{(1,11)} = 3.191$, $p = 0.0086$; for 280 μm, $F_{(1,11)} = 2.467$, $p = 0.0313$). In the right PFC, chronic stress significantly influenced apical dendritic length in a uniform manner throughout the entire apical dendritic arbor (Figure 13 B; for main effect of stress, $F_{(1,9)} = 13.22$, $p = 0.0054$; for interaction of stress and distance from soma, $F_{(22,198)} = 1.435$, $p = 0.1016$). Planned comparisons indicated that chronic stress reduced the length of apical dendritic material in both proximal and distal dendrites. Chronic stress reduced proximal dendritic length by 27% at both distances of 100 and 120 μm from the soma (for 100 μm, $F_{(1,9)} = 2.272$, $p = 0.0492$; for 120 μm, $F_{(1,9)} = 2.413$, $p = 0.0390$). Distal dendritic length decreased by 32 to 35 % at distances of 200 to 240 μm from the soma (for 200 μm, $F_{(1,9)} = 2.696$, $p = 0.0246$; for 220 μm, $F_{(1,9)} = 2.489$, $p = 0.0344$; for 240 μm, $F_{(1,9)} = 2.593$, $p = 0.029$). These results indicate that the stress-induced dendritic remodelling of callosal neurons is lateralized: chronic stress affected both proximal and distal dendrites in the right PFC, while only distal dendrites in the left PFC were affected.

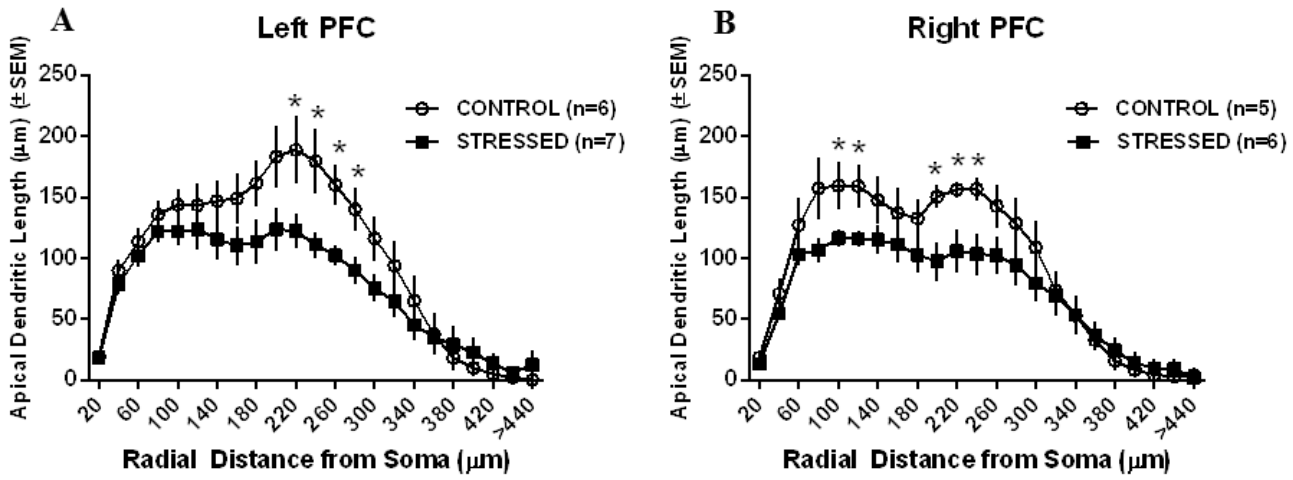

Figure 13. Sholl analysis for summed dendritic length as a function of radial distance from the soma in the left (A) and the right (B) PFC of chronically stressed and control rats. Data have been summed in 20 μm bins. A) In the left PFC, chronic restraint stress selectively reduced the length of distal apical dendrites. B) In the right PFC, chronic restraint stress caused a reduction in apical dendritic length in both proximal and distal dendrites.*$p < 0.05$, statistically significant differences relative to chronically stressed rats.

2.3.6. Branch order analyses

To further investigate any inherent hemispheric asymmetries in dendritic morphology, a branch order analysis comparing the number of branches in each order was performed. Figure 14 displays the branch number of left and right prefrontal cortical callosal neurons as a function of branch order. There were no inherent hemispheric differences in apical branch number at any branch order (Figure 14; for main effect of hemisphere, $F_{(1,9)} = 1.146$, $p = 0.3123$; for interaction between stress and branch order, $F_{(10, 90)} = 1.661$, $p = 0.1024$). These results indicate that prefrontal cortical callosal neurons had no inherent hemispheric asymmetry in branch number at any branch order.

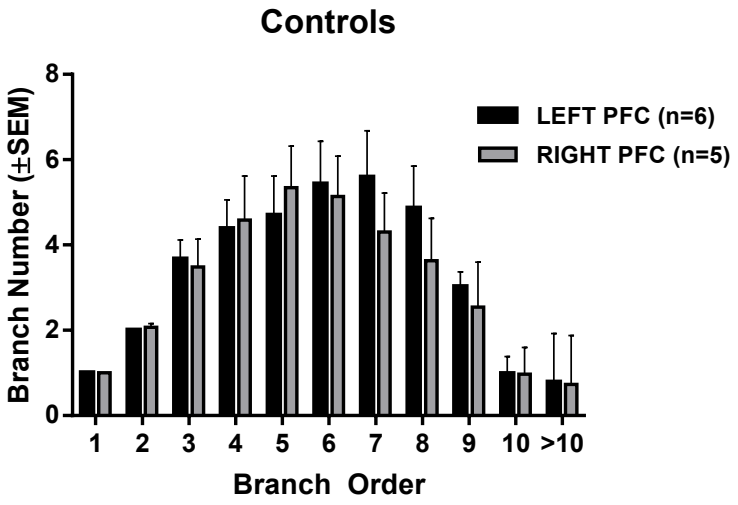

Figure 14. Mean number of apical dendritic branches at each branch order in the left and the right PFC of unstressed rats. There were no inherent differences in apical branch number across branch order between the hemispheres.

Chronic stress significantly decreased the branch number of prefrontal cortical callosal neurons; therefore, branch order analyses were performed to determine the order of the branches eliminated by chronic stress. Figure 15 shows the effect of stress on branch number as a function of branch order in the left and the right PFC. In the left PFC, chronic restraint stress significantly altered the apical branch number at certain branch orders (Figure 15 A; for main effect of stress, $F_{(1,11)} = 6.713$, $p = 0.0251$; for interaction between stress and branch order, $F_{(10, 110)} = 2.567$, $p = 0.0079$). Planned comparisons revealed that the effect of chronic stress on branch number was restricted to higher-order branches. Branch number decreased by 25 to 32% in the sixth to the eighth branch orders (for sixth branch order, $F_{(1, 11)} = 2.5$, $p = 0.295$; for seventh branch order, $F_{(1, 11)} = 2.393$, $p = 0.0357$; for eighth branch order, $F_{(1,11)} = 2.216$, $p = 0.0487$). In the right PFC, chronic restraint stress also significantly affected the apical branch number at certain branch orders (Figure 15 B; for main effect of stress, $F_{(1,9)} = 11.0$, $p = 0.009$; for interaction between stress and branch order, $F_{(10, 90)} = 2.047$, $p = 0.0373$). Planned comparisons determined that stress decreased apical branch number by 34 to 43% in the sixth to the eighth branch orders (for sixth branch order, $F_{(1,11)} = 3.14$, $p = 0.0119$; for seventh branch order, $F_{(1,11)} = 2.789$, $p = 0.0212$; for eighth branch order, $F_{(1,11)} = 2.374$, $p = 0.0416$). Therefore, chronic restraint stress reduced the number of higher-order branches of callosal neurons in both the left and the right PFC.

