Age-dependent impact of early-life stress on glia and synapses
Substrates for increased risk for Alzheimer's disease
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Effects of early-life stress on blood brain barrier morphology at basal state and in response to acute stress exposure: A preliminary report

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**Abstract**

Early-life stress (ES) is associated with lasting vulnerability to later-life disorders such as depression and Alzheimer’s disease (AD). An emerging hallmark of such psychopathologies is blood-brain barrier (BBB) dysfunction. Whether these BBB alterations have an early-life origin and/or if ES impacts the BBB, remains elusive. The BBB is established via endothelial cell expression of tight junction proteins. Maintenance of the BBB is modulated by interactions between endothelial cells and other vessel-adjacent cell types such as astrocytes, and they together form the neurovascular unit (NVU). Given that (chronic) stress exposure in adulthood impairs BBB integrity, as well as the early postnatal timing of BBB induction, we hypothesize that ES might lead to long-term impacts on BBB integrity. Considering the evidence for sex-specific effects of ES, and the strong sex bias in the prevalence and presentation of ES-related psychopathologies, we also hypothesize that ES effects on the BBB are sex-specific. To tackle these questions, we studied the long-term effects of ES on BBB properties both at basal state as well as after a second hit via restraint stress (RS) to unmask potential latent ES effects.

Male and female mice were exposed to ES using the limited bedding and nesting model from postnatal days (P) 2-9. At P120, mice were exposed to RS for 30 min while the other half remained undisturbed. Sixty minutes after restraint, mice were anesthetized and saline-perfused, with half the brain enriched for endothelial cells via magnetic-activated cell sorting, and half immersed in paraformaldehyde for immunohistochemistry. We studied BBB and NVU morphology by staining for CD31, a marker for endothelial cells, Claudin-5 (Cldn5), the main tight junction protein in the brain, and GFAP, a marker for astrocytes. We present here preliminary results regarding physiological measurements (body weight, corticosterone levels, adrenal weights) in males and females, and BBB morphology in male mice. Further analyses in female tissues are ongoing.

RS increased plasma corticosterone levels, which was not further modulated by ES or sex. Female mice had higher relative adrenal weights compared to males, regardless of stressors. ES led to increased CD31 coverage in the ventral hippocampus, accompanied by increased coverage of Cldn5 within and GFAP around endothelial cells. Additionally, RS led to increased CD31 coverage in the dorsal hippocampus CA in control, but not ES, mice. These measures were specific to the hippocampus and were not affected in the prefrontal cortex and nucleus accumbens.

Our data suggest ES induced long-term alterations to BBB integrity in male mice, which might be mediated by astrocyte-endothelial cell interactions. We also found that ES history alters the dorsal hippocampal vascular response to acute stress in adulthood. Characterization of the impact of ES on endothelial cell gene expression profiles from the same mice will provide further insights into the molecular alterations of endothelial cells that might contribute to the observed alterations in CD31. The analyses of if and how female mouse BBB and NVU are impacted by ES and RS is currently ongoing.
Introduction

Early-life stress exposure (ES) is associated with later-life vulnerability for cognitive decline, psychopathologies, and dementias\(^1\)\(^-\)\(^5\). Beyond the identification of early risk factors, progress in understanding the etiology and substrates of these diseases have implicated blood brain barrier (BBB) disruption to play an important role in disease progression\(^6\)\(^-\)\(^8\). The BBB is a physical structure formed by endothelial cells in the central nervous system that limits the exchange of molecules into and out of the brain parenchyma\(^9\). It restricts the paracellular flow of hydrophilic molecules by the expression of tight junctions proteins\(^10\)\(^-\)\(^13\), and regulates the entry of other macromolecules via a variety of transporters\(^14\)\(^,\)\(^15\).

BBB regulation is a highly dynamic process, and can be modulated by extracellular signals, including stress and its associated hormones\(^16\)\(^-\)\(^20\). This sensitivity to stress is evidenced by the ability of both acute\(^17\)\(^,\)\(^21\),\(^22\) and chronic\(^16\)\(^,\)\(^23\) stressors to modulate BBB permeability\(^24\). The BBB’s stress susceptibility\(^7\),\(^13\) is particularly pertinent in development, as proper BBB integrity and function is established by postnatal days (P) 7-9 in mice\(^25\)\(^-\)\(^27\).

