Age-dependent impact of early-life stress on glia and synapses
Substrates for increased risk for Alzheimer's disease
Kotah, J.M.

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General Discussion
A key question when studying the many consequences of early-life stress (ES), is: how can early adverse experiences lead to such long-lasting programming? In this thesis, we set out to answer this question using animal models, with the framework of the “developmental trajectory” as a key concept.

The development of the nervous system follows a program that is tightly regulated in space and time, as seen in processes such as gastrulation and the subsequent differentiation of the germinal layers, via neural tube induction, to the subsequent layering of the neocortex. Importantly, developmental processes of the brain continue into the postnatal period, and the pathways involved remain, at least to some extent, plastic and capable of shaping the organism’s phenotype throughout life.

This plasticity aids survival, and functions especially as an adaptive mechanism in response to signals from the environment, especially those pertaining to threats and danger; these adaptations are largely mediated and understood within the framework of the stress response and the phenotypic changes stress can induce. These tightly regulated processes are mediated in part via catecholamine and glucocorticoid release that follow hypothalamic-pituitary-adrenal (HPA) axis activation. Subsequently, these hormones can alter both gene expression and epigenetic levels.

Crucially, these systems are not fully mature at birth, and they can therefore be negatively impacted by perturbations during the early-life period. Resulting early epigenetic modifications can then trigger negative developmental outcomes, as seen, e.g., in altered glucocorticoid receptor methylation or in a different later-life reactivity of the HPA axis to other challenges. Over time, such aberrant feedback is thought to lead to a cumulative vulnerability to different psychopathologies. In line with this, meta-analytical studies have indicated that the behavioral phenotypes induced in rodent models of ES were stronger when the study also included a second “hit”. At the neurobiological level, alterations to e.g. neurotransmitter systems or immediate early gene expression were also more prominent in ES rodents that were exposed to additional hits.

Our work here takes inspiration from these ideas and has attempted to characterize the neurobiological systems affected by ES exposure at different ages, as we try to understand how ES can (re-)program these systems such that they respond differentially to later challenges.

**ES and the developmental trajectory**

Aging induces organ-system-specific alterations to gene and protein expression in both rodents and humans. These include, at the level of the nervous system, alterations to cognition, neurogenesis, and neuroinflammation. Aging phenotypes also manifest at the cellular level, where several molecular alterations take place, including telomere shortening after each round of cell division, genomic instability due to the loss of nuclear envelop proteins, and an accumulation of methylation sites in the epigenome of the organism, which has been used as a measure of “cellular aging.”
Our understanding of how all these systems evolve with aging have led to a distinction between the chronological and biological age\textsuperscript{28}, as well as to a search for factors that could impact “successful”, or healthier aging\textsuperscript{29,30}. Beyond increasing the risk for aging-associated disorders such as Alzheimer’s disease (AD)\textsuperscript{31,32}, ES also leads to lasting changes in cognition\textsuperscript{13}, neurogenesis\textsuperscript{33}, and (neuro)inflammation\textsuperscript{34}. Because, as mentioned, these systems are also disrupted in aging, some have hypothesized that ES alters the process of biological aging\textsuperscript{35,36}. We thus aimed, in chapters 2 and 3, to evaluate whether the overlap in ES- and aging-induced phenotypes might shed light on how the former impacts developmental trajectories.

We investigated effects of ES on the aforementioned aging-related systems (and their trajectories) by following up ES-exposed mice until the advanced age of 20 months (20M). We then compared these readouts to those of younger mice and specifically wondered whether any such effects on the aging trajectory would be of either a premature nature (meaning an earlier aged phenotype in ES mice compared to CTL ones, who then “catch up” later), or rather an accelerated nature (meaning ES and CTL mice continue to diverge with age). In chapter 2, we assessed their cognitive performance in the Morris water maze, a hippocampus dependent task, and then proceeded to investigate parameters of hippocampal neurogenesis, neuroinflammation, and telomere shortening. We continued this work in chapter 3, analyzing molecular aging in the same animals, focusing on the expression of Lamin-B1, an important senescence-associated nuclear envelope protein\textsuperscript{37}, in astrocytes.

