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An allostatic epigenetic memory on chromatin footprints after double-hit acute stress

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A B S T R A C T

Stress induces allostatic responses, whose limits depend on genetic background and the nature of the challenges. Allostatic load reflects the cumulation of these reponses over the course of life. Acute stress is usually associated with adaptive responses, although, depending on the intensity of the stress and individual differences , some may experience maladaptive coping that persists through life and may influence subsequent responses to stressful events, as is the case of post-traumatic stress disorder. We investigated the behavioral traits and epigenetic signatures in a double-hit mouse model of acute stress in which heterotypic stressors (acute swim stress and acute restraint stress) were applied within a 7-day interval period. The ventral hippocampus was isolated to study the footprints of chromatin accessibility driven by exposure to double-hit stress. Using ATAC sequencing to determine regions of open chromatin, we showed that depending on the number of acute stressors, several gene sets related to development, immune function, cell starvation, translation, the cytoskeleton, and DNA modification were reprogrammed in both males and females. Chromatin accessibility for transcription factor binding sites showed that stress altered the accessibility for androgen, glucocorticoid, and mineralocorticoid receptor binding sites (AREs/GREs) at the genome-wide level, with double-hit stressed mice displaying a profile unique from either single hit of acute stress. The investigation of AREs/GREs adjacent to gene coding regions revealed several stress-related genes, including *Fkbp5, Zbtb16,* and *Ddc*, whose chromatin accessibility was affected by prior exposure to stress. These data demonstrate that acute stress is not truly acute because it induces allostatic signatures that persist in the epigenome and may manifest when a second challenge hits later in life.

1. Introduction

"We cannot roll back the clock". This is the key tenet of the allostatic load philosophy that Bruce McEwen detailed at the end of last century (McEwen, 1998, 2000). The body responds to an acute challenge with allostatic mechanisms, activating coping strategies that promote adaptation and survival. The chronic overuse of these responses leads to allostatic load (McEwen, 2004), a point where one cannot simply "roll back the clock". The key principle that supports this metaphoric clock is the demonstration that life experiences have long-term effects that cumulate over the lifespan and shape the biological limits of stress coping (Gray et al., 2017; Lupien et al., 2009). After the discovery of steroid receptors in the brain (McEwen et al., 1968), it was clear that the brain retained the experience of stress, particularly in the limbic system. This discovery laid the groundwork to eventually understand the genomic and nongenomic effects of steroid receptors (McEwen and Plapinger, 1970; Revollo and Cidlowski, 2009), including the glucocorticoid receptors GR and MR that differ for their affinity to circulating glucocorticoids (Reul and de Kloet, 1985). More recently, evidence showed persistent molecular signatures of stress that have long-lasting genomic effects in discrete brain regions, particularly in the hippocampus (Hunter et al., 2015; Marrocco et al., 2019). Indeed, stress in early life induces vulnerability to adverse challenges later in life (Choy et al., 2008; Walker et al., 2009). The three-hit concept of vulnerability and resilience to stress elegantly summarizes the evidence that the time at which stress occurs during the lifetime combined with genetic predisposition influences the exposure to another challenge at a later timepoint (Daskalakis et al., 2013; de Kloet et al., 2007). Environmental stimuli trigger epigenetic mechanisms, such as DNA methylation, histone modification, and non-coding RNA, that regulate gene expression and function (Meaney, 2010). While one characteristic of epigenetic signatures is their reversibility, some epigenetic marks are enduring, i.e. they can happen in utero, persist or occur in adulthood (Anacker et al., 2014; Bartlett et al., 2017; Hunter et al., 2012; Hunter and McEwen, 2013; Morrison et al., 2020; Weaver et al., 2004), and even be inherited through generations (Bale, 2015). A number of studies have characterized epigenomic signatures in the brain in several preclinical models of chronic (Gray et al., 2018; Hunter et al., 2009; Mifsud and Reul, 2016; Reul, 2014) and acute stress (Mifsud and Reul, 2016; Mifsud et al., 2021). Mifsud and Reul (2018) recently advocated for further next

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generation sequencing analysis to investigate the interaction of steroid receptors, such as MRs and GRs, in the stressed genome. In this study, the hypothesis was that one stressful experience has the potential to permanently imprint molecular marks in the genome that influence a later response to stress. We investigated, in male and female mice, whether one episode of acute stress, in the form of restraint stress, epigenetically persisted over a one-week period to regulate a second exposure to acute stress, namely forced swim stress. Assay for Transposase-Accessible Chromatin sequencing (ATAC-seq), a method that uncovers the differences in open chromatin regions across the whole-genome, was used to characterize epigenetic signatures after the double-hit stress and investigate whether these epigenetic marks persisted in the absence of an applied stressor. One week after stress, we found discrete epigenetic profiles in both males and females in the absence of conclusive behavioral differences within a similar interval after stress. This epigenetic reorganization affected the genomic binding sites for the androgen receptor (AR), the glucocorticoid receptor (GR), and the mineralocorticoid receptor (MR), showing increased chromatin accessibility after one event of acute stress, albeit with a distinct degree in males and females after the second stressor occurred. These findings show that epigenetic mechanisms underlie the long-term effects of acute stress and ultimately intersect with novel stressors later in life, indicating that epigenomic investigations are a leading route to demonstrate allostatic responses to stress and their metaphoric connection to a clock that keeps moving forward.

2. Methods

2.1. Animals

C57/BL6 male and female mice were obtained by performing in-house breeding. To control for litter-specific effects, mice were selected from across multiple litters. At 2.5 months of age, mice (n = 8–9 per group) were randomly assigned to either acute restraint stress (ARS), forced swim stress (FSS), double-hit stress, namely mice undergoing FSS that were previously exposed to ARS, or control groups (Fig. 1). Animals were group housed (n = 4–5) in standard cages (28.5x17 × 13cm), which were changed weekly, and were kept on a 12-h light-dark cycle (lights off 19:00 h) in a temperature-controlled room maintained at 21 ± 2 °C. Food and water were available *ad libitum*. All procedures were performed in accordance with the National Guidelines on the Care and Use of Animals and a protocol approved by The Rockefeller University Animal Care and Use Committee.