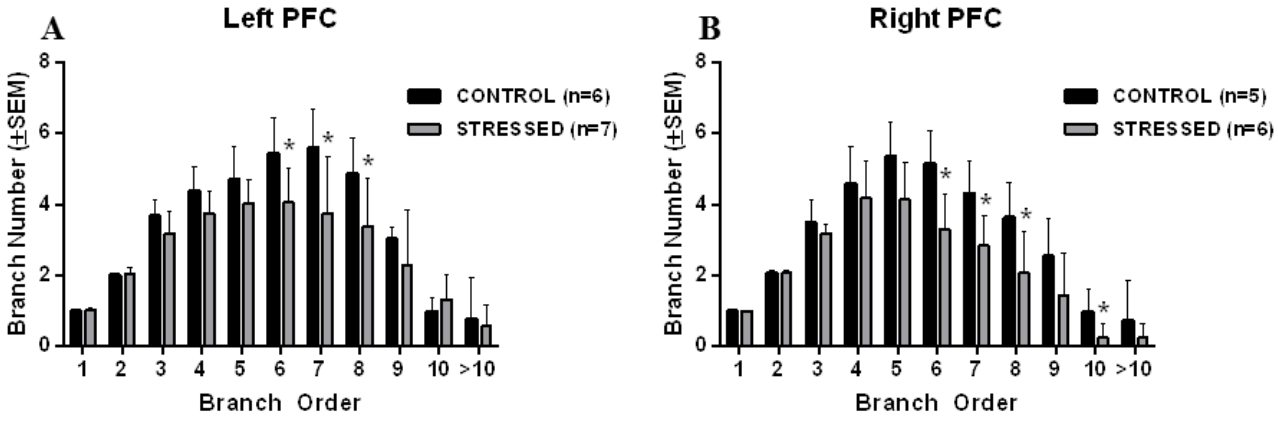

Figure 15. Mean number of branches at each branch order for apical dendrites in the left (A) and the right (B) PFC of control and chronically stressed rats. A) In the left PFC, chronic stress significantly decreased the number of higher-order apical branches. B) Similarly, chronic stress caused a significant reduction in the number of higher-order apical branches in the right hemisphere. *$p < 0.05$, statistically significant differences relative to unstressed controls.

Next, branch order analyses comparing the length of branches in each order were performed to determine if there was an inherent hemispheric asymmetry in the morphology of callosal neurons. Figure 16 shows the branch length of callosal neurons from control and stressed rats as a function of branch order. Total branch length was different between hemispheres at certain branch orders (Figure 16 A; for main effect of hemisphere, $F_{(1,9)} = 0.696$, $p = 0.4257$; for interaction between hemisphere and branch order, $F_{(10, 90)} = 1.984$, $p = 0.0441$). Planned comparisons indicated that eighth order branches were 37% shorter in the right PFC compared to the left PFC (for eighth branch order, $F_{(1,9)} = 2.45$, $p = 0.0368$). Similarly, total branch length of callosal neurons of chronically stressed animals were different between hemispheres at specific branch orders (Figure 16 B; for main effect of hemisphere, $F_{(1, 11)} = 0.908$, $p = 0.3611$; for interaction between hemisphere and branch order, $F_{(10, 110)} = 2.790$, $p = 0.0041$). Planned comparisons revealed that stressed rats maintained 47% shorter eighth order branches in the right PFC (for eighth branch order, $F_{(1, 11)} = 2.561$, $p = 0.0265$). In addition, chronic stress introduced new hemispheric asymmetries: branches in the fourth order were 30% longer while tenth order branches were 89% shorter in the right PFC (for fourth branch order, $F_{(1, 11)} = 2.675$, $p = 0.0216$; for tenth branch order, $F_{(1, 11)} = 3.107$, $p = 0.001$). These results indicate that there is an inherent asymmetry in the length of higher-order apical branches of callosal neurons in unstressed animals; eighth-order dendrites were longer in the left compared to the right PFC. Furthermore, chronic stress expanded and intensified this hemispheric asymmetry in dendritic length at several branch orders.

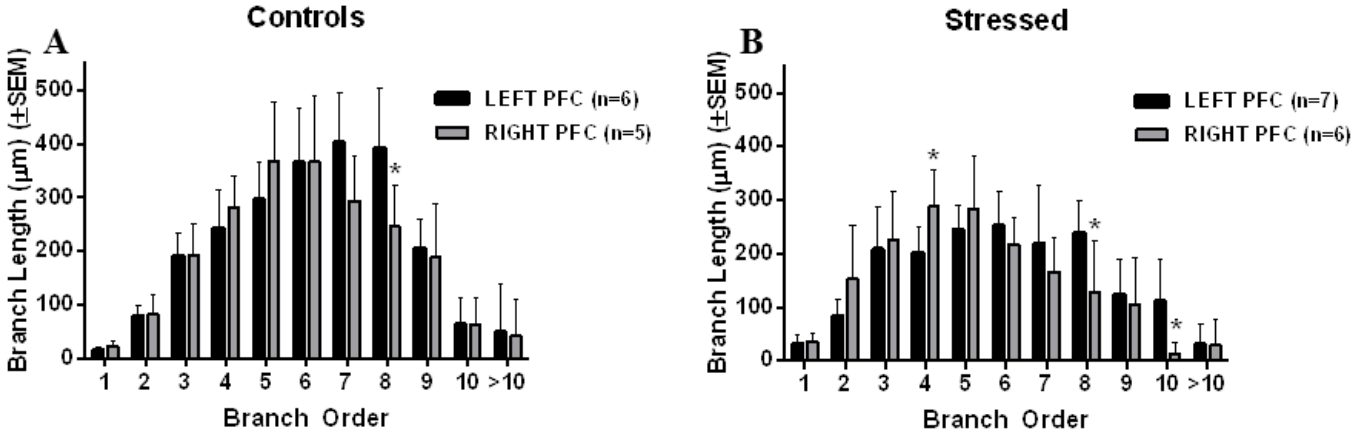

Figure 16. Mean length of branches at each branch order for apical dendrites in the left and the right PFC of control (A) and stressed (B) rats. Eighth-order dendrites are significantly shorter in the right compared to the left PFC. B) Chronic stress maintains and expands this hemispheric asymmetry across branch orders. *$p < 0.05$, statistically significant differences relative to the left PFC.

Chronic stress significantly decreased the total dendritic length of prefrontal cortical callosal neurons; therefore, branch order analyses were performed to determine the order of the branches shortened by chronic stress. Figure 17 presents the effect of stress on branch length as a function of branch order in the left and the right PFC. In the left PFC, chronic stress significantly altered apical branch length (Figure 17 A; for main effect of stress, $F_{(1, 11)} = 13.92$, $p = 0.0033$) but this effect of stress was not consistent across all branch orders (for interaction between stress and branch order, $F_{(10, 110)} = 4.278$, $p < 0.0001$). Planned comparisons indicated that changes in branch length were restricted to higher-order branches. Branch length decreased by 31 to 46% in the sixth to the ninth branch orders (for sixth branch order, $F_{(1, 11)} = 2.471$, $p = 0.0311$; for seventh branch order, $F_{(1, 11)} = 3.295$, $p = 0.0071$; for eighth branch order, $F_{(1, 11)} = 3.135$, $p = 0.0095$; for ninth branch order, $F_{(1, 11)} = 2.408$, $p = 0.0347$). Chronic stress also altered apical branch length in the right PFC (Figure 17 B; for main effect of stress, $F_{(1, 9)} = 13.24$, $p = 0.0054$) and the effect of stress was also variable across branch order (for interaction between stress and branch order, $F_{(10, 90)} = 2.516$, $p = 0.0102$). In stressed animals, branch length was reduced in higher-order branches by 41, 43, and 80% in the sixth, seventh, and tenth branch orders, respectively (for sixth branch order, $F_{(1, 9)} = 2.729$, $p = 0.0233$; for seventh branch order, $F_{(1, 9)} = 2.902$, $p = 0.0176$; for tenth branch order, $F_{(1, 9)} = 2.303$, $p = 0.0468$). These results demonstrate that chronic stress selectively decreases the length of higher-order branches of callosal neurons in both the right and the left PFC.

