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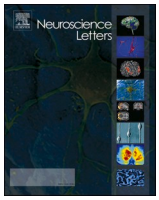

Research article

Chronic corticosterone exposure in rats induces sex-specific alterations in hypothalamic reelin fragments, MeCP2, and DNMT3a protein levels

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ABSTRACT

Women are disproportionately affected by stress-related disorders like depression. In our prior research, we discovered that females exhibit lower basal hypothalamic reelin levels, and these levels are differentially influenced by chronic stress induced through repeated corticosterone (CORT) injections. Although epigenetic mechanisms involving DNA methylation and the formation of repressor complexes by DNA methyl-transferases (DNMTs) and Methyl-CpG binding protein 2 (MeCP2) have been recognized as regulators of reelin expression *in vitro*, there is limited understanding of the impact of stress on the epigenetic regulation of reelin *in vivo* and whether sex differences exist in these mechanisms. To address these questions, we conducted various biochemical analyses on hypothalamic brain samples obtained from male and female rats previously treated with either 21 days of CORT (40 mg/kg) or vehicle (0.9 % saline) subcutaneous injections. Upon chronic CORT treatment, a reduction in reelin fragment NR2 was noted in males, while the full-length molecule remained unaffected. This decrease paralleled with an elevation in MeCP2 and a reduction in DNMT3a protein levels only in males. Importantly, sex differences in baseline and CORT-induced reelin protein levels were not associated with changes in the methylation status of the *Reln* promoter. These findings suggest that CORT-induced reelin decreases in the hypothalamus may be a combination of alterations in downstream processes beyond gene transcription. This research brings novel insights into the sexually distinct consequences of chronic stress, an essential aspect to understand, particularly concerning its role in the development of depression.

1. Introduction

Disruptions in both stress regulation and synaptic plasticity are linked to depression, but the specific mechanisms behind these changes remain unclear [1,2]. The induction of chronic stress in rats through repeated corticosterone (CORT) injections leads to the manifestation of depressive behavior and modifications in synaptic plasticity [3], including a decrease in reelin levels [4,5]. Reelin is an extracellular matrix glycoprotein known for promoting synaptic plasticity in the brain and appears to be involved in the pathophysiology of depression [6,7].

Our recent research revealed a sexually dimorphic population of cells expressing reelin in the hypothalamus, with females exhibiting lower basal levels. Furthermore, these cells demonstrate distinct responses to chronic stress induced by CORT [8,9]. Within the same study, we noted a correlation and co-expression between reelin and oxytocin in the hypothalamic paraventricular nucleus, suggesting a potential role of reelin

Abbreviations: BDNF, Brain-derived neurotrophic factor; CORT, Corticosterone; DNMT, DNA methyl transferase; GR, Glococorticoid receptor; HRM, Heterozygous reeler mice; IPTG, Isopropyl β-D-1-thiogalactopyranoside; MeCP2, Methyl-CpG-binding protein 2; N-t, N terminal; PCR, Polymerase chain reaction; PB, Phosphate buffer; PFA, paraformaldehyde; SDS, Sodium dodecyl sulfate; TSS, Transcription start site; WB, Western blot.

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in modulating the sexually dimorphic oxytocin system, known for its role in stress homeostasis through the modulation of the hypothalamic–pituitary–adrenal axis activity and its role in sex-specific behaviors. Given the higher prevalence of depression in women [10] and the underlying sex differences in stress regulation [11], it becomes crucial to explore the sex-specific mechanisms governing reelin expression *in vivo* under both basal and chronic stress-induced conditions in stress-regulatory brain regions like the hypothalamus.

Previously, sex differences have been identified in epigenetic mechanisms and their effector molecules within the brain, and it is known that epigenetic processes can alter patterns of gene expression in response to environmental stimuli [12]. Consequently, variations in epigenetic mechanisms may account for the differences in baseline reelin levels and susceptibility to CORT. The *Reln* promoter contains several CpG methylation sites that are susceptible to methylation [13]. *In vitro* studies have demonstrated that repressor complexes involving DNMT3a and MeCP2 modulate reelin expression [14–16]. DNMT3a performs *de novo* DNA methylation [17], but it has also been associated to activity-dependent changes in gene expression [18]. MeCP2, a transcriptional regulator abundant in the brain [19], selectively binds methylated DNA and interacts with corepressor or coactivator proteins to facilitate gene repression or expression, respectively [20,21]. There is also evidence indicating that methylation of the human RELN promoter leads to a reduction in reelin protein levels in individuals with schizophrenia [22,23,24] and distinct methylation patterns exist between sexes in psychiatric disorders [25].

