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UNIVERSITY OF CALIFORNIA, SAN DIEGO

**Dephosphorylation of tau in lactating rats subjected to acute restraint
stress**

A Thesis submitted in partial satisfaction of the requirements for the degree
Master of Science

in

Biology

by

Danielle G. Steinmetz

Committee in charge:

Professor Robert A. Rissman, Chair
Professor Kuo-Fen Lee, Co-Chair
Professor Brenda Bloodgood

2014

The thesis of Danielle G. Steinmetz is approved and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego
2014

DEDICATION

I dedicate this work to my family who believed in me even when I doubted myself. To my son, Asher, who was my biggest motivation to succeed – I always want to make you as proud of me as I am of you. To my mom, who has always loved and supported me – I could never have done this without you, thanks for being there for me every step of the way. To my dad who instilled in me a passion for science that can never be extinguished, thank you for making me the science nerd I am today. To my sister, who played a huge part in allowing me to stay in school - thanks for all the free babysitting! And last but certainly not least, this thesis is dedicated to my Lord Jesus for always loving me, guiding me, and blessing me unendingly.

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ABSTRACT OF THE THESIS

Dephosphorylation of tau in lactating rats subjected to acute restraint stress

by

Danielle G. Steinmetz

Master of Science in Biology

University of California, San Diego, 2014

Professor Robert A. Rissman, Chair

Professor Kuo-Fen Lee, Co-Chair

Chronic stress is a consistently implicated risk factor for neurodegenerative diseases like Alzheimer's disease (AD). Hyperphosphorylated tau tangle are a hallmark of AD pathology, and while the specific mechanisms that link stress and AD vulnerability have not been fully elucidated, our lab has shown that acute and repeated restraint stress leads to an increase in hippocampal tau phosphorylation (tau-P) in rodents, a process regulated by the

two known corticotropin-releasing factor receptors. A naturally occurring attenuation of stress response is present in female rats during the last part of pregnancy and throughout lactation. Further, the hippocampus of a lactating female is more resistant to the effects of a neurotoxin and the ratio of hippocampal Tau-P/Tau is modified by pregnancy and lactation. To test the hypothesis that the decreased sensitivity to stress during lactation modulates stress-induced tau-P, cohorts of virgin, lactating, or weaned female rats were subjected to 30 minutes of restraint stress or no stress (control) and sacrificed 20 minutes or 24 hours after the episode. Western blot was used to analyze differences at well characterized AD-relevant tau-P epitopes and relevant kinases. Lactating rats sacrificed 20 minutes after the stress episode exhibited a significant tau-P decrease compared to lactating controls as well as virgins subjected to the same stress treatment. Stressed lactating rats sacrificed 24 hours after the episode showed a significant increase in tau-P compared to the lactating stressed rats sacrificed at 20 minutes, returning to control levels. This suggests a steep, yet transient stress-induced dephosphorylation of tau in lactating rats.

INTRODUCTION

Neurodegenerative Diseases

Neurodegenerative diseases damage multiple neuronal networks in the brain causing neuronal death and leading to impairments evident in the victim's day-to-day life (Palop et al., 2006). For example, symptoms of Parkinson's disease, the second most prevalent neurodegenerative disorder in the world, often include visible body tremors due to motor neuron degeneration, and can also manifest itself through cognitive defects like dementia or depression (Mateus and Coloma, 2013). Symptoms of other neurodegenerative diseases like Huntington's disease and amyotrophic lateral sclerosis (also known as Lou Gehrig's disease or, simply, ALS) include motor dysfunction, muscle and brain atrophy, speech impairment, as well as emotional and behavioral changes (Tada et al., 2012). Neurodegenerative illnesses affect millions of people in the United States and caring for those affected costs billions of dollars annually. Parkinson's disease alone is estimated to affect one million Americans (not including caregivers) and costs \$25 billion every year (Parkinson's Disease Foundation, 2013). Alzheimer's disease (AD), the most common neurodegenerative disease in the world, affects approximately 5.2 million Americans and this number is expected to double by the year 2050 as life expectancy increases. Furthermore, the cost of caring for Alzheimer's patients is currently \$203 billion per year and is expected to increase to \$1.2 trillion annually by 2050 (Alzheimer's Association, 2013). These alarming numbers demonstrate the importance of future research to discover new and better treatments, as well as possible preventative

measures that can be taken, to impede the progressive toll that neurodegenerative diseases are taking on America.

Alzheimer's Disease

AD is the leading cause of dementia with symptoms including progressive memory loss, paranoia and delusions, confusion, speech impairment, and, in the later stages of the disease, motor coordination impairment (Selkoe, 2001). Risk factors for developing AD include ageing, the ApoE4 allele, brain trauma, low education, low mental and physical exercise, and other health problems. A familial form of AD caused by mutations in the amyloid precursor protein and presenilin 1 and 2 genes results in a much earlier onset (diagnosis before age 65) than sporadic AD, but only accounts for about 0.1% of all AD cases (Bennlow et al., 2006). Sporadic early-onset AD also occurs and accounts for about 4% of AD cases, but little is understood as to what causes the accelerated symptoms (Campion et al., 1999). In both familial and sporadic AD, multiple neurological changes occur to contribute to the disease rather than a single causal factor (Bennlow et al., 2006). In sporadic AD, disease progression is slow as neuropathological changes begin decades before any clinical symptoms may be recognized, allowing a sort of grace-period that could potentially be exploited for preventative and early treatment measures. However, a tentative diagnosis can only be made when the patient is already showing signs of dementia, and there are currently no definitive ways to diagnose a patient with AD until an autopsy is performed. For this reason, researching possible early-detection tools such as cerebral spinal fluid biomarkers is extremely important. Furthermore, there is no

known cure for AD and there are only two types of treatments for AS symptoms: NMDA receptor antagonists and cholinesterase inhibitors. These drugs do not target the disease itself; therefore, conducting experiments to better understand the underlying molecular mechanisms that contribute to AD progression could lead to better drug-treatment targets.

Alzheimer's Disease Neuropathology

AD is defined neuropathologically by the accumulation of extracellular β -amyloid plaques and intracellular neurofibrillary tangles (NFTs) composed of phosphorylated and aggregated forms of the microtubule-associated protein, tau. β -amyloid plaques are composed of filamentous products resulting from the cleavage of amyloid precursor protein by secretase proteins. The plaques are generally surrounded by damaged axons and dendrites, as well as activated microglia and astrocytes (Selkoe, 2001). Based on these observations, the "amyloid beta hypothesis" was formed which suggests increased β -amyloid plaque production as the driving force of AD development; however, a number of studies over the years have begun to question the hypothesis (Bossy-Wetzel et al., 2004; Hardy, 2009).

Amyloid plaques remain a subject of intense study, but the other defining feature of AD neuropathy, NFTs, has also been studied extensively and some studies have found positive correlations between NFT development and AD duration and severity (Arriagada et al., 1992; Gomez-Isla et al., 1997). Hyperphosphorylated tau (tau-P) has reduced ability to bind and stabilize microtubules as effectively and is thought to aggregate into paired helical

filaments (abnormal protein aggregates comprised primarily of self-polymerizing, hyperphosphorylated tau filaments) which ultimately comprise the NFTs that correlate with AD progression (Alonso et al., 1996). In one study, inhibition of cyclin-dependent kinases (known to be essential for organized progression through the cell cycle) caused a decrease in tau-P and an increase in neurite outgrowth, suggesting that the reduced ability of tau-P to stabilize microtubules may contribute to neuroplasticity (reorganization of microtubule networks for learning and memory formation) in the non-diseased brain (Schmetsdorf et al., 2009). Other studies have shown that increases in tau-P may have a neuroprotective effect against cell death as, in one study, cells expressing tau-P were resistant to induced apoptosis and, in another study, cells entering the apoptotic phase were characterized by tau dephosphorylation (Lesort et al., 1997; Mills et al., 1998). Neuroplasticity is known to play a critical role in learning and memory formation, yet in AD excess tau-P is often correlated with synaptic degeneration (Masliah et al., 1992; Wakabayashi et al., 1994). These puzzling results demonstrate the need for further research to fully elucidate the consequences of tau-P in the non-diseased and AD-brain, as well as the mechanisms by which each path (neuroplasticity vs. neuropathology) proceeds.

