Age-dependent impact of early-life stress on glia and synapses
Substrates for increased risk for Alzheimer's disease
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Early-life stress leads to age-dependent loss of astrocytes and premature astrocytic senescence in the male mouse hippocampus: A preliminary report

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Abstract

Early-life stress (ES) exposure has been proposed to accelerate the aging process, due to the convergence of neurobiological substrates that they affect. Beyond serving as a molecular hallmark of aging, the loss of the nuclear envelope protein Lamin-B1 has recently been described as a core phenotype of senescent astrocytes. Considering that astrocytic dysfunction is observed both in aging-related diseases and after ES exposure, we speculated that ES interactions with aging might occur through altered Lamin-B1 expression, especially in astrocytes. We therefore aimed to investigate ES effects on both total and astrocytic Lamin-B1 expression in the mouse hippocampus across life, and if it would impact the aging-associated changes.

In this preliminary report, we exposed C57BL/6J mice to ES by limiting their bedding and nesting material from postnatal days 2-9. Mice were then allowed to age in standard housing conditions and sacrificed via transcardial perfusion at 4, 10, and 20 months. We performed a GFAP and Lamin-B1 immunostaining to quantify total and astrocytic Lamin-B1+ expression in the molecular layer of the hippocampus.

We found that total Lamin-B1 intensity decreases from 4 to 10 months, but is not affected by 20 months. ES decreased the number of Lamin-B1+ astrocytes at 4 months, decreased the total number of astrocytes at 10 months, and increased the proportion of astrocytes expressing Lamin-B1 at 10 months. While we did not confirm previous work showing decreased total and astrocytic Lamin-B1 from 4 to 20 months, our data support the notion that Lamin-B1 expression is affected in aging, and provide preliminary evidence that ES might induce early senescent phenotypes in astrocytes. The rate and extent to which ES modulates astrocytic senescence, its functional consequences for the ES-exposed brain, and how these effects alter the aging process will require further study.
Introduction

Early-life stress exposure (ES) is associated with an increased risk to develop cognitive decline and psychopathologies1–4. While the mechanisms behind this lasting programming of later-life health are not well understood, one popular hypothesis is that this is mediated by shifts towards the aging process. We previously characterized the effects of ES exposure in advanced age (20 month-old) mice, focusing on cognition, neurogenesis, and neuroinflammation5. While we did not find evidence for ES-associated alterations in these domains, we hypothesized that these shifts might occur in other substrates of aging, particularly at the molecular level.

Aging is characterized by several molecular alterations. Among these is genomic instability, mediated in part by the loss of a family of nuclear envelope proteins, the lamins, that maintain the nuclear structure6. These intermediate filament proteins play an important role in aging7, as evidenced by the premature aging phenotype induced by mutations in Lamin-A8. On the other hand, Lamin-B1 plays a role in the development of the nervous system, more specifically in the differentiation of oligodendrocytes and neurons9–12, and its reduced expression has been proposed to be marker of cellular senescence13,14. Lamin-B1 is expressed by a variety cell types in the nervous system7, and beyond serving as a general marker of cellular aging, has recently been implicated as a defining feature of astrocytic senescence in the mouse and human brain15. Given the emerging role of astrocytes in ES-induced programming of brain function16,17, and in the ES-induced increased risk for aging related disorders such as Alzheimer’s disease17, we wondered whether ES could aggravate the aging-associated loss of Lamin-B1, particularly in astrocytes, and if this might add to our understanding of how ES alters developmental and aging trajectories.

In this study, we quantified Lamin-B1+ cell density, expression intensity, and colocalization with astrocytes in the molecular layer of the hippocampus. This was done in 4-, 10-, and 20-month-old mice exposed to ES from postnatal days 2-9. We present preliminary evidence for age-dependent alterations of astrocytic Lamin-B1 expression by ES.

