Contextual glucocorticoid signaling in-vivo: a molecular perspective

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Chapter 5:

Effects of early life stress on chromatin accessibility and genome wide transcription in the dorsal mouse hippocampus.

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(in preparation)

ABSTRACT

Exposure to excessive and/or chronic stressors during early life is a well-established risk factor for later life stress-related mood disorders. Glucocorticoids are often implied as mediators of the long term effects of early life stress (ELS), given their powerful transcriptional effects via the glucocorticoid receptor (GR). Previous work in rodents showed that the GR antagonist RU486 administered during early adulthood may reverse behavioral and cellular effects of ELS. Here, we investigated the long-term molecular effects of ELS in the form of the limited bedding and nesting (LBN) paradigm on the (dorsal) hippocampus of adult male mice. We aimed to identify alterations in chromatin accessibility and the transcriptome, and assessed the effect of an intervention with RU486. We found no evidence that ELS long-lastingly alters chromatin accessibility, but saw that it can alter the hippocampal transcriptome. We did not observe any normalization by adolescent RU486 intervention at the hippocampal chromatin or transcriptome level. The effects on the hippocampal transcriptome were found to be inconsistent across experiments, with other or no alterations at all in separate cohorts of ELS animals. These included two adolescent replication cohorts in which virtually all controllable sources of variations were eliminated, a.o. LBN protocol, laboratory and researcher. We conclude that the transcriptional effects after ELS are inconsistent and likely of stochastic nature, and that other – more comprehensive – approaches are required to unravel the molecular workings of ELS.

INTRODUCTION

When faced with an acute stressor the body's first response is to appropriately deal with the current threat, after which homeostasis has to be reinstated (1, 2). This involves activation of the hypothalamic-pituitary-adrenal (HPA) axis, resulting in the secretion of predominantly cortisol in humans and corticosterone in rodents. These steroid hormones not only regulate the later phases of the stress response that help restoration of homeostasis, but also support processes that help adaptation to future stressors (3). When challenges of homeostasis take place during critical periods of life (such as the perinatal period or adolescence), these adaptations may lead to long term changes in homeostatic setpoints, a phenomenon called allostasis (4).

A prominent example is severe early life stress (ELS; mice) or childhood trauma (humans). This may involve either multiple acute or a single very strong stressor. The adaptive changes in these cases can create a context poised to deal with similar challenges during adulthood, resulting in a brain that is "programmed" to deal with a life filled with series of adversities (5-8). This programming may involve the high endogenous glucocorticoid levels resulting from the stressor(s), activating glucocorticoid receptors expressed throughout the brain (9). While these programming effects might be beneficial to deal with future stressors, they may also negatively interfere with cognitive and emotional processes in "safe" situations, and in this way form a mismatch with later life circumstances (10-12). In fact, childhood trauma correlates with increased incidence, severity and treatment resistance across psychiatric diagnoses (13, 14). To date, merely symptomatic treatment of ELS-related psychopathology is possible. A better understanding of the long-lasting structural, behavioural and molecular changes is required to develop future preventive and/or curative interventions.

Various mouse models have been developed over the past decades to study the mechanism(s) underlying the effects of ELS, including the maternal separation and the limited bedding and nesting (LBN) paradigms (15, 16). The latter is considered a translatable model in which the absence of nesting and bedding material negatively affects maternal care, an important developmental factor that affects later life brain function and behaviour (17, 18). The LBN paradigm is hallmarked by a lower body weight at postnatal day (PND) 9, and changes in multiple behavioural domains (e.g. anxiety, learning and social) in later life (19, 20). For example, behavioural assessment after auditory fear conditioning revealed that adult male (but not female) mice were unable to differentiate between the "save" cue-off and "dangerous" cue-on periods (21). Remarkably, in two studies the ELS-induced deficit in males was normalized by a brief intervention with GR antagonist RU486 during adolescence at PND 28 – 30 or 26-28

respectively (21, 22). This demonstrated for the first time that curative interventions after ELS might be an attainable goal. Through which mechanisms ELS affects behaviour in adulthood and how this is (in part) normalized by a brief intervention in adolescence is unclear. However, given that GR is a transcription factor, and given the efficacy of the GR antagonist, the underlying mechanism may be found at the level of transcription or the epigenetic regulation thereof.

In this study we set out to investigate how ELS long-lastingly affects the male mouse hippocampus transcriptome and chromatin accessibility. To this end, we used multiple cohorts of ELS and control mice, with or without adolescent GR antagonist intervention, and assessed the corresponding chromatin accessibility and transcriptome changes. By investigating the effect of ELS in multiple cohorts of mice we were able to assess the reproducibility and replicability of the effects.

MATERIAL AND METHODS

Animals

Multiple cohorts of animals were used in the study performed in two laboratories, following the same experimental setup. C57BL6 mice were bred in-house (originally acquired from Envigo, the Netherlands and Campinas-SP, Brazil). Animals had access to *ad libitum* food and water and a light/dark cycle of 12 h/ 12 h (light period starting at 8:00 am). Litters consisted of a maximum of six pups with at least one female and were randomly assigned to ELS or control conditions. ELS was imposed using the limited bedding and nesting material (LBN) paradigm (15, 21). After PND9, the litters were transferred to standard cages and pups were kept with the dams until weaning (PND21), after which the male animals were group-housed (up to five animals per cage) and left undisturbed (apart from treatment) until sacrifice at PND31 (adolescent reproducibility cohorts) or PND120 (adult cohorts). In this study we focussed on male rodents, in view of the absence of behavioural effects earlier seen in females (21, 22). Hippocampal tissue was directly dissected after sacrifice and snap-frozen for molecular analysis. All animal experiments were approved by the national Animal Ethics Committees and carried out at the University of Amsterdam or the University of São Paulo under approval of the local Animal Welfare Body.

Limited bedding and nesting paradigm

From PND2 to PND9, control dams and pups were placed in cages with a standard amount of bedding material (a 2 cm thick layer of sawdust and a square of cotton nesting material (5 cm by 5 cm, BMI Someren, The Netherlands). ELS animals were provided with half the bedding and nesting material compared to controls and a metal

mesh covered the cage bedding (15). Body weight of the pups was assessed before (PND2) and directly after ELS (PND9) or control conditions and at weaning (for the adult cohorts) to confirm the effectiveness of the LBN paradigm.

Treatment

Animals were treated once or three times with GR antagonist RU486 (Mifepristone; 10mg/kg, Sigma) or vehicle (0.25% carboxymethylcellulose, 0.2% tween and 0.9% NaCl in water) during adolescence (PND30 or PND28-30) by intraperitoneal injection. We opted for two injection frequencies (once and three times) to be able to assess whether or not a single injection with RU486 would suffice as intervention.

Assay for transposase accessible chromatin sequencing

The Assay for transposase accessible chromatin (ATAC) sequencing protocol was adapted from Mo et al. and Corces et al. (23, 24) and carried out at 4 °C. In brief, snap-frozen dorsal right hippocampi of three times RU486 or vehicle treated ELS or control animals were homogenized in a 7 mL douncer containing 1.5 mL of cold buffer (0.25 M sucrose, 25 mM KCl, 5 mM MgCl2 and 20 mM Tricine-KOH at pH 7.8) and 90 µL of 5% IGEPAL CA-630 was added after 20 strokes with pestle A. After an additional 20-30 strokes with pestle B the homogenate was filtered through a 40 µm cell strainer into a 5 mL LoBind Eppendorf tube. 1.5 mL of 50% iodixanol was added and gently mixed with the homogenate, after which 1.5 mL of 29% iodixanol solution was layered underneath. Gradients were then centrifuged for 20 min at 2,500x g in a swinging-bucket centrifuge and the supernatant was removed. 200 µL of cold ultrapure PBS was then added to the nuclei pellet and left on ice for 5 min before resuspension of the cell nuclei. Nuclei were counted using fluorescent microscopy with DAPI and 50.000 nuclei were transferred to a new LoBind Eppendorf tube for the transposase reaction.

Nuclei were pelleted and 50 µL of ATAC-reaction mix was added as previously described (24) and incubated at 37 °C temp for 30 minutes. Sample cleaning, PCR amplification and ATAC-seq library preparation was performed according to Buenrostro protocol (25). ATAC libraries were subsequently send for single-end sequencing at the Max Planck institute in Berlin with >35 million reads/library.

ATAC-seq data analysis

Quality control was performed using FastQC and MultiQC and adapter trimming was done with Trim-galore with default parameters. Reads were mapped to mus musculus genome mm10 using Bowtie2: -very-sensitive. Peak calling was performed using MACS2 with the following parameters: -f BAM, --bdg, --nomodel, --extsize 200, --shift -100, -g mm, -qvalue 0.05. Differential accessibility analysis was performed using Diffbind (26)

and a nominal p-value of 0.05 was used to identify regions with potential differential accessibility. Differentially accessible regions were annotated to the nearest gene using Homers annotatePeaks.pl with default settings (27).

