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Sex- and stress-dependent effects on dendritic morphology and spine densities in putative orexin neurons

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ABSTRACT
We recently found that non-stressed female rats have higher basal prepro-orexin expression and activation of orexinergic neurons compared to non-stressed males, which leads to impaired habituation to repeated restraint stress at the behavioral, neural, and endocrine level. Here, we extended our study of sex differences in the orexin system by examining spine densities and dendritic morphology in putative orexin neurons in adult male and female rats that were exposed to 5 consecutive days of 30 min restraint. Analysis of spine distribution and density indicated that putative orexinergic neurons in control non-stressed females had significantly more dendritic spines than those in control males, and the majority of these were mushroom spines. This morphological finding may suggest more excitatory input onto orexin neurons in female rats. As orexin neurons are known to promote the HPA response, this morphological change in orexin neurons could underlie the impaired habituation to repeated stress in female rats. Dendritic complexity did not differ between non-stressed males and females, however repeated restraint stress decreased total dendritic length, nodes, and branching primarily in males. Thus, reduced dendritic complexity of putative orexinergic neurons is observed in males but not in females after 5 days of repeated restraint stress. This morphological change might be reflective of decreased orexin system function, which may allow males to habituate more fully to repeated restraint than females.These results extend our understanding of the role of orexin neurons in regulating habituation and demonstrate changes in putative orexin cell morphology and spines that may underlie sex differences in habituation.

Key words: Orexins, hypocretins, Stress, Morphology, Spines, Sex Differences

Introduction

An important neural substrate that mediates arousal, wakefulness, and vigilance are the neuropeptides orexins (de Lecea et al., 1998; Sakurai et al., 1998). These neuropeptides are also crucial in mediating the response to a stressful stimulus, as one must shift from a basal to a reactive state (Berridge and España, 2005). Previous studies have implicated orexins in promoting the stress response via stimulation of the sympathetic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis (Jászberényi et al., 2000; Kuru et al., 2000; Winsky-Sommerer et al., 2005; Spinazzi et al., 2006; Heydendael et al., 2011; Kuwaki, 2011; Johnson et al., 2012; Messina et al., 2014). On the other hand, stress stimulates orexin neurons. For example, direct administration of the stress regulatory peptide corticotropin releasing hormone (CRH) or exposure to a stress paradigm such as the forced swim test increases orexin activation (Winsky-Sommerer et al., 2005; Chang et al., 2007; Furlong et al., 2009; Chen et al., 2013). Notably, patients with stress-related disorders such as post-traumatic stress disorder (PTSD) show altered levels of orexins in their cerebrospinal fluid. Interestingly, females display a higher risk of developing stress-related psychiatric disorders, with depression and PTSD having a two-fold greater prevalence (Heller, 1993; Blehar, 1995; Pigott, 1999). Thus, orexins are altered in stress-related disorders in which there are marked sex differences.

One crucial adapation to repeated stress is habituation, a progressive decline in behavioral, HPA, and autonomic responses to moderately intense stressors (Grissom and Bhatnagar, 2009). Important changes in the cellular response, including decreases in neuronal firing rate, postsynaptic current, and reduction in neurotransmitter release, underlie these behavioral and physiological responses (Thompson and Spencer, 1966; Groves and Thompson, 1970; Castellucci and Kandel, 1974; Glanzman, 2006). Whether habituation takes place is dependent on the type, intensity, and duration of the stressor (Grafe and Bhatnagar, 2018). Typically, unpredictable chronic mild stress prevents habituation through variation in the type of stressor (Herman, 2013). The enhanced HPA axis drive in unpredictable stress is likely driven through stressor controllability (Maier and Watkins, 2010). Although, control of the stressor does not seem to allow habituation in females (Baratta et al., 2018).

We recently found that non stressed female rats have higher basal prepro-orexin expression and activation of orexinergic neurons compared with males, leading to impaired habituation of the HPA axis to repeated restraint stress and subsequent cognitive deficits (Grafe et al., 2017). Importantly, inhibition of orexin neurons allowed female rats to habituate more fully to repeated restraint stress at multiple levels, including reduced struggle behavior, lower activation in the paraventricular nucleus (PVN) of the hypothalamus, and decreased HPA hormones. Thus, higher orexin activity promotes the stress response and prevents habituation in female rats.