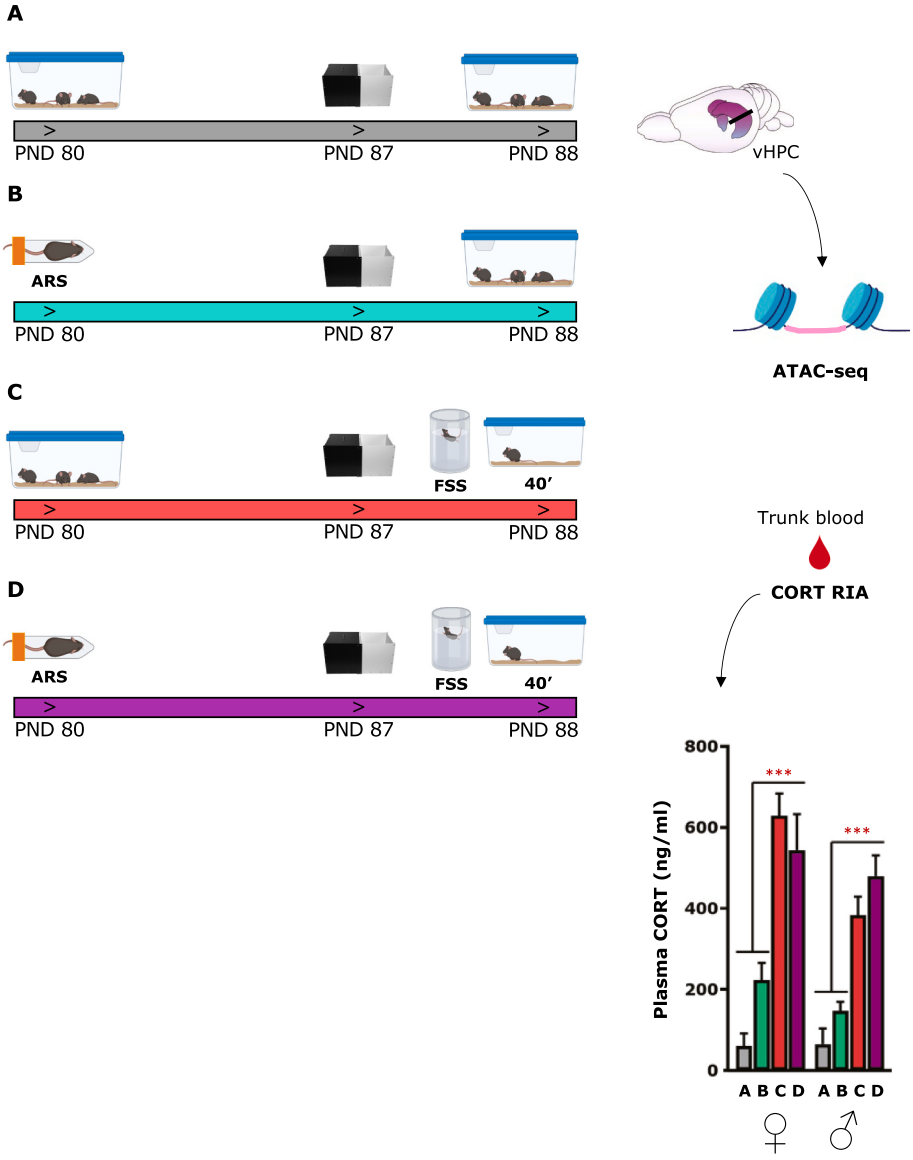

Fig. 1. Experimental timeline and plasma CORT measurements. Males (n = 8) and females (n = 9) were randomly assigned to A: CONT, B: ARS, C: FSS, and D: 2-h. At sacrifice (11:30–15:30 h), trunk blood was collected in control mice or 40-min after exposure to FSS (FSS, ARS, or 2-h mice) and the levels of plasmatic CORT were measured using RIA. Males and females exposed to FSS 40 min prior showed increased levels of CORT compared to controls, regardless of prior exposure to ARS (2-way ANOVA, stress: $F(1,20) = 96.13$, $p < 0.0001$). Bar chart shows the mean ± S.E.M. of 3 determinations per group. ***p < 0.001. CONT: control, ARS: acute restraint stress, FSS, forced swim stress, 2-h: double hit stress, vHPC: ventral hippocampus, CORT RIA: corticosterone radioimmunoassay, ♀: female, ♂: male.

2.2. Acute stress

2.2.1. Acute restraint stress

A subgroup of mice underwent acute restraint stress (ARS) 8 days prior to sacrifice between the hours of 11:00 and 15:00 (Fig. 1A). Mice were placed in a 50 mL Falcon tube restraint device that allowed mice to stretch their legs but not to move within the tube. Air holes (0.4 cm) on the restrainer allowed the mouse to breathe. Mice were kept in the restrainer for 2 h, before returning to the home cage. Unstressed mice (controls) were left undisturbed in their home cage for the same time period (Fig. 1).

2.2.2. Forced swim stress

A subgroup of control and ARS mice was exposed to forced swim stress (FSS) 40 min before sacrifice, that is 8 days after the ARS procedure between the hours of 11:00 and 15:00 (Fig. 1). Mice were subjected to a 6-min swimming task in a vertical glass cylinder (h = 25 cm; d = 12 cm) with 12 cm-deep water (23–25 °C). Mice were then moved to a novel empty standard cage for a 40-minuite recovery period, prior sacrifice. The remaining cohort of ARS and control mice was left undisturbed in their home cage (Fig. 1).

2.3. Light-dark box test

The light-dark box test was performed as previously described (Gogas et al., 2007; Hascoët and Bourin, 2009), with minor modifications. The test was performed one day prior to sacrifice between the hours of 11:00 and 15:00. The arena consisted of an open white-wall light compartment and a covered black-wall dark compartment (l = 29 cm, w = 29 cm). The compartments were connected via a small opening that enabled transition between the two boxes. Male and female mice exposed to ARS one week prior or their matched controls (n = 9–10 per group) were placed in the light compartment (50 ± 10 lux) and videotaped for 5 min using a camera fixed on the ceiling above the arena. The time spent in the light box, the latency to enter the dark box, the latency to reenter the light box, and the number of transitions between compartments were scored by an observer blind to the experimental conditions (Fig. 1).

2.4. CORT radioimmunoassay

Corticosterone levels were measured from plasma collected on the day of sacrifice (n = 3 mice/group) using the Corticosterone Double Antibody RIA kit (MP Biomedicals Inc., Santa Ana, CA, USA). Mice were rapidly decapitated and trunk blood was immediately collected in K3 EDTA (K3E) 12 mg Blood Collection Tubes (BD Vacutainer, Franklin Lakes, NJ, USA). Samples were then centrifuged at 1000 g for 10 min to collect plasma, which was rapidly frozen at −80 °C. Five microliter of plasma diluted 1:200 (100 μl total) in phosphosaline gelatin buffer (pH 7.0 ± 0.1) and 100 μL of standard calibrators were incubated for 2 h with radioactive corticosterone ^{125}I (7 μCi per vial) and then centrifuged at 1000 g for 15 min. Radioactivity was measured on the precipitant using a Hidex Automatic Gamma Counter (Turku, Finland). Corticosterone concentration was calculated using the count per minute (CPM) as a function of the logarithmic equation generated from the calibrators.

