Stress and memory in health and disease

Impact on Alzheimer's disease and memory mechanisms

Lesuis, S.L.

Publication date
2019

Document Version
Final published version

License
Other

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

UvA-DARE is a service provided by the library of the University of Amsterdam (https://dare.uva.nl)

Download date: 22 Nov 2023
Exposure to stressful experiences, either early or later in life, can have a strong impact on learning and memory in adult and ageing individuals. Early life experiences in particular have been implicated in determining the vulnerability and resilience for cognitive decline, especially when the brain is already vulnerable, such as seen in Alzheimer’s disease (AD). The first aim of this thesis was to study the effects of experiences early in life (albeit positive or negative) on ageing- or AD-related cognitive decline, and to better understand the underlying mechanisms. I particularly focused on the role of the hypothalamus-pituitary-adrenal (HPA)-axis, and on the expression and functionality of glutamate receptors in this process. Secondly, I investigated why stressful and threatening events are usually remembered so well, an effect that is a.o. attributed to the enhanced release of stress hormones. I investigate how glucocorticoid stress hormones determine memory formation, and how and where these memory traces, or “memory engrams”, are stored within the brain.
Stress and Memory in Health and Disease

Impact on Alzheimer’s Disease and Memory Mechanisms

Sylvie L. Lesuis
The studies described in this thesis were performed at the Brain Plasticity Group of the Swammerdam Institute for Life Sciences (SILS), Center for Neuroscience, University of Amsterdam (Chapter 2-9), at the Experimental Genetics Group, LEGTEGG, Department of Human Genetics, Catholic University Leuven, Belgium (Chapter 2), and at the Department of Molecular and Cellular Neurobiology, Center for Neurogenomics and Cognitive Research, Vrije Universiteit Amsterdam (Chapter 9).

The studies described in this thesis were funded by Internationale Stichting Alzheimer Onderzoek and the SILS. Printing of this thesis was kindly supported by the Graduate School Neurosciences Amsterdam Rotterdam (ONWAR), SILS, and Alzheimer Nederland.
Forget your perfect offering
There is a crack in everything
That's how the light gets in

Leonard Cohen
## Promotiecommissie

**Promotores:**
- dr. H.J. Krugers
- prof. dr. P.J. Lucassen

**Universiteit van Amsterdam**

**Overige leden:**
- dr. M.V. Schmidt
- prof. dr. M. Kindt
- prof. dr. H.W.H.G. Kessels
- dr. M.C. van den Oever
- prof. dr. D.F. Swaab

**Max Planck Institute, München**
**Universiteit van Amsterdam**
**Vrije Universiteit Amsterdam**

**Faculteit:**
- Faculteit der Natuurwetenschappen, Wiskunde en Informatica
STRESS AND MEMORY IN HEALTH AND DISEASE

IMPACT ON ALZHEIMER’S DISEASE AND MEMORY MECHANISMS

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit van Amsterdam op gezag van de Rector Magnificus prof. dr. ir. K.I.J. Maex
ten overstaan van een door het College voor Promoties ingestelde commissie, in het openbaar te verdedigen in de Agnietenkapel op donderdag 24 januari 2019, te 14.00 uur

door

Sylvie Lisa Lesuis
geboren te Rotterdam
Table of Contents

Preface 9

Chapter 1  General Introduction 21

Chapter 2  Positive and negative early life experiences differentially modulate long term survival and amyloid protein levels in a mouse model of Alzheimer’s disease

Chapter 3  Early postnatal handling reduces hippocampal amyloid plaque formation and enhances cognitive performance in APPswe/PS1dE9 mice at middle age

Chapter 4  Targeting glucocorticoid receptors prevents the effects of early life stress on amyloid pathology and cognitive performance in APPswe/PS1dE9 mice

Chapter 5  Early life stress amplifies fear responses and hippocampal synaptic potentiation in the APPswe/PS1dE9 Alzheimer mouse model

Chapter 6  Treatment with the glutamate modulator riluzole prevents early life stress-induced cognitive deficits and impairments in synaptic plasticity in APPswe/PS1dE9 mice
# Table of Content

| Chapter 7 | Early life stress impairs fear conditioning memory and synaptic plasticity; a potential role for GluN2B | 159 |

| Chapter 8 | Effects of corticosterone on mild auditory fear conditioning and extinction; role of sex and training paradigm | 183 |

| Chapter 9 | Glucocorticoids induce generalised fear by increasing the size of memory-encoding neuronal ensembles | 205 |

| Chapter 10 | General Discussion | 225 |
| Part 1 - The effects of early life experiences on AD vulnerability | 229 |
| Part 2 - The effects of stress hormones on memory | 259 |

| Summary | English summary | 279 |
| Nederlandse samenvatting | 287 |
| Samenvatting voor niet-ingewijden | 295 |

| Addenda | Acknowledgements | 303 |
| PhD portfolio/List of Publications | 314 |
| About the Author | 318 |
Positive and negative early life experiences differentially modulate long term survival and amyloid protein levels.
Preface

In our modern society, individuals are regularly exposed to stress. Stress can involve challenging and threatening (physiological and psychological) experiences that ask for adaptive, often behavioural, responses, like the “flight, fright or fight” response. Homeostasis, the establishment of a dynamic internal equilibrium, plays a central role in one’s adaptive responses upon stressful experiences. Consequently, the physiological and behavioural responses experienced in response to stress are all intended to restore the changes in homeostasis evoked by stress. Such adaptive ‘coping’ with stress requires a rapid activation of numerous bodily systems in response to a stressor as well as an adequate termination when the stressor has seized.

Glucocorticoid hormones, which are released in elevated levels from the adrenal glands in response to stressors, play a central role in such coping with stressful experiences. These hormones orchestrate the energy supply to challenged tissues and control the excitability of neuronal networks that underlie learning and memory processes. In particular, glucocorticoid hormones facilitate the acquisition and storage of new information, while promoting the extinction of less relevant information, thereby governing an adaptive and often protective response to stressors. Despite the undeniable relevance of stress for learning and memory and overall survival, exposure to stressful experiences and stress hormones have been related extensively to disruptive effects on homeostasis and to the risk for stress-related disorders such as depression, anxiety and posttraumatic stress disorder. For example, it has been hypothesised that chronic stress increases the costs of reinstating homeostasis (allostatic load), which increases the vulnerability for (mental) illnesses.

Although suffering from “stress” is considered a modern affliction (with the World Health Organisation proclaiming it to be the Health Epidemic of the 21st century), the awareness that exposure to stressors can have substantial consequences can be traced back to work done by ancient philosophers dating back as far as Aristotle and Hippocrates. Over the centuries, various nonphysical phenomena have been proposed to either cause disease, or at least contribute to the development of psychological or biological disease. The first “modern” definition of the term stress is considered to be posed by Hans Selye in the first half of the 20th century, after observing that chronic exposure to stressors, through overproduction of, at that time undefined, chemicals and hormones, resulted e.g. in ulcers and high blood pressure. Although ulcers were later shown to be caused by bacteria rather than stress, his studies laid down the groundwork for a long line of research addressing the fundamentals.
between stress and disease.

Activation of the hypothalamic pituitary adrenal (HPA) axis, resulting in the synthesis and release of glucocorticoid hormones by the adrenal glands, plays an important role in adaptation to stress, a discovery for which Kendall, Hench, and Reichstein jointly received the Nobel Prize for Physiology and Medicine in 1950. The notion that glucocorticoids could affect higher brain regions was revealed by Bruce McEwen in 1968, when he showed that corticosterone was predominantly retained in hippocampal neurons, and by the discovery by Hans Reul and Ron de Kloet of the high-affinity mineralocorticoid receptor (MR) and the lower affinity glucocorticoid receptor (GR) in the brain. We now know that the activation of MRs and GRs activates non-genomic and genomic pathways that are critical in the regulation of homeostasis, neuronal excitability and behavioural adaptation. Via activation of MRs and GRs, glucocorticoid hormones promote selective attention, memory retrieval, appraisal, and the expression of fear and emotion, as well as regulation executive function and memory storage.

Another conceptual advance in the history of early stress research came with the “Developmental Origins of Health and Disease” (DOHaD) hypothesis, which postulates that perinatal environmental factors play an important role in determining the risk to develop pathology later in life. Initially, this theory centred around the effects of e.g. perinatal malnutrition on the development of later metabolic syndromes. However, also other adverse events early in life, such as experiencing emotional neglect, physical abuse or traumatic events have been associated with the risk for developing psychopathologies like anxiety disorders and depression, cognitive dysfunction in later life, accelerated aging and an increased vulnerability to the development of age-related diseases such as Alzheimer’s disease. This suggests that programming of the brain and/or stress systems by events early in life can be a major determinant for the risk to develop later-life cognitive or emotional problems. Much effort is undertaken to identify the mediating mechanisms, and epigenetic modifications are believed to be an important mechanism by which early adversity can induce more or less persistent molecular (and behavioural) alterations, although additional mechanisms cannot be excluded.

With the development of the genomic toolbox that has become available over the past decades, the concept of genetic susceptibility to stress, or “gene x environment interactions”, has attracted considerable research interest. For instance, polymorphisms of certain genes can render individuals more or less susceptible to stressful life events. Furthermore, the ability to model
the specific genetic components of e.g. Alzheimer disease by overexpressing different variants of single or combined genes, thereby modelling different aspects of the disease, have greatly accelerated research in this field. This allows for investigating the interaction between specific genetic factors and the individual’s sensitivity to stress-related events. It should be mentioned though that the limitations of these models have by now also been appreciated, as many of the potential treatment targets arising from these genetic animal studies have subsequently failed in clinical trials, possibly due to a lack in construct validity of the models and/or timing of the interventions.

Finally, over the last decade, the advanced molecular techniques allowing for capturing and manipulating specific neurons have expanded exponentially. In particular, the discovery of opto- and chemogenetic tools to modify activity of specific neurons by the genetic or viral expression of artificial proteins (opsins or engineered receptors, respectively)\cite{30,31}, notably in predefined distinct neuronal populations, have advanced the field. Using e.g. light sources (for opsins) or exogenous compounds (for chemogenetic receptors), the activity of these neurons (and behaviour) can subsequently be manipulated. These techniques have enabled previously unimaginable levels of functional circuit mapping, and give rise to a plethora of unexplored research avenues in neuroscience.

Among these is the quest for “what is memory”, and “how are these memory represented in the brain”, including answers to questions as to where and how specific memories are stored, consolidated, and retrieved. This archaic question was first posed by Richard Semon in 1921 when he formulated the Engram Theory\cite{32}, which assumes that learning activates coordinated, and yet to be identified, “neuronal excitations” that he believed to be responsible for the formation of a memory. Building on his theory, neuroscientists now have the toolbox to recognise that learning activates a subpopulation of neurons responsible for a given memory trace, while reactivating these cells pharmacologically or by relevant cues results in the retrieval of the specific memory\cite{33}. Using these novel techniques, researchers are now finally able to capture, manipulate or reactivate memory traces in live animals, which unveils an entire new level of understanding of how the brain functions, and also allows to study in a more direct and specific manner how stress hormones can modulate these functions and the circuits that encode them.

**Outstanding questions addressed in this thesis**

Standing on the imaginary shoulders of these, and many other giants, this
thesis aims to provide insight into some of the biological mechanisms underlying the effects of how environmental experiences and stress hormones influence learning and memory processes in health and disease.

The focus will be on how early life programming of the brain during a highly sensitive developmental time window can confer lasting effects on later brain plasticity and structure, and cognitive and emotional functioning. In these studies, I will focus on the effects of early life experiences on aging and age-related disease, with a specific focus on Alzheimer’s disease. In addition, I studied the mechanisms of how stress hormones influence memory formation under healthy conditions. The following outstanding questions will be addressed:

I. Are experiences early in life critical for determining the later-life vulnerability to the development of Alzheimer’s disease related symptomatology and pathology?

II. Which biological systems and processes underlie the early life stress-induced modulation of cognitive function and Alzheimer’s disease related pathology?

III. How do stress hormones modify the properties of neurons that underlie learning and memory?

**Why animal models?**

Human literature has revealed various associations between early life experiences and later life cognitive and emotional functioning, underscoring the possible importance of the early postnatal period in shaping the brain. However, the long time lag between the early life environment and the onset of AD symptoms hampers a deeper understanding of the underlying causes and possible mechanisms. To address this, animal models allow for a more detailed investigation of this relationship that may help to identify the mechanisms by which environmental factors during the early life period can affect AD symptoms and pathology. The ability to e.g. model specific genetic (risk) factors for AD and the precise control over (timing of) life events makes such animal models highly suitable for investigating the pathological mechanisms underlying the interactions between genes, the (early) environment and AD. In addition, employing animal models enables the specific modulation of specific genes, employ viral vectors and/or other pharmacological compounds, that are required for an in-depth investigation into the abovementioned questions.
Outline of this thesis

The overarching goal of this thesis is to understand how stress, either chronically early in life or acutely after learning experiences, affect learning and memory processes in the adult and aging brain, and how such effects may arise. The first aim was to elucidate whether positive and/or negative early life experiences contribute to a different onset or exacerbation of cognitive decline in relation to Alzheimer’s Disease (AD). To answer this question, I investigate the long-lasting consequences of early life experiences on synaptic plasticity, neuropathological parameters and on behaviour in wild type mice and in relevant transgenic mouse models for AD. I further studied whether early life adversity can be prevented by targeting the hypothalamus-pituitary-adrenal (HPA)-axis later in life. In the second part of this thesis, I focus on the role of the NMDA receptor as a mediator between early life stress, AD, and aging. In the final part of this thesis I study how glucocorticoid hormones, which are released in elevated levels upon activation of the HPA-axis, alter memory formation. I therefore study how these hormones regulate the number and properties of training-activated neurons (“memory engram cells”) and whether these cells underlie the memory enhancing effects of glucocorticoid hormones.

Chapter 1 reviews literature on the effects of early life experiences on behaviour and functional plasticity of the brain, and how these effects are mediated. I discuss that early life experiences may long-lastingly alter HPA axis (re-)activity, thereby shaping behaviour and brain function during adult life and during aging, and address the hypothesis that early life experiences, either positive or negative, can alter the vulnerability for developing AD pathology.

Effects of early life experiences on AD development

In Chapter 2, I investigate the effects of positive and negative early life experiences, by manipulating the amount of maternal care that pups receive, on the lifespan of mice that express amyloid and tau AD neuropathology (biAT mice), and study whether these effects are associated with alterations in learning and memory, neuronal morphology, and in neuropathological hallmarks of AD in relevant brain regions for learning and memory.

Chapter 3 extends on these observations by investigating, in a different, well-characterised mouse model for amyloid-β-associated neuropathology (APPswe/PS1dE9 mice), whether enhanced levels of maternal care can prevent or delay the AD-induced changes in neuropathology and cognitive decline at different ages. I further investigate whether these alterations are paralleled
by alterations in HPA-axis reactivity and pathological markers in hippocampal subareas and the amygdala in adult animals.

To further investigate the role of the HPA axis in the effects of early life adversity on behavioural and pathological changes related to AD, I investigate in Chapter 4 how early life stress affects Aβ-neuropathology in APPswe/PS1dE9 mice, another genetic mouse model for AD, and I study alterations in the amyloidogenic pathway that may underlie these early effects in 6 and 12 month old mice, i.e. before and after the onset of cognitive decline. In addition, I address whether glucocorticoid signalling is altered in these mice, and whether interventions targeting the glucocorticoid receptors at older age can reverse the early life stress-enhanced AD phenotype.

The role of the NMDA receptor in early life stress, aging and AD

In Chapter 5 I test the hypothesis that the early life stress-induced cognitive deficits at 1 year of age are associated with alterations in synaptic plasticity. I investigate hippocampal synaptic plasticity in APPswe/PS1dE9 mice and wild type littermates, focusing specifically on the role of GluN2B containing NMDA receptors. I further evaluate how this affects emotional fear memory formation.

In addition, I explore the mechanisms underlying the alterations in synaptic plasticity and cognitive deficits in APPswe/PS1dE9 mice following early life stress in Chapter 6, by treating mice with the glutamate modulator riluzole. To further understand the relationship between early life stress, AD, and aging, I focus on the glutamatergic signalling pathway and the expression of the excitatory amino acid transporter 2.

In order to better understand how (early life) stress affects learning and memory processes in aging and an AD background, it is imperative to understand how these processes are regulated under non-pathological conditions. Therefore, in Chapter 7 I investigate short-term and long-term synaptic plasticity in wild type mice, and I propose a central role for the NMDA receptor subunit 2B in mediating the effects of early life stress on emotional and/or hippocampus-dependent memory formation, glucocorticoid signalling and receptor expression.

The effects of acute glucocorticoid exposure on memory formation

In Chapter 8, I investigate whether brief administration of corticosterone after training enhances consolidation of fear memories. Using different auditory
fear conditioning paradigms, I evaluate how corticosterone influences the strength of the memory trace, and the ability to extinguish fear memory. I further test whether and how these effects are sex-dependent.

In Chapter 9, I describe the effects of acute glucocorticoid exposure on memory strength and specificity. I identify and characterise the neurons responsible that may be part of a so called "engram", and determine their electrophysiological and molecular properties. By capturing and manipulating these neurons I investigate whether these neurons are responsible for the effects of acute glucocorticoid exposure on memory formation.

Finally, in Chapter 10, I summarise the main outcomes of this thesis, and discuss them in a broader perspective. First, I discuss how early life experiences can program brain (or neuronal) architecture and function persistently, and how this may impact cognition and neuropathology in an AD background. I speculate that besides a direct modulation of AD neuropathology by the HPA axis, early life experiences may also shape the "brain reserve" or "cognitive reserve", thereby rendering an individual more resilient or vulnerable to AD-associated impairments. Secondly, I discuss how glucocorticoid hormones influence memory strength and memory specificity, and in particular the role of memory engram cells. Finally, I conclude with remaining outstanding questions that can be addressed in order to move the fields of how (early life) stress alters memory formation ahead.
References

31 Urban DJ, Roth BL. DREADDs (Designer


Parts of this introduction were published in adjusted form:

Vulnerability and resilience to Alzheimer’s disease: Early life conditions modulate neuropathology and determine cognitive reserve


Introduction

The period during peri- and early postnatal life is a critical developmental phase that plays an important role in shaping adult health, a concept that has been widely described in the context of the so called Developmental Origins of Health and Disease (DOHaD) hypothesis\(^1,2\). This hypothesis centres around the notion that the early life environment impacts later health and the vulnerability to develop diseases throughout the entire lifespan, for instance in relation to mental health\(^3,4\), obesity, diabetes, coronary heart disease (e.g.\(^5,6\)). Indeed, accumulating preclinical and clinical evidence highlights the association between early life adversity and impaired cognitive function, the predisposition to develop psychopathology, and neurological disorders such as Alzheimer’s disease.

1. Early life experiences and cognitive function later in life

During early postnatal development, the brain undergoes an impressive growth that involves a massive growth and migration of glia and the proliferation of stem cells and their differentiation into young neurons, that are guided by migration along glia, their formation of early axonal connections and the functional maturation of these neural connections into (early) neuronal networks. These developmental phases involve a carefully controlled orchestration of numerous specific genes being expressed in a well-defined spatiotemporal pattern, allowing complex neuronal networks to be formed.

This delicate, developing brain is very sensitive to external influences and environmental factors, particularly during stages when massive proliferation and migration of neurons occur. As such, stressful experiences and the presence of elevated levels of glucocorticoid hormones (cortisol in humans; corticosterone in rodents) during the early life period have been reported to interfere with ongoing brain development and can exert long-lasting effects on adult brain function and behaviour. Indeed, adverse events during prenatal and early postnatal life (early life stress, ELS) are associated with stress exposure and an increased vulnerability to subsequent stressors and compromised physical and mental health later in life, both in humans and rodents\(^7\)-\(^12\). In contrast, more positive and ‘stimulating’ experiences during early life are, at least in rodents, associated with an apparent resilience to later-life challenges, with a good physical and mental health, as well as with decreased chances to develop later anxiety- and depressive-like behaviour\(^13\)-\(^17\).

The vulnerability of the brain, and in particular the hippocampus (box 1),
to an age-related loss of function seems to parallel the effects of negative or positive early life experiences on cognitive performance. For example, prenatal stress increases, while neonatal handling (which increases maternal care) decreases the rate of brain and/or hippocampal aging and plasticity. This has, in part, been attributed to lasting alterations in hypothalamic-pituitary-adrenal (HPA) axis activity that occurs following such early life experiences. The changes in reactivity of the adult life HPA axis e.g., play a major role in determining the rate of brain and body aging.

### Box 1. Brain regions most relevant to the studies in this thesis

While the stress response starts in the hypothalamus, in this thesis, the main focus will be on the **hippocampus**, an extremely plastic brain region with high relevance for cognition and regulation of the stress response. The hippocampus is highly relevant for processing contextual information and spatial learning. The hippocampus continues to develop postnatally, until two years of age in humans and up to two weeks after birth in rodents, making it particularly sensitive to events early in life. This is supported by clinical evidence showing that early life adversity results in decreased cognitive functioning in adulthood, and correlates with reduced hippocampal volume. Furthermore, hippocampus dysfunction is among the first presentation of Alzheimer’s disease. Different hippocampal subfields each exert their distinct roles in information processing, among which processing of spatial (CA1; CA3) or temporal information, novelty (CA1), and social memory (CA2). The **dentate gyrus** (DG), another hippocampal subregion, is critically involved in separation, the ability to independently represent and store similar experiences.

The **amygdala** plays a key role in the circuitry underlying emotional learning and memory, with a crucial role in auditory fear conditioning. Specific subregions have further been associated with encoding cues (basolateral amygdala (BLA)), in mediating the effects of stress hormones on memory consolidation (BLA), and with fear memory extinction (BLA; central amygdala; lateral amygdala). The amygdala further interacts closely with the **medial prefrontal cortex** (mPFC), which is involved in appraisal of threat or safety and through which, together with other brain regions, many emotional and cognitive processes are affected. The mPFC is also involved in planning, behavioural flexibility and inhibitory behaviour.
During the early postnatal period, the dam is a critical factor for her pups and a disturbance of the mother-pup interaction can have lasting effects on the HPA axis and memory of her offspring later in life (see section 1.1). In rodent models (see box 2 for a summary of the different rodent models of early life stress or enrichment), perinatal stress impairs cognitive performance at an adult age, while generally increasing emotionality and reactivity of the HPA axis and autonomic nervous system in the offspring, effects that generally also last throughout life\textsuperscript{1,2}.

1.1. Hypothalamic-pituitary-adrenal (HPA) axis

Experiences early in life can change HPA axis responsiveness in a long-lasting manner, thereby programming the (cumulative) extent of glucocorticoid exposure over life\textsuperscript{30}. Activation of the HPA axis drives glucocorticoid hormone secretion from the adrenal cortex, both in a circadian manner and in response to stress\textsuperscript{3,4}. HPA axis activity is initiated by internal and external signals that trigger the hypothalamus to release corticotropin-releasing hormone (CRH), which stimulates the anterior pituitary to secrete adrenocorticotropic hormone (ACTH). ACTH acts on the adrenal cortex to stimulate the synthesis and secretion of glucocorticoids. In turn, glucocorticoids also target the hypothalamus and anterior pituitary to inhibit the production and release of CRH and ACTH and thereby GCs control their own release via a negative feedback loop\textsuperscript{31}. Elevations of basal corticosterone levels and a reduced capacity to adapt to and recover from stressors are an inherent part of aging\textsuperscript{5,6}, and an acceleration or delay of these processes may directly influence brain and cognitive aging.

Glucocorticoid hormones can bind to the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). The MR has a high affinity for glucocorticoid hormones, is occupied under basal conditions, and is involved in the initial rise of the endocrine stress response\textsuperscript{32}. GRs, which have a tenfold lower affinity to glucocorticoids, are occupied when glucocorticoid levels are high in response to a stressor. They are involved in terminating the stress response\textsuperscript{7–12}. Both receptors are present in the cytosol and activate or suppress nuclear gene expression upon the binding of the ligand\textsuperscript{32}, important for adaptation and termination of the stress response as well as the storage of information for future use. In addition, activation of these receptors can also exert rapid, non-genomic effects\textsuperscript{13–17}. These rapid effects affect neuronal excitability and are important for the initial stress response and rapid behavioural effects\textsuperscript{22}. Combining non-genomic and genomic actions, MRs and GRs can affect behaviour over a wide time range\textsuperscript{16–20}.
Persistent increases in HPA axis activity can result in higher basal glucocorticoid levels, and in a stronger and more prolonged exposure to glucocorticoid levels following a stressor. Chronic early life stress, induced by housing dams and pups with limited nesting and bedding material (LBN, see box 2) results in elevated corticosterone level, increased adrenal gland weight\textsuperscript{33}, as well as reduced glucocorticoid receptor (GR), mineralocorticoid receptor (MR) and CRH expression\textsuperscript{21–29}. The MR is involved in the appraisal of stressful situations and in the maintenance of basal corticosterone levels, while GR regulates the stress response when endogenous GC levels are

**Box 2. Rodent models of early life stress and enhancement**

In the early life period, the brain shows massive development and is highly sensitive to environmental factors that can disturb this process and affect brain function for life. The consequences of the interferences depend on the maturity of the brain at the moment of birth. For instance, the rat brain at birth is about as mature as the human brain at mid-gestation. This programming is critically determined by, amongst others, interactions between the mother and her offspring. In animal models, the critical components shaping the local environment are; the intrauterine environment, that can be affected by specific medication or e.g. stress hormones that reach the pregnant dam and her fetus(es), as well as postnatal interactions between the dam and her offspring. This involves elements like tactile stimulation, nutrition and warmth. Both time windows can be manipulated experimentally to study the consequences of such early life experiences. We will here highlight some relevant models.

**Prenatal stress\textsuperscript{20}** is a model most commonly induced in pregnant rodents by a single or repeated session of maternal restraint stress and/or defeat during specific gestational periods (mostly during the last week of gestation, sometimes earlier).

Models in which the **naturally occurring variation in maternal care** is used to select for pups that received high amounts of maternal care compared to pups receiving low amounts of maternal care (**low vs. high licking and grooming**). This represents a model to test the consequences of ‘negative’ and stressful vs. a ‘positive’ early life environment for later brain structure and function\textsuperscript{94,96}.

*Box continues on next page*
Alterations in the postnatal mother-pup interaction can also be induced experimentally. Postnatally, early life stress is e.g. induced by a single, prolonged separation of dam and pups, called maternal deprivation\(^9\), which usually lasts for 24 hours and is conducted at postnatal day (PND) 3 or 4. Alternatively, with maternal separation\(^9\), the dam and pups are separated repeatedly for 2-5 hours/day. To introduce chronic early life stress\(^5\), a reduction in the available nesting and bedding material (limited nesting and bedding material, LBN) triggers erratic and fragmented maternal care and stress in the dam which is transmitted to her offspring.