Stress-induced Alzheimer's Disease

Stress affects everyone on a daily basis, and studies that investigated the effects of stress with respect to neurodegenerative disorders found general over-activation of the hypothalamic-pituitary-adrenal axis (part of the body's stress-response system), decreased hippocampal neurogenesis, increased

neurodegeneration, and increased cognitive impairment (Swaab et al., 2005; McEwen 2002; Carroll et al., 2011). Studies have shown that environmental factors that bring about stress can play a role in AD development and, more specifically, induce tau-P (Korneyev, 1998; Feng et al., 2005; Wilson et al., 2006; Sotiropoulos et al., 2011; Johansson et al., 2013). These studies report that both men and women who are more prone to psychological distress are more likely to develop AD, and rodent studies show increased tau phosphorylation in rodents subjected to cold water stress as well as cognitive deficits as a result of excess glucocorticoid (stress hormone) exposure (Feng et al., 2005; Sotiropoulos et al., 2011). In recent experiments, exposure to an emotional stressor (restraint) gave rise to a significant increase in tau-P in the rodent hippocampus and repeated stress led to cumulative increases in an insoluble, potentially pathogenic, form of tau-P (Rissman et al., 2007, 2012). These studies also suggest that the corticotropin-releasing-factor system (known for its role in the biological stress response) is mechanistically involved in tau-P as restraint stress-induced tau-P was only seen in mice with corticotropin releasing factor receptor 1 (CRFR1) and not in CRFR1 knockout mice. However, while this CRFR1-dependence was seen in mice subjected to emotional stress, it did not apply to physiological/immune stressors (Roe et al., 2011). These studies were conducted in male mice, so how the female rodent responds to restraint stress, particularly regarding hippocampal stress-induced tau-p, has not been entirely investigated (Rissman, 2009).

Lactation, Stress, and Neuroprotection

The changes that occur in the female physiology during pregnancy and lactation may confer neuroprotection against excitotoxins (molecules that overstimulate cells causing damage to cellular components like the cytoskeleton and DNA) and decrease sensitivity to stress (Slattery and Neumann, 2008). The morphological changes that occur in the maternal brain occur not only in areas that support lactation, but also in the CA1 region of the hippocampus (a part of the brain highly implicated in learning and memory formation), (Kinsley et al., 2006; Pawluski and Galea, 2006) and areas related to neurogenesis such as the subventricular zone and the dentate gyrus (Furuta and Bridges, 2005; Leuner et al., 2007). Another study showed that reproductive events facilitate learning and memory throughout one's lifetime and also decreases the prevalence of neuronal markers of aging (Gatewood et al., 2005). Since maternal hormones like prolactin, progesterone, and estrogen rise during pregnancy and lactation, they have become the subject of many studies to determine their relationship to neurodegeneration and AD pathology. For example, prolactin has been shown to decrease anxiety behavior in rodents as well as prevent stress-induced decreases in neurogenesis (Torner et al., 2001; Blume et al., 2009). In another study, progesterone significantly reduced tau-P and estrogen prevented β -amyloid accumulation in the hippocampus of a triple transgenic mouse model of AD (Carroll et al., 2007). The mechanisms by which these hormones regulate tau and β -amyloid are not fully understood, but these studies suggest that these maternal hormones work to attenuate the stress response and could potentially play a role in preventing AD pathology.

Stress-induced Alzheimer's Disease and Lactation Stress-Attenuation

This research project addresses the impact of reproductive events in female rats and its relationship to stress-induced tau-P and cognitive function. The neuroprotection that lactation confers to the female brain may or may not last through weaning, but if it does it may have an effect on the development and/or severity of age-related disorders. Understanding more about the mechanisms by which lactation reduces sensitivity to stress could better define the specific circuitry that may render vulnerability to AD. Understanding specific circuitries is the key to discovering new and effective therapeutic treatments for neurodegenerative disorders.

To test the hypothesis that the decreased sensitivity to stress seen during lactation can decrease stress-induced tau-P, cohorts of either nulliparous (virgin), lactating (14-18 days postpartum), or weaned (21 days postpartum) adult female rats were exposed to either 30 minutes of restraint stress or no stress (control) and sacrificed either 20 minutes or 24 hours after the episode. The 20-minute time point was chosen based on previous research that showed that stress-induced hippocampal tau-P reaches its peak 20 minutes following a 30-minute restraint episode and returns to control levels 90 minutes following the stress episode (Rissman et al., 2007). The 24-hour time point was chosen based on the Rissman et al. paper released in 2012 that used the 24-hour time point to demonstrate that repeated exposure to stress led to chronic increases in stress-induced hippocampal tau-P that was sequestered in an insoluble (potentially pathogenic) form. Therefore, for this study, an increase in tau-P seen at the 20-

minute time point is thought to be transient unless the increase is sustained through 24 hours at which point the change is considered a chronic (and potentially pathogenic) effect. Half of each group will undergo dissections of hippocampus that will be used for biochemical (Western blot) analysis of tau-P at two, well characterized N and C-terminal AD-relevant tau-P epitopes (AT8 and PHF-1), as well as three major tau kinases (GSK-3, JNK1/2, and ERK1/2). The other half of each group will be perfused for immunohistochemical experiments.

If lactation confers neuroprotection against stress-induced tau-P, then lactating rats should exhibit significantly less tau-P 20 minutes following stress compared to virgin and weaned rats. However, if this effect lasts through weaning, weaned rats would be expected to have significantly lower levels of stress-induced tau-P 20 minutes later compared to virgin rats, with lactating and weaned rats exhibiting relatively similar tau-P levels compared to each other. A single restraint stress episode is not expected to have chronic effects on tau-P (Rissman et al., 2007); therefore, 24 hours after stress, tau-P levels are expected to be similar across all reproductive stages as well as compared to non-stressed controls.

MATERIALS AND METHODS

Rats.

Adult virgin or pregnant female Wistar rats (250-300g) were housed individually under controlled temperature and lighting conditions (12-h:12-h light:dark cycle, lights on at 06:00 h), with food and water available ad libitum. One day after parturition, litter sizes were culled to 8-10. Mothers were kept undisturbed with the litters, and they were used for experiments on postpartum days 14-19 (lactating rats) or one month after weaning (weaned rats). Vaginal smears of virgin female rats were followed for at least four oestrous cycles, and they were stressed or handled in the dioestrous phase of the cycle.

Acute Restraint Stress.

Stressed rats were placed in acrylic restrainers with ventilation for 30 minutes and were sacrificed either 20 minutes or 24 hours after the restraint episode. Control rats were handled similarly; however, they were not subjected to any stress episodes.

Western blot analysis.

Tissues were homogenized in radio-immunoprecipitation assay (RIPA) buffer as performed previously (Roe et al., 2011; Rissman et al., 2012). Fifteen micrograms of each protein sample were then separated by 10% SDS-PAGE and transferred to nitrocellulose membrane at 100V for fifty minutes. Nonspecific binding was blocked by incubating membranes in 5% milk-PBS-T for thirty minutes. Membranes were then incubated with primary antibodies diluted in 5% milk-PBS-T overnight at 4°C. The primary antibodies were detected by anti-

mouse or anti-rabbit HRP-linked secondary antibodies for one hour (1:2500; EMD Biosciences, La Jolla, CA) and developed using an enhanced chemiluminescence Western blot detection kit (Supersignal West Pico; Pierce Biotechnology). Internal standard division was performed and quantitative band intensity readings were obtained using the NIH ImageJ software.

Antibodies.