2.5. ATAC- sequencing

Mice were decapitated as described above, the brains were rapidly removed and placed on a stainless-steel brain matrix for mouse (coronal repeatable sections, 1 mm spacing), and the ventral hippocampus was dissected as described by Robertson et al. (2005). Briefly, the hippocampus was placed on a flat cool surface and divided equally into the dorsal and ventral parts before being put in dry ice and transferred to −80 °C. Ventral hippocampal nuclei were isolated (50k/sample) from frozen tissue using the omni-ATAC protocol (Corces et al., 2017) and chromatin was tagmented with Tn5 transposase (Ilumina, San Diego, CA, USA). Tagmented DNA fragments were isolated to generate paired-end libraries constructed for next generation sequencing. Libraries were amplified and quantified using the KAPA Library Quantification Kit (Roche, Pleasenton, CA, USA). Three biological replicates per group (n = 3) were processed for quality control for fragment size (Tapestation) and DNA determination and then sequenced using Illumina NovaSeq 6000. Paired-end sequencing was performed to a depth of 100X coverage of the genome to ensure adequate resolution. Reads were aligned with the mm10 genome from the BSgenome.Mmusculus.UCSC.mm10 Bioconductor package (version 1.4.0), with Rsubread's alignment method in paired-end mode, with fragments of 1–5000 base pairs in length considered correctly paired (Liao et al., 2019). Duplicated pairs were removed using Picard tools (version 2.25.4). Normalized, fragment signal bigWigs were created with the rtracklayer package. Peak calls were made with MACS2 software in BAMPE mode (Feng et al., 2012). Differentially accessible motifs were discovered using ChromVAR package (version 1.14.0) (Schep et al., 2017) with the motif database JASPAR 2020 (Fornes et al., 2019). Each motif's z-score was calculated from the deviation metric, a value generated based on the accessibility of the set of peaks for each motif relative to the expectation based on equal chromatin accessibility profiles across samples, normalized by a set of background peak sets matched for guanine-cytosine content and average accessibility. This was then simplified into a single score, by calculating the standard deviation of the z-scores. Motif variability was visualized with Pheatmap R package (version 1.0.12) and motifs were aggregated to form representative motifs using motifStack (version 1.36.0). Differential ATAC-seq signals were identified using the DESeq2 package by making pairwise comparisons between groups of interest (Love et al., 2016). Significance of differentially accessible genes was set at padj <0.05, with Benjamini-Hochberg used to correct for multiple testing. Peaks were assigned to genes using ChIPseeker (version 1.26.2), and the annotation TxDb.Mmusculus.UCSC.mm10.knownGene (version 3.4.0). GO term enrichment of peaks was determined with clusterProfiler (version 1.26.2) using gene set enrichment analysis (GSEA) (Yu et al., 2012). The Transcription Start Site (TSS) meta plots were produced using the profileplyr package (Carroll and Barrows, 2021). ATAC-seq date have been deposited to GEO (GSE200670) and are fully available.

2.6. Statistics

Behavioral measurements and plasmatic CORT levels were analyzed using GraphPad Prism (GraphPad Software, Inc., USA) by performing a two-way ANOVA (sex-by-stress interaction) or applying student t-test, the latter when males and females were analyzed separately. A p-value<0.05 was set for statistical significance. Statistics used to compute the ATAC-seq data are described in the previous paragraph.

3. Results

3.1. Assessment of behavior and stress response one week after ARS

At 2.5 months of age, mice were exposed to ARS, then returned to their home cage and left undisturbed for one week. At day 7 after stress, ARS mice and their unstressed matched controls were assessed for anxiety-like behavior using the light-dark box test. When sex and stress were included as covariant, no behavioral differences were observed (Supp. Fig. 1). However, when males and females were analyzed separately, ARS males, but not females, spent more time in the light box than control males ($t = 3.40$; $df = 18$; $p < 0.05$) (Supp. Fig. 1A). No differences were observed for other variables of the same test in either males or females. The day after behavioral assessment, prior to sacrifice, a subset of mice including male and female controls, ARS males, and ARS females, was stressed using the forced-swim stress paradigm (FSS), while another subset of mice was left undisturbed during the stress procedure.

Levels of plasmatic corticosterone measured after FSS were significantly increased in both FSS mice and mice exposed to double-hit stress, namely the subset of mice undergoing FSS that were previously exposed to ARS, compared to ARS or control (never stressed) mice (Fig. 1).

3.2. Epigenetic signatures in the vHPC of males and females after double-hit stress

We sought to investigate whether males and females showed a *memory* of ARS that, although scarcely observed in the light-dark box test, was persistent in the epigenome with the potential to affect a second exposure to stress. We then studied the epigenetic response to stress in the whole genome of the vHPC by dissecting differences in open chromatin regions. ATAC-seq, a sequencing method using the Tn5 transposase to cut transcriptionally active regions of the DNA, was used to determine chromatin accessibility across the genome. Three biological replicates of control mice (never stressed), FSS mice, ARS mice, and double-hit mice were processed through ATAC-seq to obtain paired-end reads. This meets the requirement for detection of accessible regions and downstream transcription factors (Buenrostro et al., 2015). The chromatin regions of *Gapdh* and *Actb*, selected as control genes, showed normalized, consistent signal across replicates and groups (Supp. Fig. 2A). Differentially accessible chromatin regions were identified using deseq2 for pairwise comparison of multiple groups. The number of differentially accessible transcripts between controls and stress groups were quantified ($p < 0.05$, $FC > 1.3$) (Supp. Data 1). Overall, transcript accessibility showed a greater number of differentially opened transcripts in males (7,317) than females (3,254). In females, double-hit stress (1,824) had a much greater effect on transcript accessibility than either ARS or FSS alone; yet FSS females showed a greater number of differentially opened transcripts (849) compared to ARS females (581). In males, we found comparable levels of transcript changes after FSS (2,727), double-hit stress (2,520), or ARS (2,070). Deseq2 for synergistic interaction across sex and stress showed no differences in chromatin accessibility. Functional enrichment of change-ranked genes was performed using GSEA (Subramanian et al., 2005) by selecting