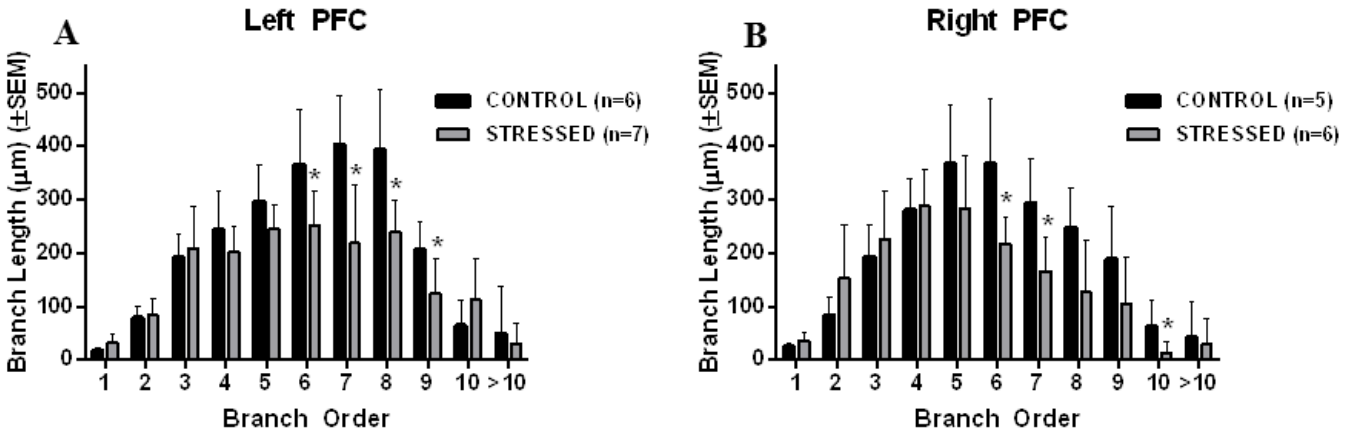

Figure 17. Mean length of branches at each branch order for apical dendrites in the left (A) and the right (B) PFC of control and stressed rats. A & B) The length of higher-order apical branches was significantly reduced by chronic stress in both hemispheres. *$p < 0.05$, statistically significant differences relative to unstressed controls.

2.4. Discussion

2.4.1. Weight gain and CORT release during chronic stress regimen

Chronic restraint stress produces various physiological, neuroendocrine, and behavioural alterations which model the symptoms reported in human stress-related neuropsychiatric disorders (Magarinos & McEwen, 1995a; Marin et al., 2007; Luine et al., 1994; Ottenweller et al., 1989). To quantify these stress-induced physiological changes, I assayed weight gain and HPA axis activation during the chronic restraint stress regimen. Chronic restraint stress caused rats to gain significantly less weight throughout the 21 days of chronic stress, corroborating the findings of numerous studies (Magarinos & McEwen, 1995a; Cook & Wellman, 2004; Radley et al., 2004). It is unclear if chronically stressed rats gained less weight because they did not have access to food during the 6 hours of daily restraint or if this decrease in weight gain was induced by stress. To assess HPA axis activation, I measured plasma CORT concentrations throughout the 6 hour restraint period on the first, tenth, and twenty-first day of chronic stress. I found that CORT levels increased from baseline throughout the 21 days of restraint; however, the CORT stress response was blunted on days 10 and 21 of chronic stress compared to the first day of restraint, indicating that habituation of the HPA axis to restraint stress had occurred by day 10 of restraint stress. Habituation of the HPA stress response to a homotypical stressor such as restraint stress is a widely reported finding (Marin et al., 2007; Magarinos & McEwen, 1995a; Cook & Wellman, 2004). When chronically stressed animals are challenged with a novel heterotypical stressor, sensitization of the HPA axis stress response can occur (Marin et al., 2007). Indeed, rats that had previously been restrained for 1 hour daily for 10 days had higher CORT levels after novelty stress than control animals (Marin et al., 2007). I used tail pinch as a heterotypical stressor to test the stress response of stressed rats during *in vivo* microdialysis recordings. It

would be interesting to determine if the more severe chronic restraint stress regimen employed in this study compared to Marin et al. also induced HPA axis sensitization. Finally, future studies should assess other physiological consequences of chronic stress such as thymus and adrenal organ weights. Perez-Cruz et al. (2007) found that chronic restraint stress causes adrenal hypertrophy and thymus atrophy in Sprague-Dawley rats; however, to my knowledge, these indices of chronic stress have never been studied in Long-Evans rats.

2.4.2. Callosal neuron morphology in unstressed animals is not lateralized

Margariños and McEwen (1995a) were the first to report that chronic restraint stress induces apical dendritic atrophy of pyramidal neurons in the hippocampus. Subsequent studies focusing on prefrontal cortical pyramidal neurons also observed stress-induced regressive changes in dendritic morphology (Cook & Wellman, 2004; Radley et al., 2004; Liston et al., 2006; Perez-Cruz et al., 2007; Liu & Aghajanian, 2008); however, these studies did not discriminate between the hemispheres. Consequently, Perez-Cruz et al. (2007) performed a detailed morphological analysis of mPFC pyramidal neurons which distinguished between hemispheres and mPFC subregions. They discovered that pyramidal neurons in the right PL and IL cortices had longer apical dendrites than in the left hemisphere. Intrinsic asymmetries in morphology have also been observed in several regions of the human cerebral cortex, such as the entorhinal cortex, language-association and auditory cortices (Simic et al., 2005; Hayes & Lewis, 1993; Hutsler, 2003).

In contrast to the hemispheric asymmetries in morphology reported in rodents and humans, we found no hemispheric asymmetry in the apical dendritic morphology of callosal neurons of unstressed control animals. There were no interhemispheric differences in length at

any distance from the soma. Furthermore, branching did not differ between the hemispheres. These opposing findings are not due to differences in the location of cells studies because callosal neurons cell bodies are typically found in the PL and IL cortices (Carr & Sesack, 2000) which are the same mPFC subregions in which Perez-Cruz et al. found hemispheric asymmetries in dendritic morphology. Prefrontal cortical pyramidal neurons undergo morphological changes in a neural circuit-specific manner: some neural circuits undergo stress-induced dendritic remodelling while others do not (Radley et al., 2013; Shanksy et al., 2009). Therefore, it is reasonable to hypothesize that the dendritic morphology of different populations of neurons could be inherently dissimilar. This hypothesis could explain why callosal neurons have no hemispheric asymmetry in their dendritic morphology even though the morphology of the general population of prefrontal cortical pyramidal neurons is inherently asymmetric in nature.

2.4.3. Remodelling of PFC callosal neurons by chronic stress

Prefrontal cortical pyramidal neurons undergo stress-induced dendritic morphological changes in a neural circuit- and hemisphere-specific manner (Radley et al., 2013; Shanksy et al., 2009; Perez-Cruz et al., 2007); thus, we investigated how 21 days of chronic restraint stress affected the dendritic morphology of callosal neurons in the right versus the left mPFC. The principal findings from our morphometric analyses were that chronic restraint stress caused a bilateral reduction in the total length, volume, and the number of branches of apical dendrites of callosal neurons. Our findings are in accordance with the 20-35% decrease in total length and branching that is widely reported in the literature (Cook & Wellman, 2004; Radley et al., 2004; Radley et al., 2005. Radley et al., 2013). Stress-induced dendritic remodelling is neural circuit-specific (Radley et al., 2013; Shanksy et al., 2009). Therefore, the prefrontal cortical callosal

neural circuit was affected by chronic restraint stress in a similar manner to the general population of pyramidal neurons in the mPFC.

