Spring 2018

A comprehensive analysis of prefrontal structural and functional changes following prolonged stress and glucocorticoid exposure in the rat

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A COMPREHENSIVE ANALYSIS OF PREFRONTAL STRUCTURAL AND FUNCTIONAL CHANGES FOLLOWING PROLONGED STRESS AND GLUCOCORTICOID EXPOSURE IN THE RAT

by

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A thesis submitted in partial fulfillment
of the requirements for the Doctor of Philosophy
degree in Psychology in the
Graduate College of
The University of Iowa

May 2018

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CERTIFICATE OF APPROVAL

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PH.D. THESIS

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To my husband and my family for their unwavering support.

ACKNOWLEDGEMENTS

I would like to think my mentor, Dr. Jason Radley, for giving me this opportunity. I have grown so much as a scientist under your mentorship. I am forever changed by the help and support you have given me. Thank you to the members of the Radley lab who became my friends. You make science fun.

Thank you to my committee members for taking time out of their schedules to provide guidance throughout my graduate career. From serving as co-sponsors, to writing endless letters of support or reference, I appreciate all you have done for me and for your contributions to this work.

Thank you to my parents, who from my earliest memories never made me feel that I wasn't capable of anything I wished to be or achieve. And thank you to my two biggest supporters, my husband and my sister. Thank you for allowing me to lean on you when I struggled and for always celebrating with me during my successes. Without you, this would not have been possible.

ABSTRACT

The hypothalamo-pituitary-adrenal (HPA) axis plays a central role in promoting adaptations to acute stress, while over activity of this system may be involved in adverse effects on physiology and behavior. Glucocorticoids, the end-products of HPA axis activation, are key mediators in adaptive and maladaptive effects following acute and chronic stress. Previous research has focused on how chronic stress and elevated glucocorticoid exposure influences hippocampal structure and functioning in the rat. The prefrontal cortex, a brain region important for executive function, is involved in inhibiting the stress response but has also been shown to be affected by repeated stress exposure. The current set of experiments were designed to get a more robust picture of how chronic stress and elevated glucocorticoids impact prefrontal structure and function in the rat.

Chapter 2 investigates how differences in basal functioning on the HPA axis impact prefrontal structure and functioning. Aging has been shown to be accompanied by disruptions in circadian functioning, so aged and young animals were investigated for basal adrenocortical activity. Individual differences were found in both young and aged groups, and animals were partitioned into high or low HPA activity. Aged animals with high glucocorticoid (CORT) secretion showed significant dendritic spine loss in prefrontal neurons, when compared to aged animals with low CORT secretion and young animals. Using a delayed alternation task using a T maze, a prefrontal dependent task, we showed that aged animals with high CORT secretion show significant working memory impairments compared to all groups.

Chapter 3 investigates the role that glucocorticoids play in the restructuring of prefrontal structure following chronic stress. As previous work has shown that chronic stress leads to regressive alterations in dendritic spines in pyramidal neurons in the medial prefrontal cortex (mPFC), this study examined the capacity of sustained increases in circulating CORT alone to alter spine density and morphology in this region. A subset of rats were implanted with subcutaneous CORT pellets to provide continuous exposure to levels approximating the circadian mean or peak for 1, 2, or 3 weeks. Pyramidal neurons in the prelimbic area of mPFC were selected for intracellular dye filling followed by high resolution three-dimensional imaging and analysis of dendritic arborization and spine morphometry. Two or more weeks of elevated CORT exposure resulted in apical dendritic retraction and dendritic spine loss, with thin spine subtypes showing the greatest degree of attrition. Finally, these alterations persisted following a 3-week washout period suggesting prolonged disruptions in HPA activity may be sufficient to induce enduring regressive structural alterations.

As the majority of studies investigating the stress response have focused on male rodents, Chapter 4 investigated sex differences in prefrontal structure and function following chronic stress, and CORT exposure in male and female rats. Adult male and female rats were exposed to two weeks of chronic variable stress (CVS) and then either tested on the delayed alternation task of the T maze or perfused for dendritic spine analyses. Both males and females showed significant impairments on the working memory task following CVS exposure compared to non-stressed animals. CVS also resulted in significant spine loss in mPFC neurons in both males and females. As chapter 2 focused on the effects of sustained CORT exposure on structural

reorganization of mPFC, female animals were implanted with subcutaneous CORT pellets and analyzed for mPFC structural alterations. Females were found to show similar effects as males in that they demonstrate decreased spine density on mPFC pyramidal neurons following 2-weeks of sustained high CORT exposure. Finally, in an attempt to generalize chronic stress effects on PFC, males and females were exposed to a chronic restraint stress (CRS) paradigm and analyzed for dendritic spine changes in PFC. Both males and females exposed to 3 weeks of CRS show significant spine loss compared to non-stressed animals.

These results show that chronic stress and subsequent glucocorticoid exposure significantly alter PFC structure and function in both male and female rats. These data enhance our understanding of how both stress and CORT specifically alter dendritic spine density and morphology and how this may lead to changes in prefrontal function.

PUBLIC ABSTRACT

Chronic stress is known to be a precursor or exacerbate some mental illnesses such as depression and anxiety, yet we know little about how chronic stress influences some brain regions that tend to be impacted by these mental illnesses. We sought to better understand how chronic stress and the stress hormone, glucocorticoids (CORT), impact the prefrontal cortex, an important brain region involved in many executive functions. Using high-resolution imaging techniques to analyze neuronal morphology, and behavioral tasks to assay for working memory, a prefrontal dependent task, we examined how prefrontal structure and function are impacted following chronic stress and CORT exposure in male and female rodents.

Our results show that chronic stress and high CORT exposure leads to regressive neuronal structure, particularly a loss of dendritic spines, important structures for post-synaptic excitatory input in the cortex, in both male and female rodents. Following chronic stress, males and females also show impairments in working memory, suggesting that the regressive plasticity experienced may play an important functional role in the prefrontal cortex.

This research is the first to carefully examine specific changes to prefrontal plasticity following stress and CORT exposure and suggests, at least with regard to the endpoints examined here, males and females are both similarly affected.

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aBST: anterior bed nucleus of the stria terminalis

ACC: anterior cingulate cortex

ACTH: adrenocorticotropin hormone

CORT: glucocorticoid

CRF: corticotropin releasing-factor

CRS: chronic restraint stress

CVS: chronic variable stress

GR: glucocorticoid receptor

HF: hippocampal formation

HPA: hypothalamo-pituitary-adrenal

IL: infralimbic area

MDT: mediodorsal thalamus

mPFC: medial prefrontal cortex

MR: mineralocorticoid receptor

PFC: prefrontal cortex

PL: prelimbic area

PVH: paraventricular nucleus of the hypothalamus

vSUB: ventral subiculum

CHAPTER 1: INTRODUCTION

Stress is commonly defined as “the response of the body to any actual or threatened disturbance to homeostasis”. However, as maintaining homeostasis is critical to an organisms survival, this definition would suggest that much of the brain’s neurocircuitry is involved in the stress response (Day, 2005). A more specific definition allows for a more refined circuit for study and allows for better interpretation of what “stress” is. Hans Selye, defined stress as the “non-specific response of the body to any demand” (Selye, 1936). A re-working of that definition that our laboratory likes is “the body’s multi-system response to any challenge that overwhelms, or is judged likely to overwhelm, selective homeostatic response mechanisms” (Day, 2005). Physiological (actual disturbance to homeostasis, such as a hemorrhage) and psychological (threat to the organism’s current or future state but cause no actual risk to well-being, such as an unfamiliar environment or presence of a predator) stressors both elicit the same physiological response in the body, though through two distinct pathways (Herman and Cullinan, 1997; Herman et al., 2003). The work of this dissertation focuses on the effects of psychological stressors on the prefrontal cortex, a brain region involved in the neurocircuitry of the stress response. In this introduction I will review:

- The hypothalamic-pituitary-adrenal axis response to acute stress
- The adaptation of this response under repeated conditions
- The role of the prefrontal cortex in the stress response

- How the variables of sex and age can modulate, or be modulated, by the stress response

The Hypothalamic-Pituitary-Adrenal axis response to acute stress

The hypothalamic-pituitary-adrenal (HPA) axis is a major neuroendocrine system that is initiated during stress. Upon the perception of stress, various limbic regions (discussed more thoroughly later on in this introduction) converge on the paraventricular nucleus of the hypothalamus (PVH), the final common pathway of the HPA axis. These neurons secrete corticotrophin releasing-factor (CRF) into the portal system which in turn triggers the release of adrenocorticotropin hormone (ACTH) from the anterior pituitary. ACTH is released into the systemic circulation and reaches the adrenal cortex, which finally, secretes glucocorticoids. Glucocorticoids are key regulators in the neuroendocrine response to stress. In rodents, corticosterone (CORT) is the common glucocorticoid; in humans, this is cortisol. These hormones serve anti-inflammatory purposes, as well as stimulate gluconeogenesis in order to provide the energy needed to mount the stressor. Under acute conditions, glucocorticoids mediate the direction of energy to systems that will be vital to overcoming the challenge. This will also, critically, leave other bodily functions, such as those involved in rest and digestion, without proper energy to function in that moment.

HPA activation is vitally important to adapt to adverse situations. During acute situations, HPA activation will be initiated and CORT will be secreted into the

blood stream within minutes and peak around 30 minutes after the onset of the stressor. Once the challenge has been overcome, the HPA axis shuts off relatively quickly. The HPA axis works in a classically negative feedback manner. Glucocorticoids will bind to mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) located throughout the limbic system and in the PVH, inhibiting the HPA axis and shutting down subsequent CORT secretion (Reul and de Kloet, 1985). Importantly, the stress response is not the only function of the HPA axis. Baseline secretion of CORT is highly involved in making sure there are adequate energy stores throughout the day (discussed below).

MR and GR receptors vary in their expression pattern and their affinity to bind CORT (Ahima and Harlan, 1990; Ahima et al., 1991). GR is expressed ubiquitously in neurons throughout the brain while MR is primarily found in the hippocampus, and to a lesser extent the prefrontal cortex and amygdala. MR has a much higher affinity to CORT than GR and so is almost entirely occupied during periods of peak CORT secretion. As such, GRs plays a bigger role in responding to stress levels of CORT secretion.

The HPA axis under repeated stress conditions

HPA axis responses tend to decline after repeated exposure to the same stressor, but may over-respond to a new challenge. These responses represent important adaptations to chronic stress and involve different neural mechanisms.

Habituation

When animals are exposed regularly to the same (homotypic) stressor for several days or weeks, reduction in the response of the HPA axis to that specific stressor is common. Our laboratory and many others have observed reduced CORT and ACTH output following repeated exposure to the same stressor (see Grissom and Bhatnagar, 2009; Herman, 2013; Radley and Sawchenko, 2015). This has also been demonstrated in humans who have been exposed to repeated challenges (Deinzer et al., 1997; Gerra et al., 2001; Schommer et al., 2003). The ability for animals to habituate to repeated stress is highly adaptive as it limits the energy and resources used, and allows those resources to be saved and used for a more life-threatening challenge.

The mechanisms involved in stress habituation are likely mediated by neural circuitry in the limbic system and is at least somewhat mediated by negative feedback. Repeated restraint stress does not seem to down-regulate GR and MR mRNA expression in the hippocampus, allowing for normal function of the negative feedback mechanisms (Girotti et al., 2006) and blocking MR or GR function prevents habituation of the HPA axis to homotypic stressors (Dallman et al., 1987; Cole et al., 2000; Jaferi et al., 2003). Reduction in c-fos mRNA following repeated stress has been observed in many regions, including the PFC and the PVH indicating reduced activity in limbic regions in response to homotypic stressors. (Radley and Sawchenko, 2015). The decreased activity following repeated stress limits the stress levels of CORT secreted into the circulatory system which may in

turn lead to fewer physiological changes (i.e. reduced weight gain, adrenal hypertrophy) and reserved energy stores.

Sensitization

Stress that is chronic, unpredictable, and varied (CVS; heterotypic) is shown to lead to sensitization of the HPA axis in animal models. In animals who have a history of this type of stress exposure, a novel stressor will elicit HPA axis hyperactivity, with a robust CORT response that is slow to decline to baseline (Ottenweller et al., 1989; Herman et al., 1995a; Radley and Sawchenko, 2015). The cumulative effects of this stress paradigm also leads to changes in behavioral, physiological, and endocrine systems that are similar to features of stress-related psychiatric disorders. While these changes appear to be maladaptive, the context in which they develop (i.e. reoccurring, unpredictable challenges) are essential to survival. Particularly relevant to this discussion, increased CORT levels promote the utilization of energy to be able to mount challenges.

Sensitization likely involves down-regulation of MR and GR in key limbic regions involved in the negative feedback regulation of the HPA axis. CVS leads to reduced MR and GR mRNA expression in hippocampus and PVH (Herman et al., 1995a) suggesting reduced ability for negative feedback in those regions. While, down-regulation of receptors involved in negative feedback likely play a large role in HPA axis sensitization following CVS, increased intrinsic excitability of CRH neurons in PVH has also been demonstrated (Franco et al., 2016). Increased activity in brain regions associated with the neural control of stress have also been

observed (Bhatnagar and Dallman, 1998). All of these data together suggest that HPA sensitivity likely results from reduced negative feedback as well as increased activity in HPA axis modulatory neurocircuitry.

People suffering from major depressive disorder exhibit increased basal levels of cortisol as well as reduced dexamethasone suppression, indicating dysregulated negative feedback of the HPA response (Sachar et al., 1973; Carrol, 1982). In humans, the cumulative effects of chronic stress exposure has been linked to many health concerns including hypertension, metabolic syndrome, immunosuppression, neurodegenerative diseases, and psychopathologies. Glucocorticoids, the end-product of the HPA axis, are implicated in many of these of symptoms.

Glucocorticoids

As touched upon briefly, glucocorticoids are the end result of HPA axis activation and are key mediators in many of the effects of stress on the body. However, glucocorticoids play necessary roles in many circadian bodily functions and are therefore secreted in a circadian rhythm throughout the day. Glucocorticoids play an important role in maintaining homeostasis and as such are increased before periods of typical high activity. In humans, a rise of glucocorticoids begins in early hours of the day and increases until about mid-day where it then declines to prepare the body for rest. As rodents are nocturnal, this daily rhythm is reversed with increased levels of glucocorticoids during the evening.

Disruption in basal levels of glucocorticoids has been associated with stress-related mental disorders, particularly in depression. People diagnosed with major depressive disorder will often show erratic rhythms and specifically appear impaired at shutting off the secretion of CORT in the evening, resulting in elevated basal levels throughout the day (Sachar et al., 1973; Carrol, 1982; Wong et al., 2000; Jarcho et al., 2013). Another pathological instance of basal glucocorticoid disruption is Cushing's Syndrome in which elevated CORT levels are a key signature of the disease (see Gabrilove and Krakoff, 1986).

Interestingly, basal CORT rhythms may become more disrupted during the process of aging. Older adults often have higher basal levels of CORT than their younger peers, most likely through disruptions of the circadian secretion (Lupien et al., 1994; Lupien et al., 1998). The role that the disruption of the daily rhythm of the HPA axis has on cognitive health is a key question that will be discussed in chapter 2 of this thesis.

The limbic system's role in the stress response

As being able to successfully mount challenges is critical to an animals' survival, the body recruits multiple systems in response to stress. Autonomic, endocrine, and behavioral responses are all regulated by multiple brain circuits. The response to physiological stressors, such as hemorrhage or hypoxia, are relayed up the brain stem to sensory areas that then directly innervate PVH to initiate the HPA axis. Psychological (or emotional) stressors require integration with cognitive areas of the brain, primarily activation of the limbic forebrain.

Hippocampus

A widely studied limbic region implicated in the stress response is the hippocampal formation. Autoradiographic evidence shows that both glucocorticoid receptors (MR and GR) are widely distributed in this region and many studies have shown that this brain region is capable of inhibiting the HPA axis (Sapolsky et al., 1984; Jacobson and Sapolsky, 1991; Herman and Cullinan, 1997). However, most of the projections from the hippocampal formation are excitatory in nature and there is no direction projection from the hippocampus to the PVH. For the hippocampus to inhibit the HPA axis, this projection is routed through the anterior bed nucleus of the stria terminalis (aBST) or the ventral subiculum (vSUB), among other areas, which in turn sends GABAergic projections to PVH, inhibiting the HPA axis.

High levels of glucocorticoids and repeated stress exposure disrupt hippocampal memory functions and this has been associated with structural and functional changes in plasticity (see McEwen and Magarinos, 1997; McEwen, 1999). The CA3 in particular has shown dendritic retraction and dendritic spine loss following chronic stress exposure. In humans, individuals suffering from stress-related disorders (typically major depressive disorder) show reduced hippocampal volume (Starkman et al., 1992; Sheline et al., 1996). As this brain region is involved in the inhibition of the HPA axis, neuroplastic changes here may play a role in impaired glucocorticoid mediated negative feedback often demonstrated in these individuals as well.

Amygdala

While the hippocampus is shown to play a key role in the inhibition of the HPA axis, the amygdala seems to plays an excitatory role (Galeno et al., 1984; Roozendaal et al., 1991, 1992; Dayas et al., 1999). This opposing role of HPA axis modulation to the hippocampus, also results in differing plasticity in amygdaloid neurons. Vyas and his group have found that after chronic stress, amygdaloid neurons show increased branching and increased synaptic connectivity (Vyas et al., 2002a; Vyas et al., 2002b; Vyas et al., 2006). This is accompanied with increased anxiety behavior following stress exposure and enhanced fear conditioning (Shors et al., 1992; Shors and Mathew, 1998; Davis and Shi, 1999).

Prefrontal cortex

Another brain region implicated in the inhibition of the HPA axis is the prefrontal cortex (PFC). The PFC is also widely connected to other limbic regions (including the hippocampus), allowing for top-down regulation of information processing in response to stress. As in the hippocampus, excitatory projections are relayed through aBST and vSUB to inhibit PVH and subsequent HPA axis activation (Herman et al., 1995b; Herman et al., 2003; Radley et al., 2009; Radley and Sawchenko, 2011). The medial prefrontal cortex (mPFC) appears to be highly susceptible to reorganization following stress. Chronic stress exposure induces regressive plasticity in the medial prefrontal cortex as has been shown multiple times from various laboratories (Cook and Wellman, 2004; Radley et al., 2004;

Liston et al., 2006; Holmes and Wellman, 2009). The prefrontal cortex is involved in many complex tasks such as working memory and behavioral flexibility and these functions have shown to be impaired following CVS (Liston et al., 2006; Hains et al., 2009; Holmes and Wellman, 2009). As with the hippocampus, individuals suffering from depression show volume reductions in prefrontal regions and abnormal prefrontal activity is common (Drevets et al., 1997; Liston et al., 2009). As this dissertation focuses on the prefrontal cortex, a more in depth analysis of the effects of stress on this brain region will be forthcoming in the following chapters.

Dendritic spines

Much of the work on stress effects on plasticity has focused on changes on dendritic arborization and dendritic spines. Dendritic spines are sites of high plasticity and make up a majority of excitatory input onto synapses in the cortex. Dendritic spines are labile structures that change rapidly in response to a variety of physiological and environmental stimuli (Nimchinsky et al., 2002; Kirov et al., 2004; Yuste and Bonhoeffer, 2004; Holtmaat et al., 2005; Hongpaisan and Alkon, 2007). Studies have shown that the shape of the dendritic spine is correlated with its function. Mushroom spines are representative of a category of larger volume, stable and mature spine phenotype that have been associated with long-lasting memories (Nimchinsky et al., 2002; Yasumatsu et al., 2008; Yang et al., 2009). Thin spines exhibit a higher degree of plasticity than other spine morphological phenotypes (Knott et al., 2006; Bourne and Harris, 2007) and have been

implicated in hippocampal and sensorimotor learning tasks (Yasumatsu et al., 2008; Yang et al., 2009; Liston et al., 2013).

Previous studies, including those from our laboratory, have shown that chronic stress is associated with remodeling of dendritic spines (Liston et al., 2006; Radley et al., 2006b; Radley et al., 2008; Radley et al., 2013). Though there is much literature showing glucocorticoid involvement in the altering of hippocampal plasticity (Watanabe et al., 1992b; Watanabe et al., 1992a; McEwen, 1998; Sousa et al., 2000; Alfarez et al., 2009), information on glucocorticoid effects on structural plasticity in mPFC has not been as extensive. A key part of the following work will be investigating the role glucocorticoids play in reorganizing prefrontal structure following stress.

Sex differences in the stress response

Sex differences in many areas of neuroscience have been forthcoming, with sex differences in the stress response being highly prevalent. In the human literature, the fact that women tend to be more prone to stress-related mental disorders such as depression and anxiety is widely accepted (Holden, 2005; Marcus et al., 2005; Kendler et al., 2006; Grigoriadis and Robinson, 2007). This warrants close investigation on the stress-response in both sexes in animal models. However, while human data would suggest women are more susceptible to stress, some research in rodents actually seems to highlight resilience in females.

In studies of hippocampal functioning, males reliably show impairments following periods of stress (see above). Females do not appear to respond the same ways as males. Depending on the hippocampal task and stress paradigm, females seem to be unaffected by the stress exposure or show enhancements in the cognitive task (Luine et al., 1998; Bowman et al., 2001; Beck and Luine, 2002; Bowman et al., 2002; Conrad et al., 2012). In CA3 dendrites, following repeated stress males show reliable decreases in dendritic arborization. However, the same stress exposure in females does not seem to affect CA3 arborization (Galea et al., 1997). This matches the cognitive consequences of repeated stress, with males showing impairments in hippocampal function while females do not (Luine et al., 1994; Bowman et al., 2001).

The reasoning behind such effects are not well delineated, though estrogen is thought to be a key player in the resilience females show following periods of stress (see Luine, 2016). The rodent female estrous cycle lasts between 4-5 days with estrogen levels varying between lower amounts during diestrus and peak levels during proestrus. Various studies have shown that animals in diestrus perform more behaviorally similar to males than when in periods of high estradiol, such as proestrus (Bowman et al., 2002; Kitraki et al., 2004; Shansky et al., 2004; Shansky et al., 2006; Wei et al., 2014). Recently, a meta-analysis of variability among male and female rodents showed that variability was no greater in females than males for any behavioral, morphological, or physiological endpoint, arguing that the potential variability added by including gonadally-intact, cycling females,

should be no greater than any variability produced in males (Prendergast et al., 2014).

Sex differences in the stress response has become widely accepted, particularly when it comes to hippocampal functioning and structure. However, much less data has been gathered on how the prefrontal cortex of female rodents may or may not differ between that of males following stress. The final chapter of this dissertation is going to focus on how stress impacts prefrontal structural organization as well as function in female rats.

Summary

The prefrontal cortex is a crucial structure in the stress response as it has been shown to modulate the HPA axis, but has importantly been shown to be adversely affected by repeated stress exposure. While more recent work has focused on this brain region and its role and response to stress, little work has paid crucial detail into the role that stress hormones and exposure plays in the restructuring of the prefrontal cortex, and how these changes in plasticity may differ in females. Therefore, the goal of the current set of experiments is to:

- Address the role glucocorticoids play in an aging context where cognitive decline is common and basal glucocorticoids are elevated
- Investigate the role glucocorticoids play in stress-induced structural plasticity in males and females

- Investigate how prefrontal function and structure are affected following chronic stress in both males and females and determine if males and females differ in response to repeated stress

CHAPTER 2: ADRENOCORTICAL STATUS PREDICTS THE DEGREE OF AGE-RELATED DEFICITES IN PREFRONTAL STRUCTURAL PLASTICITY AND WORKING MEMORY

ABSTRACT

Cognitive decline in aging is marked by considerable variability, with some individuals experiencing significant impairments and others retaining intact functioning. Whereas previous studies have linked elevated hypothalamo-pituitary-adrenal (HPA) axis activity with impaired hippocampal function during aging, the idea has languished regarding whether such differences may underlie the deterioration of other cognitive functions. Here we investigate whether endogenous differences in HPA activity are predictive of age-related impairments in prefrontal structural and behavioral plasticity. Young and aged rats (4 and 21 months, respectively) were partitioned into low or high HPA activity, based upon averaged values of corticosterone release from each animal obtained from repeated sampling across a 24h period. Pyramidal neurons in the prelimbic area of medial prefrontal cortex were selected for intracellular dye filling, followed by 3D imaging and analysis of dendritic spine morphometry. Aged animals displayed dendritic spine loss and altered geometric characteristics; however, these decrements were largely accounted for by the subgroup bearing elevated corticosterone. Moreover, high adrenocortical activity in aging was associated with downward shifts in frequency distributions for spine head diameter and length, whereas aged animals with low corticosterone showed an upward shift in these indices. Follow-up

behavioral experiments revealed that age-related spatial working memory deficits were exacerbated by increased HPA activity. By contrast, variations in HPA activity in young animals failed to impact structural or behavioral plasticity. These data implicate the cumulative exposure to glucocorticoids as a central underlying process in age-related prefrontal impairment and define synaptic features accounting for different trajectories in age-related cognitive function.

INTRODUCTION

Aging is accompanied by a gradual and progressive decline in cognitive processing ability. Nevertheless, some individuals experience significant impairment over time, whereas others retain intact functioning (Rowe and Kahn, 1987; Rapp and Amaral, 1992; Cabeza et al., 2002). Previous studies have linked elevations in the neuroendocrine system responsible for regulating adaptation to stress, the hypothalamo-pituitary-adrenal (HPA) axis and adrenocortical hormones (cortisol in humans, corticosterone [CORT] in rodents), with alterations in hippocampal neurobiology and related behaviors during aging (Landfield et al., 1978; Issa et al., 1990; Sapolsky, 1992; Lupien et al., 1994). Individual differences in HPA activity are programmed by early-life experiences and may be further modified throughout life by severe or prolonged stressful events (Zhang and Meany, 2010). Although some consideration has been given to the possibility that individual differences in HPA activity may predict vulnerability to cognitive

processes beyond the hippocampus (Lupien et al., 1999; Li et al., 2006; Franz et al., 2011), their underlying mechanisms have yet to be critically examined.

The prefrontal cortex is highly vulnerable to the effects of stress and aging (Holmes and Wellman, 2009; Mizoguchi et al., 2009; Garrido, 2010; Hara et al., 2012; McEwen and Morrison, 2013; Samson and Barnes, 2013). Many of these experience dependent network alterations in prefrontal functioning can be understood by examining dendritic spines (Goldman-Rakic, 1995; Morrison and Baxter, 2012; Dickstein et al., 2013), as these specializations encompass the vast majority of excitatory synapses made onto cortical pyramidal neurons and represent important sites for synaptic plasticity (Nimchinsky et al., 2002; Holtmaat and Svoboda, 2009). It has been widely documented that pyramidal neurons in medial prefrontal cortex (mPFC) undergo dendritic shortening, spine loss, and are associated with prefrontal functional impairment after prolonged stress and/or exposure to adrenocortical hormones (Wellman, 2001; Liston et al., 2006; Cerqueira et al., 2007; Liu and Aghajanian, 2008; Hains et al., 2009; Barsegyan et al., 2010). If the stress is abated, prefrontal neurons in young animals demonstrate an exceptional restorative capacity (Radley et al., 2005; Bloss et al., 2010). Aged animals lack such resilience and show deterioration of prefrontal dendritic spine synapses (Peters et al., 2008; Bloss et al., 2011), most notably within subtypes that are thought to result in diminished cognitive functioning (Arnsten et al., 2010; Dumitriu et al., 2010). Although this raises the possibility that the weathering of prefrontal synaptic structure and cognition may result from cumulative exposure of adrenocortical hormones throughout life (McEwen, 1998), an important implication

is that variations in HPA activity should account for different functional trajectories evident in aging. Here we characterize HPA neuroendocrine signatures in young and aged rats to interrogate the extent to which elevated adrenocortical output is predictive of age-related impairments in dendritic spine plasticity in mPFC and spatial working memory.

MATERIALS AND METHODS

Animals. The animals used in this study were male Sprague Dawley albino rats at ages of 4 and 21 months (designated as young and aged, respectively; Harlan Laboratories). Animals were housed in pairs and maintained on a 12:12 h light/dark cycle (lights on at 0600), with free access to food and water. The health of the aged animals was verified upon arrival from the supplier and monitored regularly by the attending veterinarian to ensure freedom from spontaneous tumors or other overt physiologic or immunologic signs of distress. After 2 weeks of acclimatization in the animal housing facility, rats were habituated to human contact by handling each for 5 min each day, over at least 7 d before the initiation of experiments.

Blood collection and radioimmunoassay. Basal adrenocortical activity was measured by obtaining blood samples from the tail vein of rats at 6 time points over a 24 h period starting at 8:00 A.M. For blood collection, rats were restrained briefly (15–30 s), and a small longitudinal incision was made at the distal tip of the tail with a sterile blade. Blood samples (200 μl) were collected into chilled plastic microfuge tubes containing EDTA and aprotinin, centrifuged, and fractionated for storage of

plasma at 80°C until assayed. Plasma CORT was measured without extraction, using an antiserum raised in rabbits against a CORT-BSA conjugate, and 125 I-CORT-BSA as tracer (MP Biomedicals). Assay sensitivity was 0.8μg/dl; intra-assay and interassay coefficients of variation were 5% and 10%, respectively.

Histology and tissue processing. Rats were anesthetized with chloral hydrate (350 mg/kg, i.p.) and perfused via the ascending aorta with 100 ml 1% PFA and 0.125% glutaraldehyde in 0.1 M PBS, pH 7.4, followed by 500 ml of 4% PFA and 0.125% glutaraldehyde in 0.1 M PBS, pH 7.4, at a flow rate of 55 ml/min. After postfixation, the pregenual pole of the cortex was sectioned coronally into 250-μm-thick slabs using an oscillating tissue slicer (VT-1000S, Leica) and stored in 0.1 M PBS containing 0.1% sodium azide at 4°C until the time of cell loading.

Intracellular dye injections and neuronal reconstructions. The procedures used here are based on previous reports using the same methodology (Radley et al., 2006b; Radley et al., 2008). Coronal tissue slabs were treated in the DNA-binding fluorescent stain DAPI (Invitrogen) to distinguish between nuclear lamination patterns that distinguish prelimbic from other adjacent-lying prefrontal cortical subfields. DAPI-treated sections were mounted on nitrocellulose filter paper and submerged in a tissue culture dish containing 0.1 M PBS and viewed under fluorescence using a fixed-stage microscope (Leica DM5500). Injections of 5% Lucifer yellow (Invitrogen) were made by iontophoresis through micropipettes (1–2 μm inner diameter) under a DC current of 1–6 nA for 5–10 min. Neurons in layers 2

and 3 of PL were selected for the dye injection procedure based upon the distinguishing cytoarchitectonic features of this region, notably a more densely packed layer 2 and a broader layer 5 relative to adjacent-lying cortical subfields. The general technique for cell filling involved carefully observing the passive diffusion of LY resulting from application of a negligibly small amount of current from the advancing micropipette tip under 40x magnification; LY diffuses amorphously until hitting a dendritic process or cell body, whereby the dye becomes restricted intracellularly. After several neurons were filled intracellularly, tissue sections were mounted onto glass slides and coverslipped in Vectashield (Vector Laboratories).

Neuronal reconstructions and data analyses were performed by an experimenter unaware of the treatment condition for each animal. Pyramidal neuron dendritic arbors were reconstructed in 3D using a computer-assisted morphometry system consisting of a Leica DM4000R equipped with an Applied Scientific Instrumentation MS-2000 XYZ computer-controlled motorized stage, a QImaging Blue digital camera, a Gateway computer, and morphometry software (MBF Biosciences). Neurons were visualized, and the dendritic tree was reconstructed using a Leica Apochromat 40x objective with a numerical aperture (NA) of 1.4 and Neurolucida software (MBF Biosciences).

To be considered for analysis, LY-filled PL neurons had to exhibit complete filling of the dendritic tree, as evidenced by well-defined endings. A series of strict criteria were used for inclusion of pyramidal neuron apical and basal dendrites for morphologic analysis. For apical dendrites, the fact that the primary shaft generally

coursed parallel, or gently downward from the top surface of the section (i.e., sections were flipped in instances where apical dendrites coursed upward out of the top surface of the section), optimized the probability for retaining complete dendritic arbors. However, because the dye-filling procedure performed in the sections were only 250 μm thick, it was virtually impossible to retain an entirely intact apical dendritic arbor with no truncations. Thus, apical dendrites included in the analysis retained intact secondary and tertiary branches, with truncations permitted only in collateral branches that appeared to be nearing the point of termination or deemed unlikely to make any significant bifurcations. PL neurons in layers 2 and 3 were also appreciated to exhibit some qualitative differences; deeper-lying layer 3 neurons possessed apical dendrites with elongated primary dendrites before the first truncation point (~75–125 μm; notwithstanding collateral branching), whereas more superficially situated layer 2 neurons contained shorter distances to the first branch point (~25–75 μm; e.g., compare images in Figs. 2 and 3). Nevertheless, consideration of these subsets as morphologically distinct subpopulations in ancillary analyses failed to demonstrate any quantitative differences that warranted partitioning them into distinct groupings. For basal dendrites, it was common to retain an average of 1–3 entirely intact arbors for a given LY-filled neuron, such that analyses on intact branches were performed for this category.