Surprisingly, we did not find effects of ES on these parameters at 20M. By comparing these data with those generated in younger mice, we could show that these domains were altered by aging per se, validating their use. Additionally, in chapter 3, we found ES effects on hippocampal GFAP expression at 10M of age and age-dependent ES effects ES on astrocytic LaminB1 expression. These are preliminary hints for possible early senescent phenotypes in ES mice, the ultimate trajectory and mechanisms of which needs further dissection. Based on this, we postulated that our ES effects on the aging process could reflect two possible, non-exclusive explanations:

1) Our data reflect “floor” and “ceiling” effects of aging phenotypes, beyond which no further impairments, or improvements, can be observed, specifically due to the age-associated deterioration of control (CTL) mice; and/or

2) The effects of ES, especially at later ages, need the experience of secondary challenge to become visible.

Consequently, we set out to tackle both these explanations (to varying extents), by utilizing ES-exposed mice at different ages and exposing them to a variety of secondary challenges. While the proper temporal understanding of how ES impacts the ES aging trajectory necessitates the use of mice at multiple intermediate timepoints, the generation of these cohorts, especially at advanced ages, was unfortunately logistically untenable for us. As such, we focused our next work on adult mice at discrete ages between 4-12 months of age. We aimed to tease apart aging-associated, or at least developmental, alterations by
comparing ES adult mice of different ages, as well as by comparing ES effects in pups versus adults.

In particular, we paid much attention to ES effects on developmental trajectories in the neuroimmune system, because of its role in health and disease, as well as in age-associated dysregulation. The latter phenomenon, known as immuno-senescence, involves e.g. a shift towards a more ‘pro-inflammatory’ immune phenotype, through the upregulation of genes involved in extracellular sensing and surveillance.

**Microglia**

We focused first on microglia, the resident immune cells in the nervous system. Previously, we had shown that ES altered microglial morphology in pups and adult mice and we here studied microglia for two reasons. First, across development, microglia take on three distinct clusters of functions: cell cycling, synaptic pruning, and immune surveillance, as also demonstrated in human fetal microglia. As such, their proper function is crucial for brain development. Second, microglia are sensitive to early-life experiences and are known e.g. to develop pro-inflammatory phenotypes upon maternal immune activation and early-life infection.

We set out to investigate this in chapter 4, where we characterized the transcriptomic profiles and phagocytic capacity of microglia in ES-exposed pups and adult mice. Beyond a shared switch in transcriptional profiles from cell cycling to immune surveillance, notably congruent with other reports, we identified distinct clusters of inflammatory pathways upregulated in control and ES mice. For instance, ES microglia had relatively increased expression of genes involved in the transforming growth factor beta (TGFβ) pathway and a relatively decreased expression of genes associated with the tumor necrosis factor alpha (TNFα) pathway, which are associated with pro- and anti-inflammation, respectively. Crucially, our data represent a molecular snapshot during isolation, so it is hard to pinpoint whether they indicate hypo-inflammation in ES microglia or a dampening compensation due to (chronic) hyper-inflammation. Interestingly, there are reports that TNFα activation can increase neuronal phagocytosis in vitro, and its relatively decreased expression in ES microglia is in line with the decreased phagocytic capacity we found. Similarly, TGFβ is a neurotrophic trophic factor implicated in neurogenesis, and its upregulation in ES microglia may represent a compensatory mechanism towards ES-associated alterations in neurogenesis. As we argued in the chapter, while most of the developmental microglial profile is similar between CTL and ES mice, the slight differences detected could result in (mal)adaptations that accumulate and eventually contribute to ES-associated phenotypes. These changes might, via e.g. altering synaptic or neurogenic profiles, result in very different structural synaptic landscapes over time.