In the studies described here, we extended our study of sex differences in the orexin system by examining dendritic morphology and spine densities in putative orexinergic neurons both in non-stress conditions as well as after 5 consecutive days of 30-min restraint stress in adult male and female rats. Orexin neurons were defined as fusiform cell bodies with a cross sectional area between 175-225 μm^2 in the perifornical region of the lateral hypothalamus (de Lecea et al., 1998; Cheng et al., 2003). We aimed to determine if there were observable structural differences in these putative orexinergic dendrites in males compared with females in basal conditions, and if repeated restraint stress affects any sex differences observed. As we observed greater prepro-orexin mRNA and activation in basal, non-stressed females (Grafe et al., 2017), we hypothesized that there would be more complex dendritic morphology and a greater number of spines in control females compared with males. This would be reflective of more excitable orexin cells in females. As females do not habituate as fully as males to repeated restraint stress (Grafe et al., 2017), we expected that this may remain high in females after repeated restraint. However, since males habituate more fully to repeated restraint, we

expected dendritic complexity and number of spines would decrease with 5 days of repeated restraint stress in males rats.

Experimental Procedures

Animals

Male and female Sprague-Dawley rats between 65-75 days of age were acquired from Charles River Laboratories (Wilmington, MA, USA). Rats were singly housed and food and water were available *ad libitum*, under 12-h light/dark cycle. Animals acclimated to the housing and lighting conditions for 5 days prior to any surgical or stress protocols. The Institutional Animal Care and Use Committee of The Children's Hospital of Philadelphia (CHOP) Research Institute approved all experimental procedures.

Restraint Paradigm

Animals were either assigned to a control group (and remained in their home cage) or were restrained in Plexiglas restrainers differently sized for male or female rats for 30min/day for 5 consecutive days (n = 10/group; run in two separate cohorts of 5/group). Animals were restrained within 2h after lights on so that circadian levels of orexin remained consistent (Fujiki et al., 2001). Thirty minutes after restraint ended (or in the case of controls, roughly at the same time of day as restrained rats), rats were removed from their home cage, rapidly decapitated, and brains were removed, rinsed with water, and blocked (with the cerebellum and frontal cortex removed). Brains were processed as outlined below.

Golgi-Cox Staining Method

Staining was performed as dictated by the procedure in the FD Neurotechnologies Rapid Golgi-Kit (FD Neurotechnologies, Columbia, Maryland) with minor alterations. Briefly, brains were placed in an impregnation solution containing a mixture of potassium chloride, mercury chloride, and potassium dichromate. This impregnation solution was provided as two parts (A and B) to be mixed 1:1. The impregnation solution was replaced after 24 hours, and brains remained in this solution for 14 days, with gentle agitation every 4 days to prevent precipitation of the impregnation solution. Following impregnation, the brains were placed in a rehydration solution (Solution C, FD Neurotechnologies, Columbia, Maryland) for 7 days. Brains were then frozen on dry ice, and 140 µM slices containing the lateral hypothalamus were made using a cryostat (set at -24°C) and mounted on gelatin coated slides (FD Neurotechnologies, Columbia, Maryland). Slides were dehydrated in three sessions. Each session was 4 minutes long with increasing concentrations of ethanol (50%, 75%, and 95%). Slides were cleared with three xylene washes, each 4 minutes long. Slides were then coverslipped with permount.

Imaging and Neuronal Reconstruction

Orexin neurons were reconstructed in 3-D at 40x using a Nikon Eclipse NI Microscope (Nikon, Tokyo, Japan) and Neurolucida (MBF Neuroscience, Williston, VT) by an individual blinded to treatment groups. Putative orexin neurons were identified by shape (fusiform) and cell body area (175 μm^2 – 275 μm^2) as described in (Cheng et al., 2003) and location (proximal to the fornix in the lateral hypothalamus). Each cell was randomly sampled for reconstruction. No more than three neurons per section were taken. Only neurons with complete filling and clearly defined branching were reconstructed. Dendrites that were counted in the final analysis are believed to be non-truncated. Dendrites were analyzed for total length, nodes, branching, and number of sholl radii intersections (per 10 µm concentric circles).

Dendritic Spine Counting
Putative orexin neurons were viewed using a 100x oil-immersion lens. Neurolucida was used to reconstruct a single random dendrite per one orexin neuron. All spines were marked for the entirety of the dendrite and classified as mushroom, filopodial, or stubby (Hering and Sheng, 2001). Specifically, mushroom spines were characterized by a thin neck with a bulbous head; filopodial spines were characterized as thin protuberances lacking a bulbous head; stubby spines were characterized as short structures that lack a defined neck. Spine distribution was examined as the number of spines counted per sholl radius (successive 10 µm concentric circles). Spine density was also analyzed per µm dendritic length (number of spines divided by the total length of the dendrite).