The mechanism by which chronic stress-induced dendritic retraction of pyramidal neurons within the mPFC occurs is unclear; however, one possible explanation is that dendrites retract to protect themselves from the excitotoxic effects of excessive glutamate release (Czeh et al., 2008). Decreasing the neuronal surface area would reduce the amount of synaptic inputs that the cell receives. In support of this argument, stress causes glutamate levels to increase bilaterally in the mPFC (Moghaddam, 1993; Lupinsky et al., 2010). Pharmacologically inhibiting NMDA receptor activity rescues stress-induced dendritic remodelling in the hippocampus, indicating that NMDA receptor activation is necessary for dendritic retraction to occur in this brain region (Magarinos & McEwen, 1995b). The involvement of NMDA receptor signalling in dendritic remodelling in the PFC has yet to be determined. In further support of this excitotoxic hypothesis, stress-induced apical dendritic atrophy results in blunted responses to both serotonin- and hypocretin-evoked excitatory responses (Liu & Aghajanian, 2007). It is, however, likely that there are other factors involved in addition to glutamatergic signalling. During chronic restraint stress, GC levels are dramatically elevated. Research shows that administering exogenous GCs causes regressive changes in dendritic morphology that were similar to those caused by chronic stress (Cerqueira et al., 2007). Furthermore, blocking GR activation prevented stress-induced changes in dendritic morphology (Liu & Aghajanian, 2008). Evidence also suggests that there are abnormalities in the dopaminergic and serotonergic modulation of mPFC pyramidal neurons of chronically stressed animals (Liu & Aghajanian, 2008; Mizoguchi et al., 2000; Goldwater et al., 2009). During chronic stress, dopamine levels in the PFC are elevated (Carlson et al., 1991); however, basal levels of dopamine in chronically stressed animals are reduced (Mizoguchi et al.,

2000). In addition, chronically stressed rats have decreased D1 receptor density and reduced D1 receptor-mediated induction of LTP (Goldwater et al., 2009). Stress also affects the serotonergic system: repeated stress reduces basal levels of serotonin in the PFC (Mangiavacchi et al., 2001). Moreover, chronic stress blunts serotonin-evoked excitatory responses in the PFC (Liu & Aghajanian, 2007). Therefore, stress-induced alterations in prefrontal cortical dendritic morphology are likely due to imbalances in glutamate, monoamines, and stress hormone signalling.

The majority of studies investigating the effect of stress on dendritic morphology do not specify the hemispheric location of the neurons. Perez-Cruz et al. (2007) were the first to study the effect of stress on prefrontal cortical dendritic morphology in a hemisphere-specific manner. They found that chronic stress selectively reduced the length and branching of right prefrontal cortical pyramidal neurons. In continuation with this hemisphere-specific line of research, we have found that dendritic location of the chronic stress-induced dendritic remodelling of prefrontal cortical callosal neurons was different between hemispheres: dendritic atrophy occurred in both proximal and distal apical dendrites in the right PFC, while in the left PFC only distal dendrites were affected. Thus, stress-induced remodelling was more widespread throughout the dendritic arbour of right prefrontal cortical callosal neurons. Dopamine has been hypothesized to be involved in the stress-induced dendritic remodelling prefrontal cortical neurons. When exposed to prolonged, inescapable, and severe stressors, such as restraint stress, dopamine levels are higher in the right PFC compared to the left (Carlson et al., 1991). In this study, rats were subjected to 6 hours of daily restraint stress for 21 consecutive days. During this restraint stress regimen dopaminergic transmission was likely elevated in the right PFC. Therefore, the more extensive dendritic remodelling that occurred in the right PFC could be

caused by consistent high dopamine levels during the restraint stress regimen. Taken together, this study links existing evidence of asymmetric dopamine transmission during stress to hemisphere-specific alterations in prefrontal cortical dendritic morphology.

3. Experiment 2:

The effect of chronic stress on glutamate transmission in the PFC

3.1. Rationale

The prefrontal cortical glutamate stress response primarily reflects the activation of callosal neurons (Lupinsky et al., 2010). In Experiment 1, we showed that callosal neurons undergo stress-induced dendritic remodelling in a hemisphere-specific manner. In the right PFC, the stress-induced atrophy was widespread over the entire dendritic arbour, while in the left PFC only distal dendrites were affected. Chronic stress can alter prefrontal cortical function which is presumably mediated by glutamate-containing pyramidal neurons. Therefore, stress-induced morphological changes to callosal neurons could impact their function. If so, such alterations might be reflected in altered glutamate responses to an acute stressor. Moreover, these changes in the glutamate stress response might be expected to differ in the left versus the right PFC.

In this second experiment, our objective was to determine if chronic stress affected the prefrontal cortical glutamate stress response to an acute stressor. To achieve this objective, rats were subjected to either a chronic stress or non-stress regimen. Rats were then surgically cannulated in either their left or right mPFC. After recovery, *in vivo* microdialysis was performed to measure basal concentrations of glutamate as well as the glutamate stress response to an acute tail pinch stressor.

3.2. Materials and Methods

3.2.1. Animals and chronic stress regimen

Adult male Long-Evans rats (Charles River) were also used in Experiment 2. See Experiment 1 for housing conditions. The same chronic restraint stress regimen was employed as described in Experiment 1.

3.2.2. Cannulation surgery

After completion of the chronic stress or non-stress regimens, animals were anesthetized with isoflurane by inhalation. A 22 gauge stainless steel guide cannula (Plastics One, Roanoke, VA) was stereotaxically implanted into either the left or right mPFC at a 3˚ angle off the AP plane at the following flat skull coordinates (Paxinos and Watson, 1996): AP 3.2 mm anterior to Bregma, ML ±0.7 mm from midline, DV 2.2 mm below the brain surface. Later in the experiment, this cannula would be used to insert the microdialysis probe into the mPFC. The cannula was secured using acrylic dental cement covering three screws threaded into the skull. An obturator extending 3.0 mm past the tip of the guide cannula was inserted to prevent infection, blockage, and cerebrospinal fluid (CSF) seepage. The incision was treated with antibiotic ointment (BNP Ointment; bacitracin zinc: 400 IU, neomycin sulfate: 5 mg, polymyxin B sulfate: 5000 IU) to prevent infection. Carprofen (5 mg/kg, s.c.) was injected subcutaneously as a postoperative analgesic. Animals were allowed to recover for 2-3 days before testing.

3.2.3. Microdialysis probes

I-shaped microdialysis probes of side-by-side fused silica inlet-outlet lines [internal diameter (ID): 50 μm] that were encased in polyethylene tubing (ID: 0.58-0.38 mm) were used in

this experiment. A regenerated, hollow, cellulose membrane (Spectrum, molecular weight cut-off: 1 kDa, OD: 216 μm; ID: 200 μm) was glued to the end of a 26 gauge stainless steel cannula using cyanoacrylate adhesive. The tip of the membrane was sealed with epoxy. The active cellulose membrane measured 2.5 mm. The probes were fitted with a stainless steel collar that provided a secure threaded connection to the animals' indwelling guide cannula. The probe assembly was attached to a stainless steel spring that was connected to a liquid swivel (CMA Microdialysis, Kista, Sweden). Computer-controlled microinfusion pumps (CMA Microdialysis, Kista, Sweden) were used to pump perfusate through the probes. Finally, dialysate was collected from the fused silica outlet line (dead-volume: 0.79 μl).

3.2.4. Testing procedures

Animals were tested in circular opaque compartment with a 30 cm diameter. The compartment contained 3 cm of bedding and the animals had *ad libitum* access to food and water. Before the test day, animals were habituated to the chamber for 4 hours. On the day of testing, a microdialysis probe was inserted into the animal's implanted guide cannula and perfused with sterile, artificial cerebrospinal fluid (aCSF) (26 mM $NaHCO_3$, 3mM NaH_2PO_4, 1.3 mM $MgCl_2$, 2.3 mM $CaCl_2$, 3.0 mM KCl, 126 mM NaCl, 0.2 mM L-ascorbic acid) at an initial rate of 0.5 μl/min increasing to a final rate of 1.5 μl/min during the 3 hour stabilization period. Dialysate samples were collected once the perfusion rate of aCSF reached 1.5 μl/min but were discarded; this was done to habituate the animals to the dialysate collection process and the presence of the experimenter. Samples were collected at 15 minute intervals for 60 minutes before, during, and for 30 minutes after a 30 minute exposure to tail-pinch stress. The tail pinch stressor consisted of securing a plastic clothes pin 2 cm from the base of the animal's tail. Typically, animals exhibited vocalization, defecation, and freezing behaviour during the entire

30 minute episode of tail-pinch stress. Furthermore, most animals also gnawed on the plastic clip. In our previous studies, this mild stressor reliably stimulated robust increases in extracellular PFC dopamine levels (Brake et al., 2000; Stevenson et al., 2003). The 20 µl dialysate samples were collected in a fraction vial that was preloaded with 1 µl of 0.25 M perchloric acid to prevent analyte degradation and then immediately stored at -20˚C awaiting analysis.