Coverage plots were created using Deeptools (version 3.5.0) (28) after bins per million mapped reads normalization and averaging of bw files with bamCoverage (version 3.5.0) per group.

qPCR validation

Expression levels of genes of interest based on the accessibility data were assessed by Real-time quantitative PCR (RT-qPCR). cDNA was synthesized from 1.000 ng of RNA using random hexamers and M-MLV reverse transcriptase (Promega) according to manufacturer's protocol. RT-qPCR was performed using GoTaq® qPCR Master Mix (Promega) on a CFX96 Touch™ Real-Time PCR Detection system (BioRad). mRNA expression levels were normalized to housekeeping gene *Ppia* using the 2-ΔΔCT method to the control-vehicle group.

Corticosterone measurement

Plasma corticosterone levels were determined in trunk blood of the once and three times injected parallel cohorts using a high sensitivity EIA kit (AC-15F1, Immunodiagnostic Systems).

RNA sequencing

Total RNA was isolated from dorsal hippocampal tissue of adult ELS and control animals (PND120) homogenized in the lysis buffer of the NucleoSpin RNA kit (Macherey-Nagel). Total RNA was isolated according to manufacturer's protocol and was sent for transcriptome sequencing at BGI Genomics (Hong Kong). Whole hippocampal tissue of adolescent ELS and control animals (PND31) was sent to BGI Genomics for total RNA isolation using RNeasy kit (Qiagen) and transcriptome sequencing. All samples passed quality control by BGI. Stranded mRNA libraries were constructed and 100 bp paired-end sequencing was performed on the DNBseq platform resulting in >20 million reads per sample. RNA-seq data will be deposited in NCBI's Gene Expression Omnibus.

RNA-seq data analysis

Data processing

The RNA-seq pipeline (version 4.1.0), published as part of BioWDL, was used for read quality control, alignment and quantification. BioWDL contains the main sequencing

analysis pipelines and workflows developed at Leiden University Medical Center by the sequencing analysis support core with code being accessible at https://biowdl.github.io/.

Quality control was performed using FastQC and MultiQC. Reads were aligned to Mus Musculus genome version 10 (mm10) using STAR (version 2.7.3a). The gene-read quantification was performed using HTSeq-count (version 0.12.4) on Ensembl release 97 of mm10. HTSeq-count output files were merged into a count matrix per experiment as input for differential gene expression analysis.

Differential expression analysis

DEseq2 (version 1.29.4) was used for normalization of the count data (median of ratio's method) and identification of differentially expressed genes (29). For the differential expression analysis, all genes which were expressed in a minimum of four (for the PND120 cohorts) or three (for the PND31 cohorts) replicates with >20 normalized counts for at least one of the groups were selected. One sample of the adult validation cohort was identified as an outlier based on principal component analysis (sample 21 of the ELSRU group) and excluded from subsequent analysis. Pair-wise comparisons of groups within experiments were analysed and a false discovery rate adjusted p-value of 0.05 was used as a cut-off for detection of differential gene expression (**supplementary table 1**).

Gene Ontology analysis

Gene ontology (GO) enrichment analysis of biological processes was performed on all genes differentially expressed after ELS of PND120 cohort 1 with the ViSEAGO package (version 1.4.0), using fisher's exact test with 0.01 as a significance cut-off (30) (**supplementary table 2**).

Differential exon usage analysis

Differential exon usage analysis was performed on all vehicle treated ELS and control animals of the adult validation cohort with the DEXSeq package (1.36.0) (31) with the default settings and a false discovery rate adjusted p-value of 0.01 for detection of differential exon usage (**supplementary table 3**).

Statistics

Gene expression data acquired by RT-qPCR were analysed using two-way ANOVA's followed by Tukey's multiple comparison tests. Plasma corticosterone levels were analysed using three-way ANOVA followed by Šidák multiple comparison tests. Statistical analyses were performed in GraphPad Prism 7 software (GraphPad Inc.) and sequencing data was analysed in R (version 4.0.0).

RESULTS

ELS does not strongly affect adult dorsal hippocampal accessibility.

Earlier studies in male rodents observed reversal of ELS-induced changes in behaviour by intervention with the GR antagonist RU486 (21, 22). In order to determine the long-lasting molecular effects of ELS and investigate the reported normalization by adolescent RU486 intervention, we used a two-by-two study design that followed previous work (**Fig. 1A**). ELS was imposed by the limited bedding and nesting (LBN) paradigm from PND 2-9, with control animals in standard conditions. After PND9 all animals were housed in standard conditions. RU486 or a vehicle control were administrated three times (at PND 28,29,30) or once (at PND 28). All animals were then left undisturbed and were sacrificed at PND120 (**Fig. 1B**). Bodyweight data in the first adult cohort showed a significant main interaction between stress and age of the mice [$F_{2,266}=23.12$, $P<0.0001$]. Before LBN bodyweight did not differ between ELS and control animals ($P>0.99$) and after bodyweight was significantly lower in the ELS animals compared to controls directly after ELS at PND9 ($P<0.0001$) and at weaning ($P<0.001$), confirming effectiveness of the LBN paradigm (**Sup. Fig. 1A**).

Based on the long-lasting and reversible effects of ELS we hypothesized that the chromatin accessibility of the dorsal hippocampus might be altered as a consequence of GR activation during the LBN period. With ATAC-seq we identified 119,907 accessible regions in the right dorsal hippocampus of adult male ELS and control animals. However, differential accessibility analyses did not reveal any loci with significantly altered accessibility between ELS and control animals, nor did we find any significant effects of RU486 intervention (lowest FDR corrected p-value = 0.998). The overall absence of effects on the accessibility landscape was evident by visualization of all identified accessible regions, depicted as a tornado plot (**Fig. 1C**) and by the summary profile plot (**Fig. 1D**). While this analysis showed no obvious biological effect of ELS on dorsal hippocampal accessibility, the multitude of cell types in the dorsal hippocampus (32) likely impaired the analysis by the large biological variation in chromatin accessibility between cells. This may have diluted and thereby masked cell-specific accessibility changes induces by ELS.

Chromatin accessibility data aided the identification of genes with altered expression by ELS.

To further interrogate the data, we focused on accessible regions which showed differential accessibility between control and ELS (3,077 regions, 1,532 more and 1,545 less accessible) and between ELS and ELSRU animals (7,924 regions, 2,160 more and 5,764 less accessible) at a nominal p-value <0.05. Of these, 1,062 regions (annotated to 994 genes) showed nominal differential accessibility in both contrasts in opposite directions (more accessible after ELS and less accessible after RU486 intervention or vice versa), indicating possible normalization after adolescent intervention by RU486. From this subset of accessible regions, taking into account the genomic location of the peaks (e.g. in promoter region or intronic), hippocampal expression of the associated gene and visual inspection of the ATAC-traces (e.g. *Vdac3*, **Fig. 1E**), we selected *Fhad1*, *Vdac3* (both based on altered promoter accessibility normalized by RU486) and *Efhd1* (based on altered intronic accessibility only after ELS) for follow-up.

For validation we assessed gene expression levels in a largely comparable adult cohort performed in parallel which received one injection with GR antagonist RU486 instead of three. In this parallel cohort we found a significant interaction effect for the dorsal hippocampal mRNA expression levels of *Fhad1* [F1,23=7.27, P=0.01] and *Vdac3* [F1,23=12.40, P<0.01], with significantly higher mRNA levels after ELS for *Fhad1* and significantly lower expression levels after 1x RU486 intervention compared to the ELS group for *Fhad1* and *Vdac3*. *Efhd1* showed a main effect of ELS [F1,22=5.98, P=0.02] in line with the accessibility data, but post-hoc comparison did not reach significance (**Fig. 1F-H**). These data showed that, even though the accessibility data did not reach significance for any chromatin region after multiple testing correction, the regions with altered accessibility at a nominal significance level were informative and predicted the identification of genes with altered mRNA expression levels after ELS in a parallel cohort.