Statistical Analysis
Data are presented as the mean ± the standard error of the mean. There were 4-6 cells analyzed per animal and 8-10 animals with traceable golgi staining per treatment group. Loss of some animals was due to insufficient penetration of the impregnation solution into the lateral hypothalamus. For morphology and spine density data, two-way ANOVA (Stress [Control or 5 Days Restraint] by Sex [Male or Female] treatments) was used, followed by tukey's post hoc t-tests using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Sholl analysis was also performed on all reconstructions, measuring intersections or number of spines at successive 10µm concentric circles. For these data, JMP Statistics (SAS, Cary, North Carolina) was used to run a repeated measures ANOVA of Stress (Controls or 5 Days Restraint) by Sex (Male or Female) by Distance (each sholl radii; repeated measures). Further examination of treatment group differences over all sholl distances was performed by t tests. All analyses used α=0.05 as the criterion level of significance.

Results

Stress decreases dendritic morphology (length and nodes) in putative orexin neurons more prominently in male rats
We first examined the effect of sex and stress on total dendritic length, nodes, and branching of putative orexin cells (**Figure 1**). Representative low and high resolution images of golgi-cox staining in the lateral hypothalamus are shown in **Figure 1A**. A Two-way ANOVA revealed that there was no significant main effect of sex on total dendritic length, nodes, and branching (**Figures 1B, 1C,** and **1D**, respectively). However, there was a main effect of stress in decreasing all three measures in both sexes ($F(1,136) = 4.5$, $p = 0.037$; $F(1,136) = 7.5$, $p = 0.007$; $F(1,136) = 6.8$, $p = 0.010$). Further analyses revealed that this main effect was driven by differences in males with stress significantly decreasing total dendritic length and number of nodes in male rats ($p = 0.043$ and $p = 0.010$, respectively) but not in female rats. On average, stress decreased dendritic length by 135 μm (from 725$\pm$55 μm to 590$\pm$40 μm) in male rats, whereas stress decreased dendritic length by only 61 μm (from 707$\pm$43 μm to 646$\pm$45 μm) in female rats. In summary, dendritic morphology of putative orexin neurons does not differ between non-stressed male and female rats. However, stress significantly decreases dendritic length, nodes, and branching in orexin neurons, but does so more prominently in male rats.

Stress decreases dendritic complexity across the length of putative orexin neurons in male but not female rats
We examined dendritic arbor complexity via Sholl analysis (**Figure 2**). Representative images of dendritic complexity for each treatment group are shown in **Figure 2A**. A repeated measures ANOVA revealed that while there was no significant main effect of Sex or Stress, there was the expected main effect of radial distance on the number of dendritic intersections ($F(39,89) = 11.0$, $p<0.0001$). Moreover, there was a significant interaction between distance and stress

(F(39,89) = 1.2, p<0.0001) but no significant interaction between sex and distance. Further examination of the data revealed that stressed male rats had a significantly decreased number of intersections per sholl radius than control male rats at distances of 30 to 110 μm from the cell body (**Figure 2C**, p = 0.046), but this was not observed in stressed females compared to control females (**Figure 2D**). Furthermore, stressed females exhibited higher number of intersections compared to stressed males between 30um and 50um (Figure 2E). Therefore, stress significantly decreased dendritic complexity of putative orexin neurons in male but not female rats.

Putative orexin neurons express more spines in non-stressed female compared to male rats and stress decreases spine density in females but not males

We next studied the number of spines along putative orexinergic dendrites in two ways: Spine density and Sholl analysis (Figure 3). We quantified the number of spines per μm dendritic length and the number of dendritic spines at each sholl radius. Representative images of dendritic spines are shown for each treatment group in **Figure 3A**. For total spine density, we found a main effect of sex but not stress in spine density (**Figure 3B**; F(1,87) = 6.9, p = 0.010). Post hoc analysis revealed that these putative orexin neurons have a higher total dendritic spine density in non-stressed females compared to non-stressed males (p = 0.040). Consistent with this total spine density finding, we found a main effect of sex and radial distance in dendritic spine distribution, but no main effect of stress (**Figure 3C-F**: sex F(1,87) = 3.7, p = 0.059; distance F(29,59) = 10.9, p<0.0001). However, there was a significant interaction between distance and stress (F(29,59) = 1.1, p<0.003) but no significant distance x sex interaction. Further examination of the data revealed that non-stressed female rats have more spines at the majority of sholl radii compared with non-stressed male rats (**Figure 3C;** p = 0.001). On average, females had 2 to 3 more spines per 10μm radius than males at distances of 50 μm to 130 μm from the cell body. Control males did not differ from stressed males (**Figure 3D**). However, control females had more spines than stressed females at distances 50 to 90 μm from the cell body but significantly fewer spines 140-180μm away from the cell body (**Figure 3E**, p<0.010). There were no significant differences between the sexes in the presence of stress (**Figure 3F**). In sum, our data suggest that non-stressed females have significantly more dendritic spines on putative orexin neurons compared with non-stressed males. However, with stress, the decrease number of spines in females eliminates this sex difference.