**Microglial training**

To concretely test our idea of latent ES effects that are unmasked by a later challenge, we injected some of the adult cohort with the bacterial cell wall component lipopolysaccharide (LPS). Consistent with literature, this led to an upregulation of cytokine and innate immune response genes and a downregulation of autophagy related processes. Here, we also saw distinct sets of up- and down-regulated genes after this immune challenge in CTL
and ES mice. Crucially, we found a cluster of genes that, while upregulated by LPS in both groups, were higher expressed in ES microglia. This suggested to us that ES exposure resulted in an immunological memory (specifically, microglial ‘training’), as occurs in maternal inflammation and early-life infection. These genes were distinct from previously reported datasets of “trained” microglial genes occurring in models of accelerated aging, AD, and amyotrophic lateral sclerosis, suggesting a distinct ES-associated immunological memory. The expression of this set of genes in other pre- and postnatal ES models remains to be seen.

This possible induction of microglial training after ES is intriguing given how immunological memory can modulate pathological states in a model of AD. For instance, Wendeln et al. demonstrated that pathology depended on the type of immunological memory: induction of microglial ‘training’ at 3 months (via 1x i.p. LPS injection) worsened Aβ burden at 9 months, while induction of microglial ‘tolerance’ at the same age (via 4x i.p. LPS injections) resulted in decreased plaque load later. These data support the notion that ES alters microglial trajectories both in basal and challenged states, and could thus account for ES-induced modulation of later hippocampal Aβ load.

The ability of later-life challenges to ‘unmask’ latent ES effects, is congruent with the framework of cumulative hits, but not unique to neuroimmune memory. We and others have shown before how ES effects can be ‘unmasked’ upon a secondary challenge also via other factors like e.g. diet, exercise, or another stressor. However, perhaps the most intriguing of these systems that we have used is the APPswe/PS1de9 transgenic mouse model, which aside from serving as a model for aspects of amyloid pathology, can also serve as a chronic source of inflammatory challenge. As we have discussed earlier in this thesis, AD risk is associated with ES and, given the noted trajectory of development of AD pathology in individuals and animal models, the APP/PS1 mice provided an excellent opportunity to interrogate ES interactions with trajectories in a pathological setting. In fact, we have previously shown an age-associated modulation of amyloid load in APP/PS1 mice exposed to ES. We continued this work in chapters 5 and 6, where we investigated APP/PS1 mice at 4- and 10-months of age, which represent early- and late-stages of Aβ pathology.

**Synaptic changes**

To begin with, chapter 4 suggested that ES synapses were inherently different, which led to them being phagocytosed less. We hence set out in chapter 5 to determine the proteomic composition of hippocampal synapses of both wildtype APP/PS1 mice previously exposed to ES at early and late stages of Aβ pathology. While both ES exposure and the APP/PS1 genotype at 4 months led to alterations in mitochondria and actin dynamics, there were no differences between APP/PS1-control versus APP/PS1-ES synapses at this age. We hypothesized that these might indicate convergent but non-synergistic overlaps in the proteins induced by both experimental factors, which would be in line with evidence showing ES or chronic stress induction of AD-like protein expression signatures. On the other hand, at 10 months we found a massive difference in synaptosomal proteins in ES-exposed APP/PS1 mice versus CTL APP/PS1 mice. A number of these proteins were involved with lipid metabolism, specifically their processing at the mitochondria.
Together, these effects provide a comparable picture of prominent ES effects that are ‘unmasked’ only upon conditions of more advanced, severe pathology and/or later inflammation. Importantly, while the neuroimmune system is already involved at early stages of AD\textsuperscript{97}, the morphological and functional responses of microglia to Aβ become exacerbated at advanced pathological stages\textsuperscript{88}. As oligomeric Aβ species, which prominently drive synaptotoxicity during early pathological stages\textsuperscript{89}, can impair antigen presentation\textsuperscript{90}, it could be that the modulation of the ES response to secondary (neuroinflammatory) challenges requires said challenges to be of a certain ‘strength’, or intensity, that is present at later, but not early, stages of Aβ pathology.