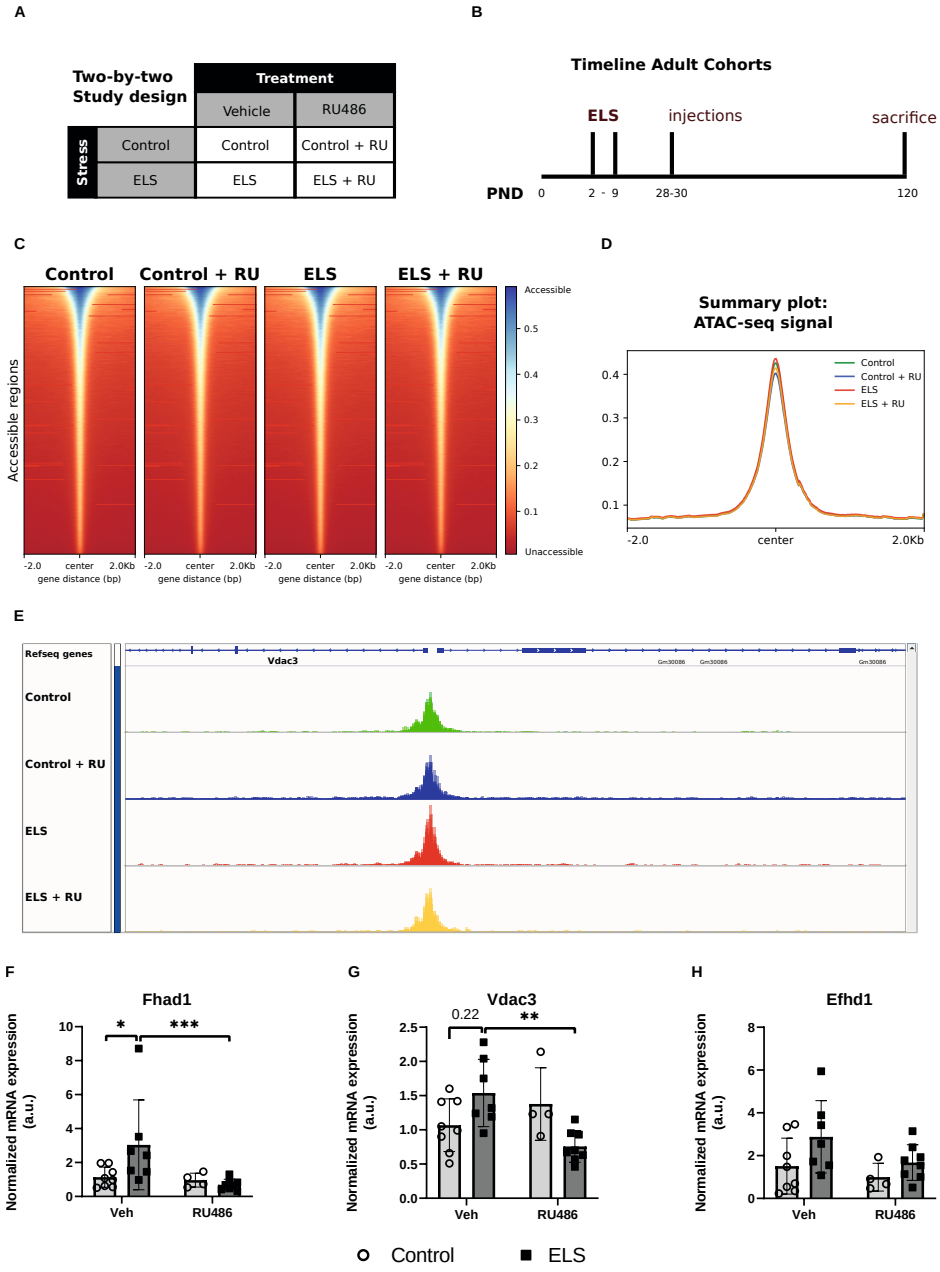


Figure 1: Early life stress did not significantly affect hippocampal chromatin accessibility, but did predict alterations in gene expression.

Figure 1 continued: (A) The two-by-two design used to study the effect of ELS and RU486 intervention. (B) Timeline followed for generation of adult cohorts. ELS was imposed by LBN paradigm between PND 2-9. RU486 intervention was administered by I.P. injections on PND 28-30 or PND 30. Animals of the adult cohort where sacrificed at PND120. (C) Tornado plots of all groups (Control, Control + RU, ELS and ELS+RU) with averaged coverage of the normalized ATAC-signal 2-kB flanking the summits of the called accessible regions (n=7-9 per group). (D) Summary profile plot with the average ATAC-signal of all called accessible regions per group (Control, Control + RU, ELS and ELS+RU). (E) Stacked ATAC-seq traces at the promotor region of *Vdac3*. ELS: early life stress, LBN: limited bedding and nesting, PND: postnatal day, RU: RU486. Validation of mRNA expression of *Fhad1* (F), *Vdac3* (G) and *Efhd1* (H) in an adult cohort with a single intervention injection of RU486 on PND 30 (n= 4-8 per group). ELS: early life stress, LBN: limited bedding and nesting, PND: postnatal day, RU: RU486.

Number of adolescent vehicle-injections does not long-lastingly effect corticosterone levels.

Because lasting effects on corticosterone levels may affect gene transcription, we next determined AM corticosterone levels in the two parallel cohorts (1x and 3x RU486) in which we performed ATAC-seq and qPCR, assessing whether these levels were differentially affected by either treatment with the GR antagonist RU486. Overall, plasma corticosterone levels were not affected by ELS [F1,77=0.45, P=0.50] and RU486 treatment resulted in non-significantly elevated corticosterone levels [F1,77=2.49, P=0.12]. A main effect for injection frequency was found [F1,77=5.26, P=0.02], likely driven by RU486 and not vehicle (**Fig. 2**). Post-hoc analysis confirmed that one vs. three vehicle injections did not result in differing AM corticosterone levels in both control (P>0.99) and ELS animals (P>0.99), thereby not hampering comparison of accessibility and transcriptome data for (at least) the vehicle groups of the parallel cohorts.

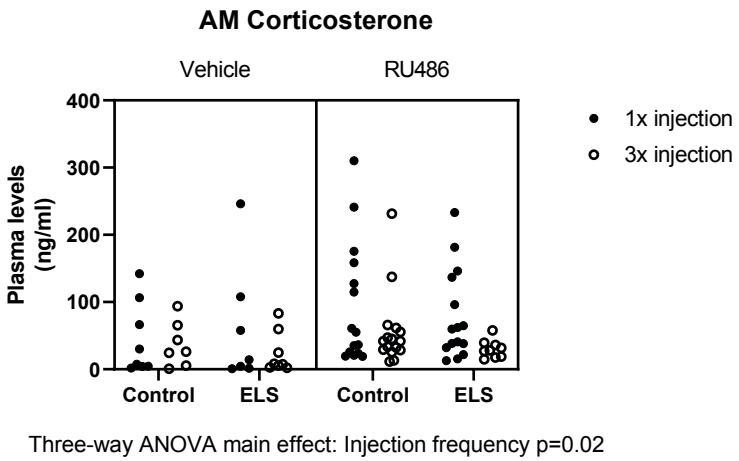


Figure 2: Corticosterone levels were not strongly affected by once versus three times RU486 intervention. AM corticosterone levels (ng/ml) determined in plasma derived from trunk blood at sacrifice (n= 7-16 per group). ELS: early life stress.

ELS can strongly alter the dorsal hippocampal transcriptome, yet not in a consistent manner.

The significantly altered mRNA expression levels of *Fhad1*, *Vdac3* and *Efhd1* confirmed that ELS can long-lastingly alter the dorsal hippocampal gene expression. To investigate the reproducibility of these and the full extent of all transcriptome changes, we analysed the right dorsal hippocampal transcriptome of an adult validation cohort with three times RU486 intervention (PND28-30). Of note, this animal experiment was performed by the same researcher, but in a different laboratory. In accordance with the first adult cohort a similar interaction effect was found [$F_{2,240}=85.13$, $P<0.0001$] and the body weight of the ELS animals in the adult validation cohort was lower at PND9 ($P<0.0001$) and PND21 ($P<0.0001$), confirming the animals were stressed during early life (**Sup. Fig. 1B**).

Differential expression analyses identified 116 DEGs after ELS (40 up- 76 downregulated, **Fig. 3A**). None of the three genes (*Fhad1*, *Vdac3* and *Efhd1*) that were differentially expressed in the first adult cohort (detected by qPCR) had altered expression levels in the adult validation cohort (supplementary table 1). To determine which processes were affected in adulthood after ELS we performed GO-term enrichment analysis, which reported 25 biological processes including the regulation of mRNA splicing via the spliceosome (**Sup. Fig. 2A, supplementary table 2**). Differential exon usage analysis between ELS and control animals identified 57 differentially used exons (in 53 genes, example gene: *Snrpc*, **Sup. Fig. 2B, supplementary table 3**), confirming that mRNA splicing was affected by ELS in the adult validation cohort. Intervention during adolescence with three times RU486 injection after ELS significantly changed the expression of 36 genes (31 up- 5 downregulated) compared to vehicle-treated ELS animals (**Fig. 3B**), while intervention with RU486 did not alter the transcriptome in control animals (**Fig. 3C**). The largest number of DEGs (715; 199 up- 516 downregulated, **Fig. 3D**) was identified between RU486-injected ELS animals and vehicle-treated controls. This suggests that RU486 amplified rather than reversed ELS-induced changes, and that the previously reported normalization of auditory fear conditioning behaviour by RU486 does likely not occur via normalization of previously instated ELS effects on the level of dorsal hippocampal gene expression (as shown schematically, **Sup. Fig. 2C**). The lack of reversal by RU486 intervention of ELS effects was confirmed by the minimal overlap of DEGs (one gene) between the contrasts ELS-RU486 vs. ELS and ELS vs. Control (**Sup. Fig. 2D**).