Confocal laser scanning microscopy and dendritic spine analysis. Two dimensional renderings for each neuron were obtained using Neurolucida software, and a radial distance of 150μm from the soma was selected as a boundary delineating proximal and distal portions of the dendritic tree. Within these regions, branches were randomly selected for each neuron for an average of 3 segments per neuron and 5 neurons for each animal. The selection criteria for confocal imaging of dendritic segments are based upon previous reports (Radley et al., 2006b; Radley et al., 2008): (1) possess a diameter of 3 μm, as larger diameter dendrites in PL pyramidal neurons exhibit greater variability in spine density values; (2) reside within a depth of 70 μm from the top surface of the section, due to the limited working distance of the optical system; (3) to be either parallel to, or course gently relative to, the coronal surface of the section (i.e., this helps to minimize *z*-axis distortion and facilitate the unambiguous identification of spines); and (4) have no overlap with other branches that would obscure visualization of spines. *z*-Stacks were collected on a Leica SP5 confocal laser-scanning microscope equipped with an argon laser and a 100X, 1.4 NA oil-immersion objective, using voxel dimensions of 0.1x0.1x0.1μm^3. Settings for pin hole size (1 airy disc), gain, and off set were optimized initially and then held relatively constant throughout the study to ensure that all images were digitized under similar illumination conditions at a resolution of 512 X 512 pixels.

Images were deconvolved with AutoDeblur (Media Cybernetics), and spine analyses were performed using the semiautomated software *NeuronStudio* (Rodriguez et al., 2006; Radley et al., 2008) (http://research. mssm.edu/cnic/tools-

ns.html), which analyzes in 3D dendritic length, spine density, and morphometric features (i.e., head/neck diameter, length, subtype) for each dendritic spine. Spines were classified as thin or mushroom if the ratio of the head diameter-to-neck diameter was >1.1. If their ratio exceeded this value, spines with a maximum head diameter >0.4 μm were classified as mushroom or else were classified as thin. Spines with head-to-neck diameter ratios <1.1 were also classified as thin if the ratio of spine length-to-neck diameter was >2.5; otherwise, they were classified as stubby. A fourth category, filopodial spines, was considered to have a long and thin shape with no enlargement at the distal tip, was very seldom observed, and was classified as thin. Finally, data readouts from the spine analysis algorithm were visually compared by the experimenter for each optical stack to verify accurate subtype classifications for dendritic spines.

Assessment of prefrontal functionality using delayed alternation. Young and aged rats were first submitted to repeated blood sampling for assessment of basal adrenocortical activity (as above) and given a week to recover while still being handled daily. Animals were then placed on a restricted diet and trained in a prefrontal-dependent spatial working memory task, delayed alternation using a T-maze (Divac, 1970; Ramos et al., 2003). Rats were first habituated to a T maze (90x65 cm; 40 cm opaque walls surrounding the perimeter) over a several day period using miniature chocolate chips as a food reward for running to either goal arm in <60 s. Next, animals were subjected to a forced alternation period of training where they were only rewarded with chocolate after entering the opposite

goal arm that they were in previously. Between trials, the maze was wiped clean with 95% ethanol to prevent olfactory cues from determining choice. After 3 consecutive days of 10 trials/day, animals were tested for spontaneous alternation (i.e., chocolate was present in both goal arms, although rats were only rewarded for entering the opposite arm from the previous trial). The delay between trials was increased until animals could successfully alternate at a 15 s interval with 70% accuracy for 10 trials. Testing involved 8 trials/day over 6 consecutive days. The delays between each trial were semirandomly varied between 30, 60, or 120 s (on a given day the same pattern was used for all animals). During delay, rats were placed in the holding cage and the maze was cleaned with 95% ethanol. The percentage of correct choice alternations made at each delay interval (30, 60, and 120 s) were obtained for each animal, and overall averages were expressed as a function of treatment group.

Statistics. Group data from the CORT radioimmunoassay were compared with a multifactorial ANOVA, followed by pairwise comparisons using Tukey HSD at each time point. Data are expressed as the mean $\pm$ SEM. Data from the dendritic branch and spine morphometric experiments were averaged from each animal (3–4 segments/PL neuron, 5 neurons/animal, 6–7 animals per group) as a function of both age and adrenocortical status. The effects on overall dendritic length, number of branch endings, dendritic spine and subtype densities, and delayed alternation performance at each interval (30, 60, and 120 s) were compared using a repeated-measures ANOVA, with factors of age (young, aged), adrenocortical status (low,

high CORT), and the delay interval as the within-subjects factor. Additional correlational tests were performed for the assessment of whether each dependent measure varied as a function of integrated CORT values for each subject. All pairwise comparisons were made using Tukey HSD, with significance set at $p < 0.05$, and data are expressed as mean ± SEM.

Cumulative distribution differences for certain spine parameters (i.e., mushroom spine head diameter, thin spine head diameter, thin spine length) were evaluated using the Kolmogorov–Smirnov test with MATLAB software (MathWorks). Because mushroom-type spine head diameters were the only subtype to reveal populations shifts as a function of treatment status, data from only these analyses are presented (see Results). Follow-up analyses were performed to determine whether changes in mushroom spine head diameters occurred equally across the entire spectrum of size measurements or were more pronounced in specific subpopulations of spines of this class (e.g., the smallest and largest). Finally, spine parameters (head diameter, length) from all subtypes were pooled, and the 25th and the 75th percentiles, and differences in proportions of spines below the 25th percentile, as well as above the 75th percentile, were examined between aged and young subgroups. The x^2 goodness-of-fit test was used to compare the proportions below the first quartile cutoffs between groups as well as the proportions above the third quartile cutoffs (Radley et al., 2008).

RESULTS

Characterization of adrenocortical activity in young and aged rats.

Adrenocortical secretory activity was characterized in 4-month-old and 21-month-old rats across the light-dark cycle. Blood samples were collected from the tail vein of animals starting at 0800 h, and were repeated at 4 h intervals, through 0400 h on the following day. For assessment of variation in adrenocortical activity as a function of age, CORT values from all six sampling intervals were averaged to a single value for each animal (Fig. 1A). Then, young and aged animals possessing plasma CORT levels above and below the median value for each group were selected to represent high and low levels of adrenocortical activity, respectively. The decision to impose a binary classification scheme for these subgroupings, instead of basing dependent measures on absolute values of CORT secretory activity from individual animals, derives from consideration that the former provides a greater degree of measurement reliability and is less sensitive to statistical outliers (Baxter and Gallagher, 1996).

Factorial ANOVA (age and CORT status as between-subjects factors; time of day of blood collection as a within-subjects factor) showed main effects for time ($F_{(5,110)} = 24.30$, $p < 0.001$) and adrenocortical status ($F_{(1,22)} = 36.18$, $p < 0.001$). Analysis of circadian release patterns in young animals revealed a nadir and peak in CORT secretion at 1200 and 2000 h, respectively (Fig. 1B). Aged animals bearing low + adrenocortical activity generally showed a pattern of CORT release

similar to the young low + CORT group, whereas the aged + high CORT animals displayed an HPA secretory pattern distinguished by a failure to reach the nadir at 1200 h relative to all three of the other groups (2.5-fold higher; $p< 0.05$ for each; Fig. 1B). Postmortem measurement of adrenal weights also revealed hypertrophy within both aged and young rats bearing high CORT animals with respect to low CORT groups (age: $F_{(1,22)}=5.32$, $p<0.03$; CORT status: $F_{(1,22)}= 19.20$, $p < 0.001$; interaction: $F_{(1,22)} =2.84$, $p<0.10$; mean $\pm$ SEM: young + low CORT 10.4 $\pm$ 0.3 μg/g; young + high CORT =11.8 $\pm$ 0.6 μg/g; aged + low CORT 11.0 $\pm$ 0.3 μg/g; aged + high CORT =13.5 $\pm$ 0.2 μg/g). Adrenal-to-body ratio in this cohort of animals also confirmed that rats with high adrenocortical status displayed a significant degree of adrenal hypertrophy, regardless of age (age: $F_{(1,16)}=1.23$,$p=0.28$; CORT status: $F_{(1,16)}=15.08$, $p<0.001$; interaction: $F_{(1,16)}=1.23$, $p=0.28$). Furthermore, the absolute values of adrenal-to-body weight ratios were comparable across cohorts (mean $\pm$ SEM: young + low CORT=10.4 $\pm$ 0.3 μg/g; young + high CORT=11.5 $\pm$ 0.4 μg/g; aged + low CORT=10.3 $\pm$ 0.5 μg/g; aged + high CORT=12.1 $\pm$ 0.2 μg/g).

Aging and adrenocortical status contribute to dendritic spine loss in mPFC.

Dendritic spine density

Individual neurons in layer 2/3 pyramidal neurons of the PL cortical subfield of mPFC were selected for intracellular injection of the fluorescent dye Lucifer yellow in young and aged rats that had been previously characterized for basal CORT activity. Different regions of the dendritic tree (<150 μm apical, >150 μm apical,

<150 μm basal) were selected for high-resolution confocal laser scanning microscopic imaging of dendritic segments (Fig. 2*A*–*C*). Digital renderings (*z*-stacks) of dendritic segments made in 3D were deconvolved, followed by the analysis of spine density and morphology using the semiautomated software *NeuronStudio* (Rodriguez et al., 2006; Radley et al., 2008) (Fig. 4*A*,*B*). In this analysis, a total of 406 dendritic segments from 92 fluorescent dye-labeled PL neurons were analyzed for spine density and morphometric analysis (199 young, 207 old), giving a total of 31,176 and 28,418 dendritic spines in young and aged animals, respectively.

We observed an 11% decrease in overall dendritic spine density in PL neurons in aged relative to young animals (Fig. 2*D*). Comparisons made between young and aged groups in the context of low and high HPA activity revealed main effects of age ($F_{(1,22)}$=9.29, p =0.006), adrenocortical status ($F_{(1,22)}$=9.14, p =0.006), and no interaction between these measures ($F_{(1,22)}$=2.69, p =0.12). Aged animals bearing high CORT levels (i.e., aged + high CORT) displayed 20% reductions in spine density relative to young subgroups and aged + low CORT animals (p <0.05 for each; Fig. 2*D*). By contrast, aged animals with low CORT levels did not show any reduction in spine density relative to either low or high CORT subgroups of young animals (p=0.75 and p=0.99, respectively). Comparisons made within different regions of the dendritic tree revealed the same downward trend of spine density in proximal apical dendrites in the aged + high CORT group or rats, showing 21% reductions in spine density relative to young low and high CORT groups, respectively (p <0.05 for each; Fig. 2*E*). Some attrition of spines, albeit

less prominent, was evident in aged + high CORT animals in both distal apical (150μm) and basal aspects of the dendritic tree (Fig.2*E*).

In a follow-up analysis, adrenocortical status was plotted as a function of dendritic spine loss in PL neurons for young and aged groups. Despite the existence of clear group differences between aged + low and high CORT groupings, no reliable correlation between these indices was noted (r = -0.46, p=0.30), whereas the analysis of young animals yielded a slightly more reliable tendency toward variations in adrenocortical activity and PL spine density (r = -0.68, p=0.10).

Dendritic arborization patterns

Given that chronic stress and exogenously administered CORT are known to induce apical dendritic atrophy in mPFC pyramidal neurons (Wellman, 2001; Radley et al., 2004; Cerqueira et al., 2007; Liu and Aghajanian, 2008), we examined dendritic arborization patterns (overall length, branch number) as a function of age and adrenocortical status in the same PL neurons analyzed for alterations in spine plasticity (Fig. 3). A two-way ANOVA design was implemented as above, with age and CORT status as the treatment variables. With regard to apical dendrites, there were no significant main effects or interactions for total length (age status: $F_{(1,19)}$=1.75, p=0.20; CORT status: $F_{(1,19)}$=1.81, p=0.20; interaction: $F_{(1,19)}$=0.96; p=0.34), or number of branch endings (age status: $F_{(1,19)}$=0.40, p= 0.85; CORT status: $F_{(1,19)}$= 0.63, p = 0.44; interaction: $F_{(1,19)}$ = 0.48; p=0.50) (Fig. 3, bottom). Basal dendritic measures of length (age status: $F_{(1,19)}$=

2.87, p=0.11; CORT status: $F_{(1,19)}$ = 0.44, p = 0.52; interaction: $F_{(1,19)}$ =0.05; p= 0.83), and the number of branch endings (age status: $F_{(1,19)}$= 0.35, p = 0.56; CORT status: $F_{(1,19)}$ = 0.01, p = 0.91; interaction: $F_{(1,19)}$ = 0.03; p = 0.87) also failed to reveal any significant differences. These data suggest that dendritic arbors remain relatively resistant to such age-related alterations, suggesting neither a homeostatic response of increased dendritic growth that could compensate for spine synaptic compromise nor further spine loss that could be compounded by dendritic shortening.

Interactive effects of age and HPA status on PL dendritic spine morphology.

Several previous studies have shown that age-related dendritic spine loss in the prefrontal cortex is most prominent within thin subtypes (Dumitriu et al., 2010; Bloss et al., 2011). Thin spines are largely representative of the immature excitatory synaptic population in cortical pyramidal neurons, exhibiting high rates of turnover compared with large spines (Matsuzaki et al., 2001b; Holtmaat et al., 2005), and have decreased rates of turnover after prolonged glucocorticoid exposure (Liston and Gan, 2011). Thus, we addressed the possibility that age-related decreases in thin spine subtypes in PL may be exaggerated in animals presenting elevated adrenocortical activity. Spine morphometric features were delineated from high-resolution optical stacks of dendritic segments in fluorescent dye-filled pyramidal neurons using *NeuronStudio* (Rodriguez et al., 2006; Radley et al., 2008) (Fig. 4*A*,*B*). It has been shown that this method of analysis produces spine density values and morphometric indices similar to EM analytic estimates in

adult cortical neurons and is substantially less prone to the population and classification sampling errors common in Golgi-based analytic approaches (Dumitriu et al., 2010).

Two-way ANOVA revealed main effects of age ($F_{(1,22)}$ = 6.28, p = 0.02), adrenocortical status ($F_{(1,22)}$ = 6.43, p = 0.02), and an interaction ($F_{(1,22)}$ = 5.41, p = 0.03) on thin spine density. *Post hoc* comparisons revealed a significant decrease (by 21%) of thin spines between aged + high CORT animals relative to the other three groups (p =0.01 for each; Fig. 4*C*). Regional analyses of thin spine density highlighted age-related losses in proximal aspects of the apical dendritic tree, with aged + high CORT animals showing a 26% decrement compared with all three groups (p <0.05 for each; Fig. 4*C*). Furthermore, some measure of thin spine loss was also noted in basal dendrites in aged + high CORT animals (Fig. 4*C*). Plots of individual values from young and aged animals failed to demonstrate a reliable inverse correlation between adrenocortical activity relative to thin spine density in PL neurons (young: r = -0.49, p = 0.26; aged: r= -0.35, p = 0.44)

Although the functional implications of stubby spines are less well understood, the available evidence suggests that this subtype reflects a distinct subpopulation of immature excitatory synapses from thin spines (Boyer et al., 1998; Petrak et al., 2005; Christoffel et al., 2011). In this analysis, main effects on overall stubby spine density were only noted for adrenocortical status ($F_{(1,22)}$= 9.19; p= 0.006), however, not for age ($F_{(1,22)}$= 3.95; p<0.06) or interaction between these variables ($F_{(1,22)}$ =3.70; p = 0.07).

Comparisons relative to aged high CORT animals revealed some diminution in overall measures of stubby spine density compared with both young low and aged low CORT groups (p =0.01 for each; p =0.051 compared with young high CORT group; Fig. 4*D*). In distal apical dendrites, aged animals bearing high HPA activity were selectively vulnerable to stubby spine loss compared with the other three groups (by 29%; p <0.05 for each; Fig.4*D*). Correlation of individual values for stubby spines as a function of CORT levels did not reveal any significant inverse relationship within young (r = -0.26, p<0.57) or aged groups (r= -0.58, p< 0.17).

Mushroom spine densities were analyzed as a function of treatment status. No main effects of age or HPA status were noted to influence mushroom spine density in PL neurons (Fig. 4*E*), nor were any trends evident within apical and basal dendritic subregions. Moreover, no group differences were observed in spine head diameter or spine length (i.e., across all spine subtypes) as a function of treatment group (Fig.4 *F*,*G*). Nevertheless, as recent evidence supports the idea that large volume/mushroom spine subtypes in cortical pyramidal neurons may undergo shrinkage in response to elevated glucocorticoids or chronic stress (Liston and Gan, 2011; Tanokashira et al., 2012; Radley et al., 2013), we examined population shifts in mushroom spine head diameter as a function of age and HPA status. In these analyses, mushroom spines from both young subgroups were treated as a single population because their frequency plots were statistically virtually indistinguishable from one another (Kolmogorov–Smirnov test; p = 0.8; Fig. 5*A*). Cumulative frequencies for mushroom spine head diameter were

significantly shifted to the left in the aged high CORT group compared with young animals (Kolmogorov–Smirnov test, p = 0.0001) (Fig. 5*B*). By contrast, aged rats with low adrenocortical activity display a rightward shift in the cumulative frequency distribution for mushroom head diameter relative to young animals (Kolmogorov–Smirnov test, p = 0.00001; Fig. 5*C*). Furthermore, examination of relative frequency distributions revealed a greater degree of variability in mushroom spine head diameters in aged relative to young subgroups of animals (Fig. 5*D*–*F*). Thus, the leftward shift of the aged + high CORT group implicates prolonged exposure to high glucocorticoid levels as a possible mechanism for mushroom spine shrinkage, whereas the rightward shift suggests that lower levels CORT during aging may be permissive for greater stability in mature spine subtypes.

Finally, to determine whether aging and adrenocortical status produce shifts throughout the entire dendritic spine population in PL neurons, relative frequency distributions were analyzed for spine head diameter and length without regard to subtype. The proportion of spines bearing head diameter or length in the upper and lower quartile of aged + high CORT and aged + low CORT groups were compared with the overall population (Table 1). Aged + high CORT animals displayed a greater proportion of spines with head diameters in the lower quartile and a smaller proportion of long spines in the upper quartile ($p < 0.01$ for each, x^2 goodness-of-fit test for comparison of proportions). By contrast, aged animals bearing low adrenocortical activity displayed a greater proportion of spine head diameters in the upper quartile, whereas spine length showed an overall rightward shift, inclusive of a smaller proportion of short spines and a greater proportion of

elongated spines (p < 0.01 for each, x^2 test; Table 1). Overall, these patterns suggest that elevated CORT levels during aging produce downward shifts in dendritic spine size and length in PL, whereas age-matched counterparts with low adrenocortical activity display upward shifts in these indices.

Interactive effects of age and HPA status on spatial working memory.

Animals were trained in a delayed alternation task using a T maze, which is dependent upon intact mPFC function (Divac, 1970; Ramos et al., 2003) (Fig. 7*A*). The number of training sessions required for each animal to reach an equivalent level of performance was used as a measure of acquisition (i.e., 70% choice accuracy at a 15 s delay) (Ramos et al., 2003). No significant differences were noted in either the rate of acquisition or in the percentage of correct choices once animals reached criterion as a function of age or adrenocortical status (Fig. 7*B*,*C*). Repeated-measures ANOVA revealed main effects for delay interval ($F_{(2,32)}$ = 4.95, p< 0.01), age ($F_{(1,16)}$ = 13.60, p = 0.002), adrenocortical status ($F_{(1,16)}$=5.77, p< 0.03), and the interaction of age and adrenocortical status ($F_{(1,16)}$= 6.10, p<0.03). Age + high CORT animals displayed deficits in delayed alternation relative to the other three groups at 60 s (p = 0.001 for each) and 120 s (p < 0.05 for each) delay intervals. Aged + low CORT animals also displayed decreased choice accuracy at the 60 s delay interval relative to young animals (p = 0.001 for each), albeit to a lesser extent than the age + high CORT group (Fig. 7*C*). Comparison of individual values for either the number of training sessions or delay interval as a function of

CORT levels failed to reveal any reliable correlation within young or aged groups (data not shown).

DISCUSSION

In the present study, we have extended previous work showing that aging is marked by a deterioration of prefrontal cognitive functions and accompanying working memory Given that aging and HPA status interact to induce regressive structural plasticity in PL neurons, we interrogated whether aged animals with high CORT also show a selective vulnerability to prefrontal cognitive impairment. Separate groups of young adult (*n* = 10) and aged (*n* =10) male Sprague Dawley rats were assayed for basal adrenocortical function and partitioned into low and high subgroups for CORT activity as described above (Fig. 6). Postmortem measurement of adrenal-to-body weight regressive synaptic changes. Although we verified that aging results in reduced dendritic spine density in PL cortical pyramidal neurons, to our knowledge this is the first to show that age related dendritic spine alterations in the rat PL can be understood in the context of elevated circulating glucocorticoids and adrenal hypertrophy. As in previous work, digital reconstruction and 3D analysis of spine geometry revealed that losses in the thin spine category were the main contributor to the overall decrease in spine density during aging (Dumitriu et al., 2010; Bloss et al., 2011); this effect was either accounted for, or exacerbated by, elevated basal HPA activity. Although some young animals in this study displayed elevated levels of plasma CORT and

adrenal hypertrophy, they failed to show any decreases in spine density in immature subtypes.

The findings of this study raise the possibility that young animals with high adrenocortical activity represent a vulnerable subpopulation, whereby the cumulative exposure to higher levels of glucocorticoids over time may render PL neurons susceptible to synaptic compromise that manifests during aging. This interpretation is consistent with evidence that prolonged elevations in circulating glucocorticoids induce structural and functional alterations in hippocampus and impair hippocampal-dependent memory function (Issa et al., 1990; Woolley et al., 1990; Lupien et al., 1998), and the prevention of age-related increases in adreno-cortical output mitigates at least some of these adverse effects (Landfield et al., 1978; Landfield et al., 1981). However, this interpretation may be complicated by the fact that PL is also capable of imparting stress-inhibitory influences via cell groups residing within the anterior (or ventrolateral) bed nuclei of the stria terminalis (Radley et al., 2009). Recent evidence suggests that chronic stress induced dendritic spine plasticity in the PL at the anterior bed nuclei of the stria terminalis HPA-inhibitory pathway may underlie sensitization of adrenocortical output (Radley et al., 2013). Therefore, diminished PL spine density in aging may instead lead to elevated HPA secretory activity. In this scenario, the fact that younger animals with elevated adrenocortical output fail to show corresponding decreases in PL spine density suggests that other limbic forebrain (i.e., hippocampus) regions may compensate for prefrontal cortical deficits, whereas impaired hippocampal function during aging may unmask detrimental effects of

diminished structural synaptic indices in PL on HPA axis output. To better understand these issues, future studies should address how alterations in hippocampal and prefrontal HPA-inhibitory pathways contribute to elevated HPA output during aging.

Our analysis failed to reveal any relationship between aging or adrenocortical status and dendritic alterations in PL pyramidal neurons. This is consistent with a previous report demonstrating that prefrontal dendritic morphology remains stable across adulthood (Bloss et al., 2010). Thus, when the observations of dendritic morphology and spine density are together, the latter provides a reliable estimate for how aging and adrenocortical status impact the overall population of axospinous synapses in prefrontal neurons. That age-related increases in adrenocortical activity are limited to dendritic spine alterations further indicates that endogenous differences in HPA activity are not sufficient to induce dendritic reorganization in PL neurons. Therefore, regressive structural remodeling in the prefrontal cortex appears to be dependent on more robust stimuli, such as chronic stress exposure or exogenously administered corticosteroids (Wellman, 2001; Cook and Wellman, 2004; Radley et al., 2004; Cerqueira et al., 2005; Perez-Cruz et al., 2007).

Dendritic spines in the cortex are constantly in a labile state and can change shape rapidly in response to a variety of physiological and environmental stimuli (Nimchinsky et al., 2002; Kirov et al., 2004; Yuste and Bonhoeffer, 2004; Holtmaat et al., 2005; Hongpaisan and Alkon, 2007; Liston et al., 2013). Thin spines exhibit a higher degree of plasticity relative to the morphological continuum of spines in

the adult cortex (Knott et al., 2006; Bourne and Harris, 2007); they have been implicated in the learning of hippocampal and sensorimotor tasks (Yasumatsu et al., 2008; Yang et al., 2009; Liston et al., 2013), and in optimal prefrontal network functioning and working memory (Kasai et al., 2003; Arnsten et al., 2010). Thin spine loss in the prefrontal cortex has been well documented after chronic stress and aging, and their losses have been hypothesized to account for age-related impairments in cognitive function (Arnsten et al., 2010; Dumitriu et al., 2010; Bloss et al., 2011). Recent evidence has shown that thin spine formation and elimination are tied to the circadian rhythmicity of corticosteroid secretory patterns and are differentially regulated by glucocorticoid and mineralocorticoid receptor-dependent mechanisms (Liston et al., 2013). Hence, even ostensibly subtle fluctuations in basal HPA activity over time may account for large-scale synaptic changes in the prefrontal cortex. Together, our results extend this body of research by highlighting a prominent role for glucocorticoids in driving these synaptic structural alterations, and direct attention to a possible mechanism accounting for differential vulnerability during aging.

At first glance, our inability to identify any group differences in mushroom spines as a function of aging or adrenocortical status appears to be consistent with evidence that attrition of thin, as opposed to mushroom spines, accounts for age- and corticosteroid-mediated functional compromise. However, when we performed population analyses for this subtype, aged animals with high HPA activity were found to have mushroom spines with smaller head diameters than aged animals with lower adrenocortical indices and in both groups of young animals. By contrast,

we also found that aged animals bearing low HPA activity displayed larger mushroom spine head diameters in PL neurons than both young groups and aged animals with high HPA activity. When the frequency analysis was expanded to include all subtypes, similar bidirectional shifts were noted in both spine head diameter and length in aged animals as a function of adrenocortical status. That glucocorticoids have recently been shown to modulate spine size via altering activity of the F-actin stabilizing protein, caldesmon (Tanokashira et al., 2012), endorses this as a possible mechanism accounting for the differential regulation of this spine subtype during aging that merits further consideration.

Although rodents lack a granular prefrontal cortical region comparable with that of primates (Wise, 2008), it is generally agreed upon that the rodent mPFC performs working memory functions analogous to those subserved by dorsolateral area 46 in the primate prefrontal cortex (Brown and Bowman, 2002). Our results verified previous work in both rodents and primates that aging results in impaired spatial working memory (Arnsten et al., 2010; Hara et al., 2012; Samson and Barnes, 2013), although similar to our structural plasticity data, these effects were largely accounted for in aged animals bearing high HPA activity. The fact that young animals bearing increased HPA activity showed no spatial working memory deficits argues against elevated glucocorticoids per se but instead implicates the cumulative exposure to corticosteroids in prefrontal cognitive impairment. However, it should be noted that aged animals bearing low HPA activity also displayed at least mild deficits in spatial working memory (i.e., 60 s delay interval;

Fig.7*C*), which suggests that glucocorticoids are not solely responsible for, but instead may exacerbate, age-related cognitive impairment.

One lingering question concerns the extent to which age and HPA-related changes in mushroom spine size vis-a`-vis thin spine loss may contribute to spatial working memory deficits. The weight of the evidence supports maintaining the population of thin spines for optimal prefrontal network function and working memory, and their disruption in cognitive deficits (Arnsten et al., 2010; Dumitriu et al., 2010). By contrast, mushroom spines may be considered as important for mediating longer-term changes in synaptic strength that are more relevant for information storage in different types of memory systems. Nevertheless, subsequent population analyses revealed broader bidirectional effects of CORT during aging, manifested by decreases in spine length and head diameter in aged high CORT animals, and increases in the proportion of larger and elongated spines in low CORT counterparts. Therefore, regardless of subtype classification, the differential effects of corticosteroids on spine size and/ or length could also provide an additional mechanism accounting for how these hormones modulate prefrontal cognitive impairment during aging. Although these data highlight a new role for glucocorticoids in the attrition of prefrontal functionality during aging, additional work is required to clarify which synaptic changes are critical for maintaining intact cognitive functioning throughout the lifespan.

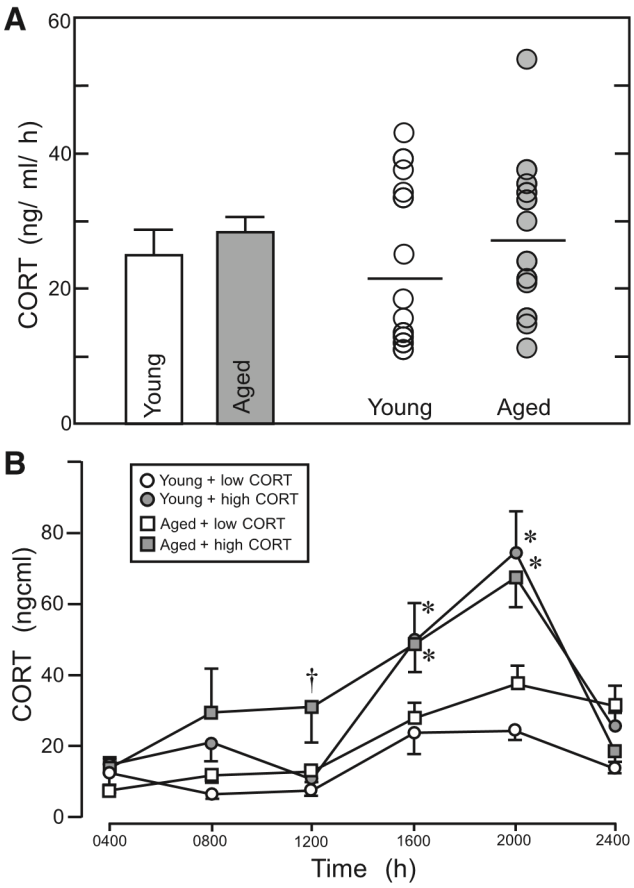

Figure 1. Basal CORT secretion in Young and Aged animals

A, Mean ± SEM for plasma CORT levels averaged across all six time points sampled (above), and plots of individual values (below). Although young and aged animals did not significantly differ in terms of overall adrenocortical activity, there exists a considerable degree of variability in glucocorticoid secretory output within each group. These differences provided the basis for dividing animals within each age category into subgroupings of high and low adrenocortical activity (horizontal line in each indicates the median value) for the assessment of age- related structural plasticity in mPFC as a function of this endocrine index. B, Mean ± SEM plasma CORT levels in young and aged animals as a function of HPA status sampled at 4 h intervals across the light-dark cycle. *p <0.05, significantly different from low CORT animals. †, p < 0.05, significantly different from both young and aged + low CORT subgroups. n = 6 –7 per group.

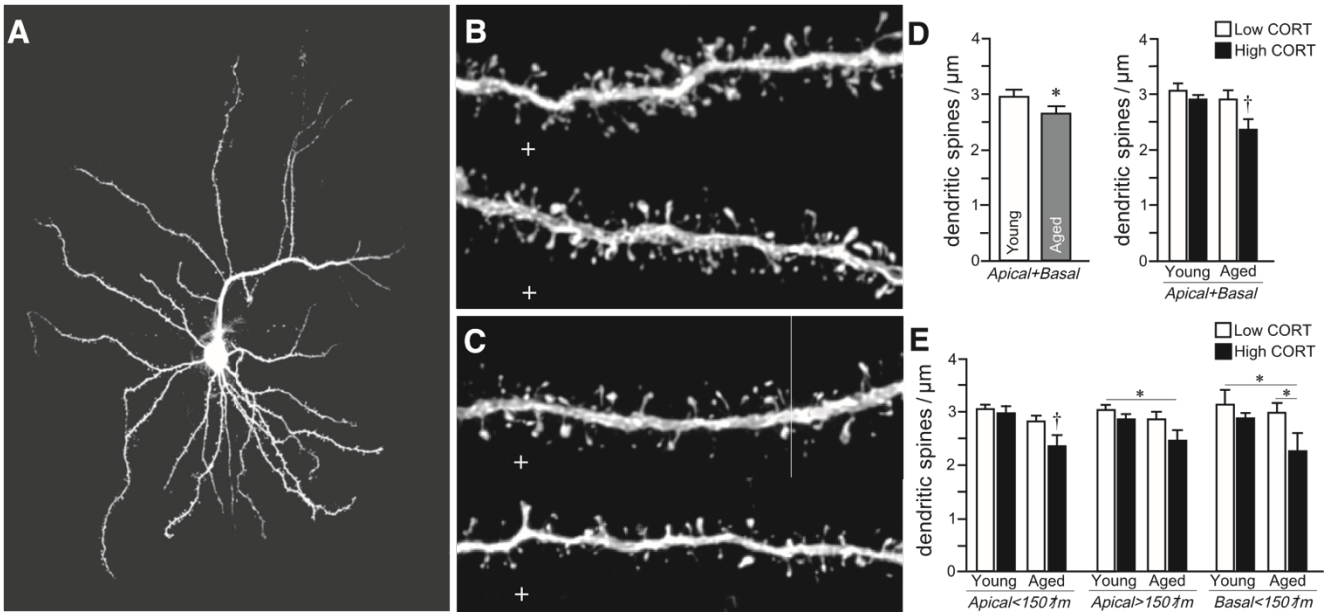

Figure 2. Dendritic spine density in Young and Aged animals

A, Example of a layer 2 PL pyramidal neuron iontophoretically filled with Lucifer yellow. The dashed circle demarcates the 150 μm boundary used to partition the dendritic tree for spine analyses. *B*, *C*, Deconvolved digital images of dendritic segments from different treatment groups. *D*, Mean ± SEM of dendritic spine density as a function of treatment group. Disregarding differences in HPA status (left), aged animals (*n*= 14) show a significant reduction in overall spine density relative to young animals(*n* =12). When aged and young animals are divided according to adrenocortical status (right), the aged + high CORT group exhibits a selective vulnerability for dendritic spine loss. *E*, Regional analyses show that dendrites throughout PL neurons are sensitive to spine loss as a function of aging and HPA status. Data represent mean SEM for each group and are based on averages from each animal. *p<0.05, significantly different for comparisons shown. †, p <0.05, significantly different from both young and aged low CORT subgroups. n = 6–7 per group. Scale bar, *B*, *C* (in *C*), 5μm.