We next examined which types of spines were present along putative orexinergic dendrites in males and females with or without exposure to repeated restraint, a stressor diminished by habituation. Specifically, we quantified the density and distribution of mushroom spines, filopodial spines, and stubby spines along dendrites in putative orexin neurons in **Figures 4, 5, and 6**, respectively). We first examined mushroom spines, characterized by a thin neck with a bulbous head; these spines are thought to indicate mature synapses (Hering and Sheng, 2001). We found a main effect of sex but not stress on mushroom spine density (**Figure 4A;** F(1,88) = 5.4, p = 0.023). Post hoc analysis indicated that these neurons have a higher mushroom spine density in non-stressed females compared to non-stressed males, but they do not differ in stressed males compared to stressed females (**Figure 4A;** p = 0.023). There was a trend for a main effect of sex and a main effect of radial distance, but no main effect of stress on dendritic mushroom spine distribution (**Figure 4B-E,** sex, F(1,88) = 2.8, p = 0.093; distance F(23,66) = 10.9, p<0.0001). Moreover, there was an interaction effect between sex and stress as well as distance by stress in dendritic mushroom spine distribution (sex x stress, F(1,88) = 3.9, p = 0.049; distance x stress (F(23,66) = 3.3, p<0.0001). Further analysis revealed that non-stressed female rats have more mushroom spines at the majority of sholl radii compared with non-stressed male rats (**Figure 4B**; p = 0.002).. Control males did not differ from stressed males

(**Figure 4C**), while control females had significantly more spines than stressed females at distances 50 to 90 μm from the cell body (**Figure 4D**, $p<0.010$). There were no significant differences between the sexes in the presence of stress (**Figure 4E**). In sum, non-stressed females have more mushroom spines along dendrites in putative orexin neurons compared to non-stressed males and compared to stressed females.

Next, we quantified filopodial spines, which are characterized as thin protuberances lacking a bulbous head (Hering and Sheng, 2001). They are transient, common on developing neurons, and may represent precursors to mature spines (Rochefort and Konnerth, 2012). We found no differences in total filopodial spine density (**Figure 5A**). There were also no significant differences in filopodial spines along the length of the dendrites between groups (**Figure 5B-E).** Lastly, we analyzed the number of stubby dendritic spines, which are short structures that lack a defined neck and are prominent between postnatal developmental stages (Hering and Sheng, 2001). There was no main effect of sex or stress on stubby spine density (**Figure 6A**) or distribution (**Figure 6B-E**). However, there was a main effect of distance on stubby spine distribution ($F(23,66) = 20.6$, $p<0.0001$), as well as an interaction between distance and stress ($F(23,66)= 2.0$, $p = 0.015$). This was due to a small difference in the number of stubby spines in control versus stressed females, depending on the distance from the cell body (**Figure 6D**). In sum, male and females do not significantly differ in their density or distribution of filopodial or stubby spines along dendrites in orexin neurons, and stress does not greatly alter these measures.

Discussion

Our previous work has revealed that the neuropeptides orexins mediate sex differences in habituation to repeated stress (Grafe et al., 2017). Specifically, elevated levels of orexins in female rats impair habituation to repeated restraint stress. The study reported here determined how sex and stress affect putative orexin neuron structure. Specifically, we were interested in examining dendritic morphology and spine density in putative orexin neurons in male and female rats both in the absence of stress and after five days of repeated restraint. We did not observe sex differences in basic dendritic morphology (such as dendrite length, nodes, and branching) in the absence of stress. However, we found that non-stressed females have more mature (mushroom) dendritic spines than non-stressed males. Thus, in basal conditions, these lateral hypothalamic neurons may have more synaptic inputs in females compared with males, which may be indicative of greater orexin system activity. As orexins are known to promote the stress response (Jászberényi et al., 2000; Kuru et al., 2000; Winsky-Sommerer et al., 2004; Spinazzi et al., 2006; Johnson et al., 2010; Kuwaki, 2011; Heydendael et al., 2012; Messina et al., 2014), this morphological finding could underlie elevated basal corticosterone levels observed in female rats or their elevated sensitivity to stress (KITAY, 1961; Grafe et al., 2017). Moreover, we found that putative orexin dendritic morphology and complexity significantly decreases after five days of repeated restraint stress in males but not females, perhaps contributing to male habituation to repeated stress, whereas females do not habituate as fully (Grafe et al., 2017). Ultimately, our data may indicate that more complex morphological characteristics of orexin cell dendrites impair habituation to repeated restraint stress in female rats.

