Neurobiology of Stress 13 (2020) 100241

Contents lists available at ScienceDirect

Neurobiology of Stress

journal homepage: www.elsevier.com/locate/ynstr

Reproductive status impact on tau phosphorylation induced by chronic stress

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ARTICLE INFO

Keywords:
Hippocampus
Female rat
PHF-1
AT8
Restraint stress

ABSTRACT

Sex and exposure to chronic stress have been identified as risk factors for developing Alzheimer's disease (AD). Although AD has been demonstrated to be more prevalent in females, sex is often overlooked in research studies, likely due to the complexity of the hormonal status. In female rats, the reproductive status can modulate the well-known increase in tau phosphorylation (pTau) caused by the exposure to acute physical and psychological stressors. To test the hypothesis that reproductive status can impact hippocampal pTau induced by chronic stress, cohorts of virgin, lactating (4–5 days pp), and post-maternal (1-month post-weaned) rats were subjected to a daily 30-min episode of restraint stress for 14 days and were sacrificed either 20 min or 24 h after their last stress/handling episode. Western blot analysis of two well-characterized AD-relevant pTau epitopes (AT8 and PHF-1) and upstream pTau mechanisms (e.g. GSK3β) analysis, showed that stressed post-maternal rats have increased pTau in comparison to stressed lactating rats 20 min after their last stress episode. Furthermore, an increase in pTau was also seen 24 h after the last stress episode in stressed post-maternal rats in comparison to their non-stressed controls in the detergent-soluble fraction. GSK3 analysis showed an increase in total levels of GSK3β in virgin rats and an increase of inactive levels of GSK3β in post-maternal rats, which suggests a different stress response in pTau after the rat has gone through the maternal experience. Interestingly, post-maternal rats also presented the more variability in their estrous cycles in response to stress. Besides no differences in pTau, non-stressed lactating rats showed an increase in inactive GSK3β 24 h after the last handling episode. Immunohistochemical detection of the PHF-1 epitope revealed increased pTau in the CA4/hilar subfield of the hippocampus of virgin and post-maternal rats exposed to chronic stress shortly after their last stress episode. Overall, lactating rats remained unresponsive to chronic restraint stress. These results suggest increased sensitivity of the virgin and post-maternal rats to hippocampal stress-induced pTau with chronic restraint stress compared to lactating rats. Because no differences were detected in response to stress by lactating rats and an exaggerated response was observed in post-maternal rats, current results support the hypothesis that lactation affects tau processing in the brain of the female.

1. Introduction

Disregulation of the hypothalamic–pituitary–adrenal axis (HPA) axis has been widely implicated as a risk factor for the precipitation and development of Alzheimer's disease (AD), with the corticotropin-releasing factor (CRF) signaling cascade emerging as a prominent player in the last decade alongside glucocorticoids (Rissman et al., 2007, 2012; Carroll et al., 2011a; Campbell et al., 2015). Studies on male rodents show that tau phosphorylation (pTau) increases in response to a variety of stressors (Rissman, 2010) and the exposure to a single episode of restraint stress transiently induces pTau in the hippocampus (Rissman et al., 2007). Moreover, chronic restraint stress increases pTau in insoluble fractions that induces pathogenic-like changes in tau (Rissman et al., 2007, 2012) and hyperphosphorylation in various AD models (Carroll et al., 2011a, 2011b).

It is estimated that around 70% of patients with AD are women (Alzheimer's Association, 2016). The basis for this prevalence points towards an interplay of different risk factors in which ovarian and other hormones play a prominent role (Christensen and Pike, 2015; Snyder et al., 2016; Grimm et al., 2016; Moser and Pike, 2016). At the same

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https://doi.org/10.1016/j.ynstr.2020.100241
Received 27 February 2020; Received in revised form 10 July 2020; Accepted 11 July 2020
Available online 20 July 2020

time, a growing body of evidence shows that females might have an inherent vulnerability to stress-related psychiatric disorders and, hence, to developing AD (Bangasser and Valentino, 2014; Yan et al., 2018). Biochemical changes occur in the brain of the female due to reproductive status. Among them, a diminished response to diverse stressors has been reported during late pregnancy and lactation (Brunton and Russell, 2009; Sze and Brunton, 2019). There is also evidence that hormones such as estrogen, progesterone, allopregnanolone and potentially prolactin can influence pTau in a wide variety of experimental models (Irwin and Brinton, 2014; Muñoz-Mayorga et al., 2018). Furthermore, changes in the expression of tau and its phosphorylation levels have been documented during pregnancy and early lactation in a tissue-dependent manner in the brain (González-Arenas et al., 2012).

We previously reported that during lactation, a decrease in pTau (dephosphorylation phenomena) occurs in response to an acute exposure to restraint stress, coupled with a reduction in overall levels of glycogen synthase kinase 3-alfa (GSK3α) (Steinmetz et al., 2015). Since chronic restraint stress has been shown to increase pTau and its change towards insolubility in males, its consequences in females at different reproductive stages might offer a deeper understanding of the role that maternal hormones and female vulnerability play in chronic stress and AD development. Due to diminished stress responses and pTau in the dam after acute stress (Steinmetz et al., 2015), in the present study we hypothesize that the hippocampus of the mother will show diminished pTau in soluble and insoluble fractions when exposed to chronic restraint stress.

2. Materials and methods

2.1. Animals

Adult (250–300 g) virgin, lactating or post-maternal (1-month post-weaned) female Wistar rats were housed individually under a 12:12 h light/dark cycle (lights on at 8:00 h), controlled temperature (20-23 °C), and with food (Purina Rodent Laboratory Chow 5001) and water ad libitum. One day after parturition, litter sizes were culled to 8–10 pups. The chronic stress treatment started at postpartum day 5 for lactating rats or at 1 month after weaning (post-maternal rats, weaning at 21 days postpartum). The Animal Care and Use Committee of the Institute of Neurobiology at the National Autonomous University of Mexico (INB-UNAM) approved all experimental protocols. Animals were handled in accordance with Official Mexican Standard NOM-062-ZOO-1999 and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Chronic restraint stress protocol

Virgin, lactating and post-maternal rats were placed in flat-bottom acrylic restrainers with ventilation (543-RR and 544-RR, Plas-Labs, Inc., Lansing, MI) for a daily episode of 30 min of restraint for 14 days and were sacrificed either 20 min or 24 h after the last restraint episode. Control rats of each reproductive condition were handled daily but not restrained. Each experimental and control group included an N of 4–5 animals. Importantly, virgin and post-maternal rats were not monitored with vaginal smears to avoid any effect of cervix stimulation during our stress protocol. Separate groups of rats were selected for this purpose.

2.3. Western blot analysis

Rats were deeply anesthetized with a lethal dose of urethane (1.5–2 g/kg bw, IP). Urethane has been shown to influence pTau although in a much longer time frame than the one used in this study (Holscher et al., 2008). Immediately after sedation, animals were decapitated, and the hippocampus was rapidly dissected and frozen on dry ice. For analysis of pTau and solubility, hippocampal tissues were homogenized using sequential fractionation of RAB and RIPA extracts as described previously (Rissman et al., 2007). Before homogenization, protease (PMSF and NaF) and phosphatase (Na_3VO_4 and okadaic acid) inhibitors were added at a concentration of 1 mM. Halt protease and phosphatase inhibitor cocktail (Pierce Biotechnology, Rockford, IL, USA) was added for a final concentration of 1%. Protein concentrations were determined using a BCA Protein Assay Kit (Pierce Biotechnology, Rockford IL). Proteins were then boiled in sample buffer containing SDS, BME and glycerol at 95 °C for 5 min. Fifteen μg of protein were then separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane using the iBlot2 system, 25 V for 7 min (0.2 μm iBlot2 Transfer Stack, Pierce Biotechnology, Rockford, IL).