Astrocytes
Given our work on microglial alterations in ES-exposed APP/PS1 mice, we wanted to also understand what the astrocytic profile in this system would look like. Astrocytes are another important component of the neuroimmune response to AD\textsuperscript{77}, and seem to be more activated as pathology develops. They participate more in the clearance of Aβ plaques at later stages of pathology than at the onset of plaque formation\textsuperscript{91}, which the temporal pattern of aberrations in their Ca2+ signaling corroborate\textsuperscript{92}. As such, we wanted to understand the temporal changes to astrocytic profiles and functions in ES animals both in wildtype and APP/PS1 conditions.

We performed this work in chapter 6, where we characterized astrocytic reactivity and astrocyte-related gene expression in ES exposed APP/PS1 mice again at 4M and 10M. We did not find any ES or genotype effects at 4 months, but at 10 months, we found decreased signal of the reactive astrocyte marker GFAP in the WT-ES hippocampus, in line with decreased GFAP+ cells at this age that we found in chapter 3. This finding, combined with increased GFAP coverage at postnatal day (P) 9, and decreased GFAP coverage at 6 months, implies a dynamic effect of ES exposure on astrocytic reactivity across the lifespan in wildtype mice. Importantly, we also presented preliminary evidence for premature aging in wildtype ES astrocytes in chapter 3, where we found decreased astrocytic expression of the senescence-associated nuclear envelop protein Lamin-B1\textsuperscript{37} at 4 months. Given that senescent\textsuperscript{93} and Lamin-B1 deficient\textsuperscript{94} astrocytes exhibit reactive phenotypes, and that reactive astrocytes increase neuroinflammation (e.g. by driving pro-inflammatory microglial activity\textsuperscript{95}), this could be a mechanism through which ES astrocytes take part in dysregulating the neuroimmune profile.

We also studied this astrocytic reactivity in 10M old transgenic mice, where we found increases in clustered GFAP signal, which we used as a proxy for the well documented clustering of astrocytes around Aβ plaque\textsuperscript{96,97}. While this was not different in ES-exposed APP/PS1 mice, we know from a previous study using series from the same brains that there are differences in amyloid load in these animals\textsuperscript{34}, suggesting that astrocytic clustering around plaques is dysregulated in ES-exposed APP/PS1 mice. Lastly, we also found evidence for differences in ES effects on non-Aβ-related astrocytic functions between WT and transgenic mice, e.g., an increased expression of fatty acid synthetase in WT-ES mice at 4M (mRNA, chapter 6) but in APP/PS1-ES mice at 10 months (protein, chapter 5). The exact temporal dynamics of these changes, and how they are impacted by ES, will have to await future studies.
For now, the overall picture from our data indicates that ES triggers different age-related trajectories, especially with respect to the (responsiveness of the) neuroimmune system. Some of these changes are evident even at basal states, but others are only unmasked during later-life challenges. Still, the factors that determine which final trajectory the ES animals end up taking is something that needs more work to understand.

**Proposed mechanism: ES effects snowball as a result of dysregulated interactions between microglia and astrocytes**

The natural follow-up question, of course, is: how would these shifts in trajectories happen? In attempting to answer this, this thesis focused on the so-called quad-partite synapse, consisting of pre- and post-synaptic neurons, astrocytes and microglia, as a substrate for ES effects. We also report preliminary work on how ES impacts the blood-brain barrier (BBB), whose physiology is coupled with activity changes at the synapse. More than anything, our data suggest that ES effects might arise not from cell-intrinsic alterations to these cell types, but rather from dysfunctional cell-cell interactions, especially between astrocytes and microglia. In this framework, dysregulated intercellular communications as a result of ES would ‘snowball’ and lead to accumulated mal-adaptations in both astrocytes and microglia, as well as systems they interact with (e.g., synapses and the BBB). This would ultimately result in an entire system that functions differently under basal, and especially under challenged, conditions (Fig. 1).