Considering the previously documented sex-specific impacts of corticosterone in the hypothalamus, the primary objective of this study was to investigate the potential existence of sex differences in reelin protein levels, *Reln* promoter methylation and epigenetic regulators under basal and stress-induced conditions in this brain region. Given the link between diminished reelin immunoreactivity and depression, as well as an increased vulnerability to stress in reelin-deficient rodents, uncovering how stress influences reelin levels in a pathological manner is crucial for identifying new therapeutic targets and developing more effective drugs for reelin-related disturbances and stress-related disorders like depression.

2. Methodology

2.1. Experimental design

For this study, Long Evans male and female rats underwent 21 days of either 40 mg/kg subcutaneous corticosterone injections or vehicle solution (0.9 % saline). Brains were collected 24 h after the last CORT or vehicle injection by decapitation after overdose with isoflurane. All animal experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care, the National Institutes of Health guide for the care and use of laboratory animals and the University of Victoria Committee on Animal Care.

2.2. Sample preparation

Fresh brains were dissected on ice to isolate the hypothalamus following the previously described method using forceps [26,27], which was subsequently snap-frozen in liquid nitrogen and stored at −80 °C.

2.3. Nuclear extraction

The hypothalamic tissue was homogenized in 2 volumes (w/v) of cell lysis buffer (0.25 M sucrose, 60 mM KCl, 15 mM NaCl, 10 mM MES (pH 6.5), 5 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.5 % Triton X-100) and incubated for 7 min on ice. At each step complete protease cocktail inhibitor [(Roche Molecular Biochemicals) Laval, Quebec, Canada] was added to buffers/suspensions in a 1:100 dilution. Samples were then centrifuged for five minutes at $2.6x10^3$ rpm at 4 °C. The supernatant was discarded, and the pellet was then resuspended in 8 volumes (w/v) of the lysis buffer and pelleted for five minutes at $2.6x10^3$ rpm at 4 °C. The pellet was resuspended in four volumes of nuclei resuspension (50 mM NaCl, 10 mM Pipes (pH 6.8), 5 mM $MgCl_2$, 1 mM $CaCl_2$) buffer and pelleted for five minutes at $2.6x10^3$ rpm at 4 °C. Finally, the pellet was resuspended in 2 volumes of nuclei resuspension buffer and the absorbance at 260 nm was read by a Varian UV Visible Spectrophotometer. The concentration of DNA was approximated using an extinction coefficient of $A_{DNA} = 20$ cm^2 and accounting for the sample being diluted 1:200 in 0.2 % SDS. Nuclei samples for use in SDS-PAGE and western blot (WB) analyses were left in suspension and were diluted in 1:1 sample buffer (250 mM Tris HCl (pH 6.8), 4 %, SDS, 40 % glycerol, 2.86 M β-mercaptoethanol, and 0.4 % bromophenol blue) and sonicated for 5 mins (10sec intervals) on ice and finally stored at −80 °C.

2.4. Electrophoresis and Western blotting

For reelin quantification: 5 µg of protein from the cytosolic fraction (SNI) was electrophoretically resolved at 110 V for 100 min in a 10 % SDS-polyacrylamide gel and then transferred onto nitrocellulose membranes (0.45 µm, Thermofisher) via wet transfer (350 mA on ice for 150 min). For epigenetic markers quantification: 5ug of protein from the nuclear fraction was resolved at 120 V for 90 min in a 10 % SDS-polyacrylamide gel and then transferred onto nitrocellulose membranes via wet transfer (400 mA on ice for 120 min). Following the transfer, ponceau staining (5 % [v/v] Glacial Acetic Acid, 0.1 % [w/v] Ponceau S) was performed and then imaged. Afterwards, membranes were blocked for 1 hr at room temperature with 5 % [w/v] bovine serum albumin (BSA). Primary antibodies diluted in blocking buffer probing for reelin (1:1000, MILLIPORE, MAB5364), MeCP2 (1:10,000, M9317, Sigma-Aldrich), DNMT3a (1:1000, Novus bio, 64B1446) and the normalization proteins or loading controls, B-actin (1:5000, Sigma-Aldrich) and H4 (1:10,000 antibody produced in house). All membranes were incubated overnight in primary antibodies at 4°C with gentle agitation. Blots were washed in tris-buffered saline with 1 % [v/v] tween then incubated with horse radish peroxidase linked goat anti-mouse (ab97023, Abcam, Cambridge, UK) or goat anti-rabbit secondary (ab205718, Abcam, Cambridge, UK) antibody (1:5,000 in 5 % [w/v] BSA) for 1 h at room temperature. Lumina Crescendo substrate was used for chemiluminescent detection with SynGene imaging system and quantified with Fiji (Image J). Density values for the protein of interest were normalized by the loading control (B-actin and H4) or by ponceau staining. Then a ratio was calculated based on the male vehicle values with the following formula: [sample X (protein interest/loading control) / average male vehicle (protein interest/loading control)].

