Age-dependent impact of early-life stress on glia and synapses
Substrates for increased risk for Alzheimer's disease
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Introduction
Age-dependent impact of early-life stress on glia and synapses: Substrates for increased risk for Alzheimer’s disease

Rationale and aim

Early-life stress (ES) exposure has lasting consequences for later-life health and disease, and is associated, based on both pre-clinical and epidemiological data, with increased rates of later cognitive decline\(^1\text{–}^3\), psychopathology\(^4\text{,}^5\) and metabolic disorders\(^6\text{,}^7\). This link between early-life experience and later-life health is perhaps most intriguingly observed in Alzheimer’s disease (AD), a yet-uncurable, age-associated neurodegenerative condition that is the leading cause of dementia in an aging global population\(^8\text{,}^9\). Epidemiological evidence indeed shows that exposure to various forms of ES is associated with an increased risk for dementia and AD\(^10\text{–}^14\).

This association with ES provides another piece to the puzzle of AD etiology, whose incidence is also associated with lifetime stress exposure\(^15\text{,}^16\). While much attention has been paid to the causal role of the characteristic neuropathological features of AD in its etiology\(^17\text{,}^18\) (especially amyloid-beta [Aβ] plaques\(^19\) ), they do not account for the majority of cases, which are non-familial, or “sporadic”, in nature\(^20\). This is further reflected by the recent lack of success of Aβ-based clinical trials to improve AD symptomatology\(^18\). This has opened up studies into additional risk factors like environmental and lifestyle factors that also contribute to disease risk\(^15\text{,}^16\text{,}^21\text{–}^23\) and new biomarkers of the disease, that can potentially be targeted\(^24\text{–}^27\).

Accordingly, the link between ES and AD can be viewed within the framework of the developmental origins of health and disease (DOHAD) hypothesis\(^11\text{,}^28\text{–}^30\). As a field that emerged following observations of a link between birthweight and later-life ischemia\(^31\), much emphasis is placed here on how early programming can lastingly alter development, and thereby later-life health\(^32\). Such a view has also been proposed for AD-associated risks, whether it be from changes occurring during critical developmental windows, the cumulative aggregation of lifetime insults that only manifest in disease, or from lasting shifts in the organism’s entire developmental trajectory\(^11\text{,}^33\). Similarly, ES is also thought to lead to differential responses to later-life challenges, which rodent studies show evidence for\(^34\text{–}^37\). Given the significant time delay between experiencing ES and the eventual emergence of an increased disease vulnerability, a common question in the field is whether this programming occurs via ES modulation of the aging process itself\(^5\).

To fully understand these mechanisms behind early-life programming of later AD risk, it is crucial to understand the neurobiological substrates that are affected by ES. At the cellular level, AD is characterized by progressive synaptic loss that is associated with cognitive deficits\(^38\text{–}^41\), and neuroinflammatory activation of astrocytes and microglia that lead to Aβ phagocytosis\(^42\text{–}^44\). Also, compromised neurovascular profiles have been implicated that dysregulate blood brain barrier permeability and impair the clearance of Aβ from the brain parenchyma\(^45\text{–}^49\).
In this thesis, we attempted to characterize how ES exposure impacts these different neurobiological substrates, and how ES can thereby modulate AD phenotypes.

General experimental design

The nature of our aim necessitates the use of animal experiments, that can help provide mechanistic insights emergent at the organismal level. Throughout this thesis, we modeled ES in mice using the limited bedding and nesting (LBN) model, first developed in the Baram lab for use in rats and mice. Dams and pups in this model are housed in cages with reduced bedding and nesting materials for the first postnatal week. These ‘impoverished’ conditions limit the dam’s ability to build nests, leading to erratic maternal behavior. Pups exposed to these conditions reared until adulthood are reported to have impairments in hypothalamic-pituitary-adrenal axis reactivity, adult neurogenesis, and cognitive performance across several domains. In the experiments we report here, mice are exposed to ES or control conditions from postnatal days (P) 2-9, and are either sacrificed at P9 to investigate the direct consequences of ES, or transferred to standard housing conditions and sacrificed in adulthood to investigate long term effects of ES.

To more specifically investigate the consequences of ES exposure in relation to AD in a controlled setting, we made use of the APPswe/PS1dE9 transgenic mouse line developed by the Borchelt lab. This model leads to Aβ processing through the prion-promoter-based overexpression of the human amyloid precursor protein (APP) harboring the Swedish mutation, along with the human Presenilin 1 gene with a deletion in exon 9. These mutations result in plaque formation by 6 months of age, and are associated with impairments in cognition, synaptic structure, and neuroinflammation, among others. Notably, the core phenotype in this model, i.e. a gradual build up of amyloid load, has been reported to be modulated by ES exposure.