3.2.5. HPLC

Glutamate levels were determined by precolumn derivatization using high pressure liquid chromatography (HPLC) with fluorescence detection. The chromatographic system was composed of a pump (ESA Inc., Sunnyvale, CA, model 582) and an injector (ESA Inc., Sunnyvale, CA, model 542) connected to an Xterra MS C18 3.0 X 50 mm 5 µm analytical column (Waters Corp., Milford, MA). The mobile phase consisted of 3.5% acetonitrile, 15% methanol, and 100 mM sodium phosphate dibasic (Na_2HPO_4) and was adjusted to a pH of 6.7 with 85% phosphoric acid. The flow rate was set at 0.5 ml/min, and the photodetector (UltiMate 300 Fluorescence Detector, Dionex, Sunnyvale, CA) was set to an excitation frequency of 350 nm and to an emission frequency of 420 nm.

Working standards (100 ng/ml) and derivatization reagents were prepared fresh daily from stock solutions and loaded with the dialysate samples into a refrigerated (10˚C) autosampler (ESA Inc., Sunnyvale, CA, model 542). Before each fraction was injected onto the analytical column, they were sequentially mixed with 20 µl of o-phthaldehyde (0.0143 mol/L) diluted with 0.1 M sodium tetraborate and 20 µl of 3-mercaptopropionic acid (0.071 mol/L) diluted with H2O and left to react for 5 minutes. After each injection, the injection loop was

flushed with 20% methanol to prevent the contamination of subsequent samples. Under these conditions, the retention time for glutamate was ~1.06 minutes. The total run time was 24 minutes per sample. Chromatographic peak analysis was performed by identifying unknown peaks in a sample according to retention times from known standards using ESA's CoulArray software (Version 3.10).

3.2.6. Histology: Probe placement

Animals were anesthetized with isoflurane by inhalation and immediately decapitated. The brains were extracted and frozen by submersion in 2-methylbutane for 5 seconds. The brains were then stored at -80˚C before being sliced. Microdialysis probe tip placements were confirmed from 35 μm cresyl violet-stained coronal sections.

3.2.7. Data format and statistical analysis

The microdialysis data are expressed as the mean (±SEM) percentage change in glutamate levels relative to the combined averages of the four baseline sampled collected immediately prior to tail-pinch stress. The data were not corrected for the probe's *in vitro* recovery.

Outlier values with z-scores surpassing 2 standard deviations were excluded from analysis. Missing or outlier values were estimated using the Cochran and Cox (1957) approximation. The degrees of freedom were corrected in statistical analyses that included these estimated values. The effects of the chronic stress treatment were tested for statistical significance using an unpaired t-test and repeated measures ANOVA using a two-factorial design with time as a within-group factor and treatment and hemisphere as between-group factors. *Post*

hoc comparisons were made using a Bonferroni correction, Tukey's honestly significant difference (HSD) test, and a simple effects analysis ($\alpha = 0.05$).

3.3. Results

3.3.1. Probe placement

A total of 34 animals with histologically confirmed mPFC cannula and microdialysis probe placements were included in the study (control right PFC: n = 9; control left PFC: n = 8; stressed right PFC: n = 9; stressed left PFC: n = 8). In all animals included in the analysis, the active (2.5 mm) portion of the microdialysis probes spanned the ventral-dorsal range of the IL and PL PFC.

3.3.2. Basal PFC glutamate levels

Figure 18 displays the effect of chronic stress on the basal (pre-stress) levels of glutamate in the left and the right PFC of control and chronically stressed rats. This analysis was performed by comparing the first four pre-stress dialysate samples from each rat across groups. In control animals, there was no significant difference in basal PFC glutamate levels between the right and the left hemispheres ($t_{(70)} = 1.281$, $p = 0.2046$). Similarly, there was no difference in basal glutamate levels in the right (n = 9) versus the left (n = 8) PFC of chronically stressed rats (Figure 18; $t_{(66)} = 0.315$, $p = 0.7537$). In contrast, chronic stress significantly reduced basal glutamate levels by 36 and 41 % in the left and the right PFC, respectively (right PFC: $t_{(70)} = 3.201$, $p = 0.0021$; left PFC: $t_{(62)} = 2.785$, $p = 0.0071$). Thus, there was no difference in basal concentrations of glutamate between hemispheres in both control and chronically stressed animals; however, chronic stress reduced pre-stress prefrontal cortical glutamate levels bilaterally.

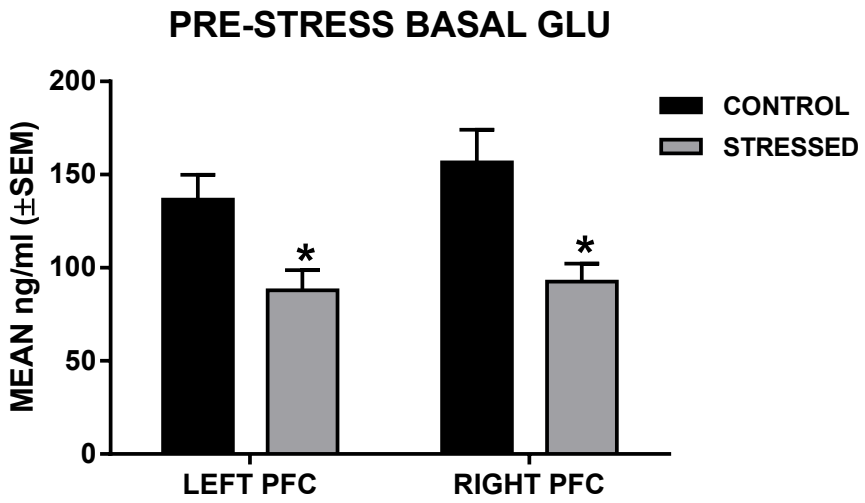

Figure 18. Basal (pre-stress) GLU levels in the left and the right PFC of control and chronically stressed rats. There are no significant hemispheric differences in basal GLU levels in stressed or control animals. Chronic restraint stressed reduced basal GLU levels in both the left and the right PFC. *$p < 0.01$.

3.3.3. PFC glutamate stress responses in control animals

Figure 19 A is a comparison of the tail pinch stress-induced increases in glutamate concentration measured in left and right PFC of unstressed animals. The data presented in this figure are the averaged percentage changes in glutamate concentration measured in the right and left PFC of unstressed control animals. There was a significant hemispheric difference in the timing of the stress-induced increases in PFC glutamate levels (two-way repeated measures ANOVA, hemisphere x time interaction, $F_{(7, 100)} = 2.35$, $p = 0.0293$). Glutamate levels in both the left and the right PFC increased significantly from pre-stress basal levels during the 30 minutes tail-pinch stress (two-way repeated measures ANOVA, effect of time, $F_{(7, 100)} = 3.73$, $p = 0.0012$). However, glutamate levels in the right PFC increased by the 15 minute time point, while glutamate levels in the left PFC only increased by the 30 minute time point. Glutamate levels at the 15 minute time point were significantly more elevated in the right compared to the left PFC (Simple effects *post hoc* test, $p = 0.0002$). These results indicate that there is an inherent hemispheric asymmetry in the timing of the prefrontal cortical glutamate stress response.

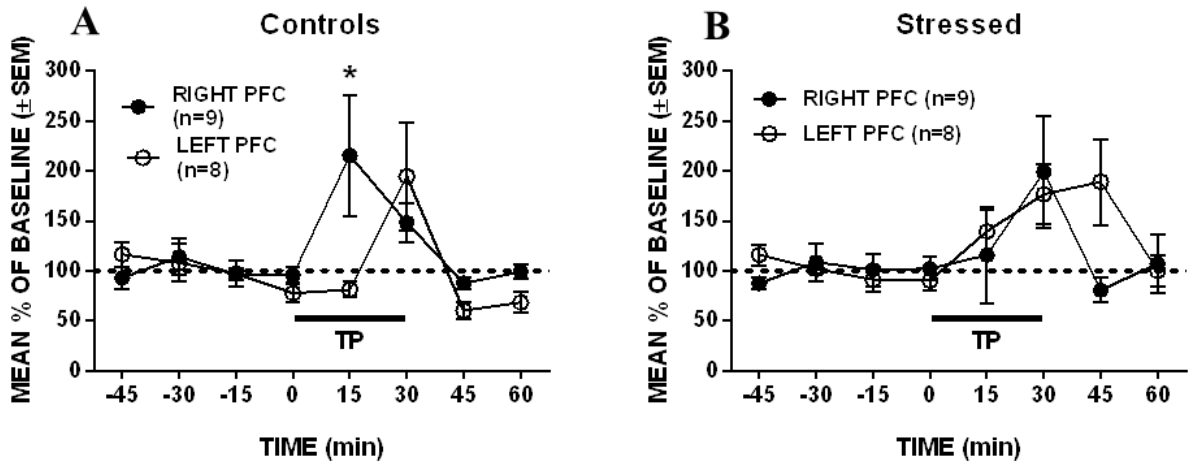

Figure 19. Comparison of stress-induced increases in dialysate GLU levels in the right versus the left PFC of control (A) and chronically stressed (B) rats. A) There was a hemispheric difference in the timing of the GLU stress response of unstressed control rats. Tail pinch (TP) stress elicited an increase in GLU transmission in the right PFC before it did in the left PFC. B) GLU levels significantly increased above baseline pre-stress values in both the right and the left PFC of stressed rats. Chronic restraint stress eliminated the hemispheric difference in the timing of the prefrontal cortical GLU stress response.*$p < 0.05$ versus time-matched sample in left PFC.