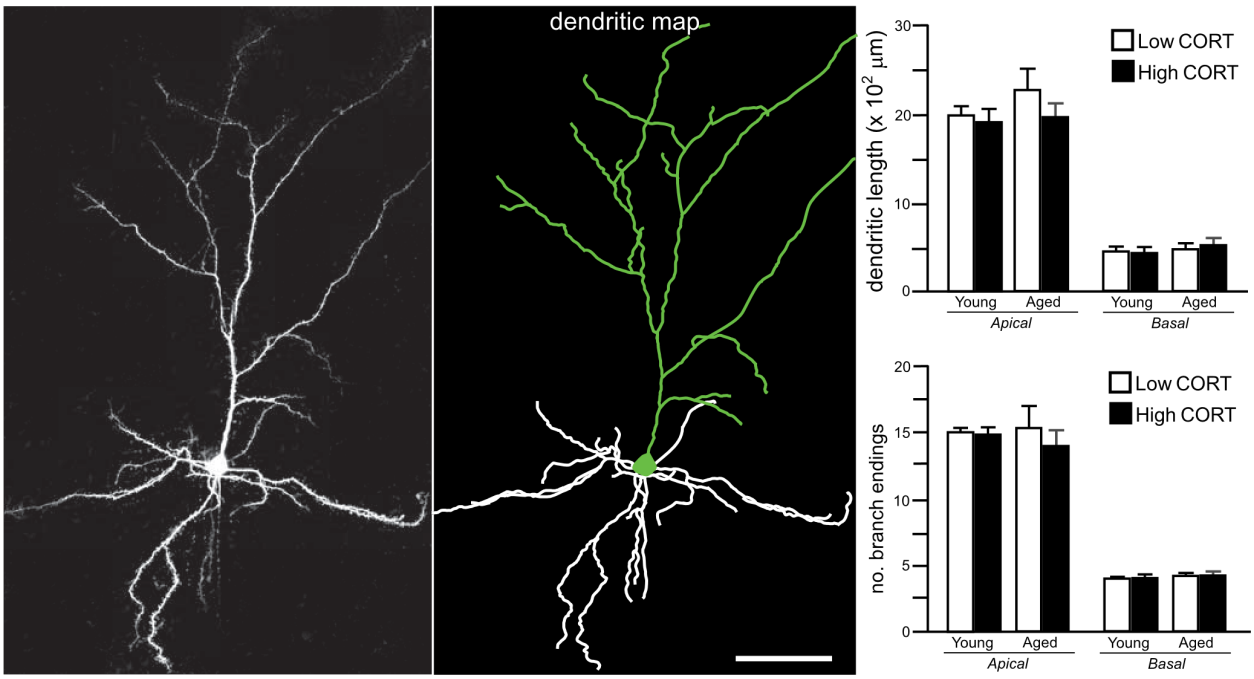

Figure 3. Dendritic arborization in Young and Aged animals

Top, Example neuron in layer 3 of PL that was iontophoretically filled (left), and the rendering of its dendritic tree (right) using computer-assisted morphometry. The apical dendritic tree is pointing upward, and basal dendrites (white) radiate from the opposite pole of the soma. Bottom, Histograms for dendritic length and number of branch endings for apical and basal dendrites. Aging or HPA status failed to result in any significant decreases in the dendritic indices examined. Data represent mean SEM for each index and are based on overall averages from each animal (i.e., *n* = 6–7 animals/group; *n* =1 arbor/neuron; *n*=5 neurons/animal). Scale bar, 75 µm.

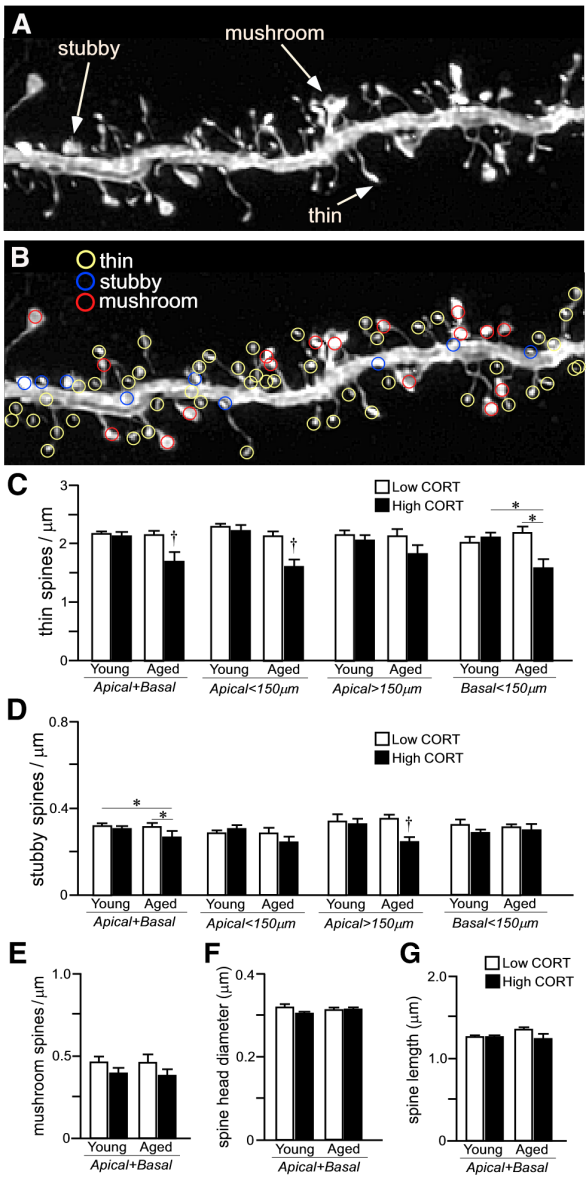

Figure 4. Dendritic spine subtype density in Young and Aged animals

A, Example of high-resolution deconvolved optical *z*-stack of a dendritic segment used for spine analysis with *NeuronStudio* software. *B*, Open colored circles represent spine subtypes based upon user-defined parameters in the software (see Materials and Methods). Histograms represent the effects of aging and adrenocortical status on thin (*C*), stubby (*D*), mushroom (*E*), spine density, and (for all three subtypes) spine head diameter (*F*) and length (*G*). Aged animals bearing high adrenocortical activity showed selective losses in thin spines and, to a lesser extent, stubby spines in overall measures throughout the

apical and basal dendritic tree.*$p < 0.05$,significantly different for comparison shown.† $p < 0.05$, significantly different from both young and aged low CORT subgroups. *N*= 6–7 per group. Scale bar, both images, 5 μm.

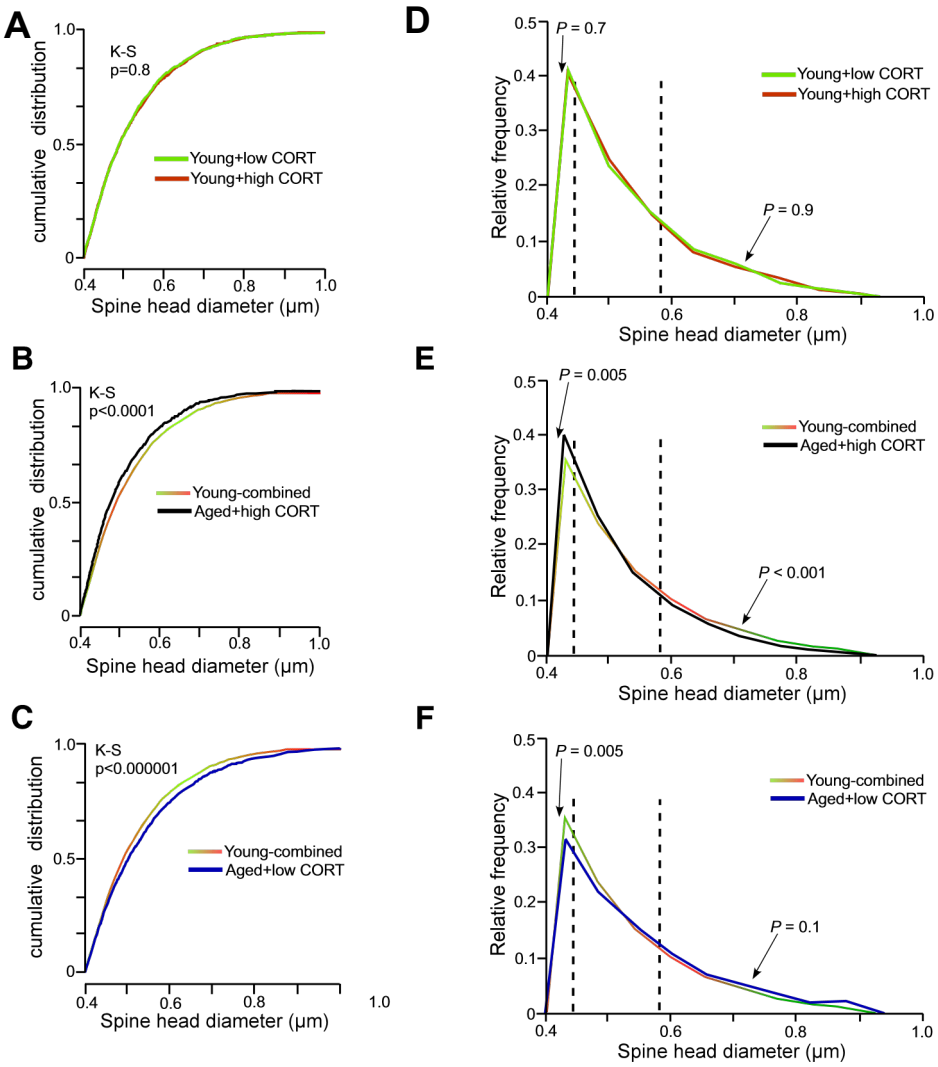

Figure 5. Cumulative frequency distributions of mushroom spine head diameters in Young and Aged subgroups.

A–C, Cumulative frequency distributions of mushroom spine head diameters in PL neurons in young and aged subgroups. *A*, Comparisons between low and high CORT subgroups in young animals revealed no differences between these distributions, thus providing the rationale for treatment as one group for

subsequent comparisons. *B*, The aged + high CORT group shows a leftward shift (i.e., decrease) in mushroom spine head diameter relative to young animals, whereas (*C*) aged + low CORT animals for this index show a cumulative frequency that is right-shifted (i.e., increased) relative to young animals, suggesting a mechanism leading to enlargement of mushroom spines relative to both young groups and aged high CORT animals. K–S, Kolmogorov–Smirnov test. *D–F*, Frequency distributions for mushroom spine head diameter in PL pyramidal neurons in young and aged animals. The dashed vertical lines in each histogram indicate the 25^{th} and 75^{th} percentiles of the entire spine population. *D*, Comparisons between low and high CORT subgroups in young animals. Because there were no differences between these distributions, subsequent analyses entailed combining these subgroups into one overall population. *E*, Comparisons between both young subgroups pooled together and aged + high CORT animals. Aged + high CORT animals show a shift in distribution toward a greater number of spines with small head diameters (lower quartile) and fewer spines with large head diameters (upper quartile) relative to young animals. *F*, Comparisons between both young subgroups pooled together and aged + low CORT animals. Aged + low CORT animals show a shift in distribution toward a greater number of spines with large head diameters (upper quartile) and fewer spines with smaller head diameters (lower quartile) relative to young animals. *P* values were obtained from the x^2 test for comparison of proportions of each distribution below or above the first or third quartile, respectively.

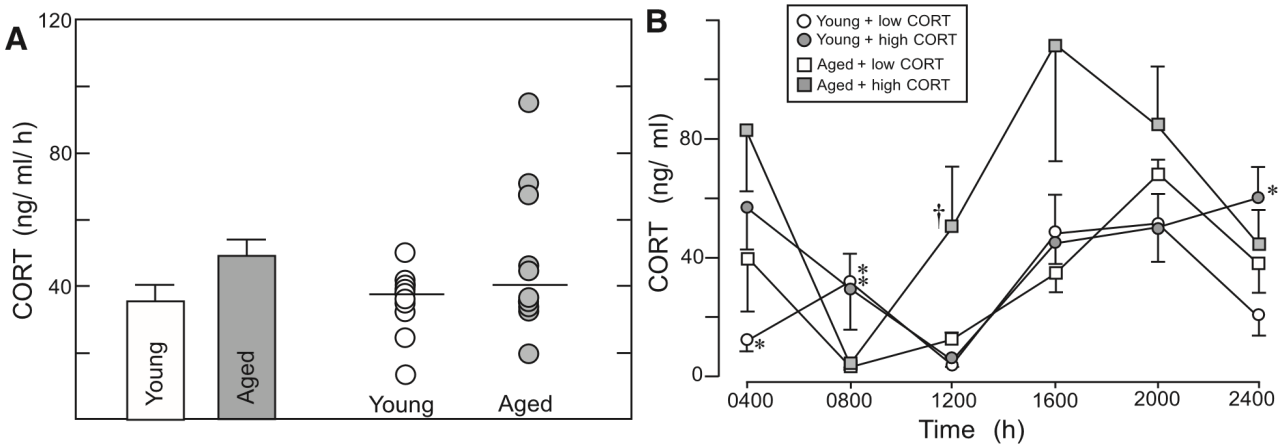

Figure 6. Basal CORT values for Young and Aged animals in the behavioral task

A, Mean SEM for plasma CORT levels averaged across all six timepoints sampled (left), and plots of individual values (right).The horizontal lines indicate median values for each age group. *B*, Mean SEM plasma CORT levels in young (*n* =10) and aged (*n* =10) animals as a function of HPA status sampled at 4 h intervals across the light-dark cycle. *,*p* <0.05, significantly different from low CORT animals. †, *p* <0.05, significantly different from young high CORT and both low CORT groups. *N*=5 per group.

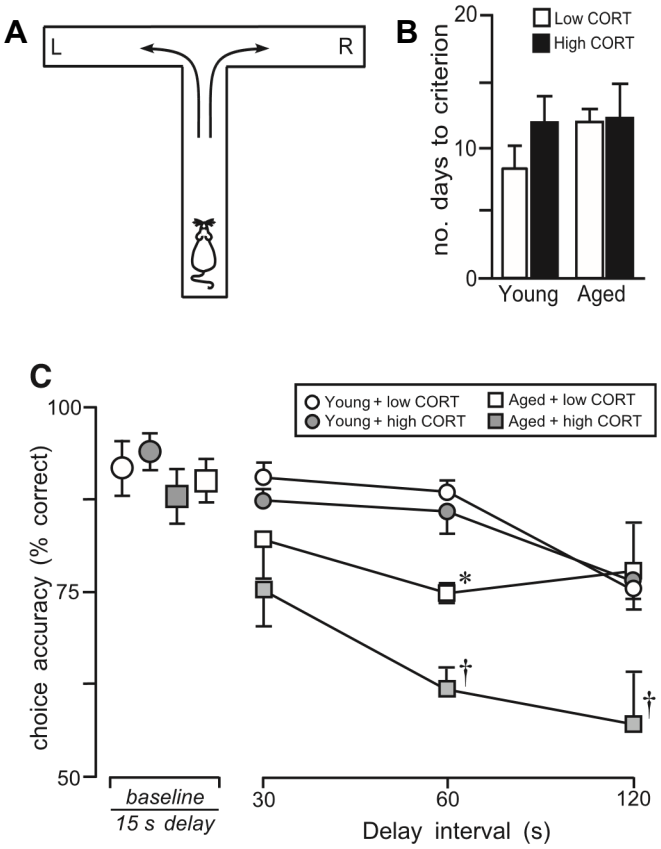

Figure 7. Working memory performance in Young and Aged animals

A, Schematic diagram of T maze (90 x 65 cm) used for delayed alternation. Rats are placed in the starting location (as shown) and were rewarded for selecting the opposite goal arm (e.g., right, R) from the previous trial (left, L). As the delay interval between each trial is increased, the percentage of correct choices provides a measure of spatial working memory. *B*, Histogram represents the average number of days that animals required to reach an equivalent level of performance in the delayed alternation task (70% choice accuracy at a 15 s delay). *C*, Graph demonstrating the percentage of correct responses for baseline performance (i.e., at 15 s delay), and delayed alternation performance. Behavioral impairments were evident in the aged high CORT group with respect to the other three groups at all three delay intervals (60, 120 s), whereas at the 60 s delay, aged low CORT animals also showed a significant decrement in performance. Data represent mean SEM and are based on overall animal averages. *$p <$ 0.05, significantly different from both young groups. †, $p<0.05$, significantly different from both young and aged + low CORT subgroups. N =5 animals per group.

Table 1. Dendritic spine dimensions in layer II/III pyramidal neurons in PL

		PERCENTILE		< 25TH (%)			>75TH (%)		
Dimension	Range	25th	75th	Y	A + low	A + high	Y	A + low	A + High
Head diameter (μm)	*0.1 – 0.895*	*0.209*	*0.383*	*24.5*	*25.3*	*26.1**	*25.4*	*25.8**	*25.4*
Length (μm)	*0.1 – 2.997*	*0.808*	*1.718*	*25.6*	*24.0**	*24.8*	*23.2*	*28.6**	*24.8**

a. For each spine morphologic parameter, minimum and maximum values (range), 25th and 75th percentiles of the entire population, the percentage of spines below the 25th percentile, and the percentage of spines above the 75th percentile are presented for each group. Y, Young; A low, aged + low CORT; A high, aged + high CORT. *, $p < 0.01$ by x^2 goodness-of-fit test (for comparisons of percentiles)

CHAPTER 3: PROLONGED CORTICOSTERONE EXPOSURE INDUCES DENDRITIC SPINE REMODELING AND ATTRITION IN THE RAT MEDIAL PREFRONTAL CORTEX

ABSTRACT

The stress-responsive hypothalamo–pituitary–adrenal (HPA) axis plays a central role in promoting adaptations acutely, whereas adverse effects on physiology and behavior following chronic challenges may result from overactivity of this system. Elevations in glucocorticoids, the end-products of HPA activation, play roles in adaptive and maladaptive processes by targeting cognate receptors throughout neurons in limbic cortical networks to alter synaptic functioning. Because previous work has shown that chronic stress leads to functionally relevant regressive alterations in dendritic spine shape and number in pyramidal neurons in the medial prefrontal cortex (mPFC), this study examines the capacity of sustained increases in circulating corticosterone (B) alone to alter dendritic spine morphology and density in this region. Subcutaneous B pellets were implanted in rats to provide continuous exposure to levels approximating the circadian mean or peak of the steroid for 1, 2, or 3 weeks. Pyramidal neurons in the prelimbic area of the mPFC were selected for intracellular fluorescent dye filling, followed by high-resolution three-dimensional imaging and analysis of dendritic arborization and spine morphometry. Two or more weeks of B exposure decreased dendritic spine volume in the mPFC, whereas higher dose exposure of the steroid resulted in apical dendritic retraction and spine loss in the same cell population, with thin spine sub- types showing the greatest degree of attrition. Finally, these structural alterations were noted to persist following a 3-week

washout period and corresponding restoration of circadian HPA rhythmicity. These studies suggest that prolonged disruptions in adrenocortical functioning may be sufficient to induce enduring regressive structural and functional alterations in the mPFC.

INTRODUCTION

A hallmark of the mammalian stress response entails activation of the hypothalamo–pituitary–adrenal (HPA) axis, a neuroendocrine cascade that stimulates glucocorticoid secretion from the adrenal gland (Antoni, 1986). A substantial body of research suggests that the capacity of environmentally salient stimuli to activate the HPA axis depends on modulatory control from a network of limbic forebrain cell groups that coordinate physiological and behavioral responses for adaptive coping (Feldman and Conforti, 1980; Diorio et al., 1993; Herman et al., 1995b; Jaferi and Bhatnagar, 2006). In rodents, the medial prefrontal cortex (mPFC) is exemplary in this regard, performing both cognitive operations important for behavioral adaptation and neuroendocrine adjustments under stressful conditions.

The prelimbic area is a centrally located subfield within the mPFC that straddles two broad functional domains, with more dorsal- and ventral-lying regions issuing projections that access distinct neural circuitries to modulate limbic-cognitive and visceromotor functions, respectively (Sesack et al., 1989; Vertes, 2004; Gabbott et al., 2005). The PL exhibits robust structural and functional plasticity in response to a variety of environmental stimuli and experiences, thus increasing its repertoire of response capabilities under changing contexts, such as chronic stress. Whereas glucocorticoids have widespread effects on prefrontal functions relevant for adaptation during acute

challenges (Shors et al., 1992; McIntyre et al., 2003; Yuen et al., 2009), perturbations in HPA activity following prolonged stress are associated with maladaptive changes in many of these responses (Mizoguchi et al., 2000; Hains et al., 2009; Hinwood et al., 2012; McKlveen et al., 2013; Radley et al., 2013).

Evidence from our laboratory and others has shown that chronic stress is associated with structural remodeling of dendritic spines in mPFC pyramidal neurons and accompanying impairments in cognitive functioning (Liston et al., 2006; Radley et al., 2006b 2013; Radley et al., 2008 2013), suggesting a role for altered adrenocortical activity in producing these changes. Although there is an extensive literature highlighting the capacity of glucocorticoids to alter hippocampal (Watanabe et al., 1992b; Watanabe et al., 1992a; McEwen, 1998; Sousa et al., 2000; Alfarez et al., 2009; Morales-Medina et al., 2009; Tanokashira et al., 2012; Wosiski-Kuhn et al., 2014) and amygdalar (Vyas et al., 2002a; Mitra et al., 2005; Vyas et al., 2006; Mitra and Sapolsky, 2008; Grillo et al., 2015) plasticity, information regarding glucocorticoid effects on structural plasticity in the mPFC has not been as forthcoming. Evidence that prolonged elevations in glucocorticoids induce regressive dendritic spine alterations in mPFC (Liu and Aghajanian, 2008; Gourley et al., 2013) and related cortical areas (Liston and Gan, 2011; Liston et al., 2013) is contrasted by conflicting reports of either no change (Cerqueira et al., 2007) or increased dendritic spine density in the mPFC (Seib and Wellman, 2003). Another complicating factor in evaluating the available evidence is that much of it is derived from Golgi-based approaches that hamper visualization of small spines that make up 50% of the total population (Dumitriu et al., 2011 2011). Therefore, we critically examine the effects of continuous corticosterone (B) exposure on rat

dendritic spine density and morphometric alterations in pyramidal neurons in the PL using a high-resolution three-dimensional (3D) analytic approach (Rodriguez et al., 2006; Radley et al., 2008). We show that continuous B exposure for at least 2 weeks induces enduring dendritic spine remodeling and loss in the PL that persists even after 3 weeks of restoration of normal HPA axis rhythmicity. These studies highlight a prominent role for alterations in the mPFC that may result from repeated stress exposure or other contexts, such as Cushing's syndrome or aging.

MATERIALS AND METHODS

Animals. The animals used in this study were 3-month-old male Sprague Dawley albino rats (Charles River Laboratories, Kingston, PA). Rats were housed singly and maintained on a 12-hour light/dark cycle (lights on at 0600 hours) with free access to food and water. All experimental protocols were approved by the institutional animal care and use committee of the University of Iowa.

Glucocorticoid treatment regimens

Experiment 1: effects of varying doses of B on structural plasticity in the PL

Rats were adrenalectomized and implanted with slow-release B pellets (i.e., 100 and 200 mg) to clamp circulating levels of this hormone to that of either the circadian mean (9 mg/dl) or the peak (14 mg/dl; see Results) over a 2-week period for the assessment of structural plasticity effects in PL neurons. The decision to perform adrenalectomies in groups of rats receiving constant B was based on the desire to provide an independent assessment of B pellet efficacy that may otherwise be masked by endogenous

adrenocortical activity in an intact animal. Rats were anesthetized with isoflurane and adrenalectomized by the dorsal approach. In the same surgical procedure, adrenalectomized rats were implanted subcutaneously in the interscapular region with 100-mg (n = 9) or 200-mg (n =15) B pellets that provided a constant and slow release into the general circulation for 3 weeks (Innovative Research of America, Sarasota, FL). An additional group of rats received a sham adrenalectomy (ADX; n = 21), which involved all of the same procedural steps except removal of the adrenal gland, and were then implanted with inert cholesterol pellets of weight equal to the B pellets. On day 14, B pellet efficacy and baseline adrenocortical activity in B-replaced and sham rats, respectively, were confirmed by collection of repeated blood samples at AM and PM times and radioimmunoassay of B, and all rats were perfused on the morning of day 15.

Experiment 2: persistence of B-induced structural alterations in the PL following a 3-week recovery period

To assess the persistence of glucocorticoid-induced alterations in PL neuronal architecture, this experiment involved replacement with high-dose B pellets (200 mg) spanning the 3-week duration of its release capability, followed by a 3-week recovery period (n =14). Rats in these experiments were not adrenalectomized to allow for the normalization of circadian HPA activity through the course of the 3-week recovery period. A second group of rats received the same treatment course of B (n = 12); however, their pellet implants were staggered to begin during the second 3-week period of the experiment so that the perfusion and cell loading procedures would be phase locked with the B + recovery group. A third group of rats received implantation of sham

cholesterol pellets (n =10), randomized between the first and second 3-week intervals to parallel procedures in the two groups of B-treated rats. On days 14 and 35, rats from all three groups were subjected to repeated AM and PM blood sampling for radioimmunoassay of plasma B levels. Pellet efficacy (on day 14) and normalization of HPA activity (on day 35) were assessed in the B + recovery group, whereas B efficacy and baseline adrenocortical activity were confirmed in B- and sham implanted rats (days 35 and 14, respectively).

Experiment 3: effects of short-term B exposure on structural plasticity in the PL

To acquire information with respect to the temporal characteristics of glucocorticoid-induced alterations in the PL, dendritic spine morphology was examined in rats exposed to high-dose B (200 mg) compared with sham-implanted controls for 7 days (n =12/group). B pellet efficacy and baseline adrenocortical activity in B replaced and sham rats were confirmed by collection of repeated blood samples at AM and PM times and radioimmunoassay of B on day 5 of steroid exposure.

Blood collection and radioimmunoassay. Adrenocortical activity was assessed by obtaining blood from the tail vein of rats at 0700 and 1700 hours on the day of sampling. Previous work from our laboratory has shown that these time points of sampling correspond well with nadir AM and peak PM values of B secretory activity in adrenally intact rats. For blood collection, rats were restrained briefly (15–30 seconds), and a small longitudinal incision was made at the distal tip of the tail with a sterile blade. Blood samples (~200 μl) were collected into chilled plastic microfuge tubes containing EDTA

and aprotinin, centrifuged, and fractionated for storage of plasma at –80 C until assayed. Plasma B was measured without extraction with an antiserum raised in rabbits against a B-BSA conjugate and 125I-B-BSA as tracer (MP Biomedicals, Santa Ana, CA). Assay sensitivity was 0.8 mg/dl; intra and interassay coefficients of variance were 5% and 10%, respectively.

Histology and tissue processing. Rats were anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused via the ascending aorta with 100 ml 1% paraformaldehyde (PFA) and 0.125% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4), followed by 500 ml of 4% PFA and 0.125% glutaraldehyde in PBS at a flow rate of 55 ml/minute. After postfixation, the pregenual pole of the cortex was sectioned coronally into 250-μm-thick slabs with a VT1000S oscillating tissue slicer (Leica, Wetzlar, Germany) and stored in 0.1 M PBS containing 0.1% sodium azide at 4 °C until the time of cell loading.

Intracellular dye injections. The procedures used in this experiment are from previous work by researchers who used the same methodology (Radley et al., 2006b; Anderson et al., 2014). Coronal tissue slabs were treated in the DNA-binding fluorescent stain 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA) to distinguish among nuclear lamination patterns that distinguish the PL from other adjacent-lying prefrontal cortical subfields. DAPI-treated sections were mounted on nitrocellulose filter paper and submerged in a tissue culture dish containing PBS and viewed under fluorescence with a DM5500 fixed stage microscope (Leica). Injections of 5% Lucifer yellow (LY; Invitrogen) were administered by iontophoresis through micropipettes (1–2-μm inner

diameter) under a DC current of 1–6 nA for 5–10 minutes. The placement of injections was evaluated with reference to standard cytoarchitectonic parcellations of the PL (Krettek and Price, 1977; Vogt and Peters, 1981). The regions are of primary interest for distinguishing the PL from the adjacent-lying regions, from dorsal to ventral, the dorsal subdivision of the anterior cingulate area (ACd), the PL, and the infralimbic area (IL). In DAPI-stained material, the ACd is characterized by a sparse layer 3 and a loosely packed and broad layer 5, which distinguishes it from the more homogeneous layer 5 and large, darkly stained cells that make up the PL. The distinction between the PL and the IL is allowed by the relatively indistinct lamination pattern that emerges ventrally and the irregular border between layers 1 and 3 that typify the IL.

In all three experiments, neurons in layers 2 and 3 the of the PL (corresponding to anteroposterior coordinates 2.9–3.5 μm relative to bregma) were selected for the dye injection procedure, whereas experiment 2 involved the selection of neurons in layers 2, 3, and 5. The general technique for cell filling involved carefully observing the passive diffusion of LY resulting from application of a negligibly small amount of current from the advancing micropipette tip at x40; LY diffuses amorphously until hitting a dendritic process or cell body, at which time the dye becomes restricted intracellularly. After several neurons were filled intracellularly, tissue sections were mounted on glass slides and coverslipped in Vectashield (Vector Laboratories, Burlingame, CA).

Dendritic morphologic analyses of PL neurons in layers 2 and 3. Neuronal reconstructions were performed for the analysis of quantitative changes in dendritic morphology as a function of the B treatment regimens employed. Apical dendrites of pyramidal neurons in layers 2 and 3 of the PL are morphologically heterogeneous and

have been shown to retain relatively consistent quantitative indices (i.e., branch length, number of branch endings), which allows pooling them into a single analysis (Cook and Wellman, 2004; Radley et al., 2004; Liston et al., 2006; Cerqueira et al., 2007). On the other hand, because layer 5 neurons have highly contrasting morphological features (Larkman and Mason, 1990; Kasper et al., 1994; Tsiola et al., 2003; Holtmaat et al., 2005; van Aerde and Feldmeyer, 2015), the dendritic morphologic analyses performed in the current study were restricted to data sets (i.e., experiments 1 and 3) that encompassed neurons in layers 2 and 3 only.

An experimenter unaware of the treatment condition for each animal performed neuronal reconstructions and data analyses. Pyramidal neuron dendritic arbors in layers 2 and 3 of the PL were reconstructed in 3D with a computer-assisted morphometry system consisting of a DM4000R (Leica) equipped with an MS-2000 XYZ computer-controlled motorized stage (Applied Scientific Instrumentation, Eugene, OR), QImaging (Surrey, British Columbia, Canada) Blue digital camera, Dell computer, and morphometry software (MBF Bioscience, Williston, VT). Neurons were visualized, and the dendritic tree was reconstructed with a Leica Apochromat x40 objective with a numerical aperture (NA) of 1.4 in Neurolucida software (RRID:SCR_001775; MBF Bioscience). To be considered for dendritic morphologic analysis, LY filled PL neurons had to exhibit complete filling of the arbor as evidenced by well-defined endings and a minimum amount of truncated branches. Optimal cases involved apical dendritic trajectories that generally coursed parallel to or gently downward from the top surface of the section. Truncations in apical dendrites were permitted only in instances in which collateral branches were deemed to be nearing the point of termination or unable to

make any further bifurcations. For basal dendrites, it was common to retain an average of one to three entirely intact arbors for a given LY-filled neuron, such that analyses on intact branches were performed for this category. Total length (in micrometers) and number of branch endings for apical and basal dendrites were obtained from each neuronal reconstruction and analyzed as described below.