In contrast, a 'positive' early life environment is typically installed by separating the dam and her pups for a brief period of up to 15 minutes on a daily basis, during a time window from PND 2-9 or until weaning. This model is generally called postnatal or neonatal handling\(^19,56,237\) and, results in increased levels of maternal care of the dam towards her pups upon reunion.

High by regulating the negative feedback of the HPA axis to stress. Naturally occurring low levels of maternal care also result in increased corticosterone levels and enhanced CRH release in response to a stressor\(^34–37\) and in a lower expression of hippocampal GRs\(^30\). Maternal separation increases corticosterone levels in response to a stressor\(^38,39\), which is accompanied by reduced GR\(^31\) and increased CRH levels\(^40–46\). Maternal deprivation also increases basal and stress induced corticosterone levels but decreases expression of GR\(^32\). Finally, prenatal stress in rats, applied during the last week of gestation, increased HPA axis responsiveness to subsequent stressors in the adult offspring\(^45,47–49\). Also, the maintenance of basal levels of HPA axis activity is altered, possibly due to a reduced hippocampal MR expression\(^32\), thus highlighting the sensitivity of the HPA axis for early life adverse experiences.

Conversely, increased levels of maternal care early in life, e.g. introduced experimentally by subjecting animals to neonatal handling (see box 2), exerts opposite effects; both corticosterone and ACTH levels are reduced following stress exposure\(^36,50\), accompanied by higher hippocampal GR\(^22\), lower MR\(^33\), and changes in CRH levels\(^31–55\). Even studies in which both prenatal stress and cross-fostering were combined, which can also be considered a form of neonatal handling as it involves intense licking of the pups upon the return of the mother\(^34–37\), show that the effects of prenatal stress on HPA axis activity can
be reversed by cross-fostering the pups to new mothers. This emphasises the strong protective effect of positive early life experiences on HPA-axis responsiveness.

Alterations in glucocorticoid hormone levels and changes in HPA axis feedback have often been associated with hippocampal aging and accelerated cognitive decline, although exceptions exist as well. In humans, those aged individuals who exhibited elevated basal cortisol levels, were the ones who displayed impaired explicit memory performance and selective attention deficits. Their hippocampus was also found to be 14% smaller than that of age-matched controls who did not show progressive cortisol increases and who were not cognitively impaired. Rats with increased HPA-reactivity show an earlier age-related decline of several hippocampus-dependent cognitive functions, while older, cognitively impaired animals also display higher HPA axis activity. Besides the deleterious effects of prolonged glucocorticoid hormone exposure, early life stress also affects the CRH and CRH receptor 1 system (see below), resulting in functional and behavioural impairments in adult life. Together, the specific effects of glucocorticoid hormones on neural development and HPA axis reactivity may change qualitatively as the nervous system matures and ages, indicating that the timing of the applied procedure and the stress hormone exposure, relative to the developmental stage of the different brain regions, is important for its later effects.

1.2. Behaviour

Early life experiences have also been correlated to behavioural alterations later in life, which may be related to HPA axis activity. Chronic early life stress (see box 2 for details) e.g. increases adult anxiety-like behaviour in the elevated plus maze, in the open field and in the light/dark box. Furthermore, various types of chronic early life stress induce memory deficits in the Morris Water Maze, novel object recognition test, and Y-maze, while conditioned fear responses are increased. Offspring that received low amounts of maternal care also shows impaired spatial memory and object recognition performance, and increased conditioned fear responses. Impaired spatial memory was also reported following maternal separation, and maternal deprivation. Furthermore, maternal separation results in more anxious animals in the light/dark exploration test and after fear conditioning. Prenatal stress has been found to increase anxiety in an open field test, and to impair spatial learning at adulthood. Neonatal handling on the other hand, has been associated with a slower rate of cognitive aging and a reduced loss of hippocampal function throughout life. It also results in improved behavioural...
performance in different learning and memory paradigms\textsuperscript{69} and a reduction in conditioned and unconditioned fear responses\textsuperscript{60,64}.

1.3. Synaptic plasticity

Cognitive and memory impairments may be related to deficits in synaptic plasticity\textsuperscript{58}. The capacity to display long-term potentiation (LTP) is one of the major cellular mechanisms thought to underlie learning and memory\textsuperscript{64,65}. Indeed, chronic early life stress results in a reduced capacity to trigger LTP in the hippocampal CA3\textsuperscript{70,71} and CA1 area\textsuperscript{77,9,66-68}. Similarly, pups that received low amounts of maternal care failed to show LTP induction in the dentate gyrus\textsuperscript{21-29} or hippocampal CA1 area\textsuperscript{14,61}. Maternal separation further impaired LTP in the prefrontal cortex (PFC)\textsuperscript{72-77}, whereas maternal deprivation impaired LTP in the dentate gyrus\textsuperscript{61} and CA1 area\textsuperscript{78}. In line with this, exposure to prenatal stress was found to impair the induction of LTP in hippocampal areas at a young adult age\textsuperscript{69}. Interestingly, neonatal handling paradigms, which lower corticosterone exposure, have also been shown to prevent age-related hippocampal and cortical neuronal atrophy and dysfunction\textsuperscript{79}, and to enhance LTP in the CA1 area of adult rats\textsuperscript{58} (more extensively reviewed by\textsuperscript{18}).

1.4. Dendritic morphology

Various studies have shown that pre- and neonatal experiences cause persistent morphological changes to individual neurons in specific limbic brain regions and the PFC\textsuperscript{70,71}. For example, following early life stress, dendritic atrophy of CA1 pyramidal cells and expansions in the CA3 mossy fibres were observed, while the number of granule cells in the hippocampal CA1 area and its innervation of CA3 pyramidal neurons was reduced\textsuperscript{28}, possibly via stress-induced increases in CRH neurons\textsuperscript{21-29}. Furthermore, exposure to chronic early life stress reduces the number of dendritic spines, i.e. the anatomical substrate for memory storage and synaptic transmission, in both CA1 and CA3 areas. Also, a reduced inhibitory synaptic density was found in the CA1 area and in parallel, a reduction in excitatory synaptic density in the hippocampal CA1 and CA3 areas\textsuperscript{80,81}.

Although less well described, also other brain regions are affected, and chronic early life stress hampers dendritic development and spine density in the PFC\textsuperscript{72-77}, whereas it increases spine density in the basolateral amygdala (see box 1)\textsuperscript{62,83}. In addition, pups that received low amounts of maternal care early in life show reduced dendritic complexity in the CA1 area and dentate gyrus at adulthood, compared to pups that received high amounts of maternal care\textsuperscript{78}.
Chapter 1

Also, the number of spines in hippocampal neurons is higher in pups that received high compared to low amounts of maternal care\(^79\). Finally, maternal separation caused atrophy of the basal dendritic tree and reduces spine density on both the apical and basal dendrites in layer II/III of the PFC\(^64,85\). Maternal deprivation reduced the number of granule cells and dendritic complexity in the dentate gyrus\(^18\), but had no effects in the amygdala\(^86\).

### 1.5. Adult neurogenesis

Aging is a prominent inhibitor of adult hippocampal neurogenesis, a form of structural plasticity referring to the continued production of new hippocampal neurons throughout adulthood. These adult-generated neurons are derived from stem cells present in the adult hippocampal dentate gyrus that go through distinct developmental stages before they become fully functional and well integrated within the hippocampal tri-synaptic circuitry\(^28\). The process of adult neurogenesis is regulated by various hormonal and environmental factors, including a stimulation by enriched environmental housing or exercise\(^52,81,87,88\) and in general an inhibition by stress\(^80,81\). Some exceptions exist as well, but in these cases, stress was often predictable, controllable and/or mild, and may actually have resulted in enrichment and could thus have been perceived as positive and rewarding experiences\(^52,54,81,88\). Neurogenesis plays a role in stress regulation\(^82,83\) and is involved in various forms of (hippocampus-dependent) learning and memory\(^87\), including pattern separation\(^84,85\).

Depending on the animal model used, early life stress generally impairs hippocampal neurogenesis at adult ages\(^51,89\), whereas ‘positive’ stimuli like (adult life) environmental enrichment or exercise increase the number of newborn cell numbers in the dentate gyrus\(^86\), but this also depends on earlier treatments and experiences, possibly in a sex-dependent manner\(^90-93\). For instance, exercise, which enhances neurogenesis in males, failed to increase neurogenesis in middle-aged female mice that had been exposed to chronic early life stress in their first week of life\(^52,81,87,88\). Thus, early life experiences can modify the aging-associated decrease in neurogenesis as well as its subsequent sensitivity to environmental stimuli applied later in life\(^94-96\).

### 2. Early life experiences and Alzheimer’s disease

Alzheimer’s disease (AD) is a neurodegenerative disorder that is highly prevalent among the elderly population. AD is characterised by progressive impairments in various behavioural and cognitive functions\(^52,54,81,88\) that have a profound impact on AD patients, their families, caregivers, and society.
Prominent neuropathological hallmarks in the brains of AD patients include amyloid-β (Aβ)-peptide-containing plaques and neurofibrillary tangles (NFTs) containing hyperphosphorylated tau. In humans and rodents, the gradual accumulation of Aβ-containing plaques and NFTs has been associated a.o. with spine loss and glial activation. Together, they may trigger the age-related cognitive decline and behavioural symptoms characteristic for AD.

Seminal genetic studies have identified mutations in the amyloid precursor protein (APP), Presenilin-1, Presenilin-2 genes and variations in ApoE in relation to early and late-onset familial AD (see e.g.97,98). While these mutations explain a small percentage, the vast majority of AD cases likely has a multifactorial aetiology, in which both age and lifestyle factors play an important modulatory role51,89. Epidemiological studies have shown that factors like higher education, a more healthy diet, more social and physical activities, bilingualism, and measures for lifelong learning and mental stimulation correlate with a slower rate of memory decline during aging, a delayed onset of mild cognitive impairment (MCI) and/or a lower incidence of AD99–102. These positive lifestyle factors may therefore delay AD onset and increase the resilience to develop AD.

On the other hand, adverse environmental experiences such as prolonged stress, have been associated with a faster progression of AD symptoms and an earlier development of pathology90–93. Stressful life events have been reported to reduce the age of onset in familial AD103, while major depression, which has a strong stress-related component, has been associated with an increased risk to develop AD earlier in life (e.g.94–96). Furthermore, glucocorticoid (GC) hormones, the main mediators of the stress response, are often increased in AD, notably already early in the disease104 and dysregulation of the hypothalamus–pituitary–adrenal (HPA) axis, i.e. the main neuro-endocrine axis controlling GC release and feedback, may increase the risk to develop AD97,98. Together, these studies highlight a possible interaction between genetic predisposition and lifestyle factors such as stress and/or low socio-economic status, in determining the vulnerability and resilience to develop AD.

In a recent study, Wang et al.31,64 have identified in particular the early postnatal period in humans as a sensitive time window for lasting effects on brain structure and function and also on the risk to develop AD105,106. Indeed, stressful and traumatic experiences during the early life period have been strongly associated with an increased vulnerability to stressors, and compromised physical and mental health in later life, both in humans and rodents99–102. On the other hand, ‘positive’ or stimulating early life experiences in humans83,107 and rodents103 have been associated with an apparent resilience
Box 3. Rodent models of AD neuropathology

Preclinical studies have so far employed transgenic and non-transgenic approaches to model aspects of Alzheimer’s Disease. These models generally reproduce various disease aspects; memory impairments, Aβ containing plaques, and/or tau/tangles, and neuronal loss (only in a few Aβ based models).

Transgenic models most frequently (over)express single or multiple mutations in the APP, presenilin (PS) and/or tau genes, or combinations of these genes, that relate to familial forms of AD. Non-transgenic models are generated by the injection of specific toxins into the brain, such as Aβ, tau or inflammatory-related compounds, or use naturalistic models of aging. Although none of these models fully captures the entire human disease profile and often model only one specific aspect of AD neuropathology, the existing models have made important contributions to our current understanding of AD pathophysiology. There are, however distinct differences in the presentation of neuropathology in transgenic models and the human presentation of dementia, in particular with regard to animal models of amyloid pathology which overall display severe hippocampal amyloidosis, which is different from the human presentation of plaque pathology. Also, no tau mutations have been identified that cause autosomal dominant AD, unlike mutations in Aβ-associated genes. Tau mutations in contrast, produce fronto-temporal dementia. The Aβ and tau-based models will be discussed in more detail in the second part of this box.

Box continues on next page

to later-life challenges and a better later physical and mental health.

Together, several human studies suggest that stressful events early in life are associated with a higher chance to develop AD whereas, in contrast, early life enrichment, longer education and more cognitive ‘stimulation’ during early periods, are correlated with a later presentation of AD symptoms. While the association between early life experiences, HPA axis responsiveness, brain structure, synaptic plasticity and memory underscores the possible importance of the early postnatal period also for AD symptomatology and neuropathology, the long time lag in between the early environment and the onset of AD symptoms has so far hampered a deeper understanding of the underlying causes and possible mechanisms. To address this, animal models
allow for a more detailed investigation of these relationships that may help to identify the mechanisms by which environmental factors during the early life...
period can affect AD symptoms and pathology. The ability to e.g. model specific genetic risk factors for AD and the precise control over (timing of) life events make such animal models highly suitable for investigating the mechanisms underlying the interactions between genes, the (early) environment and AD (see box 3 for an overview of animal models of AD neuropathology).

3. Conclusion

In conclusion, lifelong patterns of adrenocortical function and (cumulative) stress hormone exposure, in part determined by changes in set point due to early life experiences, can contribute to the rate of brain aging, at least in experimental animals. Healthy aging is often characterised by a gradient elevation of glucocorticoid levels, a process that is also modulated by early life experiences and that may influence the vulnerability or resilience of the brain to additional insults. This may be mediated by (epi)genetic alterations in GR, MR and/or CRH expression\textsuperscript{31,64}, that can have persistent consequences for glucocorticoid feedback sensitivity and the function and structure of neurons in specific brain regions. This lifelong programming of the brain by early life experiences thus contributes to the vulnerability or resilience to develop cognitive impairment and psychopathologies later in life, and may further determine the onset, severity, and/or progression of Alzheimer’s disease.
4. References


70. Maccari S, Piazza PV, Kabbaj M, Barbazanges
Chapter 1


90 Liu D, Diorio J, Day JC, Francis DD, Meaney MJ.


109 Fernández-Teruel A, Escorihuela RM, Castellano B, Gonzalez B, Tobena A. Neonatal handling and environmental enrichment effects on emotionality, novelty/reward seeking, and age-related cognitive and hippocampal
impairments: focus on the roman rat lines. Behav Genet 1997; 27.


General Introduction
Positive and negative early life experiences differentially modulate long term survival and amyloid protein levels in a mouse model of Alzheimer's disease

Sylvie L. Lesuis¹, Herve Maurin², Peter Borghgraef², Paul J. Lucassen¹, Fred Van Leuven², Harm J. Krugers¹

Oncotarget
2016, 7(26): 39118-35
Abstract

Stress has been implicated as a risk factor for the severity and progression of sporadic Alzheimer's disease (AD). Early life experiences determine stress responsivity in later life, and modulate age-dependent cognitive decline. Therefore, we examined whether early life experiences influence AD outcome in a bigenic mouse model which progressively develops combined tau and amyloid pathology (biAT mice).

Mice were subjected to either early life stress (ELS) or to 'positive' early handling (EH) postnatally (from day 2 to 9). In biAT mice, ELS significantly compromised long term survival, in contrast to EH which increased life expectancy. In 4 month old mice, ELS-reared biAT mice displayed increased hippocampal Aβ levels, while these levels were reduced in EH-reared biAT mice. No effects of ELS or EH were observed on the brain levels of APP, protein tau, or PSD-95. Dendritic morphology was moderately affected after ELS and EH in the amygdala and medial prefrontal cortex, while object recognition memory and open field performance were not affected. We conclude that despite the strong transgenic background, early life experiences significantly modulate the life expectancy of biAT mice. Parallel changes in hippocampal Aβ levels were evident, without affecting cognition of young adult biAT mice.

Key words: early life stress, early handling, biAT, Alzheimer, glucocorticoids.
1. Introduction

Alzheimer’s disease (AD) is a neurodegenerative disorder characterised by progressive impairments in cognitive functions\(^1\). Prominent neuropathological features of AD are amyloid-containing plaques and neurofibrillary tauopathy consisting of threads and tangles (NFT), which are observed throughout the brain, including areas critically involved in memory formation and emotional regulation\(^2\). The accumulation of amyloid plaques and tauopathy is believed to underlie neuronal and synaptic dysfunction, and age-related cognitive decline\(^3,4\).

While genetics, in particular specific mutations, have been implicated in rare forms of familial dementia\(^5\), the aetiology of the large majority of late-onset, sporadic AD cases remains elusive. It has however been hypothesised that gene-environment interactions, epigenetic factors, exercise, diet, stress, and life-style in general, contribute to the incidence and progression of late-onset sporadic AD\(^6,7\). Epidemiological studies support a role for chronic stress as an important environmental risk factor for AD progression\(^8,9\). Indeed, elderly individuals that are prone to psychological distress are more likely to develop AD than non-stressed individuals of the same age\(^10\). Animal model studies substantiate this role of environmental modulation of AD. Chronic stress in adulthood e.g. elevates A\(^{\beta}\)\(_{40}\) and A\(^{\beta}\)\(_{42}\) brain levels, accelerates amyloid plaque formation, increases tauopathy and neuronal atrophy, and impairs learning and memory in various mouse models\(^11-13\).

Stress during the period of early life, when the brain is still developing, often has more pronounced and longer lasting effects compared to adult stress exposure. Moreover, early life stress not only enhances sensitivity of the brain to subsequent later stressors, but also accelerates age-related cognitive decline\(^14-19\). In contrast, daily handling (EH) during the early postnatal period is known to increase maternal care, blunts the sensitivity to stressors later in life, and reduces age-related cognitive decline in wild type rodents\(^14,20\).

While early life experiences influence cognition later in life, it remains elusive whether they also affect the development of AD pathology and reduce life expectancy in AD models. We therefore imposed 'negative' versus 'positive' experiences during the early period from postnatal day (PND) 2-9 and analysed whether they differentially influenced AD-related brain changes in transgenic APP.V717I x Tau.P301L (biAT) mice. BiAT mice are well characterised to develop both amyloid plaques and neurofibrillary tau pathology over time\(^21\). We examined the effects of ELS and EH in biAT mice on (i) the life expectancy, (ii) amyloid levels and synaptic proteins in the brain, (iii) behavioural performance,
and (iv) the dendritic architecture in brain areas relevant for memory formation.

2. Results

2.1. Body weight

Housing litters in a cage with limited nesting and bedding material from PND 2-9 fragments maternal care and is a well-known model to elicit early life stress (ELS) in the offspring\textsuperscript{22,23}. In our study, offspring of biAT mice that were stressed during early life showed a 15% reduction in body weight gain from PND 2-9, compared to control-reared litters from the same genetic background (t(11)=2.96; p<0.05) (Figure 1A).

In another group of biAT mice, the pups were separated from the dam daily for 15 minutes (early handling, EH) in the same PND 2-9 timeframe. This is known to enhance maternal care upon reunion of the dam with her pups and is a commonly used model for early life enhancement\textsuperscript{24,25}. EH increased body weight gain significantly by 32% compared to control-reared mice (t(4)=-2.93; p<0.05) (Figure 1B). When reaching adulthood, these differences in body weight were no longer evident between the four groups of biAT mice (data not shown).

2.2. Life expectancy

Even biAT mice reared under control conditions, are known to suffer severe mortality between age 6-8 weeks and adulthood\textsuperscript{21} (Figure 2A). Interestingly, the survival rate of biAT mice that were stressed early in life was significantly

![Figure 1. Early life experiences acutely affect body weight of biAT mice.](image_url)

Body weight gain measured between PND 2 and 9 in A. ELS litters (Ctrl: N = 7; ELS: N = 7; t(11) = 2.96; p < 0.05), and B. EH animals (Ctrl: N = 5; EH: N = 4; t(4) = -2.93; p < 0.05) each compared to control reared biAT mice. Data are expressed as mean ± SEM.
lower compared to control biAT mice. The survival in the ELS-group remained significantly lower until the end of the experiment ($\chi^2(1) = 4.35; p < 0.05$). At P110, 40.3% of biAT control animals were still alive, while only 20.3% of the biAT animals that were exposed to ELS survived. Survival was not affected by ELS in Tau.P301L littermates during the same time window of this study ($\chi^2(1) = 1.17$, ns) (Figure 2B).

In the EH-experiment we observed a similar early mortality of the biAT control animals (Figure 2C). Most interestingly, in contrast to ELS, the EH treatment significantly increased the life expectancy of biAT mice: 82.6% were still alive at PND 110 compared to only 46.7% of the control biAT animals ($\chi^2(1) = 4.15; p < 0.05$) (Figure 2C). Again, survival of the Tau.P301L littermates undergoing exactly the same treatment was not significantly affected ($\chi^2(1) = 0.62$, ns) (Figure 2D). Given the strong effects of these early life experiences on life expectancy of the biAT mice, as opposed to the Tau.P301L littermates, all subsequent experiments were conducted in biAT mice.

**Figure 2.** ELS exacerbates early death while EH prolongs survival of young biAT mice. A. ELS significantly decreased survival of biAT mice (Ctrl: N = 32; ELS: N = 28; $\chi^2(1) = 4.35; p < 0.05$) B. EH prolonged survival compared to control mice (Ctrl: N = 15; EH: N = 23; $\chi^2(1) = 4.15; p < 0.05$). C. Tau.P301L littermates were not significantly affected by ELS (Ctrl: N = 31; ELS: N = 25; $\chi^2(1) = 1.17$, ns) or D. by EH (Ctrl: N = 17; EH: N = 6; $\chi^2(1) = 0.62$, ns).
To investigate whether ELS and EH modified AD related hallmarks differentially, we quantified protein Tau phosphorylated at serine 202 and threonine 205 (the epitope defined by Mab AT8), the levels of the amyloid peptides and their precursor APP. In addition, we measured the levels of PSD-95, a major post-synaptic protein (Figure 3A). Western blot analysis of hippocampal homogenates from the biAT mice revealed that ELS significantly increased the levels of soluble monomeric Aβ (t(6)=-2.79, p<0.05), while the levels of APP (t(10)=0.65, ns), pTau at AT8 (t(9)=-1.38, ns) and PSD-95 levels (t(10)=0.26, ns) were not altered (Figure 3B). In contrast, EH led to reduced levels of soluble, monomeric Aβ in the hippocampus (t(5)=2.63, p<0.05), while again the APP (t(6)=1.80, ns), pTau at AT8 (t(4)=-0.72, ns) and PSD-95 levels (t(4)=0.20, ns) were unaffected (Figure 3C).

**2.3. Hippocampal protein levels**

Figure 3. Early life experience alters hippocampal Aβ levels at PND 120 in biAT mice. A. Typical example of Western blots of hippocampal protein extracts. B. Aβ levels were elevated after ELS, whereas C. EH led to a reduction in Aβ levels. No effects of ELS or EH were observed on levels of APP, protein tau (AT8), or PSD-95. Experiment ELS vs. Ctrl: Ctrl: N = 7, ELS: N = 5; Experiment EH vs. Ctrl, Ctrl: N = 3, EH: N = 5. Data are expressed as mean ± SEM.
2.4. Anxiety and object recognition memory

2.4.1. Anxiety and locomotion

To investigate general locomotion and anxiety-like behaviour in young adult mice (PND 90 ± 3), the open field test was conducted. Exposure of biAT mice to ELS did not affect anxiety-like behaviour as the time spent in the centre zone of the arena was not different between ELS and control raised biAT mice (t(12) = 0.41, ns) (Figure 4A). In addition, exposure to ELS did not affect the general level of activity as no differences were detected in the total distance travelled throughout the arena (Ctrl vs ELS: t(12) = 0.86, ns) (Figure 4C). Likewise, in biAT mice EH had no effects on the time spent in the centre zone (t(35) = -0.70, ns) (Figure 4B), nor on the total distance travelled (t(35) = -0.38, ns) (Figure 4D).

Figure 4. Explorative behaviour and cognition resisted early life experiences. Time spent in the centre of the open field was not significantly different between ELS and Ctrl biAT (t(12) = 0.41, ns) (A) or between EH and Ctrl biAT mice (t(35) = -0.70, ns) (B). Total locomotor activity was also resistant to either ELS (Ctrl vs. ELS: t(12) = 0.86, ns) (C) or EH (Ctrl vs. EH: t(35) = -0.38, ns) (D). Although all groups explored the novel object significantly more than chance level (Ctrl vs. ELS experiment: Ctrl: t(5) = 17.99, p < 0.000; ELS: t(5) = 3.16, p < 0.05; Ctrl vs. EH experiment: Ctrl: t(15) = 2.96, p < 0.05; EH: t(19) = 4.60, p < 0.000), cognitive performance was comparable for all groups (Ctrl vs. ELS: t(5.58) = 1.16, ns; Ctrl vs. EH: t(34) = 0.43, ns) (E,F). ELS vs. Ctrl: Ctrl: N = 7, ELS: N = 7; EH vs. Ctrl: Ctrl: N = 15, EH: N = 21. Data are expressed as mean ± SEM.
2.4.2. Object recognition memory

In biAT mice, cognitive performance, as assessed by explorative behaviour in the novel object recognition test (PND 91 ± 3), was not affected by either ELS or EH (Figure 4E,F). When exposed to two identical objects during the training trial all animals expressed equal amounts of sniffing behaviour towards both objects, indicating no preference for the location of the object (data not shown). In the testing trial, biAT mice exposed to ELS and control rearing discriminated between the familiar and novel object, with exploration

| Table 1. Morphological alterations of the dendritic tree of neurons of subregions of the medial prefrontal cortex after ELS or EH in adult mice (PND 120). |
|---------------------------------------------------|-----|-----|-----|-----|
| **Infralimbic**                                   | Ctrl | ELS | Ctrl | EH  |
| Branch length (µm)                               |     |     |     |     |
| Basal                                             | 916 ± 32 | 651 ± 126* | 1253 ± 113 | 979 ± 73* |
| DCI                                               | 5415 ± 264 | 2662 ± 963** | 5769 ± 1017 | 4477 ± 419 |
| Branch points                                     | 6.14 ± 0.42 | 3.83 ± 1.01* | 6.82 ± 0.80  | 6.28 ± 0.46  |
| Branch length (µm)                                | 476 ± 93 | 656 ± 101 | 603 ± 85 | 478 ± 51  |
| Apical                                            | 7556 ± 1768 | 18410 ± 6607³ | 37806 ± 11205 | 14485 ± 2578 |
| DCI                                               | 3.00 ± 0.32 | 3.50 ± 0.76  | 5.58 ± 0.72 | 4.62 ± 0.57 |

| **Prelimbic**                                     |     |     |     |     |
| Branch length (µm)                               |     |     |     |     |
| Basal                                             | 924 ± 66 | 1218 ± 118* | 1397 ± 87 | 1251 ± 77 |
| DCI                                               | 4938 ± 428 | 9007 ± 2113* | 8359 ± 1428 | 7854 ± 850 |
| Branch points                                     | 5.83 ± 0.35 | 7.9 ± 0.70*  | 8.22 ± 0.92 | 8.76 ± 0.54 |
| Branch length (µm)                                | 469 ± 65 | 490 ± 60 | 733 ± 83 | 695 ± 56 |
| Apical                                            | 6848 ± 1726 | 13538 ± 4768 | 59072 ± 13985 | 56768 ± 11618 |
| DCI                                               | 3.10 ± 0.65 | 3.1 ± 0.75  | 6.91 ± 0.69 | 6.91 ± 0.66 |
| Branch points                                     |       |       |       |       |

| **Cingulate**                                     |     |     |     |     |
| Branch length (µm)                               |     |     |     |     |
| Basal                                             | 855 ± 102 | 960 ± 66 | 1400 ± 72 | 1438 ± 111 |
| DCI                                               | 7317 ± 1941 | 4496 ± 667 | 8292 ± 719 | 8433 ± 1060 |
| Branch points                                     | 7.47 ± 1.12 | 6.63 ± 1.03 | 8.46 ± 0.50 | 8.85 ± 0.62 |
| Branch length (µm)                                | 357 ± 69 | 316 ± 51 | 825 ± 53 | 665 ± 95  |
| Apical                                            | 3162 ± 1131 | 4256 ± 2075 | 70986 ± 16210 | 50530 ± 13664 |
| DCI                                               | 2.47 ± 0.72 | 2.13 ± 0.66 | 7.91 ± 0.84 | 6.35 ± 1.08 |
| Branch points                                     |       |       |       |       |

Values are presented as mean ± SEM. Significance to its relative control group is indicated as follows: **: p<0.01, *: p<0.05, #: p<0.1.
percentages being significantly higher than 50% (Ctrl: t(5)=17.99, p<0.000; ELS: t(5)=3.16, p<0.05). However, no difference in the amount of exploration was observed between ELS and control biAT mice (t(5.58)=1.16, ns) (Figure 4E). Likewise, all EH and control biAT mice displayed novel object exploration levels that were significantly higher than 50% (Ctrl: t(15)=2.96, p<0.05; EH: t(19)=4.60, p<0.000). No difference in exploration was observed between any of the groups (t(34)=0.43, ns) (Figure 4F).