Two well characterized antibodies were used to probe for specific phosphorylated residues on rat hippocampal tau: S202/T205 (AT8, 1:500; Pierce Biotechnology) and S396/404 (PHF-1, 1:1000; gift from Dr. P. Davies, Albert Einstein College of Medicine, Bronx, NY). Kinases were assessed using antibodies specific to phosphorylation or activation sites including glycogen synthase kinase-3 (GSK-3 β , 1:1000; BD Biosciences, San Diego, CA), inactivated GSK-3 β (pS⁹, 1:1000; Cell Signaling Technology, Danvers, MA), activated GSK-3 β (pY²¹⁶, 1:1000; BD Biosciences), mitogen-activated protein kinases [extracellular signal-regulated kinases 1 and 2 (ERK1/2), 1:500; Cell Signaling Technology] and phosphorylated c-Jun-N-terminal kinase (JNK, 1:1000; Cell Signaling Technology). Actin (1:2000; Sigma-Aldrich) was used as a protein-loading control.

Immunohistochemistry.

Rats were perfused with 4% paraformaldehyde and processed for immunohistochemistry, as described previously (Rissman et al., 2007). Frozen sections were cut at 30 μ m on a sliding microtome and stored at -20 °C in antifreeze until use. The PHF-1 antibody (1:5000) was used to probe for tau-p on

free-floating sections of rat hippocampus. 0.3% hydrogen peroxide was used to quench endogenous peroxidases and 0.1% sodium borohydride was used to reduce reactive free aldehydes. The staining reaction was carried out using a nickel-enhanced DAB Peroxidase Substrate kit (Vector Laboratories).

RESULTS

Basal levels of tau-p and tau kinases in female rat hippocampus of various reproductive stage

As determined by Western blot analysis and shown in Figure 1, there were no significant differences in tau-p at neither the AT8 nor PHF-1 epitope across the controls of each reproductive stage. However, as shown in figure 2, lactating controls at the 20-minute timepoint have significantly less total GSK3- α than weaned ($p = 0.016$) and virgin ($p = 0.008$) controls. This was also true for the 24-hour timepoint ($p = 0.008$ for weaned, $p = 0.036$ for virgin). There were no significant differences in ERK or JNK levels across the controls.

Effects of stress on tau-p in lactating female rat hippocampus

Western blot analysis was used to determine the effects of stress on content of hippocampal tau-p in female rats at two phospho-specific epitopes, AT8 and PHF-1. As shown in Figure 3, twenty minutes following stress, lactating rats had a significant drop in tau-p at both the AT8 ($p = 0.032$) and PHF-1 ($p = 0.032$) epitopes compared to lactating controls. This change was also significant compared to stressed virgin ($p = 0.008$) and weaned ($p = 0.008$) rats as shown in Figure 4. Twenty-four hours following stress, tau-p levels in lactating rats significantly increased compared to the twenty minute timepoint ($p = 0.008$), returning to control levels and levels similar to virgin and weaned rats.

To elucidate possible mechanisms of this stress-induced dephosphorylation of tau, changes in major tau kinases such as GSK3 were investigated using Western blot analysis. As shown in Figure 5, overall levels of

GSK3 (α and β bands) significantly decreased in lactating rats compared to their controls ($p = 0.008$) twenty minutes following stress, and these levels stayed low through the twenty-four hour timepoint ($p = 0.008$). Interestingly, active GSK3- β significantly increased twenty-four hours after the stress episode compared to lactating control ($p = 0.016$), while there were no significant changes in active GSK3- α or inactive GSK3 in lactating stressed animals compared to lactating controls. Figure 6 shows that lactating rats also displayed significantly higher levels of ERK twenty-four hours following stress compared to stressed virgin rats ($p = 0.016$). Lactating rats also have significantly less JNK 24-hours after stress compared to weaned rats ($p = 0.008$) and less than virgin rats although not significantly ($p = 0.095$). There were no significant kinase changes in stressed lactating rats between the 20-minute and 24-hour timepoints, but the significant drop in GSK3 twenty minutes following stress could play a key role in the stress-induced dephosphorylation of hippocampal tau seen in lactating rats.

Distribution of Tau-p in Female Rat Hippocampus

Immunohistochemical staining of virgin and lactating rat hippocampus was used to assess qualitative differences in tau-P (PHF-1 epitope) intensity and localization in non-stressed controls and in animals twenty minutes following stress. Figure 7 shows that lactating controls and stressed rats have less tau-p overall in hippocampus compared to their virgin counterparts. Figure 8 shows a 20x magnification of the CA3 region demonstrating a possible shift in tau-P from soma to dendrites/axons in stressed virgin and lactating rats compared to their respective controls. This is further exemplified in Figure 9, a 40x magnification of

the CA3, which clearly shows a decrease in somatic tau-p and a possible shift in labeling from the soma of pyramidal neurons in the CA3 to their dendrites and axons. This shift was also noted in hilar neurons as shown in Figure 10. Stressed lactating rats had a clear decrease in tau-P in the CA1 region following stress, but the somatodendritic shift seen in the CA3 and hilus of both lactating and virgin rats was not notable in CA1 neurons as shown in Figure 11.

DISCUSSION

Stress-induced Dephosphorylation of Tau in Lactating Rat Hippocampus

Western blot analysis demonstrated a stress-induced dephosphorylation of hippocampal tau in lactating female rats and no significant changes in virgin and rats who have weaned their young. These findings are interesting because previous studies have demonstrated significant increases in hippocampal tau-p following both acute and chronic restraint stress; however, the animals used for these experiments were male and only mice were used (Rissman et al., 2007 & 2012). Many studies have indicated that females respond differently to stress compared to males, and that the hormones involved in lactation further confer a higher tolerance for stress as regulated by the HPA stress axis, and grant a neuroprotection that extends to many regions of the brain including the hippocampus (Slattery & Neumann, 2008). Western blot analysis of major tau kinases suggest that a significant decrease in GSK3 may play a part in the stress-induced decrease in tau phosphorylation seen in lactating rats. Maternal hormones are known to play many roles in neurons during pregnancy and lactation, so it may be possible that one or more of these hormones alter GSK3 levels in response to stress.

Maternal Hormones Involved in Lactation and Stress

During lactation, the maternal brain elicits a significantly reduced response to stress possibly due to a chronic state of elevated corticosterone levels (Lightman et al., 2001). A number of hormones are also increased during

pregnancy and lactation including prolactin and oxytocin, as well as ovarian hormones, and may play crucial roles in the attenuated stress response.

Prolactin is involved in milk production and is primarily produced in the anterior pituitary during lactation, but prolactin synthesis and its receptors have been documented in the brain (Ben-Jonathan et al., 1996). Prolactin also has an experimentally documented involvement in maternal behavior, the sleep-wake cycle, grooming, sexual behavior, and stress coping (Ben-Jonathan et al., 1996). Animals treated with prolactin demonstrate an attenuated HPA-axis response to stress, but this mechanism and others involved in the overall anxiolytic effect of prolactin are not fully understood (Torner & Neumann, 2002). Our study found a stress-induced dephosphorylation of tau along with a decrease in GSK3 activity, and a few previous articles have documented relationships between prolactin and this tau kinase. One study suggests that GSK3 can work as a prolactin receptor kinase that tags the receptor for degradation and another paper described a pathway that showed prolactin increasing Akt activity which in turn inhibited GSK3 activity (Dominguez-Caceres et al., 2004; Plotnikov et al., 2008). It may be that increased prolactin levels during lactation results in decreased GSK3 activity (via Akt) in order to preserve prolactin receptors during that critical time period, and could perhaps be one explanation of the decreased baseline levels of GSK3 in lactating rats. However, more research is required to determine how stress-induced prolactin activity could cause an even further decrease in GSK3 activity resulting in a significant dephosphorylation of hippocampal tau.

Oxytocin, another hormone present at elevated levels during lactation, facilitates social behavior, maternal bonding, and milk ejection, and is known to decrease the corticosterone stress response, decrease glucocorticoid receptor expression, stimulate hippocampal neurogenesis, and modulate behavioral responses to stress (Cohen et al., 2010). In the hippocampus, oxytocin has been shown to influence a number of signaling molecules, modulate synaptic transmission, and has been shown to increase hippocampal adult neurogenesis even under stressful conditions (Petersson et al., 2003; Zaninetti et al., 2000, Leuner et al., 2012). Since oxytocin has such a large influence in the hippocampus during stress and lactation, it could potentially play a role in the stress-induced decrease in hippocampal GSK3 and tau-P seen in lactating rats. However, this line of research has not yet been explored.