Considering the early postnatal timing of BBB induction, it is plausible to assume that early-life stress might lastingly impact BBB function and integrity. Indeed, there is initial evidence that both prenatal and postnatal stressors can impact BBB maturation in rodents, as brains of rats exposed to either prenatal maternal forced swim stress, or postnatal maternal separation have increased permeability to dyes between P10-20, which were then comparable to control levels by P30\(^28\). Additionally, maternally separated rats exhibit sex-, and region-specific changes to tight junction gene expression, for instance increased Claudin-5 (Cldn5) in the hippocampus and striatum at P15\(^29\). Given that programming by ES exposure has been shown to modulate brain and physiological response to later life challenges\(^30\)\(^,\)\(^31\), and that such later-life challenges can unmask potentially latent effects of ES\(^30\),\(^32\), we aimed to characterize the BBB of adult mice exposed to ES, and investigate whether they would have differential adaptations to acute stress.

The induction and maintenance of the BBB is an active process, regulated by the interactions of endothelial cells and surrounding cell types\(^33\)\(^,\)\(^34\), which altogether form the neurovascular unit (NVU)\(^8\). Given that ES leads to early and lasting changes to the astrocytic\(^35\),\(^36\) profile, and the important role of these cells at the NVU\(^33\)\(^,\)\(^34\), we also aimed to investigate whether ES impacts endothelial cell interactions with astrocytes, and if this would contribute to ES effects on the BBB.

In the current study, we therefore investigated the effects of both early-life (by limiting the bedding and nesting material) and later-life (by acute restraint) stress exposure on BBB morphology, astrocyte-endothelial cell contact, endothelial cell gene expression, and how these potential changes might relate to HPA axis reactivity. Given the reported sex differences in susceptibility to ES phenotypes\(^37\), as well as BBB adaptations\(^29\),\(^38\), we studied these aspects in mice of both sexes. We present preliminary data concerning male mice, which suggest that ES alters vascularization, tight junction protein expression, and astrocyte coverage around blood vessels in distinct hippocampal subregions. Analyses of the impact of ES and acute stress on these same parameters in the female mice, as well as the gene
expression profile of enriched endothelial cells enriched from mice of all experimental groups, are currently ongoing.

**Materials and Methods**

**Animals and experimental design**

Wildtype mice on a C57BL/6J background were used in this study (Fig. 1A). Breeding was done as previously described. Pairs of 8-10 week old virgin females were housed with breeding males for a week, and singlehouse two weeks later. Dams were checked for pups during the first two hours of the light phase 18 days after start of breeding. If nests were found, the previous day was assigned as postnatal day (P) 0 for the pups.

Mice were placed under standard housing conditions, defined as a temperature of 20–22°C, 40–60% humidity, cage enrichment and ad libitum standard chow and water. The mice were kept on a standard 12/12 h light/dark schedule (lights on at 8 a.m.). Experimental procedures were conducted according to the Dutch national law and European Union directives on animal experiments and were approved by the animal welfare committee of the University of Amsterdam.

**Early-life stress paradigm**

Nests were randomly assigned to CTL or ELS conditions from P2-9. CTL nests were housed in standard cage environments, with 100g of sawdust and a 5x5cm nestlet for nesting. ES nests were housed on a fine-gauge stainless steel mesh on top of 1/3 amount of CTL bedding material, along with half of a 2x2 nestlet. Mice were taken out of condition into standard nests and left undisturbed aside from weaning at P21. Body weights were measured at P2, P9, and P21.

**Restraint stress paradigm**

At P120, mice were randomly assigned to the restrained (restraint stressed, RS) or non-restrained (non-RS) groups. Within the first 2h of the light phase, mice subjected to the restrained group were placed into 50ml falcon tubes with holes drilled on the sides and tip for 30min. Mice were returned to their home cages afterwards.

**Tissue collection**

Mice were generated to obtain 1) perfused fixed brains for staining, and 2) CD31-MAC-sorted cells for downstream analysis. Tail blood was similarly collected from RS mice 24h before restraint to establish a baseline, and again at 60min after end of restraint. At this time point, RS mice were injected with 120mg/kg Euthasol and perfused with cold 1x Dulbecco's Phosphate Buffer Solution (DPBS, Gibco). Mouse brains were extracted, with half a hemisphere being collected in Hanks Buffer Salt Solution (HBSS, Gibco) containing 15 mM HEPES (Lonza, BE17-737E) and 0.6% glucose (Sigma-Aldrich, G8769), and the other half drop-fixed into cold 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB, pH 7.4) overnight. Both adrenal glands were dissected and weighed. Tail blood was collected from non-RS at a similar time point to T3.5, after which they were sacrificed in a similar manner.
PFA-fixed hemispheres were cryoprotected in a 15%, then 30%, sucrose solution in 0.1M PB before being sliced into 40µm thick coronal sections using a sliding microtome. Brains were stored in antifreeze at -20°C until use.