We observed such effects first in the interactions between microglia and synapses, as shown in chapter 4. The effects of ES on the synapse are well known, e.g. how it interferes with
neurogenesis, and alters later-life synaptic structure and function. Microglia are important for shaping these developing networks, as evidenced by weakened synaptic transmission and brain connectivity when pruning is eliminated. We saw that ES exposure impaired microglial phagocytosis of prepared synaptic fractions at P9, brought about by alterations to the synapse rather than to the microglia, as also reflected in our transcriptomic data at this age. In contrast, we found microglia from adult ES mice to have deficient phagocytic capacities, regardless of whether the synapses were prepared from CTL or ES mice. While the contents of the P9 synaptosome after ES exposure is an ongoing experiment whose results we are waiting for, these data suggest that our previously described microglial phenotype in ES pups might be initially induced by cell-extrinsic factors, which nonetheless result in impaired synaptic pruning.

This impaired network formation could then lead to other downstream effects. For one, because proper neuronal activity is essential to coordinate astrocyte-neuron activity and interactions (at least in sensory domains), this might then lead to (mal)adaptive shifts in astrocytic function, providing a starting point for impaired neuron-astrocyte interactions. Taking GFAP as a marker for reactive astrocytes, its increase in certain subregions at P9 could be indicative of a shift from more developmental functions to potentially more deleterious or even neurotoxic ones. Given the interplay between reactive astrocytes and neurons, as well as microglia, this could then continue as a cycle that results in a different synaptic ‘environment’ and related properties in adulthood.

We can infer an example of this lifelong shift in “normal” functions through the context of astrocytes, whose different roles in the hippocampus, especially after ES, were covered in several chapters of this thesis. This idea was previously proposed in our group, and also by us in chapter 6, despite us finding fewer than expected ES effects on the astrocyte profile compared to our characterization of microglia in the same mice. We saw this in chapter 5, where we demonstrated that a substantial part of the synaptic proteins altered in 4-month-old wildtype ES mice is astrocytic. This was also true in 10-month-old APP/PS1 mice exposed to ES. Importantly, these deficits seemed to relate with astrocytic lipid metabolism, in part mediated by mitochondria.

Astrocytes are the main source of lipids in the brain. Beyond serving as an energy source, their lipid metabolism also plays a role in synaptogenesis, myelination, and BBB regulation. On that last note, we provide preliminary evidence in chapter 7 for increased astrocytic coverage around endothelial cells, and we hypothesize that alterations to astrocyte-BBB interactions would result in the altered hippocampal vascularization we observed.

Another possible ES-induced alteration in astrocytic function might be phagocytosis, based on our data in chapter 4. Even though we found decreased synaptosomal uptake in adult ES microglia, they actually had higher expression (versus CTLs) of Gas6, a ligand that induces microglial phagocytosis. Because Gas6 has also been shown to be expressed by microglia, we proposed this to be a possible mechanism to recruit other phagocytosis-competent cells such as astrocytes. Still, considering that astrocytes are less efficient phagocytes than microglia, that in vitro models of astrocytic senescence
describe impaired phagocytosis\textsuperscript{123,124}, and the possibility that our model induces premature astrocytic senescence (as our Lamin-B1 data in chapter 3 suggest), it is unclear whether this compensatory need will be adequately met. In any case, these potential shifts in balance might have further consequences in the context of the APP/PS1 genotype, where ES astrocytes might be too “distracted” from carrying out their otherwise protective roles, which could result in a failure to properly deal with Aβ\textsuperscript{125}, and thereby contribute to ES effects on Aβ pathology\textsuperscript{32,126}.

It remains to be seen what would instigate this snowball effect. The HPA axis is generally hyporesponsive to many, but not all, stressors during the early postnatal period\textsuperscript{9}. The fact that some models of ES can nevertheless induce HPA axis activation\textsuperscript{58,127}, suggests that ES can shift, or accelerate, the maturation of this (and probably other) brain circuits. The signals responsible are thought to be mediated by maternal care, which, even in non-ES experiments, can lead to lasting changes in pup development\textsuperscript{128,129}. This would imply that somatosensory cues also play a role in this mechanism, and some have even suggested a link between ES and later-life somatosensory processing\textsuperscript{130}. Others have also proposed that beyond maternal care, other postnatal factors (e.g. thermal stress, milk composition, sibling interactions, temperature) can all impact neurodevelopment and later-life behavior\textsuperscript{131}.