Progesterone is an ovarian hormone which has constant plasma concentrations through lactation (dropping at day 18 postpartum) and regulates reproduction, release of neurotransmitters, and neuronal development. It has also been shown to have a neuroprotective effect in the nervous system (Amorim et al., 2010). Progesterone administration has been shown to decrease tau-p and increase the dephosphorylated form of tau in the hippocampus of ovariectomized rats, but did not affect GSK3 phosphorylation (Pint-Almazan et al., 2012). Another study found that chronic progesterone treatment increased total hippocampal tau in ovariectomized rats, but this study did not address tau phosphorylation (Camacho-Arroyo et al., 2011). Interestingly, one study showed that progesterone increased tau phosphorylation at the PHF-1 epitope in rat

cerebellum associated with an increase in GSK3 activity, and another study showed a decrease in PP2A activity in the hippocampus ovariectomized rats (Guerra-Araiza et al., 2007; Amorim et al., 2010). These findings suggest a role for progesterone in regulating tau-P and associated kinases; however, these seemingly conflicting studies demonstrate the need for further research on the specific mechanisms by which progesterone regulates these proteins.

The time of lactation in the mother has been well established as a neuroprotective period, and previous studies have shown that GSK3 inhibition increases neuroprotection by inhibiting b-catenin and tau-P, modulating apoptotic pathways, promoting neurogenesis, and improving cognitive deficits in models of neurodegenerative diseases like AD and Parkinson's disease (Culbert et al., 2001; King et al., 2014). Our understanding of the role of GSK3 in the cell is continually expanding and broadening, and it has been established that it not only regulates glycogen synthase activity but also plays a role in a large number of other processes in the cell. It may be possible that one of the mechanisms by which lactation confers neuroprotection to the mother is by inhibiting GSK3 activity (via regulation by some maternal/ovarian hormone), thereby inhibiting stress-induced tau phosphorylation and apoptosis, and promoting other neuroprotective mechanisms like neurogenesis.

Somatodendritic Shift of Tau-P in Stressed Rats

Our study showed that stressed virgin and lactating rats may display a shift in tau-P localization in hippocampal CA3 pyramidal neurons from the soma to the axons and dendrites regardless of a change in tau-P concentration. A

similar change was also notable in and hilar neurons and the CA1 region. This may be a demonstration of the role tau plays in neuroplasticity; in this case specifically, possibly to facilitate learning and memory in response to this type of emotional stress. This may be mechanistically due to a stress-induced increase in levels of brain-derived neurotrophic factor (BDNF) which has been shown to regulate tau distribution in the cell during neurite outgrowth by decreasing tau phosphorylation and shifting tau content (co-distributed with tubulin) from the soma to neurites (Franklin and Perrot-Sinal, 2006; Chen et al., 2014). This effect may be exaggerated in lactating rats due to an increase in hippocampal BDNF during pregnancy and lactation, but increases in hippocampal BDNF can also be seen during the estrous cycle in non-lactating rats (Scharfman et al., 2003). Another study associated an increase in BDNF with an increase in total tau protein which accumulated in distal neurites and protected against nocodazole-induced loss of dendrites (Chen et al., 2012). In addition to this, plasma BDNF levels have been shown to be influenced by ovarian hormones such as progesterone which has been positively correlated with BDNF levels (Begliuomini et al., 2007). It may be possible that progesterone and BDNF work in concert to regulate the stress-induced tau-dephosphorylation seen in lactating rats, as well as the stress-induced somatodendritic shift observed in both virgin and lactating rat hippocampus.

Future Directions

Hormones play a complex role in the female brain throughout adolescence and motherhood. While hormones like prolactin and oxytocin are important for

lactation, their actions in the brain and throughout the body have a much larger impact than just milk production and ejection. Maternal and ovarian hormones are known to have significant anxiolytic effects in response to stress and, in some cases, increase neurogenesis. The neuroplasticity of the female brain also changes as the female goes through different reproductive stages, and tau is thought to play a role in the changes in brain circuitry that occur as part of this plasticity. The dephosphorylation of tau seen in stressed lactating rats may be a neuroprotective effect driven by elevated hormone levels. Functional adaptations in the female brain during lactation include many forms of stress-attenuation both behaviorally and molecularly, but the extent to which these changes affect the future development of neurological diseases like AD remains unclear. Further research including studies focused on chronic stress, multiple maternities, and how these variables affect behavior as well as neuropathological markers such as neurofibrillary tau tangles and amyloid-beta plaque formation are needed to better our understanding of the female brain, stress response, and how these specific circuitries play a role in the development of neurodegenerative diseases like Alzheimer's Disease.

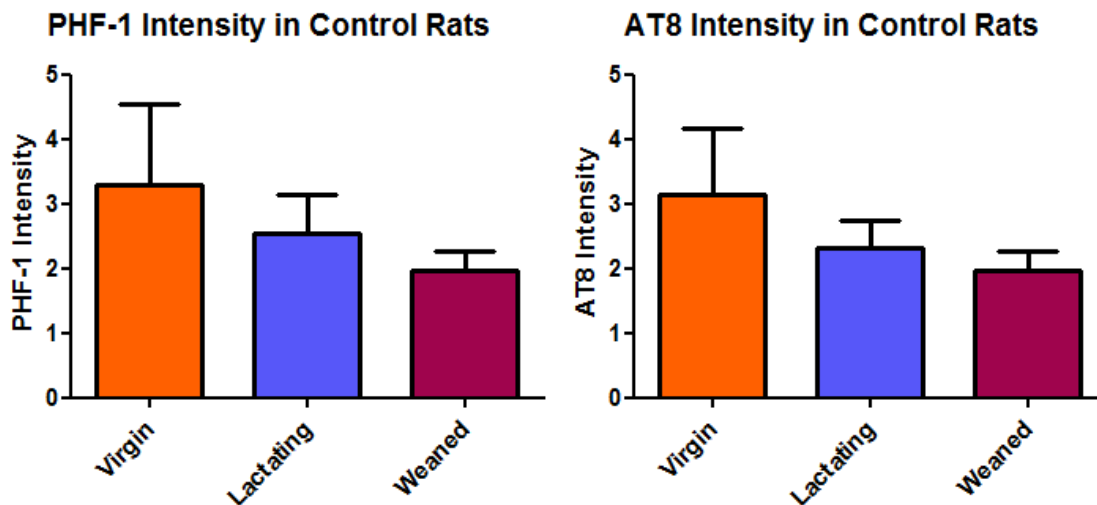


Figure 1. Western blot analysis of baseline tau-p in female rats.

To assess differences in baseline hippocampal tau phosphorylation in non-stressed virgin, lactating, and weaned rats, two tau epitopes were probed and western blot analysis was conducted by comparing band intensities with the NIH ImageJ software. No significant differences were found across the three reproductive stages at either the PHF-1 or AT8 epitope.