**CORT measurement**
We separated the plasma from the cellular component of the collected tail blood by centrifugation for 15min at 14000rpm, 4°C. Corticosterone concentrations were further measured with a high-sensitive corticosterone enzyme immunoassay (IDS Ltd, Boldon Colliery, UK).

**Magnetic activated cell sorting (MACS)**
To reduce myelin content, we removed the midbrain and below from hemispheres obtained in Cohort 2, before preparing a single-cell suspension using the neural tissue dissociation kit (P) according to manufacturer instructions (Miltenyi, 130-092-628). Brains were cut into smaller pieces using micro-scissors, before centrifuging for 2min at 300xg, RT. The supernatant was removed and replaced with a mix of enzyme P in buffer X pre-warmed to 37°C. Samples were then moved to a water bath at 37°C for 15min, at which point a mix of enzyme A and buffer Y were added to the mix. Ten minutes after enzyme A was added to the last sample, samples were homogenized in a Dounce using the loose pestle for 20 strokes before passing through a pre-wet 70µm filter. The suspension was centrifuged for 5min at 400xg, 4°C. We further removed myelin from the pellet using a 0.9M sucrose cushion as previously described\(^41\), centrifuging at 850xg and discarding the supernatant. This step was done twice.

The pellet was then resuspended in 180µl of MACS buffer (1x DPBS with 2mM EDTA, 0.5% bovine serum albumin). We then incubated the samples with 20µl of anti-mouse-CD31 microbeads (Miltenyi, 130-097-418) for 20min at 4°C in the dark. Cells were washed with 1ml MACS buffer and centrifuged for 5min at 400xg, 4°C. Samples were then passed through pre-wet 70µm cell strainer placed on top of MACS MS columns (130-042-201). The flow through was collected as the CD31\(^{\text{pos}}\) fraction, while the CD31\(^{\text{neg}}\) fraction was collected by adding 1ml MACS buffer and flushing out after removal from the magnetic stand. Both CD31\(^{\text{pos}}\) and CD31\(^{\text{neg}}\) fractions were centrifuged for 5min at 400xg, 4°C, after which we added 200µl Trizol (Thermofisher, 15596026) to collect the samples for downstream analysis.

**Validation of CD31 MACS**
We validated the enrichment of CD31 cells after MACS using both qPCR and flow cytometry (Supplementary Figure 1). RNA was extracted from CD31\(^{\text{pos}}\) and CD31\(^{\text{neg}}\) cells based on Trizol manufacturer’s instructions. CDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen, 18064022) and stored at -20°C until further processing. Relative gene expression was assessed via PCR amplification of cDNA using the Hot FirePol Evagreen qPCR supermix (Solis Biodyne, 08-24-00001). Primer sequences used are shown in Table 1 and were tested to have 90-110% efficiency in MAC-sorted samples. FACS validation was done by incubating the CD31\(^{\text{pos}}\) and CD31\(^{\text{neg}}\) fraction from one mouse, and a whole brain lysate from a second mouse (after sucrose cushion step) first in a blocking mix consisting of MACS buffer with anti-mouse Fc receptor (5 µg/ml, eBioscience, 14-0161) for 15min, then in a MACS buffer mix containing anti-mouse CD31-PE antibody (2µg/mL, Invitrogen, 12-
Stainings were done at 4°C. After adding DAPI (0.15 µg/ml, Biolegend, 422801), cells were loaded and analyzed using a BD Facs Diva, gating for single, live (DAPI-) cells and analyzing the proportion of the CD31<sup>pos</sup> cells relative to all cells.

**Immunofluorescence**

To visualize BBB morphology, we performed a sequential triple immunostaining against CD31, Claudin-5, and glial fibrillary acidic protein (GFAP). First, sections were stained free floating with anti-CD31 (1:150, rat monoclonal, BD Biosciences, 550274). Following 1h incubation at RT and overnight incubation at 4°C, sections were incubated with secondary antibody (1:800, donkey anti-Rat-A647 pre-adsorbed polyclonal, Abcam, ab150155) for 2h at RT, then mounted onto pre-coated slides (Superfrost Plus slides, Menzel). After drying, slides were subject to 15 minutes of antigen retrieval at 100°C in citrate buffer (pH 6.0), after which they were incubated with antibodies against Claudin-5 (1:250, mouse monoclonal conjugated to A488, Invitrogen, 352588) and GFAP (1:1500, rabbit polyclonal, Dako, Z0344) for 1h at RT and overnight at 4°C. A second incubation with secondary antibody (donkey anti-rabbit-A568, Invitrogen A11057) was done for 2h at RT. Brains were washed with 0.01M Tris-buffered saline (pH 7.6) between all staining steps, and aspecific binding was blocked by incubating slides in a blocking mix containing 0.3% Triton and 5% normal donkey serum in 0.01M TBS for 1h at RT. We confirmed in test sections the persistence of the CD31-A647 signal after antigen retrieval, which was necessary for proper antibody binding to Claudin-5 (Supplementary Figure 2).