2.5. DNA purification

Fresh frozen microdissected hypothalamus, stored at −80 °C, was used as starting material. Genomic DNA was extracted using a commercially available PureLink® Genomic DNA Mini Kit (Invitrogen).

2.6. Bisulfite-Sequencing and PCR

Bisulfite conversion process which we performed in the laboratory with the EZ DNA Methylation-Gold Kit from Zymo Research following the manufacturer's instructions. First methylation approach: Modified material was amplified following a standard PCR protocol using Immolase DNA polymerase and using primers designed specifically for the modified B strand of *reln* promoter. [forward, −1258,5′TTTAGGGATAGTGGTTATGTATGATATGA3′, and reverse, −948, 5′TACCCTTTCCAACCAAACCT3′]. The resulting PCR products were purified by agarose gel electrophoresis followed by a cleaning protocol using NucleoSpin Gel and a PCR Clean-up kit from Macherey-Nagel. We used pGEM®-T Vector Systems (Promega) to insert our PCR product into a plasmid containing ampicillin resistance and transformed

the plasmid into competent bacteria *E. coli* DH5α. Using isopropyl β-D-1-thiogalactopyranoside (IPTG) and X-Gal selection we identified the positive colonies (white) and proceeded to do plasmid purification by NucleoSpin 96 Plasmid kit from Macherey-Nagel. We performed in-house sequencing using Sanger technology with the 3730xl DNA analyzer from Applied Biosystems. We tried to include enough replicates to at least have 10 different clones for each of the samples. By aligning the sequences with our reference sequence, we elaborated an alignment file and we used BSmapR Mapper V2.01 to obtain a graphical representation for the sequences.

Second methylation approach: We tested a different approach to validate without sequencing the methylation state of the region of interest (CpG island) of the *Reln* promoter. Instead of designing primers avoiding CpG bases, we created them containing at least three or four of them. For each selected region we created two different pairs of primers, with the methylated or unmethylated versions. Then we performed standard PCR amplification using immolase hot start polymerase and ran the resulting PCR on a 3 % agarose gel. First pair of primers: methylated [forward, −257, 5′TGTAATCGTTCGGAGGC3′, reverse, −194, 5′AAATTACTTTAAACCGCGAAAACG3′] and unmethylated [forward, −257, 5′TTTGTAATTGTTTGGAGGT3′, reverse, −194, 5′AAATTACTTTAAACCACAAAAACA 3′]. Second pair of primers: methylated [forward, −45, 5′GATGAGAGAGCGCGCG3′, reverse, +17, 5′CCAAAACGAAAATCCGCGACG3′] and unmethylated [forward, −45, 5′GGATGAGAGAGTGTGTG3′, reverse, +17, 5′ CCAAAACAAA AATCCACAACA3′].

2.7. Statistical analysis

Statistical analysis was carried out using Statistical Package for the Social Sciences (SPSS) version 20 (IBM, USA). Data were tested for assumptions of normality and homogeneity of variance. Extreme outliers (values greater than 3xIQR's) were omitted before carrying out appropriate statistical analyses and "out" values or outliers (values greater than 1.5xIQR's) were only omitted when they affected normality or homogeneity of the sample. Group differences were considered statistically significant at $p < 0.05$ and data was expressed as mean ± 95 %CI. Two-way ANOVA with Tukey *Post-hoc* was used with effect sizes reported as partial eta-squared (η^2_p) [small (<0.01), medium (0.01–0.06), and large (>0.14)].