Nonspecific binding was blocked by incubating membranes in 5% milk-Tris buffered saline containing Tween 20 (TBS-T) for 30 min. Membranes were incubated with primary antibodies diluted in 5% albumin-TBST overnight at 4 °C. The primary antibodies were then detected by anti-mouse or anti-rabbit horseradish peroxidase-linked secondary antibodies for 1 h (dilution 1:1000; EMD Biosciences, La Jolla, CA, USA) and developed using an enhanced chemiluminescence Western blot detection kit (Supersignal West Pico; Pierce Biotechnology, Rockford, IL, USA). Quantitative band intensity readings were obtained using ImageLab Software (Bio-Rad, Hercules, CA, USA). All readings were normalized to non-stressed virgin standard band intensity readings.

2.4. Antibodies

Two well-characterized antibodies were used to probe for specific phosphorylated residues on rat hippocampal tau: S202/T205 (AT8, dilution 1:500; MN1020. Invitrogen, Rockford, IL, USA) and S396/404 (PHF-1, dilution 1:1000; gift from Dr P. Davies, Albert Einstein College of Medicine, Bronx, NY, USA). For expression and activity of GSK3 kinase antibodies specific for total GSK3α/β (0011-A, dilution 1: 1000; sc-7291. Santa Cruz Biotechnology, San Diego, CA, USA), active GSK3β (pY216, dilution 1:1000; 612312. BD Biosciences) and inactive GSK3β (pS9, dilution 1:1000; 9336. Cell Signaling Technology, Danvers, MA, USA) were used. Antibody PHF-1, which probes for phosphorylation in residues Ser396 and Ser 404 (PHF-1, dilution 1:10,000; gift from Dr P. Davies, Albert Einstein College of Medicine, Bronx, NY, USA) was used to assess the anatomical localization of pTau by immunohistochemistry. GAPDH (FL-335, dilution 1:2000; sc-25778. Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as protein-loading control.

2.5. Immunohistochemistry

A separate set of rats in the different experimental groups were anesthetized with a lethal dose of urethane (1.5–2 g/kg bw, IP), perfused with 0.9% saline (100 ml) and 4% paraformaldehyde (in sodium phosphate buffer, 250 ml) and brain tissue was processed for immunohistochemistry as described previously (Rissman et al., 2007). Coronal sections 30 μm thick, corresponding to the rostro-caudal extension of the dorsal hippocampus were cut on a freezing-sliding microtome and stored at −20 °C in antifreeze solution (30% ethylene glycol and 20% glycerol in 0.05 M sodium phosphate buffer) until use. PHF-1 antibody (dilution 1:10,000) was used to probe for pTau on free-floating sections of rat hippocampus. The staining reaction was carried out using a nickel-enhanced DAB Peroxidase Substrate kit (Vector Laboratories, Inc., Burlingame, CA, USA). Photomicrographs were taken using an LSM510 Meta microscope with a 20x objective for morphological analysis and quantification of pTau.

2.6. Morphological analysis

To quantify pTau labeling in the hippocampus, five photomicrographs were taken from five different coronal sections per animal in a rostro-caudal extension of Bregma −3.36 to −4.44. The label for pTau antibody was detected in CA3 and CA4 hippocampal subfields. Then, a

region of interest (ROI) of 213.12 μm × 248.64 μm (52,990.15 μm^2) was selected, and the percentage of area occupied by the antibody signal was quantified using ImageJ software (NIH, Bethesda, MD, USA).

2.7. Estrous cycle phase determination

To avoid the additional vaginal stimulation to experimental rats, a separate set of virgin and post-maternal rats was selected for estrous cycle determination. During the stress protocol (14 days), rats were placed in the restrainer and vaginal smears were taken. Vaginal secretion was collected with a plastic Pasteur pipette filled with approximately 1 mL of saline (NaCl 0.9%) by inserting the tip into the rat vagina. Vaginal smear was placed on glass slides. Vaginal fluid was collected with a clean pipette for each rat and analyzed under a light microscope with 10x objective lens as a wet mount preparation (direct cytology). The number and length of estrous cycles during the stress protocol were quantified as every time that proestrus or diestrus (due to the short duration of proestrus in the rat) was followed by a successful estrus and the length was counted from the first diestrus/metestrus before estrus (Cora et al., 2015). To quantify the percentage of days in a high ratio of estrogen/progesterone (High E/P), the number of days spent in either proestrus or estrus was divided by the 14 days of the stress protocol. We used the High E/P definition as described previously (Broestl et al., 2018) in which estrogen-dominant or High E/P days are proestrus and estrus, and progesterone-dominant or Low E/P days are metestrus and diestrus. Graphs of the cycles were created using Python 3.7 (Python Software Foundation. Python Language Reference).

2.8. Statistical analysis

Integrated intensity readings from Western blots were analyzed using a two-way ANOVA (factor one: reproductive condition; factor two: stress) followed by a Bonferroni post-hoc test only in the factor that resulted significant. Data (percent of control values) were plotted on bar graphs, with data expressed as the mean ± SEM. For analysis of pTau by immunohistochemistry, percent of area of positive label was analyzed by a two-way ANOVA (factor one: reproductive condition; factor two: stress) followed by a Bonferroni post-hoc test only in the factor that resulted significant. Data were plotted on bar graphs, with data (percent of positive area) expressed as the mean ± SEM. Number of cycles, length of cycles and the percent of days in High E/P ratio were analyzed using a Student t-test. Data were plotted on bar graphs, with data expressed as the mean ± SEM. Prism 8 software (GraphPad Software Inc., San Diego, CA, USA) was used to run all the statistical tests. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Post-maternal rats present increased tau phosphorylation in response to the chronic restraint protocol

Because pathologically phosphorylated tau is sequestered into insoluble cellular fractions (Iqbal et al., 1994) and chronic restraint stress promotes pTau increases and accumulation in insoluble and potentially pathogenic tau (Rissman et al., 2007, 2012); Western blot in sequentially fractionated hippocampal extracts was used to evaluate pTau in soluble and detergent-soluble fractions. Factorial analysis revealed a significant contribution only from the reproductive condition factor, as evaluated with the PHF-1 antibody (F2, 19 = 5.267, P = 0.015) and a significant contribution from both factors as evaluated with the AT8 antibody (reproductive condition factor F2, 17 = 4.685, P = 0.024; stress factor F1, 17 = 6.369, P = 0.022; interaction F2, 17 = 1.034, not significant), in the soluble fraction 20 min after handling or last stress episode. As shown in Fig. 1A and B, post-maternal rats showed a significant increase in pTau when compared to stressed lactating rats as evaluated by both PHF-1 (P = 0.0259) and AT8 (P = 0.0138) antibodies.

Furthermore, factorial analysis of the insoluble fraction, as evaluated by the PHF-1 antibody, showed a significant contribution only from the reproductive condition factor (F2, 17 = 5.593, P = 0.013; stress factor; F1, 18 = 2.687, not significant) with no interaction among factors (F2, 18 = 0.3053, not significant). Post-hoc analysis revealed a significant increase in pTau (Fig. 1C) in non-stressed post-maternal rats (P = 0.0316) in comparison to non-stressed virgin controls.

When the soluble and insoluble fractions were evaluated 24 h after the last stress episode, the factorial analysis revealed a significant contribution only from stress factor (F1, 19 = 8.116, P = 0.010) as evaluated by the PHF-1 antibody. As shown in Fig. 2C, post-hoc analysis showed that stressed post-maternal rats had significantly more pTau than their non-stressed controls in the insoluble fraction (P = 0.0193).