We attempted in chapter 8 to analyze the effect of hypothermia as a contributing factor, although we did not find evidence for this. Similarly, some work in our group focuses on the role of other maternal signals in this programming, such as breast milk.

Regardless of the specific source of these alterations, epigenetic mechanisms are a likely common mediator, and some work has already shown evidence for this in relation to ES. Supplementation with dietary micronutrients, known to be important for promoting methylation of DNA, could e.g. partially rescue cognitive phenotypes of ES\textsuperscript{132}. In addition, histone-methylation-induced restriction of gene expression in the hippocampal CA3 leads to a differential acute stress response in mice\textsuperscript{72}. Moreover, if ES would lead to microglial training, as we propose, then we should also expect to see epigenetic marks on isolated microglia, as seen e.g. in the histone acetylation pattern of microglial training in response to LPS\textsuperscript{133}. Similarly, given the epigenetic alterations that result in decreased Claudin-5 expression in the nucleus accumbens of mice susceptible to chronic social stress\textsuperscript{134}, we might also anticipate epigenetic alterations to the endothelial cells of ES-exposed mice in future studies.

The usefulness of our implemented models

“All models are wrong, but some models are useful.” – George Box

While there are calls to reduce the number of animals used in laboratory experiments, for good ethical reasons, they provide and have provided important knowledge that has helped better understand several brain mechanisms and disease substrates, and are clearly valuable for drug development and safety testing. In fact, animal models are, for disciplines like brain research, currently irreplaceable given the emergent properties they possess (e.g. behavior, brain-body interactions, etc), mechanistic insights they allow, and the intervention/
manipulation options they offer\textsuperscript{135}. However, most models often only recapitulate specific symptoms of a disorder, rather than the disease as a whole. As such, they must be used conscientiously and results interpreted carefully.

Broadly speaking, a good (animal) model has two core properties: reliability and validity\textsuperscript{136}. In the context of preclinical animal research, the former refers to the consistency of induction of experimental groups, and the latter, roughly, would correspond to whether we are truly studying what we think we are (i.e., ES in this thesis). The proper and consistent implementation of a model (within and between laboratories) is crucial for progress in the field, as can be done by meta-analytical approaches to discover “true” effects\textsuperscript{13–15}. On the other hand, the validity of a model is essential for the proper translatability of our work to the human condition\textsuperscript{137}, which we focus on in the next section.

In terms of reliability, and due to its transgenic overexpression of specific (familial) AD mutations, the APP/PS1 model is a robust, commonly-used, and reproducible model for at least some aspects of AD pathology. On the other hand, in part due to the emergence of susceptible and resilient populations to stress\textsuperscript{138}, there are intrinsic variations in our implementation of the limited bedding and nesting (LBN) model of ES. As such, to increase compatibility of data across experiments and cohorts, effective ES induction needs to be well validated. This includes the establishment of consensus readout parameters that are consistently observed in ES animals, which would ideally be assessed in each new cohort.

While differences in body weight\textsuperscript{58,69,70,127,139–141}, corticosterone levels\textsuperscript{127}, thymus weight\textsuperscript{58}, and plasma glucose levels\textsuperscript{139} are well-described phenotypes for ES-exposed pups in our currently used model, most of these measures are terminal. As we argued in chapter 8, this presents a problem for validating successful model implementation in experimental designs interested in later-life ES effects, as was the case in this thesis. Thus, we attempted to establish two non-invasive approaches as additional hallmarks of ES exposure.