2.5. Dendritic morphology

2.5.1. Medial Prefrontal Cortex (mPFC)
We first examined dendritic morphology of neurons in the mPFC, which has been shown to be sensitive to early life adversity\textsuperscript{26}. The mPFC is involved in executive functions and short-term memory, although subregions of the mPFC may respond differently as the infralimbic, prelimbic, and cingulate cortex are functionally and structurally distinct\textsuperscript{27}. Indeed, the length of the basal dendritic tree of pyramidal neurons in the infralimbic cortex was reduced both as a consequence of ELS and EH in biAT mice compared to their respective control groups (ELS vs Ctrl: t(7)=2.40, p<0.05; EH vs Ctrl: t(17)=2.42, p<0.05) (Table 1). Furthermore, after ELS exposure the dendritic complexity index (DCI) (t(7)=3.73, p<0.05), and the number of branch points (t(7)=2.56, p<0.05) of the basal dendrites were significantly reduced in the infralimbic cortex. To further investigate the effects of early life environment on cellular morphology a segmental Sholl analysis was performed which examines changes in dendritic length as a function of radial distance from the soma. The reductions in basal branch length were, albeit mildly, reflected in the segmental distribution of the basal dendrites (Figure 5A,B) with a trend towards a reduction by both EH and ELS treatments compared to their respective controls (ELS vs Ctrl: F(1,7)=5.11, p=0.058; EH vs Ctrl: F(1,17)=4.31, p<0.05). No effect of either early life manipulation were reported on apical dendrites of the infralimbic cortex (Figure 5C,D).

<table>
<thead>
<tr>
<th>Amygdala</th>
<th>Ctrl</th>
<th>ELS</th>
<th>Ctrl</th>
<th>EH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Branch length (µm)</td>
<td>941 ± 42</td>
<td>1204 ± 139*</td>
<td>1203 ± 57</td>
<td>1200 ± 88</td>
</tr>
<tr>
<td>DCI</td>
<td>5837 ± 667</td>
<td>9209 ± 3526</td>
<td>12300 ± 1244</td>
<td>11441 ± 2375</td>
</tr>
<tr>
<td>Branch points</td>
<td>8.02 ± 0.28</td>
<td>8.78 ± 1.61</td>
<td>8.78 ± 0.69</td>
<td>8.41 ± 0.84</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SEM. Significance to its relative control group is indicated as follows: *: p < 0.05.
In the basal tree of pyramidal neurons of the prelimbic cortex, ELS significantly increased the dendritic branch length \((t(10)=-2.61, p<0.05)\), the DCI \((t(9)=-3.69, p<0.05)\), and the number of branch points \((t(9)=-4.61, p<0.001)\) (Table 1). This increase was also observed in the dendritic Sholl distribution after ELS \((F(1,11)=4.92, p<0.05)\) (Figure 5E). In contrast, EH did not affect the basal dendritic tree in the prelimbic cortex (Table 1, Figure 5F). In the apical tree of pyramidal neurons of the prelimbic cortex branch length, DCI, number of branch points (Table 1), and dendritic Sholl distribution (Figure 5G,H) were not significantly altered between ELS versus control or EH versus control biAT mice.

Figure 5. Dendritic morphology of mPFC subregions is differentially affected by ELS and EH. Sholl plots of the distribution of apical and basal dendritic length at increasing distances from the centre of the cell body. In the basal dendrites of the infralimbic cortex, a trend towards a reduction in dendritic distribution was observed after both ELS and EH (ELS vs. Ctrl: \(F(1,7) = 5.11, p = 0.058\); EH vs. Ctrl: \(F(1,17) = 4.31, p < 0.05\)) (A,B). As a consequence of ELS, basal dendritic distribution of the prelimbic area was increased \((F(1,11)=4.92, p < 0.05)\), with significant post hoc differences at 60 µm from the centre of the cell body (E). No effects were observed in basal dendritic distribution in the prelimbic area after EH (F).
In the cingulate cortex, apical and basal branch length, DCI, and the number of branch points did not differ significantly between ELS versus control or EH versus control biAT mice (Table 1). Also Sholl analysis of the apical and basal dendrites revealed no significant differences in dendritic distribution in this subregion after any of the early life manipulations (Figure 5I-L).

2.5.2. Amygdala
Neurons of the basolateral amygdala, involved in emotional processing, have been reported to undergo substantial dendritic remodelling following stress in adult animals. Indeed, we observed that ELS increased the branch length of stellate neurons in the amygdala of biAT mice (t(7)=−2.72, p<0.05) (Table 2), but it did not affect the number of branch points or the DCI. Sholl analysis revealed that the differences in branch length originated mainly from the distal portion of the dendritic tree (F(1,7)=6.69, p<0.05), which was more pronounced in ELS animals in the segments between 70 to 120 µm, and 150 to 160 µm from the...
soma, whereas no differences were observed in the proximal 60 µm from the soma (Figure 6A). EH did not affect dendritic morphology in the amygdala at all (Figure 6B).

Table 3. Morphological alterations of the dendritic tree of hippocampal CA1 and CA3 pyramidal neurons after ELS or EH in adult mice (PND 120).

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>ELS</th>
<th>Ctrl</th>
<th>EH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Branch length (µm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>1017 ± 94</td>
<td>945 ± 56</td>
<td>541 ± 58</td>
<td>574 ± 45</td>
</tr>
<tr>
<td>DCI</td>
<td>25036 ± 3443</td>
<td>28390 ± 3429</td>
<td>6191 ± 1499</td>
<td>7061 ± 887</td>
</tr>
<tr>
<td>Branch points</td>
<td>9.81 ± 0.85</td>
<td>9.85 ± 0.55</td>
<td>4.88 ± 0.51</td>
<td>5.48 ± 0.36</td>
</tr>
<tr>
<td>Apical</td>
<td>Branch length (µm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCI</td>
<td>1557 ± 103</td>
<td>1440 ± 160</td>
<td>1239 ± 80</td>
<td>1380 ± 100</td>
</tr>
<tr>
<td>Branch points</td>
<td>17.19 ± 1.15</td>
<td>15.55 ± 1.95</td>
<td>13.01 ± 1.35</td>
<td>13.92 ± 1.12</td>
</tr>
<tr>
<td>Basal</td>
<td>879 ± 128</td>
<td>821 ± 83</td>
<td>339 ± 39</td>
<td>323 ± 24</td>
</tr>
<tr>
<td>DCI</td>
<td>8226 ± 2932</td>
<td>14579 ± 3586</td>
<td>1728 ± 316.69</td>
<td>1573 ± 305</td>
</tr>
<tr>
<td>Branch points</td>
<td>5.61 ± 0.85</td>
<td>8.23 ± 1.58</td>
<td>1.96 ± 0.26</td>
<td>1.66 ± 0.19</td>
</tr>
<tr>
<td>Apical</td>
<td>Branch length (µm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCI</td>
<td>614 ± 91</td>
<td>524 ± 30</td>
<td>551 ± 36</td>
<td>592 ± 39</td>
</tr>
<tr>
<td>Branch points</td>
<td>4.97 ± 0.97</td>
<td>4.07 ± 0.22</td>
<td>3.60 ± 0.36</td>
<td>4.01 ± 0.32</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SEM. Significance to its relative control group is indicated as follows: *: p<0.05.
Positive and negative early life experiences modulate AD

2.5.3. CA1 and CA3 of the hippocampus
The sensitivity of the hippocampus, which is critically involved in learning and memory, of biAT mice to early life experiences was assessed in the CA1 and CA3 areas. Representative images from pyramidal neurons in CA1 and CA3 areas show that the distribution of basal and apical dendritic length at increasing distances from the centre of the cell body is comparable between groups. In the CA3, the segmental distribution of basal branches of the neuron is not affected by ELS and EH. The segmental distribution of the apical branches is also comparable between ELS and Ctrl and EH and Ctrl animals. Data from 3-5 neurons from one animal were averaged (N = 5-12 animals/group) and expressed as mean ± SEM.

Figure 7. Neuronal morphology of CA1 and CA3 pyramidal neurons is unaffected by early life experiences. Representative images from pyramidal neurons in CA1 (A) and CA3 (F) areas from Golgi-Cox staining of coronal sections. Sholl plots indicated that the distribution in CA1 of basal (B,C) and apical (D,E) dendritic length at increasing distances from the centre of the cell body is comparable between groups. In the CA3, the segmental distribution of basal branches of the neuron is not affected by ELS (G) and EH (H). The segmental distribution of the apical branches is also comparable between ELS and Ctrl (I) and EH and Ctrl (J) animals. Data from 3-5 neurons from one animal were averaged (N = 5-12 animals/group) and expressed as mean ± SEM.
CA3 subregions (Figure 7A,F). We observed that in control and ELS-reared biAT mice, apical and basal branches of pyramidal neurons were comparable in length, in number of branch points and in DCI (Table 3). Likewise, EH did not alter hippocampal CA1 and CA3 apical and basal pyramidal dendritic morphology compared to control reared biAT mice (Table 3). Accordingly, Sholl analysis failed to reveal any effects of ELS or EH on dendritic parameters in the hippocampus of young adult biAT mice (Figure 7A-J).

3. Discussion

In order to define a possible link between early life experiences and the later development of AD, we investigated the effects of early life stress (ELS) and early handling (EH) from PND 2-9 on survival and life expectancy, on hippocampal Aβ levels, on memory, and on dendritic complexity in a bigenic mouse model of AD with transgenic co-expression of APP.V717I and Tau.P301L (biAT mice). We report that despite the strong genetic background in this model, ELS reduced long term survival and enhanced hippocampal Aβ levels at adulthood. By contrast, EH enhanced survival and reduced hippocampal Aβ levels. At PND 90, the cognition of these young adult biAT mice was not affected by ELS or EH. Dendritic complexity was slightly affected in the basal branches of the mPFC and in the amygdala, but not in the hippocampus.

3.1. Experimental model

To examine the effect of the early life environment, pups were raised in an environment with limited nesting and bedding material from PND 2 to 9 (ELS), which induces stress in the dam and pups. In contrast, a similar group of biAT mice were exposed to daily handling for 15 minutes throughout the same period, early handling (EH), which is known to enhance maternal care upon reunion. The body weight gain of pups from PND 2-9 was significantly decreased by ELS while significantly increased by EH, although these differences were no longer evident at 4 months of age. The relatively mild manipulations from PND 2-9 resulted in a comparable phenotype as reported before, and validated the effectiveness of our experimental manipulation.

We investigated transgenic mice with postnatal, neuron specific co-expression of human mutant APP.V717I and Tau.P301L protein. Both transgenes are under control of the mouse thy1 gene promoter and become expressed in the 2nd week postnatally, which coincided partially with the experimental PND 2-9 treatment period.
3.2. Life expectancy

A key finding of our study was that different early life experiences differentially affected life expectancy of biAT mice. We report a profound negative modulatory effect of ELS on later survival, leading to a two-fold lower life expectancy of biAT mice subjected to ELS when compared to control biAT mice. In contrast, EH significantly increased the life expectancy of the biAT mice by 1.6-fold. The juvenile age during which the mortality rate increased in the ELS group suggests a disturbed or delayed neurodevelopmental defect, which apparently is prevented by EH.

Increased early mortality is a phenomenon which has previously been observed in genetic mouse models of AD, in particular in the more complex models. Previous studies in the current biAT mice and in the parental Tau.P301L mice with hindbrain tauopathy revealed an inverse relationship between brainstem tauopathy and lifespan. The brainstem controls autonomous vital systems such as breathing, swallowing, and blood pressure, suggesting that the premature death of Tau.P301L mice is caused by disturbances in these processes. However, throughout the 4 months of observation in the current study, no markedly higher number of deaths occurred in the littermates that expresses Tau.P301L only. This implies the additional and specific contribution of the APP.V717I mutant protein, possibly in combination with the tau mutation, in the early death of the biAT mice. Evidence is accumulating that, similar to other models, young biAT mice are prone to epileptic activity which is also the cause of their premature death (HM, FVL, data not shown). Indeed, different transgenic AD mouse models that express mutant forms of APP were reported to die prematurely relative to wild type mice from the same strain. Various studies demonstrated that premature death is closely associated with spontaneous seizures and abnormal epileptiform electroencephalography (EEG) activities. The implicated contribution of amyloid peptides and/or APP metabolites, alone and even more when combined with mutant protein tau, in inducing neuronal hyperexcitability and epilepsy susceptibility is evident. These animal model observations are consistent with the higher incidence of epileptic activity in aged AD patients as compared to non-dementing elderly.

Interestingly, ELS was reported in various models to elicit higher incidence of spontaneous seizures and enhanced excitability. The combined data link ELS to the observed reduced survival in biAT animals by potentiating epileptic seizures, underlined additionally by the observed enhanced Aβ levels in the hippocampus of the ELS-reared biAT mice (see below). Interestingly, EH reverses this phenomenon and exerts protective effects against early mortality caused by seizure vulnerability. The combined data indicate that additional
processes, besides regulation of seizure vulnerability, are modulated by positive early life experiences. Importantly, we observed that EH reduced the Aβ levels in the hippocampus, which may contribute to reduce the epileptic activity and thereby enhance life expectancy of biAT mice.

3.3. Pathological biomarkers

Although amyloid plaques and neurofibrillary tauopathy develop from 8-10 months onwards in the current bigenic model of AD, we confirmed meaningful levels of soluble Aβ in the hippocampus of young adult biAT mice (4 months). In addition, phosphorylated protein tau was already evident at the same age in biAT mice. This combined biochemical and pathological profile warrants further investigations of early phase disease development, rather than end-stage disease consequences, which have been described most abundantly.

Interestingly, we observed that hippocampal soluble Aβ levels were further enhanced by ELS and reduced by EH in young adult biAT mice, 4 months after both the respective early life treatments. As hippocampal APP levels were unchanged, the ELS and EH treatments may affect APP-processing, respectively enhancing and inhibiting the amyloidogenic pathways. In this respect it is of interest that the promoter regions of the gene encoding β-site amyloid cleaving enzyme (BACE), which is responsible for the pathogenic cleavage of the APP protein, contains several glucocorticoid-response binding elements. The activation by glucocorticoids released during stress could thereby increase amyloid peptide production in the biAT mice, and possibly in the brain of AD patients. Thus, modulation of glucocorticoid levels by early life experiences could regulate BACE activity, thereby controlling post-translational processing of APP and the Aβ peptide levels.

Alternatively, the enzymes that are implicated in the clearance and degradation of amyloid peptides from the brain can be involved. For instance, insulin-degrading enzyme (IDE) which degrades Aβ, is claimed to be dysfunctional in AD and contributes to its pathogenesis, while IDE activity is inhibited by glucocorticoids. Another Aβ degrading peptidase, neprilysin, which reduces Aβ load, is also regulated by glucocorticoids. Finally, environmental enrichment, which is known to reduce Aβ load in animals and humans, also influences neprilysin levels and activity. It will be interesting to determine which of these mechanisms explains the observed differential effect of ELS and EH on the Aβ levels in the brain of young adult biAT mice.
3.4. Behaviour and dendritic morphology

Previous studies in young adult biAT mice revealed impaired cognitive performance in the novel object recognition tests, compared to wild type mice\(^2\). We confirm that also at 3 months of age, biAT mice successfully identified the novel object, while neither ELS nor EH modulated this capacity. Possibly, the profound effects of the transgenic background on cognition obscured any potential additional effects of ELS and EH. Alternatively, we analysed a mixed group of male and female mice, from which we know that females are more resistant to ELS than males\(^2\). Moreover, a potential bias could have been introduced in our study by the fact that the most resilient animals have been studied as more vulnerable mice had already died before the test at 3 months of age. Especially in the ELS group over 60% of the original cohort could not be included in the analyses at adult age.

Following ELS, the dendritic morphology of the basolateral amygdala and prelimbic cortex was enhanced, which may be related to increased activity in these regions based on the expression of conditioned fear after early life adversity, as reported before\(^60-62\). In basal dendrites of the infralimbic mPFC neurons, we demonstrated a reduction in dendritic complexity both after ELS and EH. In line with our findings, the ELS paradigm reduced the dendritic complexity of the mPFC\(^63\), although very little is known about effects of EH on dendritic morphology in the mPFC. In AD patients, activity in the mPFC is increased, possibly as a compensation for the decline in cognitive capacity in other brain areas\(^64\). While ELS decreased dendritic morphology of the mPFC, EH decreased pathological AD markers, which may make the compensatory overactivation of the mPFC no longer necessary, thereby potentially indirectly reducing dendritic morphology.

No major effects of ELS and EH were identified on the gross dendritic morphology in the CA1 and CA3 of the hippocampus, while also hippocampal PSD-95 levels were unchanged, indicating that hippocampal integrity was not further affected by ELS or EH in the biAT mice. Other studies have reported alterations in dendritic morphology in the hippocampus using the same stress paradigm\(^65\), however, this has never before been studied in a complex transgenic AD model.

3.5. Putative mechanism: HPA axis activity

Previous findings suggest that early life adversity can lead to persistently increased HPA axis reactivity and result in enhanced glucocorticoid secretion
Chapter 2

in response to stress\textsuperscript{22,66,67}. In contrast, early life enhancement persistently attenuates the stress reactivity in the adult brain by dampening HPA axis activity, resulting in reduced glucocorticoid secretion in response to stress\textsuperscript{24}. Furthermore, stress and elevated glucocorticoid levels have been reported to increase amyloid pathology and accelerate the development of NFT in an AD mouse model\textsuperscript{50}, and such effects could be rescued by blocking glucocorticoid receptors\textsuperscript{68}. Although this needs experimental confirmation, these studies suggest that early life experiences can accelerate or delay the appearance of AD pathology in biAT mice, possibly via changes in HPA axis activity.

There is indeed ample evidence that the HPA axis is affected in patients suffering from AD, as reflected by markedly elevated basal levels of circulating cortisol, also in early stages of the disease\textsuperscript{9,69,78,70–77} and a failure to show cortisol suppression after a dexamethasone challenge\textsuperscript{78–80}. Although AD patients show elevated basal cortisol levels, their HPA dysfunction did not worsen as the disease progressed, indicating that HPA axis dysfunction was mainly implicated in the early stages of the disease\textsuperscript{9}. This suggests that in particular early alterations in HPA axis activity could contribute to the onset and possible acceleration of AD pathogenesis. Whether this is related to early life experiences remains to be investigated.

4. Conclusion

We demonstrated here, to our knowledge for the first time, that exposure to early life stress significantly decreases the life expectancy in biAT mice, parallel to enhanced soluble A\textsubscript{ß} levels in the hippocampus. Conversely, early handling during the same period increased life expectancy and reduced soluble A\textsubscript{ß} levels. Although the studied transgenic biAT mice by their very nature do not model sporadic AD, the results do underline the importance of a modulatory role of early life experiences, superimposed on a genetic prodromal background on relevant outcome parameters of AD. Following the early life manipulations, persistent alterations in the posttranslational processing of APP may occur. While this study supports a role for early alterations in HPA axis activity in the onset of AD pathogenesis, future experiments are required to identify underlying mechanisms which may help establish more directly the causal implications of early life experiences in AD aetiology.
5. Methods

5.1. Animals

Bigenic APP.V717I x Tau.T301P (biAT) mice, and their Tau.P301L littermates, were bred in-house by crossing male heterozygous APP.V717I mice with homozygous Tau.P301L females, all in the FVB background\(^1\). One male animal was housed for 2 weeks with 2 female mice. At the beginning of the third gestational week, pregnant females were housed singly. All cages were covered with filter tops to prevent extra stress to the dams, and inspected daily between 7:00 and 10:00 AM. When a new born litter was encountered that day was assigned as postnatal day 0 (PND 0). The dams and litters were left undisturbed until PND 2 and kept under standard housing conditions (1 piece of nesting material, 12 hr light/dark cycle, lights on at 7:00 AM, humidity 40-60%, temperature 21±1°C) with unlimited access to food and water. All cages were also inspected daily for eventual deaths and the dates noted to draft the survival curves. In all groups, equal numbers of male and female mice were analysed. Animals were maintained and experiments were conducted in accordance with regulations of the KU Leuven and the European Community Council Directive (86/609/EC).

5.2. Early life stress

We examined how early life stress (ELS) affects survival, AD pathology, behaviour and dendritic morphology in biAT mice. Chronic ELS was induced by housing dams with limited nesting and bedding material from PND 2 to 9\(^2\). Dams and their litters were weighed at PND 2 and randomly assigned to the ELS or control condition. In total 7 litters were assigned to each condition, resulting in a total of 32 control mice and 28 ELS mice. Control dams were provided with normal sawdust bedding and nesting material: a square piece of cotton 5 x 5 cm (Technilab-BMI, Someren, the Netherlands). The ELS dams were provided with a strongly reduced amount of sawdust bedding and half the nesting material (2.5 x 5 cm) and with a fine-gauge stainless steel mesh placed 1 cm above the cage floor. Both control and ELS cages were left undisturbed until the end of the ELS regime at PND 9. Then all mice were weighed and returned to standard cages, with normal amounts of sawdust bedding and nesting material until weaning at PND 21. Tail biopsies were collected from all offspring mice for genotyping by standard PCR analysis. All animals were then housed with 2-6 same sex litters per cage. All experimental mice were left undisturbed, except for cage cleaning once a week, until behavioural testing.
5.3. Early life handling

In a second series of experiments we examined how early handling (EH) affects survival, AD pathology, behaviour and dendritic morphology in biAT mice. Dams and their litters were weighed at PND 2 and randomly assigned to the EH or control condition. In total 5 litters were assigned to the control condition and 4 litters to the EH condition, resulting in 15 control mice and 23 EH mice. Control mice were housed with normal nesting and bedding material and were left undisturbed between PND 2-9. EH from PND 2-9 was induced by separating the dam and pups daily for 15 minutes between 9 AM and 11 AM. The dams and pups were placed in clean separate cages and reunited after 15 minutes in their home cage which was supplemented with 2 pieces of cotton nesting materials. During the separation, pups were placed on a heating pad at 32 °C. On PND 9, all mice were weighed and placed in standard cages, with sufficient bedding and nesting material until weaning at PND 21. Upon weaning, tail biopsies were collected for genotyping, and mice were housed with 2-6 same sex littermates per cage. All experimental mice were left undisturbed, except for cage cleaning once a week, until testing.

5.4. Behavioural testing

5.4.1. Open field

On PND 90±3, the open field test was conducted between 8-12 AM in an empty arena (50 x 50 x 50 cm) with black walls and a translucent floor, dimly lit from underneath. Number of mice analysed: ELS vs Ctrl each n=7; EH vs Ctrl: n=21 and n=15 respectively. Animals were placed in a corner facing the wall, after which their exploration of the arena was monitored. The apparatus was virtually divided into an outer border and inner zone (30 x 30 cm) and the time and distance spent and travelled in each zone recorded, as well as the total time and distance each mice was mobile and travelled.

5.4.2. Object recognition

On PND 91±3, one day after the open field test, the mice were subjected to the novel object recognition task between 8 AM and 4 PM, in the same arena as the open field task. The number of mice analysed: ELS vs Ctrl, n=7 each; EH vs Ctrl: n=21 and n=15, respectively. During the training, each mouse was granted 8 minutes to explore two identical objects (blue glass marbles, 5 cm diameter) placed equidistantly from the walls and each other. After 4 hours, the test trial was conducted using one original familiar object and one novel object (red plastic cube, 5 cm diameter) placed in exactly the same locations as during the training. Mice were reintroduced into the arena for 8 minutes to explore the
novel and familiar object. The relative ratio of time spent on the novel object divided by total (novel + familiar) exploration time was used as the index. An index of over 50% preference for the novel object, was taken as recognition of the known object observed during the training trial. Mice that were immobile or spent less than 10 sec exploring the objects were excluded from the analysis.

5.5. Western blot analysis

Western blot analysis (n=4/group) was used to assess biochemical levels: amyloid peptides (6E10, Biolegend, lot no. 88718, 1:1000, 4 kDa), APP (6E10, Biolegend, lot no. 88718, 1:1000, 100 kDa), PSD-95 (D27E11, Cell Signalling, lot no. 23450S, 1:1000, 95 kDa), phosphorylated tau (AT8, Thermo Scientific, 1:200, 79 kDa), and GAPDH (14C10, Cell Signalling, lot no. 36835, 1:3000, 37 kDa). Protein brain extracts were prepared following rapid decapitation from one hemisphere which was snap frozen and stored on -80 °C until processing. Hippocampi were dissected and homogenised in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, pH=6.8) using small pellet mixers, then incubated for 10 min at room temperature and subsequently sonicated for 2x30 sec at maximum intensity, again incubated for 10 min and then centrifuged (1 min, 10,000xg, 4°C). The supernatants were collected and the protein concentrations determined by a BCA Protein Assay (Pierce, The Netherlands). An aliquot equivalent to 15 µg protein was separated by electrophoresis on 12.5% polyacrylamide-SDS gels with 5% stacking gels and proteins transferred to PVDF membranes for 2 hours at 75 V in Towbin buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH=8.3). The membranes were blocked with TBST (TBS + 0.1% Tween-20) containing 5% BSA for 1 hour, rinsed in TBST and strips were incubated with primary antibody overnight at 4°C. Blots were washed with TBST and incubated for 2 hours with secondary antibody. After thorough washing with TBS, signal was developed (Licor Odyssey FC; Leusden, the Netherlands). Signal intensities were measured using dedicated software (ImageJ; NIH; Bethesda) and normalised against GAPDH as internal marker. Individual protein levels were calculated as the mean of 3 independent replications.