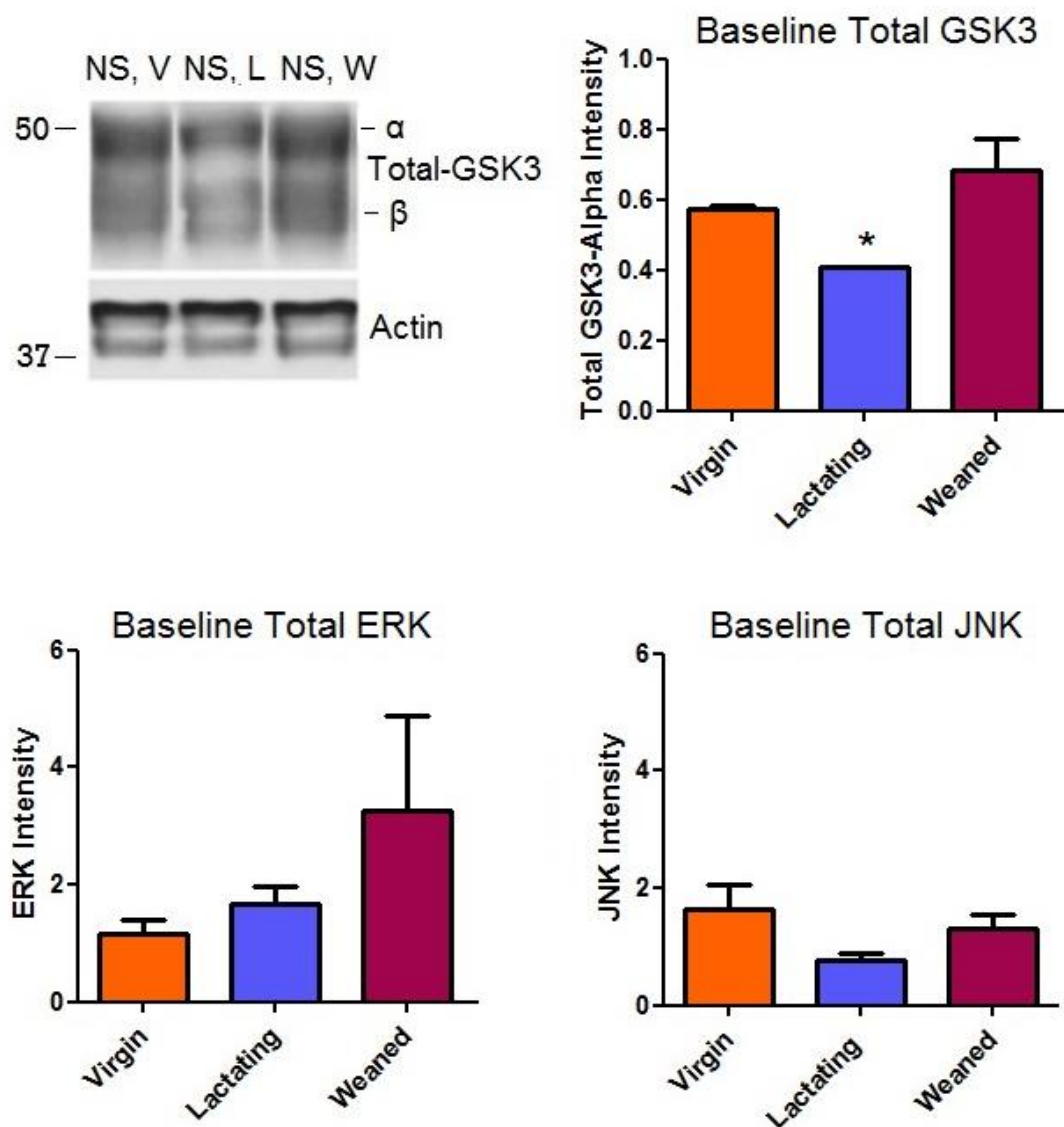


Figure 2. Western blot analysis of baseline kinase levels in female rats.

To assess differences in major tau kinases in female rat hippocampus that were non-stressed (NS) virgin (V), lactating (L), or weaned (W), Western blot analysis was performed using antibodies specific to GSK3, ERK, and JNK. We found that lactating rats have significantly less hippocampal GSK3- α compared to both virgin and weaned rats ($p = 0.016$, $p = 0.016$). There were no significant differences in GSK3- β , ERK, or JNK.

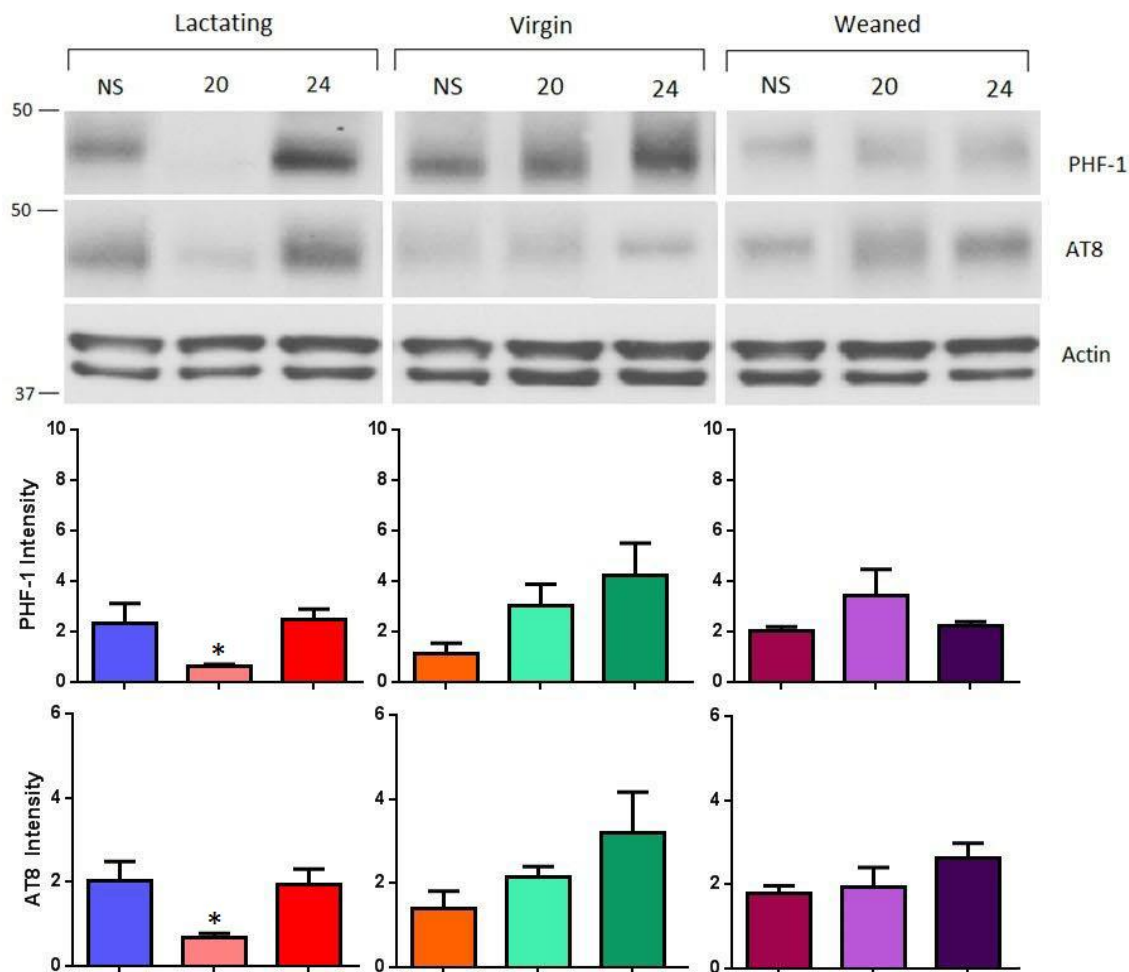


Figure 3. Acute stress-induced hippocampal tau dephosphorylation in lactating rats.

Female rats that were virgin, lactating (14-19 postpartum), or weaned (21 days postpartum) were sacrificed after no stress (NS), or 20 minutes (20) or 24 hours (24) following a single restraint episode. In lactating rats, a significant drop in tau-P at both the PHF-1 and AT8 epitopes followed 20 minutes after stress ($p=0.032$); however, tau-P rose back to control levels after 24 hours. Both virgin and weaned rats were not significantly responsive to acute stress.