The analysis of the adult validation cohort revealed the extent to which ELS can have effect on the hippocampal transcriptome and provided insight in the transcriptional effect of RU486 intervention after ELS. However, as the effect of ELS on the expression of the genes that initiated the in-depth analysis (*Fhad1*, *Vdac3* and *Efhd1*) was inconsistent, we questioned the reproducibility of all the DEGs that were identified in the adult validation cohort.

To address this issue, we performed an additional transcriptome analysis on the left dorsal hippocampus of the animals from the first adult cohort, of which the chromatin accessibility was initially assessed. This comparison relies on the assumption that the left and right dorsal hippocampal transcriptomes are comparable (see Discussion). However, in these animals we found no effect of ELS on the transcriptome (**Fig. 3E**), nor did we observe any effect of three times RU486 intervention compared to the vehicle-treated ELS group (**Fig. 3F**). Adolescent intervention with three times RU486 in control animals altered the expression of a single gene (**Fig. 3G**), and RU486-injected ELS animals compared to vehicle-treated controls also resulted in one DEG (**Fig. 3H**).

Side-by-side visualization of the ELS and RU486 intervention effects on the dorsal hippocampal transcriptome of both adult cohorts highlighted the inconsistency of the transcriptional effects. Even though the second set of transcriptome data (**Fig. 3E-H**) was derived from the same animals that were used to generate the chromatin accessibility data (**Fig. 1C-E**), the alterations observed in the once RU486 injected parallel cohort were not replicated (**Fig. 1F-H,** supplementary table 1). Of note, both these adult cohorts were generated in different laboratories by the same researcher. Altogether, these data indicate that ELS can long-lastingly alter the dorsal hippocampal transcriptome, but did not do so in a consistent and reproducible manner in both adult cohorts, despite both undergoing early life stress strictly following the LBN paradigm.

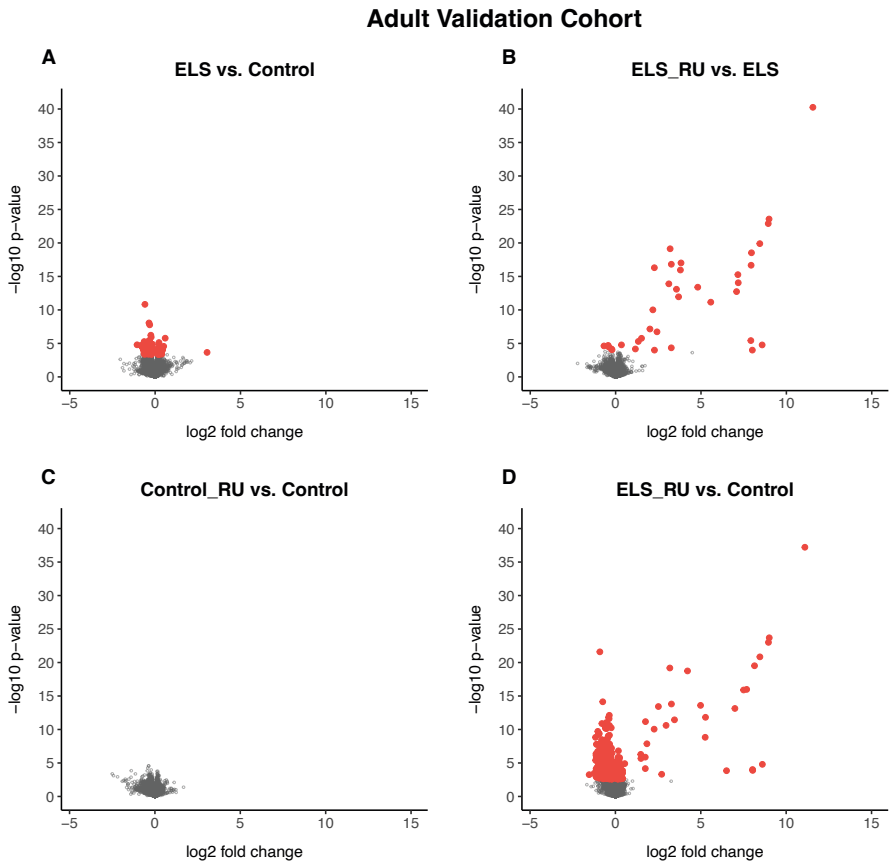


Figure 3: Inconsistent effects of early life stress on the dorsal hippocampal transcriptome.
Volcano plots visualising the DEGs per contrast of the adult validation cohort (A-D) and first adult cohort (E-H) (n=5-7 per group). Red dots represent DEGs at adjusted p-value 0.05. DEGs: differentially expressed genes, ELS: early life stress, RU: RU486.

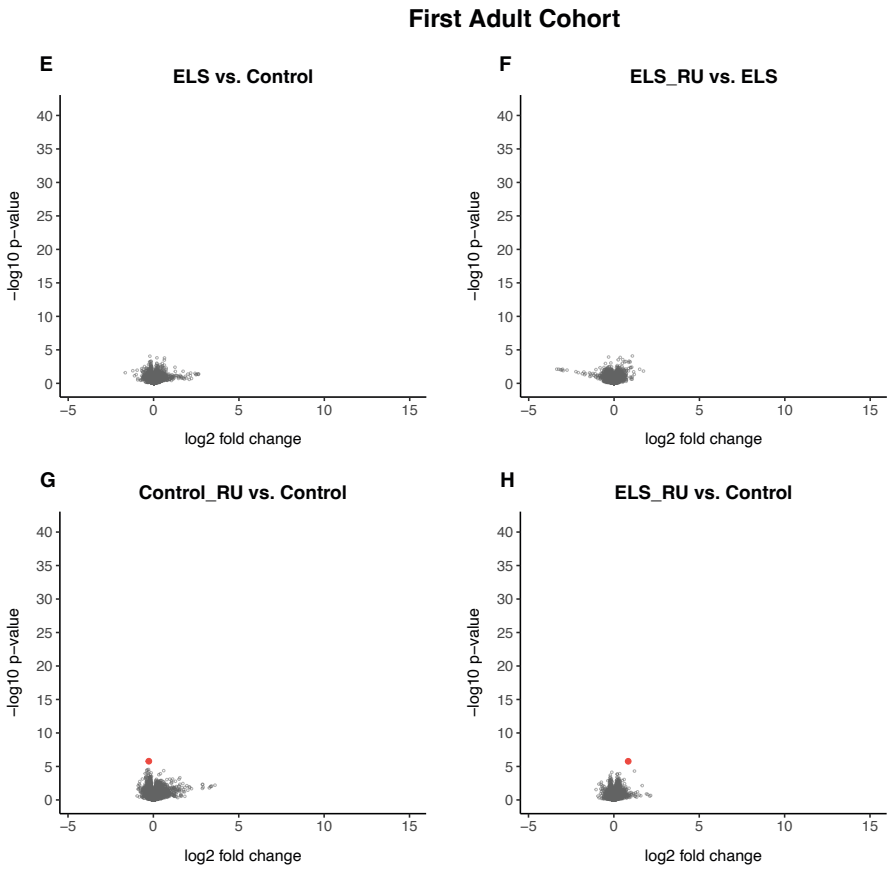


Figure 3: continued.

Adolescent effects of ELS on the hippocampal transcriptome are unreproducible.

The inconsistent results found at the transcriptome levels can be attributed to various factors, which all – to varying extent – will influence the eventual transcriptional signature obtained. Often discussed factors for ELS models are differences in background of the animals, the researcher performing the LBN and the laboratory in which the experiments were conducted (20). In addition, long-lasting effects might be transient and fade-out over time, introducing another factor which might contribute to the inconsistency we observed at adulthood (PND120). To - altogether - address these factors we performed another experiment to study the effects of ELS, and the consistency thereof, on the hippocampal transcriptome. We therefore opted to investigate the transcriptomic changes after ELS during adolescence (at PND31) under the hypothesis to detect a stronger effect closer to the intervention period (10 days after weaning when ELS effects were still apparent in the body weights). We had a single

researcher perform the two adolescent reproducibility cohorts back-to-back with an identical experimental design in the same facility, keeping all variables as comparable as possible (**Fig. 4A**).