3.2. GSK3 modulation by reproductive condition and chronic stress in the hippocampus of the female rat

To investigate possible mechanisms for the stress-induced phosphorylation seen in post-maternal rats, changes in GSK3, a well-characterized tau kinase with activation in this stress model (Steinmetz et al., 2015), were measured by Western blot in the soluble fraction. Factorial analysis showed a significant effect of only the stress factor (F1, 18 = 5.662, P = 0.029; reproductive condition factor F2, 18 = 2.150, not significant; interaction F2, 18 = 2.839, not significant) in the overall levels of GSK3β in rats that were sacrificed 20 min after handling or the last stress episode. As shown in Fig. 3A, stressed virgin rats (P = 0.0178) had significantly higher levels of total GSK3β. Interestingly, at the same time point, the factorial analysis revealed a significant contribution from the stress factor (F1, 17 = 6.658, P = 0.019; reproductive condition factor F2, 18 = 2.541, not significant; interaction F2, 18 = 1.125, not significant) when evaluated by a specific antibody for GSK3β phosphorylated in Ser^9, which renders the kinase inactive, where post-hoc analysis revealed that stressed post-maternal rats had significantly (P = 0.0357) higher levels of inactive GSK3 in comparison with their non-stressed controls (Fig. 3B).

When inactive GSK3β was evaluated 24 h after the last handling or stress episode, a significant contribution was found from the interaction reproductive condition x stress (F2, 18 = 4.935, P = 0.020), but not from the individual factors (reproductive stage factor F2, 18 = 1.263, not significant; stress factor F1, 18 = 0.3455, not significant). As shown in Fig. 4B, a significant increase was found in inactive GSK3β when non-stressed lactating rats (P = 0.0112) were compared with non-stressed virgin controls. Interestingly, stressed virgin rats had a non-significant tendency (P = 0.056) towards higher levels of inactive GSK3β when compared with their non-stressed controls. No significant differences were found in active GSK3β (Y^{216}) at any reproductive condition or time point (Supplementary Fig. 1). No differences were found in GSK3α (total, active, inactive) in any reproductive condition or time point.

3.3. Tau phosphorylation is restricted to areas CA3 and CA4 differentially affected by chronic stress and reproductive condition

Immunohistochemical staining was used to evaluate the anatomical localization of labeling and changes in the amount of pTau with a well-characterized antibody (PHF-1) that probes for the epitope Ser396/404. Representative photomicrographs in Fig. 5 and Fig. 6 show that the distribution of tau phosphorylation in the hippocampus of female rats is restricted to CA3 and CA4 areas. The PHF-1 antibody showed robust fibrillar labeling in the axons of the mossy fibers projecting to the pyramidal cell layer of the Cornu Ammonis in CA3, in *stratum lucidum* and *stratum radiatum*. The labeling in CA4 is mostly punctuate, with a handful of PHF-1-positive cell bodies, and does not extend into the granular layer of the dentate gyrus (DG). This region mostly contains mossy cells. Factorial analysis revealed a significant contribution of both factors, but not the interaction between them (reproductive condition factor F2, 22 = 3.824, P = 0.038; stress factor F1, 22 = 12.52, P = 0.002;

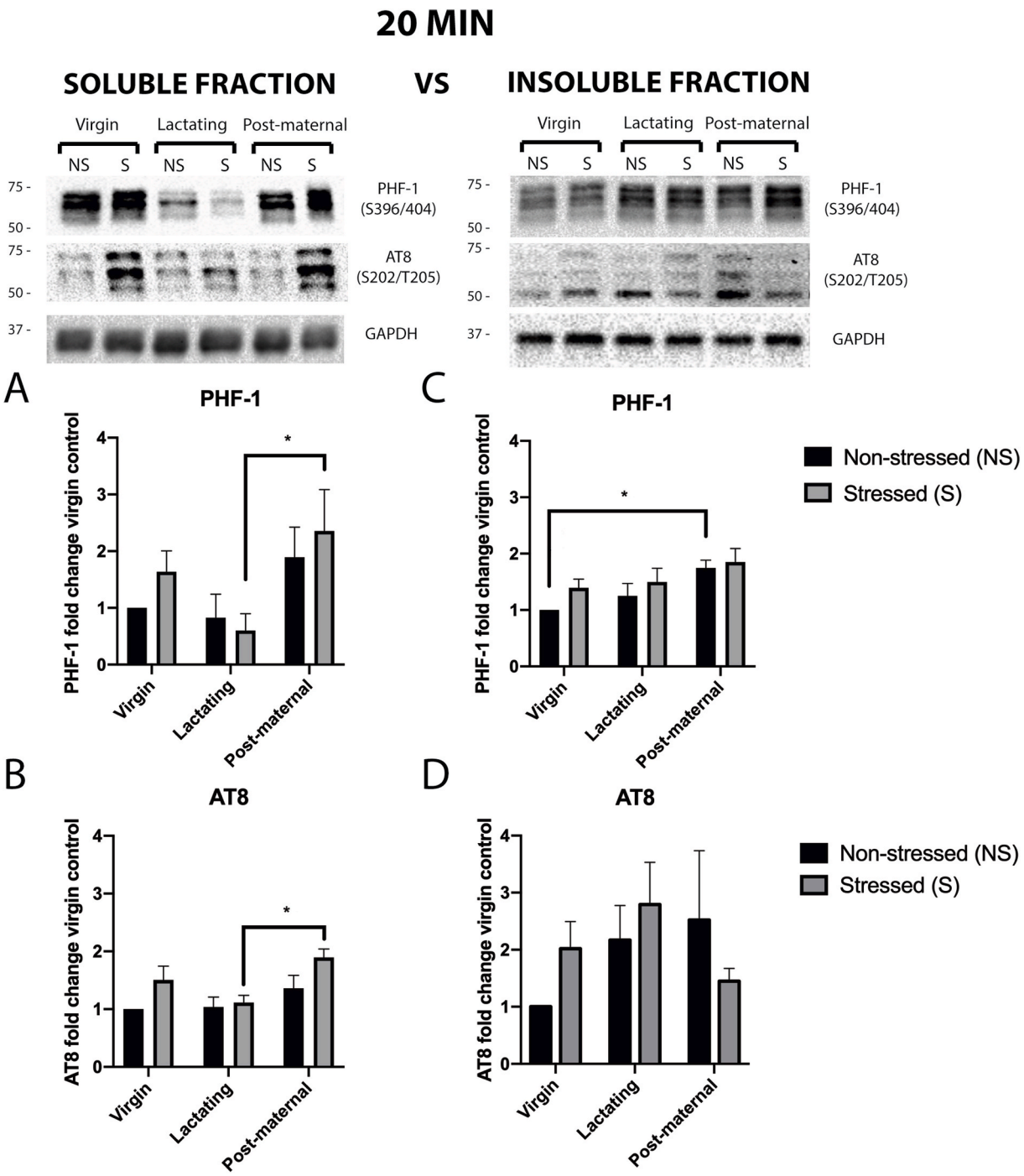

Fig. 1. Post-maternal rats show a significant increase in tau phosphorylation 20 min after the last stress episode in the soluble and insoluble fraction. Female rats in different reproductive stages (virgin, lactating and post-maternal) were sacrificed 20 min after their last stress episode (S) or after handling/no stress (NS). Stressed post-maternal rats had significantly higher phosphorylation levels when compared to stressed lactating rats as evaluated by both the PHF-1 and AT8 antibodies in the soluble fraction. Non-stressed post-maternal rats presented significantly more pTau than non-stressed virgin rats as evaluated by PHF-1 in the insoluble fraction. No significant differences were found in stressed lactating rats when compared to non-stressed controls at any time point. Data are presented as percent of virgin control group (Means ± SEM; *P < 0.05; N = 4–5).

interaction F2, 22 = 1.931, P = 0.1688, not significant). As shown in Fig. 5B, stressed virgin (P = 0.0247) and post-maternal rats (P = 0.0420) showed a significant increase in the percentage of immunoreactive area in comparison with their unstressed controls 20 min after the last stress episode only in the CA4 area. No significant changes were detected in lactating rats 20 min after their last stress episode with their non-stressed controls. No significant changes were detected in the CA3 area (Fig. 5A) in any of the reproductive conditions as evaluated by the factorial analysis, although a tendency towards the same results as CA4 area are evident (reproductive condition factor F2, 22 = 2.786, P = 0.0834; stress factor F1, 22 = 3.652, P = 0.0691; interaction F2, 22 = 0.8080, P = 0.4585).