The first approach was an optimization of the often-used maternal care observations\textsuperscript{142}, which comes with practical and logistical caveats. We proposed decreased total off-nest behavior in ES dams to be a useful readout, which could be easily and accurately implemented in addition to measures of body weight and maternal behavioral entropy. We then presented our attempts to assess differences between the surface temperature of mice in CTL and ES nest conditions, using an infrared camera. While we did not see this to be altered across cohorts investigated, I believe that this demonstrates the utility of developing and improving non-invasive readouts to further characterize features of the LBN model. Other measures in this spirit include assessing differences between CTL and ES nests in ultrasonic vocalization (e.g. by Erica Berretta et al., unpublished), as well as sexual maturation (e.g. by Jelle Knop et al.\textsuperscript{143}). Additionally, as our ability to analyze complex datasets using machine learning expands (e.g. in assessing behavior\textsuperscript{144}), we might also uncover ES-associated behavioral patterns through continuous cage monitoring. Considering the rapid growth of the ES field, efforts to consolidate this toolkit to validate successful model implementation will be crucial, especially for long-term experiments.
Translational validity in our work

While our work was focused on modeling ES in mice, it is important to keep in mind why it is relevant, and why we need to perform animal experiments in the first place. To this day, ES has a high societal cost, especially in developing countries, e.g. my homeland, the Philippines. These adverse experiences include malnutrition, neglect, and abuse, and, compounded with the high poverty rate (23.7%, or roughly 26.14 million Filipinos), can disproportionately affect later life health and well-being. The rates for experiencing ES is especially higher in urban poor segments of the country, in part exacerbated by unequal access to healthcare facilities, the effects of which are highlighted e.g. by the COVID-19 pandemic. The fact of the matter is that, for an unacceptably high number of children, ES exposure is and will continue to be a reality, with, so far, little options for prevention or intervention.

One of the main translational advantages of the LBN model is that it modeled a more translationally valid situation, i.e. that of a parent that is present but unable to provide sufficient care. Still, it is difficult to capture the full complexity and heterogeneity of ES in humans, and as such, there have been and will always be discrepancies between findings in humans and animal models. One example is the documented shorter telomere length in ES-exposed individuals, which we did not find in mice in chapter 2. This could reflect differences in cell type specific versus bulk tissue telomere measurement, but more likely reflects fundamental differences in human and rodent biology (e.g., lifespan or severity of the stressors experienced) that limit translatability. This is particularly pertinent in diseases with an advanced aging component such as neurodegenerative diseases.

Reassuringly, we also found evidence for congruence between our findings in mice with the human condition, where ES exposure is indeed associated with altered later life cognition, metabolism, and neuroinflammation. While it was the only human data collected in this thesis, one of our key findings in chapter 4 was the recapitulation of ES-increased GAS6 expression in human hippocampal microglia. In fact, there was also an increase in the amount of microglia, as assessed by the homeostatic microglial marker TMEM119. The rise of (neuro)immune alterations as a phenotype of ES-exposed individuals is crucial, given that one of the intervention strategies our group is working on, is the use of polyunsaturated fatty acids, which themselves have immunomodulatory properties.

It should be noted that there are also well-noted questions regarding the validity of the APP/PS1 model, as it e.g. does not recapture the tau pathology or massive CA1 cell loss, as seen in AD. Despite this, and given the noted limitations of the amyloid cascade hypothesis per se, we continued using the model as we still believe amyloid remains an important feature in AD, and because of its utility to test secondary challenges after ES in an AD context, as discussed above. Another important limitation regarding validity is that we did not include both sexes. There are noted neurobiological and AD-related sex differences, particularly in the (early) stress response, the latter of which we also noted in chapter 7. We have other research lines investigating the same questions we posed in this thesis in female mice, which were not included due to logistical and time constraints. The findings from these upcoming
works will be important to increase the translatability of our findings, which in my opinion is the truest test of validity in animal models.

**Outstanding questions and outlook**

Our work in this thesis has focused on the role of microglia and astrocytes in ES-induced programming effects. This was mainly due to their early developmental presence, and their role in sculpting the postnatal brain circuitry. Despite our best intentions to contribute to understanding their roles as substrates in ES programming, many outstanding questions remain. Aside from future directions as suggested in the above sections, there are five more key open questions, whose answers I believe will move the field forward.