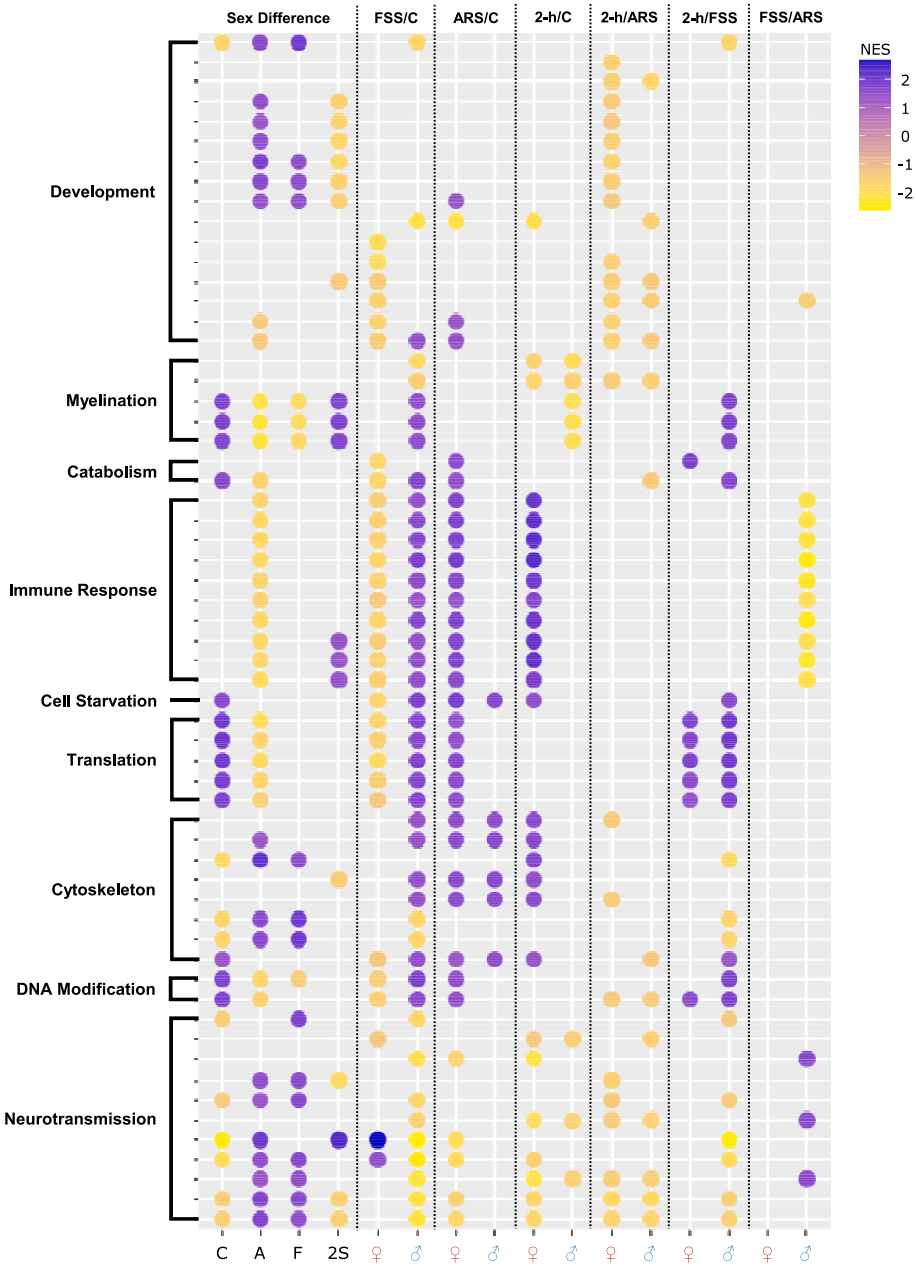

Fig. 2. Gene set enrichment analysis of ATAC-seq for multiple comparisons. GSEA shows discrete biological pathways (thick marks) enriched when comparing males and females, each stressor to controls, or stressors between them. Aggregated biological pathways were assigned a common name that reflected similar gene functions across multiple GO terms (brackets). (See Supp. Data 5 for full ordered list of pathways). Enrichment results were based on ATAC peaks annotated to genes. NES score was calculated based on the log2FC(female/male) for the sex comparisons, as well as log2FC(FSS/C), log2FC(ARS/C), log2FC(2-h/C), log2FC(2-h/ARS), log2FC(2-h/FSS), log2FC(FSS/ARS) for the stress comparisons. All presented enrichment reached a significance of padj<0.05. FSS/C: forced swim stress versus control, ARS/C: acute restraint stress versus control, 2-h/C: double-hit stress versus control, 2-h/ARS: double-hit stress versus acute restraint stress, 2-h/FSS: double-hit stress versus forced swim stress, FSS/ARS: forced swim stress versus acute restraint stress, NES: normalized enrichment score, p.adj: adjusted p-value, ♀: female, ♂: male.

regions of chromatin whose value of differential accessibility met statistical significance (padj<0.05). To summarize the overall magnitude of chromatin accessibility changes, the total GO terms enriched for each comparison were aggregated to a single value based on similarity in biological functions (Fig. 2). The greatest number of pathways whose chromatin was affected by stress was observed in FSS females (801), with fewer pathways affected by ARS (421), and even fewer networks affected in double-hit females (25). Males showed comparable levels of pathways affected by FSS (191) and double-hit stress (117), with a greater number of pathways induced after ARS (439) (Supp. Table 1; Supp. Data 2). When examining the function of these differentially enriched gene pathways across sexes, males and females displayed differences in enrichment of genes related to myelination and neurotransmission regardless of stress exposure. Unstressed males and females showed differential enrichment of genes related to development, catabolism, cell starvation, translation, cytoskeleton function, and DNA modification. ARS mice also show sex differences in the regulation of genes related to development, catabolism, translation, cytoskeleton function, and DNA modification, and, in addition to controls, sex differences in immune-related genes. Sex differences in genes related to development, the cytoskeleton, and DNA modification were similar in FSS mice and ARS mice, although FSS mice did not show significant differences in genes coding for immunity and translation functions compared to ARS mice. Double-hit mice showed similar sex differences in gene enrichment compared to FSS mice, with additional sex-biased pathways including increased enrichment of immune processes and reduced enrichment of cytoskeleton and DNA modification compared to FSS, ARS, and control mice. Functional enrichment was additionally compared for each stress condition separating males and females. In females, FSS induced enrichment of genes implicated in development, catabolism, the immune response, cell starvation, translation, cytoskeleton function, DNA modification, and neurotransmission. In males FSS induced an enrichment pattern alike FSS females, with a substantial increased enrichment in myelination-, cytoskeleton-, and neurotransmission-related pathways. In females, ARS induced an enrichment profile similar to FSS, in addition to an enrichment of genes that regulate development, cytoskeleton, and neurotransmission processes. Unlike females, ARS in males only induced regulation of genes involved in cell starvation and cytoskeleton processes. Interestingly, double-hit females and ARS females, shared similar enrichment patterns including development-, immune-, cell starvation-, cytoskeleton-, and neurotransmission-related genes, although reduced enrichment of catabolism- and translation-related pathways and increased enrichment of myelination pathways was unique to double-hit females. Males undergoing double-hit only showed enrichment of myelination- and neurotransmission-related pathways. While comparing enriched pathways between double-hit and ARS mice, males and females showed similar expression patterns for processes related to development, myelination, the cytoskeleton, DNA modification, and neurotransmission, with females showing distinct enrichment of catabolism-related genes. When FSS mice were compared to double-hit mice, males and females showed similar regulation of genes involved in translation and DNA modification, with males showing distinct enrichment of development-, myelination-, catabolism-, cell starvation-, cytoskeleton-, and neurotransmission-related pathways. Finally, when comparing the effects of FSS and ARS, males showed differences in enrichment of genes related to development, immunity, and neurotransmission, with no major differences observed in females, regardless of stress (Fig. 2; Supp. Data 1).

3.3. Acute stress and reorganization of chromatin accessibility of androgen, glucocorticoid, and mineralocorticoid transcription factor binding sites in the vHPC

3.3.1. Double-hit acute stress changes chromatin accessibility for androgen, glucocorticoid, and mineralocorticoid receptor binding