5.6. Dendritic morphology

Golgi-Cox impregnation was performed as described previously82. Immediately after decapitation, one hemisphere was immersed in Golgi-Cox solution (5% K₂CrO₇, 5% HgCl and 5% K₂Cr₂O₇) for 14 days, after which they were embedded
in celloidin and cut coronally into 200 µm thick sections. The dendritic tree of neurons in the amygdala (bregma -2.0 mm to -3.2 mm), the CA1 and CA3 area of the hippocampus (bregma -2.0 mm to -3.2 mm), and the IL, PRL, and CG of the mPFC (bregma 2.2 mm to 4.2 mm) were analysed by obtaining Z-stacks (step-size 1 µm) using a microscope (LSM510, Zeiss, Germany) with a 20x magnification. Cells were reconstructed using dedicated software (Image Pro Analysis and Neurodraw reconstruction). Neurons were included according to criteria as previously described: (1) the presence of untruncated dendrites, (2) consistent and dark impregnation along the entire extent of all dendrites, and (3) relative isolation from neighbouring impregnated neurons. A total of 3-5 neurons from each animal were averaged (3-12 animals/group). Structural measures included total branch length, number of branch points, and dendritic complexity index (DCI). The DCI was calculated by the formula: \( \frac{\text{∑ branchtip orders + # of branch tips}}{\text{# of primary dendrites}} \times \text{total arbour length} \). In addition, for each reconstructed neuron, the 3D Sholl analysis was performed using dedicated software (NeuronStudio).

5.7. Statistical analysis

Statistical analysis was performed using SPSS 21.0 except for outlier analysis, which was conducted using Grubb’s test (Graphpad Prism 5). All data are presented as mean ± SEM, with \( p< 0.05 \) considered statistically significant. All experimental groups were compared to their respective control group.

Analysis of mouse survival was conducted using the log rank test. Body weight and behaviour were analysed using an independent samples t-test, or by the non-parametric equivalent Mann Whitney U test if one of its assumptions was violated. Data from the novel object recognition test were analysed by one-sample t-test to compare the exploration percentage to 50 (no discrimination). For the analysis of the dendritic morphology, 3 to 5 neurons from one animal were averaged and compared using independent samples t-test. Statistical significances for segmental dendritic plots (Sholl analysis) were obtained from repeated measures ANOVA with adequate corrections (Greenhouse-Geisser) on the significant values when the sphericity assumption was not met, and post hoc t-test was conducted to determine the distance at which differences occurred.

6. Acknowledgements

The authors thank Benoit Lechat for his assistance with the experimental animal work, Gideon Meerhoff and Jan den Blaauwen for their technical
Positive and negative early life experiences modulate AD

support with the preparation of the Golgi-Cox staining, and Judith Kok, Amber Brands, Nicole Breeuwsma, and Mariska Willemsen for their contribution to the analysis of the brain tissue. This study was supported by a grant from Internationale Stichting Alzheimer Onderzoek (ISAO)/Alzheimer Nederland (grant: #12534 to HJK). PJL is supported by NWO, the ISAO/Alzheimer Nederland.
7. References


22. Rice CJ, Sandman CA, Lenjavi MR, Baram TZ. A
Positive and negative early life experiences modulate AD


45 Salzberg M, Kumar G, Supit L, Jones N. Early postnatal stress confers enduring vulnerability...


67 Martisova E, Aisa B, Guerenu G, Ramirez M. Effects of early maternal separation on biobehavioral and neuropathological markers of Alzheimer’s disease in adult male rats. *Curr
Positive and negative early life experiences modulate AD


Early postnatal handling reduces hippocampal amyloid plaque formation and enhances cognitive performance in APPswe/PS1dE9 mice at middle age

Sylvie L. Lesuis, Beryl A.C.E. van Hoek, Paul J. Lucassen, Harm J. Krugers

Neurobiology of Learning and Memory
2017, 144:27-35
Abstract

In rodents, fragmented and low levels of maternal care have been implicated in age-related cognitive decline and the incidence and progression of Alzheimer's pathology. In contrast, enhancing early postnatal maternal care has been associated with improved cognitive function later in life. Here we examined whether early postnatal handling of mouse pups from postnatal days 2-9 enhanced maternal care and whether this affected cognition and Alzheimer pathology at 5 and 11 months of age in the APPswe/PS1dE9 mouse model for Alzheimer's disease.

Brief, 15 minute daily episodes of separating offspring from their dams from postnatal days 2-9 (early handling, EH) increased maternal care of the dam towards her pups upon reunion. At 11 (but not five) months of age, EH APPswe/PS1dE9 mice displayed significantly reduced amyloid plaque pathology in the hippocampus. At this age, EH also prevented short-term working memory deficits while restoring impairments in contextual fear memory formation in APPswe/PS1dE9 mice. EH did not modulate amyloid pathology in the amygdala, nor did it affect auditory fear conditioning deficits in APPswe/PS1dE9 mice.

We conclude that increased levels of maternal care during the early life period delays amyloid accumulation and cognitive decline in an Alzheimer's mouse model, involving the hippocampus, but not the amygdala. These studies highlight the importance of the early postnatal period in modulating resilience to develop Alzheimer's pathology later in life.

Key words: early handling, Alzheimer's disease, cognitive reserve, stress, enrichment

Brain Plasticity Group, Swammerdam Institute for Life Sciences, Center for Neuroscience, University of Amsterdam, Science Park 904, 1098 XH Amsterdam, The Netherlands
1. Introduction

Alzheimer’s disease (AD) is a neurodegenerative disorder characterised by progressive impairments in cognitive and emotional functioning). Prominent neuropathological features of AD are amyloid-containing plaques and neurofibrillary tangles, which are present in brain areas critical for memory formation and emotion regulation, such as the hippocampus and amygdala. While genetic mutations are associated with rare familial variants of AD, the vast majority of AD cases are sporadic and have no genetic cause. Epidemiological studies have shown that lifestyle factors are important for the incidence and progression of AD. For example, stress exposure has been associated with an increased incidence of AD and AD pathology in humans and rodents. In rodents, environmental stimulation has been reported to improve learning and memory later in life, and to protect from brain pathology.

Exposure to environmental enrichment has particularly strong and long-lasting effects on cognition during the early postnatal period, i.e. when the brain is still developing. For instance, early handling (EH), which involves the separation of the dam and offspring for 15 minutes per day during at least the first week of life, produces a variety of long-term neuro-behavioural effects. Later in life, EH reduces conditioned and unconditioned fear and anxiety, blunts behavioural and endocrine sensitivity to stressors, and reduces age-related cognitive decline in rodents.

In the current study, we examined whether early life handling from postnatal days (PND) 2-9 modifies cognition and amyloid plaque pathology in the classic APPswe/PS1dE9 mouse model for AD, both in young (5 months) and middle-aged (11 months) animals.

2. Materials and methods

All mice were kept under standard housing conditions (temperature 20-22°C, 40-60% humidity, standard chow and water ad libitum, a 12/12h light schedule (lights on at 8 a.m.)) and background noise was provided by a radio to control for unexpected auditory cues and as described before. All experimental procedures were conducted under national law and European Union directives on animal experiments and were approved by the animal welfare committee of the University of Amsterdam. For the current experiments, wild type (WT) and APPswe/PS1dE9 male littermates of 5 and 11 (± 1) months old were used. To obtain mice, two 10 weeks old C57Bl/6J virgin wild type (WT) females (Harlan Laboratories B.V., Venray, The Netherlands) and one heterozygous male...
APPswe/PS1dE9 mouse were housed together for one week to allow mating. Pregnant females were housed individually in a standard cage which was covered with a filter top and monitored daily for the birth of pups\textsuperscript{32-34}. When a litter was born before 10.00 a.m., the previous day was considered the day of birth (postnatal day 0; PND 0), after which the early handling paradigm was initiated from PND 2-9. At PND 21, mice were weaned and ear biopsies were collected for identification and genotyping. Mice were housed with 2-6 same sex littermates per cage. All experimental mice were left undisturbed (except for cage cleaning once a week) until the start of the experimental procedures at 5 and 11 (± 1) months of age.

2.1. Early handling

At PND 2 litters were culled to 6 pups per litter to decrease the variation in maternal care that individual pups received\textsuperscript{33,35,36}, and dams and their litters were weighed and randomly assigned to the early handling (EH) or control condition. In total, 23 litters were assigned to the control condition and 26 litters were assigned to the EH condition, resulting in 14 EH-WT mice, 11 EH-APPswe/PS1dE9 mice, 12 Ctrl-WT mice, 10 Ctrl-APPswe/PS1dE9 mice of 5 months old, and 12 Ctrl-WT mice, 9 Ctrl-APPswe/PS1dE9 mice, 15 EH-WT mice and 9 EH-APPswe/PS1dE9 mice of 11 months old.

Control dams were housed with a standard amount of sawdust bedding and nesting material (one square piece of cotton nesting material (5 x 5 cm; Technilab-BMI, Someren, the Netherlands)), and were left undisturbed from PND 2-9. Early Handling (EH) from PND 2-9 was induced by separating the dam and pups daily for 15 minutes between 9 a.m. and 11 a.m. The dams and pups were placed in clean separate cages and reunited after 15 minutes in their home cage, which was supplemented with 2 pieces of cotton nesting material\textsuperscript{32,37,38}. During the separation pups were placed on a heating pad at 32 °C. At PND 9 all mice were weighed and placed in standard cages, with sufficient bedding and nesting material until weaning at PND 21.

2.2. Maternal behaviour

Maternal behaviour was observed two times per day from PND 3 until PND 8, i.e. in the light phase (between 9.00 a.m. and 11.00 a.m.) and in the dark phase (8.30 p.m.) during 48 minute observation sessions. Levels of activity of the dam were scored every third minute, resulting in 16 one-minute epochs per observational session. Behaviours that were scored were: licking and grooming behaviour, nursing behaviour, and the time that the dam spent off the pups.
2.3. Behavioural testing

At 5 and 11 (± 1) months, APPswe/PS1dE9 and WT male mice were tested in a behavioural test battery which involved the following tasks (in order of testing): T-maze, fear conditioning, and forced swim test as an acute stressor to determine stress-responsiveness, with 1 week between each test. During testing, mice were recorded by a video camera connected to a computer with Ethovision software (Noldus, The Netherlands) and subsequently, if applicable, manually scored by an experimenter blinded to the experimental condition of the animal using Observer 6.1 (Noldus, The Netherlands). Because mice are nocturnal animals, one month prior to behavioural testing the mice were housed under a reversed light/dark schedule (lights on at 8 p.m.), and testing was conducted during the dark (active phase) between 1 and 6 p.m. in a clean testing room lit by two red spots (EGB, 25 Watt). One week before the first test mice were housed in the testing room, and three days prior to the start of the test battery mice were handled for five minutes per day. Mice were single housed 1 week prior to fear conditioning. Arenas and test apparatus were cleaned between each trial with 25 % EtOH to dissipate odour cues, unless stated otherwise.

2.3.1. T-maze
Spontaneous alternation in a T-maze was measured to assess short term working memory\(^3\). Each trial in the T-maze consisted of a test phase and a sample phase. During the test phase, mice were placed in the start arm (base) of the T-maze and had 30 seconds to choose between the left and right arm. Mice were confined in the arm of their choice for 30 seconds. Immediately thereafter, mice were reintroduced into the start arm for the sample phase. The arm of choice of both phases was recorded, and when this was different within one trial, an alternation was scored. Three trials per day for two consecutive days were performed with an inter-trial interval of 2 hours, and the percentage of alternations was calculated from six trials. Trials in which mice failed to make a choice within 30 seconds were excluded, if a mouse failed to make a choice in >2 trials, the mouse was excluded from further analysis in this task.

2.3.2. Fear conditioning
5 and 11 (± 1) month APPswe/PS1dE9 and WT male mice were tested for contextual and auditory fear memory using a fear conditioning paradigm. On day 1 mice were placed in a chamber which had a stainless steel grid floor connected to a shock generator\(^4\), which had been cleaned with 1% acidic acid to create a recognisable and standardised odour trace. Mice were allowed to explore the context for three minutes, after which a 30 second tone was used. During the last 2 seconds mice received a single mild foot shock (0.4 mA) for
2 seconds. After this shock, the mice remained in the chamber for 30 seconds. 24 hours later, the mice were reintroduced in the same chamber for 3 minutes (“contextual fear memory”). 1 hour later, mice were brought to a different room by a different experimenter, and placed in a different chamber (round transparent Perspex walls with saw dust bedding on the floor, cleaned with 25 % EtOH). After 3 minutes exploration, mice were exposed 6 times to the 30 seconds tone, with an interval of 1 minute between the tones (“auditory fear memory”). Freezing behaviour during every trial was scored by an observer blind to the experimental condition, with “freezing” being defined as “no body movements except those related to breathing”\(^1\).

### 2.4. Stress responsiveness

A 6 minute forced swim test was used as an acute stressor to determine stress responsiveness. Blood samples were taken by a tail cut at 30 minutes (stress response) and 90 minutes (stress recovery) after the onset of the stressor\(^2\). Samples were collected in EDTA-coated tubes (Sarstedt, Etten-leur, The Netherlands), placed on ice and centrifuged at 14,000 rpm for 15 minutes after which the plasma was stored at -20 °C. Plasma corticosterone (CORT) levels were measured using a commercially available radioimmunoassay kit (MP Biomedicals, Eindhoven, The Netherlands) according to the manufacturer’s instructions.

### 2.5. Tissue Preparation

One week after finishing the behavioural testing, mice were sacrificed by quick decapitation between 8.00 and 9.00 p.m. (i.e. at the beginning of the inactive phase), when plasma corticosterone levels are low. Trunk blood was collected and plasma CORT levels were determined as described above.

Brains were removed, and the hippocampus from the right hemisphere was dissected, snap frozen on dry ice, and stored at -80 °C until further processing. The hippocampus from the left hemisphere was immersion-fixed in 4 % paraformaldehyde (PFA) in phosphate buffer (0.1 M PB, pH 7.4) for 48 hours and stored in 0.1M PB with 0.01% sodium-azide at 4 °C until further processing. Paraformaldehyde-fixed tissue was overnight cryo-protected in 30% sucrose/0.1M PB. Frozen brains were cut in 40 µm thick coronal sections in six parallel series using a sliding microtome and stored in antifreeze solution (30% Ethylene glycol, 20% Glycerol, 50% 0.05M PBS) at -20 °C until immunohistochemical staining.
2.6. DAB Immunocytochemistry

Immunocytochemistry was used to identify amyloid plaques. All stainings were performed on parallel series from the same brains within an age group. Prior to staining, sections were mounted on glass (Superfrost Plus slides, Menzal, Braunschweig, Germany) and antigen retrieval was performed by heating the sections in 0.1M citrate buffer (pH 6) in a standard microwave (Samsung M6235) to a temperature of ± 95 °C for 15 min (5 min at 800 Watt, 5 min at 400 Watt, and 5 min at 200 Watt). Sections were incubated with 0.3% H₂O₂ for 15 min to block endogenous peroxidase activity. After 3 x 5 min rinsing in wash buffer (0.01% Triton X-100 in 0.05 M TBS), sections were incubated for 30 min in blocking buffer (1% BSA, 0.3% Triton X-100 in 0.05 M TBS) and subsequently incubated with the primary antibody 6E10 (1:1500, BioLegend) for two hours at room temperature followed by overnight incubation at 4 °C. Then, sections were rinsed 3 x 5 min with wash buffer and incubated with biotinylated secondary antibody in Supermix (1:200, sheep anti-mouse, GE Healthcare) for two hours at room temperature followed by a 90 min incubation with avidin-biotin complex (ABC kit, Elite Vectastain Brunschwig Chemie, Amsterdam, 1:800). Subsequent chromogen development was performed with diaminobenzidine (DAB; 20 mg/100 mL 0.05M Tris, 0.01% H₂O₂).

2.7. Imaging and quantification

Plaque load was quantified for all APPswe/PS1dE9 mice by an experimenter blinded to the experimental procedures. Quantification was performed on coronal sections of the left hemisphere on 8-10 sections/animal of matched anatomical levels along the rostro-caudal axis. Using a Nikon DS-Ri2 microscope, representative images of 10x magnification were systematically captured. Using ImageJ software, the pictures were binarised to 8-bit black-and-white pictures, and a fixed intensity threshold was applied defining the DAB staining. Measurements were performed for the percentage area covered by DAB staining⁴³,⁴⁴.

2.8. Western blot

To compare hippocampal protein levels between the groups, hippocampi were homogenised in RIPA buffer (150 mM NaCl, 1% Triton X100, 0.5% Sodium deoxycholate, 0.1% SDS at pH 7.6) using a small syringe. The samples were incubated on ice for 30 min and then centrifuged for 20 min at 16,000 rpm at 4 °C. Protein lysate was stored at −20 °C. For each sample the protein concentration was measured using a BCA Protein Assay (23225, Pierce (Thermo
Fischer), The Netherlands). Samples containing between 10-30 μg protein in sample buffer were denaturised at 95 °C for 5 min. A polyacrylamide-SDS gel (Biorad, The Netherlands) was used for protein separation by electrophoresis. The proteins were transferred to a PVDF membrane (162-0177, Biorad, The Netherlands) in a Tris-glycine buffer. The membrane strips were blocked in TBST containing 5% BSA for 1 h. After blocking, blots were washed with TBST and incubated with primary antibodies at 4 °C overnight. Primary antibodies included 6E10 (1:1000, mouse; BioLegend), and GAPDH (1:3000, rabbit; 2118S, Cell Signaling (Bioke), The Netherlands). After washing with TBS, blots were incubated with secondary antibodies for 2 h at room temperature (HRP conjugate, Biorad, The Netherlands). Blots were washed again and bands were visualised by chemiluminescence using an ECL Prime kit (RPN2232, Amersham (GE Healthcare), The Netherlands). A Li-COR machine was used to measure the chemiluminescence. Optical density was determined in ImageJ. Measurements of the protein of interest were corrected for total protein (GAPDH band).

2.9. Statistical analysis

Data were analysed using SPSS22.0 (IBM software) and graphs were constructed using Graphpad Prism 6 (Graphpad software). All data are expressed as mean ± standard error of the mean (SEM). Data were considered statistically significant when p<0.05. Outliers were determined using a Grubb’s test. Animals from multiple litters were included in each experiment and nested under the condition factor. Models with and without litter included as random factor were compared to assess the degree to which litter effects influenced the outcome variables. Litter effects were negligible for all endocrine, histological and behavioural outcomes. A repeated measures ANOVA was performed to assess differences in maternal care behaviour. Greenhouse-Geisser correction was applied when the assumption of sphericity was violated. One-sample t-tests were performed to assess pathological markers in APPswe/PS1dE9 mice. Appropriate corrections were applied when assumption of homogeneity of variance was not met. Two-way analysis of variance (ANOVA) was performed for comparison between groups accounting for the main and interaction effects of genotype (WT vs. APPswe/PS1dE9) and condition (control vs. EH). If a significant difference was detected, post hoc analyses were performed using Tukey multiple comparison tests.
3. Results

3.1. Maternal behaviour

Observations of maternal behaviour during both the light phase (i.e. shortly after the early handling (EH)) and the dark phase, revealed that licking and grooming behaviour of the dam towards her pups was increased after EH (light phase: F(1,19)=129.49, p<0.001; dark phase: F(1,13)=14.46, p=0.002) (Figure 1A,D). Total nursing time (Figure 1B,E) or the time that the dam spend off the nest (Figure 1C,F) was left unaffected. The EH procedure did not affect body weight at P9 (Ctrl: 3.5 ± 0.1 gram; EH: 3.6 ± 0.1 gram) (t(48)=1.14, p=0.26) or at P21 (Ctrl: 8.8 ± 0.2 gram; EH: 9.2 ± 0.3 gram) (t(48)=1.17, p=0.25). Together, this indicates that EH was effective in enhancing maternal care of the dam towards her offspring.

3.2. Corticosterone levels

At 5 months of age, no significant differences in plasma corticosterone (CORT) levels were present between EH and control offspring under basal conditions (Figure 2A). Exposure to an acute stressor, the forced swim test, did not affect the response or recovery of CORT levels (Figure 2B,C). At 11 months of age,
basal CORT levels were again not significantly different between the groups (Figure 2D) but following the exposure to swim stress, CORT response levels were significantly affected by the interaction between early life and genotype $\,(F(1,39)=7.14, \ p=0.01)$. Post hoc tests revealed that EH significantly increased the CORT response in the APPswe/PS1dE9 mice ($p=0.03$), while no effect was observed in the EH-WT mice (Figure 2E). CORT recovery levels (90 minutes after the swim stress exposure) remained elevated in EH-APPswe/PS1dE9 mice relative to control APPswe/PS1dE9 mice ($F(1,40)=13.22, \ p=0.001$; post hoc Tukey: $p=0.003$) and to EH-WT mice ($p=0.01$) (Figure 2F).

### 3.3. Amyloid pathology

At 5 months of age no differences were observed in the % of surface area covered by plaques in the CA region, dentate gyrus or subiculum of the hippocampus, nor in the amygdala (Figure 3A,C), or in the number of plaques (CA: $t(7)=0.60, \ p=0.57$; DG: $t(7)=0.55, \ p=0.60$; Sub: $t(2.13)=0.89, \ p=0.47$; amygdala: $t(11)=-0.11, \ p=0.92$) (data not shown). At 11 months of age EH-APPswe/PS1dE9 mice showed a reduction in plaque load in the CA region ($t(15)=2.14, \ p=0.049$), dentate gyrus ($t(16)=2.81, \ p=0.044$) and subiculum ($t(13)=2.47, \ p=0.028$) (Figure 3B,D), as well as in the number of plaques (CA: $t(15)=2.16, \ p=0.047$; DG: $t(14)=2.46, \ p=0.028$; subiculum: $t(14)=2.27, \ p=0.039$) (data not shown). At this
Figure 3. Amyloid pathology in the hippocampus and amygdala. A, B. Typical example of plaque load in the CA area, dentate gyrus (DG), subiculum (Sub), and amygdala (Amy) of 5 (A) and 11 (B) months old mice. C. At 5 months, no differences in the plaque load in the CA area, dentate gyrus, subiculum or amygdala were observed. D. At 11 months, plaque load is reduced in the CA area, the dentate gyrus, and the subiculum after early handling, but not in the amygdala. E, F. No difference in the level of full-length APP, as measured at 100 kDa with the 6E10 antibody, is present at 5 months (E) or 11 months (F).

age, no differences were observed in plaque load in the amygdala (t(8)=0.10, p=0.92). These differences are not likely to originate from differences in APP production, as the full length APP protein levels were not different between control and EH mice at either 5 or 11 months of age (Figure 3E,F).
3.4. Behavioural testing

Prior to behavioural testing, no differences in overall locomotor activity were present between the experimental groups (5 months: condition effect: $F(1,36)=0.71$, $p=0.44$; genotype effect: $F(1,36)=0.02$, $p=0.88$; 11 months: condition effect: $F(1,45)=1.86$, $p=0.18$; genotype effect: $F(1,45)=1.77$, $p=0.19$). Also, no differences in anxiety-like behaviour were present between the experimental groups as measured during the habituation phase, i.e. prior to the shock, of the fear conditioning paradigm (5 months: condition effect: $F(1,34)=0.71$, $p=0.40$; genotype effect: $F(1,34)=0.02$, $p=0.88$; 11 months: condition effect ($F(1,44)=0.60$, $p=0.44$; genotype effect: $F(1,44)=0.24$, $p=0.63$).

3.4.1. T-maze

Working memory was assessed using the spontaneous alternation rate in the T-maze. The alternation rate was comparable between the groups at 5 months of age (Figure 4A). At 11 months of age, Ctrl-APPswe/PS1dE9 mice alternated significantly less when compared to WT mice ($F(1,42)=7.72$, $p=0.008$, post hoc test: $p=0.003$) (Figure 4B). This impairment was not present in APPswe/PS1dE9 mice exposed to EH ($p=0.005$).

3.4.2. Fear conditioning

Following a mild foot shock, 5 month old control Ctrl-APPswe/PS1dE9 mice showed increased freezing behaviour when compared to Ctrl-WT mice when placed back in the same context ($F(1,33)=6.66$, $p=0.01$, post hoc: $p<0.001$) (Figure 5A). This response was normalised by rearing APPswe/PS1dE9 mice under EH conditions ($p=0.005$). Re-exposure to the tone in a novel, safe context revealed a main effect of genotype (average: $F(1,33)=15.46$, $p<0.001$) (Figure 5B,C), where
APPswe/PS1dE9 mice froze less when compared to WT mice. This was not modulated by early life conditions.

An interaction effect was found in 11 month old mice in contextual fear memory (F(1,38)=8.43, p=0.006) (Figure 5D). Post hoc tests revealed that Ctrl-APPswe/PS1dE9 mice displayed significantly less freezing behaviour when compared to Ctrl-WT mice (p=0.043). However, EH-APPswe/PS1dE9 mice displayed significantly higher freezing levels (p=0.035), comparable to those
of WT mice. A genotype effect was also observed after re-exposure to the tone (average: F(1,43)=8.35, p=0.006), where Ctrl-APPswe/PS1dE9 mice displayed less freezing behaviour relative to WT mice reared under similar conditions (p=0.007) (Figure 5E,F).

4. Discussion

In this study, we investigated whether early handling (EH) from postnatal days 2-9 was able to modify amyloid pathology and cognition at later life in a transgenic mouse model for AD. We report that EH increased maternal care of the dam towards her offspring, which subsequently reduced amyloid plaque pathology in the hippocampus of middle-aged transgenic APPswe/PS1dE9 mice. In parallel, EH reduced short-term working memory deficits and contextual fear memory deficits in APPswe/PS1dE9 mice at middle age.

After exposure of APPswe/PS1dE9 and WT littermates to daily handling episodes of 15 minutes from PND 2-9, maternal care was strongly enhanced. Interestingly, the elevated licking and grooming behaviour remained present even up until 12 hours after the handling procedure, indicative of a substantial increase in maternal care towards handled mice not only immediately following the EH procedure, but also throughout the day. There is substantial evidence that maternal licking and grooming behaviour exerts a major influence on the development of emotional and cognitive behaviours later in life. For instance, Meaney and colleagues have reported that naturally occurring variations in maternal care in rats predict later alterations in spatial learning and memory processes, emotional learning and memory processes, anxiety-like behaviour, social behaviour and stress-reactivity46,47. These aforementioned studies indicate that enhanced levels of maternal care early in life are associated with reductions in age-related cognitive decline29,48–50.