Materials and Methods

Early-life stress paradigm, and experimental design
We subjected wildtype C57BL/6J mice (Envigo, the Netherlands) to early-life stress (ES) from postnatal days (PND) 2-9 as previously reported18. After a two-weeks of acclimatization in the animal facility, 8-10 week old virgin female mice were bred in-house with 4-6 week old male mice. Male mice were taken out after one week, and females were single-housed a week later. We started checking for pups between the first two hours of the light phase 18 days after the start of breeding. Nests found at this time had the previous day denoted as PND 0. Early-life stress (ES) was induced from postnatal days (PND) 2-9 as previously reported18. At PND2, mice were randomly assigned to control (CTL) or ES conditions. CTL litters were housed in standard cage settings (around 100 g sawdust bedding, along with a piece of 5x5 cm nesting material [Technilab-BMI, Someren, the Netherlands]), while ES litters were housed on a fine-gauge stainless steel mesh on top of 33 g of sawdust, along
with 2.5x5 cm nesting material. At PND9, mice were transferred to standard cage settings, and aged until 4-, 10-, or 20-months of age (n per group – 4M-CTL: 6, 4M-ES: 5, 10M-CTL: 5, 10M-ES: 4, 20M-CTL: 8, 20M-ES: 8). The mice used in this report were previously used in another study, where other measures (physiological measurements, behavior, neurogenesis, neuroinflammation, and telomere length) have been reported.

Throughout the experiment, mice were housed at a temperature of 20-22°C, 40-60% humidity, with cage enrichment and ad libitum standard chow and water. The animal facility maintained 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.

**Tissue collection and processing**

At the appropriate experimental age, mice were i.p. injected with 120mg/kg Euthasol and transcardially perfused with ice cold saline, and then cold 4% paraformaldehyde in 0.1M tris buffer. Brains were cryoprotected in 15%, then 30% sucrose, then coronally sliced into 40µm sections on a freezing microtome. Tissue was stored in antifreeze at -20°C until use.

**Immunostaining for Lamin-B1 and quantification**

We performed a double immunostaining against Lamin-B1 (rabbit polyclonal, 1:1000, Abcam ab65986) and GFAP (mouse monoclonal, 1:2000, Merck G3893). Before staining, brain sections were washed in 0.05M Tris buffered saline (TBS, pH 7.6), and then mounted onto pre-coated glass slides (Superfrost, Menzel). Brains were washed with 0.05M TBS in between all steps. We performed antigen retrieval in citrate buffer (pH 6.0) for 15 minutes in a microwave at a temperature of ±95°C. After cooling to room temperature, slides were incubated at room temperature for 30 min with blocking mix (1% bovine serum albumin, 5% normal donkey serum, 0.3% triton in 0.05M TBS). Slides were then incubated with antibodies diluted in the same blocking mix for 1h at room temperature, then overnight at 4°C. Secondary antibodies (donkey anti-rabbit-A488, 1:1000, Invitrogen; goat anti-mouse-A568, 1:1000, Invitrogen) were diluted in blocking mix, and applied on the slides for 2h at room temperature. We then added Vectashield containing DAPI (Thermofisher Scientific, USA) and coverslipped the slides.

**Epifluorescence and confocal microscopy**

We took 20x epifluorescent images using a Nikon DS-Ri2 microscope (Nikon Instruments, Europe), as well as 40x confocal images using a Nikon A1-confocal laser microscope (Nikon Instruments, Europe). Epifluorescent images were used to count Lamin-B1 positive cells in the molecular layer of the dentate gyrus, while confocal images were used to quantify the intensity of the Lamin-B1 signal, as well as colocalization of GFAP and Lamin-B1.

Epifluorescent images were taken at 8 different bregma points (from -1.34mm to -3.88mm) to characterize the dorsoventral axis of the hippocampus. We took and stitched together images (Dorsal: 3 tall x 2 wide; Ventral: 2 tall x 3 wide) at 20x magnification. Confocal images were taken in the middle of the molecular layer at 4 different bregma points (from -1.58mm to -3.88mm).
until -3.28mm), including two dorsal and two ventral regions. A z-stack of 11 steps (total range 33µm) was captured and stitched into a 2 tall x 1 wide image.