On the other hand, the factorial analysis 24 h after the last stress episode showed only a significant contribution of reproductive condition factor (F2, 20 = 4.538, P = 0.0237) but not for stress factor (F1, 20 = 0.2887, not significant) or the interaction (F2, 20 = 1.299, not

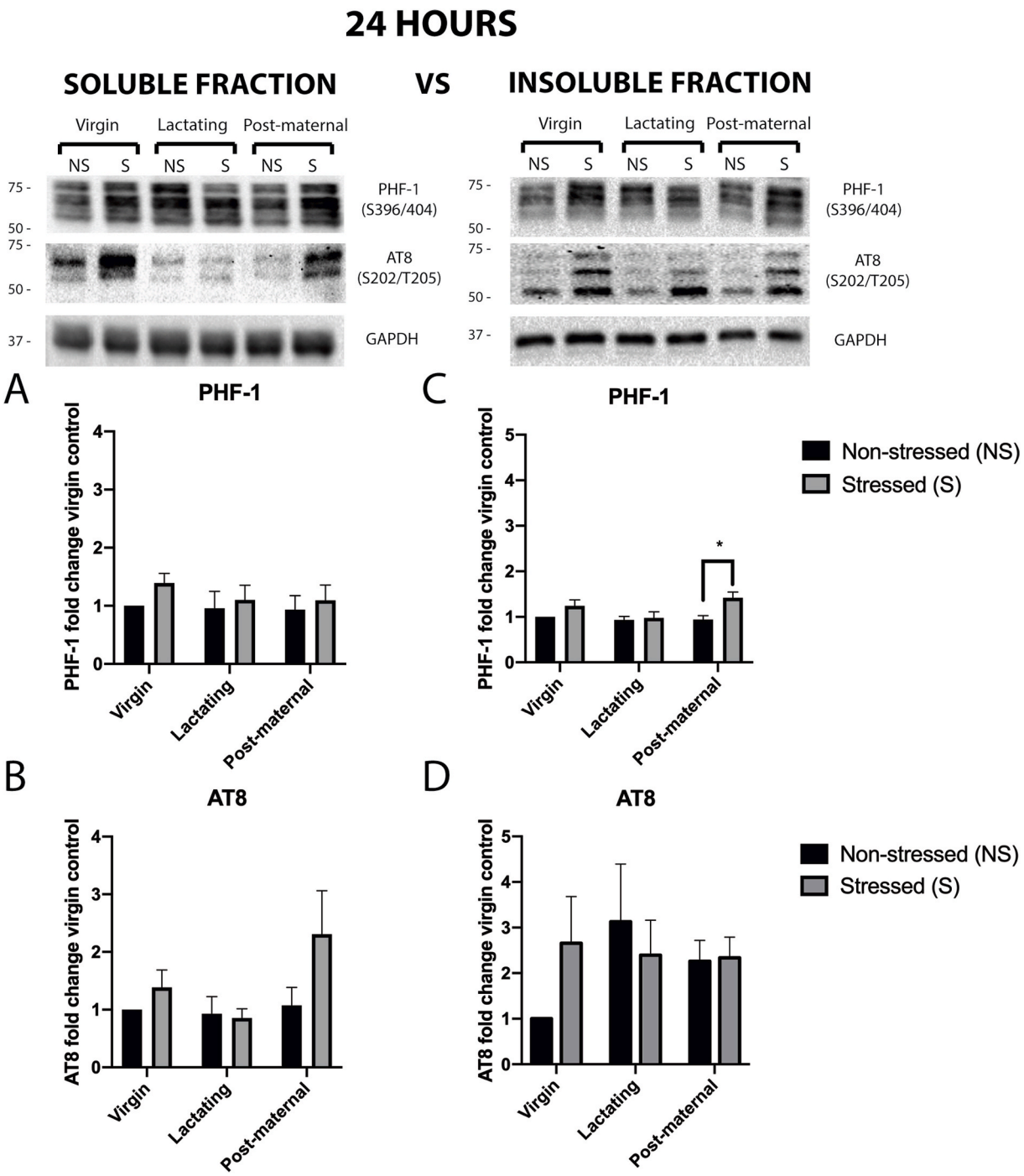

Fig. 2. Stressed post-maternal rats show a significant increase in tau phosphorylation 24 h after the last stress episode in the insoluble fraction. Female rats in different reproductive stages (virgin, lactating and post-maternal) were sacrificed at either handling (NS) or 24 h (S) after their last stress episode. Stressed post-maternal rats had significantly higher phosphorylation levels than their non-stressed controls in the insoluble fraction as evaluated by the PHF-1 epitope. No significant differences were found in stressed virgin or lactating rats at either the soluble or insoluble fraction. Bars represent mean ± SEM; *P < 0.05; N = 4–5.

significant). Fig. 6A shows that stressed lactating rats had a significant increase in the pTau immunoreactivity area percentage in comparison with stressed virgin (P = 0.0288) and post-maternal (P = 0.0236) rats 24 h after their last stress episode. No significant changes were detected in lactating rats in comparison with their non-stressed controls. No significant changes were detected in the CA4 area (Fig. 6B) in any of the reproductive conditions at this time point.

3.4. Post-maternal rats showed more individual variability in estrous cycle than virgin rats

Vaginal cytology in wet mount preparations throughout the stress protocol was used to evaluate if stress affected or altogether abolished the estrous cycle. As shown in Fig. 7A, this chronic stress protocol does not suppress the estrous cycle. Furthermore, post-maternal rats presented the most individual variation in the number of their estrous cycles (Fig. 7C) and spent a significantly higher (P = 0.0259) percentage of days in High E/P ratio (Fig. 7B) than virgin rats during the stress