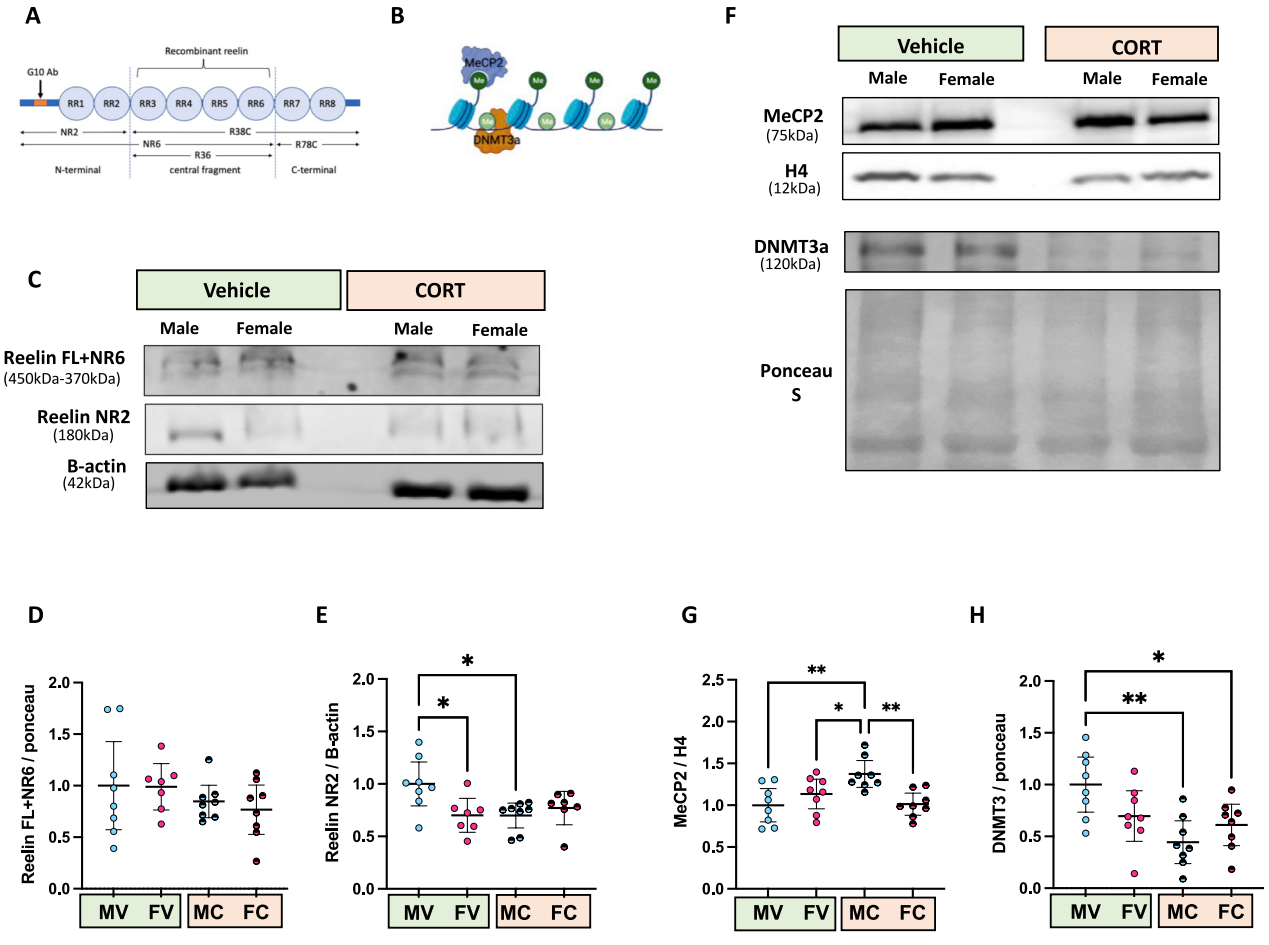

Fig. 1. Sexually dimorphic effects of CORT on reelin, MeCP2 and DNMT3a protein levels in the hypothalamus. A) Structure and fragments of the reelin protein. **B)** Simplified representation of MeCP2 and DNMT3a interaction with chromatin. **C)** WB results on reelin protein levels; **D)** full length (FL) and N-terminal repeat 6 fragment (NR6) and **E)** N-terminal repeat 2 (NR2) fragment. Separated on a 10 % acrylamide gel and normalized by B-actin. **F)** WB results on **G)** MeCP2 and **H)** DNMT3a protein levels. Separated on a 10 % acrylamide gel and normalized by H4 or total protein with Ponceau. Each data point represents the average density of 2 technical replicates expressed as the ratio over the average density in male vehicles. MV = Male Vehicle, FV = Female Vehicle, MC = Male CORT, FC = Female CORT. Error bars show 95 %CI. Statistical significance *$p < 0.05$, **$p < 0.01$.