Given the age-dependent nature of these effects, we studied the ES and APP/PS1 interaction at both early (4 months of age) and late (10-12 months of age) stages of Aβ pathology. Importantly, beyond serving as a model for AD neuropathology, our APP/PS1 mouse model, which displays a strong neuroimmune response to Aβ, can also be viewed as a chronic neuroinflammatory challenge that could unmask latent ES effects. We also similarly implemented other forms of secondary challenges, such as aging or restraint, in this thesis in order to unmask ES effects in wildtype mice.

Neurobiological substrates of interest

We focused in this thesis on the effects of ES and Aβ overexpression on the hippocampus, a brain region important for cognition, in which adult neurogenesis occurs as well.

The hippocampus is strongly affected in AD, exhibiting extensive synaptic loss. It is one of the first regions showing both amyloid and later also tau pathology that spreads through the brain. Importantly, the hippocampus contains several cell types that have each been...
shown to contribute to AD pathology\textsuperscript{41,75,76}. This region is also highly sensitive to stress, due to its relatively enriched expression of glucocorticoid receptors\textsuperscript{77,78}. In fact, various types of stress alter hippocampal size\textsuperscript{58}, synaptic structure\textsuperscript{79,80}, and neurogenesis\textsuperscript{81,82}. Altogether, the hippocampus is an interesting brain region due to the consistent disruption of hippocampus-related spatial learning tasks\textsuperscript{60} and neurogenesis\textsuperscript{83,84} by ES and AD.

In this work, we studied the effects of ES in a transgenic AD mouse model on hippocampal synapses, glial cells, and blood brain barrier features. Below, we first give an overview of these systems, as to how we understand them to be affected by our experimental variables.

**Synapses**
As the main functional unit through which neurons communicate, the synapse is an important substrate for all neurobiological questions that is often approached via an investigation of its structural and functional alterations. In particular, in the context of memory, the synapse can be viewed as a neural correlate of learning\textsuperscript{85}. Much attention has further been paid recently to the study of synaptic plasticity\textsuperscript{86,87}, neurogenesis\textsuperscript{88,89}, and the representation of “memory traces” in specific cell ensembles, called engrams\textsuperscript{90}.

As mentioned, synaptic loss is a prominent feature of AD pathology, both in early and late stages of the disease\textsuperscript{40,91–93}. These effects are partly modulated by direct effects of Aβ on synaptic proteins\textsuperscript{38}. While work has been done showing both structural and functional alterations to synapses in rodent models of AD\textsuperscript{41,64,72}, the trajectory of these associated alterations is still unknown. On the other hand, ES alters synaptic structure, by reducing synaptic protein levels\textsuperscript{94–96}, spine numbers\textsuperscript{97}, and neurogenesis\textsuperscript{58,98} and function\textsuperscript{58,94,99}. However, it remains unknown how ES induces alterations to the overall proteomic profile in hippocampal synapses, and how it would interact with the trajectory of Aβ overexpression-induced synaptic changes.

**Astrocytes**
The formation, maturation, and maintenance of synapses is aided by astrocytes\textsuperscript{100–103}. While these cells have classically been viewed as passive supporting cells to the neurons, astrocytes can release gliotransmitters that modulate synaptic activity\textsuperscript{104,105} and synchronize neuronal network firing\textsuperscript{106}. These cells support synaptic functions of neurons largely via their surveillance of the extracellular milieu. This is mediated through different astrocytic receptors, which allow for regulation, e.g., of the levels of neurotransmitters\textsuperscript{107,108}, ions\textsuperscript{109}, water\textsuperscript{110}, and nutrients\textsuperscript{101,111}.

Astrocytes also respond to extracellular challenges and play a role in the neuroinflammatory response, e.g. via cytokine release\textsuperscript{112}. In such states, they exhibit a “reactive” profile, marked by the upregulation of the astrocytic marker GFAP that underlies the process of astrogliosis\textsuperscript{113,114}. Notably, these states can lead to a neurotoxic phenotype\textsuperscript{115}, further driving disease states. This is the case in AD, where astrogliosis occurs in response to Aβ plaques\textsuperscript{116–119}. These morphological alterations are widespread throughout the main hippocampal subregions and accompanied by spatially heterogeneous astrocytic transcriptomes in the AD brain\textsuperscript{120}. Beyond this, astrocytes are also highly sensitive to glucocorticoids and stress\textsuperscript{121,122}, which can alter their morphology\textsuperscript{123,124} and glucose metabolism\textsuperscript{125,126}. However,
while there is emerging evidence for astrocytes as a substrate for ES effects\(^\text{127}\), it is unclear how ES impacts the astrocytic profile across the lifespan, and how they might be involved in ES modulation of AD.