Confocal laser scanning microscopy and dendritic spine analysis. 2D renderings for each neuron were obtained in Neurolucida from the neuronal reconstruction procedures described above, and a radial distance of 150 μm from the soma was selected as a boundary delineating proximal and distal portions of the dendritic tree. Within these regions, branches were randomly selected for imaging from each neuron for an average of three segments per neuron and five neurons for each animal. The selection criteria for confocal imaging of dendritic segments were based on previous reports (Radley et al., 2006b; Radley et al., 2013; Anderson et al., 2014) and required that segments 1) possess a diameter of <3 μm because larger diameter dendrites in PL pyramidal neurons exhibit greater variability in spine density values, 2) reside within a depth of 70 μm from the top surface of the section because of the limited working distance of the optical system, 3) either be parallel to or course gently relative to the coronal surface of the section (i.e., this helps to minimize z-axis distortion and facilitate the unambiguous identification of spines), and 4) have no overlap with other branches that would obscure visualization of spines. In contrast to the dendritic arborization analyses described above, dye-filled neurons containing truncated branches were permissible for their inclusion into the dendritic spine imaging and analysis as long as they exhibited complete filling of the dendritic arbor and well-defined branch tips. z-Stacks were

collected on a Leica SP5 confocal laser scanning microscope equipped with an argon laser and a x100, 1.4 NA oil-immersion objective, with voxel dimensions of 0.1 x 0.1 x 0.1 μm^3. Settings for pinhole size (one airy disc), gain, and offset were initially optimized and then held relatively constant throughout the study to ensure that all images were digitized under similar illumination conditions at a resolution of 512 x 512 pixels. Images were deconvolved with AutoDeblur (Media Cybernetics, Silver Spring, MD), and spine analyses were performed in the semiautomated software NeuronStudio (RRID:SCR_013798; Computational Neurobiology and Imaging Center; http:// research.mssm.edu/cnic/tools-ns.html; Rodriguez et al., 2006, 2008, 2009; Radley et al., 2008; Anderson et al., 2014), which analyzes dendritic length, spine density, and morphometric features (i.e., head/neck diameter, volume, subtype) in 3D for each dendritic spine. Spines were classified as thin or mushroom shaped if the ratio of the head diameter to neck diameter was >1.1. If their ratio exceeded this value, spines with a maximum head diameter >0.4 μm were classified as mushroom shaped; otherwise, they were classified as thin. Spines with head-to-neck diameter ratios < 1.1 were also classified as thin if the ratio of spine length to neck diameter was >2.5; otherwise, they were classified as stubby. A fourth category, filopodial spines, exhibited a long and thin shape, with no enlargement at the distal tip. Because these were observed very seldom, they were classified as thin subtypes. Finally, data readouts from the spine analysis algorithm were visually compared by the experimenter for each optical stack to verify accurate subtype classifications for dendritic spines.

Statistical analysis. Group data from the B radioimmunoassay were compared by repeated-measures ANOVA, followed by pairwise comparisons with Fisher's least

significant difference (LSD) at both morning and evening times. Data are expressed as mean ± SEM. Dendritic branch and spine morphometric data are averaged from each animal (i.e., three to five segments/neuron, five to seven neurons/rat) as a function of treatment. The final group sizes in both experiments are lower than our starting sample sizes reported above. This difference is due to the fact that the success rate from perfused rats that yield suitable numbers of fluorescent dye-filled neurons for inclusion into the analysis was 55%. The effects on overall dendritic length, number of branch endings, and dendritic spine and subtype densities were compared by one-way ANOVA. Population analysis of spine volume as a function of subtype and experimental treatment were analyzed via comparison of cumulative frequency distributions with the KolmogorovSmirnov (K-S) test in Matlab (MathWorks, Natick, MA). Additional correlational tests were performed to determine whether each dependent measure varied as a function of integrated B values for each subject; however, because no significant trends were identified, these results are not reported here. All pairwise comparisons were made with Fisher's LSD with significance set at $P< 0.05$, and data are expressed as mean with SEM. Significance for the K-S test was set at $P< 0.01$.

RESULTS

Experiment 1: effects of varying doses of B on structural plasticity in the PL

Characterization of plasma B levels

Two weeks after pellet implantation (i.e., 100 mg, 200 mg, or cholesterol), blood samples were collected from the tail vein of rats at times approximating the circadian

nadir (0700 hours) and zenith (1700 hours), determined from our previous characterization of the sex, strain, age, and housing conditions of these rats (Anderson et al., 2014), to verify pellet efficacy and plasma B concentrations relative to intact rats (Fig. 8A). Repeated-measures ANOVA showed main effects for time of day ($F_{(1,19)}$ = 57.2, $P< 0.05$) and interaction between time of day and treatment group ($F_{(2,19)}$= 10.5, $P< 0.05$). Although B levels held constant throughout the day in ADX + 100B (i.e., 100 mg B) and ADX + 200B (i.e., 200 mg B) groups, sham ADX rats showed lower AM and increased PM plasma B levels (Fig. 8). Pairwise comparisons revealed plasma levels of B in ADX +100B and ADX + 200B that were lower in PM and higher in AM, respectively, compared with sham ADX rats ($P< 0.05$ for each; Fig. 1B). These data suggest resultant plasma B levels in the 100B group that roughly approximated the circadian mean (9 mg/dl), with the 200B group displaying closer to circadian peak ADX + 100B and ADX + 200B that were lower in PM and higher in AM, respectively, compared with sham ADX rats ($P< 0.05$ for each; Fig. 8B). These data suggest resultant plasma B levels in the 100B group that roughly approximated the circadian mean (9 mg/dl), with the 200B group displaying closer to circadian peak values (14 mg/dl) compared with sham ADX rats (11 and 16 mg/dl, respectively).

Chronic elevations in plasma B induced dendritic spine loss in PL pyramidal neurons.

Figures 9 and 10 illustrate groups of LY-filled pyramidal neurons in layers 2 and 3 of the PL that were subjected to dendritic spine morphometric analyses (198, 106, and 109 dendritic segments in sham, 100B, and 200B groups, respectively). These segments yielded a total of 54,267 dendritic spines subjected to density, subtype, and morphologic analysis (sham, n = 10 rats/27,225 spines; 100B, n = 6 rats/14,458 spines;

200B, n = 6 rats/12,554 spines; Table 2). One-way ANOVA performed at different portions of the dendritic tree revealed most pronounced main effects in distal apical dendrites (i.e., > 150 mm; $F_{(2,20)} = 3.873$, P = 0.04; Fig. 4). ADX 1 200B rats displayed a 21% decrease in dendritic spine density within this region and a 13% decrease when combined across all three regions compared with sham ADX rats (P< 0.01 for each). By contrast, ADX + 100B treatment failed to produce any reliable differences in dendritic spine density relative to control or 200B-dose groups (Fig. 11, Table 2).

Additional analyses of dendritic arborization patterns (overall length, branch number) in the same set of PL neurons were undertaken to assess the extent to which dendritic spine loss may be accounted for by larger scale changes in dendritic morphology. These analyses revealed a main effect for apical dendritic length ($F_{(2,20)} = 3.5$, P< 0.05). Post hoc comparisons revealed a 23% reduction in apical dendritic length in B200 rats relative to sham control rats (P= 0.02), whereas no significant reduction was evident between 100B and sham control groups (P= 0.2; Fig. 12). Prolonged B exposure failed to alter the number of branch endings in apical dendrites and also had no effect on either index in basal dendrites (Fig. 12, Table 2). When apical dendritic shortening and decreases in spine density are taken together, this compounds the net loss of dendritic spines in layers 2 and 3 PL neurons to levels approximating 67% of control values following prolonged high dose exposure of glucocorticoids.

Effects of high levels of B on PL dendritic spine morphology

Dendritic spines can be distinguished by geometric characteristics (i.e., thin, mushroom, stubby) that have proved to be useful for inferring synaptic structure–function relationships (Kasai et al., 2003; Bourne and Harris, 2007; Yang et al., 2009;

Dumitriu et al., 2010; Lee et al., 2012; Fig. 13A). In cortical pyramidal neurons, spines classified as thin represent the majority of the population (60–70%; Bourne and Harris, 2007), and previous studies have identified this subtype as important for long-term potentiation and learning-related plasticity (Arnsten et al., 2010; Anderson et al., 2014). In our analysis of spine subtype (Fig. 13A), thin spines were the most vulnerable to prolonged high-dose glucocorticoid exposure, particularly within more distal apical dendrites ($F_{(2,20)} = 3.6$, $P < 0.05$). Post hoc comparisons revealed a significant 15% decrement of thin spines in combined apical and basal segments in the 200B group compared with control rats ($P < 0.05$), with the most significant loss on the most distal aspects (by 24%, $P < 0.05$; Fig. 13B). In contrast, examination of thin spine densities in the 100B group failed to demonstrate any reliable shifts relative to control or 200B rats (Fig. 13B, Table 1).

Evaluation of other spine subtypes (mushroom, stubby) did not uncover any differences following prolonged glucocorticoid exposure (Fig. 13C,D). Moreover, additional analyses of several spine parameters (length, head diameter, volume) did not reveal any group differences as a function of glucocorticoid treatment (data not shown). However, population analysis of spine volume revealed cumulative frequency shifts following both regimens of glucocorticoid treatment. Comparison of frequency distributions of overall spine populations revealed downward trends (i.e., leftward shifts) in volume in both 100B and 200B rats relative to control groups ($P = 0.02$ and $P < 0.01$, respectively, K-S test), with the 200B spine population showing an even greater shift relative to 100B ($P < 0.01$, K-S test; Fig. 14A). Examination of frequency shifts in subtypes revealed the greatest effects in thin spines (Fig. 14B); 100B showed a leftward

shift relative to controls (P< 0.01, K-S test), whereas 200B showed significant shifts relative to both control and 100B populations (P< 0.01 for each, K-S test). Cumulative frequencies plotted for mushroom spine subtypes also revealed a leftward shift in the 200B treatment group relative to 100B and control groups (P< 0.01 for each, K-S test), whereas the 100B group failed to show any differences in this index relative to the control population (P= 0.7, K-S test; Fig. 14C). These observations support the interpretation that prolonged B exposure induces dendritic spine shrinkage in PL neurons even in the absence of any frank loss of spines in the case of low-dose B, whereas high-dose B results in more generalized decreases in spine volume across subtypes as well as attrition of thin spines.

Experiment 2: persistence of B-induced structural alterations in the PL following a 3-week recovery period

Characterization of plasma B levels

Next, we examined whether a washout period following prolonged B exposure would ameliorate the regressive structural alterations in PL dendritic spines (Fig. 15A). In this experiment, we extended our spine morphologic analysis to include neurons in layers 2, 3, and 5 to assess the extent to which glucocorticoid-induced spine loss generalizes across different laminae in the PL. On day 14 after steroid implantation, blood samples were collected from the tail vein of rats in the control and 200B + recovery groups to verify pellet efficacy and plasma B concentrations, and blood samples were collected again on day 35 (i.e., in 200B and 200B + recovery groups) to verify the normalization of

adrenocortical activity during the washout period. Repeated-measures ANOVA showed main effects for treatment group ($F_{(3,45)} = 5.3$, $P < 0.05$), time of day ($F_{(1,45)} = 7.2$, $P < 0.05$), and interaction ($F_{(3,45)} = 24.0$, $P < 0.05$). B levels remained consistently elevated near the circadian zenith during the 21-day B exposure time frame in both 200B and 200B + recovery groups compared with sham controls (Fig. 15B). 200B + recovery rats displayed normalization of AM and PM levels of plasma B when assayed during the washout period on day 35 (Fig. 15B).

Lack of normalization in PL dendritic spine morphology following recovery from B exposure

In this experiment, 460 dendritic segments from 153 fluorescent dye-labeled PL neurons in layers 2, 3, and 5 were analyzed for spine density and morphometric analysis (120, 160, and 180 dendritic segments in control, 200B, and 200B + recovery groups, respectively). These segments yielded a total of 61,600 dendritic spines subjected to density, subtype, and morphologic analysis (sham, n = 6 rats/17,400 spines; 200B, n = 7 rats/ 20,800 spines; 200B + recovery, n = 8 rats/23,400 spines; Table 2). One-way ANOVA of spine density in layers 2, 3, and 5 pyramidal neurons revealed a main effect as a function of experimental treatment ($F_{(2,18)} = 4.6$, $P < 0.05$; Fig. 9A–C). Post hoc analyses revealed significant decreases in 200B and 200B 1 recovery groups relative to controls (by 16%, $P < 0.05$ for each; Fig. 16C), suggesting that a 21-day washout period was insufficient to reverse glucocorticoid-induced decrements in spine density, even after normalization of circadian adrenocortical activity. Regional analysis of apical and basal subdivisions demonstrated downward trends throughout, with statistically significant reductions evident in the proximal region of the apical tree in the 200B group

(P< 0.05) and basal dendrites of the 200B + recovery group (P< 0.05) relative to controls. In contrast, no trends or statistically significant differences in spine density were evident in the 200B and 200B + recovery groups. Examination of spine subtype revealed generalized downward trends in both 200B and 200B + recovery groups. These effects were most noteworthy in overall mushroom ($F_{(2,18)}$ = 3.5, P = 0.052) and overall thin ($F_{(2,18)}$ = 2.8, P= 0.08) categories; however, neither was statistically significant (Fig. 16D–F). Follow-up analysis of population shifts in spine volume revealed significant decrements in both 200B and 200B + recovery groups compared with control animals (P< 0.01 for each, K-S test), whereas the frequency distributions between 200B and 200B + recovery groups were not statistically different (P= 0.11, K-S test; Fig. 17A). Population analyses performed within thin and mushroom spines also revealed similar leftward (downward) shifts in volume in 200B and 200B + recovery groups relative to controls (P< 0.01 for each, K-S test), whereas no statistically significant differences were noted via comparison of cumulative distributions between 200B and 200B + recovery groups (Fig. 17B,C). These data endorse the results from the first experiment that high-dose glucocorticoid exposure may induce a persistent attrition of dendritic spines throughout pyramidal neurons in the PL, likely via one or multiple mechanisms involving spine pruning and shrinkage.

Experiment 3: effects of short-term B exposure on structural plasticity in the PL

Because the first two experiments had demonstrated that 2 weeks or longer of prolonged high-dose glucocorticoid exposure induces regressive structural plasticity in PL neurons, in a final experiment we addressed whether a shorter duration (i.e., 1

week) of high-dose B exposure could produce similar changes. In these studies, we again restricted our analyses to PL neurons within layers 2 and 3 to compare both dendritic morphology and density. Nevertheless, this analysis failed to reveal any effects on any of the structural indices examined (Fig. 18, Table 4). These data, when taken together with the other two experiments, suggest that >1 week of continuous exposure to glucocorticoids is required for the induction of regressive modifications in PL neurons.

DISCUSSION

It has been widely documented that chronic stress leads to the reorganization of neuronal architecture in the mPFC. Here, we expand on these findings by showing that increasing plasma levels of B over a period of at least 2 weeks induces apical dendritic shortening, spine loss, and morphological alterations in PL pyramidal neurons. A more surprising result is that these structural alterations persist even after a washout period and its attendant restoration of circadian B secretory activity. These data generally endorse the view that chronic stress-induced effects on prefrontal structural plasticity involve a glucocorticoid-dependent component. However, they contrast with the effects of repeated stress, whereby recovery periods restore dendrite and spine morphologic indices (Radley et al., 2005; Bloss et al., 2010; Bloss et al., 2011). It is worth emphasizing that prolonged elevations in glucocorticoids are not taken to be equivalent to stress. In fact, elevated glucocorticoids may be more broadly representative of dysregulated homeostatic parameters produced by systemic diseases or by prolonged pharmacotherapeutic regimens under certain chronic conditions. Therefore, our data implicate disrupted adrenocortical function in the induction of protracted synaptic

reorganization in the mPFC that may follow prolonged stress or other diseases involving endocrine disruptions.

Methodological considerations

Many repeated stress manipulations are known to decrease dendrite and spine morphologic indices reliably throughout mPFC subfields in the rat (Cook and Wellman, 2004; Radley et al., 2004; Liston et al., 2006; Michelsen et al., 2007; Perez-Cruz et al., 2007; Liu and Aghajanian, 2008; Radley et al., 2008; Dias-Ferreira et al., 2009; Hains et al., 2009; Holmes and Wellman, 2009; Shansky et al., 2009b). However, previous work investigating the effects of repeated B exposure on mPFC structural plasticity has produced mixed results. In one series of studies, repeated daily glucocorticoid injections for 3 weeks differentially altered mPFC pyramidal neuron dendritic morphology, marked by increased and decreased dendritic branching patterns in proximal and distal aspects of the dendritic tree, respectively, and increased dendritic spine density in the same cell types (Wellman, 2001; Seib and Wellman, 2003). Another study showed decreased dendrite length, branching patterns, and anatomical regional volumes in mPFC subfields, with no change in spine density after 1 month of daily glucocorticoid injections (Cerqueira et al., 2005; Cerqueira et al., 2007). In contrast, studies that have administered corticosteroids through drinking water for 10–20 days have also observed mPFC spine density decrements comparable to those induced by stress (Liu and Aghajanian, 2008; Gourley et al., 2013). Although it is possible that some of these discrepancies are attributable to varying modes of glucocorticoid administration (i.e., injections, drinking water, subcutaneous implants), different approaches for visualization and quantification of spines are also likely contributors. For instance, much of the

information from this field of study is derived from the Golgi impregnation method, yet recent evidence suggests that this approach undersamples dendritic spine densities (by > 50%) and specifically biases against the identification of thin subtypes (Dumitriu et al., 2011; Dumitriu et al., 2012; Radley et al., 2015). Nevertheless, the present results and the weight of prior evidence support our interpretation that prolonged glucocorticoid exposure leads to regressive and persistent dendritic spine alterations in mPFC neurons.

The current study also highlights the importance of applying more refined technical approaches to understand glucocorticoid effects on structural plasticity better. In another line of investigation, Liston and Gan (2011) applied two-photon microscopy for time-lapse imaging of dendritic spines in the mouse somatosensory cortex (S1) and showed that prolonged glucocorticoid exposure concurrently eliminated existing spines and prevented new spine formation. Although there are some constraints in the level of resolution that is currently achievable with in vivo two-photon microscopy, the ability to perform repeated sampling has provided fundamentally important information with respect to how glucocorticoids influence spine dynamics (Liston et al., 2013). Electron microscopic approaches will continue to provide important ultrastructural detail for understanding glucocorticoid effects on synaptic alterations, and emerging developments in automated technologies may soon allow higher throughput screening at this level of resolution (Kasthuri et al., 2015). In the meantime, the dendritic spine morphometric approach employed here balances the attainment of high-quality resolution with high-throughput that cannot yet be achieved with other methods.

Interpretive considerations

Comparing and contrasting data from each of the experiments presented in this report raises some interesting questions with respect to possible differences in effects of glucocorticoids on dendritic spine alterations. First, we failed to observe consistent decreases in thin spine subtypes as a function of glucocorticoids across experiments. In the initial experiment, high-dose treatment with B for 2 weeks induced significant decrements in spine density that were manifested by a clear loss of thin spines in PL neurons, whereas, in the second experiment, 3-week B exposure failed to result in a statistically significant reduction in this subtype (e.g., Fig. 16D). This likely is due to the fact that the former experiment examined only layer 3 PL neurons, and the latter involved the analysis of both layer 3 and layer 5 PL neurons. Because we often observe lower overall spine densities in layer 5 relative to layer 3 pyramidal neurons in the mPFC (Anderson and Radley, unpublished observations), combining these into one analysis might have increased the variance enough to reduce the reliability of the observed effects. Given that experience-dependent dendritic spine plasticity has been shown to vary as a function of apical dendritic morphology in layer 5 neurons in S1 (Holtmaat et al., 2005), it is possible that our failure to examine dendritic morphology in layer 5 PL neurons or partition subpopulations accordingly added an additional source of variability in experiment 2.

A second interpretive issue concerns whether rats that were subjected to a longer period of B exposure (i.e., 3 weeks in the second experiment as opposed to 2 weeks in the first) might have shown greater spine loss in the PL. Although there were no frank differences in the overall proportion of spines lost between experiments (by

13% and 16% in experiments 1 and 2, respectively), the second experiment revealed more expansive downward trends in both thin (P= 0.08) and mushroom (P= 0.052) subtypes, whereas the first displayed only decreases in thin and no downward trend in mushroom subtypes. Because mushroom spines are representative of a category of larger volume, stable, and mature synaptic phenotypes (Harris and Stevens, 1989; Kasai et al., 2003; Holtmaat et al., 2005; Knott et al., 2006; Yasumatsu et al., 2008), one possibility is that B-induced spine shrinkage of this subtype requires longer intervals of steroid exposure. In this scenario, mushroom spine shrinkage could fall below our threshold criteria for being classified as mushroom (i.e., spine head diameter > 0.4 μm; see Materials and Methods), resulting in their reclassification as thin. The idea that an extended period of glucocorticoid exposure can lead to the selective targeting of large-volume spines is supported by at least two studies (Tanokashira et al., 2012; Liston et al., 2013) and may involve rearrangement of the F-actin network in the spine head via downregulation of the stabilizing protein caldesmon (Tanokashira et al., 2012). This caldesmon-dependent signaling pathway underlying spine shrinkage is also distinct from the aforementioned mechanisms for spine formation/elimination, raising the possibility that the spine loss and shrinkage that we observed following high-dose B treatment may be regulated via distinct mechanisms. Support for this idea might also derive from the observation that prolonged lower dose B treatment in the first experiment resulted in reliable decreases only in spine volume.

Our results in the second experiment show that, after 3 weeks of repeated B exposure, dendritic spine alterations were evident even in rats provided with a 3-week washout period that allowed for the normalization of HPA rhythmicity after high-dose B

exposure. At least one previous study has shown that dendritic spine decreases in prefrontal subfields exhibit regionally differentiated patterns of partial recovery following prolonged B exposure (i.e., in infralimbic but not orbitofrontal cortex) after a 7-day washout period (Gourley et al., 2013). One possibility is that the PL may be less resilient to corticosteroid-induced dendritic spine alterations, even the restoration of HPA rhythmicity, although more work is required to understand better how stressful or traumatic experiences may induce long-lasting disruptions in prefrontal networks.

The data presented here may also help to conceptualize how hypercortisolism in Cushing's syndrome or aging could lead to enduring regressive structural and functional changes in mPFC synaptic networks. Patients with Cushing's syndrome display cognitive and prefrontal functional deficits that linger after the normalization of glucocorticoids (Tiemensma et al., 2010a; Tiemensma et al., 2010b; Andela et al., 2015). Recent evidence suggests that diminished prefrontal functional connectivity and cortical thinning in Cushing's syndrome persist even after long-term remission of elevated glucocorticoids (Crespo et al., 2014; Bas-Hoogendam et al., 2015). The cumulative exposure to glucocorticoids in rats has also recently been implicated in age-related decreases in dendritic spine density in mPFC and prefrontal functioning (Anderson et al., 2014), although many studies have linked elevated glucocorticoids in aging with hippocampal and prefrontal impairments (Landfield et al., 1978 1978; Issa et al., 1990; Sapolsky, 1992; Lupien et al., 1994; Mizoguchi et al., 2009; Garrido, 2010).

Role of disruptions in the adrenocortical rhythm

One lingering question concerns the extent to which B-mediated prefrontal structural alterations may be explained by elevated plasma levels, per se, or the resultant disruption of circadian rhythmicity resulting from the continuous release of steroid into the circulation achieved with subcutaneous implants. Because HPA axis dysregulation may often reflect a flattening of the B secretory rhythm in a variety of human clinical disorders (Miller et al., 2007 2007; Hall et al., 2015), this may have relevance for the repeated glucocorticoid regimen used in the present study. Studies utilizing other methods of repeated B administration that likely enhance circadian peaks of B have also observed similar effects on structural plasticity (Liu and Aghajanian, 2008; Liston and Gan, 2011; Gourley et al., 2013; Swanson et al., 2013), although information is not yet forthcoming regarding the precise morphological differences in spine volume and subtype under these varying regimens of glucocorticoid treatment. Several recent studies have linked glucocorticoid circadian rhythmicity to natural variations in spine formation and elimination (i.e., in somatosensory cortex and hippocampus; Liston et al., 2013; Ikeda et al., 2015). In two other studies, disruption of the circadian photoperiod induced regressive structural changes in the hippocampus and the mPFC along with corresponding cognitive impairments (Pyter et al., 2005; Karatsoreos et al., 2011). These latter studies warrant consideration with respect to whether changes in adrenocortical secretory patterns underlie cognitive impairments following circadian disruption and the extent to which changes in circadian secretory patterns vs. increased levels of B account for prefrontal structural and functional alterations in other experimental and clinical contexts.

Functional considerations

Thin spine subtypes in prefrontal neurons have proved to be highly vulnerable to pruning in response to a variety of contexts, including aging and chronic stress (Dumitriu et al., 2010; Bloss et al., 2011; Radley et al., 2013; Anderson et al., 2014). More recently, thin spine loss in the prefrontal cortex has been implicated in psychiatric illnesses, such as schizophrenia, and psychostimulant use (Arnsten et al., 2010; McEwen and Morrison, 2013; Radley et al., 2015), and our data implicate altered adrenocortical functioning as a contributing factor to prefrontal synaptic reorganization in these settings. One important question concerns whether prolonged glucocorticoid exposure decreases thin spines through a mechanism involving impaired spine formation and/or increased elimination. Recent evidence suggests that short-term exposure to glucocorticoids mediates both processes via separate signaling pathways, spine formation via glucocorticoid receptor (GR) type I activation via an LIM kinase-cofilin pathway and spine elimination through a GR type II transcriptionally dependent mechanism (Liston et al., 2013). Future studies are required to understand better the precise mechanisms underlying prefrontal spine attrition in response to prolonged periods of glucocorticoid exposure, chronic stress, and other contexts involving altered adrenocortical functioning.

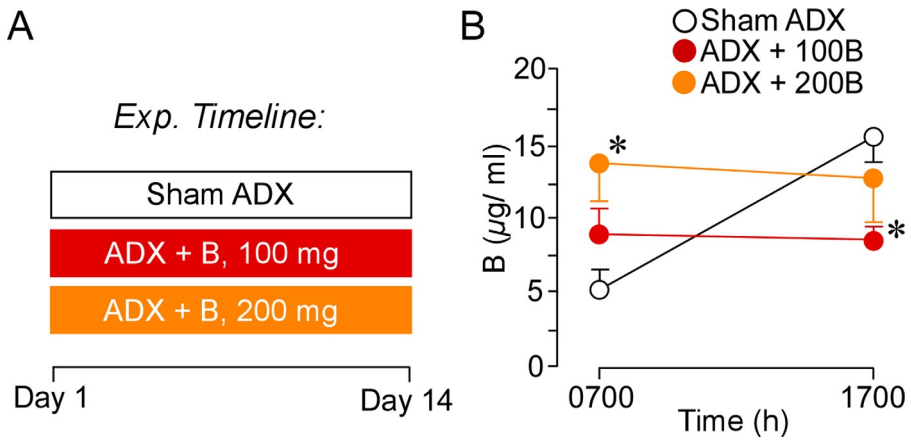

Figure 8. Basal CORT values for B- and sham-treated animals

A: Time line of the first experiment. On day 1, animals underwent ADX surgeries and B pellet implantation or sham ADX and cholesterol pellet implantation. On day 14, blood was collected to assay for effectiveness of pellet increasing B levels. B: Graph depicting mean ± SEM plasma B levels at AM and PM sampling of B as a function of treatment group. These data reveal that 200-mg B (200B) pellets clamp levels to peak circadian levels, whereas 100-mg B (100B) pellets clamp B levels to values approximating the circadian mean. Control, n = 10; 100B and 200B, n = 6. *$P < 0.05$, significantly different from sham-treated animals.

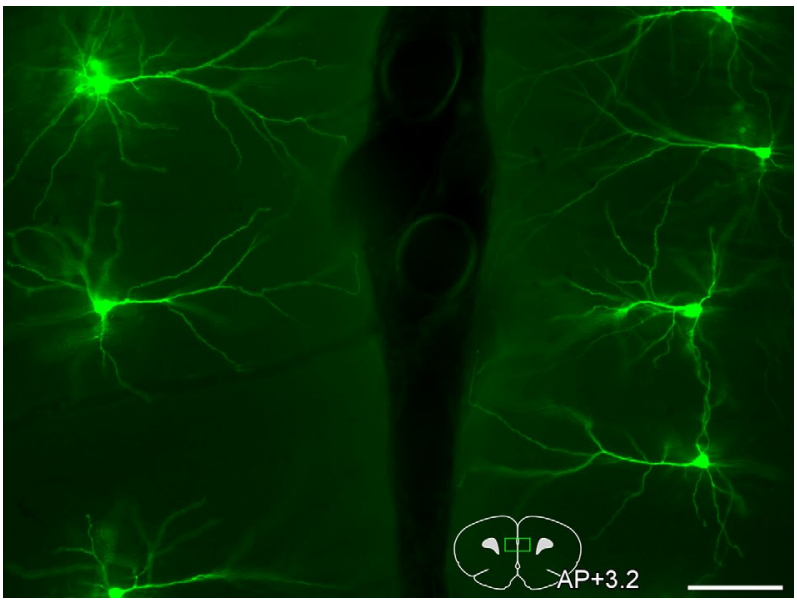

Figure 9. Darkfield photomicrograph depicting an array of bilateral layer II/III pyramidal neurons

Darkfield photomicrograph depicting an array of bilateral layer 2 and 3 pyramidal neurons in the PL targeted for intracellular dye injection with LY (pseudocolored green). An atlas plate (lower right) depicts the approximate region within the PL in which neurons were filled for morphologic analyses. Distance in millimeters relative to bregma is indicated. AP, anteroposterior. Scale bar = 100 μm.

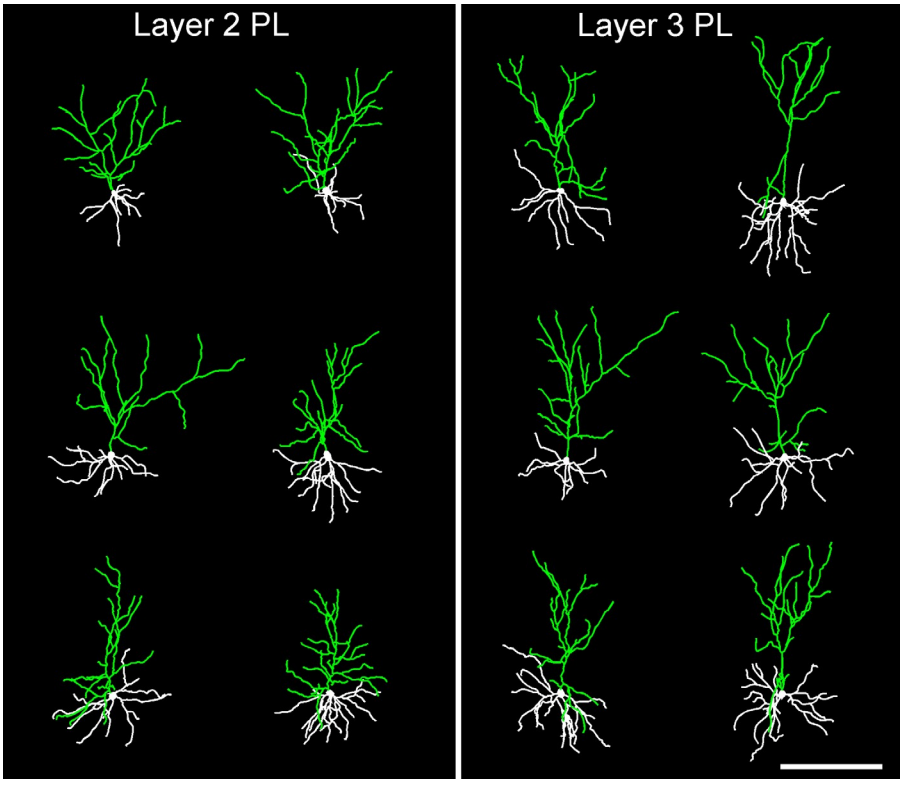

Figure 10. Examples of 3D digital reconstructions of layer II/III pyramidal neurons

Examples of 3D digital reconstructions showing LY-filled layer 2 (left) and layer 3 (right) pyramidal neurons in the PL. Contrasting apical dendritic morphologies between these neuronal subtypes are highlighted in green, with layer 3 neurons exhibiting apical dendrites with an initial bifurcation point more distal to the cell body than in layer 2. Scale bar = 200 μm.

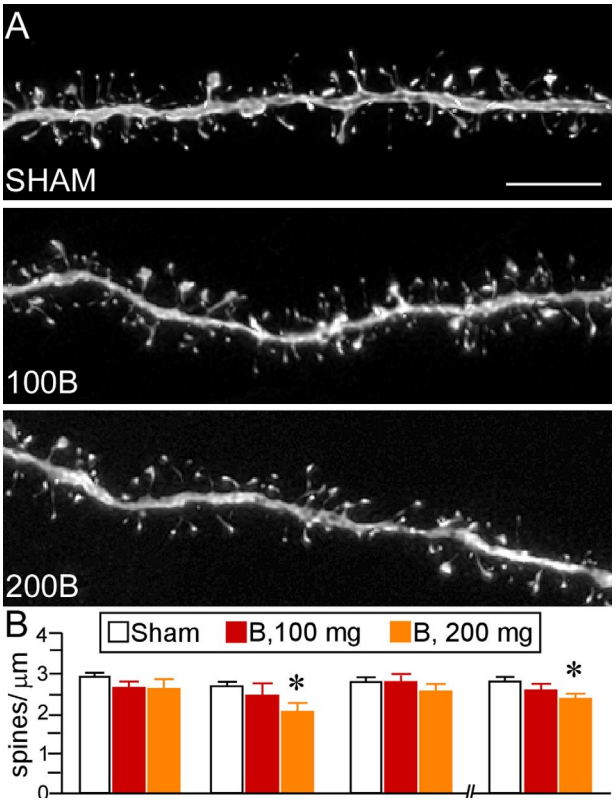

Figure 11. Dendritic spine density in B- and sham-treated animals

A: Deconvolved images of dendritic segments from layer 2 and 3 pyramidal neurons in the PL from different treatment groups. B: Mean and SEM of dendritic spine density as a function of B treatment. B100 (i.e., 100 mg B) animals show no differences in spine density relative to controls within any region of the dendritic tree. B200 (i.e., 200 mg B) rats show overall spine loss, notably in the more distal aspects of the apical tree. Control, n = 10; B100 and B200, n = 6. *$P < 0.05$, significantly different from sham rats. Scale bar = 5 µm.