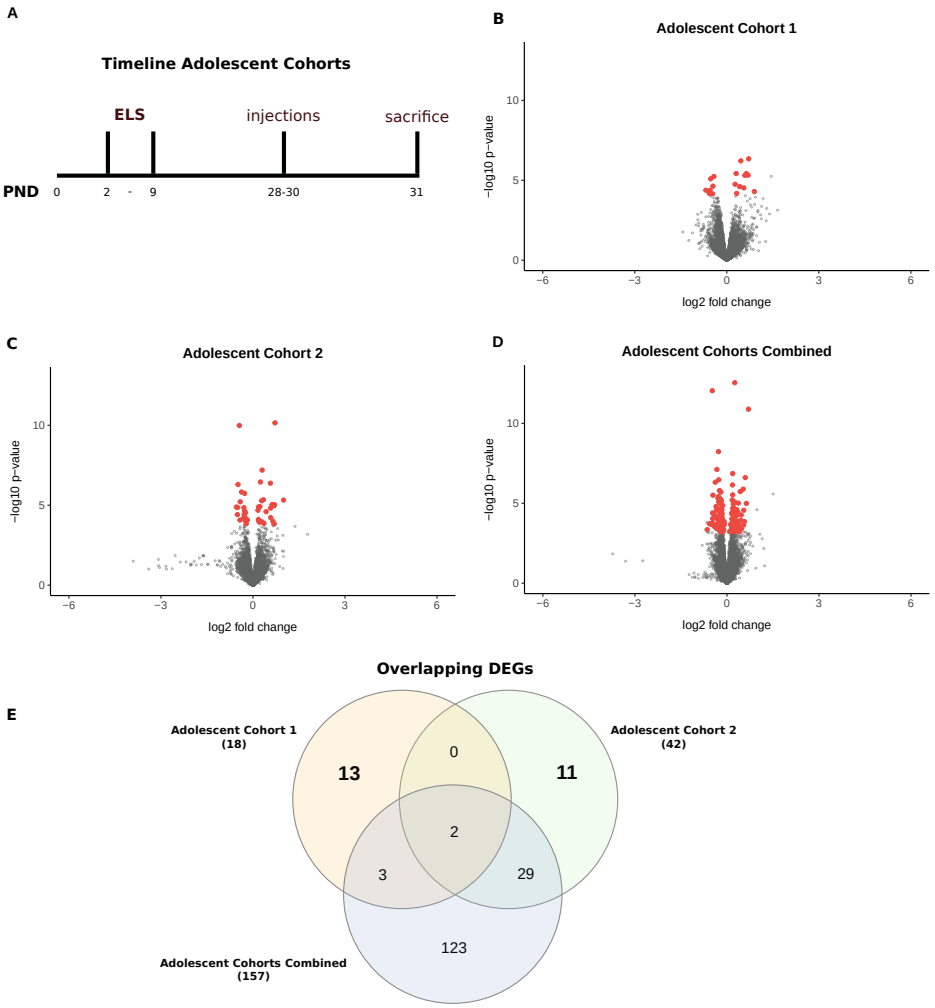


Figure 4: Effect of early life stress on the adolescent hippocampal transcriptome.

(A) Timeline followed for generation of adolescent cohorts. ELS was imposed by LBN paradigm between PND 2-9. RU486 intervention was administered by I.P. injections on PND 28-30. Animals of the adolescent cohort where sacrificed at PND31. Volcano plots visualising the DEGs between ELS and control animals of adolescent cohort 1 (B, n= 4 per group), adolescent cohort 2 (C, n= 6 per group) and both adolescent cohorts combined (D, n= 10 per group). Red dots represent DEGs at adjusted p-value 0.05. (E) Venn diagrams displaying the number of overlapping DEGs of both adolescent cohorts separate and the combined cohort. DEGs: differentially expressed genes, ELS: early life stress, LBN: limited bedding and nesting, PND: postnatal day, RU: RU486.

Bodyweight again revealed an interaction effect between stress and age in both adolescent cohorts (adolescent cohort 1: [F1,34=8.72, P=0.0057] and adolescent cohort 2: [F1,44=19.69, P<0.0001]) and was significantly lower in ELS animals compared to their respective controls at PND9 (adolescent cohort 1: (P<0.0001) and adolescent cohort 2: (P<0.0001)), with no difference at onset of the LBN paradigm at PND2 (adolescent cohort 1: (P=0.81) and adolescent cohort 2: (P>0.99)) (**Sup. Fig. 1C&D**). Bodyweights at weaning were not obtained in these adolescent cohorts. Transcriptome analyses of ELS vs. control animals in the adolescent cohorts revealed 18 DEGs in adolescent cohort 1 (11 up- and 7 downregulated, **Fig. 4B**) and 42 DEGs (24 up- and 18 downregulated, **Fig. 4C**, **supplementary table 1**) in adolescent cohort 2. Two genes (*Lct* and *Meg3*) were differentially expressed in both adolescent cohorts. Overall there was very limited overlap of DEGs between these two highly comparable adolescent reproducibility cohorts.

The adolescent reproducibility cohorts were performed in series and the samples were processed simultaneously. Therefore the groups could be pooled to increase the power of the analysis and assess the contribution of group size. Pooling increased the number of identified DEGs to 157 (66 up- and 91 downregulated, **Fig. 4D, supplementary table 1**), which showed that larger group sizes indeed aided the detection of DEGs. However, overlap-analysis of all DEGs revealed that the expression of 24 genes was no longer significantly altered after pooling both adolescent reproducibility cohorts. This may be an indication that ELS differently affected both cohorts - or part thereof - and that the inconsistent results were not merely driven by lack of power (**Fig. 4E**). This experiment showed that the effects of ELS on the hippocampal transcriptome were inconsistent even in tightly controlled conditions and indicated inherent variability. With a bigger sample size, the number of significant differences increased, but the fold-change of these genes was very modest.

DISCUSSION

We set out to investigate how ELS long-lastingly affects the mouse hippocampus at the chromatin and transcriptome level and subsequently assessed the reproducibility thereof. We initially found nominally significant effects on dorsal hippocampal chromatin accessibility that aided the identification of genes with altered expression. In line with these findings, follow-up studies using transcriptome analysis of multiple cohorts confirmed that ELS/LBN can alter the dorsal hippocampal transcriptome, but did not do so consistently. Assessment of the hippocampal transcriptome in two separate yet highly comparable adolescent reproducibility cohorts showed that controlling external variables (lab environment, researcher, animal supplier etc.) did not improve consistency in the results at cohort level. Altogether our data raise caution with respect to the

reproducibility and replicability and thereby usability of the LBN paradigm in its current form and practice – e.g. using relatively small groups of animals - to study molecular ELS effects in the hippocampus of male mice.

Effects of ELS and normalization by RU486

We opted to study the long-lasting molecular effects of ELS in male mice based on the reported behavioural effect after auditory fear conditioning and reversal thereof by GR antagonist RU486 intervention (21). The normalization of adult behaviour by adolescent intervention indicates a mechanism of programming that involves the GR in this reversal phenomenon and therefore likely also in the initial effects of ELS. To study these long-lasting effects we exposed the animals solely to ELS and an adolescent treatment intervention, but not to other stressors or any behavioural task before sacrifice which might alter the programming. We hypothesized hippocampal chromatin accessibility (rather than gene expression) would be affected by ELS, since mice function relatively "normal" until facing another stressor (second hit). Following this reasoning we assessed the overall hippocampal accessibility using ATAC-seq as it summarizes the state of the chromatin landscape (33), and may define responsiveness of the genome to and after an acute stressor as shown in the ventral hippocampus (34). However, this approach did not identify any regions with significant differential accessibility in the dorsal hippocampal, potentially reflecting a technical limitation of the technique rather than a definitive biological result. Since the dorsal hippocampus is composed of various cell types and subsets thereof, small changes that could be expected were likely diluted in the bulk approach (32). Nevertheless, follow up on nominally significant effects did identify differentially expressed genes by qPCR in a comparable cohort (1x RU – first adult cohort), which was ran in parallel to the cohort used for ATAC-seq (3x RU – first adult cohort). This validation suggested I) reproducibility within an experimental setting and II) focus on specific genes of interest (therefore not involving correction for multiple comparisons) did yield significant results, pointing to limited group size as one of the confounding factors. We could however not validate the gene expression data in an adult validation cohort performed by the same experimenter following the same LBN paradigm, though carried out in a different laboratory and in another hemisphere of the same animals that initially implicated the genes (3x RU – first adult cohort).