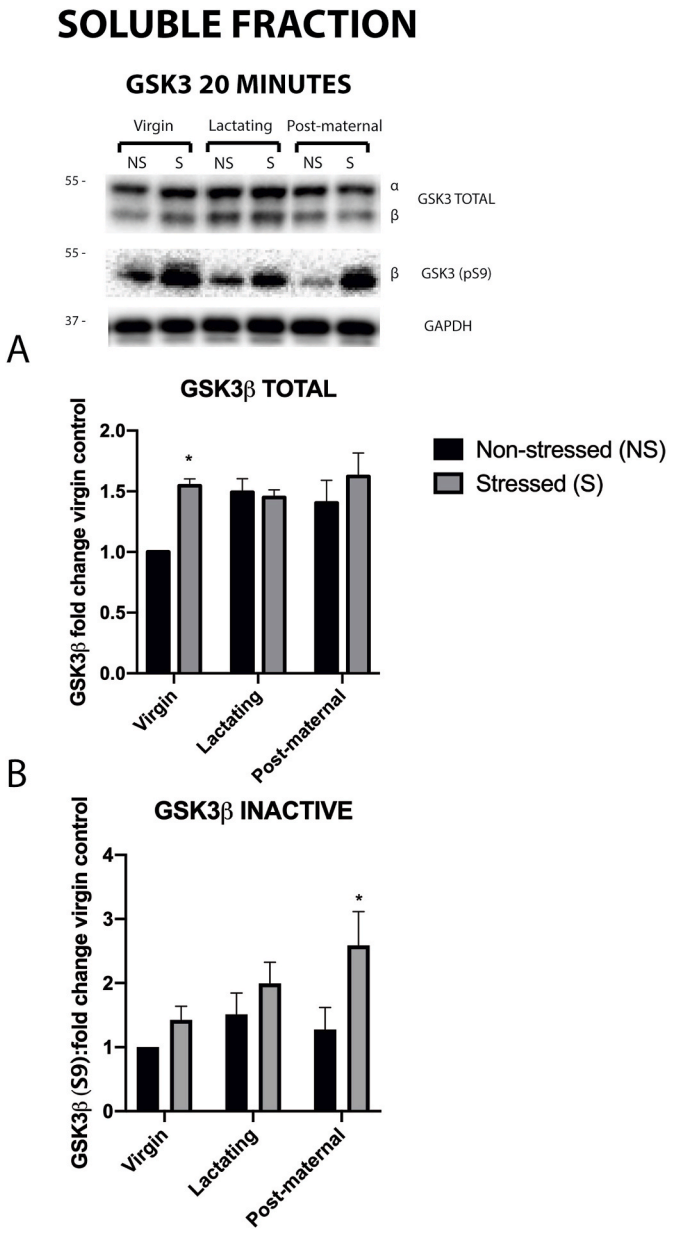

Fig. 3. Stressed virgin rats show a significant increase in total levels of GSK3β whereas stressed post-maternal show increased inactive GSK3β 20 min after the last stress/handling episode. Western blot was performed using antibodies specific to GSK3 to analyze possible mechanisms of stress-induced pTau in virgin, lactating and post-maternal rats sacrificed 20 min after either handling (NS) or their last stress episode (S). (A) Stressed virgin rats and showed a significant increase in the overall levels of GSK3β 20 min after the last handling or stress episode. (B) Stressed post-maternal rats showed a significant increase in GSK3β(S9) at the same time point. Data are presented as percent of virgin control group (mean ± SEM; *P < 0.05; N = 4–5).

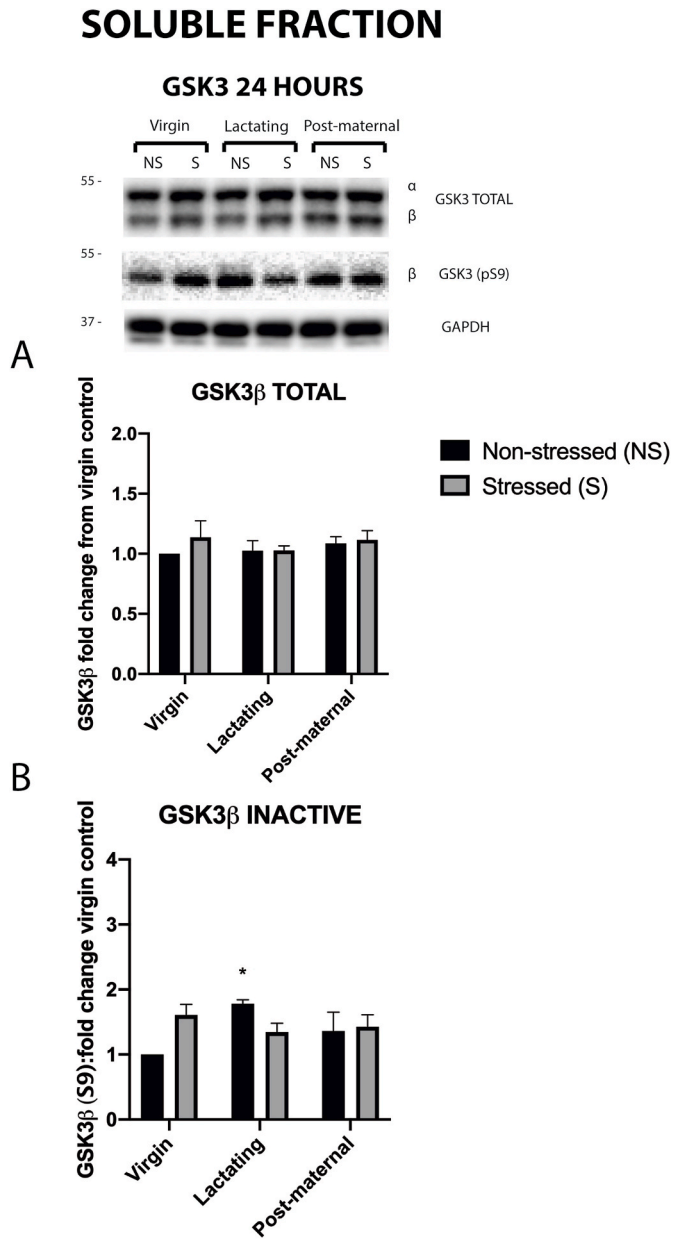

Fig. 4. Non-stressed lactating rats show a significant increase in inactive GSK3β 24 h after the last stress/handling episode. Western blot was performed using antibodies specific to GSK3 to analyze possible mechanisms of stress-induced pTau in virgin, lactating and post-maternal rats sacrificed 20 min or 24 h after either handling (NS) or their last stress episode (S). Non-stressed lactating rats showed an increase in inactive GSK3β 24 h after handling or the last stress episode. No significant differences were found in overall GSK3β levels 24 h after the last stress episode. Data are presented as percent of virgin control group (mean ± SEM; *P < 0.05; N = 4–5).

protocol.

4. Discussion

In this study we tested the hypothesis that female rats undergoing lactation have diminished pTau responses in both soluble and insoluble fractions after chronic restraint stress. Our findings are that i) stressed virgin and post-maternal rats showed increased pTau in response to chronic restraint stress; ii) GSK3β levels and activity are affected by the reproductive condition and chronic restraint treatment; and iii) the CA4 subfield in the hippocampus is the most sensitive to chronic restraint stress in female rats.

4.1. pTau in the hippocampus of post-maternal rats increased after chronic restraint stress

It has been demonstrated previously that chronic restraint stress can trigger pTau and induce a change towards insolubility and tau aggregation, which can be potentially pathogenic (Rissman et al., 2007, 2012). Our groups have also shown that lactating rats exposed to a single episode of restraint stress show a dephosphorylation phenomenon, coupled with reduction of overall levels of GSK3α (Steinmetz et al.,