We then assessed which transcription factors were associated with differential chromatin accessibility across groups, that is unstressed male and female mice (controls), ARS mice, FSS mice, or double-hit mice. The total number of motifs across the genome that bind transcription factors was investigated using ChromVAR. The motifs were then organized based on their degree of accessibility calculated using a variability score across groups (Supp. Fig. 2D). The top 50 motifs with the highest variability scores were organized into a heatmap for hierarchical clustering based on patterns of deviation (Fig. 3A; Supp. Fig. 3). The chromatin accessibility for androgen (AREs) or glucocorticoid response elements (GREs), that is motifs across the entire genome that bind the androgen receptor (AR) or glucocorticoid/mineralocorticoid receptor (GR/MR), respectively, showed the highest change in accessibility across stress conditions compared to all other transcription factor binding sites. Pairwise testing using the chromVAR approach revealed that each experimental group showed discrete levels of chromatin accessibility when comparing between stressors, and between males and females (padj<0.05). Chromatin accessibility at ARE/GRE sites was increased in both males and females after FSS. ARS also increased chromatin accessibility at ARE/GRE sites, yet to a lesser extent than FSS, in both sexes. FSS, ARS, and control females showed greater chromatin opening at AREs/GREs compared to their matched males. Curiously, ARE/GRE sites were opened after double-hit in both sexes, yet double-hit females showed lower chromatin opening than FSS females but more than ARS females, while double-hit males showed greater chromatin opening than FSS males. This demonstrated that exposure to ARS one week prior to FSS regulated the opening of ARE/GRE sites in both males and females. The top 50 selected motifs were clustered together to assess similar binding site patterns, showing that AR, MR, and GR clustered based on a similar binding motif within the genome (Fig. 3B). Variation in chromatin accessibility for ARE/GRE sites was investigated to exclude the likelihood that a single experimental group was driving the overall findings. The variability in chromatin accessibility for transcription factor binding sites was then calculated in a pairwise manner using males and females from each stress condition (ARS, FSS, or double hit) compared to controls. FSS mice and double-hit stressed mice showed the greatest changes in ARE/GRE chromatin accessibility, because these sites ranked the highest when the level of chromatin accessibility was calculated comparing FSS mice and controls, or double-hit mice and controls. Conversely, ARE/GRE sites were not found among the top 50-ranked transcription factor binding sites with changing accessibility when comparing ARS and controls (Supp. Data 3). Thus, motif clustering along with chromatin accessibility pattern on ARE/GRE sites demonstrated that ARE/GRE sites across the genome were epigenetically regulated by FSS or double-hit stress and that this effect was present in both males and females.

3.3.2. Double-hit acute stress changes accessibility for AREs/GREs within the coding genome

Based on changes in chromatin accessibility at ARE/GRE sites, we selected AREs/GREs proximity coding regions on the genome, that is regions <2 kilobases from the transcription start site (TSS) that allow to study gene expression changes associated to chromatin accessibility. TSS meta plots were generated to visualize the TSS signal, compiling the total reads or levels of open chromatin adjacent to the TSS across AREs/GREs, which visualizes the accessibility of AR, GR, and MR binding sites throughout the coding region of the genome (Supp. Fig. 4A–C). When comparing matching males and females subjected to ARS, FSS, double-hit, or controls, we found differences in TSS signal across the genome for GREs (Fig. 4B and C), while TSS signal for AREs differed between

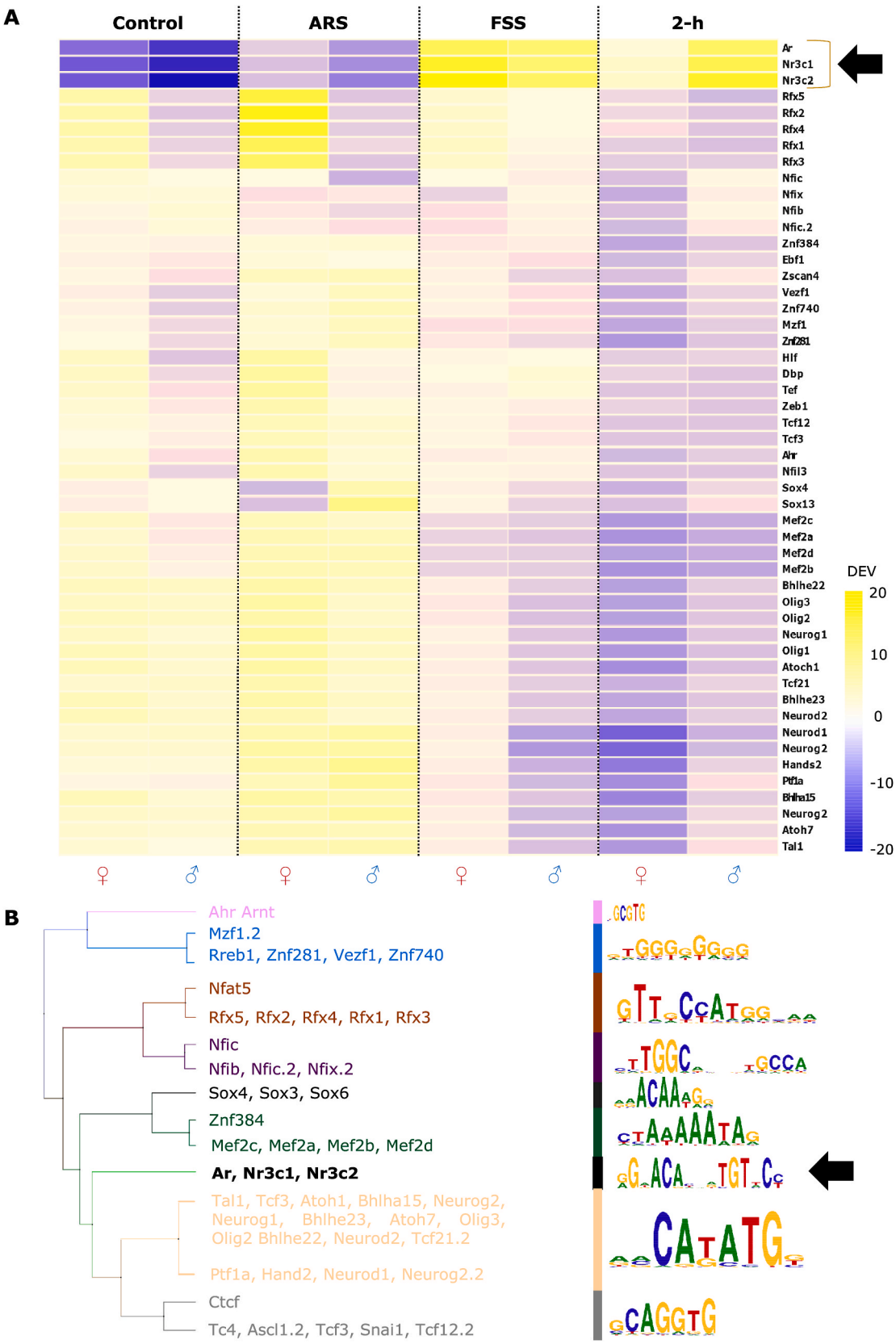

Fig. 3. Identification of the most variable motifs across stress conditions. A. The top fifty motifs with the most significant variability across stress groups are plotted in a heatmap. Differential chromatin accessibility of each motif was calculated from a deviation score representing how accessible each motif site was relative to the expectation based in equal chromatin accessibility profiles across samples, normalized by a set of background peak sets matched for GC and average accessibility. B. Clustering of motifs into transcription factor families based on similar binding motifs. Transcription factors AR, GR, and MR ranked the top three and clustered together into the same transcription factor family. ARS: acute restraint stress, FSS: forced swim stress, 2-h: double hit stress, AR: androgen receptor, GR/Nr3c1: glucocorticoid receptor, MR/Nr3c2: mineralocorticoid receptor, DEV: deviation score, ♀: female, ♂: male.