To extend these observations, we here investigated whether EH could delay cognitive decline and amyloid pathology using APPswe/PS1dE9 mice, a transgenic model for AD. Earlier studies have reported that these mice develop progressive cognitive decline starting from 6-7 months of age in various cognitive tasks51–53. In line with this, we found that APPswe/PS1dE9 mice developed age-dependent decline in spontaneous alternation behaviour in the T-maze at 11 months, an effect that was absent in 5 months old APPswe/PS1dE9 mice. The T-maze task is thought to test short-term and working memory, and is highly sensitive to dysfunction of the hippocampus, although brain areas like the prefrontal cortex may also be involved54. Previous studies using different behavioural tests in AD mouse models have indeed
shown that these memory domains are particularly impaired\textsuperscript{55,56}. These results are consistent with the observation in humans that short-term memories, and consequently working memory, are among the first domains impaired in AD\textsuperscript{57,58}. We found that cognitive decline was completely prevented by EH, as APPswe/PS1dE9 mice exposed to EH displayed alternation behaviour that was comparable to WT mice. In addition, 11 months old APPswe/PS1dE9 mice were impaired in contextual fear memory formation, comparable to previous reports\textsuperscript{59–62}. Also here, we show this cognitive impairment to be completely prevented by rearing APPswe/PS1dE9 mice under EH conditions. Somewhat unexpected, we observed that at 5 months, control reared APPswe/PS1dE9 mice showed enhanced freezing behaviour when compared to WT mice in the contextual fear-conditioning paradigm. Although the nature of this effect needs to be investigated in more detail, EH rearing was again able to also normalise these changes in contextual freezing.

APPswe/PS1dE9 mice further showed impairments in auditory freezing responses that started at 5 months of age and remained present at 11 months of age. In contrast to the data on the contextual fear conditioning paradigm, EH rearing did not further modulate freezing behaviour. One explanation for this could be that EH modifies contextual and hippocampal function, but is less able to modify amygdala function, which is strongly related to auditory fear conditioning\textsuperscript{63}. The possibility that EH specifically affects hippocampal function rather than amygdala function is emphasised by the amyloid plaque pathology in our mice. EH reduced amyloid plaque load in the hippocampus, but not in the amygdala. This region-specific effect of EH on plaque pathology became apparent at 11 months and was not seen at 5 months of age, whereas behavioural alterations were already observed in contextual fear conditioning at this age. Plaque accumulation is thought to result from altered processing of the amyloid precursor protein (APP), which may precede plaque pathology at 5 months. Accordingly, previous studies have shown that already in a phase prior to the emergence of cognitive deficits, EH can affect Aβ levels\textsuperscript{32}. As the effects of EH on hippocampal amyloid pathology were not due to alterations in APP levels, it remains to be investigated how EH reduced amyloid levels. Potential underlying mechanisms are alterations in APP processing\textsuperscript{64} or in clearance of Aβ\textsuperscript{65}. Why EH affects amyloid levels and function of the hippocampus, but leaves the amygdala unaffected, remains elusive. These effects can possibly be attributed to a difference in developmental trajectory, since the hippocampus and amygdala display clear differences in postnatal growth and maturation\textsuperscript{66}.

Various studies have furthermore demonstrated that EH decreases stress-responsiveness, which may, at least in part, underlie the rescuing effects of EH on hippocampal function\textsuperscript{26,29,67–69}. In our studies, control APPswe/PS1dE9
mice showed normal stress-responsiveness, while EH enhanced stress-responsiveness in these animals. This suggests that the EH effects on spatial memory are not related to long-lasting reductions in corticosteroid hormone levels. This observation is peculiar in light of the previous literature on stress responsiveness after EH in WT mice, and contradicts the hypothesis that alterations in stress sensitivity and glucocorticoid levels underlie cognitive decline. Therefore, further research on this and on the HPA axis function in AD mouse models is warranted.

As no effects of EH were observed in WT mice at both 5 and 11 months, EH does not simply affect the formation of emotional or working memory, but may specifically protect against alterations in behaviour induced by the APPswe/PS1dE9 genetic background of these mice at both ages. One explanation could be that the selected tasks were not sufficiently challenging to allow for discrimination between control and EH reared WT mice (“ceiling/floor” effects). Only when control reared mice were already compromised, for instance as a consequence of the APPswe/PS1dE9 gene expression, could EH improve cognition. This is in line with the theory of cognitive reserve/flexibility, which proposes that the brain may adapt to pathological effects by maintaining and prolonging proper cognitive function despite already ongoing detrimental neural alterations. Since enrichment throughout adult and particularly early life has been suggested to increase cognitive reserve, and since EH and adult life environmental enrichment may be mediated by similar mechanisms, it is tempting to speculate that cognitive reserve may be an important factor in our current findings.

Taken together, our results show that early handling delays cognitive impairment and decreases pathological markers associated with AD. This protection is most pronounced in the hippocampus, while the amygdala remains largely resilient to early life enhancement. The current experiments point towards a mechanism through which individual differences in the resilience to develop AD may arise. These studies further highlight the importance of the early postnatal time window in determining possible resilience to develop AD.
5. References


26 Fernández-Teruel A, Escorihuela RM, Castellano B, Gonzalez B, Tobena A. Neonatal handling and environmental enrichment effects on emotionality, novelty/reward seeking, and age-related cognitive and hippocampal impairments: focus on the roman rat lines. Behav Genet 1997; 27.


36 Yam KY, Naninck EFG, Abbink MR, La Fleur SE, Schipper L, van den Beukel JC et al. Exposure to chronic early-life stress lastingly alters the adipose tissue, the leptin system and changes the vulnerability to western-style diet later in life in mice. Psychoneuroendocrinology 2017; 77: 186–195.


44 Marlatt MW, Potter MC, Bayer TA, van Praag H,


Scullion GA, Kendall DA, Marsden CA, Sunter D, Pardon M-C. Chronic treatment with the α2-adrenoceptor antagonist fluparoxan prevents age-related deficits in spatial working memory in APP+PS1 transgenic mice without altering β-amyloid plaque load or astrocytosis. Neuropharmacology 2011; 60: 223–234.


89
beta peptide deposition by increasing the expression of APP and BACE1 and decreasing the expression of amyloid-beta-degrading proteases. *Endocrinology* 2011; **152**: 2704–2715.


72 Nithianantharajah J, Hannan AJ. The neurobiology of brain and cognitive reserve: Mental and physical activity as modulators of brain disorders. *Prog Neurobiol* 2009; **89**: 369–382.
Targeting glucocorticoid receptors prevents the effects of early life stress on amyloid pathology and cognitive performance in APPswe/PS1dE9 mice

Sylvie L. Lesuis¹, Sacha Weggen², Sandra Baches², Paul J. Lucassen¹, Harm J. Krugers¹

Translational Psychiatry
2018, 8(1):53
Abstract

Exposure to chronic stress or elevated glucocorticoid hormone levels in adult life has been associated with cognitive deficits and an increased risk for Alzheimer's disease (AD). Since exposure to stress during early life enhances stress-responsiveness and lastingly affects cognition in adult life, we here investigated; i) whether chronic early life stress (ELS) affects AD pathology and cognition in middle-aged APPswe/PS1dE9 mice, and ii) whether it is still possible to rescue these late effects by briefly blocking glucocorticoid receptors (GRs) at a translationally relevant, middle age. Transgenic APPswe/PS1dE9 mice were subjected to ELS by housing dams and pups with limited nesting and bedding material from postnatal days 2-9. In 6 and 12 month old offspring, this resulted in enhanced hippocampal amyloid-β (Aβ)-40 and -42 levels, and in reduced cognitive flexibility, that correlated well with the Aβ-42 levels. In parallel, corticosterone levels and BACE1 levels were significantly elevated. Surprisingly, blocking GRs for only 3 days at 12 months of age, reduced corticosterone levels, reduced hippocampal Aβ-40 and -42, and β-site APP-cleaving enzyme 1 (BACE1) levels, and notably rescued the cognitive deficits in 12 month old APPswe/PS1dE9 mice. These mouse data demonstrate that exposure to stress during the sensitive period early in life influences later amyloid pathology and cognition in genetically predisposed, mutant mice, and as such, may increase AD vulnerability. The fact that a short treatment with a GR antagonist at middle age lastingly reduced Aβ levels and rescued the cognitive deficits after ELS, highlights the therapeutic potential of this drug for reducing amyloid pathology.
1. Introduction

The mechanisms that underlie sporadic Alzheimer’s disease (AD) remain largely elusive, which hampers the development of successful intervention strategies for AD. While familial forms of AD can be explained by genetic causes - often related to changes in amyloid-β (Aβ) e.g.1,2 - sporadic AD likely has a multifactorial aetiology, in which, next to amyloid, also lifestyle factors play an important role3-5.

Stress is an important environmental risk factor that has been implicated in AD progression6,7. Clinical observations e.g. suggest that stressful life events can reduce the age of onset in AD6, while stress-related disorders like depression can promote AD symptoms and neuropathology (see8). Glucocorticoid hormones (GCs; cortisol in humans, corticosterone (CORT) in rodents) are powerful steroids released in response to stress. They are often increased in AD, notably already in early stages of the disease9,10, and dysregulation of the hypothalamus–pituitary–adrenal (HPA) axis is also associated with a higher AD risk8,10,11. Rodent studies further demonstrate that exposure to stress and/or elevated GC levels at an adult age impairs cognition and enhances Aβ levels both in mutant12-16 and in wild type animals17,18.

The early postnatal period is a particularly sensitive time window that determines sensitivity to stress and cognitive function in later life. As exposure to early life stress (ELS) in wild type mice is well known to accelerate cognitive decline9,20, we here tested the hypothesis that ELS – induced by housing mice with limited nesting and bedding material from postnatal days 2-921-24 – increases the development of AD pathology and cognitive decline in APPswe/PS1dE9 mice, a classic mouse model for amyloid pathology25. Secondly, in order to study whether glucocorticoids are instrumental, we tested whether briefly targeting glucocorticoid receptors (GR) could rescue late effects of ELS on AD-related pathology and cognitive performance. We therefore treated animals with mifepristone, which is an FDA-approved drug that selectively blocks GRs at high concentrations and is prescribed to treat Cushing’s disease. It has further been tested in preliminary studies on (aspects of) AD26 and psychotic depression27,28 (see 29 for a review about the function and applicability of mifepristone in humans).
2. Materials and Methods

2.1. Mice and breeding

In this study we conducted experiments under Dutch national law as well as under European Union directives on animal experiments. The animal welfare committee of the University of Amsterdam approved all experiments. Mice were housed at a temperature of 20-22 °C. Humidity was between 40–60%, and animals were fed *ad libitum* with standard chow and water. Lights were on between 8.00 a.m. and 8.00 p.m. unless stated otherwise. Wild type (WT) and APPswe/PS1dE9 male littermates of 6 and 12 (± 1) months of age were used. To obtain mice, two 10 weeks old C57Bl/6J virgin wild type (WT) females (Harlan Laboratories B.V., Venray, The Netherlands) and one heterozygous male APPswe/PS1dE9 mouse were housed together for one week to allow mating. Individually housed pregnant females were monitored daily for the birth of pups. For litters born before 10.00 a.m., the day of birth (postnatal day 0; PND 0) was considered the previous day, after which the early life stress paradigm was initiated from PND 2-9. At PND 21, mice were weaned and ear tissue was collected for identification and genotyping. Littermates were housed with 2-6 mice per cage. All animals were left undisturbed (except for cage cleaning once a week) until the start of the experimental procedures. Number of mice: 6 months: Ctrl-WT: 12, Ctrl-APPswe/PS1dE9: 10, ELS-WT: 11, ELS-APPswe/PS1dE9: 14; 12 months: Ctrl-WT: 16, Ctrl-APPswe/PS1dE9: 11, ELS-WT: 19, ELS-APPswe/PS1dE9: 12. Group sizes were chosen to ensure sufficient statistical power.

2.2. Early life stress

At postnatal day (PND) 2, litters were culled to 6 pups per litter, and dams and their litters were randomly assigned to the early life stress (ELS) or control condition until PND 9, after which all mice were treated equally, as described before. Briefly, the control condition consisted of cages with standard amounts of nesting and bedding material (one piece of nesting material (5x5 cm; Technilab-BMI, Someren, the Netherlands)). In the ELS condition, a fine-gauge stainless steel mesh was placed in the cage, with a small amount of sawdust bedding and ½ piece of nesting material.

2.3. Maternal behaviour

Maternal behaviour was observed daily from PND 3 until PND 8 at 9.00 a.m.
Targeting glucocorticoid receptors in APPswe/PS1dE9 mice

and 8.30 p.m as described previously\textsuperscript{30}. Briefly, the level of activity of the dam was scored in 16 one-minute epochs per day, spread over 48 min observation sessions. The behaviours that were scored were: licking and grooming behaviour, nursing behaviour, and the time that the dam spent off the pups.

\textbf{2.4. Barnes maze}

Six and twelve month old APPswe/PS1dE9 and WT male mice were tested for spatial memory in the spatial Barnes maze task. Testing occurred during the dark, active phase in the afternoon (1 p.m.). A classic set up was used (110 cm diameter, 12 exit holes) in which mice were placed in the centre of the maze twice daily (inter-trial interval of 30 minutes) for 4 consecutive days and allowed to navigate to the exit hole leading to the home cage (acquisition learning). A probe trial (all holes closed) was conducted 24 hours after the last trial. Following the probe trial, behavioural flexibility was tested by relocating the exit hole to another location on the maze (150 degrees) for 4 days (reversal learning), followed by a probe trial. Cages containing used bedding material were placed at equal distances under the maze to avoid guidance by odour cues, the board was rotated after each trial, and the maze was cleaned with 25 % EtOH to dissipate odour cues. Distal extra-maze cues were always fixed relative to the exit hole. Performance of the mouse was assessed by an observer blind to the experimental condition of the mouse.

\textbf{2.5. Stress response}

Two acute stressors were used to determine stress responsiveness; a six-minute forced swim test (original experiment) or an acute 0.4 mA foot shock (mifepristone experiment). Blood samples were collected by a tail cut at 30 minutes (peak stress response) and 90 minutes (stress recovery) after the stressor. A commercially available radioimmunoassay kit (MP Biomedicals, Eindhoven, The Netherlands) was used to measure plasma CORT levels.

\textbf{2.6. Tissue preparation}

Following behavioural testing, mice were sacrificed by quick decapitation, between 8.00 and 9.00 p.m. (beginning of the inactive phase), i.e. when plasma CORT levels are low. Plasma blood was collected and CORT levels were determined as described above.
2.7. DAB immunohistochemistry

For DAB immunohistochemistry, pre-mounted sections (40 µm) on glass slides (Superfrost Plus slides, Menzel) were dried overnight. 15 min of 0.3% H$_2$O$_2$ was used to block endogenous peroxidase activity, after which sections were boiled in a microwave (±95 °C) in citrate buffer (0.01 M, pH 6.0, 15 min). To block non-specific staining, slices were incubated for 1 h in blocking mix (0.05 M TBS containing 1% bovine albumin serum (BSA) and 0.1% triton), and primary antibody mix (6E10 (1:1500, Bioline) in blocking mix) was applied for 2 h at room temperature and overnight at 4 °C. Secondary antibody (1:200, sheep anti-mouse biotinylated (GE Healthcare)) was applied for 2 hours, after which sections were treated with avidin-biotin complex (ABC, 1:800, Vectastain elite ABCc-peroxidase kit, Brunschwig Chemie). Chromogen development was conducted by incubation in 0.05 M TB containing 0.01% H$_2$O$_2$ and 0.2 mg/ml diaminobenzidine (DAB).

2.8. Imaging and quantification

Plaque load was quantified for all APPswe/PS1dE9 mice by an experimenter who was blind to the experimental conditions. Using a Nikon DS-Ri2 microscope, representative images (10x magnification) were captured and analysed using ImageJ. A fixed intensity threshold was applied to 8-bit binarised pictures to define the DAB staining. The percentage of area covered by DAB staining was analysed as described previously.

2.9. Western blotting

Following quick decapitation and dissection, hippocampi were snap frozen and homogenised in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, pH = 6.8). Homogenates were sonicated for 2x30 sec (max intensity), and centrifuged for 1 min (10000 g, 4 °C). Protein concentration was determined in the supernatant by BCA Protein Assay (Pierce, The Netherlands). 15 µg protein was separated on 12.5% polyacrylamide-SDS gels using electrophoresis, and proteins were transferred to PVDF membranes at 75 V in Towbin buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH = 8.3). 5% BSA in TBST (0.1 M TBS + 0.1% Tween-20) was used to block the membranes, and membranes were incubated overnight at 4 °C with primary antibody. Proteins studied were: APP (6E10, 1:1500, BioLegend, 100 kDa), β-site amyloid precursor protein cleaving enzyme 1 (BACE1) (D10E5, 1:1000, Cell Signalling, 70 kDa), actin (A2066, Sigma-Aldrich, 42 kDa), and GAPDH (14C10, 1:3000, Cell Signalling, 37 kDa). Secondary antibodies were incubated for 2 hours, and signal was
Targeting glucocorticoid receptors in APPswe/PS1dE9 mice

developed (Licor Odyssey FC; Leusden, the Netherlands). Using ImageJ (NIH; Bethesda) signal intensities were measured and normalised against GAPDH or actin as internal marker. 3 independent replications were made, and protein levels were analysed as the mean of these replicates, and expressed as % of Ctrl-APPswe/PS1dE9 levels.

2.10. SDS-soluble Aβ levels

Aβ40 and -42 peptide levels were determined in whole hippocampal homogenates using a sandwich ELISA assay as described previously.

2.11. Mifepristone treatment

Mifepristone (Sigma) (40 mg/ml dissolved in 99.9 % EtOH and diluted 20x in arachide oil) was injected i.p. for 3 consecutive days (final dose: 10 mg/kg, injection volume: 5 µl/g body weight) between PND 339-341 (± 7 days). The appropriate vehicle solution was administered to control mice accordingly.

2.12. Statistical analysis

Data were analysed using SPSS 22.0 (IBM software). Data are expressed as mean ± standard error of the mean (S.E.M.). Data were considered statistically significant when p<0.05 (two-sided testing). Animals/observations were excluded in case of technical failures, following spontaneous death, and/or if identified as a significant outlier using a Grubb’s test. Unpaired student’s t-tests (or non-parametric equivalent) were performed to assess differences between two groups. To compare between groups accounting for the main and interaction effects of genotype (WT vs. APPswe/PS1dE9) and condition (control vs. ELS), a two-way analysis of variance (ANOVA) was performed, with a post hoc Tukey test. A repeated measures ANOVA was performed to assess Barnes maze learning curves over the different trials, and to measure differences in plasma CORT response values. Greenhouse-Geisser correction was applied when the assumption of sphericity was violated. One sample t-test was used to compare performance on the probe trials of the Barnes maze against chance level (25%). Pearson’s correlation test was conducted to determine correlations.
3. Results

3.1. ELS results in fragmented maternal care for the offspring.

We subjected APPswe/PS1dE9\textsuperscript{25} and WT mice to the well-characterised paradigm of housing with limited nesting and bedding material from PND 2 to 9. This resulted in fragmented maternal care, as indicated by increased exits of the dam from the nest, increased numbers of pups outside the nest and a reduced body weight gain of the pups between PND 2 to 9, consistent with previous reports\textsuperscript{21–24} (Supplementary Table 1). Since the effects of ELS are particularly sex-specific\textsuperscript{24}, all experiments were further conducted with male mice.

3.2. ELS increases amyloid pathology

ELISA analysis of whole hippocampal homogenates revealed that the levels of both Aβ40 and the more aggregation-prone Aβ42 peptide were strongly elevated in ELS-APPswe/PS1dE9 transgenic mice at 6 months of age (Aβ40: $t(10.35) = -2.39$, $p = 0.038$; Aβ42: $t(10.74) = -2.74$, $p = 0.02$; Figure 1A). At 12 months of age, when the amyloid plaque pathology had progressed substantially, SDS-soluble Aβ42, but not Aβ40, levels were significantly elevated in ELS-APPswe/PS1dE9 mice (Aβ40: $t(16) = -0.80$, $p = 0.44$; Aβ42: $t(8.36) = -2.97$, $p = 0.017$; Figure 1B). Protein levels of full-length APP were not different between Ctrl and ELS APPswe/PS1dE9 mice at these ages (6 months: $t(8) = 0.66$, $p = 0.53$; 12 months: $t(13) = 0.59$, $p = 0.57$; Figure 1C,D). Stereological quantification revealed that the percentage of area covered by Aβ plaques was significantly increased in the subiculum of 6-month-old ELS-APPswe/PS1dE9 mice ($t(7) = 2.52$, $p = 0.04$) relative to Ctrl-APPswe/PS1dE9 mice, but was not different in the dentate gyrus (DG) ($t(11) = 0.57$, $p = 0.57$) or cornu ammonis (CA) subregions ($t(9) = 0.19$, $p = 0.85$; Figure 1E). In 12-month-old AD mice, no differences were observed in the subiculum ($t(15) = 1.60$, $p = 0.13$), DG ($t(15) = 1.89$, $p = 0.08$) or CA areas ($t(16) = 0.88$, $p = 0.39$) between Ctrl and ELS conditions (Figure 1F). As no Aβ peptides, APP or plaques were detected in Ctrl and ELS WT mice (data not shown), these results reveal that ELS lastingly increases hippocampal SDS-soluble Aβ peptide levels in APPswe/PS1dE9 mice, starting from a relatively early age onwards. As a potential mechanism by which ELS could increase Aβ levels, we examined the expression of BACE1. In 6 and 12 month-old-mice of age, ELS significantly increased BACE1 expression in APPswe/PS1dE9 mice relative to Ctrl-APPswe/PS1dE9 mice (6 months: $t(13) = 2.46$, $p = 0.03$; 12 months: $t(11) = 2.72$, $p = 0.01$; Figure 1G,H).
3.3. ELS does not alter cognitive performance in 6-month-old APPswe/PS1dE9 mice

To assess whether the alterations in amyloid levels after ELS were accompanied
by behavioural changes, we tested spatial navigation and cognitive flexibility in the Barnes maze (see time line in Figure 2A). At 6 months of age, mice from all groups were able to locate the exit hole comparably (acquisition learning: condition effect: $F(1,38) = 0.02$, $p = 0.90$; genotype effect: $F(1,38) = 1.03$, $p = 0.32$; interaction effect: $F(1,38) = 0.02$, $p = 0.88$), (average: condition effect: $F(1,43) = 0.13$, $p = 0.72$; genotype effect: $F(1,43) = 0.54$, $p = 0.47$; interaction effect: $F(1,43) = 0.03$, $p = 0.87$; Figure 2B,C). All mice could identify the exit quadrant in the probe trial, as assessed by significantly more than 25% (chance level) of time spend in the exit quadrant (Ctrl-WT: $t(10) = 10.51$, $p < 0.001$; Ctrl-APPswe/PS1dE9: $t(6) = 3.69$, $p = 0.01$; ELS-WT: $t(9) = 11.97$, $p < 0.001$; ELS-APPswe/PS1dE9: $t(14) = 5.33$, $p < 0.001$; Figure 2D). However, during the probe trial, ELS-APPswe/PS1dE9 mice performed less well compared to ELS-WT mice (genotype effect: $F(1,39) = 11.56$, $p < 0.001$; ELS-WT vs. ELS-APPswe/PS1dE9: $p < 0.001$). After acquisition learning, the same mice were trained in a reversal paradigm, in which the exit hole was relocated $150^\circ$ (reversal learning). No differences in the time required to locate the exit hole were present between the groups (reversal learning: condition effect: $F(1,39) = 1.97$, $p = 0.17$; genotype effect: $F(1,39) = 3.61$, $p = 0.07$; interaction effect: $F(1,39) = 0.02$, $p = 0.88$), (average: condition effect: $F(1,41) = 1.52$, $p = 0.23$; genotype effect: $F(1,41) = 2.85$, $p = 0.10$; interaction effect: $F(1,41) = 0.00$, $p = 0.98$; Figure 2E,F), and no between-group effects were present on the probe trial either (condition effect: $F(1,40) = 0.12$, $p = 0.74$; genotype effect: $F(1,40) = 2.03$, $p = 0.16$; inter- action effect: $F(1,40) = 0.04$, $p = 0.84$; Figure 2G).