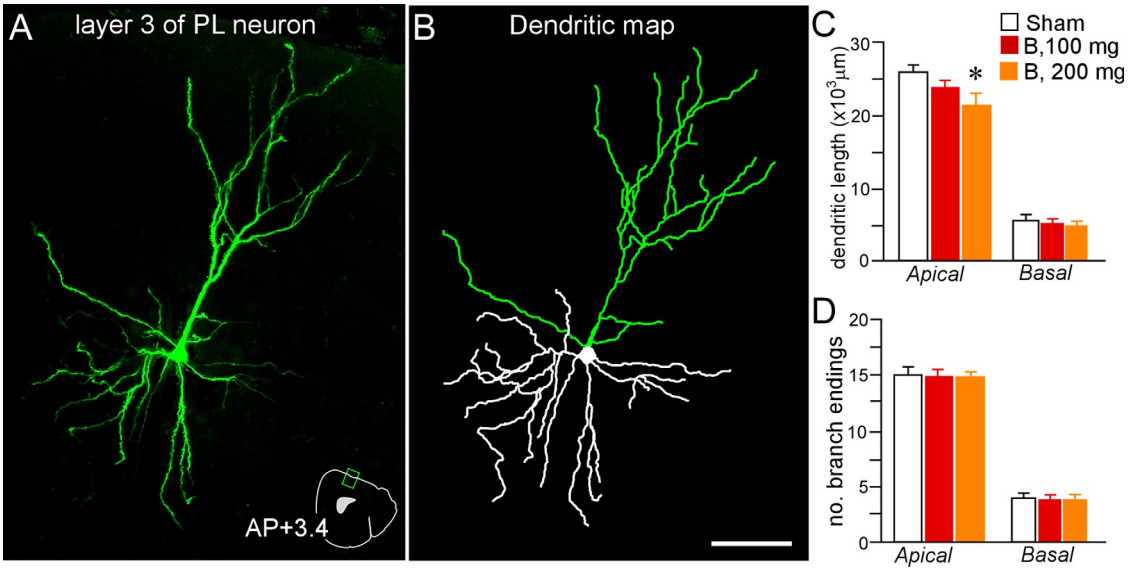

Figure 12. Dendritic arborization in B- and Sham-treated animals

3D digital reconstruction of LY-filled layer 2 and 3 PL neuron (pseudocolored green) with confocal laser-scanning microscopy (A) and the rendering of its dendritic tree with computer-assisted morphometry (B). An atlas plate (A, lower right) depicts the neuron's approximate location and angle of orientation within the PL. Distance in millimeters relative to bregma is indicated. AP, anteroposterior. Mean and SEM for dendritic length (C) and number of branch endings (D) for apical and basal dendrites as a function of treatment group. Only rats that received high-dose glucocorticoids (200 mg) displayed apical dendritic shrinkage. Control, n = 10; B100 and B200 (i.e., 100 and 200 mg B, respectively), n = 6. *$P < 0.05$, significantly different from sham rats. Scale bar = 75 µm

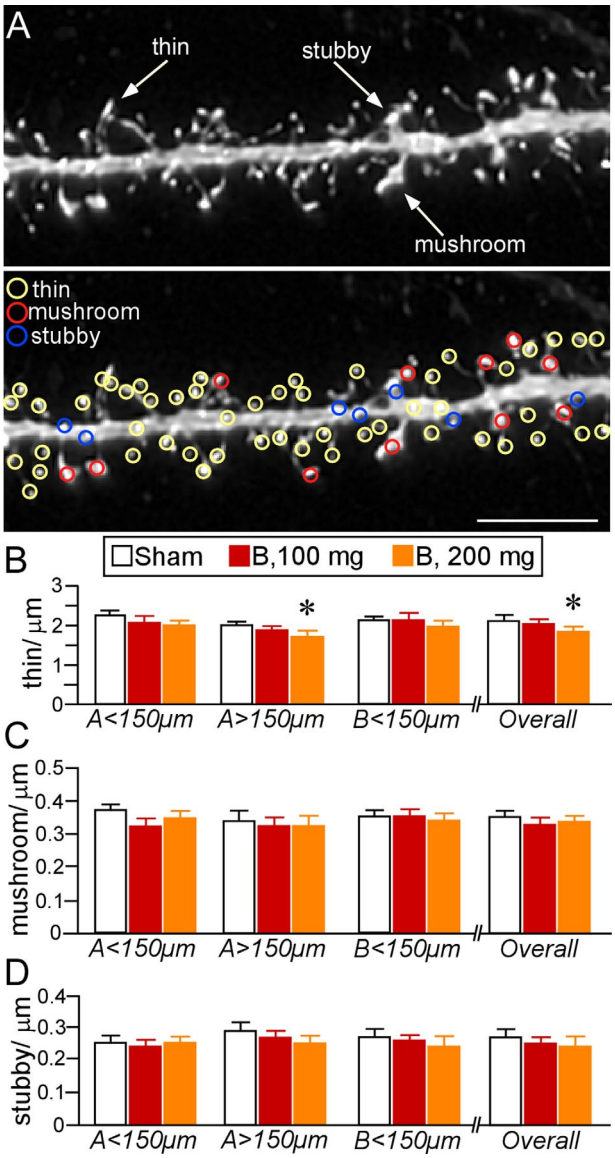

Figure 13. Dendritic spine subtype density in B- and Sham-treated animals

A: Example of high-resolution deconvolved optical z- stack of a dendritic segment used for spine analysis in NeuroStudio. Circles designate spine subtypes based on user-defined parameters in the software. B–D: Mean ± SEM of dendritic spine subtypes. Thin spine loss was evident in B200 (i.e., 200 mg B) rats (B), especially at radial distances >150 µm. No other effects were evident in other subtypes (C,D). Control, n =10; B100 and B200, n = 6. *$P < 0.05$, significantly different from sham rats. Scale bar = 5 µm.

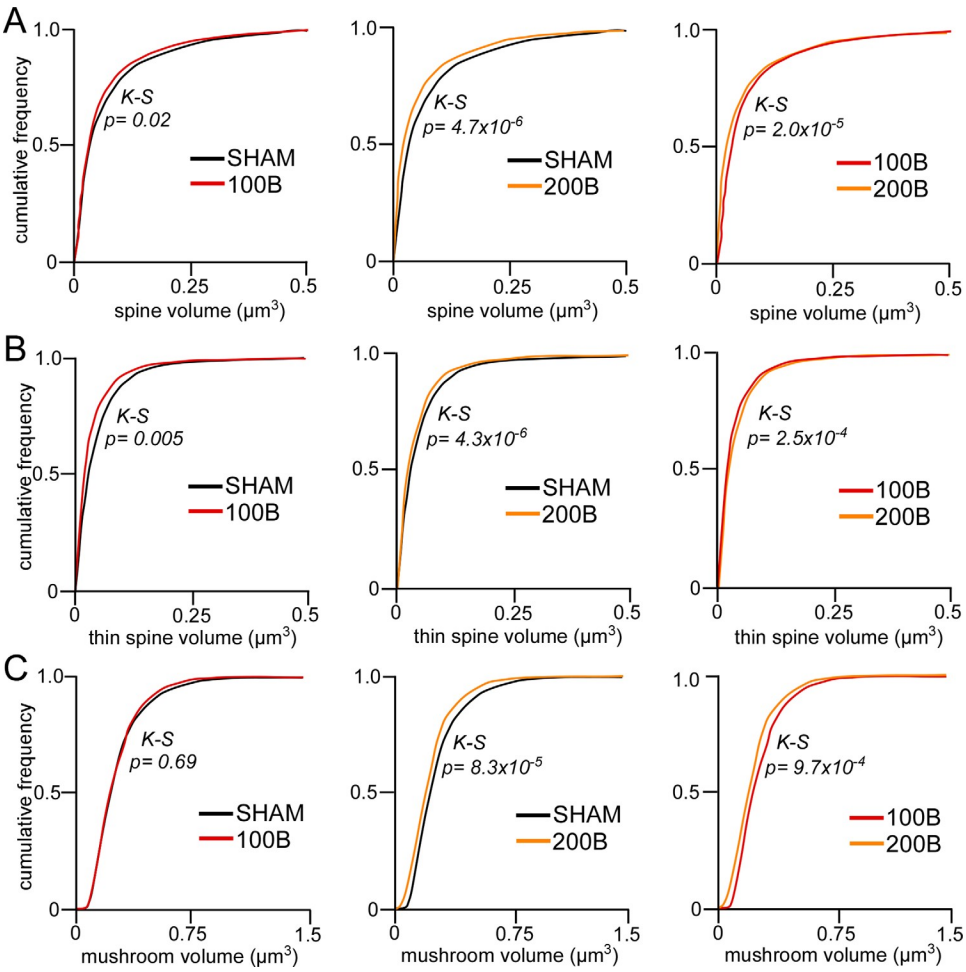

Figure 14. Cumulative distribution frequencies of B- and Sham-treated animals

A: Cumulative frequency distributions of overall spine volume in PL neurons reveal graded leftward shifts (i.e., decrease) in spine volume in 100B and 200B (i.e., 100 and 200 mg B, respectively) rats. B: This trend is recapitulated in thin spine volumes. C: In contrast, B200 rats show selective decreases in mushroom spine volumes relative to B100 and sham groups. Significance set at $P < 0.01$, K-S test

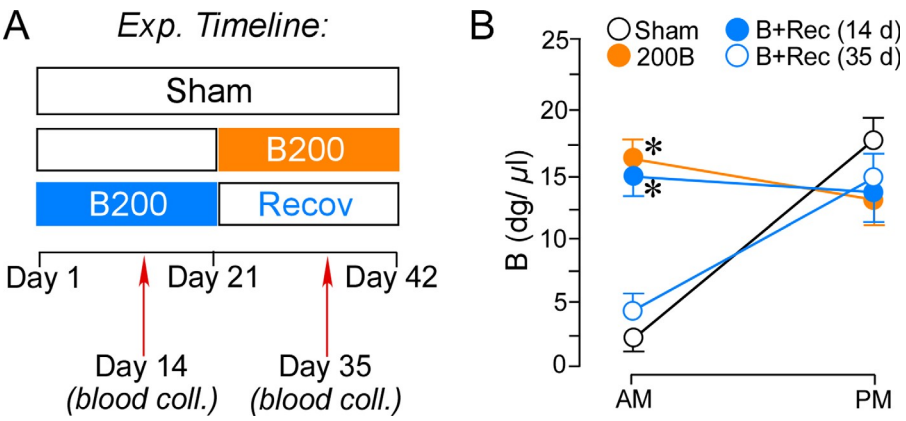

Figure 15. Basal CORT in B-, B-recovery, and Sham-treated animals

A: Time line of the second experiment for high-dose B treatment (B200) and comparison with groups of rats given a 3-week recovery period (B + Recov) to restore HPA rhythmicity. On day 14, blood was collected in sham and B + Recov animals to assay for effectiveness of pellet increasing B levels. On day 35, blood was collected to assay for effectiveness of pellet increasing B levels in B200 animals and in B + Recov to assay for HPA activity restoration. B: B200 and B200 + Recov (Rec) groups show elevated B, and B + Rec rats show a normalization of adrenocortical function after the cessation of B exposure. N = 6–8 rats/group. *$P < 0.05$, significantly different from sham group

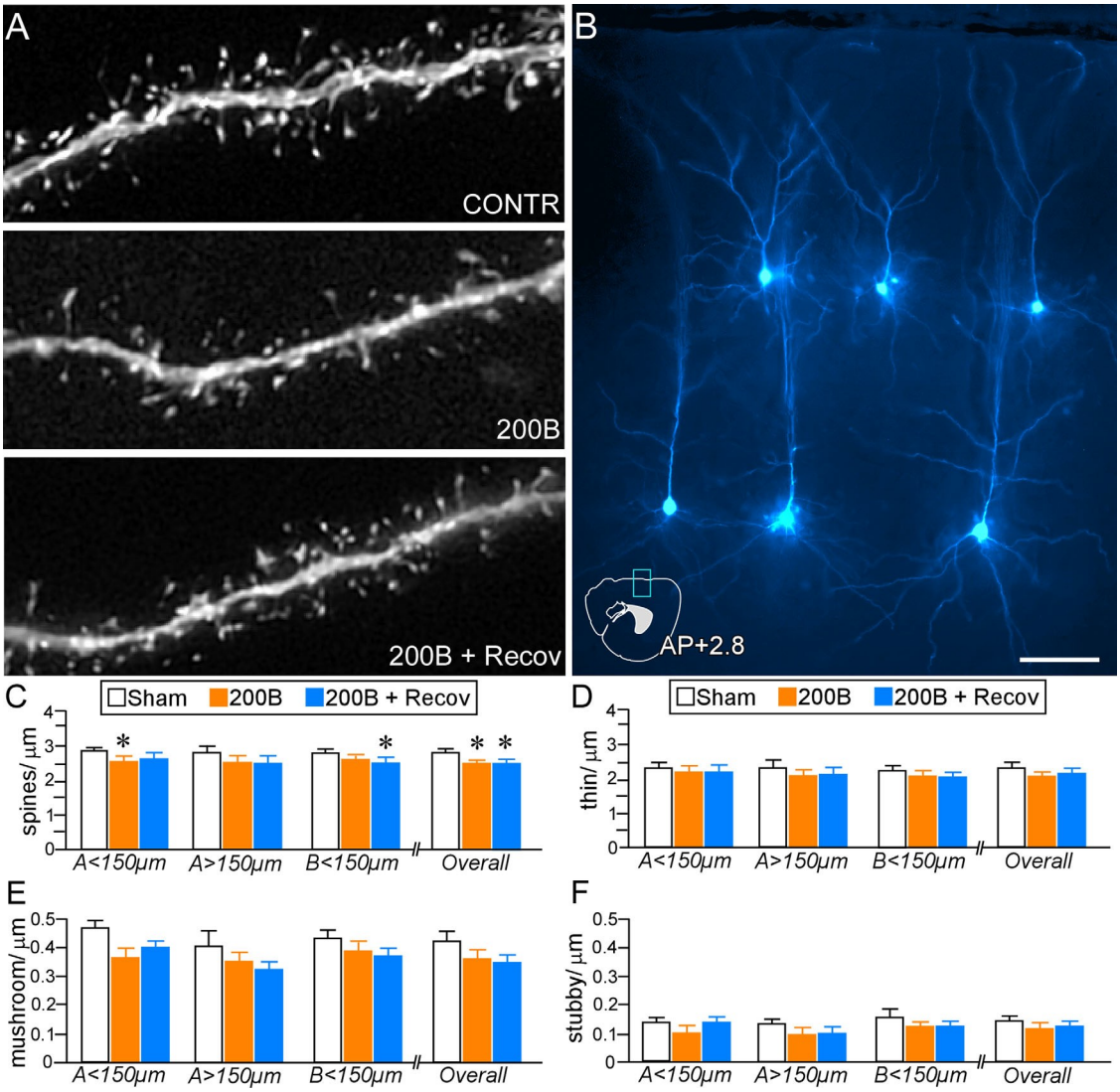

Figure 16. Dendritic spine density in B-. B-recovery, and Sham-treated animals

A: Deconvolved images of dendritic segments from pyramidal neurons in the PL from different treatment groups. B: Image depicts several layer 2, 3, and 5 dye-filled PL pyramidal neurons (pseudocolored cyan). An atlas plate (lower left) depicts the approximate location and orientation within the PL of the dye-filled neurons as shown. Distance in millimeters relative to bregma is indicated. AP, anteroposterior. Mean and SEM of dendritic spine density (C) and thin subtype density (D) as a function of experimental treatment. Both 200B and B200 + recovery (Recov) groups show overall decreases in density relative to sham rats, whereas only 200B rats show significant reductions in overall thin subtypes. Mean and SEM of mushroom (E) and stubby (F) spine densities in treatment groups. Both 200B and 200B + Recov groups display evidence of mushroom spine loss at various regions of the dendritic tree,

whereas 200B + Recov rats show overall decreases, relative to sham control rats. N = 6–8 rats per group *$P < 0.05$, significantly different relative to sham group. Scale bar = 5 μm.

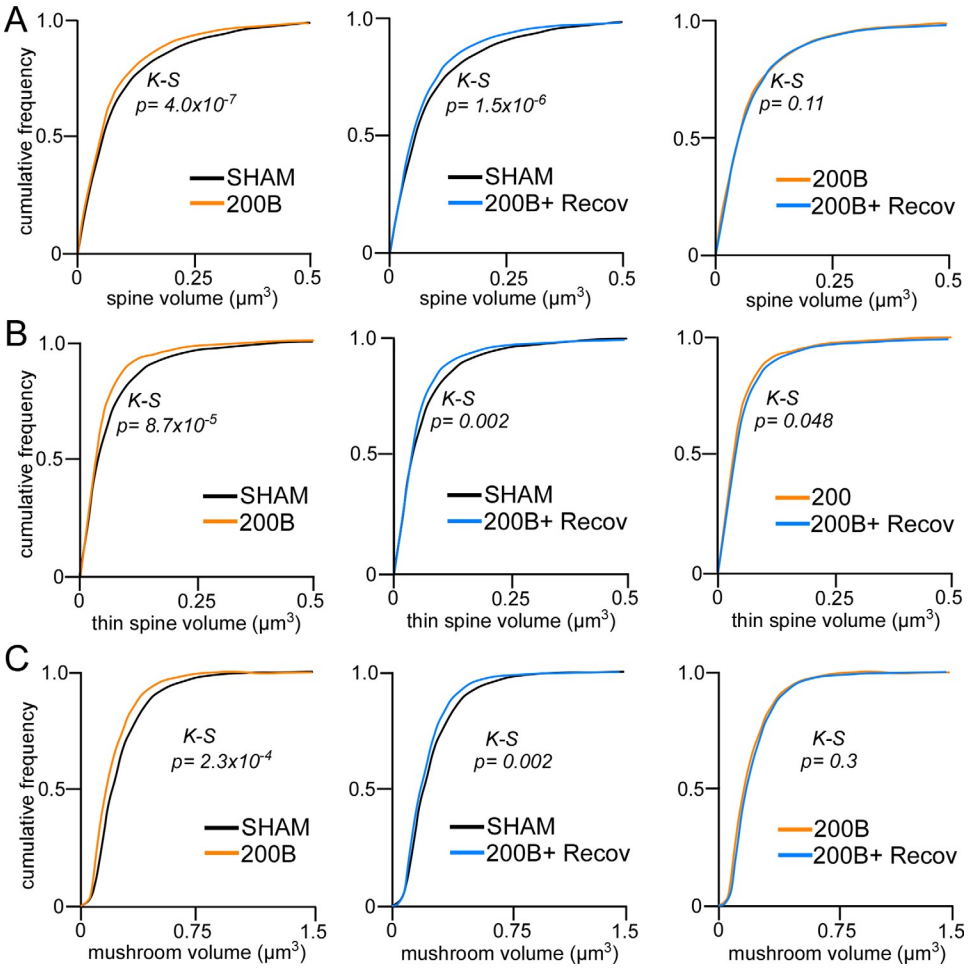

Figure 17. Cumulative frequency distributions in B-, B-recovery, and Sham-treated animals

A: Cumulative frequency distributions of overall spine volume in PL neurons reveal leftward shifts (i.e., decrease) in spine volume following 200B treatment, even after a 21-day recovery period. This trend is also evident in thin (B) and mushroom (C) subtypes with respect to sham control rats. Significance set at $P < 0.01$, K-S test.

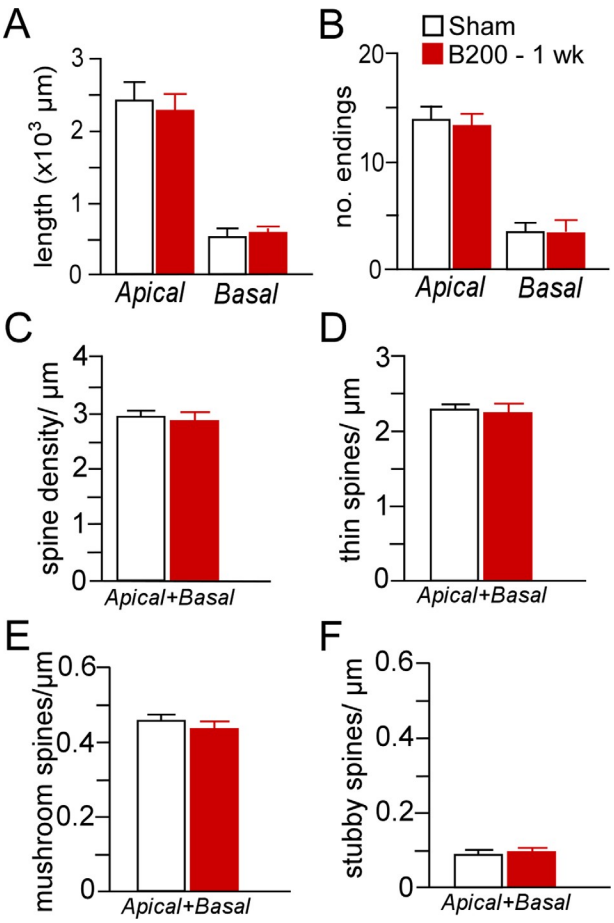

Figure 18. Dendritic spine density in 1 week B-treated animals

Mean and SEM for dendritic length (A) and number of branch endings (B) after 1 week of exposure to high-dose B (200- mg pellet, s.c.). Mean and SEM for overall (C), thin (D), mushroom (E), and stubby (F) spine densities as a function of B200 or sham pellets. This duration of glucocorticoid exposure failed to induce any frank differences in the dendritic or spine morphologic indices examined in PL neurons. N = 5–6 rats per group.

Table 2. Data summary for Exp. 1

TABLE 2.

Data Summary for Experiment 1: Effects of Varying Doses of B on Structural Plasticity in PL

Treatment	Sham	100B	200B
Animals	10	6	6
Neurons	66	36	36
Neurons/animal	6–7	6	6
Laminae analyzed (layers)	2, 3	2, 3	2, 3
Spines	27,225	14,458	12,554
Overall spine density 6 SEM	2.75 6 0.06	2.63 6 0.17	2.47 6 0.12[1]
Thin spine density 6 SEM	2.13 6 0.06	2.03 6 0.15	1.89 6 0.12[1]
Mushroom spine density 6 SEM	0.35 6 0.02	0.34 6 0.02	0.33 6 0.02
Stubby spine density 6 SEM	0.27 6 0.02	0.25 6 0.01	0.25 6 0.01
Neurons/animal; dendritic arborization analysis	5, 6[2]	4, 5[2]	4–6[2]
Apical dendrite length	2,819 6 135	2,467 6 255	2,164 6 214[1]
Apical branch endings	15 6 1.1	14 6 1.2	14 6 0.7
Basal dendrite length	617.8 6 45	568.4 6 56	519.8 6 50
Basal branch endings	4.9 6 0.46	4.5 6 0.38	4.46 6 0.38

1 $P < 0.05$, compared with control treatment group.

2 Because of strict inclusion criteria (see Materials and Methods), the number of neurons analyzed in the dendritic arborization analysis is smaller than the number of neurons analyzed in the dendritic spine analysis.

Table 3. Data summary for Exp. 2

TABLE 3.

Data Summary for Experiment 2: Persistence of B-Induced Structural Alterations in PL Following a 3-Week Recovery Period

Treatment	Sham	200B	200B1 recovery
Animals	6	7	8
Neurons	40	53	60
Neurons/animal	6, 7	7, 8	7, 8
Laminae analyzed (layers)	2, 3, 5	2, 3, 5	2, 3, 5
Spines	17,400	20,800	23,400
Overall spine density 6 SEM	2.87 6 0.07	2.62 6 0.08[1]	2.61 6 0.08[1]
Thin spine density 6 SEM	2.31 6 0.07	2.12 6 0.07	2.11 6 0.07
Mushroom spine density 6 SEM	0.43 6 0.01	0.4 6 0.02	0.38 6 0.02
Stubby spine density 6 SEM	0.13 6 0.01	0.11 6 0.01	0.12 6 0.01
Neurons/animal; dendritic arborization analysis	N/A	N/A	N/A
Apical dendrite length	N/A	N/A	N/A
Apical branch endings	N/A	N/A	N/A
Basal dendrite length	N/A	N/A	N/A
Basal branch endings	N/A	N/A	N/A

1 P< 0.05 compared with control treatment group.

Table 4. Data summary for Exp. 3

TABLE 4.
Data Summary for Experiment 3: Effects of Short-Term B Exposure on Structural Plasticity in PL

Treatment	Sham	200B 1 week
Animals	6	5
Neurons	34	26
Neurons/animal	5, 6	5, 6
Laminae analyzed (layers)	2, 3	2, 3
Spines	13,095	10,763
Overall spine density 6 SEM	2.91 6 0.13	2.87 6 0.10
Thin spine density 6 SEM	2.37 6 0.11	2.35 6 0.07
Mushroom spine density 6 SEM	0.45 6 0.02	0.42 6 0.03
Stubby spine density 6 SEM	0.09 6 0.01	0.10 6 0.01
Neurons/animal; dendritic arborization analysis	4, 5[1]	4, 5[1]
Apical dendrite length	1,814 6 261	1,679 6 167
Apical branch endings	12 6 1.5	11 6 1.1
Basal dendrite length	329 6 38	340 6 30
Basal branch endings	2.8 6 0.22	2.9 6 0.17

[1]Because of strict inclusion criteria (see Materials and Methods), the number of neurons analyzed in the dendritic arborization analysis is smaller than the number of neurons analyzed in the dendritic spine analysis.

CHAPTER 4: EVIDENCE THAT CHRONIC STRESS-INDUCED PREFRONTAL DENDRITIC SPINE LOSS AND WORKING MEMORY IMPAIRMENTS ARE NOT SEXUALLY- DIFFERENTIATED IN ADULT RATS

ABSTRACT

Stress exposure is widely implicated in psychiatric illnesses, although in recent years attention has been directed toward the fact that females show a higher incidence of stress-related affective disorders than males. Hippocampal and prefrontal dysfunction are commonly associated in stress-related disorders, and laboratory rodent models of chronic stress have advanced our understanding of the resultant structural and functional impairments in these cortical regions. Paradoxically, much of the rodent literature shows intact, and in some cases even enhanced cognitive functioning in females following chronic stress. Work and others have previously shown that chronic stress in male rats leads to dendritic spine attrition in the medial prefrontal cortex (mPFC) and accompanying working memory impairments, however, we have not previously critically evaluated these effects in female rats. Here we examine dendritic spine morphological alterations in the prelimbic region of mPFC, and working memory functioning, in both female and male rats exposed to 14 days of chronic variable stress (CVS; daily exposure to different stressors at unpredictable times over 14 days). To follow up, females were implanted with slow-releasing corticosterone (CORT) pellets to assess changes in dendritic spines as a result of high CORT, as we have previously shown that loss of spines is glucocorticoid dependent. Finally, to look at the generalizable effects of stress, males and females underwent a chronic restraint stress

(CRS) paradigm of 6 hour restraint for 21 days and again assessed for prefrontal structural plasticity. These studies extend our previous work showing that the adverse effects of chronic stress on prefrontal structural and functional plasticity have broad effects that are not well-differentiated between adult male and female rats, and suggest that cognitive resilience shown in female rodents following prolonged stress exposure does not generalize to the prefrontal endpoints examined here.

INTRODUCTION

Chronic stress has been shown to precipitate or exacerbate many psychological disorders, such as depression and anxiety (Kendler et al., 1999; Monroe et al., 2007b, a). The hypothalamo-pituitary-adrenal (HPA) axis is a key component of the body's response to stress and has been shown to be dysregulated in some stress-related mental illnesses (Sachar et al., 1973; Carrol, 1982; Wong et al., 2000; Holsen et al., 2013). Women are more susceptible to these stress-related illnesses, being diagnosed twice as often as men (Holden, 2005; Marcus et al., 2005; Grigoriadis and Robinson, 2007). Therefore, it is critical to understand any sex differences that may exist in the stress response.

Chronic variable stress (CVS) is a widely used paradigm that involves daily exposure to different stressors at unpredictable times of the day for 2 – 3 weeks (Ottenweller et al., 1989; Willner, 1997; Grippo et al., 2003). This paradigm typically involves alterations that are related to some of the symptomology involved in stress-related mental illnesses (Willner, 1997). Animals exposed to CVS reliably show

sensitized HPA activity following exposure to a novel stressor (Ottenweller et al., 1989; Herman et al., 1995a; Radley and Sawchenko, 2015). This is in contrast to animals who have been exposed to the same stressor at predictable times of day over a similar length of time. For example, in chronic restraint stress, animals are restrained for 6 hours a day for 3-weeks (although there are many iterations of this paradigm; see Cook and Wellman, 2004; Garrett and Wellman, 2009; Shansky et al., 2009b; Gomez and Luine, 2014). Following this, animals show dampened HPA activity in response to new stressors (Bhatnagar and Dallman, 1998; Girotti et al., 2006; Jaferi and Bhatnagar, 2006; Grissom and Bhatnagar, 2009; Radley and Sawchenko, 2015). Both of these paradigms have been shown to reliably impact corticolimbic structures such as the hippocampus, and the prefrontal cortex (PFC). In male rats, CVS decreases hippocampal and PFC dendritic arborization, as well as dendritic spine density (Watanabe et al., 1992a; Magarinos and McEwen, 1995; McEwen and Magarinos, 1997; McEwen, 1999; Cook and Wellman, 2004; Liston et al., 2006; Liu and Aghajanian, 2008; Holmes and Wellman, 2009; McEwen and Morrison, 2013; Radley et al., 2013). Chronic restraint stress (CRS) has also been shown to induce regressive plasticity in these brain regions in males (Watanabe et al., 1992a; Luine et al., 1994; Magarinos and McEwen, 1995; Cook and Wellman, 2004; Radley et al., 2005; Liston et al., 2006; Radley et al., 2006b; Radley et al., 2008; Shansky et al., 2009b). While regressive plasticity is robustly observed in males following stress, in females CRS has been shown to increase dendritic spine density and arborization in the hippocampus (Galea et al., 1997; McLaughlin et al., 2009; Conrad et al., 2012), suggesting a sex-differentiated response. Though most of these studies have investigated hippocampal

changes following CRS, fewer studies have looked at how CRS or CVS impact female PFC plasticity. In one study, after one week of CRS, females show increased dendritic arborization (Garrett and Wellman, 2009), suggesting sex-differentiation in the PFC's response to stress. In another, following 10-days of CRS, females show little dendritic arborization reorganization, and no changes in dendritic spine density (Moench and Wellman, 2017). The structural reorganization following CRS may be circuit specific as Shansky and colleagues (2010) show that enhanced dendritic arborization following CRS is limited to BLA-projecting neurons in females.

As stress has been shown to impact behavioral and cognitive endpoints, many studies have investigated the role of chronic stress on hippocampal and PFC-dependent behaviors in males and females. In males, chronic stress has been shown to lead to behavioral deficits in a variety of hippocampal-dependent tasks, such as object placement (Beck and Luine, 2002), radial arm maze (Luine et al., 1994), and Y-maze (Conrad et al., 1996). PFC-dependent tasks such as working memory, attentional set-shifting, and other executive tasks, are also impaired in males following stress (Arnsten, 2000; Liston et al., 2006; Holmes and Wellman, 2009; Devilbiss et al., 2012). Females again show differences from male that correspond with structural endpoints and show either no change (Beck and Luine, 2002) or enhancements in hippocampal dependent tasks such as radial arm maze (Luine et al., 1998; Bowman et al., 2001; Kitraki et al., 2004; Conrad et al., 2012; Luine and Frankfurt, 2012). In contrast, prefrontal-dependent tasks appear impaired in some paradigms of stress in females (Shansky et al., 2004; Shansky et al., 2006) suggesting differential sensitivity of brain regions to stress. Though the mechanism behind this sexual differentiation is not clear, it has been

hypothesized that estradiol may be a key mediator in some of these differences. Many of the structural and behavioral effects observed in females following stress are shown to be mediated by estrogen (Luine et al., 1998; Bowman et al., 2002; Shansky et al., 2004; Shansky et al., 2006; Wallace et al., 2006; Shansky et al., 2009a; Conrad et al., 2012; Luine and Frankfurt, 2012; Wei et al., 2014).

As glucocorticoids (CORT), the end result of the HPA axis, have been shown to be key players in many of the regressive structural plasticity observed following stress in both the hippocampus and PFC in male animals (Woolley et al., 1990; Watanabe et al., 1992b; Wellman, 2001; Cerqueira et al., 2005; Alfarez et al., 2009; Morales-Medina et al., 2009; Tanokashira et al., 2012; Gourley et al., 2013; Anderson et al., 2016), a goal of this study is to investigate if structural rearrangement following stress in females also shares a CORT component. To do this, we examined the effects of continuous elevated CORT on dendritic spine density and morphometry in PFC neurons in female rats.

Along with investigating the role of CORT on PFC structural plasticity in females, a primary goal of this current study is to examine PFC structural and behavioral endpoints following CVS in both males and females, to provide a side-by-side comparison. To assess the generality of chronic stress on prefrontal structure, we also look at CRS effects on PFC structural plasticity in both males and females. By looking at both of these paradigms concurrently in males and females, we hope to provide a rigorous analysis and comparison of the effects of stress on the prefrontal cortex between sexes.