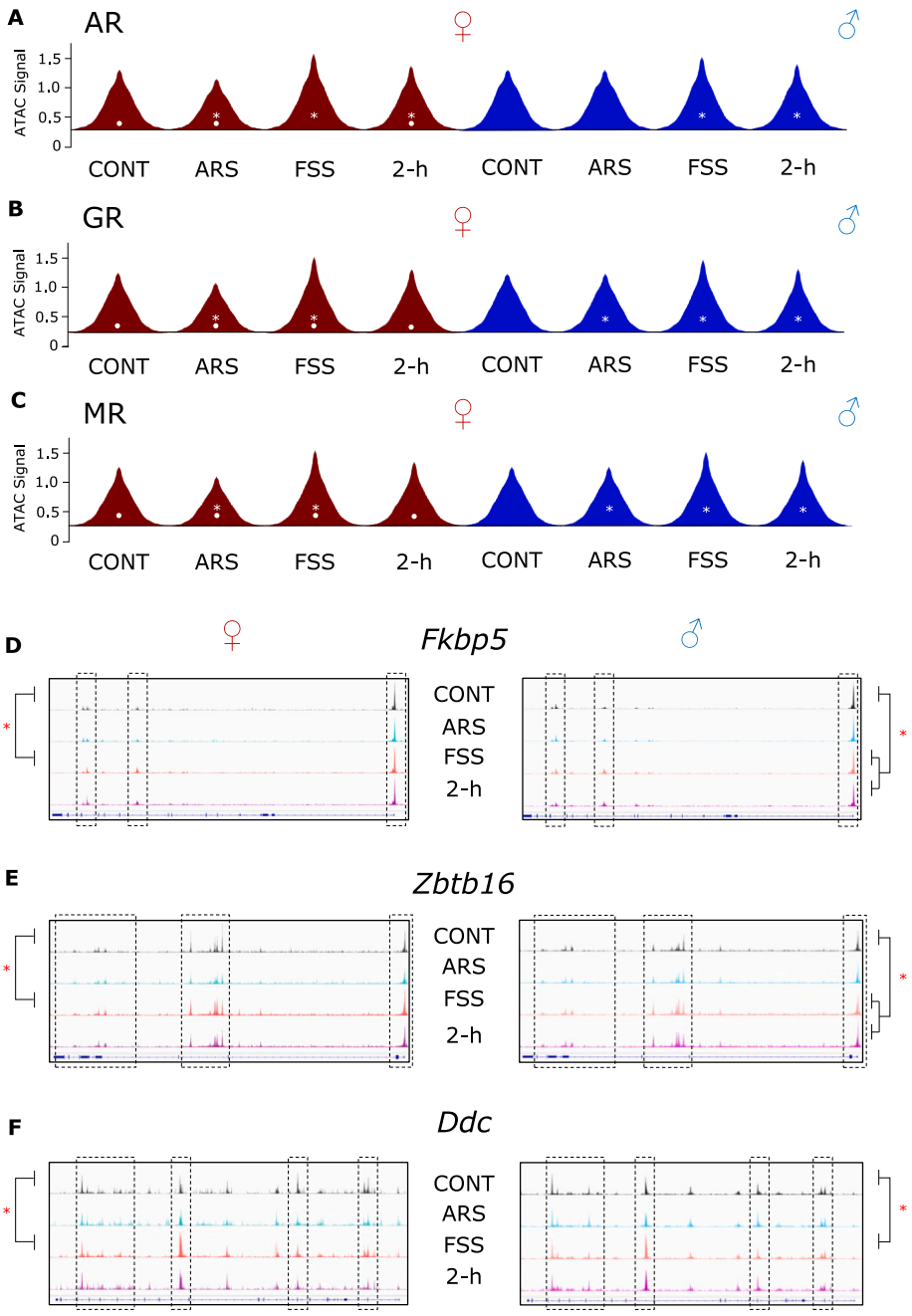

Fig. 4. Changes in open chromatin for TSS binding AR, GR, and MR. Meta plots of the mean ATAC signal at TSS sites with transcription factor motif binding sites for A. AR, B. GR, and C. MR. All plots were centered around the TSS ± 2 kb. Wilcox testing identified significant differences in TSS mean signaling across groups. ●p < 0.05 versus same stress between sex, *p < 0.05 versus control within sex. D. *Fkbp5*, E. *Zbtb16*, and F. *Ddc* show chromatin ATAC profiles of differentially accessible genes computed by paired comparison with controls in females (left) and males (right) (padj < 0.05; fold change > 1.3), where padj = adjusted p value. TSS: transcription start site, CONT: control, ARS: acute restraint stress, FSS, forced swim stress, 2-h: double-hit stress, ♀: female, ♂: male.

males and females in controls, in ARS mice, and in double-hit mice, but not in FSS mice (Fig. 4A). Namely, TSS signal associated to AREs was significantly increased after FSS or double-hit stress in males and females, decreased in ARS females, and unaffected in males (Fig. 4A). The TSS signal at GR and MR GRE sites was decreased in ARS mice and increased in FSS mice in both males and females, yet double-hit stress increased TSS signal only in males (Fig. 4B and C). Selected ARE and GRE sites (Supp. Data 4) were associated with genes with differential chromatin accessibility after stress (padj<0.05, FC > 1.3), including *Fkbp5*, *Zbtb16*, and *Ddc*, chosen among the topmost differentially accessible genes (Supp. Data 1). Altered expression of *Fkbp5*, a feedback regulator of the GR signaling (Binder, 2009), *Zbtb16*, involved in neural progenitor cell maturation (Usui et al., 2021), and *Ddc*, an essential gene for the synthesis of dopamine and serotonin (Eisenberg et al., 2016), has been described in several neuropsychiatric conditions. The ATAC signal of *Fkbp5* and *Zbtb16* was increased in FSS males, double-hit males, and FSS females (Fig. 4D and E), while *Ddc* showed increased signal only in FSS males and FSS females (Fig. 4F). Together, these data demonstrate that the time at which stress occurs, and the number of acute stress events change the accessibility of AREs/GREs across the coding genome and that this is associated with changes in chromatin accessibility of genes crucial in the stress response and in the control of mental health.

4. Discussion

Acute stress activates multiple responses that prime the body for survival and homeostasis, yet, when dysregulated, survival mediators may contribute to stress susceptibility (McEwen, 2005). The severe consequences of acute stress depend on the type, intensity, and time in which the stress occurs, and canonically follow an inverted-U shape response (Lupien and McEwen, 1997; Lupien et al., 2009). By capitalizing on the allostatic clock metaphor and sex differences often observed

in the stress response, we report for the first time that, in male and female mice, a previous exposure to acute stress induces epigenetic reorganization after a second exposure to acute stress, and that the epigenetic memory of acute stress persist one week after stress. This reorganization included transcription factor binding sites of AR, GR, and MR whose accessibility was increased after one event of acute stress and after double-hit stress in both males and females. When narrowing the investigation of these AREs and GREs adjacent to gene coding regions, we identified AR/GR/MR binding genes that were differentially regulated across different stress conditions in both males and females.