3. Results

3.1. CORT has sexually dimorphic effects on reelin fragments, MeCP2 and DNMT3a protein levels in the hypothalamus

Hypothalamus tissue from either vehicle or CORT-treated rats was used to quantify changes in reelin, MeCP2 and DNMT3a protein levels. Western blot semiquantitative analyses showed that sex [$F(1,29) = 2.719$, $p = 0.0.111$, $\eta_p^2 = 0.095$] and CORT [$F(1,29) = 2.752$, $p = 0.109$, $\eta_p^2 = 0.096$] alone did not cause significant effects on the N-terminal repeat 2 (NR2) reelin fragment levels. Yet the combination of sex and CORT treatment caused a significant effect on the levels of NR2-reelin [$F(1,29) = 7.073$, $p = 0.013$, $\eta_p^2 = 0.214$] **(**Fig. 1**, C-E)** and male vehicle rats had significantly more NR2 reelin than female vehicles ($p = 0.025$) and male CORT rats ($p = 0.019$). Full-length reelin and NR6 fragments were quantified together and we observed that neither sex [$F(1, 30) = 0.145$, $p = 0.706$, $\eta_p^2 = 0.005$], CORT treatment [$F(1,30) = 2.444$, $p = 0.130$, $\eta_p^2 = 0.083$] or an interaction of the two [$F(1,30) = 0.081$, $p = 0.778$, $\eta_p^2 = 0.003$] significantly affected these reelin fragments.

Sex alone did not cause significant effects on MeCP2 levels [$F(1, 31) = 2.509$, $p = 0.124$, $\eta_p^2 = 0.082$], neither CORT treatment [$F(1,31) = 0.121$, $p = 0.088$, $\eta_p^2 = 0.400$]. However, the combination of sex and CORT treatment caused significant changes in MeCP2 levels with a large effect size [$F(1,31) = 12.104$, $p = 0.002$, $\eta_p^2 = 0.919$] **(**Fig. 1**, F-G)**. Post-hoc analyses showed that male CORT rats had significantly more MeCP2 than male vehicles ($p = 0.0011$), female vehicles ($p = 0.0418$) and female CORT rats ($p = 0.0015$). CORT treatment also had an effect on DNMT3a levels [$F(1,31) = 10.765$, $p = 0.003$, $\eta_p^2 = 0.278$] **(**Fig. 1**, H)** with male CORT rats having significantly lower DNMT3a than male vehicles ($p = 0.0083$) and there was an interaction effect with sex too [$F(1,31) = 5.789$, $p = 0.023$, $\eta_p^2 = 0.171$] so that female CORT rats also had lower DNMT3a than male vehicle ($p = 0.041$).

3.2. Alterations in reelin protein levels were not paralleled by changes in Reln promoter methylation

With two different experimental approaches, we investigated the methylation status of the *Reln* promoter in 4 male samples (2 vehicles and 2 CORT) and 2 female samples (1 vehicle and 1 CORT).

3.2.1. Bisulfite sequencing revealed that 15 different CpG sites were unmethylated in all samples

With the first method, we obtained an amplicon between −1258 to −948 bp before the transcription start site (TSS), and sequencing results showed that all samples presented 15 different CpG sites and less than 8 % were methylated (considered unmethylated) with no significant differences between sexes or treatment conditions **(**Fig. 2**)**.

3.2.2. Methylation-sensitive primers revealed that the two CpG islands close to the TSS were unmethylated in all samples

To validate that the previous results were representative of the two CpG islands closer to the TSS we designed two new sets of primers assuming methylation was present or not **(**Fig. 3**A)** with the principle that depending on the status of the sequence, only one pair of primers would amplify the region. As observed in Fig. 3**B**, only the unmethylated pairs of primers were successfully amplified and detected in an agarose gel, with no significant differences between groups.