**Microglia**

Besides astrocytes, microglia, the innate immune cells in the nervous system, also play a key role in maintaining proper brain function\(^\text{128}\). In healthy states, they survey the extracellular environment\(^\text{129}\), releasing cytokines in response to indicators of extracellular damage such as ATP\(^\text{130,131}\), or phagocytosing apoptotic cells and toxic molecules\(^\text{132}\). Microglia also play a prominent role in a variety of disease states, leading to distinct expression and functional profiles\(^\text{133}\). This microglial adaptation is most evident in neurodegenerative conditions like AD\(^\text{134}\), where mutations in microglial genes can lead to progression of sporadic AD\(^\text{135}\). In fact, microglia can similarly drive disease, as their neuroinflammatory responses\(^\text{42}\) can become maladaptive, e.g. worsening pathology by phagocytosing healthy synapses\(^\text{136}\).

Microglia are also among the first functional cell types present in the brain, migrating from the yolk sac during embryonic development\(^\text{137}\). They express distinct gene expression profiles throughout development\(^\text{138,139}\), playing more of a role in sculpting the neuronal architecture and synaptic landscape than inflammatory regulation early in life\(^\text{140,141}\). This early presence of microglia makes them sensitive to early alterations in the brain milieu\(^\text{142}\). Similarly, ES exposure affects microglia, as seen in the transcriptomic changes associated with maternal separation in mice\(^\text{143}\). Studies using the LBN model have also illustrated this, as shown in our work characterizing age-dependent ES effects on microglial morphology and density in the hippocampus in both wildtype and APP/PS1 mice\(^\text{67}\), as well as a study from the Baram lab illustrating ES impairment of microglial synaptic pruning the hypothalamus in wildtype mice\(^\text{144}\).

Important for our research aims, microglia are sensitive to “priming” by prior experiences, as they form an “immunological” memory in their responses to later stimuli\(^\text{145}\). Repeated exposure to immune-activating substances contribute to either milder (i.e. “tolerant”) or exaggerated (i.e., “trained”) responses\(^\text{146}\), the direction of which can be dictated e.g. by the frequency of the immune challenge\(^\text{147}\). Crucially, priming can be induced already even early in life; as seen in the later life response of mice exposed to a prenatal inflammatory environment\(^\text{148,149}\), or in mice injected postnatally with adjuvants consisting of bacterial cell wall extracts\(^\text{150}\). Given the high sensitivity of microglia to stress\(^\text{151}\), one of the outstanding questions in the field is whether ES can also prime later-life microglial function, and whether priming might mediate its effects on the neuroimmune system.

**Blood-brain barrier**

Lastly, we wanted to investigate the blood brain barrier (BBB), a physical barrier serving as the interface between brain and periphery\(^\text{152}\). The BBB is formed via endothelial cell expression of specific tight junction proteins such as Claudin-\(^\text{5}\)\(^\text{153}\). The surrounding vessel-adjacent cell types are collectively referred to as the neurovascular unit (NVU)\(^\text{154}\). Among the most important processes at the NVU is neurovascular coupling, i.e. the dynamic changes to vascular properties in response to neuronal activity, that are important for energy balance\(^\text{155}\). Disruptions of this balance are notably associated with AD-like cognitive impairments, can
lead to vascular dementias, and may result in brain metabolic alterations, such as seen in AD.

Beyond regulating nutrient entry into the brain, the BBB is also implicated in AD through its role in the clearance of Aβ. This process is largely mediated by pathways involving apolipoprotein E, a prominent risk factor for AD. Work on this and other related proteins have demonstrated an emerging role for brain cholesterol metabolism in AD pathology. Crucially, there are strong alterations to both the central and peripheral lipid profiles in ES. However, despite evidence for stress-associated alterations to BBB function, it is currently not known whether and how the BBB is modulated by ES exposure.

**Interactions between substrates**

Beyond our interest in the ES effects on each system, we also understand that these components are interdependent, and that the effects of ES might be in modulating emergent interactions.