**Image Analysis**

We imaged 0.5µm-step Z-stacks (total Z range: Prefrontal Cortex and Nucleus Accumbens – 10µm, Hippocampus – 5.5µm, Fig. 1B) at 40x magnification using a Nikon A1 confocal microscope. Images were taken from two sections of the PFC and NAcc, and six sections along the dorsoventral axis hippocampus (Bregma point -1.34mm to -3.88mm) to obtain an even representation. Each image was a stitched composite (2 wide, 4 tall) such that all hippocampal subregions (Stratum Oriens in the Cornu Ammonis [CA] to the Hilus in the dentate gyrus [DG]) were visible in each image.

Images were analyzed with FIJI (v1.53q) by first drawing regions of interest to define the cornu ammonis (CA) and dentate gyrus (DG) regions. Per slice, binary masks of CD31 signal were generated using an automated threshold followed by particle analysis to reduce noise. Hippocampal CD31 area, as well as the automated thresholded area of Cldn5 and GFAP, was measured, with coverage being defined as (Clbn5 or GFAP area/CD31 area). While Clbn5 coverage analysis was similarly analyzed in the PFC and NAcc, we were not able to measure CD31 coverage in these regions due to their ambiguous spatial boundaries in the DAPI channel. GFAP coverage was also not measured in the PFC and NAcc due to the low basal expression of the protein in these regions<sup>35,42,43</sup>. Statistical analyses and plotting were done using R.
Results

Effects of ES and RS on body weight, CORT levels, and adrenal gland weight

We confirmed in our mice the reported\textsuperscript{39,44} decreased body weight gain between postnatal days (P) 2-9 as a result of early-life stress (ES) exposure (Two-way ANOVA – Early-life condition: F(1,19)=43.419, p<0.001; Sex: F(1,19)=0.1718, p=0.6832; Interaction: F(1,19)=0.0107, p=0.9189; Fig. 1C).

![Figure 1](image-url)

\textbf{Figure 1.} Physiological readouts from the experiment
(A) Experimental design. Mice were exposed to early-life stress using the limited bedding and nesting model from postnatal days (P) 2-9. At P120, mice were subject to restraint stress for 30min, and then sacrificed 60min thereafter.
(B) Brain regions analyzed in the current immunofluorescence analysis. Reference atlas from the Allen Mouse Brain Atlas, atlas.brain-map.org. (C) Decreased body weight gain between P2-9 as a result of early-life stress exposure.
(D) Increased plasma corticosterone levels in restrained (RS) vs non-restrained (nonRS) mice.
(E) Increased plasma corticosterone levels in restrained mice 60 min after end of stress exposure. Basal CORT was taken 24h before the experiment.
(F) Female mice have increased relative adrenal weights regardless of early-life condition or restraint exposure. *, early-life condition effect, p<0.05; #, restraint stress effect, p<0.05; @: sex effect, p<0.05
Moreover, in line with previous observations⁴⁵, plasma CORT levels were elevated in restrained (RS) vs non-restrained (non-RS) mice, with female mice having higher CORT levels than males (Three-way ANOVA – early-life condition: $F(1,58)=0.1450$, $p=0.7048$; restraint: $F(1,58)=46.4287$, $p<0.001$; sex: $F(1,58)=4.0844$, $p=0.0479$; early-life condition*restraint: $F(1,58)=0.3927$, $p=0.5333$; early-life condition*sex: $F(1,58)=0.4504$, $p=0.5048$; restraint*sex: $F(1,58)=2.8286$, $p=0.0980$; early-life condition*restraint*sex: $F(1,58)=1.7713$, $p=0.1884$; Fig. 1D). This was not affected by ES. There was no effect of early-life condition or sex on the increase in CORT levels within RS mice (Two-way ANOVA – Early-life condition: $F(1,29)=0.1015$, $p=0.7523$; sex: $F(1,29)=3.3914$, $p=0.0758$; Interaction: $F(1,29)=3.5037$, $p=0.0714$; Fig. 1E).