Even in the absence of stress, we observed that females had more dendritic spines in putative orexin neurons than males. When we categorized the type of spines on the dendrite more closely, we found that the majority of these spines were classified as mushroom spines. As mushroom spines are thought to indicate mature synapses (Hering and Sheng, 2001), more mushroom spines are likely indicative of more synaptic input (Matsuzaki et al., 2001), which

may contribute to the higher orexin system activation in females that we observed in previous studies (Grafe et al., 2017). It should be noted that we currently do not know what connections are being made with the orexin dendrites. However, dendritic spines typically receive excitatory synaptic input from axons (Gray, 1959; Tønnesen and Nägerl, 2016). Thus, more mature dendritic spines likely indicate more excitatory input onto these neurons, potentially increasing orexin system activity in females and leading to their impaired habituation and cognitive flexibility that we have previously observed (Grafe et al., 2017).

The result of more orexin dendritic spines in non stressed females compared with males is not surprising; Sex differences in synaptic plasticity have been shown before in other brain areas like the hippocampus and amygdala (Woolley and McEwen, 1992; Bender et al., 2017). Previous studies have indicated that estradiol may be sufficient to account for these sex differences, potentially increasing the amount of spines present or synaptic connections made. Additional studies have found that estradiol increases dendritic spines by enhancing presynaptic glutamate release in the hippocampus and hypothalamus (Schwarz et al., 2008; Oberlander and Woolley, 2016). It is unclear if this is the mechanism by which sex differences are observed in our studies. Alternatively, as estrogen also potentiates the HPA response (Carey et al., 1995), it is possible that estrogen increases CRH, whose terminals are known to make direct contact with orexin neurons (Winsky-Sommerer et al., 2004), potentially increasing synaptic strength. However, future studies are required to explore the types of connections that are being made with orexin neurons in non-stressed males and females. Certainly, a greater number of excitatory inputs onto orexin neurons in females would explain higher orexin system activity in females compared to males in the absence of stress (Grafe et al., 2017).

With exposure to repeated restraint, the extent to which one habituates to the stressor becomes important in determining how synaptic plasiticity in a given region will ultimately be affected. Habituation is defined operationally to be a response decrement as a result of repeated stimulation (Thompson and Spencer, 1966; Groves and Thompson, 1970). In the seminal work on aplysia, habituation of the gill withdrawal reflex, explained as a form of synaptic depression, was driven by a reduction in glutamate release with repeated stimulation (Castellucci and Kandel, 1974). This leads to decreased dendritic complexity and, eventually, elimination of synapses.

Habituation in the field of stress neurobiology is defined as the reduction in physiological and behavioral responses elicited by exposure to a repeated homotypic stressor (Grissom and Bhatnagar, 2009). Of course, this observable decrease in behavior is likely mediated by cellular changes in the brain such as decreases in neuronal firing, receptor density, postsynaptic currents, and neurotransmitter release (Thompson and Spencer, 1966; Groves and Thompson, 1970; Castellucci and Kandel, 1974; Glanzman, 2006; Herman, 2013). In experiments where habituation to a stressor occurred, there were observable decreases in the central response to the stressor (measured by cFos, a marker of neural activation, in the parvocellular PVN) (Girotti et al., 2006). In addition, habituation to repeated stress is blocked by pre-stress injections of a mineralocorticoid receptor (MR) antagonist, suggesting that adaptation to stress is regulated in part by the MR (Cole et al., 2000). Other studies focusing on structural changes in the brain have found that repeated restraint causes retraction of basal dendrites in the prefrontal cortex, which may then affect the HPA response (Brown et al., 2005).

As the relationship between HPA hormones and orexins is bidirectional (orexins increase HPA hormone release, and stress hormones such as CRH promote orexin action), it can be hypothesized that with habituation, both CRH and orexin activity are ultimately dampened. While the precise mechanism(s) underlying sex differences in habituation to stress is unclear, one particular study using 7 days of repeated restraint stress found that ovariectomized rats given 17-β estradiol replacement displayed higher levels of dendritic branching in the prefrontal cortex compared to ovariectomized rats given vehicle (Garrett and Wellman 2009). Similarly, in our studies, estrogen could counteract the effects of habituation to

repeated restraint stress in females. In short, estrogen may prevent the reduction of dendritic branching that we observed in males habituating to restraint stress. Thus, analogous to the gill withdrawal reflex, habituation to stress results in a reduction of excitatory neurotransmitters (like glutamate), but estrogen counters this by enhancing presynaptic glutamate (Schwarz et al., 2008; Oberlander and Woolley, 2016). Alternatively, estrogen or CRH may act on interneurons that then modulate another neurotransmitter type, which then would affect lateral hypothalamic neurons, analgous to mechanisms involved in sensitization (Brunelli et al., 1976). However, little is known about gonadal hormone regulation of orexin neuron morphology or function. Regardless, there is an interplay between sex and stress that ultimately leads to the morphological effects on the dendrites of putative orexin neurons, which may regulate habituation to stress.