1. **What triggers the ES phenotype in glial cells?**

   From the work presented here, it evident that ES exposure impacts both glial cell types in the hippocampus at P9. One salient candidate pathway for the ES-induced programming of these cells is glucocorticoid signaling. As such, it would be interesting to see how the ES phenotype might emerge when their receptors (GRs are highly expressed in both astrocytes and microglia) are selectively ablated. Additionally, to test my hypothesis that ES effects are mediated by the cyclical transmission of the phenotype between cells at the quad-partite synapse, it would be interesting to see whether global ablation of one of these components (e.g. microglia via CSF1R inhibition) would impact the ES phenotype.

2. **Do our effects on the hippocampus generalize to other parts of the brain?**

   We focused here on the hippocampus, due to its sensitivity to stress, as well as the prominent alterations in spatial learning in ES mice. However, stress occurs as a body- and brain-wide experience. It is thus important to not only extend our investigations of these different systems into other regions susceptible to stress, (e.g., prefrontal cortex, amygdala, and hypothalamus), but also to understand ES effects on the connections between and within these areas.

3. **How late can these effects be induced? Until when can they be rescued?**

   Our work corroborates that of others implying that early postnatal development is a sensitive period for the development of stress-related phenotypes. Importantly, another sensitive period in development is puberty. Whether the epigenetic alterations imposed by ES, as we hypothesize occurs, can be erased during this period, is yet to be seen. On the other hand, work from our group has demonstrated the potential of using dietary supplementation to rescue ES phenotypes, perhaps by acting on the same substrates. What is unknown is how late these interventions can be effective, and what factors would mediate this.
4. Are ES-induced shifts in trajectories beneficial or harmful?

The alteration of developmental trajectories by ES, that we propose in this thesis, could be seen as adaptive responses, to some extent evolutionarily in nature. One interesting observation from our group is that ES-exposed APP/PS1 mice have less cell-associated amyloid at 4 months, yet more Aβ plaques at 10 months\(^\text{14}\). We have long speculated that this might represent an early adaptive response that runs out of steam in the face of insurmountable amyloid deposition, not unlike the concept of cognitive reserve\(^\text{174}\). However, the facilitation of Aβ clearance in a transgenic AD mouse model has been shown to worsen cognitive outcomes, as microglia end up phagocytosing more healthy synapses, too\(^\text{175}\). As such, future attempts to modulate ES-associated Aβ phenotypes (e.g. via dietary supplementation with nutrients that have been suggested to increase phagocytic capacity\(^\text{162}\)) should also consider which direction would be most beneficial for the organism’s long-term well-being.

5. What determines resilience or susceptibility to ES?

Both in our implementation of the model and in the human situation, there are individual differences in the response to ES. In fact, beyond resilience (which can be defined by non-negative responses to stress), some have proposed the concept of anti-fragility, or thriving amidst the uncertainty of stress\(^\text{176}\), as might be described for individuals who undergo post-traumatic growth\(^\text{177}\). This is especially interesting in the context of altered aging trajectories: which factors could place individuals back “on track,” and would these occur via inhibiting the processes that lead to the shift in the first place, or by initiating distinct, opposite process to counteract them?

These and more questions are the subject of intense and hard work by colleagues at the Lucassen lab who I’ve been fortunate to collaborate with over the years, especially Niek Brosens, Jorine Geertsema, and Jeniffer Sanguino-Gomez. To anyone who might be reading this a bit further into the future, I enjoin you to look up their theses and see what wonderful clues and answers they might hold to these and more questions.

**Concluding remarks**

Our work over the past several years has led to some exciting insights into the effects of ES on the developmental trajectories of different neurobiological substrates. Still, there is clearly much more to do, especially regarding the understanding of how these effects come about and how these different systems interact with each other. I look forward to seeing how the field will grow in the coming future, and hope that we never lose sight of why this work is important along the way.
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