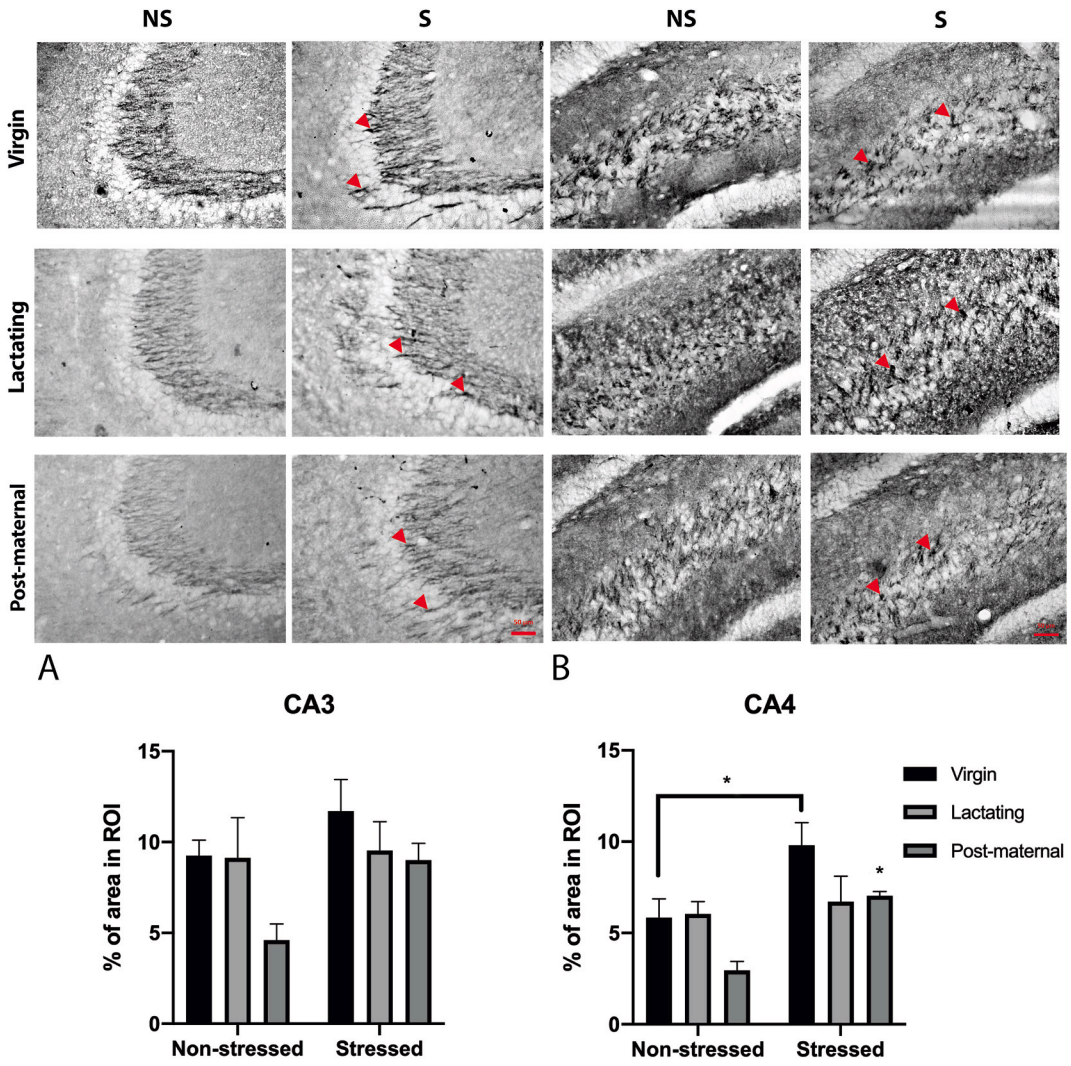

Fig. 5. Chronic stress significantly increased pTau immunoreactivity in the CA4 subfield of stressed virgin and post-maternal rats sacrificed 20 min after their last stress episode in comparison to their non-stressed controls. Representative photomicrographs and corresponding quantification demonstrating positive immunoreactivity of pTau (PHF-1) in the CA3 and CA4 hippocampal subfields of stressed and non-stressed rats (control) at different reproductive stages (virgin, lactating and post-maternal). Stressed virgin and post-maternal rats had a significantly larger area of immunoreactivity for pTau in the CA4 subfield in comparison with their respective non-stressed controls. We found no significant differences in pTau immunoreactivity in stressed lactating rats 20 min following their last stress episode in the CA4 subfield in comparison with non-stressed controls. No significant differences were found in the CA3 subfield at any reproductive condition. Bars represent mean ± SEM; *P < 0.05; N = 4–5. Scale bar = 50 μm.

2015). The present study focused on the chronic exposure to the same stressor and found a significant increase in pTau in post-maternal rats in the soluble fraction 20 min after the last stress episode in comparison with stressed lactating rats. Interestingly, non-stressed post-maternal rats had significantly more pTau in the insoluble fraction when compared to non-stressed virgin controls. Most importantly, stressed post-maternal rats showed a significant increase in pTau 24 h after the last stress episode in comparison with their non-stressed controls in the insoluble fraction. The fact that stressed post-maternal rats showed increased pTau levels in the soluble fraction, which translates to the insoluble fraction, indicates that the sustained pTau in response to repeated stress promotes the change of tau to a detergent-soluble fraction where the bulk of aberrantly phosphorylated tau in paired helical fragments (PHF) and neurofibrillary tangles (NTF) resides in AD (Iqbal et al., 1984, 1994, Rissman et al., 2007, 2012).

The fact that we found a significant increase in inactive GSK3β 20 min after the last stress episode in stressed post-maternal rats despite the clear pTau increase strongly suggests that this might be a mechanism trying to prevent pTau triggered by chronic stress, but it is surpassed by the action of other kinases or the failure of phosphatase mechanisms (such as PP2A) that may contribute to the sustained pTau and its accumulation in the insoluble fraction. Kinases such as ERK 1/2 and JNK, are increased after restraint stress in male mice and contribute to stress-induced pTau (Rissman et al., 2007).

At this point, it is important to acknowledge the small sample size as a limitation to our study. Our current GSK3 data suggests that an increase in overall levels of GSK3β 20 min after the last stress episode may play a role in the stress-induced phosphorylation observed in virgin rats 20 min after their last stress episode by immunohistochemistry. This increase was not found significant by Western blot. Furthermore, stressed virgin rats also show a tendency towards an increase in inactive GSK3β, in comparison to non-stressed virgin controls 24 h after the last handling/stress episode. Taken together, these observations suggest that virgin rats are affected by stress but might be able to activate mechanisms to reduce pTau in response to chronic stress.

Remarkably, these observations were not seen in lactating dams. Stressed lactating rats showed no significant differences at any time point in pTau in comparison to their non-stressed controls with any of