MATERIALS AND METHODS

Animals. The animals used in this study were 3-month-old male Sprague Dawley albino rats (Charles River Laboratories). Rats were pair-housed, unless otherwise noted, and maintained on a 12:12 h light/dark cycle (lights on at 0600), with free access to food and water. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Iowa. After 7 d of acclimatization in the animal housing facility, rats were habituated to human contact by handling each for 5 min each day, over at least 7 d before the initiation of experiments.

Experiment 1: Evaluation of sex differences in the effects of chronic variable stress on prefrontal functioning and dendritic spine density and morphology in PL neurons

General procedures. In this experiment, separate cohorts of rats were used to assess CVS effects on a prefrontal-dependent behavioral task and dendritic spine morphology in prefrontal neurons. The rationale for not examining prefrontal function and spine morphology in the same cohort is based upon consideration that the effects and learning may have interactive effects on prefrontal spine morphology (Knott et al., 2002; Holtmaat et al., 2008; Dumitriu et al., 2010; Liston et al., 2013), that could potentially confound the interpretation of CVS effects.

In cohort 1, male (N = 22) and female (N = 20) rats were first submitted to repeated blood sampling for assessment of baseline adrenocortical activity. Rats were then placed on a restricted diet (~20 g of chow per day for male and ~15 g/ day for female rats) and trained in a prefrontal-dependent spatial working memory task, delayed alternation using a *T* maze (Divac, 1970; Ramos et al., 2003; Anderson et al., 2014). In

order to appropriately control for food intake, rats in this experiment were single-housed. Delayed alternation training ensued over a 6-day period, whereafter a subset of rats (N = 10/ sex) were subjected to 14 d of CVS. Controls were handled but not subjected to any of the stress procedures. During the 14-day CVS period, both CVS and control groups were provided food and water *ad libitum.* On the day following CVS, AM and PM blood samples were again collected in CVS and control groups for the assessment of CORT via radioimmunoassay. One male rat that was removed since it underwent significantly greater weight decreases following CVS exposure relative to the cohort.

In cohort 2, analysis of sex and CVS effects on prefrontal dendritic spine density and morphology were conducted in groups of male (N = 20) and female (N = 40) rats. Each group was divided in half and then either subjected to 14 days of CVS or remained as unstressed controls. AM and PM blood samples were collected for assessment of plasma CORT one day prior to CVS exposure in both CVS and control groups. A second series of blood samples were collected in both groups of rats on the evening on the last day of CVS (PM time point). The AM sample was collected on the morning of day 15, and rats were perfused thereafter. Brains were harvested and tissue was prepared for intracellular dye injection of prefrontal neurons. Attrition in the final sample sizes reported in the results are based upon the strict criteria required for inclusion of neuronal segments into the dendritic spine analysis (described below) and the sensitivity of the cell loading procedure to optimal fixation conditions (males: N = 5, control; N = 5 CVS; females: N = 10 control; N = 13 CVS).

Chronic variable stress (CVS). CVS involved daily exposure to either two brief or one sustained stressor over a 14-d period, in semi-randomized order, presented at different

times each day. Brief stressors included open-field exposure for 10 min, placement of cage on orbital shaker for 30 min at 100 rpm, tail suspension for 10 min, forced swim for 10 min in 23°C water, and cold exposure for 4 h (4°C). Sustained stressors included overnight exposure (12-16 h duration) to wet bedding or isolation.

Assessment of prefrontal functionality using delayed alternation. The training period began by initially habituating rats to a *T* maze (90 X 65 cm; 40 cm opaque walls surrounding the perimeter) over a several day period using miniature chocolate chips as a food reward for running to either goal arm in under 60 s. Next, rats were subjected to a forced alternation period of training where they were only rewarded with chocolate after entering the opposite goal arm that they were in previously. Between trials, the maze was wiped clean with 95% ethanol to prevent olfactory cues from determining choice. After 3 consecutive days of 10 trials/ day, rats were tested for spontaneous alternation (i.e., chocolate was present in both goal arms, although rats were only rewarded for entering the opposite arm from the previous trial). The delay between trials was increased until animals could successfully alternate at a 15 s interval with >80% accuracy for 10 trials. After all rats reached the same criterion level of performance, some were subjected to 14 days of CVS. On the second day following CVS exposure, rats were tested in delayed alternation, involving 8 trials/ day over 6 consecutive days. The delays between each trial were semi-randomly varied between 30, 60, or 120 s (on a given day the same pattern was used for all animals). During delay, rats were placed in the holding cage and the maze was cleaned with 95% ethanol. The percentage of correct choice alternations made at each delay interval (30, 60, and 120 s) were

obtained for each animal, and overall averages were expressed as a function of treatment group.

Experiment 2: effect of prolonged CORT exposure on dendritic spine structural alterations in PL neurons in female rats

Female rats were implanted with slow-release CORT (200 mg) to clamp circulating CORT levels of this hormone to the circadian peak (~250 ng/ml) over a 14-day period. Rats were anesthetized with isoflurane and were implanted subcutaneously in the interscapular region with 200-mg (N = 12) CORT pellets that provide a constant and slow release into the general circulation for up to 3 weeks (Innovative Research of America, Sarasota, FL). A second group of rats (N = 12) was implanted with inert cholesterol pellets of weight equal to the CORT pellets. On day 14, CORT pellet efficacy and baseline adrenocortical activity in CORT-replaced and sham rats, respectively, were confirmed by collection of repeated blood samples at AM and PM times for radioimmunoassay of CORT, and all rats were perfused on the morning of day 22. Two rats containing CORT implants were thrown out of the study due to ineffective CORT pellet functioning. Additional rats not included in the data analysis were due to insufficient fixation parameters or quality of dye-filling, yielding final group sizes of N = 7 CORT and N = 5 Control.

Experiment 3: effect of chronic restraint stress (CRS) on dendritic spine structural alterations in PL neurons in female rats

Male and female rats (N = 10/ sex) were subjected to 6 hours of restraint (beginning at 0800) for a 21-day period. Restraint was performed in Plexiglas restrainers (Braintree Scientific, Braintree, MA) that have adjustable enclosures to account for individual differences in sizes and rates of growth. Control animals (N = 10/ sex) were left undisturbed during throughout this period. AM and PM blood samples were collected for assessment of plasma CORT one day prior to CRS exposure in both CRS and control groups. A second series of blood samples were collected in both groups of rats on the evening on the last day of CRS, and the AM sample collected on the morning of day 22. To assess how animals responded to the repeated restraint paradigm, blood was collected from the tail vein of CRS rats right before the beginning of restraint, 30 min into restraint, and at the end of restraint (6 hours) on both the first day of restraint (day 1) and on the last day (day 21). All rats perfused on the morning of day 22, and brain tissue was prepared for intracellular dye injection of prefrontal neurons. Final group sizes due to attrition from the cell loading procedure were as follows: males: N = 8 Control, N = 6 CRS; females: N = 8 Control, N = 7 CRS.

Blood collection and radioimmunoassay. Basal adrenocortical activity was measured by obtaining repeated blood samples from the tail vein of rats at morning (0500) and evening time points (1700) before and after the chronic stress/ CORT treatments in each experiment. Rats were briefly restrained (15 – 30 s), and a small longitudinal incision was made at the distal tip of the tail with a sterile blade. Blood samples (~200

ul) were collected into chilled plastic microfuge tubes containing EDTA and aprotinin, centrifuged, and fractionated for storage of plasma at -80°C until assayed. Plasma corticosterone (CORT) was measured without extraction, using an antiserum raised in rabbits against a CORT-BSA conjugate, and ^{125}I-CORT-BSA as tracer (MP Biomedicals). Assay sensitivity was 8 ng/ ml; intra-assay and interassay coefficients of variation were 5% and 10% respectively.

Histology and tissue processing. Rats were anesthetized with Fatal-Plus (100 mg/kg, i.p.) and perfused via the ascending aorta with 100 ml 1% PFA and 0.125% glutaraldehyde in 0.1 M PBS, pH 7.4, followed by 500 ml of 4% PFA and 0.125% glutaraldehyde in 0.1 M PBS, 7.4, at a flow rate of 55 ml/min. The descending aorta was clamped to limit the flow of fixative to the head and upper extremities, and to prevent fixation of the adrenal glands. Blood was taken via cardiac puncture before perfusion and centrifuged and fractionated for storage of plasma at -80°C for later radioimmunoassay of estradiol and testosterone. Immediately following perfusion, the adrenal glands were extracted and weighed, and brains were removed and postfixed for 4 hours. After post-fixation, the pregenual pole of the cortex was sectioned coronally into 250 µm-thick slabs using an oscillating tissue slicer (VT-1000S, Leica) and stored in 0.1 M PBS containing 0.1% sodium azide at 4°C until the time of cell loading.

Intracellular dye injection procedure. The procedures used in these experiments are based on previous reports using the same methodology (Radley et al., 2006b; Anderson

et al., 2014). Coronal tissue slabs were treated in the DNA-binding fluorescent stain DAPI (Invitrogen) to distinguish between nuclear lamination patterns that distinguish PL from other adjacent-lying prefrontal cortical subfields. DAPI-treated sections were mounted on nitrocellulose filter paper and submerged in a tissue culture dish containing PBS and viewed under fluorescence using a fixed-stage microscope (Leica DM5500). Injections of 5% Lucifer yellow (LY; Invitrogen) were made by iontophoresis through micropipettes (1–2 μm inner diameter) under a DC current of 1– 6 nA for 5–10 min. Neurons in superficial and deep layers of PL were selected for the dye injection procedure based upon the distinguishing cytoarchitectonic features of this region, notably a more densely packed layer 2 and a broader layer 5 relative to adjacent-lying cortical subfields. The general technique for cell filling involved carefully observing the passive diffusion of LY resulting from application of a negligibly small amount of current from the advancing micropipette tip under 40x magnification; LY diffuses amorphously until hitting a dendritic process or cell body, whereby the dye becomes restricted intracellularly. After several neurons were filled intracellularly, tissue sections were mounted onto glass slides and coverslipped in Vectashield (Vector Laboratories).

Imaging, and dendritic spine morphometric analyses. An experimenter unaware of the treatment condition for each animal performed neuronal reconstructions and data analyses. Pyramidal neuron dendritic arbors were reconstructed in 3D using a computer-assisted morphometry system consisting of a Leica DM4000R equipped with an Applied Scientific Instrumentation MS-2000 XYZ computer-controlled motorized stage, a QImaging Blue digital camera, a Gateway computer, and morphometry

software (MBF Biosciences). Neurons were visualized, and the dendritic tree was reconstructed using a Leica Apochromat 40X objective with a numerical aperture (NA) of 1.4 and Neurolucida software (MBF Biosciences). To be considered for dendritic spine morphometric analyses, LY-filled PL neurons had to exhibit complete filling of the dendritic tree, as evidenced by well-defined endings. However, because the dye-filling procedure performed in the sections were only 250 μm thick, it was virtually impossible to retain an entirely intact apical dendritic arbor with no truncations, and the data set were not deemed as suitable for the analysis of dendritic branching patterns.

All fluorescent dye-filled neurons were analyzed by individuals unaware of the experimental treatment. Two-dimensional renderings for each neuron were obtained using Neurolucida software, and two radial distances at 150 μm increments from the soma were selected as a boundary delineating proximal, distal, and tufted portions of the apical dendritic tree. Sampling of basal dendrites was only carried out within the proximal region (<150 μm). Within these regions, branches were randomly selected for imaging from each neuron for an average of 3-5 segments per neuron and 5-7 neurons for each animal. The selection criteria for confocal imaging of dendritic segments are based upon previous reports (Radley et al., 2006b; Radley et al., 2013; Anderson et al., 2014): (1) possess a diameter of <3 μm, as larger diameter dendrites in PL pyramidal neurons exhibit greater variability in spine density values; (2) reside within a depth of 70 μm from the top surface of the section, due to the limited working distance of the optical system; (3) to be either parallel to, or course gently relative to, the coronal surface of the section (i.e., this helps to minimize *z*-axis distortion and facilitate the unambiguous identification of spines); and (4) have no overlap with other branches that would obscure

visualization of spines. *z*-Stacks were collected on a Leica SP5 confocal laser-scanning microscope equipped with an argon laser and a 100X, 1.4 NA oil-immersion objective, using voxel dimensions of 0.1 x 0.1 x 0.1 μm^3. Settings for pinhole size (1 airy disc), gain, and offset were optimized initially and then held relatively constant throughout the study to ensure that all images were digitized under similar illumination conditions at a resolution of 512 X 512 pixels.

Images were deconvolved with AutoDeblur (Media Cybernetics), and spine analyses were performed using the semiautomated software *NeuronStudio* (Rodriguez et al., 2006; Radley et al., 2008; Anderson et al., 2014) (http://research. mssm.edu/cnic/tools-ns.html), which analyzes in 3D dendritic length, spine density, and morphometric features (i.e., head/neck diameter, volume, subtype) for each dendritic spine. Spines were classified as thin or mushroom if the ratio of the head diameter-to-neck diameter was >1.1. If their ratio exceeded this value, spines with a maximum head diameter >0.4 μm were classified as mushroom, or else were classified as thin. Spines with head-to-neck diameter ratios <1.1 were also classified as thin if the ratio of spine length-to-neck diameter was >2.5; otherwise, they were classified as stubby. A fourth category, filopodial spines, exhibited a long and thin shape with no enlargement at the distal tip. Since these were very seldom observed, they were classified as thin subtypes. Finally, data readouts from the spine analysis algorithm were visually compared by the experimenter for each optical stack to verify accurate subtype classifications for dendritic spines.

Statistics. Group data from the CORT radioimmunoassay were compared with a multifactorial ANOVA, followed by pairwise comparisons using Fisher's LSD at both morning and evening time points. Data are expressed as the mean ± SEM. Spine morphometric data were averaged from each animal (i.e. 3-5 segments/neuron, 5-7 neurons/ rat) as a function of treatment. As delineated in each experiment, the final group sizes in each spine morphology experiment are lower than our starting sample sizes (~55% yield). This was due to the fact that the success rate from perfused rats that yield suitable numbers of fluorescent dye-filled neurons for inclusion into the analysis. The judgment of whether individual cases did not meet our standard of quality for dendritic spine labeling or contained a sufficient number of dendritic segments imaged was made by an experimenter unaware of the specific treatment. The effects on dendritic spine and subtype densities were compared using a two-way ANOVA with treatment and sex as factors. Population analyses of spine volume as a function of subtype and experimental treatment were analyzed via comparison of cumulative frequency distributions using the Kolmogorov-Smirnov test with MATLAB software (MathWorks). Delayed alternation performance at each interval (30, 60, and 120 s) were compared using a repeated measures ANOVA. All pairwise comparisons were made using Fisher's LSD with significance set at $p < 0.05$ and data are expressed at mean ± SEM. Significance for the Kolmogorov-Smirnov test was set at $p < 0.01$.

RESULTS

Experiment 1: Evaluation of sex and CVS on prefrontal functioning and dendritic spine density and morphology in PL neurons

CVS exposure induces adrenocortical hyperactivity in both sexes. CVS and similar repeated stress regimens (a.k.a., chronic unpredictable stress, chronic mild stress, chronic intermittent stress) are known to produce a constellation of allostatic physiological, endocrine, and behavioral alterations (Ottenweller et al., 1989; Papp et al., 1991; Herman et al., 1995a; Willner, 1997; Grippo et al., 2003) among which are decreased body weight gain and adrenocortical hyperactivity. Thus, for each cohort of rats, we assayed for changes in pre- and post-CVS body weight gain and plasma levels of CORT, and we measured adrenal weights post mortem to obtain adrenal-to-body weight ratios. CVS produced consistent and reliable decrements in the percentage of weight gain ($F_{(1,38)} = 86.8$, $P < 0.05$) in both sexes over the 14-d stress period as compared with unstressed control rats. Significant increases in adrenal weight (grams) by percentage of body weight (kilograms) ($F_{(1,38)} = 16.1$, $P < 0.05$) were also noted in all CVS-exposed rats regardless of sex. Main effects of sex were noted in each of these analyses (weight gain: $F_{(1,38)} = 946.1$, $P < 0.05$; adrenal weight: $F_{(1,38)} = 138.6$, $P < 0.05$) but no interaction (weight gain: $F_{(1,38)} = 1.3$, $P = 0.3$), adrenal weight: $F_{(1,38)} = 0.4$, $P = 0.5$).

Comparison of AM and PM CORT levels prior to CVS exposure between males and females revealed main effects of time of day ($F_{(1,38)} = 20.2$, $P < 0.05$), sex ($F_{(1,38)} = 15.2$, $P < 0.05$) and interaction for time and sex ($F_{(1,38)} = 12.1$, $P < 0.05$) (Fig. 19A). These

main effects are consistent with the expectations that evening levels of plasma CORT should be substantially higher than in the morning, and that female rats exhibit higher basal levels of plasma CORT relative to males. Following CVS, plasma levels of CORT were elevated in CVS-treated male and female rats, most notably at the PM time point. Main effects were observed for sex ($F_{(1,38)} = 18.5$, $P < 0.05$), time of day ($F_{(1,38)} = 36.9$, $P < 0.05$), and interaction of time of day with CVS exposure ($F_{(1,38)} = 5.6$, $P < 0.05$) (Fig. 19B). Assessment of integrated values of AM and PM CORT (i.e., area under the curve) between pre- and post-stress days of measurement also revealed main effects for sex ($F_{(1,38)} = 27.8$, $P < 0.05$), day ($F_{(1,38)} = 25.8$, $P < 0.05$), and interaction between day with CVS exposure ($F_{(1,38)} = 4.1$, $P < 0.05$) (Fig. 19C). The general trend highlighted by analysis of integrated CORT is that, although females display higher levels of CORT than male rats regardless of treatment, CVS reliably enhances adrenocortical output in both sexes.

CVS impairs spatial working memory in male and female rats. To assess the effects of sex and CVS on prefrontal functioning, rats were trained and tested in a delayed alternation task using a *T* maze. This task has been shown to be dependent upon intact mPFC functioning (Divac, 1970; Ramos et al., 2003; Hinwood et al., 2012) and reveals behavioral impairments that are associated with dendritic spine loss in PL neurons (Hains et al., 2009; Anderson et al., 2014; Radley et al., 2015). Rats were trained to an equivalent level of performance (i.e., 80% choice accuracy at a 15 s delay) prior to CVS exposure and tested in the days following the completion of the CVS regimen. This also enabled us to evaluate sex differences in the rate of acquisition, as based upon the

number of training sessions required for each animal to reach criterion (Ramos et al., 2003). Female rats required fewer days to reach the criterion level for task acquisition than males ($F_{(1,40)} = 3.3$, $P < 0.05$), with the mean number of sessions (one per day) for a female of 4.2 and males 5.5. Following 14 days of the CVS epoch, both stress and control groups were tested in the delayed alternation task at increasing delay intervals (30, 60, or 120 sec) over a course of three days. Repeated measures ANOVA revealed main effects only for delay interval ($F_{(2,74)} = 89.9$, $P < 0.05$), stress treatment ($F_{(1,37)} = 11.3$, $P < 0.05$), and interaction between delay and stress treatment ($F_{(2,36)} = 13.9$, $P < 0.05$). At the 60 s and 120 s time points, CVS-treated rats performed significantly worse than controls, regardless of sex ($P < 0.05$) (Fig. 20). Comparison of individual values for delay interval as a function of CORT levels (AM, PM, or integrated values) failed to reveal any reliable correlation as a function of sex or stress (data not shown).

CVS exposure induces dendritic spine loss in adult male and female rats. A separate cohort of rats were used to assess CVS effects on dendritic spine morphology in prefrontal neurons, based upon evidence of their exquisite degree of sensitivity to environmental experience and learning (Knott et al., 2002; Holtmaat et al., 2008; Dumitriu et al., 2010; Liston et al., 2013), that could have interactive effects with CVS. Adrenocortical hyperactivity in CVS-treated male and female rats was verified as described above. On the day after CVS, all rats were perfused, and tissue was prepared for intracellular dye injections in pyramidal neurons in layer 2/3 of the PL. Different regions of the dendritic tree (apical: proximal, <150 µm, distal, 150-300 µm, tufts, >300 µm; basal: <150 µm) were selected for high-resolution confocal laser scanning

microscopic imaging of dendritic segments. Digital renderings (*z*-stacks) of dendritic segments made in three dimensions were deconvolved, followed by the analysis of spine density and morphology using the semi-automated software *NeuronStudio*. In this study a total of 504 dendritic segments were digitally imaged and approximately 80,000 dendritic spines were reconstructed and analyzed.

Multifactorial ANOVA revealed main effect of CVS ($F_{(1,31)} = 12.5$, $P < 0.05$) but no main effect of sex ($F_{(1,31)} = 0.2$, $P = 0.7$) or CVS by sex interaction ($F_{(1,31)} = 0.1$, $P = 0.8$) on dendritic spine density. We observed an 11% decrease (11.5% in males, 10% in females) in overall dendritic spine density in PL neurons in CVS animals relative to control animals. Comparisons made between CVS and control animals revealed a significant loss of spines on most segments of the neuronal trees in CVS animals (basal: $F_{(1,31)} = 5.3$; proximal: $F_{(1,31)} = 5.4$; distal: $F_{(1,31)} = 12.1$; $P < 0.05$ for all; Fig. 21)

Effects of CVS on PL dendritic spine morphology. Dendritic spines can be distinguished by geometric characteristics (i.e., thin, mushroom, stubby) that have proved to be useful for inferring synaptic structure-function relationships (Kasai et al., 2003; Bourne and Harris, 2007; Yang et al., 2009; Dumitriu et al., 2010; Lee et al., 2012). In cortical pyramidal neurons, spines classified as thin represent the majority of the population (60-70%; Bourne and Harris, 2007), and previous studies have identified this subtype as important for long-term potentiation and learning-related plasticity (Arnsten et al., 2010; Anderson et al., 2014). CVS produced consistent decrements in the overall density of thin spine subtypes in both male and female rats ($F_{(1,31)} = 6.2$, $P < 0.05$; 8.5%), whereas there was no effect of sex ($F_{(1,31)} = 0.6$, $P = 0.4$), or CVS by sex interaction ($F_{(1,31)} = 0.1$,

P= 0.9; Fig. 22). Consideration of the spatial characteristics of thin spine loss in CVS-treated rats revealed consistent non-significant trends throughout all aspects of the dendritic tree that were sampled (apical proximal, P = 0.06; apical medial, P = 0.1; apical distal, P = 0.09; basal, P = 0.1).

CVS was not found to produce as robust alterations in overall density in other spine subtypes examined (Fig. 23). Overall mushroom spine densities showed downward trends as a function of CVS ($F(1,31) = 2.8$, $P = 0.1$; Fig. 23), that were largely accounted for in the more distal regions of apical dendritic arbors (medial: $F(1,31) = 6.3$, $P < 0.05$; distal: $F(1,31) = 1.6$, $P = 0.2$). Overall densities of stubby subtypes showed downward trends following CVS exposure ($F(1,31) = 3.0$, $P = 0.09$; Fig. 23). The decreasing trend of stubby spine density in PL neurons following CVS were accounted for by decrements in basal ($F(1,31) = 3.8$, $P = 0.06$), and more distally within apical dendrites (medial: $F(1,31) = 7.9$, $P < 0.05$; distal: $F(1,31) = 5.7$, $P < 0.05$). We gave consideration to whether filopodial spine densities in PL neurons varied as a function of CVS or sex, however, these were too seldom observed to allow for quantitative analysis. Finally, analyses of several spine parameters (length, head diameter, volume) did not reveal any group differences as a function of glucocorticoid treatment (data not shown). Nevertheless, population analysis of dendritic spine volumes revealed that CVS exposure may differentially affect spine size as a function of sex. Consistent with past work of ours showing that chronic stress or prolonged corticosterone administration may lead to downward population shifts in spine volume in PL neurons (Radley et al., 2008; 2013; Anderson et al., 2014, Anderson et al., 2016), comparison of frequency distributions of overall spine populations revealed downward trends (i.e., leftward shift)

in volume in CVS-treated as compared with unstressed control male rats (Fig. 24A.). By contrast, female rats subjected to CVS displayed upward trends in spine volume (i.e., rightward shift in the cumulative distribution frequency curve) related to unstressed controls (Fig. 24B). The increases in spine volume in CVS-treated females could not be accounted for by the fact that CVS-induced decreases in overall spine density that were largely due to losses within thin subtypes, that tend to have smaller overall volumes, since population analyses within thin subtypes also revealed similar increases (K-S test, $P = 0.004$).

In summary, these observations suggest that CVS leads to dendritic spine loss and effects on subtypes within PL neurons that generally do not lend support to the possibility that these effects are sexually-differentiated. By contrast, CVS appears capable of producing bimodal effects on spine volume, leading to decreases in male and increases in female rats.

Experiment 2: effect of prolonged CORT exposure on dendritic spine structural alterations in PL neurons in female rats

Previous studies have shown that glucocorticoids play a prominent role in chronic stress-induced limbic and neocortical structural alterations in male rodents (Magarinos and McEwen, 1995; Wellman, 2001; Cerqueira et al., 2005; Cerqueira et al., 2007; Liu and Aghajanian, 2008; Liston and Gan, 2011; Gourley et al., 2013; Anderson et al., 2016). Moreover, CVS leads to increased adrenocortical output that is paralleled by impaired prefrontal functioning and dendritic spine loss in prefrontal neurons in both

male and female rats. These data raise the possibility that CVS-induced increases in corticosterone may have an important contribution in the reorganization of prefrontal synaptic networks. In follow-up to our first experiment, we next examined whether sustained increases in CORT in female rats is capable of inducing a similar pattern of alterations in prefrontal dendritic spine density and morphology as following CVS exposure in both sexes, and as we and others have previously observed in male rats (Liu and Aghajanian, 2008; Gourley et al., 2013; Anderson et al., 2016).

Characterization of plasma CORT levels. Two weeks after pellet implantation (200 mg or cholesterol) blood samples were collected from the tail vein of CORT- and sham-implanted rats at times in the morning (0500) and evening (1700) to verify pellet efficacy and plasma CORT concentration relative to control animals. Repeated measures ANOVA did not show main effect of treatment ($F(1,22) = 0.5$, $P = 0.5$.; Fig. 25), as females generally show high levels of CORT. As *a prior* assumptions were made that CORT pellets should level off the circadian rhythm, planned comparisons revealed a main effect of treatment at the AM time point ($P < 0.05$), but no significant differences in the PM time point ($P= 0.2$; Fig. 25). This suggests that CORT pellets level off the rhythm, whereas sham animals show the expect PM rise in CORT values.

Chronic elevations in plasma CORT induce dendritic spine loss in PL pyramidal neurons in female animals. Individual neurons in layer 2/3 pyramidal neurons of the PL cortical subfield of mPFC were selected for intracellular injection in CORT and control animals.

Different regions of the dendritic tree (apical: proximal, < 150 μm, distal, 150 – 300 μm, tufts, > 300 μm; basal: < 150 μm) were selected for high-resolution confocal laser scanning microscopic imaging of dendritic segments. Digital renderings (z-stacks) of dendritic segments made in 3D were deconvolved, followed by the analysis of spine density and morphology using the semi-automated software *NeuronStudio*. In this study a total of 287 dendritic segments were analyzed for spine density (~43,000 dendritic spines).

We observed a 14% decrease in overall dendritic spine density in PL neurons in female CORT animals relative to control animals (Fig. 26). Comparisons made between CORT and control animals revealed significant loss of spines at most segments of the dendritic tree (proximal, $F_{(1,11)} = 5.099$, $p < 0.05$; distal, $F_{(1,11)} = 5.6$, $P < 0.05$; basal, $F_{(1,11)} = 7.4$, $P < 0.05$); all segments averaged, $F_{(1,11)} = 7.7$, $P < 0.05$; Fig. 26). This length of exposure to CORT results in similar deficits that we have previously observed in males (Anderson et al., 2016).

Effects of high levels of CORT on PL dendritic spine morphology in females. As in the first experiment, we investigated changes in spine morphology, to see if thin spines would be vulnerable to high CORT exposure. In males, this spine phenotype was significantly decreased following two-weeks of CORT exposure, while the other spine phenotypes (mushroom and stubby) remained relatively unchanged (Anderson et al., 2016).

One-way ANOVAs revealed significant decreases of thin spine populations, on most aspects of the neuronal tree (proximal, $F_{(1,11)} = 7.0$, $P < 0.05$; distal, $F_{(1,11)} = 5.9$, $P < 0.05$; basal, $F_{(1,11)} = 11.1$, $P < 0.05$; all segments averaged, $F_{(1,11)} = 10.5$, $P < 0.05$; Fig. 27). Similarly to males, stubby and mushroom spines did not show changes in density following CORT exposure (Fig. 28).

While no main effects of treatment status were noted to influence mushroom spine density in PL neurons, as we have previously shown that CORT and CVS exposure in males leads to shrinkage of mushroom spines, but that CVS enlarges mushroom spines in females we carried out population analyses of spine volumes in this subtype, as well as in stubby and thin spines. Following CORT treatment, mushroom spine populations revealed increased trends (i.e. rightward shifts) in volume relative to control groups (Kolmogorov-Smirnov test, K-S; $p < 0.001$; Fig. 29). While no other spine subtype revealed changes in volume, these observations support the results from the previous experiments, showing that CORT influences spine volume in a bi-directional manner in males and females.

Taken together, these observations suggest that CORT leads to dendritic spine loss and effects on subtypes within PL neurons that are similar to what we observe using the same paradigm in male animals (Anderson et al, 2016) and generally do not support sex-differentiation. By contrast, CORT appears capable of producing bimodal effects on spine volume, leading to decreases in male and increases in female rats, which parallels the effects of CVS on spine volume. This indicates that the CVS effects we observe are modulated by CORT exposure.

Experiment 3: effect of chronic restraint stress (CRS) on dendritic spine structural alterations in PL neurons in female rats

Much of the existing information regarding the effects of chronic stress on limbic cortical structural alterations in male and female rodents has been derived from the chronic restraint (a.k.a., immobilization) stress regimen (Watanabe et al., 1992a; Luine et al., 1994; Magarinos and McEwen, 1995; Galea et al., 1997; Bowman et al., 2001; Bowman et al., 2002; Vyas et al., 2002a; Cook and Wellman, 2004; Radley et al., 2004; Radley et al., 2006b; Garrett and Wellman, 2009; Shansky et al., 2010; Conrad et al., 2012). This has raised uncertainty regarding whether sex differences in response to chronic stress may vary as a function of the specific regimen employed. To address the generality of effects of chronic stress on male and female rats, we employed a commonly used stress regimen, CRS, involving exposure of rats to restraint for 6 h/ day for 21 days (Watanabe et al., 1992a; Luine et al., 1994; Galea et al., 1997; Bowman et al., 2001; Bowman et al., 2002; Radley et al., 2004; Radley et al., 2006b; Radley et al., 2008).

Characterization of adrenocortical activity in male and female rats as a function of three weeks of repeated restraint. Basal CORT values (AM and PM samples) were taken before repeated restraint began (Fig. 30A) and taken again following the cessation of the 21-day period (Fig. 30B) to assess changes in circadian rhythmicity following CRS. Repeated measures revealed a main effect of sex ($F_{(1,36)} = 17.4$; $P < 0.05$) but no effect of treatment ($F_{(1,36)} = 0.2$, $P = 0.9$) and no interaction ($F_{(1,36)} = .4$; $P = 0.5$). Females had higher CORT levels in the first blood collection, prior to any future manipulations, again supporting research in the past that has shown females to have higher basal CORT

secretion when compared to males (P<0.05 for al; Fig. 30A). Following repeated restraint, females (regardless of treatment) had higher CORT values than control males at the evening time point (P <0.05; Fig. 30B) but not the males who had undergone repeated restraint. While there is no statistical different between stressed males and control males, stressed males trended towards higher PM values following repeated restraint (P = 0.08). Assessment of integrated values of AM and PM CORT (i.e., area under the curve) between pre- and post-stress days of measurement also revealed main effects for sex ($F_{(1,36)}$ = 15.7, P < 0.05), but no effect of CRS ($F_{(1,36)}$ = 0.1, P = 0.7), or CRS by treatment interaction ($F_{(1,36)}$ = 0.01, P = 0.9; Fig. 30C). The general trend highlighted by analysis of integrated CORT is that both males and females habituated to the CRS procedure and reveal no changes in basal CORT secretion over the 21-day paradigm.

To assess how CORT secretion in response to restraint in both males and females changed over the 21 day period, blood was taken via tail vein right before the start of the beginning of restraint, 30 minutes into the restraint, and right before the cessation of restraint (6 hours) on the first day of restraint and on the last day (day 21). Repeated measures revealed a main effect of time of blood draw ($F_{(5,90)}$ = 15. 1, P <0.05) and sex ($F_{(1,18)}$ = 13.7, P < 0.05) but no interaction of time and sex ($F_{(1,18)}$ = 0.8, P = 0.5). On the first day of restraint, females had higher CORT values at the 30 min and 6 h time point. In both sexes, there was higher CORT secretion 30 minutes into the restraint than in the beginning (P <0.05 for all; Fig. 31A). The CORT values at the 6 hour time point were higher than the start of the restraint (P < 0.05) but lower than the 30 min time point (P <0.05), indicating some habituation throughout the first restraint

period. On day 21, the 30 min and 6 h CORT values were higher than the beginning of restraint for that session for both males and females ($P < 0.05$ for both; Fig. 31B). Females had higher values CORT values than males on the last day of restraint at the 30 min and 6 hour time point, as shown on the first day of restraint ($P < 0.05$).