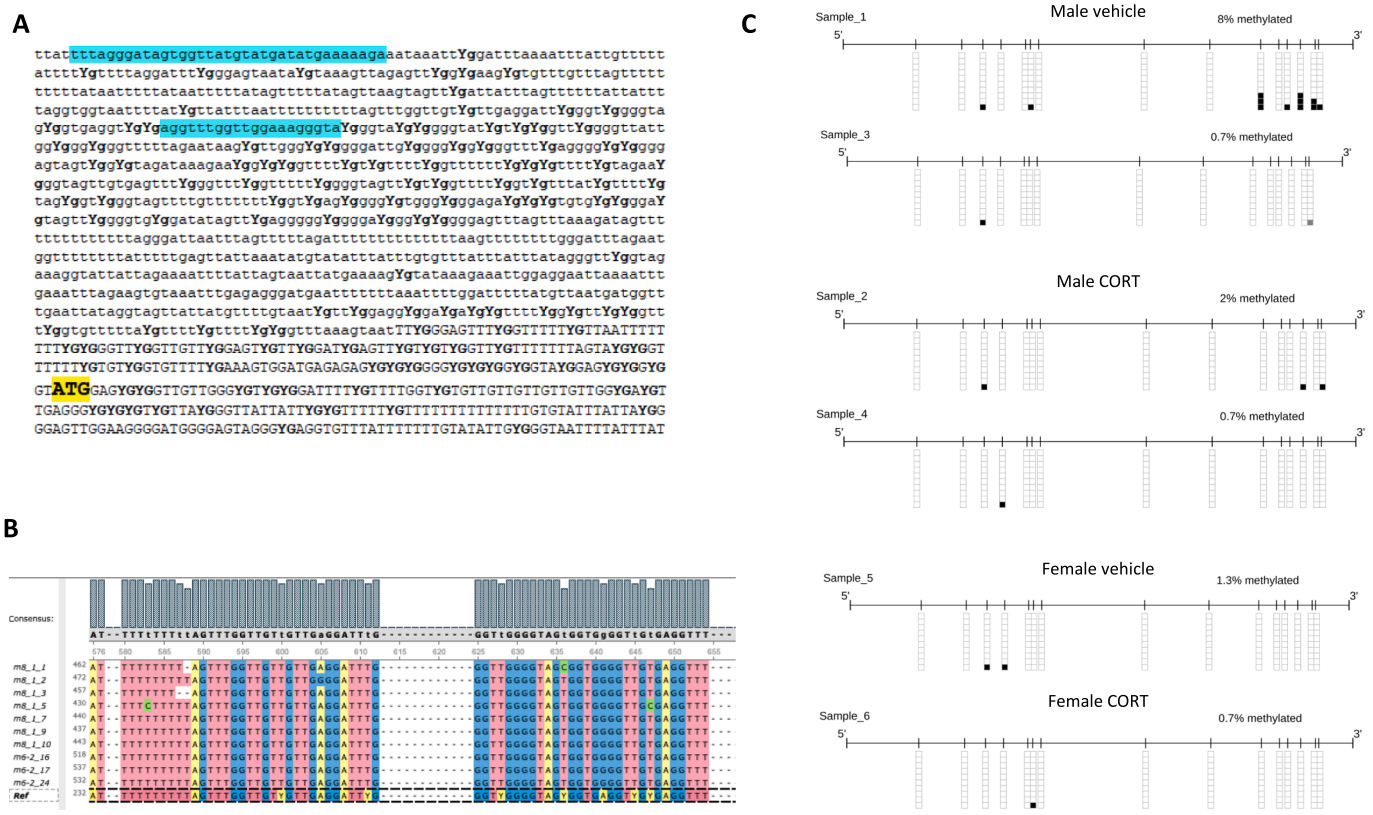

Fig. 2. Bisulfite sequencing shows no significant differences in *Reln* methylation in the amplified sequence. A) Highlighted in blue are the sequences used to design the forward and reverse primers, and in yellow the transcription starting site (TSS). **B)** Example of the alignment performed to determine the methylated CpG sites using Unipro Ugene software. **C)** Representation of the methylation results obtained from each analysed sample (1–3 = Male vehicle, 2–4 = Male CORT, 5 = Female vehicle, 6 = Female CORT). Black squares indicate a methylated CpG. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