3.3.4. PFC glutamate stress responses in chronically stressed animals

Figure 19 B is a comparison of the tail pinch stress-induced increases in glutamate concentration measured in left and right PFC of chronically stressed animals. Tail pinch stress resulted in an increase in both the left and the right PFC of stressed animals (two-way repeated measures ANOVA, effect of time, $F_{(7, 103)} = 2.76$, $p = 0.0113$). There was no difference in the timing of the PFC glutamate stress response between hemispheres (two-way repeated measures ANOVA, effect of hemisphere, $F_{(1, 13)} = 0.64$, $p = 0.4394$). Thus, chronic restraint stress eliminated the inherent lateralization in the timing of the PFC glutamate stress response measured in control animals.

Figure 20 A shows the glutamate stress response in the left PFC of chronically stressed and control rats. Glutamate concentrations in the left PFC increased in response to tail pinch stress in both chronically stressed and control animals (two-way repeated measures ANOVA, effect of time, $F_{(7, 94)} = 4.18$, $p = 0.0005$). However, the glutamate stress response in chronically stressed rats was significantly prolonged compared to control rats (two-way repeated measures ANOVA, stress x time interaction, $F_{(7, 103)} = 2.29$, $p = 0.0333$). At the 45 minute time point, after the 30 minute tail pinch stress episode was terminated, glutamate levels in the left PFC of chronically stressed rats were still significantly elevated (Simple effects *post hoc* test, $p < 0.0001$). Thus, chronic restraint stress extended the left PFC glutamate response to the heterotypical tail pinch stressor.

Figure 20 B displays the glutamate stress response in the right PFC of chronically stressed and control rats. In both stressed and control rats, glutamate concentrations in the right PFC increased in response to tail pinch stress (two-way repeated measures ANOVA, effect of

time, $F_{(7, 109)} = 3.04$, $p = 0.0059$). In contrast to the left PFC, however, chronic stress did not significantly alter the glutamate stress response in stressed rats compared to controls (two-way repeated measures ANOVA, effect of hemisphere, $F_{(1, 13)} = 0.15$, $p = 0.7003$). There did appear to be an inverse trend compared to the left PFC, with chronic stress delaying the right PFC glutamate stress response. However, this trend did not meet statistical significance. In all, the effect of chronic stress on the glutamate stress response is lateralized: chronic stress prolonged the glutamate stress response in the left PFC but had no effect in the right hemisphere.

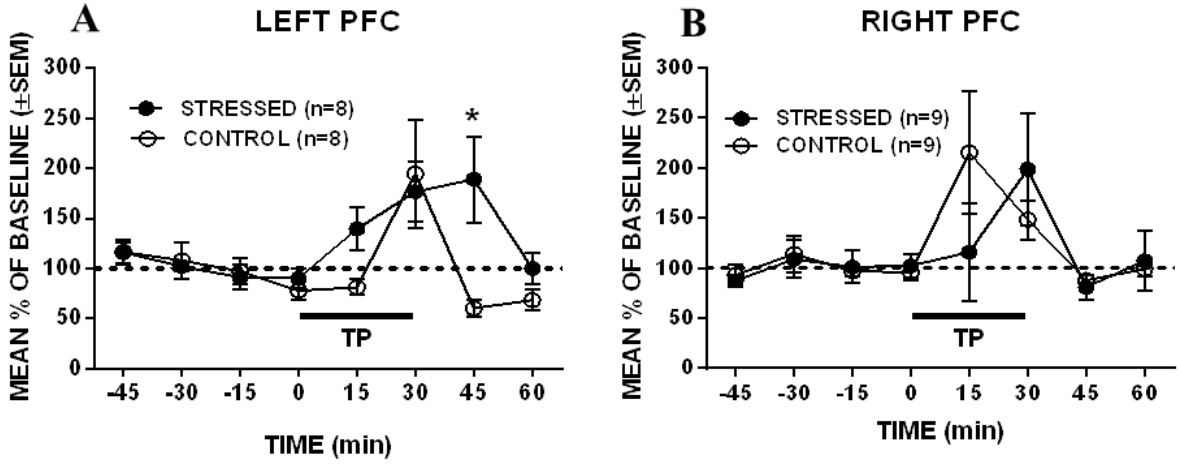

Figure 20. GLU stress response in the left (A) and the right (B) PFC of chronically stressed and control rats. A) Tail pinch (TP) stress significantly increased GLU levels in the left PFC of both chronically stressed and control animals. Chronically stressed animals had a prolonged left prefrontal cortical GLU stress response compared to controls. B) TP stress significantly increased GLU levels in the right PFC of both chronically stressed and control animals. Chronic stress did not significantly alter the GLU stress response in the right PFC.*$p < 0.0001$ versus time-matched sample in control group.

3.4. Discussion

3.4.1. Lateralization of the glutamate stress response in unstressed rats

During exposure to stressors, the hemispheres must communicate in order to mount an appropriate stress response. We have hypothesized that the prefrontal cortical glutamate stress response plays a role in exchanging stress-relevant information between the hemispheres. Glutamate levels increase in both the right and the left PFC in response to acute stressors (Moghaddam, 1993; Lupinsky et al., 2010). We reported an inherent asymmetry in the timing of the glutamate stress response: glutamate levels increased in the right before the left PFC in response to tail-pinch stress. Our results suggest that glutamatergic transmission behaves in a similar but inverse manner to dopamine. Dopamine transmission in the PFC shifts from an initial left hemisphere bias to a right hemisphere bias in response to more intense or prolonged stressors (Carlson et al., 1991). Moreover, dopamine exerts an indirect D1 receptor-mediated inhibitory influence on callosal neurons (Lupinsky et al., 2010). Thus, when exposed to a moderate uncontrollable stressor, such as tail-pinch, dopaminergic transmission could increase to prevent the over-excitation of glutamate-containing callosal neurons. This hypothesis is in line with our results, since the glutamate stress response reflects the activity of callosal neurons whose cell bodies are located in the opposite hemisphere. In the first 15 minutes of tail pinch stress, glutamate levels increase in the right but not the left PFC. Concurrently, in response to physical stressors, dopamine transmission initially increases in the left PFC, where it would exert a D1-mediated indirect inhibitory influence on callosal neurons projecting to the opposite hemisphere. After 30 minutes, the stressor was likely perceived as being prolonged and uncontrollable. At this time period, we measured an increase in glutamate transmission in the left PFC. It is possible that the increase in dopamine levels in the right PFC in response to prolonged and uncontrollable

physical stressors occurs in part to prevent the over-excitation of callosal neurons. These results indicate that there were hemispheric biases in prefrontal cortical signalling during stress. Furthermore, this asymmetry in the timing of the glutamate stress response could underlie how the hemispheres communicate while mediating stress responses.