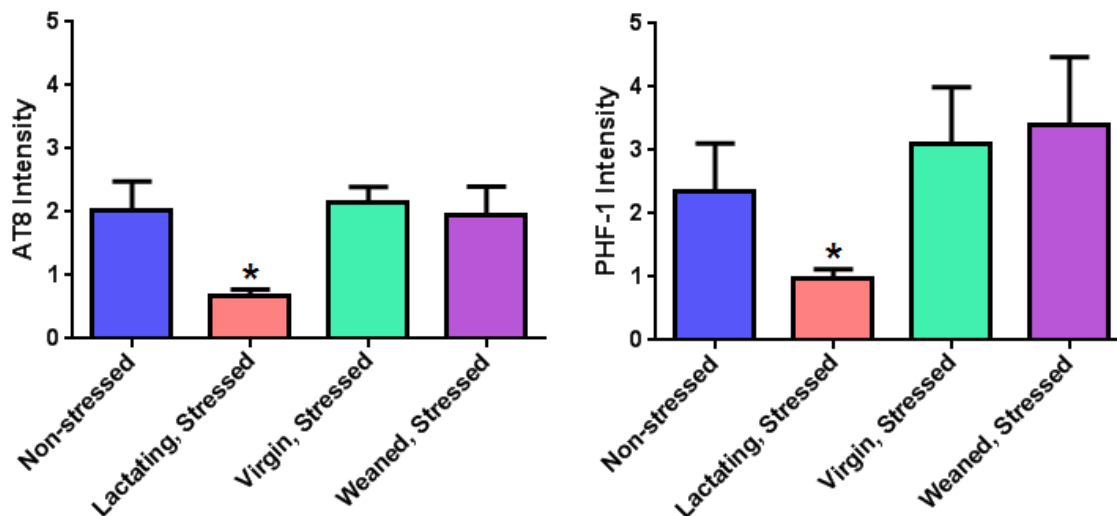


Figure 4. Comparison of stress-induced tau phosphorylation across three reproductive stages.

Female rats that were virgin, lactating (14-19 postpartum), or weaned (21 days postpartum) were sacrificed 20 minutes following a single restraint episode. Lactating rats showed a significant decrease in tau phosphorylation at both PHF-1 and AT8 epitopes compared to virgin ($p=0.008$) and weaned rats ($p=0.008$). The control shown is that of non-stressed (20-minute group) lactating rats.

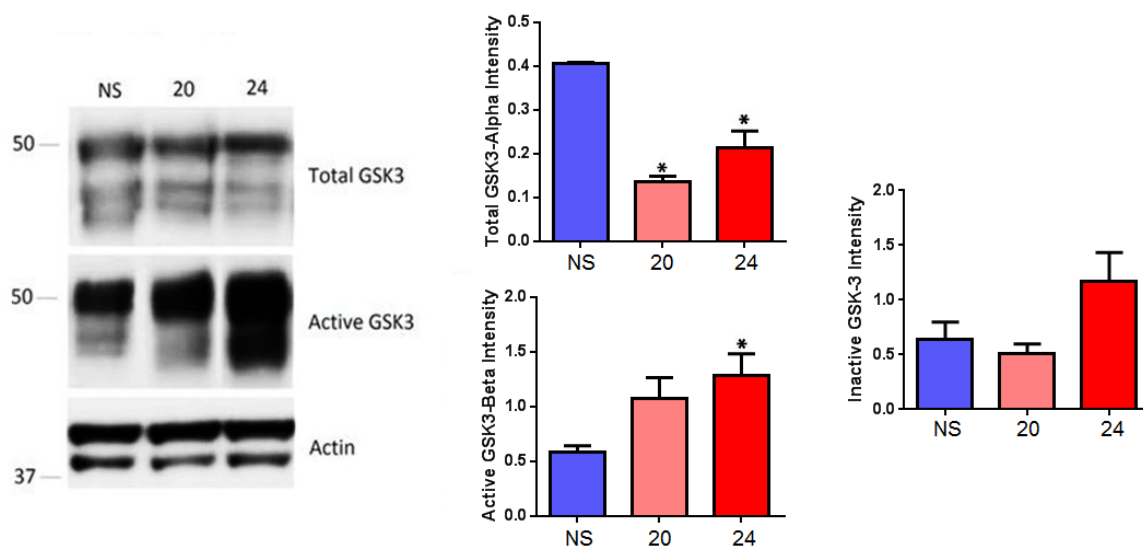


Figure 5. GSK3 levels in control and stressed lactating rats.

Lactating rats were sacrificed after no stress (NS), or 20 minutes (20) or 24 hours (24) following acute restraint stress. Twenty minutes after stress, lactating rats had significantly lower levels of total hippocampal GSK3 compared to their controls ($p=0.008$) and these low levels were sustained 24 hours later ($p=0.008$). However, 24 hours following stress, active GSK3- β levels rose compared to control ($p=0.016$), although active GSK3- α levels stayed relatively the same. There were no significant changes in inactive GSK3 levels.

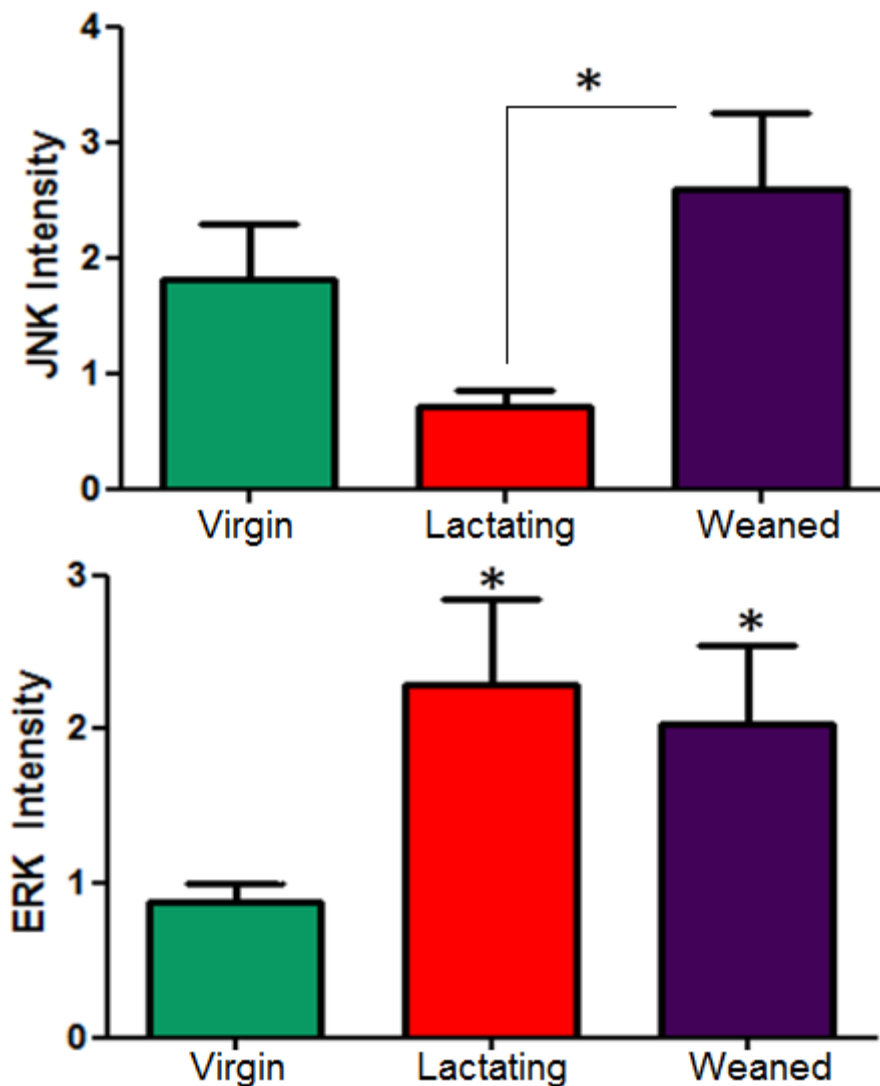


Figure 6. JNK and ERK levels in stressed female rats sacrificed 24 hours after the stress episode.

Two major tau kinases, JNK and ERK, were probed for using hippocampus protein extract for Western blot and band intensities were quantified. In lactating female rats sacrificed 24 hours after stress, JNK levels were significantly lower compared weaned rats ($p=0.008$) sacrificed 24 hours after stress, and lower than virgin rats although not significantly ($p=0.095$). In virgin rats sacrificed 24 hours after stress, ERK levels were significantly lower compared to lactating and weaned rats treated similarly ($p=0.016$).