We also did not observe any normalization of gene expression as hypothesized, i.e. gene upregulation by ELS and downregulation after RU486 intervention or vice versa. Normalization by RU486 treatment after stress has been reported in other stress paradigms extending beyond the first weeks of life (22, 35-37), but the underlying mechanism remains elusive. Molecular normalization (if present) might involve other brain regions (38-40), or take place at a level different from mRNA expression or

chromatin accessibility. To reveal this a more comprehensive and integrated approach – encompassing multiple levels of regulation and different brain areas, and at cellular resolution – is likely required. Therefore, the aim of our project shifted from unravelling the molecular mechanism underlying ELS effects and normalization thereof, towards assessing the reproducibility of the transcriptome alterations after ELS.

Reproducibility of molecular changes after ELS

Given that this project gradually shifted towards assessing reproducibility of ELS effects based on progressive insight, the study has some limitations. Firstly, initial gene expression changes were validated in a cohort (1x RU – first adult cohort) that was performed in parallel, but was injected once during adolescence instead of three times. Despite this difference in treatment, the AM corticosterone levels of the vehicle groups were similar. This is reminiscent of earlier studies on chronic-stress induced changes in hippocampal neurogenesis. In these studies, multiple RU486 injections had very comparable effects as a single injection (41), suggesting that RU486 likely acts as a switch resetting the stress system. The differing injection frequencies may therefore not have largely affected the outcome of our study. Secondly, we compared data (between and within cohorts) acquired from the left and right hippocampus. Lateral difference has been described for adult hippocampal volume and activity-dependent c-fos expression in one study using 24h maternal deprivation paradigm on PND3 in rats (22), with strongest effects in the right hemisphere, similar to earlier findings after chronic stress in the prefrontal cortex (42). To the best of our knowledge no such hemispheric differences were reported for the transcriptome, but they cannot be excluded. Lastly, the adult cohorts were performed in different labs (Netherlands and Brazil) and the adolescence cohorts were performed by a different experimenter compared to the adult cohorts (female vs. male). Despite these differences, the key stress inducing components of the LBN paradigm were strictly followed for all cohorts. Therefore, the variable results at least indicate that the outcome of the model is sensitive to secondary (external and uncontrolled for) factors and cannot be compared between (and even within) laboratories. We cannot rule out that the relatively limited cohort sizes play a role, as was earlier indicated by meta-analyses on behavioural data after ELS (19, 43). Although one would expect less variability when studying processes close to the biological origin (e.g. gene transcription as opposed to behaviour), it is likely that studies involving expensive analyses such as the ATAC-seq are systematically underpowered. Of note, our current group sizes were relatively large compared to other studies using similar technologies.

Given the observed inconsistent effects of ELS on the hippocampal transcriptome we assessed two adolescent reproducibility cohorts. These cohorts were designed in a

manner to reduce variation that was thought to contribute to the inconsistency, e.g. different laboratory, experimenter or animal supplier. These experiments identified two transcripts that were differentially expressed in the whole hippocampus of both adolescent cohorts: long non-coding RNA *Meg3* and *Lct*. *Meg3* was earlier found to be upregulated after cued fear conditioning and has been shown to modulate the surface expression of AMPA receptors in primary cortical neurons, together indicating a functional role in synaptic plasticity (44). Increased *Meg3* mRNA levels – as we observed during adolescence after ELS - were also associated with neurological impairments after cerebral ischemia-reperfusion injury, which could be restored by MEG3 silencing (45). *Lct* (encoding for lactase) is well-known for its function in the intestines (metabolizing lactose to galactose and glucose) and its expression in enterocytes is regulated by epigenetic programming (46). The role of *Lct* in the brain has however not yet been studied, despite its very exclusive expression in the mouse hippocampus (47). A nutritional hypothesis could be postulated as stressing the dams with LBN could interfere with dietary lactose intake by nursing. However, assessment of maternal care did not show significant differences in nursing after LBN (20, 48) and it is even uncertain whether dietary lactose would reach the mouse brain. While *Meg3* and *Lct* are interesting targets for future studies, we argue the main finding is not the overlap but the extensive lack therefore. This raises the question whether LBN is too subtle a model for ELS (given the variable and inconsistent results at multiple levels, even when investigating processes in relatively homogenous tissue) or whether the variability is inherent to what is being modelled, reflecting a stochastic process. In the latter case we should adjust our investigative approaches accordingly, perhaps by empowering our experiments by using historical control data (43) or exploiting the variation rather than striving for consistency.

Despite thorough investigation of multiple cohorts, our transcriptome analyses of the (dorsal) hippocampi of ELS animals did not indicate any genes consistently regulated after ELS. A similar approach in *rats* (no additional disturbance besides LBN) was reported to alter the hippocampal expression of 142 genes, of which eight were validated in a separate cohort (49). None of these validated genes, but six other genes (*Bcar1*, *Lrtm2*, *Camk1g*, *Epha6*, *Myh11* and *Fndc4*) were also significantly altered in one of our ELS-cohorts (either adolescent or adult). Of the six overlapping with the Bolton study, only *Camk1g* and *Epha6* were altered in the same direction by ELS in one of our cohorts, which emphasizes variability of the ELS effects in the same model (although across species, laboratories and researchers).

The majority of animal studies in the field of neuroscience are underpowered (43). The effect of / necessity for a larger cohort was also evident from our adolescent

reproducibility study, as pooling of both cohorts identified over two-fold the number of DEGs. However, pooling also resulted in the loss of significance for transcripts that were identified as such in the separate per cohort analyses. These might entail false positives, which were identified due to the increased power, but they can also indicate that the transcriptome effects of ELS can differ per cohort. We hypothesize that ELS might influence the expression of two sets of genes: a tightly regulated set of genes (e.g. small effect sizes) underlying the core ELS effects and a set of genes that stochastically differ per cohort.

Variability of ELS effects - whether it concerns behaviour, corticosterone levels or gene expression - is well described and has previously been critically assessed (20, 50). From a translational perspective, not every individual who experiences adversity in early life goes on to later-life behavioural and/or emotional deficits, yet at a group level early life adversity is a well-established risk factor for psychiatric disorders (51-53). We showed transcriptional ELS effects display high degrees of inconsistency in tightly controlled mouse experiments, evident in our adolescent reproducibility cohorts. In addition, alterations differ depending on duration of ELS, the model used and the age of the animals (20). However, meta-analysis of behavioural ELS data showed there is a consistent, albeit small, effect on predominantly hippocampal and amygdala driven behaviours, most consistently found in rats (19). Large scale analysis of behavioural data also showed that the effects of ELS are strongest after a second hit (additional negative experience) and this may also be true for the effects on the hippocampal transcriptome. While this was out of scope for our study, as we essentially aimed to find what renders mice more sensitive to a second hit, the molecular alterations after a second hit might also be more robust as was shown for the behavioural outcomes.

Altogether, we conclude that variation is inherent to ELS as currently carried out, especially when investigating in a single – relatively small – cohort. Therefore we should aim to better design future studies to properly address and perhaps overcome this variability. As the LBN paradigm is based on aberrant maternal care, linking the quality/quantity of maternal care directly to the other outcome measures (e.g. gene expression, behaviour etc.) could proof beneficial as previously demonstrated with the licking and grooming model (54). Such a technically challenging approach will reduce inter-individual variation and may enable the use of feasible cohort sizes per condition. Moreover, it is more likely that a more comprehensive and integrated approach is required if we want to understand the mechanism underlying the long-lasting effects of ELS. Such a more comprehensive approach could consist of multiple omics approaches per animal at a cellular resolution, enabling to dissect the potential ELS effect in more depth – likely requiring machine learning approaches due to the expected and non-linear complexity.