When CORT values were averaged for the first day and last day of restraint, there are clear trends towards habituation over the 21 day period. One-way ANOVA shows a main effect of sex for both day 1 ($F_{(1,18)} = 16.9$, $P < 0.05$) and day 21 ($F_{(1,18)} = 6.5$, $P < 0.05$), with females showing higher CORT secretion than males. Both sexes show clear trends towards decreased CORT secretion on day 21 compared to day 1 ($P = 0.06$ for females; $p = 0.07$ for males) indicating some habituation over the repeated restraint period (Fig. 31C).

Repeated restraint induces similar effects on dendritic spine density in adult male and female rats. Following the 21 day period of repeated restraint, or control treatments, animals were perfused for dendritic spine analysis. Pyramidal neurons in PL were filled with Lucifer Yellow dye and imaged on a confocal microscope. In this experiment, 417 dendritic segments were imaged (~58,000 dendritic spines).

Multifactorial ANOVA revealed main effect of CRS at the most proximal portion of the apical tree ($F_{(1,16)} = 5.49$, $P < 0.05$, Fig. 32) but no main effect of sex ($F_{(1,16)} = 1.7$, $P = 0.1$) or CRS by sex interaction ($F_{(1,16)} = 0.3$, $P = 0.9$) on dendritic spine density. We observed a 11% decrease (13% in males, 9.4% in females) in overall dendritic spine density on proximal segments in PL neurons in CRS animals relative to control animals.

Regional analyses failed to show dendritic spine changes on any other part of the apical tree. The loss of spines observed in CRS animals is primarily accounted for by thin spines. Thin spines show the most decrements following CRS, particularly at the proximal part of the neuronal tree ($F_{(1,16)} = 5.9$, $P < 0.05$, Fig. 33), with no other region showing thin spine changes. Mushroom and stubby spines appear relatively stable as no significant changes in density were observed in these subtypes (Fig. 34). As in previous experiments, population analyses were carried out to investigate changes in spine morphometry. No significant changes were observed in any spine subtype (data not shown).

Taken together, these results suggest that CRS leads to differential HPA activity than the CVS paradigm, with no obvious changes to basal adrenocortical activity in males or females exposed to CRS, likely due to habituation over the 21 day period. While dendritic spine loss is still evident after CRS, this appears to be relegated to the proximal portion of the apical tree, instead of widespread spine loss observed following CVS. Again, these data generally do lend support to the possibility that these effects are sexually-differentiated.

DISCUSSION

The results presented here suggest that chronic stress leads to similar behavioral deficits and dendritic spine structural alterations in the prefrontal cortex of male and female rats. CVS exposure in male and female rats induced working memory impairments and a significant loss of dendritic spines in PL pyramidal neurons. These

structural changes are likely to involve at least some glucocorticoid dependency, as male rats (Liu and Aghajanian, 2008; Gourley et al., 2013; Anderson et al., 2016) and female rats (presented here) show a similar pattern of regressive structural plasticity in PL neurons following chronic CORT exposure. Male and female rats subjected to CRS also showed similar dendritic spine changes in PL as following CVS, indicating a generalizable effect of chronic stress exposure on prefrontal structure. Taken together, our data suggest little sexual-differentiation in prefrontal indices following stress.

In all three experiments, the majority of the spine loss observed is accounted for by decrements in thin subtypes. Thin spines show a much higher degree of plasticity compared to other spine phenotypes (Knott et al., 2006; Bourne and Harris, 2007). This subtype has been implicated in optimal prefrontal functioning as well as working memory (Kasai et al., 2003; Arnsten et al., 2010; Anderson et al., 2014). Our results indicate that thin spines are vulnerable to different stress exposures (variable and repeated) as well as to 14 days of elevated CORT, in both males and females. As the majority of the aforementioned studies have been carried out in male rats, this extends the body of literature supporting the vulnerability of thin spines in females as well.

Our data collected here show that female prefrontal plasticity following stress and CORT is similar to males, however, other studies have shown sexual dimorphism in this region following stress. In particular, one week of repeated restraint has been shown to induce increased dendritic arborization in females (Garrett and Wellman, 2009). Interestingly, when this period of stress is increased to 10 days, females show marginally significant reduced dendritic arborization but no changes in dendritic spine density (Moench and Wellman, 2017). Our stress paradigms used here are more

prolonged, which may be part of the reason we see regressive plasticity in females as well as males. It is also worth noting that we did not present dendritic arborization data in these studies. Given strict inclusion data, very few intracellularly filled neurons fit our criteria for analysis. Cells that fill well for dendritic spine analysis tend to show truncations of primary apical shafts that would not provide enough detail to show changes to dendritic arbors following our manipulations. Although previous studies from our laboratory have carried out these analyses (Radley et al., 2004; Radley et al., 2008; Anderson et al., 2014; Anderson et al., 2016) our focus has changed to elucidate changes to dendritic spines.

Impairments in working memory tasks following chronic stress has been reliably shown in male rodents (Arnsten, 2000; Liston et al., 2006; Hains et al., 2009; Devilbiss et al., 2012). However, there has not been much investigation on this in adult females. Here we show that females also display deficits in working memory following two weeks of CVS. Studies of hippocampal functioning female rats often show no impairment to enhancement following chronic stress (Bowman et al., 2001; Bowman et al., 2002; Kitraki et al., 2004; McLaughlin et al., 2009; Conrad et al., 2012; Wei et al., 2014), though a few studies looking at acute (Maeng and Shors, 2013; Gomez and Luine, 2014) and pharmacologically induced stress (Shansky et al., 2004; Shansky et al., 2009a) indicate that working memory in females is susceptible to impairment. Our results indicate that prolonged psychological stress also leads to impaired working memory in females and suggests that the prefrontal cortex may not be as resilient to stress as the hippocampus has been purported to be.

Gonadal hormone influences on structural plasticity

When discussing sex differences it is essential examine the role gonadal hormones may play in our behavioral and structural endpoints that we analyzed here. Estrogen in particular has been thought to play a neuroprotective role following stress (see McLaughlin et al., 2009; Luine, 2016). Estrogen appears to increase dendritic spine density with higher levels of estradiol levels associated with higher spine density, particularly in the hippocampus (Woolley and McEwen, 1992; Luine and Frankfurt, 2012). Here we took blood samples on the day of perfusion to assess estradiol levels and attempted to correlate them with levels of spines. In our studies, no clear correlation was found between estradiol and spine density (data not shown). We also assayed testosterone in males on the day of perfusion with spine density, as testosterone has been previously shown to influence spine density in the hippocampus (Jacome et al., 2016) but again, no clear correlation was found between the two (data not shown).

Gonadal hormone levels have also been shown to influence behavior in various tasks (Luine et al., 1998; Kritzer et al., 2001; Luine et al., 2003; Wallace et al., 2006; Kritzer et al., 2007; Luine and Frankfurt, 2012). Again, we correlated both estrogen and testosterone with our working memory task but found no clear relationship. For females, the stage of estrus cycle did not appear to influence performance across testing day. (data not shown). This should not be taken to mean that there is no effect of estradiol on our results as our sample size for animals in diestrus and proestrus was likely too small to reveal any relationship, however it does suggest that the cyclic variation of estradiol does not lead to overt behavioral differences within sex. Our findings are consistent with the recent meta-analysis that the estrous cycle of rats does not intrinsically add more variability in research (Prendergast et al., 2014).

A particularly interesting finding from our data is the bi-directional effect of CVS and CORT on mushroom spine volume. As we have demonstrated previously (Anderson et al., 2014; Anderson et al., 2016), and is presented here, males show reductions in mushroom spine volume following elevated CORT. However, in females, enlargements in mushroom spine volume following both CVS and CORT manipulations are revealed. It is possible that estrogen is playing a neuroprotective role in this case in potentially compensating for spine loss by increasing spine volume as large spine volume has been associated with enhanced spine efficacy and increased stability (Harris et al., 1992; Grutzendler et al., 2002; Nimchinsky et al., 2002; Mizrahi and Katz, 2003; Nimchinsky et al., 2004; Holtmaat et al., 2005; Ashby et al., 2006). Though we are unable to determine the functionality of these changes as animals who underwent prefrontal testing on the T maze were a different cohort of animals than the ones used for structural analyses (see methods), it is interesting to speculate the role that both volume and spine density play in our functional endpoints. As both males and females demonstrate impaired working memory following CVS, any benefit females may receive from enlarged spine volumes do not appear to compensate for the spine loss that they experience.

CORT involvement in structural changes following CVS and CRS

One lingering question from these studies is whether changes in circadian levels of CORT following chronic stress are involved in the prefrontal plasticity observed in all experiments. Following 14 days of CVS, males and females show enhanced HPA output, particularly at the PM time point. This supports the hypothesis that spine changes following CVS are glucocorticoid dependent. However, data from our repeated

restraint paradigm provide an interpretive challenge to that hypothesis. Following 21-days of repeated restraint, no significant changes in basal CORT secretion were observed in males or females, yet these animals still demonstrated significant spine loss. Though CRS may not have led to alterations in basal CORT secretion, this doesn't necessarily exclude a glucocorticoid component in prefrontal dendritic spine compromise. Previous research from the McEwen, Magariños and colleagues have demonstrated that preventing biosynthesis of glucocorticoids during 21-days of repeated restraint blocks reductions in dendritic arborization (Magarinos and McEwen, 1995). Our lack of observed basal CORT changes also may not indicate a lack of adrenocortical alteration during the CRS paradigm. As blood was only collected before and after the 21-day period we do not have a clear idea of how basal adrenocortical activity may have changed in the interim. Some previous work has suggested that following 7 days of repeated restraint, animals show enhanced resting adrenocortical activity (Daviu et al., 2014). It is possible that our animals also experienced increased basal HPA axis activity during the middle of the 21-day repeated restraint period, and then habituated to the stress over the course of the exposure. Previous work from our laboratory has shown that regressive spine plasticity in PL following high CORT exposure in male rats persists following 3-week of normalized HPA activity (Anderson et al., 2016), so the spine changes we observe in these animals may not have recovered following increased HPA activity, even though basal adrenocortical activity was found to be normalized at the end of this period.

Though it is likely that glucocorticoids played a role in the dendritic spine changes observed following CRS, there are other factors that may also mediate this

effect. Blocking excitatory amino acid release during stress exposure has been shown to be sufficient to prevent reductions in dendritic arborization following CRS (Watanabe et al., 1992b; Magarinos and McEwen, 1995). It has been hypothesized that the effects of glucocorticoids on dendritic reorganization in this instance are primarily due to the potentiated effect they play on release of excitatory amino acids (Magarinos and McEwen, 1995). Future studies should investigate the role of glutamate antagonism on dendritic spine plasticity following CRS in both males and females.

Altogether, these data suggest that male and female rats show similar sensitivity to chronic stress exposure when it comes to prefrontal-dependent behavior and structure and provides evidence that this brain region may differ in comparison to the hippocampus when it comes to the neuroprotective effects of estrogen on hippocampal functioning.

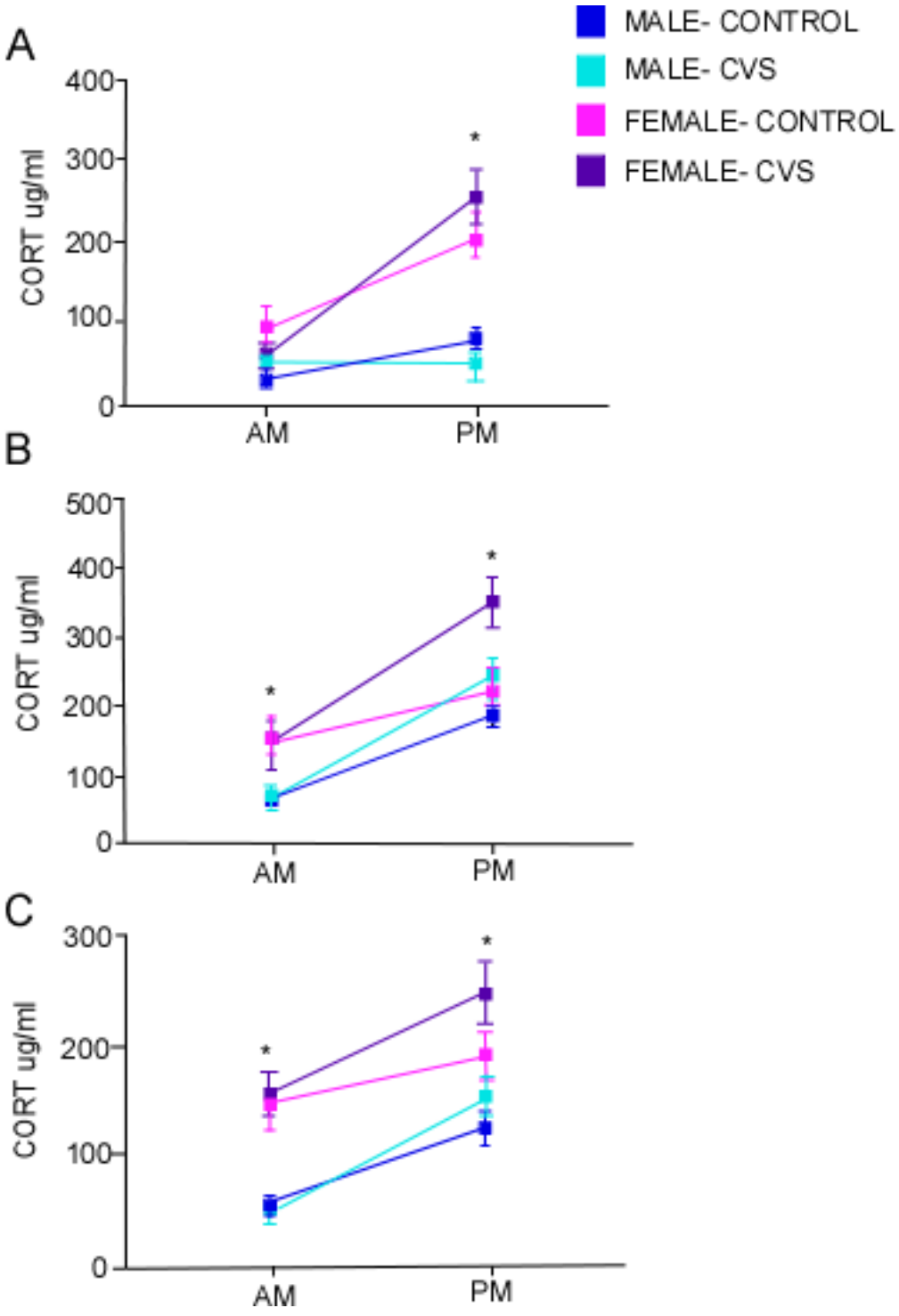

Figure 19. Basal CORT values in M + F CVS animals

Graph depicting mean ± SEM plasma CORT levels at AM and PM sampling as a function of treatment group. A., Before CVS, females show higher CORT values in the PM time point than males. B., Following

CVS, CVS animals show increases of CORT in the PM time point, regardless of sex. Females still show higher CORT values when compared to males in the AM time point. C., Integrated values (area under the curve, AUC) from the sampling before (AUC1) and after stress (AUC2) show trends towards increased CORT values in CVS animals following the CVS paradigm. *, $P < 0.05$ compared to males; $P < 0.05$ compared to controls where indicated. N = 5 males per treatment, N = 10-13 females per treatment

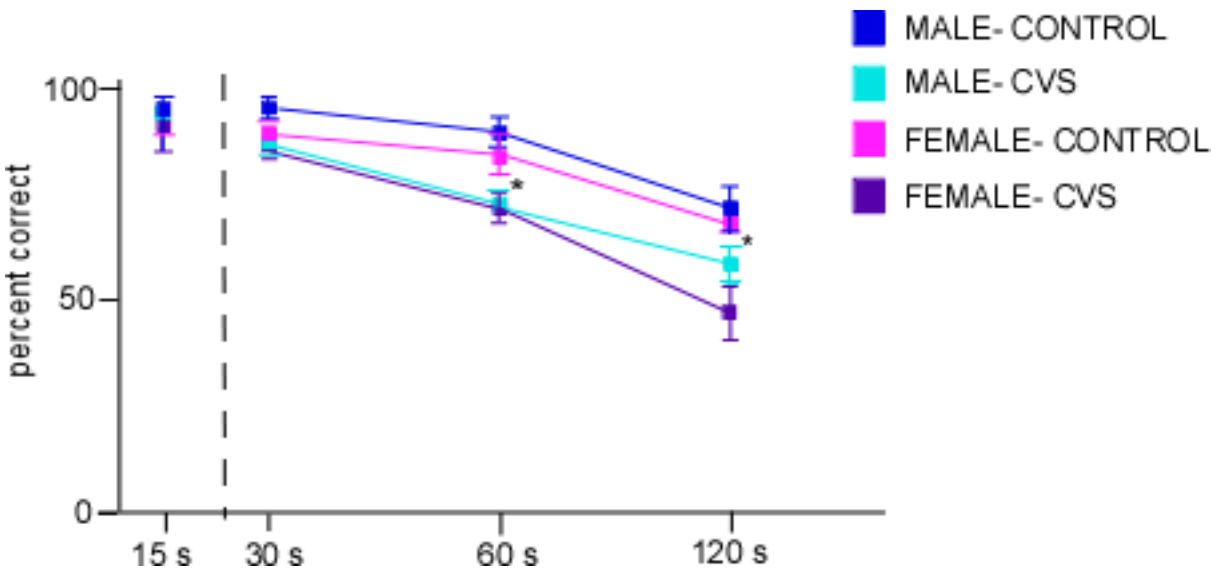

Figure 20. Working memory in M +F CVS animals

Graph demonstrating the percentage of correct responses for delayed alternation performance. Before stress (indicated before the dotted line) all animals performed equivalently at 15 s delay (criterion to continue on in the task). Following CVS (after dotted line), working memory impairments were evident in CVS animals (male and female) with respect to control animals at the longer delay intervals (60, 120 s), Data represent mean ± SEM and are based on overall animal averages. *, $P < 0.05$ compared to controls. N = 10 per group

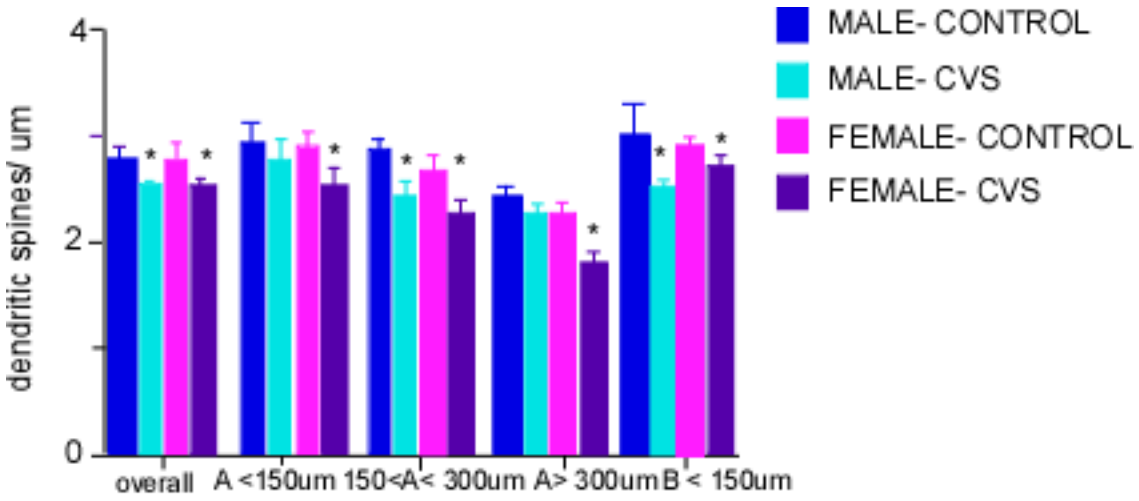

Figure 21. Dendritic spine density in M + F CVS animals

Mean and SEM of dendritic spine density as a function of treatment. CVS animals of both sexes show decreases in dendritic spine density in PL pyramidal neurons, particularly at distal portions of the apical tree. *, $P < 0.05$ compared to controls. N = 5 males per group; N = 10-13 females per group.

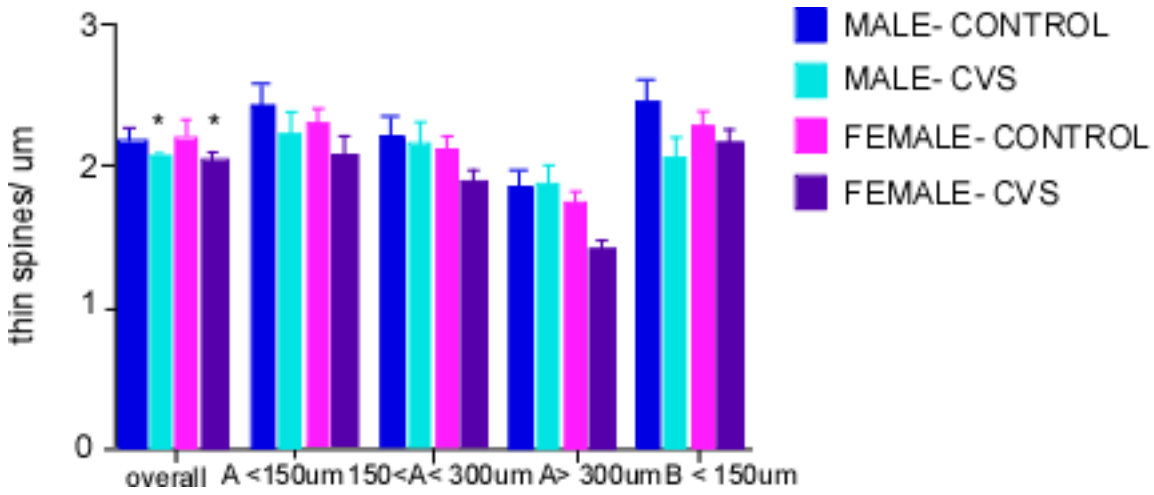

Figure 22. Thin spine density in M + F CVS animals

Mean and SEM of thin spine density as a function of treatment. CVS animals of both sexes show decrements in thin spine density compared to control animals. Decrease in thin spine trends are observable at individual sections of the apical tree, but do not reach statistical significance. *, $P < 0.05$ compared to controls. N = 5 males per group; N = 10-13 females per group.

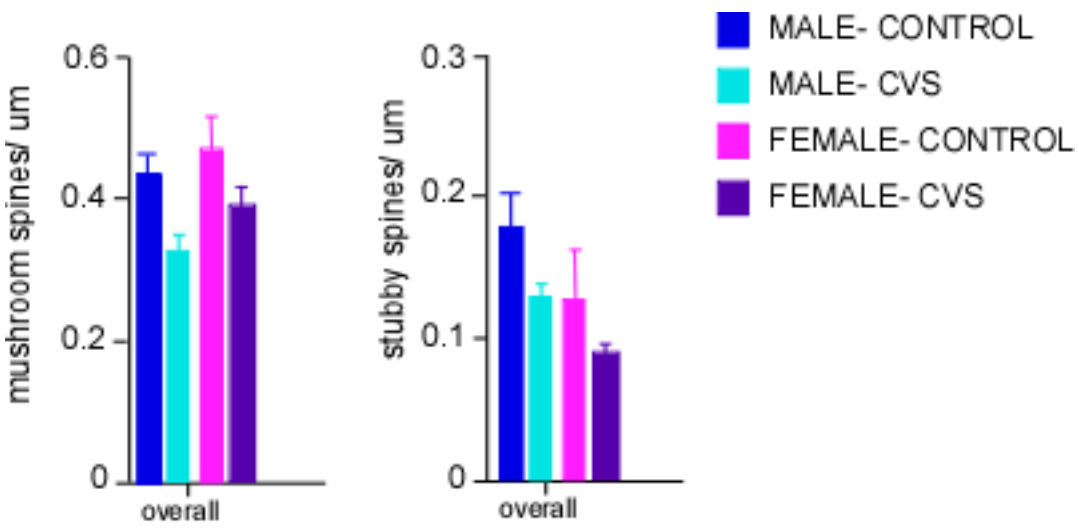

Figure 23. Mushroom & Stubby spine density in M + F CVS animals

Mean and SEM of mushroom (left) and stubby (right) spine density as a function of treatment. CVS animals of both sexes show trends in decreases in these two subtypes but these do not reach statistical significance. N = 5 males per group; N = 10-13 females per group.

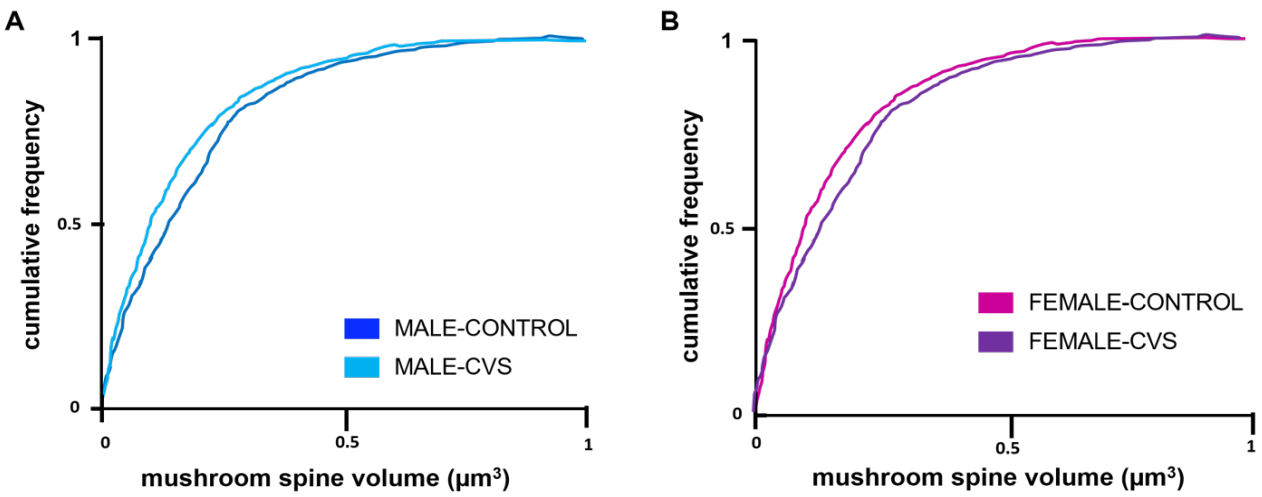

Figure 24. Mushroom spine volume in M + F CVS animals

Cumulative frequency distributions of mushroom spine volume in PL neurons in male (A) and females (B). Male CVS animals reveal graded leftward shifts (i.e., decrease) in mushroom spine volume while female CVS animals reveal graded rightward shifts (i.e., increase). Significance set at $P < 0.01$, K-S test.

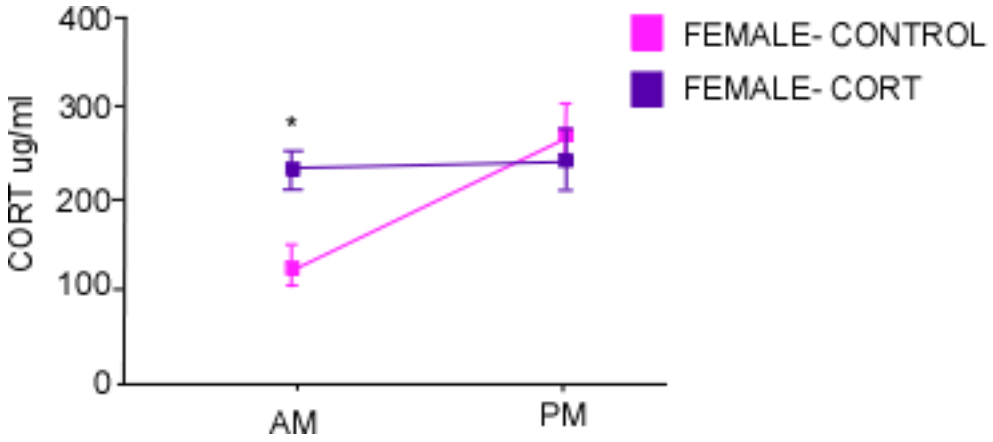

Figure 25. Basal CORT values in female CORT and Sham treated animals

Graph depicting mean ± SEM plasma CORT levels at AM and PM sampling as a function of treatment group. CORT treated animals have higher AM values, while there is no difference in PM values between groups. *, P <0.05 compared to controls. N = 5 - 7 per group.

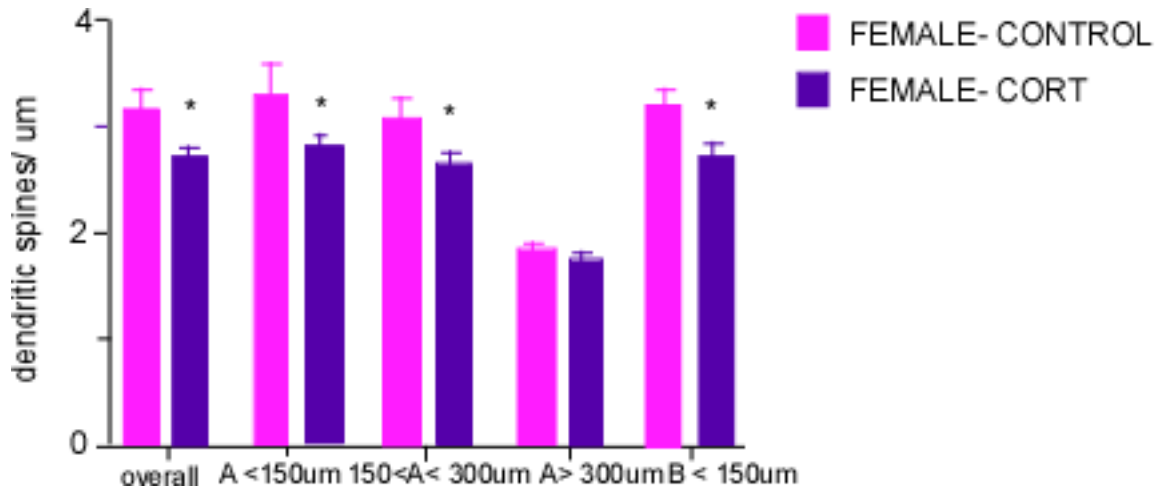

Figure 26. Dendritic spine density in Female CORT and sham treated animals

Mean and SEM of dendritic spine density as a function of treatment. Female CORT animals show decreases in dendritic spine density in PL pyramidal neurons, at all but the most distal (A > 300 μm) portion of the apical tree. *, P < 0.05 compared to controls.

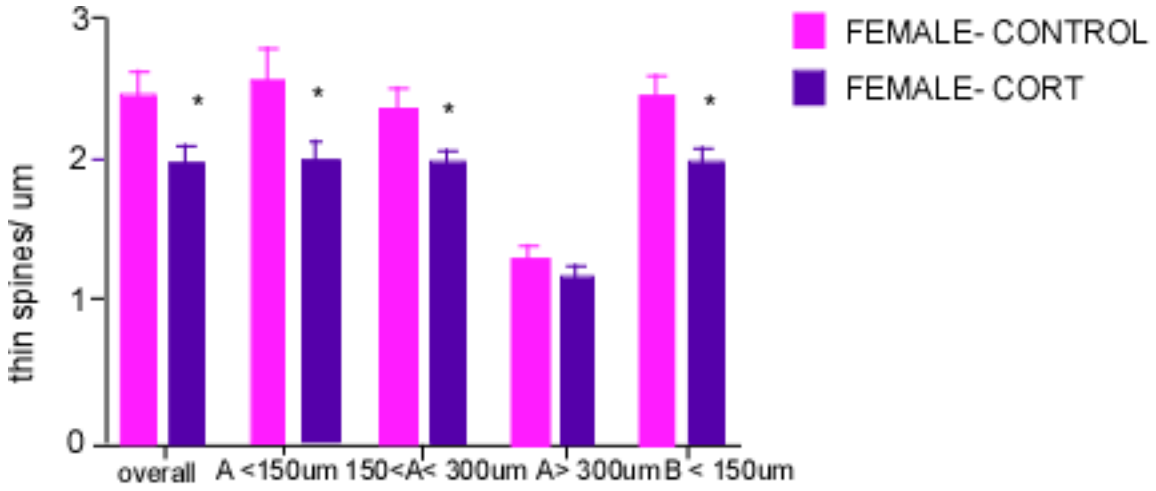

Figure 27. Thin spine density in Female CORT and sham treated animals

Mean and SEM of thin spine density as a function of treatment. Female CORT animals show decrements in thin spine density compared to control animals. Decrease in thin spines are observable in all but the most distal sections of the apical tree. *, $P < 0.05$ compared to controls. N = 5 - 7 per group.