For instance, beyond the well-known recognition of the role of astrocytes in supporting and maintaining the pre- and post-synapse (which together form the “tripartite synapse”), this view has been recently expanded to include microglia (i.e., the “quad-partite” synapse). This framework views the long term maintenance of synapse as a result of sculpting from both glial cell types. Beyond the microglial pruning of synapses, the bi-directional signaling between microglia and astrocytes is also important, given e.g. the astrocytic stimulation of microglial phagocytosis, and the observation that activated microglia can in turn, induce neurotoxic, reactive astrocytes.

Similarly, the induction and integrity of the BBB is actively mediated by cells around the neurovascular unit. This is classically done through the signaling of pericytes or astrocytes, whose end feet wrap tightly around endothelial cells. In fact, astrocyte secretion can regulate both vascularization and BBB permeability, facilitating the coupling of neuronal activity with vascular perfusion discussed above. Crucially, microglia have also recently been shown to be able to impact BBB maintenance in health and disease states.

We expect that these interactions will all contribute to the puzzle of how ES affects the progression of AD pathology. While we were not always able to directly interrogate these interactions, we nonetheless attempted to take them into account in the interpretation of our findings.

**Aims and outline of this thesis**

As introduced above, this thesis aims to contribute to our understanding of how ES leads to shifts in the developmental trajectories and properties of microglia, synapses, astrocytes, and the blood brain barrier. This will help to understand how ES modulation of AD pathology might occur through these substrates. This thesis is organized as follows:
In **chapter 2**, we test the hypothesis that ES exposure shifts the aging process by comparing wildtype ES and control mice at 20 months of age. We assessed the physiology and cognitive performance behavior of these mice, as well as age-associated hippocampal alterations in neurogenesis, neuroinflammation, and telomere length. By comparing these data with those of younger mice, we attempted to integrate our data into a better understanding of the ES “trajectory”.

In **chapter 3**, we continued our investigation into ES effects on the developmental trajectory by focusing on molecular aspects of aging. In particular, we investigated the hippocampal expression of LaminB1, a nuclear envelop protein whose declining expression is proposed to be a hallmark of aging. We compared how ES exposure affected the overall and astrocytic expression of this protein at 4-, 10-, and 20-months.

In **chapter 4**, we characterized the short- and long-term effects of ES on the hippocampal microglial profile by performing mRNA sequencing on fluorescently-sorted microglia, both at P9 and in adulthood. In line with our questions on developmental trajectories, we also compared how ES altered the pattern of microglial transcriptome changes between P9 and adulthood, as well as how ES modulates the microglial transcriptomic response to a later-life immune challenge. We compliment these findings by adapting an ex vivo flow cytometry assay to assess synaptic phagocytosis in ES microglia, as well as by validating one of our gene expression targets in a postmortem human cohort exposed to childhood abuse.

In **chapter 5**, we investigated the quad-partite synapse, and how ES and Aβ pathology affect this at early and later stages of pathology. We isolated hippocampal synaptosomal fractions and measured the synaptic proteomic composition via mass spectrometry in WT and APP/PS1 mice exposed to ES. We then used electron microscopy and immunofluorescence to validate our findings.

In **chapter 6**, after a better understanding how ES modulated the APP/PS1-induced synaptic alterations, we turned to the last component of the quad-partite synapse, and characterized astrocytes across ages, using qPCR and immunohistochemistry. We compared how these were modulated by ES across ages in the wildtype condition, as well as how ES and APP/PS1 genotype affected these at early and late pathological stages.

In **chapter 7**, we generated a cohort to understand the effects of ES and later-life restraint on the BBB in wildtype mice. We enriched brain endothelial cells to characterize ES effects on blood vessel gene expression, as well as generated tissue for immunofluorescence analysis. We present preliminary data of our immunofluorescence investigations of BBB structure and morphology in ES-exposed mice. We further studied whether this might be a system in which ES alterations of astrocytes also manifest.

In **chapter 8**, we present observations about the ES model used over the past five years, analyzing our data from several rounds of maternal care observation data, and proposing a new readout to validate successful implementation of the LBN model. Additionally, we tackled the well-noted question of how the restriction of nesting material in our model
might alter the temperature of the animals, and whether these might also play roles in the ES phenotype.

Lastly, in chapter 9, we provide an interpretation of the different findings in the thesis, and attempt to contextualize these data with the current state of the field. Based on this discussion, we hope to convince the reader that the ES modulation of AD pathology is in part mediated by alterations to these substrates we investigated, as well as their interactions.
References

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