Lastly, we found that female mice had increased relative adrenal weights, with no interactions with either ES or RS (Three-way ANOVA – early-life condition: $F(1,56)=0.5762$, $p=0.4510$; restraint: $F(1,56)=1.5711$, $p=0.2153$; sex: $F(1,56)=96.3070$, $p<0.001$; early-life condition*restraint: $F(1,56)=0.0142$, $p=0.9057$; early-life condition*sex: $F(1,56)=0.0887$, $p=0.7670$; restraint*sex: $F(1,56)=0.0007$, $p=0.9784$; early-life condition*restraint*sex: $F(1,56)=0.9950$, $p=0.3228$; Fig. 1F).

### Early-life stress and restraint stress alter CD31 immunoreactivity in a hippocampal subregion specific manner

To visualize alterations to vascular morphology in the hippocampus after ES and RS exposure, we quantified the immunoreactivity of CD31, a commonly used marker for endothelial cells⁴⁶ (Fig. 2A). We found interaction effects between early-life condition and later-life restraint on CD31 signal in the dorsal hippocampus (Fig. 2B, Dorsal-CA – early-life condition: $F(1,24)=2.8624$, $p=0.1036$; restraint: $F(1,24)=0.0316$, $p=0.8605$; interaction: $F(1,24)=5.9368$, $p=0.0226$; Dorsal-DG – early-life condition: $F(1,23)=0.1414$, $p=0.7104$; restraint: $F(1,23)=0.0024$, $p=0.9611$; interaction: $F(1,23)=9.0939$, $p=0.006$).

These interaction effects are explained by an RS-induced increase in CD31 vessel area in control but not ES mice, especially in the especially in the DG (Tukey corrected posthoc tests, CA – CTL-nonRS vs CTL-RS, $p=0.0771$; ES-nonRS vs ES-RS, $p=0.123$; DG – CTL-nonRS VS CTL-RS, $p=0.0278$; ES-nonRS vs ES-RS, $p=0.0713$).

In the ventral hippocampus, ES led to an increase in the amount of CD31 immunoreactivity, which was not affected by RS (Ventral-CA – early-life condition: $F(1,25)=4.9634$, $p=0.0351$; restraint: $F(1,25)=0.5982$, $p=0.4463$; interaction: $F(1,25)=3.5975$, $p=0.0695$; Ventral-DG – early-life condition: $F(1,26)=5.5532$, $p=0.0263$; restraint: $F(1,26)=0.0449$, $p=0.8339$; interaction: $F(1,26)=1.811$, $p=0.1899$).

### Early-life stress increases Claudin-5 coverage within endothelial cells in the ventral CA

To investigate effects of ES and RS on BBB integrity, we measured differences in the immunoreactivity for Claudin-5 (Cldn5), the major tight junction protein in the brain¹³. We found that ES exposure increased Cldn5 coverage inside CD31 blood vessels specifically in the ventral CA region of the hippocampus, which was not further affected by RS (Fig. 3A, 3B, Ventral-CA – early-life condition: $F(1,22)=9.8425$, $p<0.01$; restraint: $F(1,22)=4.007$, $p=0.0578$; interaction: $F(1,22)=0.3279$, $p=0.5727$).
Blood-brain barrier alterations after early-life stress and later-life restraint

A

![Representative images show dorsal CA CD31 images from each experimental group.](image1)

B

**Figure 2.** Early-life stress and restraint stress alter hippocampal CD31 coverage

(A) Representative images show dorsal CA CD31 images from each experimental group. (B) In the dorsal hippocampus, restraint stress (RS) leads to increased CD31 coverage in control (CTL) but not early-life stressed (ES) mice. ES leads to increased CD31 coverage in the ventral hippocampus, without interaction with RS. *, early-life condition effect, p<0.05; &, restraint stress effect, p<0.05

This was not present in the dorsal CA, dorsal dentate gyrus (DG), or ventral DG (Fig. 3B, Dorsal-CA – early-life condition: F(1,26)=1.8117, p=0.1899; restraint: F(1,26)=0.2399, p=0.6284; interaction: F(1,26)=0.2983, p=0.5896); Dorsal-DG – early-life condition: F(1,24)=0.6463, p=0.4293; restraint: F(1,24)=3.0913, p=0.0915; interaction: F(1,24)=7.246, p=0.0431; Ventral-DG – early-life condition: F(1,26)=0.4027, p=0.5312; restraint: F(1,26)=0.3351, p=0.5676; interaction: F(1,26)=0.6850, p=0.4154).