Lupinsky et al. (2010) performed an *in vivo* microdialysis experiment similar to the present study and did not report a hemispheric asymmetry in the timing of the glutamate stress response. Differences in the experimental protocols employed in the two studies could explain these contrasting findings. In the study by Lupinsky et al. rats were not exposed to any experimental condition before surgery, while in our study control animals were handled and weighed daily for 21 consecutive days. Lupinsky et al. also housed these rats in a reverse 12 hour light/dark cycle (lights off at 12:00 PM), whereas in our study rats were on a normal 12 hour light/dark cycle (lights on at 10:00 AM). Lupinsky et al. collected microdialysis samples at 10 minute intervals with a flow rate of 2 μl/min, while we collected samples at 15 minute intervals with a flow rate of 1.5 μl/min. Moreover, we used a 30 minute period of tail pinch to elicit a glutamate stress response, whereas Lupinsky et al. subjected animals to 20 minutes of tail-pinch stress. Finally, Lupinsky et al. measure glutamate levels by HPLC using electrochemical detection. In contrast, we used HPLC with fluorescence detection to quantify glutamate levels, which is a more sensitive method of detection. Thus, we propose that the dissimilar results obtained in the two studies are due to differences in the experimental protocols employed.

3.4.2. Effect of chronic stress on glutamate transmission in the PFC

Basal levels of glutamate were reduced in both the right and the left PFC of chronically stressed animals. Chronic stress is known to cause basal levels of monoamine neurotransmitter to

decline in the PFC: concentrations of dopamine and serotonin are reduced in chronically stressed rats (Mizoguchi et al., 2000; Mangiavacchi et al., 2001). These findings suggest that chronic stress causes a state of hypotransmission of glutamate and monoamines in the PFC. This decrease in neurotransmission could be due to a stress-induced augmentation of inhibitory mechanisms. During stress, glutamate transmission is elevated in the PFC (Moghaddam, 1993; Lupinsky et al., 2010). It is possible that during the chronic stress regimen, homeostatic inhibitory mechanisms were employed to prevent negative effects of this stress-induced increase in glutamate release such as excitotoxicity. If these inhibitory mechanisms were still in place after chronic stress, this could explain why glutamate levels were still reduced. A possible mechanism for this inhibitory homeostatic activity is GABAergic release. Therefore, future experiments should measure basal GABA levels in chronically stressed animals.

Chronic stress abolishes the inter-hemispheric difference in the timing of the prefrontal cortical glutamate stress response. Lateralization appears to be important for normal prefrontal cortical function. Right hemisphere lesions of the IL and PL cortices reduce stress-induced HPA-axis activation and stomach ulcer formation (Sullivan & Gratton, 1999). Furthermore, these right sided lesions cause a decreased level of anxiety during behavioural testing (Sullivan & Gratton, 2002). These studies suggest that deficits in right PFC activity result in the disregulation of the neuroendocrine, autonomic, and behavioural responses to stress. Therefore, it is reasonable to assume that a reduction in the lateralization of the glutamate stress response could impact the stress response. Indeed, chronically stressed animals show hyperactivation of the HPA axis and long-lasting enhancement in anxiety-like behaviour (Marin et al., 2007; Vyas et al., 2004). In addition to these abnormalities in stress responsivity, chronically stressed animals also have cognitive deficits in attentional set-shifting, spatial working memory, and extinction retrieval

(Liston et al., 2006; Hains et al., 2009; Wilber et al., 2011). Lateralization is important for optimal PFC function; therefore, the stress-induced elimination in the asymmetry of the glutamate stress response could contribute to the deficits in prefrontal activity observed in chronically stressed animals.

In humans, lateralization also appears to be vital for normal prefrontal cortical performance. In older adults, decline in cognitive performance is linked to a reduction in lateralization of fronto-cortical activity (Dolcos et al., 2002). Imbalances in left versus right PFC activity have also been implicated in the psychopathology of depression and anxiety disorders, mental illnesses which are strongly associated with impaired stress responsivity. Damage of the left frontal lobe and a reduction in activity in the left PFC are found in individuals with depression (Gainotti, 1972; Schaffer et al., 1983). In addition, depressed patients display reduced volume and metabolic activity in the left PFC (Botteron et al., 2002; Martinot et al., 1990). Anxiety disorders are generally associated with right PFC hyperactivation: patients with generalized anxiety disorder display enhanced right PFC activity when presented with threatening stimuli (Monk et al., 2008). Therefore, depressive disorders generally exhibit left prefrontal cortical hypofunction, while anxiety disorders demonstrate right prefrontal cortical hyperfunction. Chronic stress is a major risk factor for neuropsychiatric disorders, including both depression and anxiety disorders (McEwen, 2004). In this study, I report a reduction in the lateralization of the prefrontal cortical glutamate stress response in chronically stressed animals. It is possible that similar stress-induced reductions in asymmetric PFC activity occur in humans and play a role in the pathophysiology of mental disorders.

4. General Discussion

4.1. Overview

This study is the first report of both circuit- and hemisphere-specific effects of stress on prefrontal cortical morphology and function. Our first experiment investigated the effect of 21 days of 6 hour daily restraint stress on the morphology of retrogradely-labelled callosal neurons. Callosal neurons are prefrontal cortical pyramidal neurons that project to the opposite hemisphere (Ferino et al., 1987) and are responsible for the majority of the contralateral prefrontal cortical glutamate stress response (Lupinsky et al., 2010), suggesting that they are involved in the interhemispheric exchange of stress-relevant information. We found that the apical dendrites of callosal neurons underwent regressive structural changes. Furthermore, these alterations were more widespread throughout the apical dendritic arbour of right PFC callosal neurons. In our second study, *in vivo* microdialysis was utilized to compare the left versus right PFC glutamate stress response in chronically stressed and control animals. The stress-induced alterations to the prefrontal cortical glutamate stress response was also hemisphere-specific: the glutamate stress response was prolonged in the left PFC while there was no change in the response in the right PFC. Since the glutamate stress response is mainly due to the activity of glutamatergic callosal neurons projecting from the opposite hemisphere (Lupinsky et al., 2010), the widespread regressive changes in dendritic morphology of right PFC callosal neurons could underlie the enhanced glutamate stress response in the left PFC. Together, these experiments tie apical dendritic atrophy to altered glutamatergic transmission in the PFC, defining a mechanism by which chronic stress-induced regressive structural changes in callosal neurons alter the interhemispheric exchange of stress-relevant information.

4.2. Chronic stress affects both the function and morphology of the rat PFC in a hemisphere-dependent manner

We report both a retraction in dendritic material and augmentation in activation in PFC callosal neurons. To explain these results, we postulate that there was a stress-induced reduction of inhibitory GABAergic inputs to right PFC callosal neurons. Left PFC callosal neurons only showed stress-induced retraction of distal apical dendrites and there was no change in the glutamate stress response in the right hemisphere. Thus, this loss of inhibitory synaptic inputs in the right PFC was likely localized to the proximal dendritic arbour. Indeed, GABAergic inhibition of hippocampal pyramidal neurons occurs proximally to the cell body (Soltesz et al., 1995). Lupinsky et al. (2010) demonstrated that dopamine exerts a D1-mediated inhibitory influence on the activity of callosal neurons. GABAergic neurons are thought to express D1 receptors; therefore, when dopamine is released, these neurons are activated and release inhibitory GABA on callosal neurons. Dopamine transmission is elevated in the right compared to the left PFC during chronic restraint stress (Carlson et al., 1991). Under these conditions, GABAergic interneurons expressing D1 receptors are activated. During the 21 days of restraint stress, it is possible that a portion of the GABAergic inhibition of callosal neurons was eliminated to maintain homeostasis, resulting in a loss of proximal inhibitory synapses between GABAergic interneurons and right PFC callosal neurons. It follows that when animals are no longer stressed, dopamine levels in the right PFC would return to basal levels. This would leave callosal neurons in a state of reduced inhibition, which could explain the enhancement in the duration of the left PFC glutamate stress response.