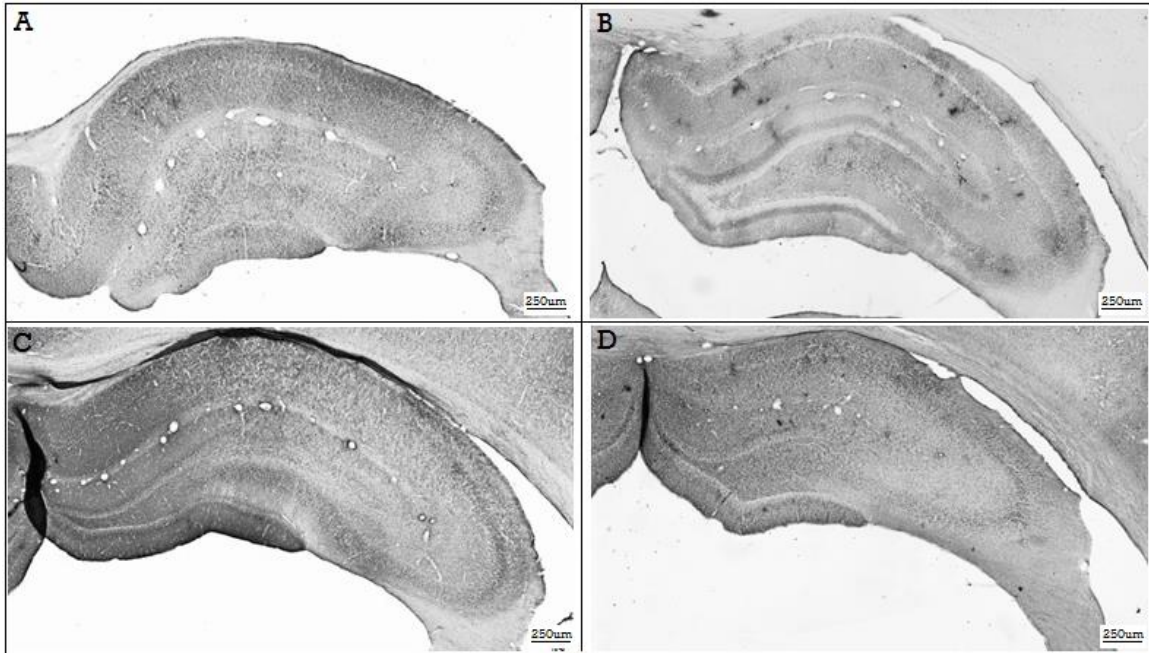


Figure 7. 5x magnification of virgin and lactating rat hippocampus. Lactating control (A) and stressed (B) rats have a visibly lower amount of PHF-1 staining in hippocampus compared to virgin control (C) and stressed (D) rats twenty minutes following the stress episode.

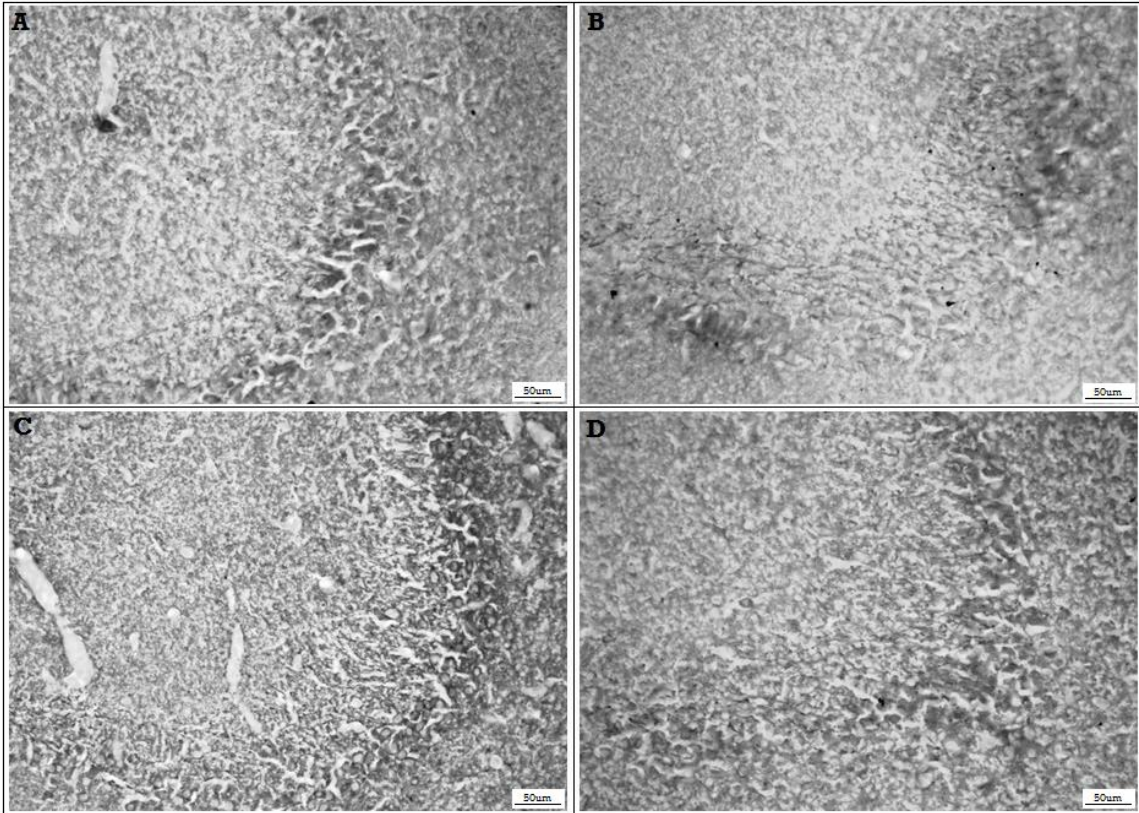


Figure 8. 20x magnification of virgin and lactating rat CA3 region of hippocampus.

At 20x magnification of CA3 pyramidal neurons in hippocampus, a dramatic shift from somatic staining in lactating controls (A) to dendritic and axonal staining in lactating rat hippocampus can be seen twenty minutes following a single restraint episode (B). A similar shift can be seen in stressed virgins (D) compared to their non-stressed controls (C), although not as remarkably.