4.1. Anxiety-like behavior one week after stress

Mice were tested in the light-dark box one week after ARS to assess anxiety-like behavior. No behavioral differences were observed when considering the interaction between sex and stress. However, if males and females were analyzed separately, males displayed reduced anxiety-like behavior compared to controls whereas no behavioral differences were found in females. The literature suggests that long-term effects of acute stress are paradigm dependent. For example, Mitra and Sapolsky showed that one acute injection of corticosterone induces anxiety-like behavior 12 days later in male mice (Mitra and Sapolsky, 2008), yet, a single episode of restraint stress delays the expression of anxiety-like behavior until 10 days post-stress (Mitra et al., 2005). Acute stress can often reveal resilient or susceptible behavioral responses that differ in males and females. For example, spatial memory in the Y-maze is impaired in males and facilitated in females after acute stress (Conrad et al., 2004). Others have shown that, 10 days after acute stress, males show increased anxiety-like behavior and females do not (Gupta and Chattarji, 2021). Also, exposure to multiple acute stressors has opposite effects on classical eyeblink conditioning in males and females, a difference dependent on circulating gonadal hormones (Shors et al., 2001; Wood and Shors, 1998). Similar stressors do, indeed, elicit behavioral strategies that differ in males and females, as also extensively discussed in the recent literature (Bangasser and Cuarenta, 2021; Hodes and Epperson, 2019; Marrocco and McEwen, 2016; Rincón-Cortés et al., 2019), which could explain why males showed a phenotype that was not found in females. In the context of double-hit stress, cumulative stressful experiences either compound, leading to psychopathology (McEwen, 1998), or induce adaptive plasticity which may mismatch with what is necessary to cope with the current environment, also leading to psychopathology (Gluckman et al., 2009; Nederhof and Schmidt, 2012). Notably, epigenetic modifications broadly contribute to behavioral lability and resilience resulting from the intersection of genetic factors and early-life environment (Daskalakis et al., 2013; Schmidt, 2011). However, given that we only have one exposure to stress before behavioral assessment, we cannot exclude that other type of stressors may induce different behavioral phenotypes. In addition, it is likely that mice would show phenotypic differences when tested in other behavioral paradigms.

4.2. Distinct gene pathways induced by acute stress and double-hit stress

If the long-term effects of acute stress are weakly observable using behavioral measurements, where would the *memory* of the stress experience be located? We found that this *memory* was epigenetic. ATAC-seq was used to detail the chromatin reorganization in the vHPC after double-hit stress. The vHPC is a functionally distinct from the dorsal hippocampus (Fanselow and Dong, 2010), and also shows a discrete epigenome (Zhang et al., 2018) and distinct role in the regulation of the stress response (Maggio and Segal, 2009). Importantly, chromatin organization in the vHPC differs in males and females and fluctuates across the estrous cycle (Jaric et al., 2019). The chromatin landscape of the vHPC displayed several biological pathways that were affected by acute stress, in both males and females. Fewer stress-enriched gene pathways were identified in males compared to females, consistent with previous gene expression analysis in the hippocampus after acute stress (Marrocco et al., 2017). Differentially enriched networks after stress in males and females included pathways involved in developmental, immune, cell starvation, translation, cytoskeletal, and DNA modifier whose genes showed the greatest change in chromatin accessibility. Previously, we reported that acute stress alone also induces differential expression of DNA-binding and transcription-related pathways in males and females that was specific to CA3 pyramidal neurons (Marrocco et al., 2017). Consistent with the current findings, we recently showed that models of stress susceptibility generated using pharmacological, environmental, or genetic approaches share enrichment in pathways implicated in immune signaling, cytoskeleton function, and growth factor signaling (Caradonna et al., 2021). Notably, processes related to development, immune function, cell starvation, translation, the cytoskeleton, and DNA modification have all been reported in the molecular characterization of stress-related disorders (Bulik et al., 1997; Czarny et al., 2017; Evans et al., 2004; Miller and Raison, 2016; Schmauss, 2003; Wong et al., 2013). These biological processes also impact other circuits and cell types within limbic system. Indeed, acute stress has broad epigenomic impact beyond the vHPC and affects the epigenetic organization in other brain regions (Häusl et al., 2021; Reed et al., 2012). Further investigation is warranted to understand how double-hit acute stress alters the reorganization of chromatin across diverse neuronal and glial cell types, and across different brain regions.

4.3. Adrenal steroid receptors in the vHPC

Adrenal steroids canonically bind the genome both directly at GREs (McEwen and Plapinger, 1970) and indirectly via other transcription factors (Datson et al., 2011; Gray et al., 2018; Revollo and Cidlowski, 2009). Nuclear receptors can access their hormone-responsive elements in promoter regions, unlocking nucleosomal structures and rendering them accessible (Hebbar and Archer, 2003; Kinyamu and Archer, 2004), serving as focal points to orchestrate broad biological functions, such as energy metabolism (Scholtes and Giguère, 2022). The hippocampus is a brain region that is particularly sensitive to stress-induced genomic changes (McEwen, 1999), and necessitates a fine regulation of adrenal steroid receptors whose imbalance may associated with increased risk for stress-related disorders (De Kloet et al., 1998; Caradonna et al., 2021). Using ATAC-seq, we demonstrated that double-hit acute stress reorganizes chromatin accessibility at discrete transcription factor binding sites in the vHPC. After double-hit stress, the chromatin regions binding AR, GR, and MR, namely the AREs and GREs, were among the most differentially accessible sites. This signifies that chromatin accessibility induced by double-hit acute stress at ARE/GRE sites persists for at least 7 days after one event of stress. Although we did not investigate direct binding of nuclear receptors, our study presents the first evidence that a double-hit acute stress epigenetically modulates the accessibility for the sites within the hippocampal genome that bind AR, GR, and MR. Mifsud and Reul (2016) have shown that exposure to ARS or FSS alone increases the binding of GR and MR to GREs in the chromatin isolated from the hippocampus. This increased binding at GREs directly affects a mosaic of gene expression changes involved in neuroplasticity processes, learning and memory, and several neuropsychiatric disorders (Mifsud et al., 2021). Notably, the action of androgens, glucocorticoids, and mineralocorticoids on their respective targets is biphasic, with timing being a major factor for stress coping (Lupien et al., 2009). For example, timed elevation of glucocorticoids prior to stress exposure protects from detrimental effects of stress (Rao et al., 2012), but repeated high doses rather mimic the effects of chronic stress (Mitra and Sapolsky, 2008; Woolley et al., 1990). The time-dependent effects of glucocorticoid exposure are also demonstrated in clinical data from subjects with PTSD, where glucocorticoids administered at the time of trauma may prevent the pathophysiological manifestation of PTSD (Schelling et al., 2004; Zohar et al., 2011). Thus, steroid hormones imprint an epigenetic memory of an acute stress event that reorganizes

coping strategies later in life. Further investigations are needed to understand how the levels of chromatin accessibility affect the binding to the DNA, whether this impacts the spatial epigenetic organization at the single-cell level, or how this genomic reorganization affects translation and protein expression.