4.3. Chronic stress and PFC function: Clinical significance

An increasing body of evidence from studies in rats demonstrates that the PFC not only plays a critical role in coordinating the behavioural and systemic responses to stress, but that neurons in the rodent PFC are very susceptible to stress and undergo dendritic remodelling following stress exposure. These findings corroborate the idea that stress-induced alterations in PFC function significantly contribute to the neural insult underlying the deficits in prefrontal cortical function found in chronically stressed rodents, and the cognitive aspect of many neuropsychiatric diseases. Risk for several neuropsychiatric diseases is strongly linked to psychological stress. In post-traumatic stress disorder (PTSD), stressful life events that occur after the initial traumatic episode exacerbate PTSD symptomatology (King et al., 1998). Early parental loss, an environmental stressor, significantly increases the likelihood of developing major depression, bipolar disorder, and schizophrenia during adult life (Agid et al., 1999). Ventura et al. (1989) found that stressful life events augmented the risk of psychotic relapse and exacerbated symptoms of schizophrenia. Finally, stress is a risk factor for the development of drug addiction and strongly enhances the vulnerability to drug relapse (Sinha, 2008). Intuitively, the psychiatric diseases most notably associated with impaired coping ability, enhanced sensitivity to stress and deficits in regulating stress are the anxiety and depressive disorders. Significant alterations in HPA function are present in both classes of disorders. In patients with panic disorder, salivary cortisol levels are significantly higher than control subjects (Bandelow et al., 2000). In contrast, no difference in salivary cortisol levels is found between patients with social phobia and control subjects (Uhde et al., 1994). In many but not all studies, PTSD patients have low cortisol levels and enhanced cortisol suppression in response to dexamethasone (Yehuda, 2006). Depressive disorders are associated with increased CRH activity and the

continuous elevation of cortisol (Wong et al., 2000). Furthermore, patients with depression have deficits in the negative feedback regulation of the HPA axis, as reflected by the failure of dexamethasone to suppress cortisol levels (Rybakowski & Twardowska, 1999). Despite the obvious differences in the specific symptoms of these two classes of disorders, the similarities in stress sensitivity and compromised coping ability suggest that, generally these disorders should exhibit a pathological imbalance favouring activity in the right PFC.

Imbalances in right versus left PFC activity have been implicated in the psychopathology of psychiatric disorders. This is of particular importance to our research, since chronic restraint stress abolished the inherent asymmetry in the timing of the glutamate stress response. Given this finding, it can be conceptualized that the deficits in PFC-mediated functions are due to reductions in the lateralization of PFC neural activity. Indeed, in older adults, decline in cognitive performance is linked to a reduction in lateralization of fronto-cortical activity (Dolcos et al., 2002). Pathological imbalances in right versus left prefrontal activity are also present in several mental disorders such as depressive and anxiety disorders, as well as attention-deficit/hyperactivity disorder and drug addiction. Depressive disorders have predominantly left-sided PFC hypofunction, while anxiety disorders show right-sided PFC hyperfunction. Depressed conditions are associated with left frontal brain damage and reduced EEG activity in the left PFC (Gainotti, 1972; Schaffer et al., 1983). Furthermore, reductions in volume and metabolic activity are present in the left ventromedial PFC of depressed individuals (Botteron et al., 2002; Martinot et al., 1990). Depending on the frequency employed, transcranial magnetic stimulation (TMS) can stimulate (high frequencies) or suppress (low frequencies) neural activity. Antidepressant effects have been reported from rapid rate TMS over the left PFC or from low frequency TMS over the right PFC (George et al., 1997; Klein et al., 1999); that is enhancing

neural activity in the left PFC or suppressing right PFC function is beneficial in treating depression. Interestingly, ameliorations in depression rating scores following TMS or beneficial antidepressant treatments are correlated with normalization of the dexamethasone suppression test, indicating that proper HPA feedback regulation mechanisms had been reinstated (Pridmore, 1999). Right PFC hyperactivity is reported in patients with anxiety disorders. When youth with generalized anxiety disorder (GAD) were presented with threatening stimuli, functional magnetic resonance imaging (fMRI) showed hyperactivation of the right PFC and amygdala (Monk et al., 2008). Furthermore, when low frequency TMS was applied to the right dorsolateral PFC, EEG activity in the left PFC increased and patients reported decreased anxiety levels (Schutter et al., 2011). These findings in patients with psychiatric disorders imply that both left and right PFC play distinct, yet complementary, roles in the maintenance of optimal executive function. Most of the evidence for prefrontal cortical lateralization focuses on the role of the right PFC, since it has more of a straightforward role in regulating stress responses. However, it is important to remember that stressors initially activate the left PFC and that activity in the right PFC only increases when stressors are prolonged or perceived as uncontrollable (Carlson et al., 1991; Carlson et al., 1993). This initial left PFC activation has been hypothesized to prevent mild stressors from becoming major stressors (Sullivan & Szechtman, 1995). This concept is especially applicable for the lateralization deficits seen in patients with depressive and anxiety disorders, where mildly stressful situations become overwhelming.

4.4. Final remarks and future directions

In the present study, we determined that chronic stress altered the function and dendritic morphology of prefrontal cortical callosal neurons in a hemisphere-specific manner. Although this work has furthered our understanding of how stress affects the prefrontal cortical callosal

neural circuit, more research is needed to fully understand the mechanisms underlying these changes. The two future experiments we propose can be realized using the dataset of LY-filled neurons collected in this study. They are possible because when LY-filled cells are immunocytochemically stained with DAB, they can be stored on slides and viewed with brightfield microscopy for an indefinite period of time. The first forthcoming study should determine if there are hemispheric asymmetries in basilar dendritic morphology of callosal neurons of unstressed control rats. The effect of chronic stress on basilar dendritic morphology should also be investigated. Although most studies have found no stress-induced change in basilar dendritic morphology (Cook & Wellman, 2004; Perez-Cruz et al., 2007; Radley et al., 2006; but see Perez-Cruz et al., 2009), we know that the effect of chronic stress is neural circuit-specific (Shanksy et al., 2009; Radley et al., 2013); therefore, it would still be worthwhile to examine the basilar dendrites of callosal neurons. The second future experiment should study the density and type of spines on the dendrites of callosal neurons. This experiment would determine if spine density and type are inherently lateralized in the PFC of unstressed animals. Furthermore, the effect of stress on spine density and type of callosal neurons should be investigated. Studying spines gives insight to the hemispheric and stress-induced changes in synaptic inputs to callosal neurons. These proposed experiments could be completed with the brightfield microscope and Neurolucida program which were utilized in this present study.

In addition to examining the dendrites and spines of PFC callosal neurons, we propose the characterization of the synaptic appositions on the cell bodies and dendrites of these cells. Allard et al. (2012) used LY intracellular labelling in conjunction with immunohistochemistry techniques to characterize the glutamatergic and GABAergic appositions on the cell bodies of pyramidal neurons. Using similar methodology as Allard et al., this experiment would give

insight into how the pre-synaptic inputs to callosal neurons are altered between hemispheres and by chronic stress. Retrogradely labelled callosal neurons would be iontophoretically loaded with LY dye. Then, sections would be incubated with fluorescent antibodies for either VGluT1 or GAD65 which label glutamatergic and GABAergic presynaptic appositions, respectively. Using fluorescent confocal microscopy, presynaptic appositions would be quantified on the dendrites and cell bodies of LY-filled callosal neurons. This experiment would determine how glutamatergic and GABAergic inputs are altered by chronic stress, which could allow us to better understand the glutamate stress response data. Finally, I propose using an anterograde tracer to label the axonal terminals of callosal neurons. I would inject an anterograde tracer into the right or the left PFC and then quantify the number of labelled axon terminals in the opposite hemisphere of chronically stressed and unstressed controls. This study would reveal if chronic stress alters the axonal projections of PFC callosal neurons as well as their dendritic morphology. Together, these future experiments would depict a more complete mechanism of the effect of chronic stress on callosal neuron function and morphology.

Stressful life events are major risk factors for numerous neuropsychiatric diseases (McEwen, 2004). Moreover, many of these diseases are also associated with abnormal prefrontal cortical structure and function (Holmes & Wellman, 2009). In the case of prefrontal cortical dysfunction and risk for mental illness, it is next to impossible to single-out the causative effect of stress from other factors, such as genetic predisposition. However, our research, as well as many other studies, demonstrates that the PFC is a main target of chronic stress, which gives weight to the idea that stress-induced prefrontal cortical dysfunction is involved in the pathophysiology of these diseases. Future studies investigating the mechanisms by which stress affects the PFC will ameliorate our understanding of neuropsychiatric diseases. Furthermore,

these advances could lead to novel therapeutic options which have the potential to prevent stress-induced prefrontal cortical dysfunction and treat existing conditions.

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