**Image analysis**
All image analyses were performed in ImageJ. As the immunostaining was done in two separate rounds, all data were normalized to a sample present in both rounds. All measurements were averaged along the dorsoventral hippocampus; analyses were performed by an experimenter blind to the experimental conditions. Cell counts were obtained by drawing a region of interest (ROI) around the molecular layer of the dentate gyrus in the DAPI channel, and counting Lamin-B1+ cells within this ROI with the Cell Counter plugin. Density was calculated as the total number of cells divided by ROI area. Intensity analysis was done as follows: after merging z-stacks into a single plane using sum, molecular layer ROIs were drawn on the DAPI channel. After background subtraction, intensity within the ROI was measured in the Lamin-B1 channel using the mean grey value. Lastly, Lamin-B1-positive astrocytes, as well as total astrocytes, were counted manually by merging the Lamin-B1 channel and the GFAP channel on ImageJ.

**Statistical analysis**
Data analysis was performed on RStudio v1.4.1717\(^{19}\), and data visualization was done using the ggplot2 package\(^{20}\). After testing for significant outliers using the 1.5*IQR method, we performed a two-way ANOVA using age and condition as factors. When applicable, exploratory post-hoc analyses were performed using the emmeans package\(^{21}\), correcting for multiple comparisons using Tukey’s method. Aging effects were analyzed post-hoc by comparing each age with the other two pairwise, while aging x condition interactions were analyzed post-hoc by testing for ES effects within each age point. Data were considered statistically significant when p<0.05.

**Results**

**Hippocampal Lamin-B1+ intensity is reduced at 10 but not at 20 months, without modulation by ES**
We found an age-dependent effect on Lamin-B1 cell number in the hippocampus, which was not affected by ES (Fig. 1A, 1B, condition: F(1,27)=1.673, p=0.2069; age: F(2,27) = 5.108; p=0.0131; interaction: F(2,27)=0.8094, p=0.4556). Post-hoc analyses indicate that this age effect is due to an increase of Lamin-B1+ cells from 10 to 20 months (p=0.025).