3.4. ELS hampers reversal learning in 12-month-old APPswe/PS1dE9 mice

At 12 months of age, APPswe/PS1dE9 mice overall required more time to locate the exit hole when compared to WT type mice (genotype effect: $F(1,47) = 37.25$, $p < 0.001$), and displayed significantly longer latencies to find the exit hole over training days 2–4 (day 2: $F(1,47) = 31.22$, $p < 0.001$; day 3: $F(1,47) = 27.28$, $p < 0.001$; day 4: $F(1,47) = 16.33$, $p < 0.001$; Figure 3A). These findings were also reflected in the average time that the mice required to locate the exit hole, which was higher in both groups of APPswe/PS1dE9 mice when compared to WT mice (F (1,47) = 32.72, $p < 0.001$; post hoc: Ctrl-WT vs. Ctrl-APPswe/PS1dE9, $p = 0.02$; ELS-WT vs. ELS-APPswe/PS1dE9, $p < 0.001$; Figure 3B). During the probe trial, Ctrl- APPswe/PS1dE9 mice spend less time in the exit quadrant than Ctrl-WT mice (genotype effect: $F(1,45) = 7.54$, $p = 0.01$; post hoc: $p = 0.04$; Figure 3C). Importantly, no differences were observed between the groups in walking distance or speed during habituation (genotype effect: $F(1,46) = 2.66$, $p = 0.11$; condition effect: $F(1,46) = 2.81$, $p = 0.10$), ruling out a priori differences in locomotor activity (data not shown).
During reversal learning, only ELS-APPswe/PS1dE9 mice were unable to locate the exit hole, as indicated by a significant overall difference in reversal learning (interaction effect: F(1,43) = 9.52, p = 0.004) and their failure to decrease the time to the exit hole over the trials (Ctrl-WT: F(1.58,18.90) = 12.11, p < 0.001; ELS-WT: F(1.54,26.15) = 13.64, p < 0.001; Ctrl-APPswe/PS1dE9: F(3,21) = 4.25, p = 0.02; ELS-APPswe/PS1dE9: F(3,21) = 0.35, p = 0.79; **Figure 3D**) and higher average escape latencies across all trials (interaction effect: F(1,43) = 9.52, p < 0.001; post hoc test: ELS-WT vs. ELS-APPswe/PS1dE9, p < 0.001; Ctrl-APPswe/PS1dE9 vs. ELS-APPswe/PS1dE9, p = 0.02; **Figure 3E**). This was confirmed by the probe trial, where ELS-APPswe/PS1dE9 mice spend less time in the exit quadrant than ELS-WT mice (genotype effect: F(1,45) = 4.32, p = 0.04; post hoc
Figure 3. Barnes maze performance of 12-month-old mice. A. Acquisition learning was significantly slower in APPswe/PS1dE9 mice compared to WT mice on days 2, 3 and 4. All groups showed a significant learning curve over the trials. B. The average time to locate the exit hole was higher in APPswe/PS1dE9 mice, with significant post hoc differences between Ctrl-WT and Ctrl-APPswe/PS1dE9 mice, and between ELS-WT and ELS-APPswe/PS1dE9 mice. C. On the probe trial, Ctrl-APPswe/PS1dE9 mice performed significantly worse than Ctrl-WT mice. D. During reversal learning, there was a significant difference between the groups on day 2, 3 and 4 in the latency to find the exit hole. Overall, all groups showed a significant learning curve, except for ELS-APPswe/PS1dE9 mice. E. The average time to locate the exit hole was higher in the ELS-APPswe/PS1dE9 mice compared to Ctrl-APPswe/PS1dE9 mice, and compared to ELS-WT mice. F. On the probe trial, ELS-APPswe/PS1dE9 mice were the only group, which did not spend more than 25% of the total time in the exit quadrant. G. A typical search pattern of APPswe/PS1dE9 mice reared under control (left) or ELS (right) conditions. H. Performance on the final trial during acquisition learning of the Barnes maze correlated significantly with Aβ42 levels \( r = 0.69, p < 0.05 \). I. Performance on the second trial of reversal learning of the Barnes maze correlated significantly with Aβ42 levels in APPswe/PS1dE9 mice \( r = 0.62, p < 0.05 \). N = 8–18 mice/group. * indicates a significant post hoc Tukey test. # indicates a significant learning curve over the days. ^ indicates significant performance compared to chance level.
test: $p = 0.02$), and only the ELS-APPswe/PS1dE9 mice spend $<25\%$ of the time (chance level) in the escape quadrant (Figure 3F). This effect is illustrated by the movement trajectories of the ELS-APPswe/PS1dE9 mice, which showed little improvement in these animals over the trial days compared to Ctrl-APPswe/PS1dE9 mice (Figure 3G). Notably, the latency to locate the exit hole on the final trial of the acquisition phase of the Barnes maze (trial 4.2), which is the most reliable trial to assess the extent to which mice have learned to successfully navigate to the exit hole, correlated positively with hippocampal Aβ42 levels in 12-month-old APPswe/PS1dE9 mice ($r = 0.69$, $n = 16$, $p < 0.001$; Figure 3H). Moreover, hippocampal Aβ42 levels correlated positively with the latency to locate the exit hole on the second trial of the reversal learning phase (trial 1.2; $r = 0.62$, $n = 16$, $p = 0.01$; Figure 3I), which is the first trial in which the ability of the animal to adapt to the relocated position of the exit hole becomes apparent (i.e., the trial in which the mice need cognitive flexibility). Since the behavioural tests revealed a phenotype only in ELS-APPswe/PS1dE9 mice at 12 months of age, all sub-sequent experiments were conducted with this age group.

### 3.5. CORT levels and ELS

To evaluate whether alterations in responsiveness of the HPA axis were indeed induced by ELS in APPswe/PS1dE9 mice, we measured plasma CORT levels under basal conditions, and at 30 and 90 min after a forced swim stress. Basal plasma CORT levels were comparable between the groups (Figure 4A). Thirty minutes after acute stress, ELS-APPswe/PS1dE9 mice had higher plasma CORT levels than Ctrl-APPswe/PS1dE9 mice, while ELS-WT mice had lower plasma CORT levels than Ctrl-WT mice (interaction effect: $F(1,40) = 14.54$, $p < 0.001$; post hoc test: ELS-WT vs. ELS-APPswe/PS1dE9, $p < 0.001$; Ctrl-APPswe/PS1dE9 vs. ELS-APPswe/PS1dE9, $p < 0.001$; Ctrl-WT vs. ELS-WT, $p = 0.03$).

Ninety minutes after stressor onset, plasma CORT levels in ELS-APPswe/PS1dE9 mice were still elevated when compared to Ctrl-APPswe/PS1dE9 mice (interaction effect: $F(1,41) = 8.22$, $p = 0.01$; post hoc Tukey test: Ctrl-WT vs. ELS-WT, $p = 0.04$; ELS-WT vs. ELS-APPswe/PS1dE9, $p = 0.02$). Determining the total area under the curve of the plasma CORT response from 0 to 90 min after stressor onset revealed that ELS-APPswe/PS1dE9 mice were exposed to elevated plasma CORT levels after stress, relative to Ctrl-APPswe/PS1dE9 mice, and relative to ELS-WT mice (interaction effect: $F(1,40) = 22.67$, $p < 0.001$; post hoc Tukey test: ELS-WT vs. ELS-APPswe/PS1dE9, $p < 0.001$, Ctrl-APPswe/PS1dE9 vs. ELS-APPswe/PS1dE9, $p < 0.001$; Figure 4B). Plasma CORT levels (area under the curve) further correlated positively with the latency on trial 1.2 of the...
reversal learning test (r = 0.40, n = 44, p = 0.01), i.e., the trial in which cognitive flexibility is best reflected (Figure 4C).

3.6. Brief treatment with mifepristone reduces Aβ pathology and rescues spatial memory deficit

To investigate whether blocking the GR, i.e., the receptor that becomes selectively occupied by CORT only during stress, could interfere with Aβ pathology and cognitive decline in ELS-APPswe/PS1dE9 mice, animals were treated for 3 days with mifepristone at 12 months of age (Figure 5A). To validate the acute effects of mifepristone treatment on relevant parameters, APPswe/PS1dE9 mice were sacrificed 24 h after the last treatment. Mifepristone reduced both basal plasma CORT levels (treatment effect: $F(1,31) = 111.46, p < 0.001$; post hoc: Ctrl-WT-veh vs. Ctrl-WT-Mif, $p < 0.001$; ELS-WT-veh vs. ELS-WT-Mif, $p < 0.001$;
Targeting glucocorticoid receptors in APPswe/PS1dE9 mice

Ctrl-APPswe/PS1dE9-veh vs. Ctrl-APPswe/PS1dE9-Mif, p < 0.001; ELS-APPswe/PS1dE9-veh vs. ELS-APPswe/PS1dE9-Mif, p < 0.001) and the elevation in plasma CORT levels after foot shock stress (F(1,56)=53.53, p < 0.001; post hoc: Ctrl-WT-veh vs. Ctrl-WT-Mif, p < 0.01; ELS-WT-veh vs. ELS-WT-Mif, p=0.001; Ctrl-APPswe/PS1dE9-veh vs. Ctrl-APPswe/PS1dE9-Mif, p=0.05; ELS-APPswe/PS1dE9-veh vs. ELS-APPswe/PS1dE9-Mif, p < 0.001; ELS-WT-veh vs. ELS-APPswe/PS1dE9-veh, p= 0.04; Figure 5B,C). Twenty-four hours after mifepristone treatment, hippocampal levels of Aβ40 and of Aβ42 were reduced by 60% and 45%, respectively (Aβ40: t(4) =4.81, p= 0.01; Aβ42: t(4) =3.52, p= 0.02; Figure 5D), while BACE1 levels in the hippocampus were also reduced (t(6) =3.12, p=0.02; Figure 5E). Twenty-one days after mifepristone treatment, no differences were present in basal plasma CORT levels between any of the groups (t(15) =0.89, p= 0.39; Figure 5F). However, the levels of Aβ42 remained significantly reduced in ELS mice that had been treated with mifepristone (F(1,18) =15.08, p < 0.001; post hoc: ELS-mifepristone vs. ELS-vehicle, p < 0.001), while Aβ40 levels were comparable between vehicle and mifepristone-treated APPswe/PS1dE9 mice (treatment effect: F(1,20) = 0.73, p= 0.40; condition effect: F(1,20) = 0.56, p= 0.46; Figure 5G). No effects of mifepristone on BACE1 were present at twenty-one days after treatment (condition effect: F(1,20) = 1.62, p=0.22; treatment effect: F(1,20) = 2.57, p= 0.12; interaction effect: F(1,20) = 4.32, p =0.051; Figure 5H). During acquisition learning, APPswe/PS1dE9 mice were slower in acquisition learning on the Barnes maze (F(1,32) = 14.94, p < 0.001; Figure 5J,3). Notably, mifepristone improved cognitive performance in both ELS- APPswe/PS1dE9 and Ctrl-APPswe/PS1dE9 mice (acquisition learning: treatment effect, F(1,25) = 11.78 and p < 0.001; probe trial treatment effect, F(1,58) = 4.66 and p = 0.035; no post hoc Tukey differences detected; Figure 5J,K), while the treatment did not affect performance in WT mice (F(1,38) = 1.05, p = 0.31; Figure 5I). Upon reversal learning, mifepristone also rescued the cognitive impairments specifically in ELS-APPswe/PS1dE9 mice, but did not alter performance in WT mice (WT: F(1,38) = 0.21, p = 0.65; APPswe/PS1dE9: F(1,60) = 4.80, p = 0.03; post hoc Tukey: p = 0.01; Figure 5L-O).

4. Discussion

We report that exposure of genetically predisposed mice to early life stress (ELS) from PND 2-9 exacerbates the development of amyloid pathology and accelerates cognitive decline, notably in close association with enhanced HPA axis activity. Blocking glucocorticoid receptors (GRs) with the GR antagonist mifepristone for only 3 days normalised the elevated plasma corticosterone levels, reduced Aβ levels and BACE1, and rescued the cognitive impairments in 12 month old ELS-APPswe/PS1dE9 mice.
Acquisition learning in the Barnes maze was clearly impaired in 12 month old APPswe/PS1dE9 mice, consistent with earlier observations. Only the ELS-APPswe/PS1dE9 mice, however, were hampered in their reversal learning/cognitive flexibility, a domain also specifically affected in early stages of AD. This reduction in cognitive flexibility was not observed in ELS-WT mice. Since the ELS-APPswe/PS1dE9 mice also showed enhanced Aβ pathology, this suggests these behavioural deficits are mediated by elevations in brain amyloid levels. In line with this, the Aβ42 levels and Barnes maze performance were strongly correlated, both during acquisition and retrieval. ELS may thus specifically promote increases in Aβ42 levels, likely via changes in BACE1, which, over time, could accelerate the reduction in cognitive flexibility. Clearly, age is a relevant factor too as increases in hippocampal Aβ42 levels and plaque load were already found after ELS in 6 month old APPswe/PS1dE9 mice, but reduced cognitive flexibility was not apparent yet at that age. Possibly, the absolute Aβ levels may need to be further increased before they affect cognition.
**Long-term effects (21 days)**

**F.** Basal CORT levels

<table>
<thead>
<tr>
<th>CORT (ng/ml)</th>
<th>WT</th>
<th>APP/PS1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ctrl-veh</td>
<td>ELS-veh</td>
</tr>
</tbody>
</table>

![Graph showing CORT levels over time](image)

**G.** SDS-soluble fraction

<table>
<thead>
<tr>
<th>Aβ40</th>
<th>Aβ42</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl-veh</td>
<td>ELS-veh</td>
</tr>
</tbody>
</table>

![Graph showing Aβ40 and Aβ42 levels](image)

**H.** BACE1

<table>
<thead>
<tr>
<th>% of control</th>
<th>Ctrl</th>
<th>ELS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mif.</td>
<td>Mif.</td>
</tr>
</tbody>
</table>

![Graph showing BACE1 levels](image)

**I.** Acquisition - wild type mice

<table>
<thead>
<tr>
<th>Time (s)</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ctrl-veh #</td>
<td>ELS-veh #</td>
<td>Ctrl-Mif. #</td>
<td>ELS-Mif. #</td>
</tr>
</tbody>
</table>

![Graph showing time to locate the exit hole](image)

**J.** Acquisition - APP/PS1 mice

<table>
<thead>
<tr>
<th>Time (s)</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ctrl-veh #</td>
<td>ELS-veh #</td>
<td>Ctrl-Mif. #</td>
<td>ELS-Mif. #</td>
</tr>
</tbody>
</table>

![Graph showing time to locate the exit hole](image)

**K.** Probe trial

<table>
<thead>
<tr>
<th>% of time in exit quadrant</th>
<th>WT</th>
<th>APP/PS1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ctrl-veh</td>
<td>ELS-veh</td>
</tr>
</tbody>
</table>

![Graph showing time spent in exit quadrant](image)

**L.** Reversal - wild type mice

<table>
<thead>
<tr>
<th>Time (s)</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ctrl-veh #</td>
<td>ELS-veh #</td>
<td>Ctrl-Mif. #</td>
<td>ELS-Mif. #</td>
</tr>
</tbody>
</table>

![Graph showing time to locate the exit hole](image)

**M.** Reversal - APP/PS1 mice

<table>
<thead>
<tr>
<th>Time (s)</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ctrl-veh #</td>
<td>ELS-veh #</td>
<td>Ctrl-Mif. #</td>
<td>ELS-Mif. #</td>
</tr>
</tbody>
</table>

![Graph showing time to locate the exit hole](image)

**N.** Probe trial

<table>
<thead>
<tr>
<th>% of time in exit quadrant</th>
<th>WT</th>
<th>APP/PS1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ctrl-veh</td>
<td>ELS-veh</td>
</tr>
</tbody>
</table>

![Graph showing time spent in exit quadrant](image)

**O.**

![Image showing search patterns](image)

**Figure 5 (continued).** F. Twenty-one days after the last treatment, no differences in basal CORT levels were observed. G. Twenty-one days after the last treatment, there were no differences in the levels of Aβ40 between the groups. Aβ42 levels remained lower after mifepristone treatment, with significant post hoc tests between the ELS groups. H. Twenty-one days after the mifepristone treatment, no differences are observed in the expression of BACE1. I. In WT mice, mifepristone treatment had no effect on acquisition learning on the Barnes maze, and all mice learned to locate the exit hole. J. In APPswe/PS1dE9 mice, mifepristone treatment resulted in a decreased time to locate the exit hole. K. During the probe trial, all groups spend significantly more than 25% of the time in the exit quadrant. L. In WT mice, mifepristone treatment had no effect on reversal learning. M. In APPswe/PS1dE9 mice, mifepristone treatment resulted in a decreased time to locate the exit hole, specifically in ELS-APPswe/PS1dE9 mice. All mice treated with mifepristone learned to locate the exit hole; vehicle treated mice did not. N. In the probe trial of the reversal learning, mifepristone treatment resulted in an increase in the time spend in the exit quadrant. Post hoc Tukey revealed a significant difference between ELS-APPswe/PS1dE9-veh and ELS-APPswe/PS1dE9-mifepristone mice. O. A typical search pattern of ELS-APPswe/PS1dE9 mice with and without mifepristone. All experiments at 21 days used N= 7-13 mice/group. Mif. mifepristone. * indicates a significant post hoc Tukey test. # indicates a significant learning curve over the days. ^ indicates significant performance compared to chance level.
Chapter 4

Age-related cognitive decline has previously been associated with age-related increases in HPA-axis activity\(^\text{35,36}\). In addition, elevated basal levels of circulating cortisol in the early disease stages\(^\text{37–39}\), as well as the failure to suppress cortisol after the dexamethasone challenge\(^\text{40–42}\), indicates that HPA axis activity is altered in AD patients. Notwithstanding, HPA dysfunction does not seem to worsen further with disease progression in patients\(^\text{43,44}\), implying that early alterations in HPA axis, likely acting via glucocorticoids, in particular contribute to the onset and subsequent acceleration of AD pathogenesis. When this occurs in the context of an early life stress history, such changes may possibly be amplified with increasing age\(^\text{45–47}\). In agreement, stress-induced corticosterone levels were exclusively enhanced in the ELS-APPswe/PS1dE9 mice.

In addition, and in line with the hypothesis that changes in HPA-axis activity contribute to the accelerated cognitive decline in ELS-APPswe/PS1dE9 mice, we could rescue the reduction in cognitive flexibility by a brief treatment with the glucocorticoid receptor antagonist mifepristone, an effect that, notably, was most prominent in the ELS-APPswe/PS1dE9 mice. Moreover, ELS caused increases in both A\(\beta\)40 and A\(\beta\)42 levels at 6, and in A\(\beta\)42 levels at 12 months of age (Figure 1A,B), while mifepristone treatment at 12 months of age strongly reduced both A\(\beta\) species (Figure 5E,F) in the APPswe/PS1dE9 mice, again indicative of an amyloid-related mechanism. This is supported by earlier studies that used a much longer treatment period or a much higher dosage in other AD mouse models\(^\text{12,48}\). Because of its short treatment duration and relatively low dose, our current 3 day mifepristone treatment provides considerable practical advantages. The rescue effects we report here occur at an age at which the cognitive impairments and amyloid pathology have already manifested. Moreover, this short treatment regime not only rescued the cognitive and neuropathological phenotypes, but, importantly, these effects also persisted for at least 3 weeks.

An outstanding question remains how early stress and mifepristone treatment could affect amyloid pathology in the APPswe/PS1dE9 mice. ELS did not affect the levels of full-length APP, indicating that the changes in A\(\beta\) levels result either from altered processing of APP, or from altered A\(\beta\) clearance. The rate-limiting enzyme involved in processing of APP to A\(\beta\) is \(\beta\)-site amyloid precursor protein cleaving enzyme 1 (BACE1), an enzyme that also contains glucocorticoid binding sites\(^\text{49}\). Hence, increases in BACE1 expression following elevated CORT levels could, in time, lead to a higher and prolonged A\(\beta\) production, and hence an earlier plaque accumulation. Indeed, BACE1 expression was significantly enhanced following ELS in APPswe/PS1dE9 mice, and also mifepristone treatment rapidly reduced BACE1 expression, indicative
of a common mechanism via which ELS and mifepristone affect Aβ levels.

Although the exact cellular mechanisms through which mifepristone reduces Aβ levels and prevents cognitive decline remain to be further investigated, blocking GC actions is an attractive option for possible future therapeutic interventions. So far, a small clinical trial in AD patients and old macaques monkeys reported improvements in cognition after mifepristone treatment\textsuperscript{26,50}, although the short time window and small sample size warrants caution in interpreting these results. Furthermore, AD patients with the highest baseline cortisol levels benefited most from a mifepristone intervention and showed persistent memory improvements up to 8 weeks after discontinuation of the treatment\textsuperscript{26}. This is in line with our findings that after 3 weeks, Aβ42 levels were still strongly reduced in the ELS-APPswe/PS1dE9 mice treated for 3 days with mifepristone. While this highlights an interesting translational potential of the drug and suggests it can 're-set' earlier established changes, it also calls for further study of the mechanisms underlying these intriguing lasting effects already induced after such a short treatment with mifepristone.

Taken together, exposure to stress early in life, likely via the associated alterations in HPA axis activity, can exacerbate amyloid pathology at a later age. ELS upregulates Aβ levels and BACE1 expression, which may underlie cognitive deficits such as reversal learning, and as such may be a risk factor for AD in vulnerable individuals. Given the current rescue effects on both Aβ levels and cognitive flexibility in middle aged mice already after a short mifepristone treatment, interventions with this classic drug, or other compounds targeting the HPA axis, may provide potential therapeutic benefits for AD, even at ages when symptoms have already manifested.

5. Funding and disclosure

HJK and SLL are supported by a grant from Internationale Stichting Alzheimer Onderzoek (ISAO)/Alzheimer Nederland (grant: #12534 to HJK). PJL is supported by ISAO/Alzheimer Nederland (grant WE.03.2012.041). The authors declare no conflict of interest.

6. Acknowledgements

The authors thank B.A.C.E van Hoek and L.A.E. Catsburg for their assistance with the experimental animal work.
7. References

Targeting glucocorticoid receptors in APP/PS1 mice


8. Supplementary data

Supplementary table 1. Effects of chronic ELS exposure on pups (PND 9), 6 and 12 month old mice.

<table>
<thead>
<tr>
<th>Maternal care</th>
<th>Ctrl</th>
<th>ELS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light phase</td>
<td>Dark phase</td>
</tr>
<tr>
<td>Duration nursing (s)</td>
<td>2667 ± 46 (8)</td>
<td>484 ± 45 (8)</td>
</tr>
<tr>
<td>Duration off pups (s)</td>
<td>182 ± 40 (8)</td>
<td>561 ± 43 (8)</td>
</tr>
<tr>
<td>Number of exits</td>
<td>0.5 ± 0.2 (8)</td>
<td>2.4 ± 0.4 (8)</td>
</tr>
<tr>
<td># pup(s) out of nest</td>
<td>0.0 ± 0.0 (8)</td>
<td>0.0 ± 0.0 (8)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pups</th>
<th>Ctrl</th>
<th>ELS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain PND 2-9 (g)</td>
<td>3.47 ± 0.11 (24)</td>
<td>2.60 ± 0.09 (27)*</td>
</tr>
<tr>
<td>Body weight PND 21 (g)</td>
<td>8.80 ± 0.23 (24)</td>
<td>8.42 ± 0.23 (27)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>6 months</th>
<th>Ctrl - WT</th>
<th>Ctrl – APP/PS1</th>
<th>ELS – WT</th>
<th>ELS – APP/PS1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>29.4 ± 0.89 (12)</td>
<td>29.7 ± 0.34 (10)</td>
<td>28.9 ± 0.55 (11)</td>
<td>29.7 ± 0.38 (14)</td>
</tr>
<tr>
<td>Thymus weight (% of BW)</td>
<td>0.159 ± 0.01 (8)</td>
<td>0.154 ± 0.03 (6)</td>
<td>0.147 ± 0.01 (10)</td>
<td>0.148 ± 0.00 (6)</td>
</tr>
<tr>
<td>Adrenal gland weight (% of BW)</td>
<td>0.0062 ± 0.00 (8)</td>
<td>0.0076 ± 0.01 (7)</td>
<td>0.0096 ± 0.00 (11)*</td>
<td>0.0085 ± 0.00 (7)*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>12 months</th>
<th>Ctrl - WT</th>
<th>Ctrl – APP/PS1</th>
<th>ELS – WT</th>
<th>ELS – APP/PS1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>39.43 ± 1.50 (13)</td>
<td>39.13 ± 1.24 (9)</td>
<td>38.65 ± 1.13 (17)</td>
<td>42.43 ± 1.75 (9)</td>
</tr>
<tr>
<td>Thymus weight (% of BW)</td>
<td>0.109 ± 0.01 (13)</td>
<td>0.092 ± 0.04 (7)*</td>
<td>0.090 ± 0.01 (16)*</td>
<td>0.107 ± 0.01 (9)*</td>
</tr>
<tr>
<td>Adrenal gland weight (% of BW)</td>
<td>0.013 ± 0.00 (14)</td>
<td>0.013 ± 0.00 (9)</td>
<td>0.011 ± 0.00 (15)*</td>
<td>0.011 ± 0.01 (7)*</td>
</tr>
</tbody>
</table>

Data expressed as mean ± S.E.M. (N).
*: p<0.05, t-test compared to Ctrl mice
**: p<0.05, two-way ANOVA, main effect for condition
***: p<0.05, two-way ANOVA, interaction effect
Early life stress amplifies fear responses and hippocampal synaptic potentiation in the APPswe/PS1dE9 Alzheimer mouse model

Sylvie L. Lesuis, Paul J. Lucassen, Harm J. Krugers

Submitted
Abstract

Cognitive deficits and alterations in emotional behaviour are typical features of Alzheimer's disease (AD). Moreover, exposure to stress or adversity during the early life period has been associated with an acceleration of cognitive deficits and increased AD pathology in transgenic AD mouse models. Whether and how early life adversity affects emotional behaviour in AD mice remains elusive.

We therefore investigated whether exposure to early life stress (ELS) alters fear learning in APPswe/PS1dE9 mice, a classic mouse model for AD, and whether this is accompanied by alterations in hippocampal synaptic potentiation, an important cellular substrate for learning and memory.

Transgenic APPswe/PS1dE9 mice were subjected to ELS by housing the dams and her pups with limited nesting and bedding material from postnatal days 2-9. Following a fear conditioning paradigm, 12 month old ELS-exposed APPswe/PS1dE9 mice displayed enhanced contextual freezing behaviour, both in the adverse training environment and in a novel, “potentially safe” environment. ELS-exposed APPswe/PS1dE9 mice also displayed enhanced hippocampal synaptic potentiation, even in the presence of the GluN2B antagonist Ro25-6981 (which prevented synaptic potentiation in control mice). No differences in the level of PSD-95 or synaptophysin were observed between the groups.

We conclude that ELS disrupts fear memory formation in APPswe/PS1dE9 mice, which is accompanied by aberrant hippocampal synaptic potentiation. These results may help to understand how individual differences in the vulnerability to develop AD pathology arise and emphasise the importance of the early postnatal time window in determining the later vulnerability to AD.

Keywords: plasticity, memory, Alzheimer’s disease, Ro25-6981, LTP, early life stress, fear, emotionality
ELS amplifies fear in AD

1. Introduction

The sensitivity and responsiveness to stressors at adult age is largely determined during the early postnatal period\textsuperscript{1–4}. In general, different models of early life adversity during the postnatal time window enhance the sensitivity to stress later in life, reduces spatial cognitive function, and enhances fear responses\textsuperscript{5–7}. For instance, offspring that received low amounts of maternal care showed impaired spatial memory and object recognition performance\textsuperscript{4,8–10} and increased conditioned fear responses\textsuperscript{11–13}, while impaired spatial memory was reported following maternal separation\textsuperscript{14,15}, and maternal deprivation\textsuperscript{16–19}. Furthermore, maternal separation results in more anxious animals in the light/dark exploration test\textsuperscript{20} and after fear conditioning\textsuperscript{21}. In addition, we have recently reported that exposure to limited nesting and bedding material from postnatal day (PND) 2-9 in transgenic Alzheimer (AD) mice enhances Aβ levels\textsuperscript{22,23} and enhances spatial reversal learning deficits\textsuperscript{23}. Yet, whether and how early life adversity affects emotional learning in AD mice remains elusive.

AD is an age-related neurodegenerative disease characterised by progressive memory loss and enhanced emotionality\textsuperscript{24}. Amyloid-β (Aβ) containing plaques are a major hallmark of AD and Aβ oligomers have been implicated in loss of synaptic function and cognitive decline\textsuperscript{25,26}. While genetic mutations result in familial forms of AD\textsuperscript{27,28}, environmental factors may contribute to the progression of AD pathology in sporadic AD. Exposure to stressors has frequently been implicated in AD\textsuperscript{29,30}. Major stressful events accelerate the age of onset of familial AD\textsuperscript{31} and amplify the progression of AD-related symptoms and neuropathology in sporadic AD\textsuperscript{32,33}. In line with this, rodent studies have reported that stress exposure in AD transgenic mice results in stronger cognitive decline, increased amyloid precursor protein (APP) misprocessing, reduced Aβ clearance and enhanced tau hyperphosphorylation\textsuperscript{22,23,34–37}, whereas environmental enrichment mitigates these effects\textsuperscript{22,38,39}.