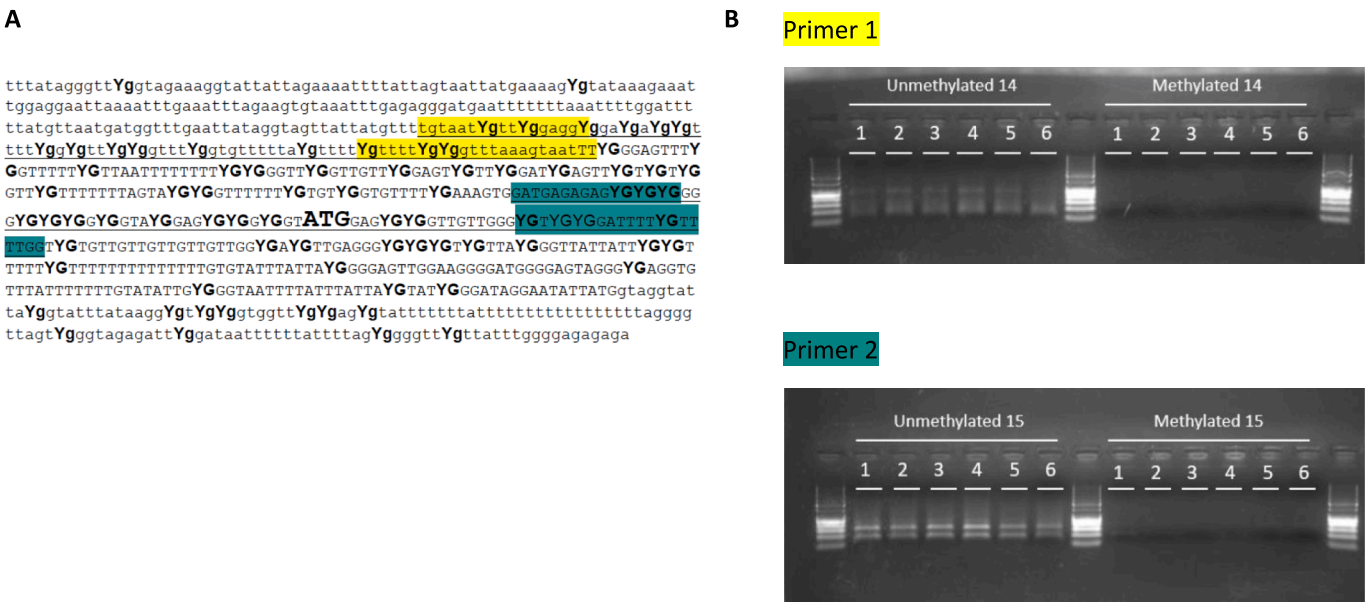

Fig. 3. CpG islands closer to the TSS of the *Reln* promoter are unmethylated. A) Bisulfite converted portion of the *Reln* promoter showing the two sequences used to design the promoters for the second methylation approach (primer 1 in yellow and primer 2 in green). **B)** Agarose gel with the amplified PCR products with methylated and unmethylated primers, showing that only unmethylated *Reln* promoter was detected in all samples (1–3 = Male vehicle, 2–4 = Male CORT, 5 = Female vehicle, 6 = Female CORT). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

The main findings of this report indicate that CORT has sexually dimorphic effects on distinct epigenetic regulators in the hypothalamus that align with alterations in reelin protein fragments. We observed inherent sex-related variations in basal reelin NR2 protein levels within the rat hypothalamus, and prolonged corticosterone exposure induced sex-specific changes in reelin NR2, MeCP2, and DNMT3a protein levels. Importantly, these modifications were not linked to alterations in the methylation status of the *Reln* promoter, which remained unmethylated across all groups, irrespective of sex or treatment. While various studies have demonstrated the epigenetic regulation of reelin expression through different mechanisms *in vitro*, there is limited understanding regarding stress-induced epigenetic modulation of reelin *in vivo* in adulthood. Given the significance of sex-specific susceptibilities to stress-related pathologies such as depression, it is crucial to investigate the mechanisms that underlie these sexual dimorphisms. Our work is the first to study this *in vivo* using the chronic CORT model of depression.

In our previous studies using this same animal model, we identified sex-specific reductions in the density of reelin-positive cells in the hypothalamus [9] paralleling the observed reductions in NR2 reelin fragments in this study. Expanding on these, our observations revealed that male rats treated with CORT exhibit lower DNMT3a and higher MeCP2 levels in the hypothalamus compared to vehicle-treated rats, whereas no effects of CORT were noted in females. This suggests that CORT acts on these epigenetic regulators in a sex-specific manner, potentially influenced by the actions of estrogen and progesterone since these hormones impede glucocorticoid receptor (GR) actions within the cell. Moreover, variations in GR expression in this region between sexes exist and might underlie differences in CORT susceptibility [28,29].