We next analyzed Lamin-B1 intensity using the mean grey value (Fig. 1A, 1C). We similarly found an age effect in the intensity of Lamin-B1 signal in the molecular layer of the hippocampus independent of ES exposure (condition: F(1,26)=0.000, p=0.9897; age: F(2,26)=6.757, p=0.0043; interaction: F(2,26)=0.956, p=0.3977). Post-hoc analyses reveal that this age effect is due to a significant decrease in Lamin-B1 intensity between 4 versus 10 months (p=0.0024), and an increase from 10 to 20 months (p=0.0316). Lamin-B1 intensity was not significantly different between 4 and 20 months (p=0.267).
Figure 1. Early-life stress exposure age-dependently alters astrocytic, but not total, Lamin-B1 expression.
(A) Representative images of Lamin-B1 immunostaining in the molecular layer of the hippocampus, where all analyses were performed. (B) Total Lamin-B1 cell counts are affected by age, but not early-life stress (ES) exposure. (C) Aging leads to non-linear loss of total Lamin-B1 intensity, without modulation by ES. While total Lamin-B1 intensity decreases between 4- and 10-months, this measure was comparable between 4- and 20-months. (D) Representative images of Lamin-B1 expression in astrocytes, as marked by GFAP. (E) ES age-dependently decreases the total number of astrocytes, specifically at 10 months. (F) ES leads to age-dependent loss of the number of Lamin-B1-expressing astrocytes at 4 months. (G) ES increases the proportion of Lamin-B1-expressing astrocytes at 10 months. #, aging effect, p<0.05; &Interaction effect, p<0.05; a, post-hoc difference between 10- and 20-months, p<0.05; b, post-hoc difference between 4- and 10-months, p<0.05; c, post-hoc difference between CTL and ES, p<0.05.
ES age-dependently modulates astrocytic cell counts and Lamin-B1 expression
We observed an interaction between (but not main effects of) ES exposure and aging regarding the number of astrocytes in the molecular layer of the hippocampus (Fig. 1E, condition: F(1,28)=2.112, 0.1573; age: F(2,28)=0.371, p=0.6934; interaction: F(2,28)=3.642, p=0.0393), with post-hoc tests revealing the interaction to be due to fewer GFAP+ astrocytes in ES mice specifically at 10 months of age (p=0.0167). Investigating the Lamin-B1 expression in these astrocytes, we found that, while ES and aging alone did not have significant effects, there was an interaction effect of both variables on the total number of Lamin-B1+ astrocytes (Fig. 1F, condition: F(1,28)=1.367, p=0.2522; age: F(2,28)=0.155, p=0.8574; interaction: F(2,28)=5.368, p=0.0106). Post-hoc analyses revealed that ES decreased the total number of Lamin-B1+ astrocytes at 4M (p=0.0339), with a trend in the same direction at 10 months (p=0.0575) and a trend in the opposite direction at 20 months (0.0871). These effects in total cell count were accompanied by similar interaction (but not main) effects of ES and aging regarding the proportion of astrocytes expressing Lamin-B1 (Fig. 1G, condition: F(1,28)=0.4630, p=0.5018; age: F(2,28)=0.5669, p=0.5737; interaction: F(2,28)=3.3722, p=0.0487). This interaction was due to an increased proportion of Lamin-B1+ astrocytes in ES mice at 10 months (p=0.0277).

Discussion
We present here our data investigating the total and astrocytic expression of the senescence marker Lamin-B1 in the molecular layer of the hippocampus, and how this was impacted by early-life stress (ES) exposure at three different ages. We were able to confirm an initial aging-associated decrease (independent of ES) in total Lamin-B1 intensity between 4- and 10-months of age in the hippocampus, which was absent by 20-months. While aging did not lead to overall changes in astrocytic numbers or Lamin-B1 expression, we found that it interacted with ES history. Specifically, ES exposure decreased the total number of astrocytes at 10 months, as well as the number of Lamin-B1+ astrocytes at 4 months (with a similar trend at 10 months). These changes were associated with an increased proportion of Lamin-B1 expressing astrocytes at 10 months. We discuss below possible implications of our findings for the ES modulation of the aging trajectory via astrocytic senescence.

Aging leads to non-linear loss of Lamin-B1+ intensity in the molecular layer of the hippocampus
The declining expression of Lamin-B1, and the consequences thereof on nuclear envelope integrity, is one of the hallmarks of senescent cells. Loss of Lamin-B1, in part induced by autophagic processes, mediates age-associated loss of hippocampal neurogenesis, and aggravates other senescent phenotypes, especially in the context of cell division. Matias et al. recently reported a decline in total and astrocytic Lamin-B1 expression in the hippocampus of 18–24-month-old mice compared with 3–4 month-old mice. While we did not detect differences in Lamin-B1 cell count and intensity between our 4- and 20-month mice, we observed an aging-associated decrease in the total intensity of Lamin-B1 expression at 10 months, a timepoint wherein aging-associated phenotypes already emerge in mice. We also were not able to confirm the aging-associated decrease in astrocytic Lamin-B1
expression, although visual inspection suggests that the number of Lamin-B1+ astrocytes might be decreased in CTL mice by 20 months of age.