In contrast, we did not observe effects of ES or RS on Cldn5 coverage within CD31 endothelial cells in the prefrontal cortex (early-life condition: F(1,22)=0.236, p=0.632; restraint: F(1,22)=1.178, p=0.29; interaction: F(1,22)=0.068, p=0.797; Fig. 3C,D) or the nucleus accumbens (early-life condition: F(1,23)=0.570, p=0.458; restraint: F(1,23)=0.131, p=0.721; interaction: F(1,23)=0.014, p=0.906; Fig. 3E,F).
Early-life stress leads to increased expression of Claudin-5 in CD31+ endothelial cells in the ventral CA subregion hippocampus

(A) Representative images show Claudin-5 (Cldn5) expression inside CD31+ endothelial cells in the ventral cornu ammonis (CA) from non-restrained (non-RS) control (CTL) and early-life stressed (ES) mice. (B) ES decreases Cldn5 coverage inside CD31+ endothelial cells specifically in the ventral CA region of the hippocampus. Cldn5 coverage inside CD31+ endothelial cells in the prefrontal cortex (PFC, C-D) or nucleus accumbens (NAcc, E-F) is not affected by ES or later-life restraint (RS). *, early-life condition effect, p<0.05

Early-life stress increases astrocytic coverage around endothelial cells in the ventral hippocampus

To visualize astrocyte-endothelial cell contacts, we next assessed GFAP coverage within CD31-generated masks. ES increased GFAP coverage in the ventral hippocampus, specifically in the CA region, without further impact of RS (Fig. 4A, 4B, Ventral-CA – early-life condition: F(1,26)=4.3784, p=0.0463; restraint: F(1,26)=0.0916, p=0.7646; interaction: F(1,26)=0.7789, p=0.3856; Ventral-DG – early-life condition: F(1,26)=3.576, p=0.0698; restraint: F(1,26)=0.1157, p=0.7365; interaction: F(1,26)=1.1237, p=0.7279).

Neither ES or RS affected GFAP coverage in the dorsal hippocampus (Fig. 4B, Dorsal-CA – early-life condition: F(1,24)=0.0084, p=0.9278; restraint: F(1,24)=0.0303, p=0.8633;
interaction: $F(1,24)=0.1487$, $p=0.7032$; Dorsal-DG – early-life condition: $F(1,23)=0.0018$, $p=0.9669$; restraint: $F(1,23)=0.8611$, $p=0.3631$; interaction: $F(1,23)=0.4096$, $p=0.5285$).

![Figure 4](image)

**Figure 4.** Early-life stress increases GFAP coverage around CD31+ endothelial cells in the ventral CA subregion of the hippocampus. (A) Representative images showing GFAP around CD31+ endothelial cells in the ventral cornu ammonis (CA) region of non-restrained (non-RS) control (CTL) and early-life stressed (ES) mice. (B) GFAP coverage is increased after ES exposure specifically in the ventral CA region of the hippocampus. *, early-life condition effect, $p<0.05$

**Discussion**

In this project, we aimed to investigate the effects of early-life stress (ES) exposure via the limited bedding and nesting (LBN) paradigm, later-life restraint stress (RS), and their interaction on the morphology of the neurovascular unit (NVU) and the blood-brain barrier (BBB). ES increased the immunoreactivity of CD31+ endothelial cells in the ventral hippocampus, accompanied by increased Claudin-5 (Cldn5) and GFAP reactivity within the endothelial cells. We also found that RS increased CD31 coverage in the dorsal hippocampus of control (CTL) but not ES mice. While we are still conducting our analyses on the female BBB morphology, we expect to find sex-dependent ES effects of ES considering the sex-dependent effects of adult stress on brain vasculature and BBB.

**ES did not affect HPA axis reactivity**

We did not see ES modulation of either the increase in plasma CORT within RS animals or the difference in CORT between RS and non-RS mice 60 min post-restraint. While the latter is in line with the findings from Bonapersona et al. when male mice were studied 50 min post-RS, there are notable discrepancies in literature regarding the stress response in ES-exposed rodents. For instance, in two studies using the same limited bedding and nesting model, one reported a blunted CORT response after a 30 min restraint in male P35 mice, while another reported increased CORT response to a 20 min RS in P70 female rats. While these could reflect differences in models and model implementation, it could also of course be due to individual differences in stress susceptibility.
ES leads to lasting effects to the NVU, specifically in the ventral hippocampus in male mice