REFERENCES

1. Tsigos C, Kyrou I, Kassi E, Chrousos GP. Stress: Endocrine Physiology and Pathophysiology. In: Feingold KR, Anawalt B, Boyce A, Chrousos G, de Herder WW, Dhatariya K, et al., editors. Endotext. South Dartmouth (MA)2000.
2. Chrousos GP. Stress and disorders of the stress system. Nat Rev Endocrinol. 2009;5(7):374-81.
3. Herman JP, McKlveen JM, Ghosal S, Kopp B, Wulsin A, Makinson R, et al. Regulation of the Hypothalamic-Pituitary-Adrenocortical Stress Response. Compr Physiol. 2016;6(2):603-21.
4. McEwen BS, Wingfield JC. The concept of allostasis in biology and biomedicine. Horm Behav. 2003;43(1):2-15.
5. Godoy LD, Rossignoli MT, Delfino-Pereira P, Garcia-Cairasco N, de Lima Umeoka EH. A Comprehensive Overview on Stress Neurobiology: Basic Concepts and Clinical Implications. Front Behav Neurosci. 2018;12:127.
6. de Kloet ER, Joels M, Holsboer F. Stress and the brain: from adaptation to disease. Nat Rev Neurosci. 2005;6(6):463-75.
7. Chen Y, Baram TZ. Toward Understanding How Early-Life Stress Reprograms Cognitive and Emotional Brain Networks. Neuropsychopharmacology. 2016;41(1):197-206.
8. Champagne DL, Bagot RC, van Hasselt F, Ramakers G, Meaney MJ, de Kloet ER, et al. Maternal care and hippocampal plasticity: evidence for experience-dependent structural plasticity, altered synaptic functioning, and differential responsiveness to glucocorticoids and stress. J Neurosci. 2008;28(23):6037-45.
9. Gans IM, Coffman JA. Glucocorticoid-Mediated Developmental Programming of Vertebrate Stress Responsivity. Front Physiol. 2021;12:812195.
10. Schmidt MV. Animal models for depression and the mismatch hypothesis of disease. Psychoneuroendocrinology. 2011;36(3):330-8.
11. Gluckman PD, Hanson MA, Beedle AS. Early life events and their consequences for later disease: a life history and evolutionary perspective. Am J Hum Biol. 2007;19(1):1-19.
12. Daskalakis NP, Bagot RC, Parker KJ, Vinkers CH, de Kloet ER. The three-hit concept of vulnerability and resilience: toward understanding adaptation to early-life adversity outcome. Psychoneuroendocrinology. 2013;38(9):1858-73.
13. Kuzminskaite E, Vinkers CH, Milaneschi Y, Giltay EJ, Penninx B. Childhood trauma and its impact on depressive and anxiety symptomatology in adulthood: A 6-year longitudinal study. J Affect Disord. 2022;312:322-30.
14. Stanton KJ, Denietolis B, Goodwin BJ, Dvir Y. Childhood Trauma and Psychosis: An Updated Review. Child Adolesc Psychiatr Clin N Am. 2020;29(1):115-29.
15. Rice CJ, Sandman CA, Lenjavi MR, Baram TZ. A novel mouse model for acute and long-lasting consequences of early life stress. Endocrinology. 2008;149(10):4892-900.

16. Millstein RA, Holmes A. Effects of repeated maternal separation on anxiety- and depression-related phenotypes in different mouse strains. Neurosci Biobehav Rev. 2007;31(1):3-17.

17. Meaney MJ. Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations. Annu Rev Neurosci. 2001;24:1161-92.

18. Orso R, Creutzberg KC, Wearick-Silva LE, Wendt Viola T, Tractenberg SG, Benetti F, et al. How Early Life Stress Impact Maternal Care: A Systematic Review of Rodent Studies. Front Behav Neurosci. 2019;13:197.

19. Bonapersona V, Kentrop J, Van Lissa CJ, van der Veen R, Joels M, Sarabdjitsingh RA. The behavioral phenotype of early life adversity: A 3-level meta-analysis of rodent studies. Neurosci Biobehav R. 2019;102:299-307.

20. Walker CD, Bath KG, Joels M, Korosi A, Larauche M, Lucassen PJ, et al. Chronic early life stress induced by limited bedding and nesting (LBN) material in rodents: critical considerations of methodology, outcomes and translational potential. Stress. 2017;20(5):421-48.

21. Arp JM, ter Horst JP, Loi M, den Blaauwen J, Bangert E, Fernandez G, et al. Blocking glucocorticoid receptors at adolescent age prevents enhanced freezing between repeated cue-exposures after conditioned fear in adult mice raised under chronic early life stress. Neurobiol Learn Mem. 2016;133:30-8.

22. Loi M, Sarabdjitsingh RA, Tsouli A, Trinh S, Arp M, Krugers HJ, et al. Transient Prepubertal Mifepristone Treatment Normalizes Deficits in Contextual Memory and Neuronal Activity of Adult Male Rats Exposed to Maternal Deprivation. eNeuro. 2017;4(5).

23. Mo A, Mukamel EA, Davis FP, Luo C, Henry GL, Picard S, et al. Epigenomic Signatures of Neuronal Diversity in the Mammalian Brain. Neuron. 2015;86(6):1369-84.

24. Corces MR, Trevino AE, Hamilton EG, Greenside PG, Sinnott-Armstrong NA, Vesuna S, et al. An improved ATAC-seq protocol reduces background and enables interrogation of frozen tissues. Nat Methods. 2017;14(10):959-62.

25. Buenrostro JD, Wu B, Chang HY, Greenleaf WJ. ATAC-seq: A Method for Assaying Chromatin Accessibility Genome-Wide. Curr Protoc Mol Biol. 2015;109:21 9 1- 9 9.

26. Ross-Innes CS, Stark R, Teschendorff AE, Holmes KA, Ali HR, Dunning MJ, et al. Differential oestrogen receptor binding is associated with clinical outcome in breast cancer. Nature. 2012;481(7381):389-93.

27. Heinz S, Benner C, Spann N, Bertolino E, Lin YC, Laslo P, et al. Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. Mol Cell. 2010;38(4):576-89.

28. Ramirez F, Ryan DP, Gruning B, Bhardwaj V, Kilpert F, Richter AS, et al. deepTools2: a next generation web server for deep-sequencing data analysis. Nucleic Acids Res. 2016;44(W1):W160-5.

29. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15(12):550.

30. Brionne A, Juanchich A, Hennequet-Antier C. ViSEAGO: a Bioconductor package for clustering biological functions using Gene Ontology and semantic similarity. BioData Min. 2019;12:16.

31. Anders S, Reyes A, Huber W. Detecting differential usage of exons from RNA-seq data. Genome Res. 2012;22(10):2008-17.

32. Viho EMG, Buurstede JC, Berkhout JB, Mahfouz A, Meijer OC. Cell type specificity of glucocorticoid signaling in the adult mouse hippocampus. J Neuroendocrinol. 2022;34(2):e13072.

33. Buenrostro JD, Giresi PG, Zaba LC, Chang HY, Greenleaf WJ. Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. Nat Methods. 2013;10(12):1213-8.

34. Caradonna SG, Paul MR, Marrocco J. An allostatic epigenetic memory on chromatin footprints after double-hit acute stress. Neurobiol Stress. 2022;20:100475.

35. Ding J, Chen X, da Silva MS, Lingeman J, Han F, Meijer OC. Effects of RU486 treatment after single prolonged stress depend on the post-stress interval. Mol Cell Neurosci. 2020;108:103541.

36. Ding J, da Silva MS, Lingeman J, Chen X, Shi Y, Han F, et al. Late glucocorticoid receptor antagonism changes the outcome of adult life stress. Psychoneuroendocrinology. 2019;107:169-78.

37. Papilloud A, Veenit V, Tzanoulinou S, Riccio O, Zanoletti O, Guillot de Suduiraut I, et al. Peripubertal stress-induced heightened aggression: modulation of the glucocorticoid receptor in the central amygdala and normalization by mifepristone treatment. Neuropsychopharmacology. 2019;44(4):674-82.

38. Bhatnagar S, Viau V, Chu A, Soriano L, Meijer OC, Dallman MF. A cholecystokinin-mediated pathway to the paraventricular thalamus is recruited in chronically stressed rats and regulates hypothalamic-pituitary-adrenal function. J Neurosci. 2000;20(14):5564-73.

39. Yang XD, Liao XM, Uribe-Marino A, Liu R, Xie XM, Jia J, et al. Stress during a critical postnatal period induces region-specific structural abnormalities and dysfunction of the prefrontal cortex via CRF1. Neuropsychopharmacology. 2015;40(5):1203-15.

40. Malter Cohen M, Jing D, Yang RR, Tottenham N, Lee FS, Casey BJ. Early-life stress has persistent effects on amygdala function and development in mice and humans. Proc Natl Acad Sci U S A. 2013;110(45):18274-8.

41. Hu P, Oomen C, van Dam AM, Wester J, Zhou JN, Joels M, et al. A single-day treatment with mifepristone is sufficient to normalize chronic glucocorticoid induced suppression of hippocampal cell proliferation. PLoS One. 2012;7(9):e46224.

42. Czeh B, Muller-Keuker JI, Rygula R, Abumaria N, Hiemke C, Domenici E, et al. Chronic social stress inhibits cell proliferation in the adult medial prefrontal cortex: hemispheric asymmetry and reversal by fluoxetine treatment. Neuropsychopharmacology. 2007;32(7):1490-503.

43. Bonapersona V, Hoijtink H, Consortium R, Sarabdjitsingh RA, Joels M. Increasing the statistical power of animal experiments with historical control data. Nat Neurosci. 2021;24(4):470-7.