While we can only speculate about why we see such reductions at 10, but not 20, months of age, a possible explanation might be the regions studied. Our work focused on the molecular layer of the hippocampus due to the high expression of Lamin-B1 in neural progenitors24. As Lamin-B1 is highly expressed in immature cells25, their data on decreased total Lamin-B1 intensity by 18-24 months might also partly reflect decreased neurogenesis with aging26. On the other hand, the absence of further aging-associated effects on Lamin-B1 intensity at 20 months might reflect aging-associated compensatory proliferation by non-neuronal cell types, as has been reported for aged microglia27. Alternatively, this absence of Lamin-B1 loss by 20 months could also be indicative of other compensatory mechanisms against age-induced alterations to genomic stability, as has been shown to occur via telomerase activity14,28. Regardless, this increase in Lamin-B1 intensity in advanced aging could also have detrimental consequences, as its overexpression in neurons can induce neurodegenerative phenotypes29. Similarly, Lamin-B1 overexpression in astrocytes results in a reactive phenotype30, and could thus signify different parallel mechanisms to induce senescent phenotypes.

**ES exposure leads to accelerated senescence in astrocytes**

Given the important role of astrocytes in the physiology of the nervous system31, much recent work has been done to understand how their functions and profiles change with aging32–35. Loss of Lamin-B1 is a recently described marker of astrocytic aging associated with astrocytic secretion of the senescence related proteins15. We found in our data that ES decreased the number of Lamin-B1-expressing astrocytes by 4 months, an effect that also seems to be present at 10 months (albeit as a trend). Curiously, we also found that at 10 months of age, ES both decreased astrocytic cell numbers and increased the proportion of Lamin-B1-expressing astrocytes, suggesting decreased survival of ES astrocytes that lack Lamin-B1. These data are in line with our previous report of decreased GFAP immunoreactivity in the hippocampus of ES-exposed mice at 10 months17, and lend credence to the hypothesis that Lamin-B1 loss contributes to apoptosis via ruptures in the nuclear membrane12,36.

Beyond initiating cell death, Lamin-B1 deficits have been associated with systemic inflammation37,38, and it is intriguing to consider whether its deficiency in astrocytes might contribute to aging-associated alterations to the neuroimmune system39. Aging impairs the immune system40,41, which is thought to underlie the increased risk for several pathologies in old age42,43. While much focus on this in the neuroimmune context has been placed on microglia44–48, there is also evidence for astrocytic involvement in neuroimmune aging49. For instance, astrocytes isolated from 2-year-old mice have a transcriptomic profile characteristic of reactive astrocytes33,34, which can also be induced by the secretion of microglial factors50. In addition, astrocytes from 18–20-month-old mice reduce IL-10 signaling and cholesterol biosynthesis in response to an LPS challenge, driving pro-inflammatory microglial activity51. These shifts in astrocytic phenotypes might ultimately contribute to their involvement in a variety of neuropathological conditions with age52,53.
A central question in the field of ES is whether it affects the aging process\textsuperscript{5}. Our data here, in addition with our findings of age-dependent ES modulation of GFAP reactivity\textsuperscript{27}, would suggest that ES exposure leads to a premature switch to a senescent phenotype in astrocytes. This might contribute to the age-dependent modulation by ES exposure on amyloid-beta pathology, which we and others have reported\textsuperscript{54,55}. Given the reported senescence-induced disruptions to astrocytic functions such as synaptic maintenance\textsuperscript{15,34} and inflammation\textsuperscript{33,34}, it remains to be seen whether ES-exposed astrocytes would exhibit in similar functional alterations, and if they are able to exhibit other aging-associated features at younger ages.

**Conclusion**

In this preliminary report, we report aging associated alterations to Lamin-B1 expression, especially in astrocytes. We show evidence that this trajectory is altered in the ES hippocampus. Given the link between genomic instability (maintained by Lamin-B1 and its related proteins) and aging phenotypes, our data show a link between ES exposure and disrupted molecular aging in astrocytes. The functional consequences of this ES modulation, both on astrocytic function and astrocytic interactions with other cell types in the brain throughout the lifespan remain to be seen.
References

Astrocytic Lamin-B1 expression in aged early-life stressed mice