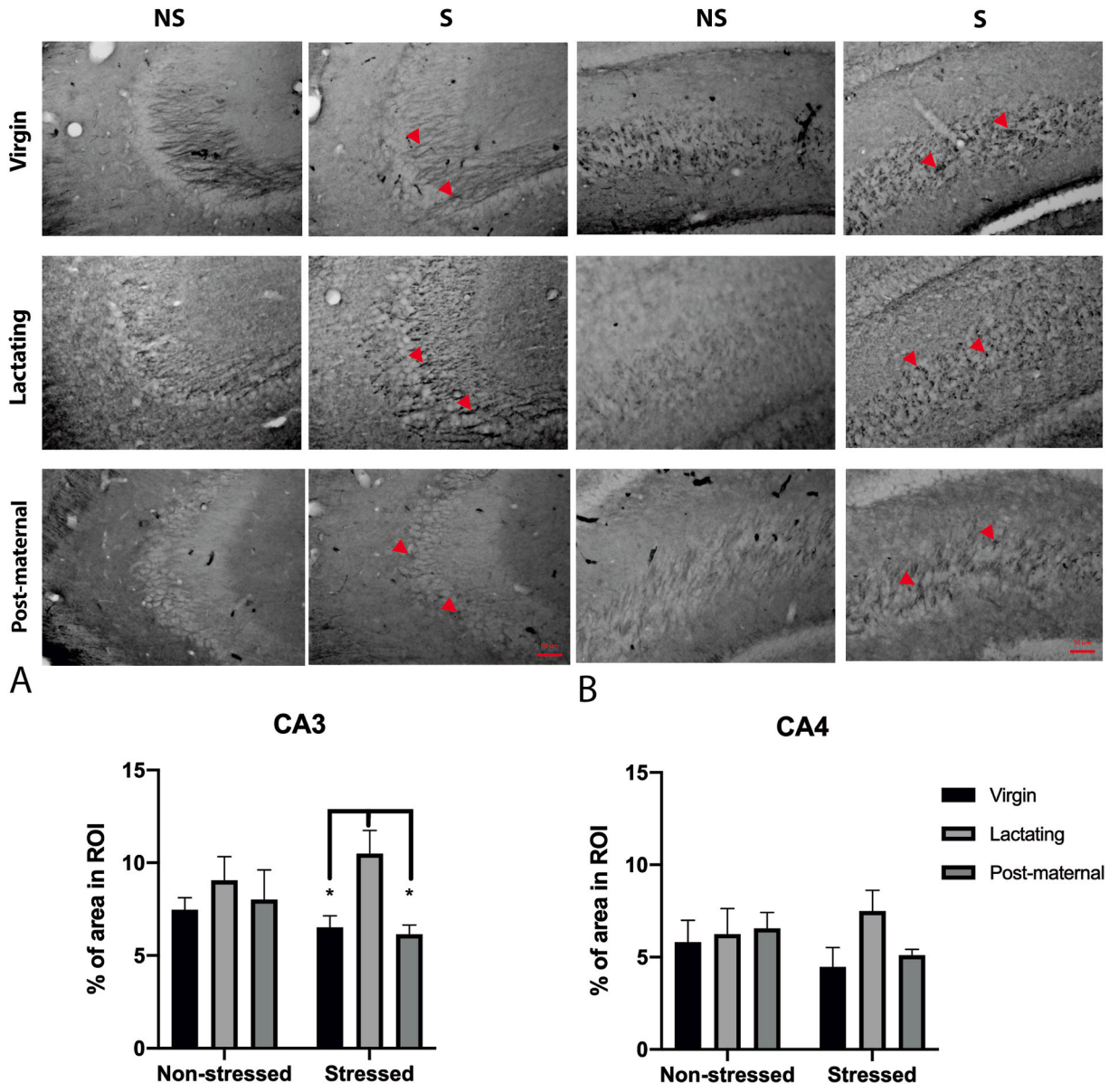

Fig. 6. Chronic stress significantly increased p-tau immunoreactivity in the CA3 subfield of lactating rats sacrificed 24 h after the last stress episode compared to stressed virgin and post-maternal rats. Representative photomicrographs and corresponding quantification demonstrating positive immunoreactivity of pTau (PHF-1) in the CA3 and CA4 hippocampal subfields of stressed and non-stressed rats (control) at different reproductive stages (virgin, lactating and post-maternal). Stressed lactating rats had a significantly larger area of immunoreactivity for pTau than stressed virgin and post-maternal rats in the CA3 subfield. We found no significant differences in pTau immunoreactivity in any group compared to their non-stressed controls in CA3. No significant differences were found in the CA4 subfield at any reproductive condition. Bars represent mean ± SEM; *P < 0.05; N = 4–5. Scale bar = 50 μm.

our techniques, which suggests that they can buffer the effects of the chronic restraint treatment in pTau but not reverse them as observed using an acute stress episode (Steinmetz et al., 2015). Twenty-four hours after the last stress episode, a significant increase in inactive GSK3β (phosphorylated in Ser9) was seen in non-stressed lactating rats. These results suggest that lactation activates mechanisms by itself and in response to stress that diminish their susceptibility to pTau but only as long as this period lasts by the regulation of GSK3 activity.

4.2. The CA4 area in the hippocampus of virgin and post-maternal female rats is more susceptible to the effects of chronic restraint stress

The labeling pattern observed is in concordance with a previous study of endogenous tau in male mice using a phosphorylation-independent antibody and in which tau was mostly found in non-myelinated axons, not in dendritic compartments or synapses, and to a lesser degree in oligodendrocytes (Kubo et al., 2018). A second immunohistochemical analysis was performed with another phosphorylation-dependent antibody (S422), with an identical pattern and similar results (data not shown). Previous studies using restraint stress or corticotropin-releasing factor receptor (CRFR) overexpression report significantly more markers of neurodegeneration (Jeong et al., 2006) and pTau (Rissman et al., 2007, 2012; Campbell et al., 2015) in CA1, CA3 and the DG, specially CA4. We did not find pTau in the CA1 subfield or the molecular layer of the DG like some of the aforementioned reports using the same antibody and stressor, which indicates that female rats respond differently in comparison with male mice. There is evidence that gender, sex and reproductive status can influence the response to different stressors and the recruitment of different circuitries and biochemical pathways activated by them, thus explaining

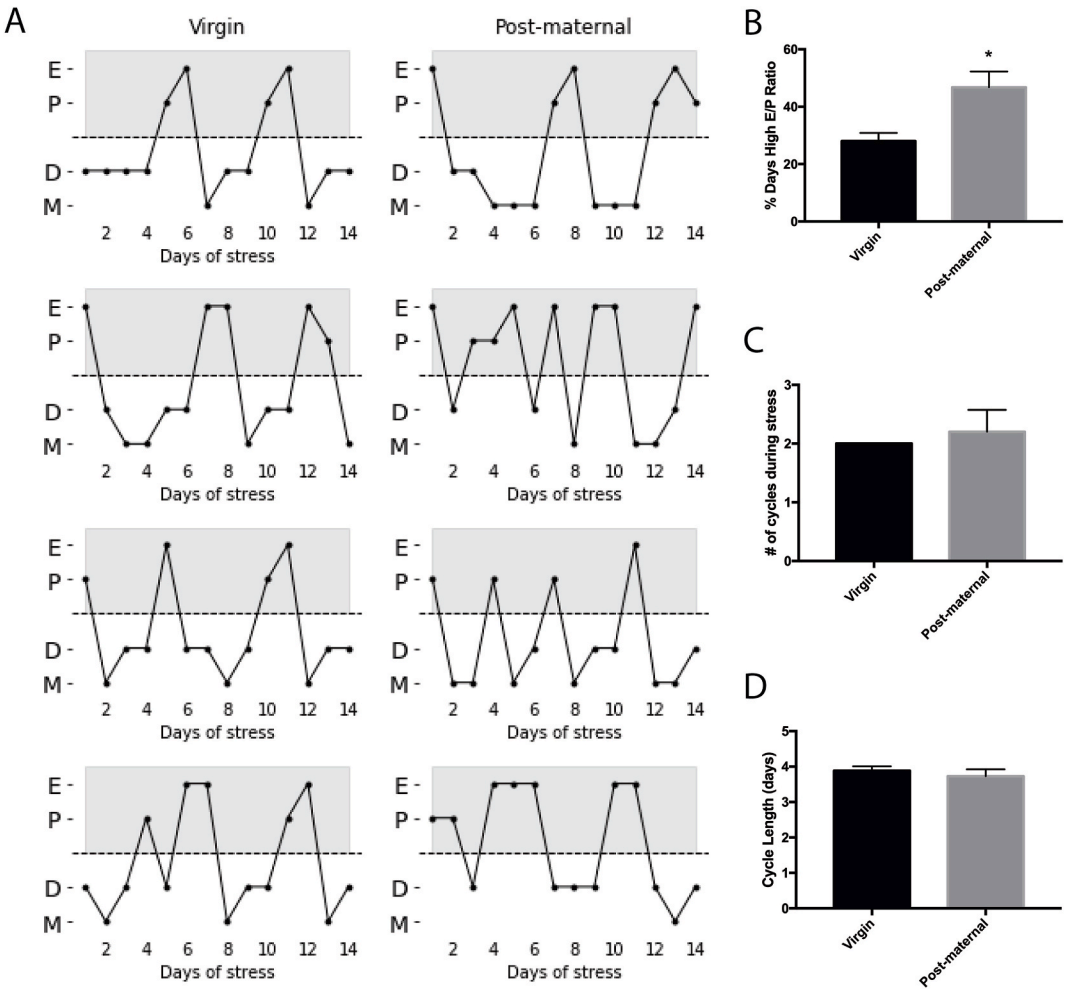

Fig. 7. Post-maternal rats spent significantly higher percentage of days in stages of the cycle with a high ratio of estrogen/progesterone (High E/P). The graph shows the stage of the estrous cycle for virgin and post-maternal rats during the 14 days of the stress protocol. A) Post-maternal rats showed erratic cycles during the stress protocol although the cycle was not abolished completely. B) Post-maternal rats spent significantly higher percentage of days in stages of the cycle with a High E/P. *Abbreviations:* M = Metestrus, D = Diestrus, P = Proestrus, E = Estrus. Bars represent mean ± SEM; *P < 0.05; N = 4–5.

this difference with previous studies (Bangasser et al., 2010, 2017; Salvatore et al., 2018; Rincón-Cortés et al., 2019).