44. Tan MC, Widagdo J, Chau YQ, Zhu T, Wong JJ, Cheung A, et al. The Activity-Induced Long Non-Coding RNA Meg3 Modulates AMPA Receptor Surface Expression in Primary Cortical Neurons. Front Cell Neurosci. 2017;11:124.

45. You D, You H. Repression of long non-coding RNA MEG3 restores nerve growth and alleviates neurological impairment after cerebral ischemia-reperfusion injury in a rat model. Biomed Pharmacother. 2019;111:1447-57.

46. Oh E, Jeremian R, Oh G, Groot D, Susic M, Lee K, et al. Transcriptional heterogeneity in the lactase gene within cell-type is linked to the epigenome. Sci Rep. 2017;7:41843.

47. Allen Brain Atlas. Mouse Brain Atlas - *Lct* expression 2022 [Available from: https://mouse.brain-map.org/experiment/show/70218745.

48. Molet J, Heins K, Zhuo X, Mei YT, Regev L, Baram TZ, et al. Fragmentation and high entropy of neonatal experience predict adolescent emotional outcome. Transl Psychiatry. 2016;6:e702.

49. Bolton JL, Schulmann A, Garcia-Curran MM, Regev L, Chen YC, Kamei N, et al. Unexpected Transcriptional Programs Contribute to Hippocampal Memory Deficits and Neuronal Stunting after Early-Life Adversity. Cell Reports. 2020;33(11).

50. Murthy S, Gould E. Early Life Stress in Rodents: Animal Models of Illness or Resilience? Front Behav Neurosci. 2018;12:157.

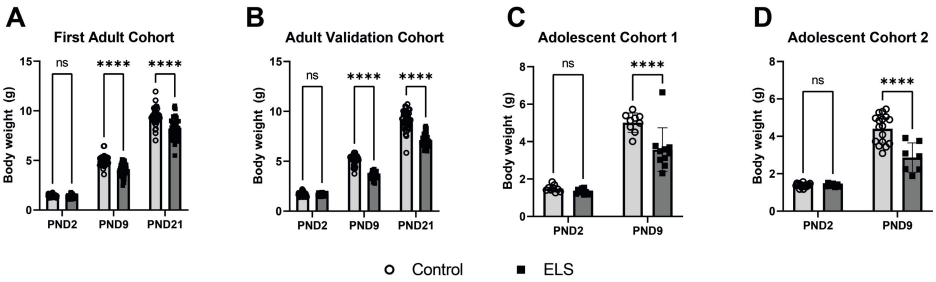
51. Kessler RC, McLaughlin KA, Green JG, Gruber MJ, Sampson NA, Zaslavsky AM, et al. Childhood adversities and adult psychopathology in the WHO World Mental Health Surveys. Br J Psychiatry. 2010;197(5):378-85.

52. LeMoult J, Humphreys KL, Tracy A, Hoffmeister JA, Ip E, Gotlib IH. Meta-analysis: Exposure to Early Life Stress and Risk for Depression in Childhood and Adolescence. J Am Acad Child Adolesc Psychiatry. 2020;59(7):842-55.

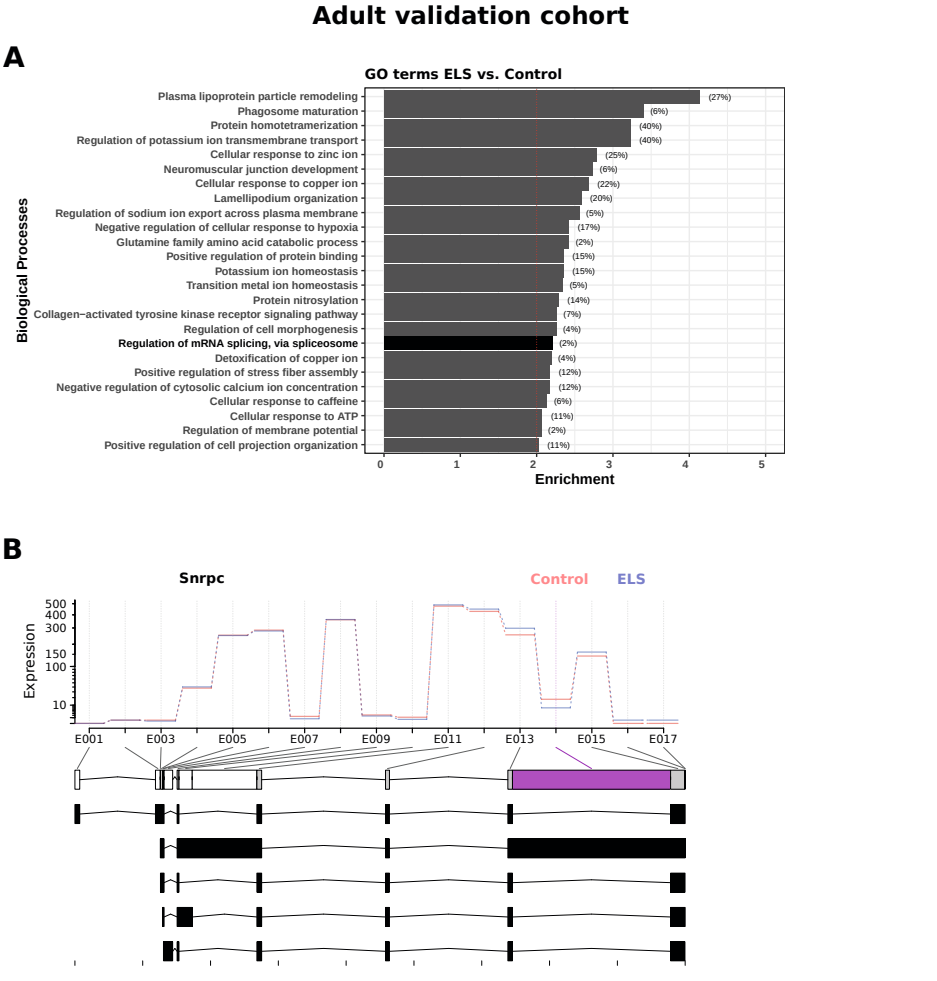
53. Teicher MH, Samson JA, Anderson CM, Ohashi K. The effects of childhood maltreatment on brain structure, function and connectivity. Nat Rev Neurosci. 2016;17(10):652-66.

54. Liu D, Diorio J, Tannenbaum B, Caldji C, Francis D, Freedman A, et al. Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. Science. 1997;277(5332):1659-62.

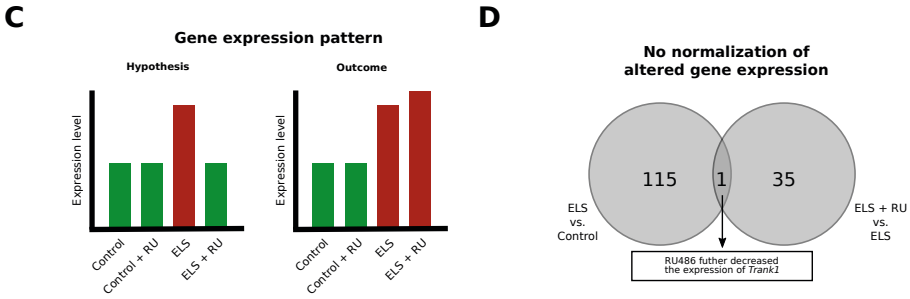
SUPPLEMENTARY FIGURES:



Supplemental Figure 1: Early life stress consistently resulted in a significantly lower body weight. Body weights (in grams) of the studied ELS cohorts: first adult cohort (A), adult validation cohort (B), adolescent cohort 1 (C) and adolescent cohort 2 (D). ELS: early life stress, PND: postnatal day.



Supplemental Figure 2: Early life stress induced alternative splicing was not normalized by adolescent RU486 intervention.



Supplemental Figure 2 continued: (A) Results of the GO term enrichment analyses for biological processes on all DEGs after ELS versus controls of the adult validation cohort. Regulation of mRNA splicing is highlighted in bold. (B) Example of significant differential exon usage of exon 14 of *Snrpc*, confirming different mRNA splicing after ELS. (C) Gene expression patterns for the groups of the adult validation cohort. Hypothesized expression pattern illustrating normalization by RU486 intervention (left) and schematic summary of the actual outcome on the hippocampal transcriptome without normalization (right). (D) Venn diagrams confirming absence of normalization for any gene after RU486 normalization in the context of ELS. DEGs: differentially expressed genes, ELS: early life stress, GO: gene ontology, RU: RU486.

Supplementary Table 1: Output of all transcriptome analyses performed, sorted per cohort analysed.

Not included

Supplementary Table 2: Results of the GO term analysis – biological processes on the DEGS between ELS and control animals of the adult validation cohort.

Not included

Supplementary Table 3: Results of the differential exon usage analysis performed on the ELS and control animals of the adult validation cohort.

Not included