These experiments are important in that, for the first time, they examine how sex and stress affect structural changes in lateral hypothalamic neurons. The literature provides evidence for stress effects on dendritic morphology and dendritic spine density in the prefrontal cortex (Brown et al., 2005; Liston et al., 2006; Radley et al., 2006), hippocampus (Gould et al., 1990; Magariños et al., 1996; Diamond et al., 2006), and amygdala (Vyas et al., 2004; Mitra et al., 2005), but not much work has been done on hypothalamic regions. Moreover, there is some evidence of sex differences in dendritic morphology (specifically, how stress differentially affects these measures in males and females) in the prefrontal cortex (Garrett and Wellman, 2009; Shansky et al., 2009) and hippocampus (Galea et al., 1997; McLaughlin et al., 2010), but this has also not been explored in hypothalamic regions such as the lateral hypothalamus.

In this study, stress resulted in less spines proximal to the cell body, but more spines distal to the cell body in females. Spines closer to the cell body likely have stronger effects on total activity of the neuron (Hao et al., 2009), but the types of connections made from the proximal or distal spines of orexin neurons are not known. These results suggest that in females, stress may reduce local connectivity in favor of connections to distant populations of neurons. The net effect may be reduced signal strength to the female orexinergic neurons following stress, or may instead reflect alterations in the relative contribution of region-specific or neurochemical-specific signals. The striking decrease in spines proximal to the soma in stressed females vs non-stressed females is due primarily to mushroom spines, thought to indicate mature, active synapses. Distal to the soma, we noted small increases in both mushroom and stubby spines in stressed vs non-stressed females that may underlie the stress-induced increase in total spines more distally in females. The clear differences between males and females in mushroom spines and the effects of stress on mushroom spines suggests that there are synaptic inputs to these regions of orexin dendrites that have relevance to both sex differences and the effects of stress in females.

Our examination of the distribution of spines along dendrites in both sexes with or without stress (in addition to the typically reported measure of dendritic spine density) represents snapshots of these treatment groups. Thus, we don't know exactly when changes are taking place during the five days of repeated restraint (or how things dynamically change throughout the stressor), but we do have evidence of how spines are affected at the end of this habituated stress paradigm. In order to see spine growth and retraction in real time, live imaging techniques such as two photon microscopy would need to be employed (Denk et al., 1990). However, this technique is currently only able to penetrate depths of about 1 mm of tissue, with most studies only collecting data from the apical tuft of layer 5 pyramidal neurons in different cortical areas (Rochefort and Konnerth, 2012). Thus, a reliable live imaging technique of spines that can penetrate deeper tissue (such as in the lateral hypothalamus, where orexin neurons are located) is required to analyze dynamic spine data of this nature. As dendritic spines can show rapid motility, changing shape over a timescale of seconds to minutes (Hering and Sheng, 2001), live imaging during stress would certainly give us more insight to how the orexin system plays a role in responding to habituated repeated restraint stress.

We did not observe any sex differences in dendritic morphology (eg dendritic length, number of nodes, and number of branches) in the absence of stress. Thus, male and female rats showed similar dendritic morphology; females simply had more mature spines on the dendrites sampled. Interestingly, habituated repeated restraint stress resulted in significant changes in dendritic morphology, decreasing dendritic length, number of nodes, and number of branches. However, stress decreased these measures in males but not in females. Dendritic structure and function are inseparably linked, defining the dendritic integration of synaptic signals and their capability to evoke action potentials (Häusser et al., 2000; Magee, 2000). Therefore, a decrease in dendritic morphological measures likely indicates decreased orexin system activity in males after habituation to stress due to changes in regional connectivity. Decreased dendritic complexity in the absence of decreased spine density likely indicates that, following stress, the male orexin neurons "fine-tune" their connectivity, eliminating dendritic connections without decreasing synaptic strength in the remaining connections. As the orexin system is known to potentiate the stress response, decreased orexin system activity in males may contribute to their habituation to repeated restraint stress (whereas females do not show these decreased measures to the same extent as males, and do not habituate as fully to repeated restraint, as we have previously shown) (Grafe et al., 2017). However, as we are only able to assess these measures post mortem, we cannot determine if these changes in dendritic morphology allowed males to habituate to repeated restraint, though we believe this to be the case. Examining orexin dendritic morphology after each subsequent day of restraint may give us more information as to how orexin neuron structure changes over habituation to repeated stress exposure. It should be noted that while spines are extremely dynamic (changing in a matter of seconds to minutes), morphological plasticity typically takes hours to days to be observed (Hering and Sheng, 2001), thus, it is not likely that one single 30-minute restraint would cause these changes in dendritic morphology. Rather, it is more likely that several days of habituation are causing these decreases in dendritic length, branching, and nodes observed in male rats. It would be interesting to see how many days of restraint stress cause significant decreases in orexinergic dendritic morphology in males compared to that of females, and if this correlates with the observed HPA habituation to repeated restraint stress.