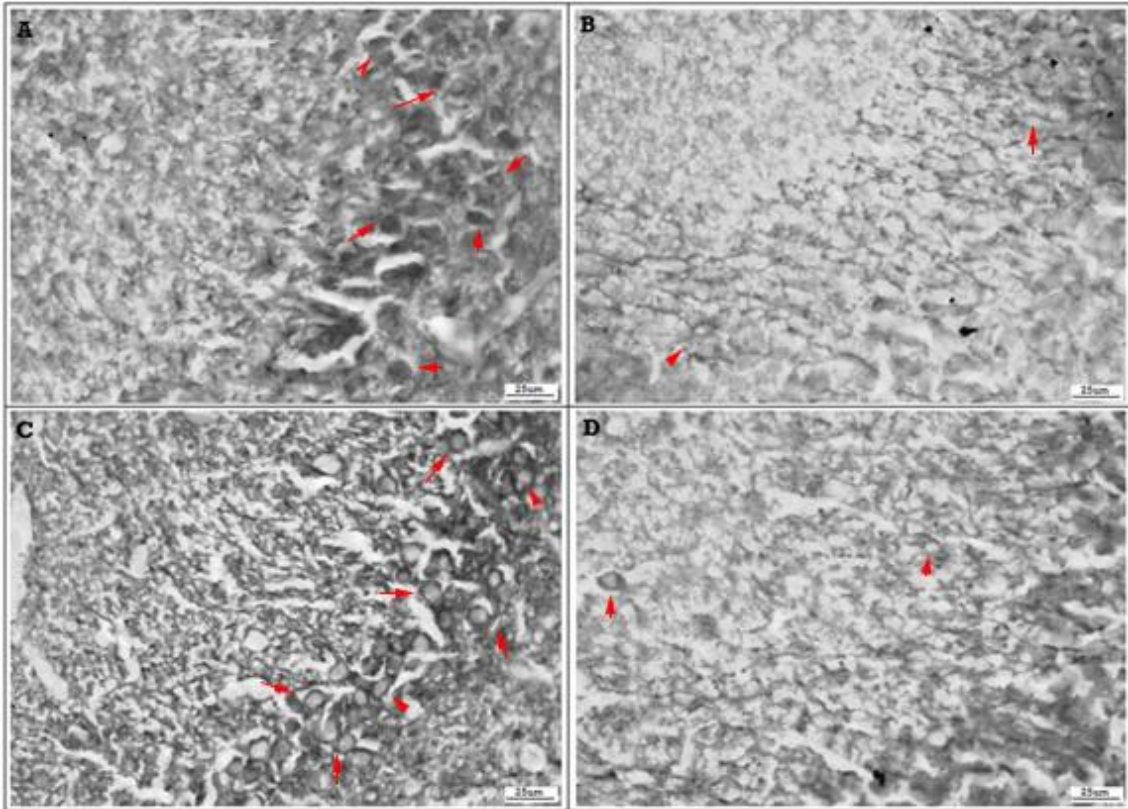


Figure 9. 40x magnification of CA3 pyramidal neurons in virgin and lactating rat hippocampus.

A 40x magnification further demonstrates the striking shift from tau-phosphorylation in the soma of CA3 pyramidal neurons (red arrows) to dendritic/axonal tau-phosphorylation following stress in lactating and virgin rats (B, D) compared to their respective controls (A, C).

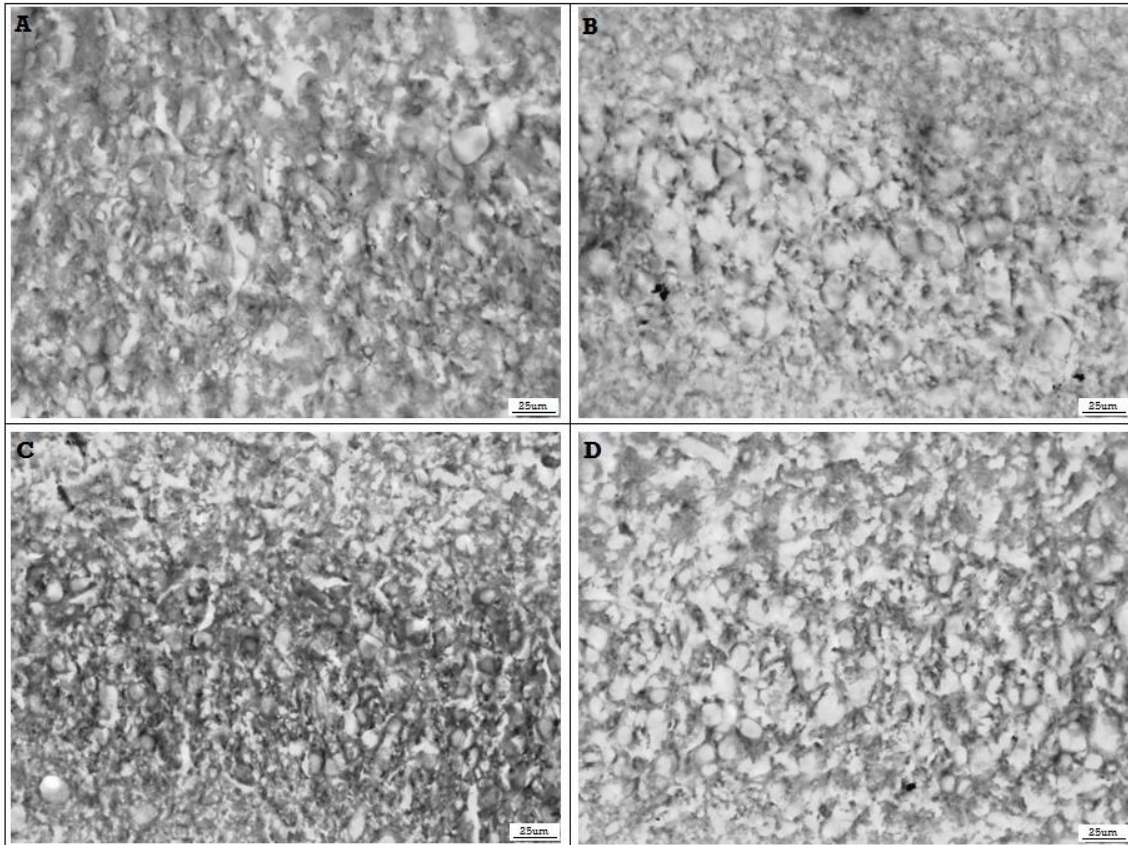


Figure 10. 40x magnification of hilar neurons in virgin and lactating rat hippocampus.

A stress-induced somatodendritic shift is clearly seen in hilus of stressed virgin and lactating rats (B, D) compared to their respective controls (A, C).

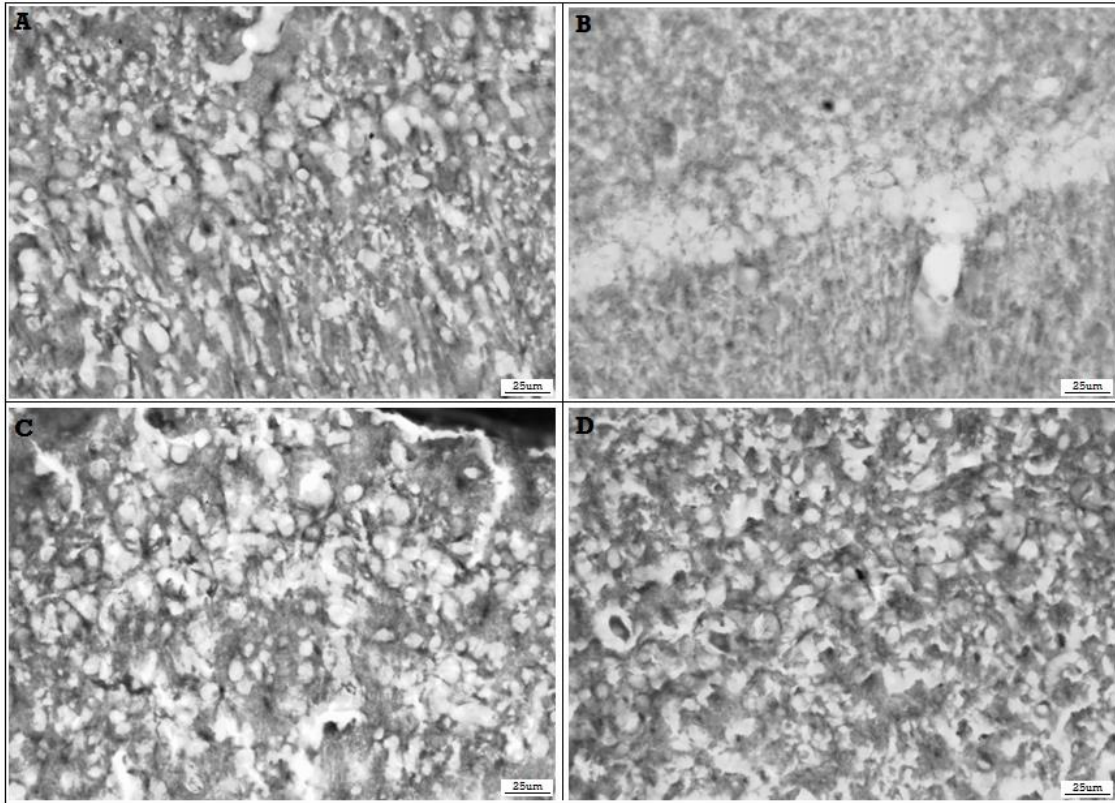


Figure 11. 40x magnification of CA1 neurons in virgin and lactating rat hippocampus.

A strong decrease in phosphorylated tau is notable in stressed lactating rats (B) compared to lactating controls (A). There is no notable differences in virgin rats (C and D), and no pronounced somatodendritic shift in the CA1 region.

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