Furthermore, these epigenetic molecules play a role in regulating gene expression in adult neurons and are essential for synaptic plasticity [18]. Phosphorylated MeCP2 facilitates BDNF transcription during activity-dependent synaptic plasticity in the adult brain, a process susceptible to impairment by stress, as observed in previous animal models [30]. In our investigation, a reduction in reelin correlated with an elevation in MeCP2 levels in CORT-treated male rats, suggesting a MeCP2-mediated repression of reelin expression in a sex-specific manner. Although prior studies demonstrated MeCP2 transcriptional repression of the human RELN promoter with hypermethylation [16,31], we did not observe an increase in the methylation status of the *Reln* promoter with the CORT paradigm. While the complete roles of MeCP2 in the adult brain are not fully understood, some observations suggest its involvement in alternative splicing, specifically intron retention, associated with gene expression downregulation [32]. This hints that MeCP2 might induce reelin alterations through this less-explored mechanism. Regardless of the underlying mechanisms, our study suggests a potential association between MeCP2, DNMT3a, and reelin, warranting further exploration in future research due to their crucial roles in synaptic plasticity.

Modifications in reelin protein levels induced by stress might also stem from downstream epigenetic events or protein processing mechanisms. Notably, we observed no significant alterations in full-length reelin; however, changes were evident in NR2 (N-terminal fragment), with male vehicle rats displaying significantly higher protein levels compared to vehicle females and CORT-treated rats. It has previously been proposed that N-t cleavage allows the release of the active fragment from the inactive or reservoir full-length reelin [33,34]. Additionally, cleavage at the N-terminal (N-t) site is believed to enhance the diffusion of central and NR6 (N-terminal with central) reelin fragments throughout the brain, facilitating more efficient reelin signaling [35], especially since the N-t fragment also mediates the non-canonical actions of reelin [36]. This discovery holds significance as distinct reelin fragments have been linked to diverse biological functions, and variations in proteolytic cleavage of reelin have been previously identified in the cerebrospinal fluid of individuals with neurodegenerative diseases [37]. Subsequent studies should investigate whether the CORT-induced changes in reelin fragments in the hypothalamus result from an upsurge in the proteolytic activity of specific metalloproteinases that cleave reelin [38], or if they are due to modifications in reelin diffusion and secretion originating from other distal regions.

This study focused on the effects of stress and sex on *Reln* promoter methylation in the hypothalamus. However, a comparative analysis with other brain regions, such as the hippocampus, where CORT also has a robust effect on reelin-positive cells would be interesting to ascertain whether these mechanisms are region-specific. Moreover, it is essential

to acknowledge the limitations of this study before making definitive conclusions. On the one hand, our analysis focused on the entire hypothalamus rather than specific nuclei. Given the broad diversity of populations within this brain region, the observed alterations in epigenetic markers may be linked to their functions on other cell types. Therefore, caution is warranted in interpreting our findings, and future investigations should replicate this study by looking at more specific nuclei like the paraventricular nucleus, to provide a more nuanced understanding of the observed changes. On the other hand, while this study suggests that the methylation status of the *Reln* promoter in the hypothalamus is not changed with chronic CORT treatment, this should be replicated with a larger sample size. Nevertheless, to our knowledge, we are the first group to study in detail the methylation status of the *Reln* promoter in adult rats in the context of chronic stress, which despite being similar to mice in terms of gene sequence, its transcriptional regulation might be different.

5. Conclusion

This report shows preliminary evidence of sexually dimorphic effects of CORT on various epigenetic regulators in the hypothalamus that parallel with changes in reelin protein fragments but not *Reln* promoter methylation. This provides further insights into the different mechanisms underlying the sex-specific effects of chronic stress on reelin levels in the hypothalamus, a key region of the brain for stress and mood regulation. Future studies should continue to investigate these mechanisms as they could be used to develop novel pharmacological targets to prevent and treat stress-related pathologies and reelin-related disturbances. Moreover, these findings underscore the distinct utilization of epigenetic mechanisms by males and females to regulate brain function in response to environmental stimuli, suggesting that sex differences in epigenetic regulation and function may contribute to the differential vulnerabilities to stress-related disorders like depression.

CRediT authorship contribution statement

Carla Liria Sánchez-Lafuente: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Laura Martinez-Verbo:** Validation, Methodology, Formal analysis, Data curation. **Jenessa N. Johnston:** Investigation, Methodology. **Jennifer Floyd:** Investigation, Methodology. **Manel Esteller:** Supervision, Writing – review & editing. **Lisa E. Kalynchuk:** Funding acquisition, Supervision. **Juan Ausió:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Hector J. Caruncho:** Writing – review & editing, Supervision, Conceptualization, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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