An important limitation of our study is that putative orexin neurons were identified by size, shape, and location (as in (Cheng et al., 2003)), rather than neurochemically, because our Golgi staining method precludes immunohistochemical or other methods of co-staining. Thus, we were unable to confirm the neurochemical specificity of these neurons. It should be noted that orexin neurons coexpress many neurotransmitters, including dynorphin (Chou et al., 2001) and glutamate (Rosin et al., 2003; Torrealba et al., 2003; Henny et al., 2010), thus neurochemical identification of orexin would not eliminate the possible contributions of these cotransmitters. To our knowledge, MCH neurons are the only other neurochemically distinct neurons from orexinergic neurons in perifornical area of the lateral hypothalamus (Cheng et al., 2003). Interestingly, little is known about sex differences in neurons of the lateral hypothalamic perifornical region so the present results may indicate sex differences in multiple cell types in this region. Nevertheless, it will be important in future studies to use cell filling technqiues that allow for neurochemical typing of cells to confirm that they are orexinergic.

Future studies are required to explore the types of connections that are being made with putative orexin neurons in non-stressed males and females and how these change with exposure to repeated restraint stress. A greater number of excitatory inputs onto putative orexin neurons in females would explain higher orexin system activity in females compared to males in the absence of stress. Ultimately, this higher orexin system activity may potentiate the response to repeated restraint stress, preventing habituation in females. As CRH is known to bidirectionally interact with orexins, and terminals of CRH have been observed onto orexin neurons (Winsky-Sommerer et al., 2004), this stress neuropeptide may mediate the sex differences we observed in this study. Indeed, sex differences in the CRH receptor have been

observed in the locus coeruleus (Bangasser et al., 2018), and this has yet to be examined in the lateral hypothalamus. Other known inputs to orexin neurons include GABA (Ferrari et al., 2018), acetylcholine (Zhou et al., 2015), serotonin (Tabuchi et al., 2013), endocannabinoids (Cristino et al., 2013), and cholecystokinin (Giardino et al., 2018), though sex differences or effects of stress on these inputs on orexin neurons have not been studied. Ultimately, it is important to better understand how sex and stress can influence structural differences, and thereby, functional differences in the orexin system. As the orexins display sex differences in expression and action (Grafe et al., 2017; Jöhren, 2018), potentiate the stress response (Kuru et al., 2000; von der Goltz et al., 2011), and are altered in psychiatric disorders (James et al., 2017; Grafe and Bhatnagar, 2018), this peptidergic system may be an important biological substrate in contributing to sex differences in stress-related mental illness.

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Figure 1. Repeated restraint stress decreases dendritic length, nodes, and branching in putative orexin neurons more prominently in male compared to female rats
Panel A. Left: Representative 4x picture of golgi staining in the lateral hypothalamus. 3V denotes the third ventricle and Opt denotes the optic tract. Right: Representative 40x picture of a golgi stained neuron in the lateral hypothalamus. Based on its size, shape, and location, it was characterized as a putative orexin neuron. **Panel B.** Dendritic length (μm) in putative orexin neurons in non-stressed and stressed male and female rats. There was a main effect for repeated restraint stress in decreasing dendritic length in putative orexin neurons. **Panel C.** Number of dendritic nodes in putative orexin neurons in non-stressed and stressed male and female rats. There was a main effect for repeated restraint stress in decreasing the number of dendritic nodes in putative orexin neurons. **Panel D.** Number of dendritic branches in putative orexin neurons in non-stressed and stressed male and female rats. There was a main effect for repeated restraint stress in decreasing the number of dendritic branches in putative orexin neurons. Further analyses revealed that the main effects in length and nodes was driven by differences in males with stress significantly decreasing total dendritic length and number of nodes in male rats but not in female rats. 8 animals per group and 4 cells per animal were analyzed.
*p<0.05, **p<0.01; ***p<0.05 significant differences between control males and stressed males

Figure 2. Stress decreases dendritic complexity in putative orexin neurons more prominently in males compared to females
Panel A. Representative Sholl reconstructions of each treatment group (control male, control female, stressed male, stressed female). **Panel B.** Number of dendritic intersections along sholl radii (10 μm concentric circles) in control male and female rats. Dendritic complexity does not differ between non-stressed males and females. **Panel C.** Number of dendritic intersections along sholl radii (10 μm concentric circles) in control and stressed male rats. Stress significantly decreases dendritic complexity of orexin neurons in male rats at distances of 30 to 110 μm from the cell body. **Panel D.** Number of dendritic intersections along sholl radii (10 μm concentric circles) in control and stressed female rats. Stress does not significantly decrease dendritic complexity of orexin neurons in female rats. **Panel E.** Number of dendritic intersections along sholl radii (10 μm concentric circles) in stressed male and female rats. Stressed male and female rats do not display significantly different dendritic complexity.
*p<0.05