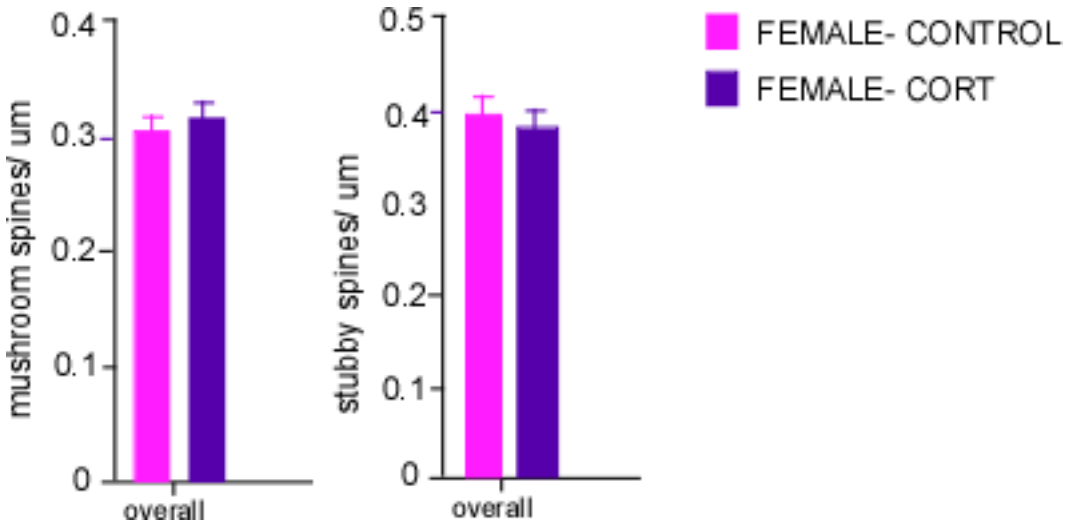

Figure 28. Female CORT mushroom & stubby spine density

Mean and SEM of mushroom (left) and stubby (right) spine density as a function of treatment. CORT exposure did not influence mushroom or stubby spine density. N = 5 - 7 per group.

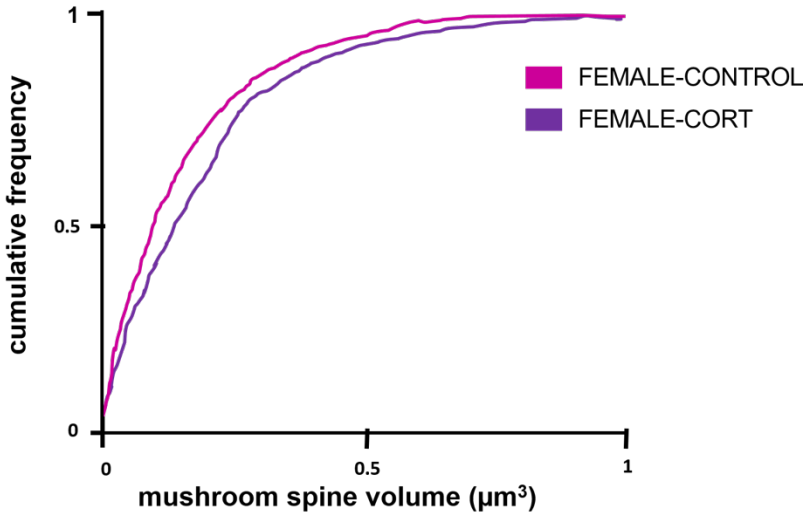

Figure 29. Cumulative frequency distribution of mushroom spine volume in female CORT and sham treated animals

Cumulative frequency distributions of mushroom spine volume in PL neurons reveal rightward shifts (i.e., increase) in spine volume following CORT treatment. Significance set at $P < 0.01$, K-S test.

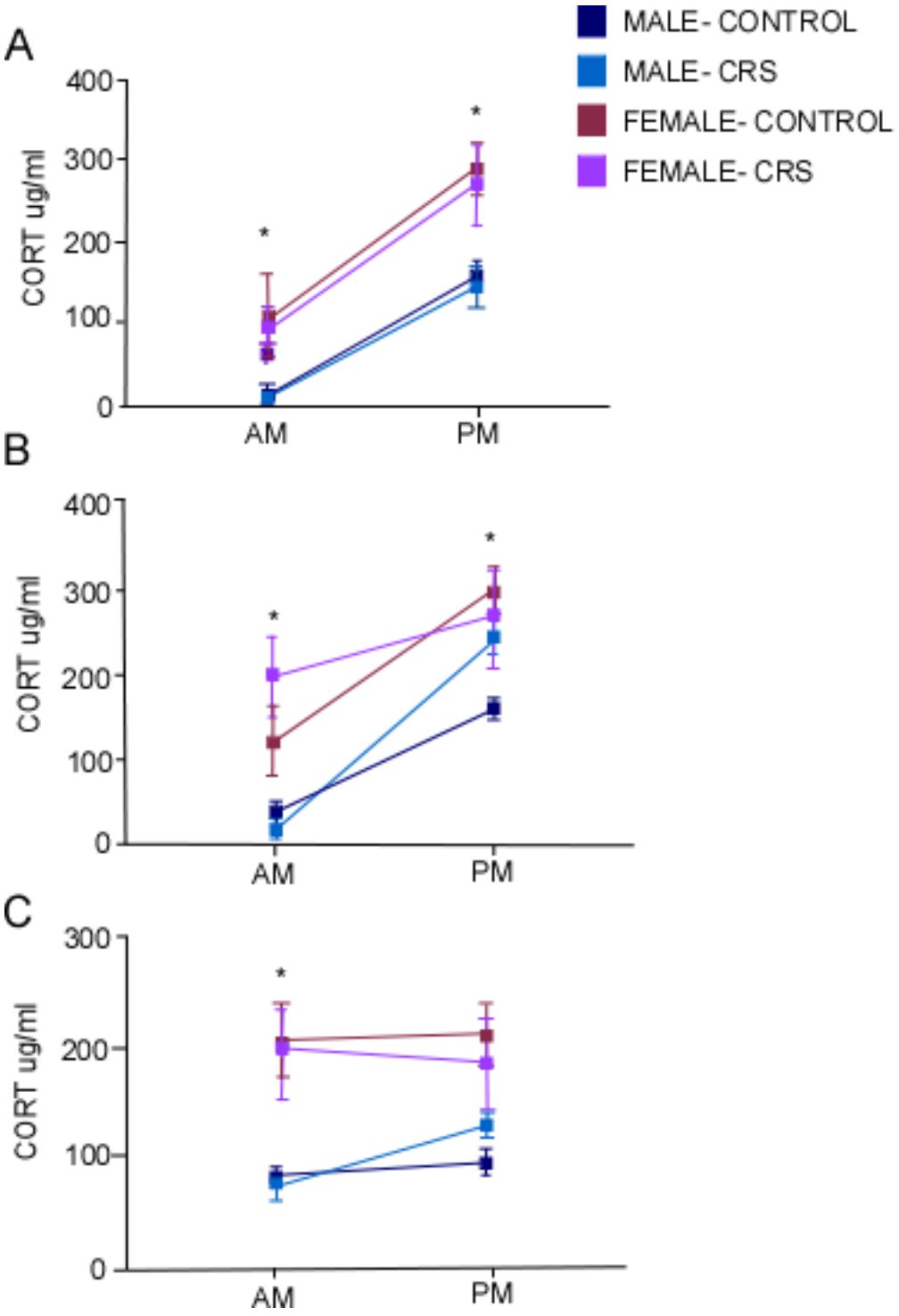

Figure 30. Basal CORT for CRS animals

Graph depicting mean ± SEM plasma CORT levels at AM and PM sampling as a function of treatment group. A., Before CRS, females show higher CORT values in the AM and PM time points than males. B., Following CRS, this observation remained, with females showing higher values in the AM than males,

regardless of treatment. In the PM time point, females had higher values than control males, though not CRS males. CRS males also trend towards higher PM values than control males (P = 0.08) C., Integrated values (area under the curve, AUC) from the sampling before and after CRS reveal that females had higher CORT secretion prior to CRS, but no significant changes within sex due to treatment. *, P < 0.05. N = 10 per group.

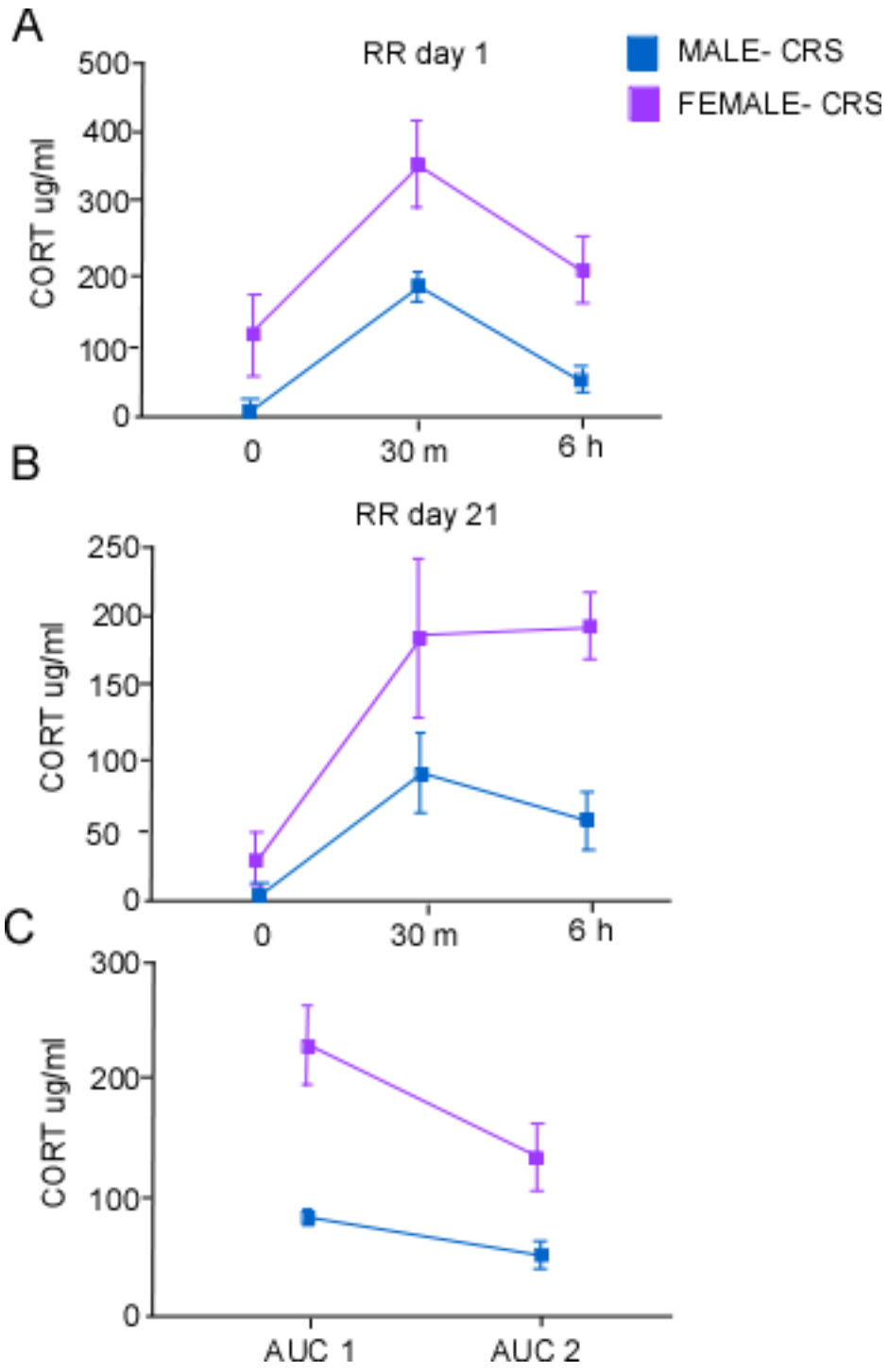

Figure 31. CORT secretion in CRS animals in response to restraint on day 1 and day 21

Graph depicting mean ± SEM plasma CORT levels throughout the repeated restraint paradigm. Blood was taken prior to the beginning of restraint, 30 min into restraint, and at the end of restraint (6 h) on the

first day of restraint (A) and on day 21 (B). A., On the first day of restraint, both males and females show increased CORT values 30 min into the restraint, compared to time point 0. By the end of restraint, CORT secretion has decreased to CORT values similar to the beginning of restraint. B., By the last day of restraint (day 21), CORT values still rise within 30 min of restraint, but not to the levels of day 1. C., Integrated values (area under the curve, AUC) from the first day of restraint and the last day of restraint show trends towards decreased CORT values in both males and females, though this does not reach significance. N = 10 per group.

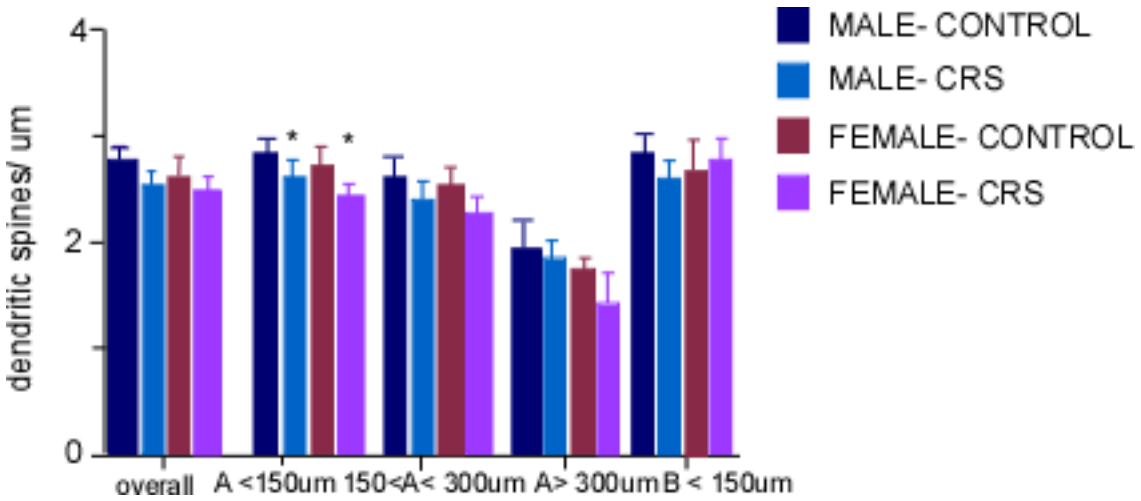

Figure 32. Dendritic spine density in M + F CRS animals

Mean and SEM of dendritic spine density as a function of treatment. CRS animals show decreased spines relative to control animals at the most proximal (A < 150 μm) portion of the apical tree. N = 6- 8 per group.

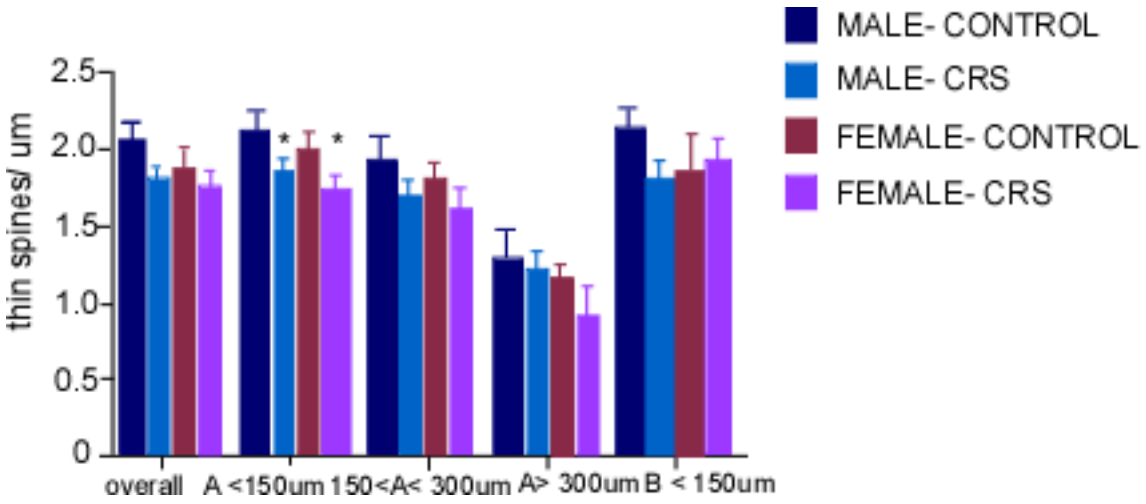

Figure 33. Thin spine density in M+F CRS animals

Mean and SEM of dendritic spine density as a function of treatment. CRS animals show decreased thin spines relative to control animals, particularly at the proximal portion (A < 150 μm) of the apical tree. *, P < 0.05 compared to control animals. N = 6- 8 per group.

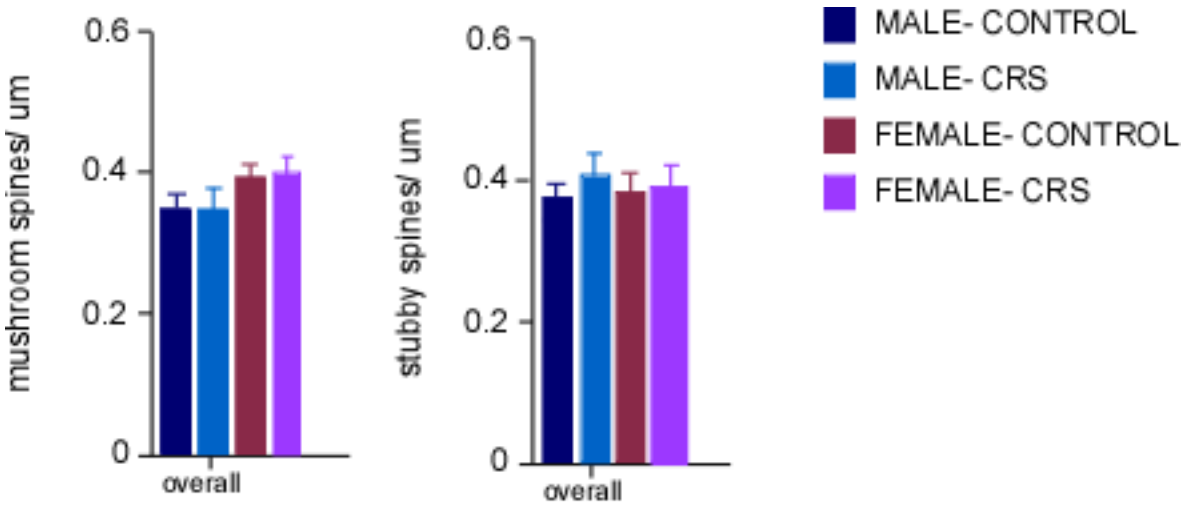

Figure 34. CRS mushroom and stubby spine density

Mean and SEM of mushroom (left) and stubby (right) spine density as a function of treatment. CRS did not significantly change mushroom or stubby spine density. N = 6- 8 per group.

CHAPTER 5. GENERAL DISCUSSION

The prefrontal cortex is critically involved in a wide variety of executive functions that allow for planning and directing motivated behavior. This brain region is also crucially important for mediating behavioral and physiological responses to stress. The PFC has been shown to be adversely impacted by repeated stress in animal models as well by stress-related illnesses in humans, though a thorough analysis of these changes has not been forthcoming. This work is an attempt to provide a detailed look at how chronic stress and high glucocorticoid (CORT) exposure impacts prefrontal function and structure in the rat and hopefully will provide insight into how the PFC is affected in stress-related mental illnesses and other instances where hypothalamo-pituitary-adrenal (HPA) axis activity is disrupted.

In chapter 2, we examined how circadian disruptions of HPA activity may be involved in age-related cognitive decline. Aged animals with high CORT secretion showed a significant loss of dendritic spines in PFC pyramidal neurons compared to aged animals with low CORT secretion and young animals. Follow-up behavioral analyses revealed that spatial working memory deficits were exacerbated by increased HPA activity in aged animals. As younger animals with elevated basal HPA activity did not show changes in PFC indices (relative to their peers), this may argue for a wearing down of plasticity over prolonged periods of time.

In a follow up with the results from chapter 2, we examined the role that glucocorticoids play in the regressive prefrontal plasticity shown after chronic stress, as well as in instances of elevated HPA activity. Male animals who were chronically exposed to CORT levels that mimicked peak CORT circadian activity showed significant

decreases of dendritic spines in PFC pyramidal neurons, with the greatest degree of attrition in thin spine phenotypes. These data generally support the view that chronic stress-induced effects on prefrontal structural plasticity involve a glucocorticoid-dependent component.

In chapter 4, structural and functional analyses were carried out in both male and female rodents in attempt to clarify whether there are sex differences in the effects of chronic stress relative to prefrontal cortical endpoints. Our results indicate that PFC structural and functional indices are affected similarly in males and females following chronic stress. Both males and females showed significant working memory impairments as well as decreased dendritic spines following 14 days of chronic variable stress (CVS; a heterotypic stress paradigm). Follow-up analyses in females showed that this is similarly glucocorticoid dependent (as shown in males in chapter 3). Finally, these changes seem to generalize to various types of repeated stress. Both males and females exposed to 21 days of chronic restraint stress (CRS; a homotypic stress exposure) show decreases in dendritic spine density compared to unstressed controls. Together, the results indicate that when it comes to PFC measurements of function and plasticity, males and females do not seem to be sexually-differentiated following stress.

Anatomy and connectivity of the rodent PFC

The focus of this work has been on the pyramidal neurons in PFC and hasn't given much careful consideration of the neural circuits that may be involved in the changes reported in these studies. As dendritic spines make up the majority of sites of excitatory

input in the cortex, it is imperative to discuss potential inputs that may be impacted by stress that are involved in the regressive changes we have observed.

The medial prefrontal cortex (mPFC) is comprised of various subregions including the anterior cingulate cortex (ACC), infralimbic (IL) and prelimbic areas (PL; the area focused on in this work). The majority of neural connections in the mPFC are between these various subregions and layers (Jones et al., 2005). However, in the context of modulating stress, there are a couple of pathways to PL that may be playing important roles in the effects observed and recorded here. The hippocampal formation (HF) sends excitatory projections to PL (Hoover and Vertes, 2007) which appear to be involved in the integration of spatial and cognitive processes. This projection has shown to be critically involved in the performance of delayed-response tasks (Floresco et al., 1997). The ventral tegmental area, the basolateral amygdala, and midline thalamus also send excitatory projections to PL (Hoover and Vertes, 2007). Whereas the HF allows for spatial integration of executive functions, these subcortical limbic projections likely are important for affective components of goal directed behavior.

Though we did not assess function in animals who underwent structural analyses (as not to influence plasticity measures), it is possible that the decrements in spines observed following stress and CORT exposure disrupt inputs from HF and other subcortical regions and subsequently disrupt prefrontal function. As dendritic spines make up the majority of sites of excitatory input in the cortex, the logical conclusion is that following stress there is reduced excitatory input on these PL pyramidal neurons. This likely accounts for disruptions in working memory, as these neurons are critical for

integration of delayed-response tracking and goal-directed behavior and previous work has correlated the loss of spines with prefrontal tasks (Dumitriu et al., 2010).

Evidence for disrupted excitatory afferent projections to PFC and subsequent behavioral disruptions comes from Morilak and colleagues (2017). Following chronic stress, rats show impairments on the prefrontal dependent attentional set-shifting task (AST). The same impairments could be recapitulated in control animals by blocking glutamate receptors, indicating that dysfunction of these excitatory receptors is sufficient to impair prefrontal function. Chronically stressed animals also showed compromised c-fos expression and reduced local field potentials in PFC following afferent activation of the mediodorsal thalamus (MDT), a primary excitatory input. These results suggest that the excitatory afferent projection from MDT is compromised following stress and that this plays a role in some prefrontal impairments (Jett et al., 2017).

A question remains as to whether increasing spine density (or preventing loss in the first place) would rescue (or prevent) working memory impairments. Though we have not carried out these experiments, evidence suggests that this could be a possibility. Ketamine is a fast-acting NMDA antagonist that has been used to therapeutically reduce depression symptoms (Berman et al., 2000; Zarate et al., 2006). Ketamine in pre-clinical models increases dendritic spines and this is correlated with antidepressant behavioral effects (Li et al., 2011), indicating that spine density is important in some of the observed symptomology. Optogenetically stimulating IL neurons using parameters similar to what ketamine produces on post-synaptic neurons is also sufficient to increase spine density and ameliorate depressant behaviors, such as immobility on the forced swim task. (Fuchikami et al., 2015). Though these studies

did not examine if increasing dendritic spines could prevent or rescue cognitive function, the ability to prevent depression-like behaviors leads one to question if they could. Future studies should focus on optogenetically stimulating afferent pathways known to be important for prefrontal function (such as MDT or HF) to see if stimulation is capable of increasing dendritic spine density and subsequent working memory performance following or during stress.

Effects of glucocorticoids on PFC

The PFC is not only is modulated by stress but also plays a role in modulating HPA activity. As mentioned in the introduction, the PFC sends excitatory projections to aBST and vSUB that then send inhibitory projections to PVH, leading to inhibition of the HPA axis. A key interpretive question that results from our studies is to what extent are changes in CORT cause or consequence of chronic stress? In other words: does elevated CORT release following stress cause the prefrontal structural changes observed here or does stress cause structural changes that leads to elevated CORT secretion? The data presented here cannot fully address these questions, however my inclination is that both are involved, though to the extent to which they are may vary.

Glucocorticoids are known to be involved in dendritic spine plasticity as others have shown and as we have presented here. By blocking glucocorticoid synthesis, some of the structural alterations that occur from elevated CORT can be prevented (Magarinos and McEwen, 1995). This data may suggest that chronic CORT release following stress is capable of inducing dendritic spine changes, instead of structural

changes leading to increased CORT. This is also supported by data from Chapter 3. Animals implanted with CORT pellets for 3 weeks show reductions in dendritic spine density. However, following the absorption of the pellet, dendritic spine loss persists, yet HPA activity normalizes. If structural rearrangement was sufficient on its own to elevate HPA activity, we should expect HPA activity to still be high in these animals.

Although there is much support for elevated CORT impacting dendritic spine density, it is still likely that regressive plasticity may also lead to impaired ability to inhibit HPA activity. Damage to the PL prolongs HPA axis responses to acute stress (Diorio et al., 1993; Figueiredo et al., 2003; Radley et al., 2006a) and stimulation of these neurons are capable of inhibiting stress response (Jones et al., 2011). As dendritic spines play important roles in integrating excitatory inputs in pyramidal neurons, a loss of spines could be involved in reduced neuronal excitability and subsequent impaired HPA inhibition, though there has been little work done to investigate this.

Another interpretive question that results from our data is what constitutes “high” CORT and how does this effect dendritic spine density. In the second chapter, aged animals classified as high CORT animals (above the median split for aged animals) show decreases in spine density as well as working memory impairments. However, young animals with high CORT secretion (relative to other young animals) do not show the same impairments. This is in direct contrast with young animals who were implanted with slow-releasing CORT pellets (chapter 3) as these animals do show spine loss. Again, our data do not provide the answer to why there is this discrepancy, but one hypothesis to explain our findings in chapter 3 may be that the circadian rhythm was disrupted in these animals but not in our young animals from chapter 2. Several studies

have linked glucocorticoid circadian rhythmicity with spine formation and elimination (Liston et al., 2013; Ikeda et al., 2015) and disruptions in this rhythm has been associated with regressive structural changes (Pyter et al., 2005; Karatsoreos et al., 2011). As suggested by these data, our changes in spines in experiment 3 may be partially due to the disrupted circadian rhythm of CORT induced by the pellet and our young + high CORT animals in chapter 2 may be protected by their intact circadian rhythms.

Relatedly, animals exposed to chronic restraint stress (CRS) do not show changes in basal CORT secretion (unlike CVS animals) but still show dendritic spine loss (similarly to CVS animals). Again, our data do not allow us to interpret why these differences exist. As briefly mentioned in the discussion to chapter 4, a lack of change in basal CORT secretion observed at the end of the 21-day period of restraint does not indicate any potential changes to basal adrenocortical activity in the interim. Regardless of any potential basal change in HPA activity or elevated CORT exposure, the structural changes observed following stress are likely not solely due to glucocorticoids. Many signaling pathways are involved in mediating dendritic spine structure, and many of them have shown to be influenced by stress (for examples see Andres et al., 2013; Skrzypiec et al., 2013; Bennett and Lagopoulos, 2014). It is possible in instances such as our CRS paradigm where changes in basal HPA activity are not noticeable, that stress is influencing these indices through different mechanisms.

Sex differences

Studies have observed sex differences in all levels of the stress response (Kitay, 1961; Critchlow et al., 1963; Turner and Weaver, 1985; Handa et al., 1994; Carey et al., 1995; Iwasaki-Sekino et al., 2009), with a majority of the work focusing on hippocampal functioning following stress (Daniel et al., 1997; Luine et al., 1998; Bowman et al., 2001; Bowman et al., 2002; Dalla et al., 2009; Conrad et al., 2012) The work presented here focused on stress and CORT exposure in adulthood in both males and females and finds no drastic difference in prefrontal behavioral or structural endpoints. Though this may appear in opposition to previous studies, we believe that the findings here highlight that brain regions may differ in their stress susceptibility in females.

Though we demonstrate here that males and females show similar responses to chronic stress and CORT treatment, we do not believe that our data necessarily indicate a lack of sex differences. Many studies have shown that the mechanism behind certain behavioral or structural endpoints in females and males are different, even if the end result is the same (see De Vries, 2004). As this work does not investigate the molecular underpinnings of our changes, we cannot rule out the idea that there are molecular differences that have led to our results in males and females. For instance, corticotropin-releasing factor (CRF), the neuropeptide involved in the neuroendocrine response to stress, has been shown to be excessively released in females when compared to males during stress (see Valentino et al., 2013). CRF receptors also appear to be more sensitive in females, which may lead to reduced ability to respond and habituate to chronic stress and may be involved in some of the effects we have observed here.

Females also reliably show higher basal and stress-induced CORT levels than male rats (Kitay, 1961; Weinstock et al., 1998; Viau et al., 2005). This may be in part due to ovarian hormone levels as females in proestrus have higher CORT levels than females in diestrus (Viau and Meany, 1991; Carey et al., 1995). We also see higher CORT secretion in females in our data, supporting this previous work. The physiological impact behind this is unclear, though it could contribute to enhanced susceptibility of stress-related mental illness in humans (for discussion see Bangasser and Valentino, 2014), though studies have shown that the corticosteroid-binding globulin is elevated in females, resulting in similar net result in free circulating CORT (Tinnikov, 1999).

Functional considerations

Our data demonstrate loss of dendritic spines following chronic stress and elevated glucocorticoids in the PFC. A question remains about the functional implications of this regressive plasticity. As spines are highly labile structures that change quickly in response to various physiological and psychological stimuli, we did not analyze dendritic spines in animals who had undergone behavioral testing. As we are not able to correlate spine density with impairments on our working memory task, we are not able to say with any certainty the functional implications of stress or glucocorticoids on the prefrontal cortex. However, other work has suggested that thin spines in particular are important for proper prefrontal functionality (Arnsten et al., 2010; Dumitriu et al., 2010) and that glucocorticoids are key mediators of spine plasticity (Liston and Gan, 2011; Tanokashira et al., 2012; Liston et al., 2013).

Numerous studies have examined cortical tissue for changes after a variety of learning or long-term potentiation using electron microscopy and have shown increases in dendritic spine density as well as enlargement of spines (Bailey and Kandel, 1993; Gray et al., 2006; Liston et al., 2013; Roy et al., 2016) as a function of learning. In even more refined techniques, dendritic spine growth and enlargement have been visualized directly in the mouse neocortex following learning tasks (Knott et al., 2002; Holtmaat et al., 2008). As mentioned earlier, increasing spine density optogenetically or pharmacologically rescues depressant-like behaviors in rodents. These data together point to critical functional importance of dendritic spines giving support to some of the impairments observed in our studies resulting from loss of dendritic spines in PL pyramidal neurons.

Mushroom spines represent a more stable spine phenotype. Electron microscopy data have shown that mushroom spines contain more α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and that spine volume is associated with synaptic strength (Harris et al., 1992; Matsuzaki et al., 2001a; Nimchinsky et al., 2004; Ashby et al., 2006). Studies also suggest that spine stability is associated with spine size, indicating that larger spine phenotypes will persist longer in the cortex (Grutzendler et al., 2002; Nimchinsky et al., 2002; Mizrahi and Katz, 2003; Holtmaat et al., 2005). This supports our data that tend to show that mushroom spine density does not change in response to stress or elevated glucocorticoids. However, these spines are not immune to these challenges. We have consistently shown that mushroom spine volume decreases following stress manipulations and exposure to high CORT in males. A striking sex-difference in our data is that in females, stress and high CORT

manipulations result in increased mushroom volume. Though the mechanism behind this difference is not revealed in our analyses, estrogen is thought to play a neuroprotective role and has been shown to induce dendritic spine growth (Woolley and McEwen, 1992; Luine and Frankfurt, 2012; Srivastava et al., 2013; Luine, 2016). Given the role it plays in spine plasticity it is possible that estrogen may be involved in the enlargement of spines observed in females following stress reported here.

Conclusions

The current findings provide a rigorous and detailed account how glucocorticoids and stress affect prefrontal function and structure in male and female rats. Collectively, they show that a chronic exposure of CORT or stress (homotypic or heterotypic) leads to dendritic spine loss in mPFC pyramidal neurons and impaired prefrontal-dependent working memory. Importantly, the effects of these manipulations are similar in males and females suggesting that this brain region is not differentially susceptible to stress relative to other areas that have shown sex differences. Together, this work highlights a critical role of glucocorticoids in prefrontal structural rearrangement following stress which may help explain prefrontal functional deficits in stress-related mental illnesses or other diseases where HPA axis activity is disrupted.

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