Our data suggest that ES leads to lasting changes in the vasculature, especially in the ventral hippocampus. To our knowledge, this is the first description of such long-term impact of ES on brain vasculature. Importantly, this in line with the evidence that both prenatal stress and postnatal maternal separation accelerated microvessel growth at the expense of capillary thickness in rat pups\textsuperscript{28,50}. Vascularization can be seen as an adaptive process, essential in the response to insults such as hypoxia\textsuperscript{51}, lesions\textsuperscript{52} and ischemia\textsuperscript{53}. While the effects of (early) stress on this process is not well studied, data from a cohort of individuals exposed to early life famine indicate lasting decreases in brain perfusion\textsuperscript{54}. In this context, our data might suggest increased vascularization as a compensatory mechanism. Considering the emerging role of angiogenesis in regulating neurogenesis\textsuperscript{55}, as well as the key role of the vasculature in delivering important substrates such as oxygen to the brain\textsuperscript{56}, it will be important to study whether these alterations could be related to the neuro-metabolic phenotypes previously described\textsuperscript{57,58}.

The consequences of increased vascularization, especially with respect to nutrient and substrate delivery, of course also depend on tight junctions, of which Cldn5 is the most important in the brain\textsuperscript{13,59,60}. The increase in Cldn5 within endothelial cells in the ES hippocampus, along with the increased amount of CD31 area, suggests an overall increased expression of tight junctions in the ES-affected BBB. This observation is in line with the described increase in Cldn5 mRNA expression in the hippocampus of maternally separated rats at P15 and P70\textsuperscript{29}. Interestingly, our data contrast with studies on the effects of chronic stress during adulthood on the BBB. Repeated restraint stress decreased Cldn5 expression in the rat hippocampus\textsuperscript{17} and amygdala\textsuperscript{22}, and chronic social stress increased BBB permeability, accompanied by decreased Cldn5 expression, especially in stress susceptible mice\textsuperscript{23}. Another difference between the effects of early-life vs adult stress exposure on the BBB is the affected brain region. In fact, while chronic stress effects on the BBB have been mostly reported to take place in the prefrontal cortex\textsuperscript{29,38}, and nucleus accumbens\textsuperscript{16,23}, the ES effects we found were restricted to the hippocampus. This specificity might be, in part, a consequence of the increased hippocampal vulnerability to developmental stressors, due to its relatively enriched expression of the glucocorticoid and mineralocorticoid receptors\textsuperscript{61}, especially during the early postnatal period\textsuperscript{62}.

In line with this, while we can only speculate about the mechanisms underlying such divergent effects of early versus adult stress exposure, it is important to note that the ES we induce occurs during a sensitive developmental period for both NVU and BBB induction\textsuperscript{25–27} and hippocampal development\textsuperscript{63,64}. Previous findings in adult mice exposed to chronic social stress have demonstrated epigenetic alterations to be key to stress-induced decrease expression of Cldn5 in brains of stress susceptible mice\textsuperscript{16}. Given the evidence for ES-induced epigenetic modifications in hippocampal gene expression\textsuperscript{65}, we might expect to find epigenetic alterations in endothelial cells that match our results. Further work (including e.g., our ongoing characterization of endothelial cell gene expression, and future studies on vascular perfusion, permeability, nutrient sensing, or transporters) will be needed to determine more details on how ES lastingly alters BBB morphology and function. Moreover,
our effects were specific to the ventral hippocampus, which has a distinct molecular, connectomic, and functional profile compared to the dorsal hippocampus\textsuperscript{66–69}. Considering the evidence for differential roles of each region in behavior (e.g., the dorsal region is associated with cognition, while the ventral part with affective processes\textsuperscript{67}), which have been shown to be differentially affected by ES\textsuperscript{70}, it is intriguing to speculate whether vascular adaptations might have a role for certain ES behavioral phenotypes.

**Possible role of astrocytes in the ES effects on the male hippocampal NVU**

We report here an ES-induced increase in GFAP reactivity within endothelial cell masks in the ventral hippocampus. We considered this assessment to be a valuable proxy for the contact between the astrocytic endfeet and vasculature. As we have reported that overall hippocampal GFAP coverage is not affected in ES mice at this age\textsuperscript{35}, our data imply increased astrocytic surveillance of blood vessels in the ES brain.