Figure 3. Non-stressed females have more dendritic spines on putative orexin neurons compared to non-stressed males and stress reduces dendritic spine density in females but not in males
Panel A. Representative spine images of each treatment group (control male, control female, stressed male, stressed female). **Panel B.** Total spine density (spines/μm dendritic length) in non-stressed and stressed male and female rats. In the absence of stress, the dendritic spine density in orexin neurons is higher in female rats compared to male rats. There was no main effect of stress on spine density. **Panel C.** Number of spines along sholl radii (10 μm concentric circles) in control male and female rats. Non-stressed female rats have more spines at the majority of sholl radii compared with non-stressed male rats. **Panel D.** Number of spines along sholl radii (10 μm concentric circles) in control and stressed male rats. Stress did not significantly change dendritic spine distribution in orexin neurons in males. **Panel E.** Number of spines along sholl radii (10 μm concentric circles) in control and stressed female rats. Stress significantly reduced dendritic spine distribution in orexin neurons in females. **Panel F.** Number of spines along sholl radii (10 μm concentric circles) in stressed male and female rats. Stressed male and female rats did not have significantly different dendritic spine distribution.

*P<0.05, **P<0.01

Figure 4. Non-stressed females have more mushroom spines on putative orexin neurons compared to non-stressed males and stress reduces this density in females but not in males

Panel A. Mushroom spine density (spines/μm dendritic length) in non-stressed and stressed male and female rats. In the absence of stress, the dendritic mushroom spine density in orexin neurons is higher in female rats compared to male rats. There was no main effect of stress on mushroom spine density. **Panel B.** Number of mushroom spines along sholl radii (10 μm concentric circles) in control male and female rats. Non-stressed female rats have more mushroom spines at the majority of sholl radii compared with non-stressed male rats. **Panel C.** Number of mushroom spines along sholl radii (10 μm concentric circles) in control and stressed male rats. Stress did not significantly change dendritic mushroom spine distribution in orexin neurons in males. **Panel D.** Number of mushroom spines along sholl radii (10 μm concentric circles) in control and stressed female rats. Stress significantly reduced mushroom spine distribution proximal to the cell body in putative orexin neurons in females. **Panel E.** Number of mushroom spines along sholl radii (10 μm concentric circles) in stressed male and female rats. Stressed male and female rats did not have significantly different dendritic mushroom spine distribution.

*P<0.05, **P<0.01

Figure 5. No effects of sex or stress on dendritic filopodial spines on putative orexin neurons

Panel A. Filopodial spine density (spines/μm dendritic length) in non-stressed and stressed male and female rats. **Panel B.** Number of filopodial spines along sholl radii (10 μm concentric circles) in control male and female rats. **Panel C.** Number of filopodial spines along sholl radii (10 μm concentric circles) in control and stressed male rats. Stress did not significantly change dendritic filopodial spine distribution in orexin neurons in males. **Panel D.** Number of filopodial spines along sholl radii (10 μm concentric circles) in control and stressed female rats. Stress did not significantly change dendritic filopodial spine distribution in orexin neurons in females. **Panel E.** Number of filopodial spines along sholl radii (10 μm concentric circles) in stressed male and female rats. Stressed male and female rats did not have significantly different dendritic filopodial spine distribution.

Figure 6. Males and females do not differ in dendritic stubby spine distribution or density on orexin neurons regardless of stress

Panel A. Stubby spine density (spines/μm dendritic length) in non-stressed and stressed male and female rats. Neither sex nor stress affect the density of stubby dendritic spines.

Panel B. Number of stubby spines along sholl radii (10 μm concentric circles) in control male and female rats. Non-stressed males and females do not differ in their dendritic stubby spine distribution. **Panel C.** Number of stubby spines along sholl radii (10 μm concentric circles) in control and stressed male rats. Stress did not significantly change dendritic stubby spine distribution in orexin neurons in males. **Panel D.** Number of stubby spines along sholl radii (10 μm concentric circles) in control and stressed female rats. Stress significantly changed dendritic stubby spine distribution in orexin neurons in females at some radial distances. **Panel E.** Number of stubby spines along sholl radii (10 μm concentric circles) in stressed male and female rats. Stressed male and female rats did not have significantly different dendritic stubby spine distribution.

*P<0.05