4.4. Chromatin accessibility for AREs/GREs in males and females

We narrowed the investigation of accessibility at ARE/GRE sites adjacent to gene coding regions of the genome. Males and females displayed different regulation of GREs when compared at baseline or within the same stress treatment, suggesting that GR binding may be affected in both sexes. AREs accessibility differed in males and females at baseline, after ARS or the double-hit, but not after FSS. This suggests that while sex differences in chromatin organization after acute stress are negligible in the short term, a prior history of stress induces different degrees of epigenetic reorganization in males and females. Females showed differences in AREs opening across all stress conditions, while in males AREs changed only after FSS males and double-hit stress. Evidence shows that testosterone signaling in the vHPC mediates stress resilience differently in males and females (Goel and Bale, 2008; Williams et al., 2020), thus it is likely that homeostatic feedbacks that reset stress-mediated androgen signaling one week after ARS exist in males but not in females. Similarly, GRE-binding GR and MR were affected by ARS and FSS in both males and females, yet double-hit changed GREs accessibility only in males. One possible explanation is that ARS has a priming effect in females that induces a homeostatic chromatin organization at GRE binding, while this phenomenon is absent in males. Interestingly, GR expression in the hippocampus is associated with different stress susceptibility in males and females, with GR deletion conferring higher susceptibility in males than females (Solomon et al., 2012). Double-hit stress in females reversed the changes in GRE opening observed after FSS, suggesting an allostatic epigenetic response that was unique to females. Both males and females showed increases in chromatin accessibility after FSS for ARE/GRE sites, yet decreased accessibility after ARS. Others reported that despite inducing distinct glucocorticoid responses, ARS or FSS result in largely similar GR and MR binding to GREs (Mifsud and Reul, 2016). These observations suggest that the time elapsed between the stress exposure and the epigenetic analysis is crucial to understand chromatin accessibility over the short versus the long term. Sites adjacent to ARE/GRE, whose accessibility was affected by double-hit stress, coded for genes implicated in stress-related disorders such as *Fkbp5*, *Zbtb16*, and *Ddc*. *Fkbp5*, *Zbtb16*, and *Ddc* were selected among the topmost differentially accessible genes with ARE and GRE biding sites. Consistent with our results, Mifsud and Reul (2016) used chromatin immunoprecipitation to show that diverse types of acute stress, including restraint and forced swim, increase GR and MR binding to GREs with *Fkbp5*. Notably, the human ortholog of *Fkbp5* is one biomarker whose altered expression is critical for the diagnosis of post-traumatic stress disorder (PTSD) (Yehuda et al., 2009). We found increased chromatin accessibility of *Fkbp5* in double-hit males but not in females. This may explain why levels of *Fkbp5* after acute stress return to *homeostatic* levels sooner in females than males (Bourke et al., 2013). It is likely that increased chromatin accessibility of *Fkbp5* in double-hit males is the result of a latent impact of the first hit of stress, and could itself confer protection against anxiety-like phenotypes, consistent with findings showing that overexpression of *Fkbp5* in discrete brain regions induces anxiolytic phenotypes (Engelhardt et al., 2021). Chromatin accessibility of *Zbtb16* was also different in males and females, with double-hit stress inducing significant chromatin remodeling in males but not in females. Changes in the expression of *Zbtb16* have been observed in parallel with increased levels of glucocorticoids (Austin et al., 2021) and higher risk-taking behavior (Usui et al., 2021), consistent with the increased exploratory activity of ARS males observed in the light-dark box test. *Fkpb5* and *Zbtb16* were not affected by double-hit stress in females, despite an increase in chromatin accessibility induced by FSS alone. One hypothesis is that epigenetic mechanisms unique to females organized in response to the first stressor, buffer the epigenomic response to the second stressor, a priming mechanism that may be absent males. Chromatin accessibility after FSS was also changed at the *Ddc* gene, which is involved in the signaling response to a novel antidepressant in models of stress (Wang et al., 2022). Finally, chromatin remodeling after stress is undoubtedly the key to understand how experiences "get under the skin" (McEwen, 2012) and = nurture our DNA.

4.5. Is acute stress strictly acute?

We have shown that the effects of acute stress epigenetically persist in the long term in both males and females. Many animal models commonly employ chronic stress paradigms to investigate the stress response in the brain, as the observed behavioral, neurological, and genomic effects are more extreme and persist into the long-term. Yet, this overlooks the question as to how the brain moves from a physiological to a maladaptive state since initial fight-or-flight responses are the molecular foundations of subsequent allostatic (mal)adaptations. We may give the example of PTSD where epigenetic memories imprinted during the trauma underlie the hypermnesia of the event and the inability to restrict fear to an appropriate context (Al Jowf et al., 2021; Ross et al., 2017; Zovkic and Sweatt, 2013). Verbitsky et al. (2020) recently reviewed the complexity of recapitulating PTSD in rodent models, and we acknowledge that our study meets some criteria of construct validity for PTSD (Verbitsky et al., 2020). Indeed, we shed light on epigenomic marks of stress across the genome, particularly in ARE and GRE sites, that differentially persist over time in males and females and that epigenetically respond to a subsequent stressor occurring in a different context. Since hormonal activation of neuronal steroid receptors defines sex differences in gene expression (Gegenhuber et al., 2022), further studies are needed to isolate the role of gonadal hormones in chromatin reorganization after double-hit stress. One speculation is that the higher epigenomic reactivity that these findings show in females compared to males may underlie the genomics behind the likelihood to develop PTSD in humans, whose prevalence is twice higher in women than in men (Olff, 2017). Ultimately, we must recognize that the pathophysiology of complex neuropsychiatric disorders cannot be limited to binary interpretations of sex, as in humans this would imply the study of a broader sexual and gender spectrum.

5. Conclusion

The bell has been rung as the clock strikes the hour; we cannot roll back. Acute stress leaves its hidden marks in the epigenome, only to be uncovered by a future stress exposure. The key promise of epigenetics is its reversibility, as factors in our environment, such as diet, exercise, or drug intervention, can counterbalance toxic impact on the epigenome. Yet epigenetic alterations left unchecked feed forward to impact neuronal architecture and circuits, leaving more lasting changes in our physiology and behavior. The implications of these mechanisms are broad. Could there be lasting changes induced by the genomic machinery leading to the manifestation of disease? Thus, is hope all lost? While Bruce McEwen seemed to dispute *reversal*, he believed in *resilience*. Every physiological alteration impacted by epigenetics cannot be undone, but Bruce McEwen trusted that an everchanging brain meant that there are treasures surrounding our DNA that we can hunt with the objective to heal. Bruce McEwen's work lit the way, and brought us here to tell a new story that we wish we could add to the infinite library that lived in his brain. At the time when he found steroid receptors in the hippocampus, little did he know that through this discovery of the unexpected, Bruce McEwen had turned on the *allostatic clock*, moving the dial forward, and leaving a lasting, irreversible signature to master the neurobiology of stress.

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CRediT authorship contribution statement

Salvatore G. Caradonna: Conceptualization, Methodology, Investigation, Formal analysis, Investigation, Writing – original draft, Visualization. **Matthew R. Paul:** Software, Data curation, Formal analysis, Writing – review & editing, Visualization. **Jordan Marrocco:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Supervision, Project administration, 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

ATAC-seq data have been deposited to GEO (GSE200670) and are fully available. Supplemental data are also available.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ynstr.2022.100475.

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