These findings are interesting given that the ventral hippocampus in ES mice also had more CD31 coverage, which suggest an increased growth or branching of blood vessels. These processes (i.e., vascularization and angiogenesis), are known to be influenced by astrocytes\textsuperscript{71,72}, which secrete factors such as vascular endothelial growth factor-A\textsuperscript{71,72}, the matrix metalloprotease 9\textsuperscript{73}, and apolipoprotein E\textsuperscript{74,75} under both physiological states and in response to injuries\textsuperscript{76}. Given the high stress sensitivity of astrocytes\textsuperscript{77,78}, their proposed role in ES phenotype\textsuperscript{35,36}, and how shifts in astrocytic profiles (e.g. towards reactivity) can disrupt their homeostatic roles\textsuperscript{79}, it is intriguing to consider how ES modulation of hippocampal astrocytes might impact their role in BBB regulation and maintenance. Beyond investigating possible ES effects on astrocyte secreted proteins, it will also be important to characterize the extent to which astrocytic contact with endothelial cells is affected, e.g. by using a marker enriched in astrocytic endfeet, such as the water homeostasis regulator Aquaporin-4\textsuperscript{80}.

While we initiated here an investigation into astrocytic involvement in the ES and RS effects on brain vasculature, other cell types at the NVU, namely the microglia and pericytes, are also important to BBB induction and maintenance\textsuperscript{34,81,82}. Given the described effects of ES on microglia by us and others\textsuperscript{83,84}, it will be important to also investigate if and how ES impacts microglia and pericyte interaction with the BBB and NVU. These neuroimmune\textsuperscript{85} and vascular\textsuperscript{6} changes are emerging hallmarks of diseases like Alzheimer’s (AD); given how ES can serve as a risk factor for AD\textsuperscript{4,86,87}, it is interesting to speculate that ES-induced changes in BBB and NVU might be at the origin of the changes also observed in this disease.

**Minimal effects of 30 min RS on BBB morphology**

Contrary to our expectations, we did not see much evidence for the modulation of the BBB profile by RS. Our main finding is that a 30 min RS led to an increased amount of CD31 coverage in the dorsal hippocampus of CTL but not ES mice. Notably, these effects are in line with another study showing no alteration in BBB permeability in mice restrained for 6h\textsuperscript{24}.

We had expected RS to affect BBB morphology given that acute-stress has been described to lead to alterations to the multiple aspects of hippocampus both at the level of transcriptome
and proteome. The relatively minor effect we observed might be due to the timing of our experiment and the relatively short interval between restraint stress and sacrifice of only 30 minutes, as longer durations of restraint stress (5-6h per day, or repeated across days) have been reported to lead to progressive alterations in tight junction expression in the rat hippocampus and amygdala, accompanied by stress-induced increase of the glucose transporter Glut-1 in the PFC and hippocampus. Further analysis of the gene expression profile of endothelial cells from the same mice will give further insight into this.

**Sex as a biological variable in later-life stress reactivity**

Lastly, although the analysis of the female brains is ongoing, we already observed higher CORT levels and adrenal weight at sacrifice in female mice compared to males independent of ES and later-life RS. This sex difference in the HPA axis is well-documented, has been described across species, and is thought to underlie sex differences in incidence of stress-associated psychopathologies. The early developmental origin of differences in male-female HPA axis reactivity is well described, along with sex differences in susceptibility to ES. The emerging evidence as well for sex-specific effects of BBB susceptibility to other chronic forms of stress, underscores the importance of taking sex as a biological variable when investigating the developmental trajectories of systems such as the BBB.

**Conclusion**

In this preliminary report, we present initial evidence for early-life stress induced alterations to BBB morphology, tight junction coverage, and astrocytic coverage of blood vessels in the hippocampus of male mice. Additional experiments are currently ongoing to study in more detail the possible modulation of other aspects of the BBB, such as endothelial cell gene expression, whether these would be impacted by restraint stress, and whether these effects are sex specific.
References


Blood-brain barrier alterations after early-life stress and later-life restraint


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Supplementary Figure 1. Validation of CD31+ endothelial cell enrichment after magnetic activated cell sorting using FACS and qPCR

(A) Gating strategy for FACS validation. A single cell live (DAPI-) population was gated for CD31 expression. (B) CD31+ MACS fraction had a higher proportion of CD31-PE+ cells compared to the CD31- MACS fraction or whole brain lysate. (C) CD31+ MACS fraction had increased relative expression of endothelial genes CD31, Claudin-5 (Cldn5), and Occludin (Ocln) compared to whole brain lysate. (D) CD31+ MACS fraction had decreased relative expression of classical markers of non-endothelial cell types. qPCR analysis was normalized to Ct values in whole brain lysate sample.
Supplementary Figure 2. Validation of staining strategy
(A) CD31 signal after free floating staining. (B) Survival of free floating CD31 signal after 15 min antigen retrieval in citrate buffer (pH 6.0). (C) Negative control signal, free floating.