Synaptic function is critically involved in (emotional) learning and memory\textsuperscript{40–42}, and impairments in synaptic function, as present in mouse models for AD, are increasingly viewed as an early manifestation of AD\textsuperscript{43,44}. Since long-term potentiation (LTP), an important cellular model for learning and memory\textsuperscript{40,45}, is impaired in mouse models with transgenic overexpression of Aβ\textsuperscript{46,47}, we here examined whether early life stress (ELS) modulates synaptic plasticity and fear memory formation in APPswe/PS1dE9 transgenic mice, which develop progressive spatial cognitive deficits and Aβ accumulation in the brain\textsuperscript{48,49}. Mice were exposed to ELS by housing them with limited nesting and bedding material from PND 2-9, a well-described model for ELS\textsuperscript{6,22,23,50},
Chapter 5

after which we examined synaptic potentiation and fear memory in 12 month old APPswe/PS1dE9 animals and their appropriate controls.

2. Materials and Methods

2.1. Mice and breeding

All mice were kept under standard housing conditions (temperature 20-22 °C, 40-60% humidity). Standard chow and water were available ad libitum. Mice were housed on a 12/12 h light/dark schedule (lights on at 8 a.m.) in standard cages (325x170x140 mm, Tecnilab-BMI, Someren, The Netherlands). A radio provided background noise. Wild type-like (hereafter referred to as “WT”) and APPswe/PS1dE9 male mice at the age of 12 months were used. To obtain mice, two 10-weeks old C57BL/6J virgin WT females (Harlan Laboratories B.V., Venray, The Netherlands) and one heterozygous male APPswe/PS1dE9 mouse were housed together for one week to allow mating. After another week of paired-housing, pregnant females were housed individually in a standard cage covered with a filter top and monitored daily for the birth of pups. When a litter was born before 10.00 a.m., the previous day was considered as the day of birth (postnatal day 0; PND 0), after which the early life paradigm was initiated at PND 2. At PND 21, the male mice used for this study were weaned and ear biopsies were collected to identify and genotype the mice. Mice were housed with 2-5 male littermates per cage after weaning. All experimental mice were left undisturbed (except for cage cleaning once a week) until start of experimental procedures. All experiments were conducted under the EU directive 2010/63/EU for animal experiments and were approved by the animal welfare committee of the University of Amsterdam.

2.2. Early life stress

At PND 2, litters were culled to 6 pups per litter, and dams and their litters were weighed and randomly assigned to the early life stress (ELS) or control condition as described before. Eighteen litters were assigned to the control condition and 17 litters were assigned to the ELS condition. Control dams were provided with a standard amount of sawdust bedding and nesting material (one square piece of cotton nesting material (5 x 5 cm; Tecnilab-BMI, Someren, the Netherlands)). As described and discussed before, the ELS dams were provided with reduced amounts of sawdust bedding (approximately 10% of control cages) and half the nesting material (1/2 square piece of cotton nesting material (2.5 x 5 cm)), and a fine-gauge stainless steel mesh was placed
ELS amplifies fear in AD

1 cm above the cage floor. Both control and ELS cages were left undisturbed until PND 9, after which all litters were weighed and placed in standard cages, with standard amounts of bedding and nesting material until weaning at PND 21. The average body weight gain between PND 2 to 9 of all males in a litter was analysed.

### 2.3. Fear conditioning

One month prior to behavioural testing male mice were weighed and housed under a reversed light/dark schedule (lights on at 8 p.m.) and testing was conducted during the dark (active phase) between 1 and 6 p.m. in a testing room that was illuminated by two red spots (EGB, 25 Watt). Mice were single housed one week prior to fear conditioning. During testing, mice were recorded by a camera connected to a computer with Ethovision software (version 6.1, Noldus, The Netherlands) and videos were manually scored by an experimenter blind to the experimental condition of the mouse.

Twelve month old (± 7 days) APPswe/PS1dE9 and WT male mice were tested for contextual fear memory. On day 1, mice were placed in a chamber which had a stainless steel grid floor connected to a shock generator, which had been cleaned with 1% acidic acid to create a recognisable odour trace. Mice were allowed to explore the context for three minutes, after which a tone was administered (2.8 kHz, 76 dB). During the last 2 seconds mice received a single mild foot shock (0.4 mA) for 2 seconds. After this shock, the mice remained in the chamber for 30 seconds. Twenty-four hours later, the mice were reintroduced in the same chamber for 3 minutes. One hour later, mice were introduced in a completely novel chamber (cleaned with 25% EtOH). Freezing behaviour was scored by an observer blind to the experimental condition, with “freezing” being defined as “no body movements except those related to breathing”.

### 2.4. Field potential recordings

Field potential recordings were conducted in a separate batch of animals. At PND 360 ± 14, male mice were sacrificed between 9 and 10 a.m. through quick decapitation. Immediately after decapitation, the brain was rapidly removed, and collected in ice-cold oxygenated (95% O₂/5% CO₂) solution containing (in mM): Cholinechloride (120), glucose (10), NaHCO₃ (25), MgSO₄ (6), KCl (3.5), NaH₂PO₄ (1.25), CaCl₂ (0.5). Coronal slices (350 μm) were cut using a microtome (Leica VT1000S). For recovery, slices were incubated for 20 minutes in warm (32 °C) oxygenated standard artificial cerebrospinal fluid (aCSF) containing (in
mM): NaCl (120), KCl (3.5), MgSO$_4$ (1.3), Na$_2$PO$_4$ (1.25), CaCl$_2$ (2.5), glucose (10), NaHCO$_3$ (25), after which the sections were maintained at room temperature (22 °C). Sections containing the dorsal hippocampal CA1 area (bregma -2.0 mm to -3.2 mm) were placed in a recording chamber with a constant flow of oxygenated aCSF. Field excitatory synaptic potentials (fEPSPs) were recorded as described previously$^{57-59}$. fEPSPs were evoked using a stainless steel bipolar stimulation electrode (60 µm diameter, insulated except for the tip) positioned on the Schaffer collaterals and recorded through a glass electrode (2-5 MΩ impedance, filled with aCSF) positioned in the CA1 stratum radiatum. A stimulus-response curve was generated by gradually increasing the stimulus intensity to define a level that generated the half-maximal response that was used for the remainder of the experiment. Once the input-output curve for each recording was established, baseline synaptic transmission was monitored (0.033 Hz) during 10 minutes. When recordings were stable, afferent fibres were stimulated at 10 Hz for 90 seconds$^{59,60}$. Next, the degree of potentiation was determined by recording of fEPSPs every 30 seconds during 1 hours (0.033 Hz). The magnitude of the fEPSP was assessed by analysing the slope of the signal. The average baseline value was normalised to 100% and all values of the experiment were normalised to this baseline average. Synaptic potentiation was measured in the presence and absence of the GluN2B antagonist Ro25-6981 (3 µM; Sigma) in order to investigate plasticity under conditions that normally do not elicit potentiation (i.e. in the presence of Ro25-6981).

2.5. Plasma corticosterone levels

At 9 a.m., when plasma corticosterone levels are low, mice that were used for electrophysiology experiments were taken from the stables and within 90 seconds decapitated, after which trunk blood was collected for the determination of basal plasma corticosterone (CORT) levels. Samples were collected in ice cold EDTA-coated tubes (Sarstedt, Etten-leur, The Netherlands), placed on ice and centrifuged at 14.000 rpm for 15 minutes after which plasma was stored at -20 °C. Plasma CORT levels were measured using a commercially available radioimmunoassay kit (MP Biomedicals, Eindhoven, The Netherlands).

2.6. Western blots

To compare hippocampal protein levels between the groups, male 12 month old mice were decapitated and hippocampi were dissected in saline on ice. Tissue was stored at -80 °C. For protein extraction, hippocampi were homogenised in RIPA buffer (150 mM NaCl, 1% Triton X100, 0.5% Sodium deoxycholate, 0.1% SDS
at pH 7.6) using a small syringe. The samples were incubated on ice for 30 min and then centrifuged for 20 min at 16,000 rpm at 4 °C. Protein lysate was stored at −20 °C. For each sample the protein concentration was measured using a BCA Protein Assay (23225, Pierce (Thermo Fischer) The Netherlands). Samples containing between 10-30 μg protein in sample buffer were denaturised at 95 °C for 5 min. A polyacrylamide-SDS gel (Biorad, The Netherlands) was used for protein separation by electrophoresis. The proteins were transferred to a PVDF membrane (162-0177, Biorad, The Netherlands) in a tris-glycine buffer. The membranes were cut for incubation with different antibodies. The membrane strips were blocked in TBST containing 5% BSA for 1 h. After blocking, blots were washed with TBST and incubated with primary antibodies at 4 °C overnight. Primary antibodies included PSD-95 (1:3000, D27E11, Cell Signaling), synaptophysin (1:3000, SY38, Abcam), α-tubulin (1:1000, 10D8, Santa Cruz) and GAPDH (1:3000, 2118S, Cell Signaling (Bioke) The Netherlands). After washing with TBS, blots were incubated with secondary antibodies for 2 h at room temperature (HRP conjugate, Biorad, The Netherlands). Blots were washed again and bands were visualised by chemiluminescence using an ECL Prime kit (RPN2232, Amersham, (GE Healthcare) The Netherlands). A Li-COR machine was used to measure the chemiluminescence. Optical density was determined in ImageJ. Measurements of the protein of interest were corrected for total protein (GAPDH band).

2.7. Statistical analysis

Data were analysed using SPSS 22.0 (IBM software). All data are expressed as mean ± standard error of the mean (SEM). Data were considered statistically significant when p<0.05. Outliers were determined using a Grubbs's test. Independent-samples t-tests were performed to assess differences in body weight up until PND 21. Appropriate corrections were applied when assumption of homogeneity of variance was not met. When assumption of normality was not met, Mann-Whitney test was conducted. To determine the effects of condition and genotype on the degree of LTP, a three factor repeated-measures ANOVA was performed using condition (control vs. ELS) and genotype (WT vs. APPswe/PS1dE9) as between-subject factors and slope of the pre- and post-stimulation fEPSP as the within-subject factor. We compared the baseline (-10 to 0 min) with total LTP (0 – 60 min after 10 Hz stimulation) and late LTP (50 – 60 min after 10 Hz stimulation). When significant, a post hoc Tukey test was performed to compare groups. Two-way analysis of variance (ANOVA) was performed for comparison between groups accounting for the main and interaction effects of genotype (WT vs. APPswe/
Chapter 5

3. Results

3.1. Early life stress

Housing APPswe/PS1dE9 and WT litters in a cage with limited nesting and bedding material from PND 2-9 reduced body weight gain in the male early life stress (ELS) offspring compared to control-reared litters over this period (t(33)=2.57, p=0.015) (Table 1). At 21 days and at 12 months of age, no differences in body weight were present between the groups (P21: t(17)=0.24, p=0.81; 12 months: genotype: F(1,26)=1.38, p=0.25; condition: F(1,26)=0.72, p=0.40). No differences in basal plasma CORT levels (genotype: F(1,25)=0.38, p=0.54; condition: F(1,25)=2.60, p=0.12) were observed at 12 months of age. These findings are consistent with previous reports of the model\(^{23,50,52,61}\), validating the effective application of the ELS paradigm. Since male mice are more sensitive to the effects of ELS\(^{7,50,62}\), all experiments were further conducted with male mice.

Table 1. Effects of ELS on body weight, CORT levels, locomotion and anxiety-like behaviour.

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>ELS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PND 2-9 (g)</td>
<td>3.20 ± 0.12 (18)</td>
<td>2.78 ± 0.11 (17)*</td>
</tr>
<tr>
<td>Body weight PND 21</td>
<td>8.54 ± 0.28 (30)</td>
<td>8.45 ± 0.20 (29)</td>
</tr>
<tr>
<td>12 months:</td>
<td>Ctrl – WT</td>
<td>Ctrl – APP/PS1</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>39.43 ± 1.50 (8)</td>
<td>39.13 ± 2.01 (7)</td>
</tr>
<tr>
<td>Basal CORT (ng/ml)</td>
<td>6.6 ± 1.9 (9)</td>
<td>7.7 ± 1.7 (6)</td>
</tr>
<tr>
<td></td>
<td>ELS – WT</td>
<td>ELS – APP/PS1</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>38.65 ± 1.13 (8)</td>
<td>42.43 ± 1.75 (7)</td>
</tr>
<tr>
<td>Basal CORT (ng/ml)</td>
<td>3.9 ± 1.1 (8)</td>
<td>4.9 ± 1.8 (6)</td>
</tr>
</tbody>
</table>

Data expressed as mean ± S.E.M (N). * p<0.05, t-test compared to Ctrl mice.
3.2. Fear conditioning

Mice were trained in a fear conditioning paradigm to assess emotional memory formation (Figure 1A). Freezing behaviour before the foot shock was comparable between all experimental groups (genotype: F(1,26)=0.13, p=0.72; condition: F(1,26)=0.24, p=0.63) (Figure 1B). Twenty-four hours later, when animals were placed back in the same context as where the foot shock was received, Ctrl-APPswe/PS1dE9 mice showed reduced freezing compared to Ctrl-WT mice (genotype x condition interaction effect: F(1,26)=12.48, p=0.002; post hoc: 0.004) (Figure 1C). ELS-APPswe/PS1dE9 mice showed higher freezing levels than Ctrl-APPswe/PS1dE9 mice (p=0.02). To determine whether this freezing behaviour was specific for the context, mice were placed in a non-familiar and non-aversive context B one hour later. Interestingly, here, too, ELS-APPswe/PS1dE9 mice showed higher freezing levels than any of the other groups (interaction effect: F(1,26)=14.90, post hoc Tukey compared to WT-APPswe/PS1dE9: p<0.001, compared to Ctrl-APPswe/PS1dE9: p=0.007) (Figure 1D).
Figure 2. Synaptic potentiation of is enhanced in 12 month old APPswe/PS1dE9 mice exposed to ELS after 10 Hz stimulation for 90 seconds. A. Typical examples of fEPSP traces, with signal before (black) and after (gray) the 10 Hz stimulation. B. There was an interaction effect between the groups in the slope of the fEPSP over the entire 60 minutes after stimulation ($F(1,52)=4.22, p=0.045$). ELS-APPswe/PS1dE9 mice show stronger potentiation than Ctrl-APPswe/PS1dE9 mice (post hoc Tukey: $p=0.02$). C. During the last 10 minutes of recording, there was an interaction effect in the potentiation ($F(1,52)=5.05, p=0.03$), with a post hoc Tukey difference between Ctrl-APPswe/PS1dE9 and ELS-APPswe/PS1dE9 mice ($p=0.02$). The post-stimulation fEPSPs during this period were significantly higher than 100% in Ctrl-WT ($t(17)=2.376, p=0.030$), ELS-WT ($t(18)=3.32, p=0.004$), and ELS-APPswe/PS1dE9 ($t(8)=3.58, p=0.007$), but not in Ctrl-APPswe/PS1dE9 mice ($t(10)=1.118, p=0.29$). D. In the presence of Ro25-6981, there was a main condition effect, and an interaction effect (interaction effect: $F(1,31)=5.68, p=0.02$), with significant post hoc Tukey tests between ELS-APPswe/PS1dE9 and Ctrl-APPswe/PS1dE9 mice ($p=0.022$), and ELS-APPswe/PS1dE9 and ELS-WT mice ($p=0.035$). E. In the presence of Ro25-6981, there was a significant condition*genotype effect ($F(1,30)=10.1, p=0.003$), with post hoc differences between Ctrl-APPswe/PS1dE9 and ELS-APPswe/PS1dE9 mice ($F(1,30)=10.15, p=0.003$). No synaptic potentiation is induced in Ctrl-WT and ELS-WT mice 50 to 60 minutes after the stimulation (average of last 50-60 min compared to 100: Ctrl-WT: $t(6)=1.65, p=0.15$, ELS-WT: $t(12)=1.11, p=0.29$). Ctrl-APPswe/PS1dE9 mice show synaptic depression (average of last 50-60 min compared to baseline: $t(8)=3.46, p=0.01$), whereas ELS-APPswe/PS1dE9, synaptic potentiation can still be induced (average of last 50-60 min compared to 100: $t(4)=4.97, p=0.008$). *: significantly different from baseline (100%). #: post hoc Tukey: $p<0.05$.
3.3. Synaptic potentiation

We first assessed the effects of early life stress and the APPswe/PS1dE9 background on baseline synaptic properties of hippocampal CA1 neurons. Input output curves were fitted with a Boltzmann equation (Table 2). There was a main genotype and an interaction effect on the maximal slope of the fEPSP (genotype effect: F(1,119)=7.58, p=0.01; interaction effect: F(1,119)=4.17, p=0.04). Post hoc tests indicated that ELS-WT mice showed an increased maximal slope compared to Ctrl-WT mice, while it was reduced in ELS-APPswe/PS1dE9 mice compared to ELS-WT mice (post hoc tests: Ctrl-WT vs. ELS-WT: p=0.02; ELS-WT vs. ELS-APPswe/PS1dE9: p=0.004). No differences in the half-maximum stimulus intensity were observed (genotype effect: F(1,119)=0.77, p=0.38; condition effect: F(1,119)=2.01, p=0.15). The slope of the input-output curve (interaction effect: F(1,119)=9.95, p=0.002, post hoc: p=0.009) was also reduced in ELS-APPswe/PS1dE9 mice compared to ELS-WT mice.

Next, we investigated synaptic potentiation in the CA1 of the hippocampus using a mild stimulation paradigm (10 Hz, 90 seconds) (Figure 2A). Following 10 Hz stimulation, ELS-APPswe/PS1dE9 mice showed stronger potentiation of the fEPSP over the entire 60 minutes after stimulation (F(1,52)=4.22, p=0.045, post hoc Tukey: Ctrl-APPswe/PS1dE9 vs. ELS-APPswe/PS1dE9: p=0.02) (Figure 2B), as well as during the 50-60 min after stimulation (F(1,52)=5.05, p=0.03, post hoc: p=0.016) (Figure 2C). To assess whether post-stimulation fEPSPs were different from baseline (100%), we compared the signal during the last 10 minutes to 100. While it was possible to induce significant amounts of LTP in all WT mice (Ctrl-WT: 123.3 ± 9.8%, ELS-WT: 124.1 ± 7.3%; average of last 50-60 min compared to 100: Ctrl-WT: t(17)=2.376, p=0.030; ELS-WT: t(18)=3.324, Table 2. Basal field potential characteristics for hippocampal CA1 area.

<table>
<thead>
<tr>
<th></th>
<th>Ctrl-WT</th>
<th>ELS-WT</th>
<th>Ctrl-APP/PS1</th>
<th>ELS-APP/PS1</th>
<th>Statistics:</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R_{\text{max}}$ (mV/ms)</td>
<td>-0.17 ± 0.02 $b$</td>
<td>-0.39 ± 0.09</td>
<td>-0.21 ± 0.04</td>
<td>-0.12 ± 0.02 $a$</td>
<td>G, CxG</td>
</tr>
<tr>
<td>$I_{h}$ (µA)</td>
<td>3.41 ± 0.13</td>
<td>3.37 ± 0.12</td>
<td>3.44 ± 0.13</td>
<td>3.12 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>$S$</td>
<td>0.10 ± 0.01 $a$</td>
<td>0.15 ± 0.02 $a$</td>
<td>0.13 ± 0.02</td>
<td>0.08 ± 0.01 $a$</td>
<td>CxG</td>
</tr>
<tr>
<td>$N$ (mice (slices))</td>
<td>9 (35)</td>
<td>8 (35)</td>
<td>6 (24)</td>
<td>6 (29)</td>
<td></td>
</tr>
</tbody>
</table>

Data expressed as mean ± S.E.M (N). Maximal slope of the fEPSP ($R_{\text{max}}$), half-maximum stimulus intensity ($I_{h}$), and the slope of the input-output curve (slope factor $S$) in the CA1 area. C: main condition effect, G: main genotype effect, CxG: interaction effect, $a$, $b$: equal letters indicate a significant post hoc Tukey difference between the two groups.
Chapter 5

p=0.004), it was not possible to significantly induce potentiation in the Ctrl-APPswe/PS1dE9 mice (107.3 ± 7.4%; Ctrl-APPswe/PS1dE9: t(10)=1.118, p=0.290). Interestingly, we observed that APPswe/PS1dE9 mice that had been exposed to ELS displayed enhanced potentiation (154.3 ± 14.3%; average of last 50-60 min compared to 100: t(8)=3.579, p=0.007), which was also post hoc significantly higher than in Ctrl-APPswe/PS1dE9 mice (p=0.016) (Figure 2B,C).

To assess mechanisms that may underlie changes in synaptic potentiation we applied Ro25-6981, which blocks the GluN2B subunit of the NMDA receptor. When measuring LTP in the presence of Ro25-6981 (3 mM), ELS-APPswe/PS1dE9 displayed more potentiation than Ctrl-APPswe/PS1dE9 mice (p=0.022), and also more than ELS-WT mice (p=0.035) (interaction effect: F(1,30)=5.68, p=0.02) (Figure 2D). In the presence of Ro25-6981, ELS-APPswe/PS1dE9 mice also showed higher levels of potentiation during the last 10 minutes of recording (F(1,30)=10.1, p=0.003) (Figure 2E). Under these conditions, LTP was absent in WT mice (Ctrl-WT: 92.2 ± 4.7%, ELS-WT: 95.6 ± 3.9%; average of last 50-60 min compared to 100: Ctrl-WT: t(6)=1.65, p=0.15, ELS-WT: t(12)=1.11, p=0.29), while Ctrl-APPswe/PS1dE9 mice even showed synaptic depression (81.5 ± 5.3 %). However, in the presence of Ro25-6981, synaptic potentiation was still present in slices of APPswe/PS1dE9 mice that had been exposed to ELS (116.6 ± 3.3%; average of last 50-60 min compared to 100: t(4)=4.97, p=0.008). Together, this indicates that ELS amplifies synaptic potentiation in APPswe/PS1dE9 mice, even under conditions where LTP is absent in control animals.

Figure 3. Synaptic proteins in the hippocampus of 12 month old mice. A, B. The levels of the postsynaptic protein PSD-95 (A) and presynaptic synaptophysin (B) were unaltered following either rearing condition or APPswe/PS1dE9 background.
3.4. Synaptic protein expression

To assess potential effects on synaptic proteins, we also examined the expression of PSD-95 and synaptophysin. In the hippocampus of twelve month old mice, no differences in the expression of PSD-95 (condition effect: F(1,12)=0.26, p=0.62; genotype effect: F(1,12)=0.13, p=0.73) or synaptophysin were present (condition effect: F(1,12)=0.03, p=0.87; genotype effect: F(1,12)=1.14, p=0.31) (Figure 3A,B).

4. Discussion

In this study, we examined whether APPswe/PS1dE9 mice, a classic model for AD, displayed alterations in fear memory formation and hippocampal synaptic potentiation, in particular when animals were exposed to early life stress. We report that contextual fear memory formation was impaired in 12 month old APPswe/PS1dE9 mice. Interestingly, following ELS exposure, contextual fear expression was enhanced in APPswe/PS1dE9 mice. In addition, ELS enhanced hippocampal synaptic potentiation in APPswe/PS1dE9 mice, even under conditions where synaptic potentiation was absent in control animals (through the presence of Ro25-6981). These observations coincide with enhanced amyloid pathology after ELS at this age. We conclude that ELS enhances fear responsiveness in response to aversive and non-aversive contexts, accompanied by aberrantly enhanced synaptic hippocampal synaptic potentiation.

The enhanced freezing levels of ELS-APPswe/PS1dE9 mice in the neutral context suggests that ELS-APPswe/PS1dE9 mice, rather than forming a stronger specific memory of the aversive event, exhibit a stronger anxiety-like response to novel environments following the aversive foot shock than Ctrl-APPswe/PS1dE9 or WT mice. This is not due to differences in basal anxiety-like behaviour, but becomes only apparent after animals had received a mild foot shock. It has previously been reported that APPswe/PS1dE9 mice display disinhibitory tendencies (Lalonde et al., 2004), a phenotype that may have been amplified by ELS exposure. Alternatively, the enhanced fear responsiveness following ELS in APPswe/PS1dE9 mice could reflect enhanced responsiveness and adaptation to fearful experiences. The enhanced fear behaviour in ELS-APPswe/PS1dE9 mice therefore indicates a gene x environment interaction in the expression of fear, in line with observations in humans that emotional memories are relatively spared or even enhanced in AD patients, when compared to matched controls.

To further understand the mechanisms that underlie these differences in
fear memory, we investigated synaptic potentiation in the hippocampal CA1 area, which is known to be strongly correlated to contextual fear memory. Using a mild 10 Hz stimulation protocol, we found that Ctrl-APPswe/PS1dE9 mice displayed no synaptic potentiation. This is in line with other studies showing that synaptic potentiation is hampered in these mice and with reports that Aβ down-regulates the strength of excitatory glutamatergic synaptic transmission, results in loss of dendritic spines, and hampers NMDA-receptor-dependent LTP. In our current study we did not observe that ELS affected synaptic potentiation in the hippocampal CA1 area of WT mice. In rats however, deficits in synaptic potentiation have been observed after ELS at middle age (Brunson et al., 2005), which may indicate either a timing and/or species difference. Intriguingly, we further found a strong potentiation (>50% an hour after the stimulation) in APPswe/PS1dE9 mice exposed to ELS. We consider this degree of synaptic potentiation atypical since other studies using 10 Hz stimulation paradigms in mice, typically report potentiation levels of not more than approximately ±25%. This suggests that either an enhanced potentiation or a reduced depression of synapses occurs in ELS-APPswe/PS1dE9 mice. Moreover, synaptic potentiation in APPswe/PS1dE9 mice that were exposed to ELS was still present when the GluN2B antagonist Ro25-6981 was applied. In control animals, Ro25-6981 completely prevented synaptic potentiation, but it failed to block LTP in ELS-APPswe/PS1dE9 mice. This supports the notion that plasticity mechanisms may be impaired in ELS-APPswe/PS1dE9 mice resulting in enhanced synaptic potentiation.