The immunohistochemical data from rats sacrificed 20 min after the last stress episode support our biochemical data indicating that virgin and post-maternal rats are more susceptible to the effects of chronic restraint stress. The CA4 hippocampal subfield is where the stress effects are most significant. Although the effects of chronic stress in this subfield are still not well understood, it is known that neurogenesis in the DG is negatively affected by stress and glucocorticoid administration (Snyder et al., 2011; Schoenfeld and Gould, 2012). Furthermore, evidence suggests that decreased neurogenesis could translate to changes in CA3 atrophy and hippocampal volume (Schoenfeld et al., 2017). Non-stressed post-maternal rats had significantly higher levels of pTau than non-stressed virgin rats as analyzed by Western blot, although the same was not observed as measured by immunohistochemistry. Despite the antibodies employed for Western blot and immunohistochemistry are the same, the two methods are different. Whereas this last one is detecting the antigen directly in the fixed tissue at specific regions of the hippocampus, for Western blot proteins undergo an extraction and further steps before been exposed to the antibody.

Interestingly, stressed lactating rats had significantly more pTau in CA3 24 h after the last stress episode in comparison with both stressed virgin and post-maternal rats. We believe that this seemingly conflicting result is due to more soluble tau being available for the antibody to detect in lactating rats, in contrast with virgin and post-maternal rats where tau might be increasingly changing into an insoluble form not easily detected by the antibody. This mechanism has been proposed previously in studies with the same restraint protocol in male mice (Rissman et al., 2007, 2012) and further highlights the fact that pTau mechanisms are altered during lactation.

4.3. Hormones and response to chronic restraint stress

Although corticosterone levels (a canonical parameter of the stress response) were not measured, we have reasons to believe that corticosterone is not pivotally involved in stressed-induced pTau, as adrenalectomized males still show this response (Rissman et al., 2007). Moreover, in a previous study using an acute episode of restraint stress, no differences were found in corticosterone levels between stressed and non-stressed rats (Steinmetz et al., 2015). As for stress habituation, our overall results, the variation in estrous cycle and our own observations (urination and defecation) during the stress protocol support the fact that our rats did not experience habituation (Girotti et al., 2006). It is well documented that hormones, such as estrogen and progesterone (and its metabolites), can influence pTau in different experimental models generally proposing protective actions for both hormones (reviewed in Muñoz-Mayorga et al., 2018). In this study, we observed that virgin and post-maternal rats have a stress-induced increase in pTau and that post-maternal rats seem to be the most sensitive to these effects, as they accumulate tau in an insoluble and potentially pathogenic

fraction.

During the stress protocol, post-maternal rats had the most individual variation in estrous cycle number, ranging from 1 to 3 in contrast to the 2 cycles observed in virgin rats. Furthermore, stressed post-maternal rats spent a significantly higher percentage of days in estrogen-dominant (High E/P ratio) cycle stages in response to the stress protocol when compared to stressed virgin rats. Females of an AD model spent a higher percentage of days in a High E/P ratio when compared to non-transgenic controls (Broestl et al., 2018). Furthermore, subjects in days with a High E/P ratio had neural network alterations, cognitive impairment and higher production of pathogenic beta amyloid (Aβ1-42) when compared to subjects in Low E/P ratio or gonadectomized (Broestl et al., 2018). Interestingly, no changes in total tau or phosphorylated tau (PHF-1) were observed in this study.

According to our data, the maternal experience seems to render the rat brain more susceptible to the effects of stress-induced pTau. Some studies point to the number of pregnancies as a risk factor for AD in humans (Ptok et al., 2002; Colucci et al., 2006) and an interesting study evaluating phosphorylation-dependent tau antibodies and their correlation with Braak stages observed that all of the brain tissue examined in Braak stages V/VI came from women (Neddens et al., 2018). Other studies correlate late pregnancy with better cognitive performance (Fox et al., 2013) and prolonged periods of breastfeeding with a reduced risk of developing AD (Karim et al., 2016). In rodents it has been reported that the number of maternal experiences has a positive effect on brain aging (Gatewood et al., 2005). Further research is needed to clarify these observations.

The decreased sensitivity of the lactating dam to stressors (Brunton et al., 2008), in which oxytocin and prolactin, play an important role has been widely documented (Torner and Neumann, 2002). High levels of prolactin during the lactation period are known to contribute to the stress hyporesponsiveness characteristic of this state (Torner and Neumann, 2002; Torner et al., 2002). We have previously proposed that one of the mechanisms by which prolactin could protect the lactating rat from the effects of restraint stress is by increasing the activity of the Akt pathway that can phosphorylate GSK3 in the residue Ser9, which inactivates the kinase, preventing it from tagging the prolactin receptor for degradation and also preventing stress-induced pTau (Domínguez-Cáceres et al., 2004; Plotnikov et al., 2008; Steinmetz et al., 2015; Muñoz-Mayorga et al., 2018). Although our biochemical results fit and support the aforementioned mechanism, these observations, until our study, were done in lymphoid and breast cancer cells (Domínguez-Cáceres et al., 2004; Plotnikov et al., 2008). Thus, more research is needed to prove its existence in the brain. This study and others build upon the existing literature and lay the foundation to keep researching prolactin as a potential regulator of tau phosphorylation. Notwithstanding, how prolactin regulates the stress response in different brain regions is not well understood, and the contribution of other cellular pathways involving phosphatase regulation or interaction with CRFR receptors in the hippocampus cannot be excluded.

Due to the paramount reproductive importance of the maternal experience (Arbeitman, 2019), the array of hormones involved such as allopregnanolone in late pregnancy and prolactin during lactation, result in a decreased response to stress (Brunton et al., 2008; Sze and Brunton, 2019). Furthermore, progesterone has been reported to increase between days 12 and 16 of lactation (Vanoye-Carlo et al., 2008) and oxytocin possesses documented anxiolytic actions and has high levels during lactation (Brunton et al., 2008). We must now stress the fact that it is the entire lactation period, its associated hormonal changes and the interplay between them that result in pTau changes during this period, so several yet unknown hormonal mechanisms might be involved.

Overall, our results show that lactation diminishes the effects of chronic stress on hippocampal pTau only for as long as this stage lasts; subsequently, the female is more vulnerable to stress-induced pTau. The fact that lactating rats are more resistant to stress-induced pTau can have profound physiological implications, such as a more stable cytoskeleton and synaptic communication needed in the material brain for the care of the offspring. Further studies focusing on the overall female aging process, multiple maternities and different stressors are needed to better understand how these changes translate into an increased or decreased risk for developing neurological diseases, especially AD.

Data sharing

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of competing interest

The authors of the manuscript have no conflicts of interest to declare.

CRediT authorship contribution statement

Daniel Muñoz-Mayorga: Conceptualization, Investigation, Formal analysis, Writing - original draft. **Robert A. Rissman:** Conceptualization, Formal analysis, Writing - review & editing, Funding acquisition. **Teresa Morales:** Conceptualization, Formal analysis, Writing - review & editing, Funding acquisition.

Acknowledgements

These studies were supported by grants IN204718 from UNAM-DGAPA-PAPIIT and A1S8948 from CONACYT to Dr. Teresa Morales; and BX003040, AG018440 and AG065385-from National Institutes of Health to Dr. Robert Rissman. This work is part of Daniel Muñoz-Mayorga's PhD studies in the Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México (UNAM). DMM studies were supported by CONACyT scholarship (CVU/Recipient: 662482/574065), CONACyT Becas Mixtas scholarship program and UNAM Programa de Apoyo para los Estudios de Posgrado. The authors are grateful to Jessica Gonzalez for proofreading the English version of this manuscript, Biol. Eugenia Ramos for technical assistance, Julio Reyes-Mendoza for statistical assistance and helpful discussions, Dr. Martín García and Dr. Alejandra Castilla assistance for animal care. We thank to Dr. Peter Davies (Albert Einstein College of Medicine, Bronx, NY, USA) for the generous donation of the PHF-1 antibody.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ynstr.2020.100241.

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