Networks composed of synapses that exhibit aberrant synaptic potentiation, rather than synapses in which synaptic strength is bi-directionally well controlled, decrease their storage capacity and may increase errors, thereby failing to adequately store memories. Indeed, APPswe/PS1dE9 mice exposed to ELS displayed impaired cognitive flexibility in a hippocampus-dependent spatial navigation task previously, supporting the idea that the observed aberrant synaptic potentiation in ELS-APPswe/PS1dE9 mice may contribute to impaired cognitive performance. Yet, excessive synaptic potentiation has also been related to impaired memory performance before. One interpretation could be that the currently observed increase in synaptic potentiation in ELS exposed APPswe/PS1dE9 mice hampers information processing, which possibly results in enhanced fear responsiveness. It is unlikely that differences in the total number of synapses underlie these effects, as the hippocampal levels of PSD-95 and synaptophysin were unaffected by either condition or genotype. While PSD-95 and synaptophysin most likely reflect synaptic protein levels given the fact that their proteins are known to be enriched in synaptic fraction, studies on dendritic spines need to be carried out in more detail to examine potential alterations in synaptic connections.
The atypical potentiation observed in ELS-APPswe/PS1dE9 is not the mere result of exposure to ELS, as wild type mice exposed to ELS did not display enhanced levels of potentiation. From PND 2 to 9, when the ELS was applied, both glutamatergic and GABA-ergic transmission are still under development\textsuperscript{56-80}, which ultimately determines network activity. Potentially, exposure to early life adversity during this period can lead to profound and long lasting changes in synaptic potentiation throughout life\textsuperscript{5,81,82}. The early life period appears to be particularly sensitive in this respect, as later life stress induced a reduction in synaptic potentiation in transgenic AD mice\textsuperscript{83-85}. Secondly, we have recently reported that, using the same early life stress paradigm and age of the mice, ELS enhances the level of soluble Aβ\textsubscript{42} in the hippocampus of APPswe/PS1dE9 mice\textsuperscript{23}. Since Aβ has been reported to impair LTP, this suggests that another mechanism besides Aβ-induced effects on synaptic plasticity underlies the enhanced synaptic potentiation in ELS-APPswe/PS1dE9 mice. In this respect, it will be interesting to investigate not only excitation, but also effects on inhibition.

Taken together, exposure of APPswe/PS1dE9 mice to stress early in life exaggerates the expression of fear behaviour and synaptic potentiation. This novel gene x environment interaction may be associated to the decreased cognitive flexibility and enhanced expression of fear behaviour, observed specifically in APPswe/PS1dE9 mice exposed to ELS. As such, it bears considerable relevance for AD aetiology and further emphasises the importance of the early postnatal time window in determining the later vulnerability to develop AD pathology.

5. Funding
This work was supported by the Internationale Stichting Alzheimer Onderzoek (ISAO)/Alzheimer Nederland (grant: #12534 to HJK).

6. Author contributions
SLL and HJK contributed to the conception and design of the study; SLL organised the database; SLL conducted the experiments, SLL performed the statistical analysis; SLL wrote the first draft of the manuscript; SLL, HJK and PJL contributed to manuscript revision, read and approved the submitted version.
Chapter 5

7. References


ELS amplifies fear in AD


Chapter 5


61 Yam KY, Naninck EFG, Abbink MR, la Fleur SE, Schipper L, van den Beukel JC et al. Exposure to chronic early-life stress lastingly alters the adipose tissue, the leptin system and changes the vulnerability to western-style diet later in life in mice. *Psychoneuroendocrinology* 2017; 77: 186–195.


66 Maren S, De Oca B, Fanselow MS. Sex differences in hippocampal long-term potentiation (LTP) and Pavlovian fear conditioning in rats: positive
ELS amplifies fear in AD


69 Turner PR, O’Connor K, Tate WP, Abraham WC. Roles of amyloid precursor protein and its fragments in regulating neural activity, plasticity and memory. Prog Neurobiol 2003; 70: 1–32.


Treatment with the glutamate modulator riluzole prevents early life stress–induced cognitive deficits and impairments in synaptic plasticity in APPswe/PS1dE9 mice

Sylvie L. Lesuis, Paul J. Lucassen, Harm J. Krugers

Submitted
Abstract

Environmental factors like stress affect age-related cognitive deficits and promote Alzheimer’s disease (AD)-related pathology in mice. Excess glutamate has been proposed as a possible mediator underlying these effects in the hippocampus, a vulnerable brain region implicated in learning and memory.

Here, we examined a) whether stress applied during a sensitive developmental period early in life affects later synaptic plasticity, learning and memory and plaque load in the APPswe/PS1dE9 mouse model for Alzheimer’s disease and b) whether these effects could be rescued using long-term treatment with the glutamate modulator riluzole.

Our results demonstrate that ELS impairs synaptic plasticity, increases plaque load, and impairs reversal learning in 12 months old APPswe/PS1dE9 mice. Notably, reversal learning correlated well with hippocampal expression of the transporter EAAT2, which is important for extracellular glutamate uptake. The changes in LTP, plaque load and cognition after ELS were all prevented by riluzole treatment from post-weaning onwards.

These results suggest that normalising glutamate signalling may be a viable therapeutic strategy for treating vulnerable individuals at risk of developing stress-aggravated AD, particularly in relation to adverse early life experiences.

Keywords: riluzole, Alzheimer’s disease, early life stress, EAAT2, LTP, Barnes maze, glutamate
Riluzole rescues ELS-induced deficits in APPswe/PS1dE9 mice

1. Introduction

Alzheimer’s disease (AD) is an age-related neurodegenerative disorder characterised by progressive cognitive decline. In view of current human life expectancy, an increase in the numbers of AD patients is expected. While familial forms of AD are linked to rare genetic mutations, the cause of sporadic AD remains elusive. Various recent lines of evidence suggest that environmental factors play a role in AD risk. One of these environmental factors is exposure to stress, particularly when experienced during the sensitive period of early life. For instance, individuals with a history of childhood adversity have a higher probability to develop later diseases, and a higher prevalence and severity of mild cognitive impairment at an older age. Likewise, also evidence from rodent studies indicates that early life stress (ELS) triggers an age-related cognitive decline. ELS-induced accelerations of cognitive decline are often accompanied by changes in (neuro)biological markers of aging, such as a reduced telomere length, reductions in adult hippocampal neurogenesis, and enhanced neuro-inflammatory profiles. In line with the hypothesis that ELS may affect the course of AD related changes, ELS has been shown to worsen cognitive decline in various genetic mouse models for AD both following pre- and postnatal stress.

Studies in transgenic animal models for AD have implicated glutamatergic N-methyl-D-aspartate (NMDA) receptors in AD and reveal that glutamatergic synapses are particularly affected. Whereas synaptic NMDA activity is critical for long-term potentiation (LTP) and memory formation, excessive extrasynaptic NMDA activation has been associated with the induction of long-term depression and even excitotoxicity. Glutamate uptake by the excitatory amino acid transporter 2 (EAAT2, (GLT-1 or Slc1a2)) is the primary mechanism via which extracellular glutamate regulates physiological glutamatergic neurotransmission in the brain. Interestingly, the expression of glutamate transporters, including EAAT2, is decreased after early life stress, in aging as well as in AD, and has been associated with neurodegeneration.

Since (early life) stress can disturb glutamatergic signalling and function, the effects of ELS and AD may thus converge at glutamatergic transmission. In the present study we therefore tested in APPswe/PS1dE9 mice, a commonly used mouse model for AD, whether ELS affects mechanisms which critical for the uptake of glutamate from synapses (i.e. EAAT2), synaptic plasticity, and whether these effects can be modulated by the glutamate modulator riluzole. This drug alters glutamatergic neurotransmission by decreasing presynaptic glutamate release, and by facilitating glial glutamate uptake via increases in...
EAAT2 expression\textsuperscript{48–52}. Riluzole increases synaptic connectivity, strengthens neural connectivity\textsuperscript{53}, and enhance LTP\textsuperscript{54}. Moreover, riluzole prevented age-related cognitive decline in rodents\textsuperscript{55} and AD related changes in gene expression\textsuperscript{52}. Our present results show not only that ELS affects synaptic plasticity and learning and memory processes, in close correlation with EAAT2 expression in the hippocampus, but also that these deficits in LTP and cognitive performance in 12 month old AD mice were completely prevented by prolonged riluzole treatment.

2. Materials and Methods

2.1. Mice and breeding

All experimental procedures were conducted under Dutch national law and European Union directives on animal experiments, and were approved by the animal welfare committee of the University of Amsterdam. Wild type (WT) and APPswe/PS1dE9 male littermates\textsuperscript{56} of 6 and 12 (± 1) months of age were used. To obtain mice, two 10 weeks old C57BL/6J virgin wild type (WT) females (Harlan Laboratories B.V., Venray, The Netherlands) and one heterozygous male APPswe/PS1dE9 mouse were housed together for one week to allow mating. Pregnant females were housed individually in a standard cage covered with a filter top and monitored daily for the birth of pups\textsuperscript{27, 57–59}. When a litter was born before 10.00 a.m., the previous day was considered the day of birth (postnatal day 0; PND 0), after which the early life stress paradigm was initiated from PND 2-9. At PND 21, mice were weaned and ear biopsies were collected for identification and genotyping. Mice were housed with 2-6 same sex littermates per cage. All experimental mice were left undisturbed (except for cage cleaning once a week) until the start of the experimental procedures at 6 and 12 months of age. Number of mice used: 6 months old: 56 mice; 12 months old: 57 mice.

2.2. Early life stress

At postnatal day (PND) 2, litters were culled to 6 pups per litter, and dams and their litters were randomly assigned to the early life stress (ELS) or control condition until PND 9, after which all mice were treated equally, as described before\textsuperscript{22, 27, 57–59}. Briefly, control dams were provided with a standard amount of sawdust bedding and nesting material (one square piece of cotton nesting material (5 x 5 cm; Tecnilab-BMI, Someren, the Netherlands)). ELS dams were provided with a strongly reduced amount of sawdust bedding and half the
nesting material (1/2 piece of nesting material), and a fine-gauge stainless steel mesh was placed 1 cm above the cage floor.

2.3. Riluzole treatment

Riluzole (Selleckchem, The Netherlands) was added to the drinking water from weaning (PND 28) onwards, and provide fresh every 3-4 days. Bottles were shielded from light to prevent light exposure. A dosage of 4.0 mg/kg per day per animal (adapted from\(^5\)) was dissolved in tap water and stirred until the water was completely transparent.

2.4. Field potential recordings

Field potential recordings were conducted in 6 month old male animals. At PND 180 ± 14 mice were sacrificed between 9 and 10 a.m. through quick decapitation. Immediately after decapitation, the brain was rapidly removed, and collected in ice-cold oxygenated (95% \(\text{O}_2/5\% \text{ CO}_2\)) solution containing (in mM): Cholinechloride (120), glucose (10), NaHCO\(_3\) (25), MgSO\(_4\) (6), KCl (3.5), NaH\(_2\)PO\(_4\) (1.25), CaCl\(_2\) (0.5). Coronal slices (350 \(\mu\)m) were cut using a microtome (Leica VT1000S). For recovery, slices were incubated for 20 minutes in warm (32 °C) oxygenated standard artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl (120), KCl (3.5), MgSO\(_4\) (1.3), NaH\(_2\)PO\(_4\) (1.25), CaCl\(_2\) (2.5), glucose (10), NaHCO\(_3\) (25), after which the sections were maintained at room temperature (22 °C). Sections containing the dorsal hippocampal CA1 area (bregma -2.0 mm to -3.2 mm) were placed in a recording chamber with a constant flow of oxygenated aCSF. Field excitatory synaptic potentials (fEPSPs) were recorded as described previously\(^60–62\). fEPSPs were evoked using a stainless steel bipolar stimulation electrode (60 \(\mu\)m diameter, insulated except for the tip) positioned on the Schaffer collaterals and recorded through a glass electrode (2-5 MΩ impedance, filled with aCSF) positioned in the CA1 stratum radiatum. A stimulus-response curve was generated by gradually increasing the stimulus intensity to define a level that generated the half-maximal response that was used for the remainder of the experiment. Once the input-output curve for each recording was established, baseline synaptic transmission was monitored by stimulating at 0.033 Hz for 10 minutes. When recordings were stable, afferent fibres were stimulated at 10 Hz for 90 seconds\(^62,63\). Next, the degree of potentiation was determined by recording fEPSPs every 30 seconds for 1h. Synaptic transmission was measured by determining the slope of the fEPSP. The average baseline value was normalised to 100% and all values of the experiment were normalised to this baseline average.
2.5. Barnes maze

Mice (12 months) were transferred to a reversed light/dark cycle (lights on 8 p.m., lights off 8 a.m.) one month before behavioural testing commenced and were single-housed in the behaviour room for one more week before testing. Three days prior to testing, mice were handled for five minutes per day. Testing was conducted during the dark, active phase of the mice between 12 and 6 p.m. During testing, recording was done with a video camera connected to a computer with Ethovision software version 14 (Noldus, The Netherlands). Twelve month old APPswe/PS1dE9 and WT male mice were tested for spatial memory in the spatial Barnes maze task. A classic set up was used (110 cm diameter, 12 exit holes) in which mice were trained for one (day 1 and 2) or two (day 3 and 4) sessions a day (adapted from\(^2\)). During training, mice were placed in the centre of the maze twice (inter-trial interval of 30 minutes) and were allowed to navigate to the exit hole leading to the home cage (acquisition learning). Behavioural flexibility was tested by relocating the exit hole to another location on the maze (180 degrees) for two sessions per day on two consecutive days (reversal learning). Cages containing used bedding material were placed at equal distances under the maze to avoid guidance by odour cues, the board was rotated after each trial, and the maze was cleaned with 25 % EtOH to dissipate odour cues. The location of the exit hole was always fixed relative to the distal extra-maze cues in the room. The distance the mice travelled until the exit hole was reached was analysed.

2.6. Tissue preparation

One week after behavioural testing, mice were sacrificed by quick decapitation, between 8.00 and 9.00 p.m. (beginning of the inactive phase). The brains were removed, and the left hemisphere was immersion-fixed in 4% paraformaldehyde in phosphate buffer (0.1 M PB, pH 7.4) for 48 h and then stored in 0.01% sodium-azide in 0.1 M PB at 4 °C until further processing. Paraformaldehyde-fixed tissue was overnight cryoprotected in 30% sucrose/0.1 M PB. Frozen hemispheres were cut in 40 µm thick coronal sections in six parallel series using a sliding microtome and stored in antifreeze solution (30% Ethylene glycol, 20% Glycerol, 50% 0.05 M PBS) at -20 °C until immunohistochemical staining.

2.7. DAB immunohistochemistry

Immunocytochemistry was used to visualise amyloid plaques. All stainings were performed on parallel series from the same brains within an age group. Prior to staining, sections were mounted on glass (Superfrost Plus slides,
Menzal, Braunschweig, Germany) and antigen retrieval was performed by heating the sections in 0.1 M citrate buffer (pH 6) in a microwave (Samsung M6235) to a temperature of ± 95 °C for 15 min. Sections were incubated with 0.3% H₂O₂ for 15 min to block endogenous peroxidase activity, and were next incubated for 30 min in blocking buffer (1% BSA, 0.3% Triton X-100 in 0.05 M TBS). Primary antibody 6E10 (1:1500, BioLegend) was incubated for two hours at room temperature and overnight at 4 °C. Sections were incubated with biotinylated secondary antibody (1:200, sheep anti-mouse, GE Healthcare) for 2h at room temperature followed by a 90 min incubation with avidin-biotin complex (ABC kit, Elite Vectastain Brunschwig Chemie, Amster- dam, 1:800). Subsequent chromogen development was performed with diaminobenzidine (DAB; 20 mg/100 mL 0.05 M Tris, 0.01% H₂O₂).

2.8. Fluorescent immunohistochemistry

For EAAT2 immunohistochemistry, sections were incubated with blocking mix containing goat anti-mouse Fab fragments (1:200) in 0.1 M PBS. Primary mouse anti-EAAT2 (1:250, Cell Signalling) was incubated for 1h at RT followed by incubation at 4 °C overnight. Sections were incubated in the secondary antibodies (1:200 sheep anti-mouse) for 2h, and mounted and coverslipped with Vectashield.

2.9. Imaging and quantification

Quantification was performed on coronal sections of the left hemisphere on 8–10 sections per animal of matched anatomical levels along the rostro-caudal axis. Using a Nikon DS-Ri2 microscope, representative images of 20x magnification were systematically captured. For images from DAB staining, ImageJ software was used to binarise the pictures to 8-bit black-and-white pictures, and a fixed intensity threshold was applied defining the DAB staining. Measurements were performed for the percentage area covered by DAB staining. EAAT2 fluorescence was measured using ImageJ in 50 µm intervals from the cellular layer in the CA1 of the hippocampus. All images were quantified by an experimenter blinded to the experimental procedures and animals.

2.10. Statistical analysis

Data were analysed using SPSS 22.0 (IBM software). Data are expressed as mean ± standard error of the mean (S.E.M.). Data were considered statistically...
significant when p<0.05. Outliers were determined using a Grubb’s test, which identifies a maximum of one value to be excluded from the analysis. Repeated measures ANOVA was performed to assess Barnes maze learning curves over the different trials, and to assess synaptic plasticity. Greenhouse-Geisser correction was applied when the assumption of sphericity was violated. To enhance the readability of the graphs, the repeated measures data for the LTP and Barnes maze have been split up in separate graphs (Figure 1A,B and Figure 2A-D), although statistical analysis was performed on all data combined. To compare between groups accounting for the main and interaction effects of genotype (WT vs. APPswe/PS1dE9), condition (Ctrl vs. ELS), and treatment (water vs. riluzole), a 2x2x2 ANOVA was performed, with planned contrasts as post hoc tests to correct for the relevant comparisons conducted. Pearson’s correlation test was conducted to determine correlations.

3. Results

APPswe/PS1dE9 and WT littermates were housed with limited nesting and bedding materials from PND 2 to 9 in order to induce ELS. In line with previous reports (e.g. 27) this procedure reduced body weight gain (Ctrl: 3.6 ± 0.11; ELS: 2.5 ± 0.08; t(55)=8.06, p=0.001), indicative of effective stress exposure. Since effects of ELS are particularly sex-specific22,67, all experiments were further conducted with male mice. From PND 28 onwards, half of the mice received riluzole supplementation to their drinking solution. No difference in water consumption was observed between any of the groups (treatment effect: F(1,49)=0.54, p=0.47).

3.1. Hippocampal synaptic plasticity

To investigate whether ELS and/or an APPswe/PS1dE9 background affected synaptic plasticity, we measured hippocampal long-term potentiation (LTP) at 6 months of age, and tested whether effects could be rescued by riluzole treatment. We found no differences of condition, genotype or treatment on maximum slope or the half-maximum stimulation intensity, as determined from the input-output curve (Table 1). There was a main effect of treatment (F(1,97)=30.84, p<0.001) on the slope factor.

In water treated mice, both condition and genotype reduced LTP (condition: F(1,40)=4.47, p=0.04; genotype: F(1,40)=7.86, p=0.008) (Figure 1A). When combining all data, riluzole treatment increased LTP in all groups (main treatment effect: F(1,63)=61.62, p<0.001) (Figure 1A,B). However, these effects were most pronounced in APPswe/PS1dE9 mice (genotype*treatment:
Riluzole rescues ELS-induced deficits in APPswe/PS1dE9 mice

Figure 1. Riluzole treatment rescues ELS-induced impairments in hippocampal LTP in APPswe/PS1dE9 mice after 10 Hz stimulation for 90 seconds. A. LTP in water-treated mice. Both genotype and condition decrease the slope of the fEPSP over the entire 60 minutes after stimulation, resulting in LTD in ELS-APPswe/PS1dE9 mice. B. Riluzole significantly increases the fEPSP, most strongly in APPswe/PS1dE9 mice. C. During the last 10 minutes of recording, riluzole increased the fEPSP significantly in ELS-WT, Ctrl-APPswe/PS1dE9 and ELS-APPswe/PS1dE9 mice. Ctrl-WT-water: N=18; ELS-WT-water: N=13; Ctrl-APPswe/PS1dE9-water: N=10; ELS-APPswe/PS1dE9-water: N=5; Ctrl-WT-riluzole: N=4; ELS-WT-riluzole: N=6; Ctrl-APPswe/PS1dE9-riluzole: N=4; ELS-APPswe/PS1dE9-riluzole: N=10. *: p<0.05.
Chapter 6

Table 1. Basal field potential characteristics for hippocampal CA1 area

<table>
<thead>
<tr>
<th></th>
<th>R\textsubscript{max} (mV/ms)</th>
<th>I\textsubscript{h} (µA)</th>
<th>S</th>
<th>N (mice (slices))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctrl – WT</td>
<td>-0.24 ± 0.03</td>
<td>2.27 ± 0.05</td>
<td>-0.22 ± 0.05</td>
<td>10 (27)</td>
</tr>
<tr>
<td>ELS – WT</td>
<td>-0.27 ± 0.03</td>
<td>2.29 ± 0.04</td>
<td>-0.23 ± 0.04</td>
<td>8 (21)</td>
</tr>
<tr>
<td>Ctrl – APPswe/PS1dE9</td>
<td>-0.26 ± 0.04</td>
<td>2.36 ± 0.05</td>
<td>-0.24 ± 0.05</td>
<td>10 (17)</td>
</tr>
<tr>
<td>ELS – APPswe/PS1dE9</td>
<td>-0.16 ± 0.04</td>
<td>2.25 ± 0.10</td>
<td>-0.15 ± 0.04</td>
<td>6 (14)</td>
</tr>
<tr>
<td>Riluzole</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctrl – WT</td>
<td>-0.36 ± 0.03</td>
<td>2.10 ± 0.05</td>
<td>-0.54 ± 0.15</td>
<td>6 (8)</td>
</tr>
<tr>
<td>ELS – WT</td>
<td>-0.45 ± 0.04</td>
<td>1.87 ± 0.03</td>
<td>-0.54 ± 0.07</td>
<td>5 (6)</td>
</tr>
<tr>
<td>Ctrl – APPswe/PS1dE9</td>
<td>-0.33 ± 0.05</td>
<td>2.14 ± 0.07</td>
<td>-0.58 ± 0.12</td>
<td>5 (7)</td>
</tr>
<tr>
<td>ELS – APPswe/PS1dE9</td>
<td>-0.30 ± 0.05</td>
<td>2.06 ± 0.11</td>
<td>-0.32 ± 0.05</td>
<td>6 (9)</td>
</tr>
</tbody>
</table>

Main/interaction effects  ns  ns  T*  

Data expressed as mean ± S.E.M (n). Maximal slope of the fEPSP (R\textsubscript{max}), half-maximum stimulus intensity (I\textsubscript{h}), and the slope of the input-output curve (slope factor S) in the CA1 area. C: condition effect, G: genotype effect, T: treatment effect.

F(1,63)=22.62, p<0.001; post hoc difference between: Ctrl-APPswe/PS1dE9 water vs. riluzole: p<0.001; ELS-APPswe/PS1dE9 water vs. riluzole p<0.001), while there was also an interaction between condition and treatment (F(1,63)=4.40, p=0.04) (Figure 1A,B). The average of the signal during the last 10 minutes was analysed separately (Figure 1C). Here, too, riluzole treatment significantly increased synaptic potentiation (F(1,63)=62.41, p<0.001), most strongly in APPswe/PS1dE9 mice (F(1,63)=15.34, p<0.001). Post hoc testing revealed a significant effect of riluzole treatment in ELS-WT mice (p=0.01), Ctrl-APPswe/PS1dE9 mice (p<0.001), and ELS-APPswe/PS1dE9 mice (p<0.001).

3.2. Barnes maze

We next investigated whether ELS-induced changes in synaptic plasticity also affect spatial memory performance in WT and APPswe/PSdE9 mice, and whether such effects could be prevented by riluzole in 12 month old mice. For acquisition learning, there was a mild but significant effect of treatment, in which riluzole resulted in a shorter distance to locate the exit hole (F(1,55)=6.23, p=0.02) (Figure 2A,B). However, neither genotype nor condition affected performance on acquisition learning (genotype effect: F(1,55)=0.24, p=0.63; condition effect: F(1,55)=1.18, p=0.28). No effects were observed when examining,
Riluzole rescues ELS-induced deficits in APPswe/PS1dE9 mice

Figure 2: Riluzole-treated aged APPswe/PS1dE9 mice were protected against ELS-induced deficits in Barnes maze performance. A,B. The distance travelled before the mice located the exit hole was comparable between all groups (water-treated mice: full line; riluzole-treated mice: dashed line). C. The distance travelled during the last trial of acquisition learning was also comparable between all groups. D. When the exit hole was relocated to a novel location, in WT mice, long-lasting riluzole treatment (dashed line) resulted in a slight improvement in the distance travelled to the exit hole, compared to water-treated mice (full line). E. Water-treated APPswe/PS1dE9 mice took longer to locate the exit hole compared to WT mice, especially when exposed to ELS. The distance travelled was improved in all groups after riluzole treatment. F. The distance travelled to the exit hole during reversal learning was reduced by riluzole treatment in all groups, except for Ctrl-WT mice. Ctrl-WT-water: N=7; ELS-WT-water: N=9; Ctrl-APPswe/PS1dE9-water: N=7; ELS-APPswe/PS1dE9-water: N=9; Ctrl-WT-riluzole: N=7; ELS-WT-riluzole: N=7; Ctrl-APPswe/PS1dE9-riluzole: N=8; ELS-APPswe/PS1dE9-riluzole: N=9. *: p<0.05.
During reversal learning, i.e. when the exit hole was relocated to a new location, riluzole again improved performance, resulting in a shorter distance travelled to the exit hole (F(1,55)=23.76, p=0.001) (Figure 2D,E). In addition, APPswe/PS1dE9 mice took a longer distance to find the exit hole (F(1,55)=10.36, p=0.002). Analysis of the last trial, as an indication of how well mice had learned to locate the exit hole, revealed an effect of treatment, genotype and condition, as well as a condition x genotype interaction effect (treatment:
Riluzole rescues ELS-induced deficits in APPswe/PS1dE9 mice

F(1,55)=34.46, p=0.001; genotype: F(1,55)=8.26, p=0.006; condition: F(1,55)=5.012, p=0.029; genotype x treatment: F(1,55)=7.65, p=0.008) (Figure 2F). Post hoc testing revealed that in APPswe/PS1dE9 mice, ELS resulted in a longer distance to the exit hole than Ctrl animals. Riluzole treatment also resulted in a shorter travelling distance to the exit hole in both groups.

3.3. EAAT2 expression

Immunocytochemical labelling revealed that EAAT2 was reduced in the distal portion of the CA1 area with age (F(1,34)=81.38, p=0.001) (Figure 3A). We further found that EAAT2 expression in aged riluzole treated animals was enhanced when compared to untreated young and aged mice (treatment effect: F(1,34)=250.22, p=0.001). Moreover, in water-treated animals, genotype reduced EAAT2 expression at all ages (F(1,34)=5.6, p=0.025). We found an interaction effect between condition x treatment (F(1,34)=14.42, p=0.001) and genotype x treatment (F(1,34)=8.76, p=0.006), reflecting the enhanced EAAT2 expression following riluzole treatment in aged ELS and APPswe/PS1dE9 mice.

Importantly, EAAT2 expression correlated significantly with cognitive performance of the last reversal learning trial of the Barnes maze in aged mice (r=-0.75, n=32, p=0.001) (Figure 3B), which suggests a potential mechanism by which riluzole may rescue cognitive performance.

3.4. Hippocampal plaque load

Finally, we investigated plaque load, an important pathological hallmark of AD, and we found a significant interaction effect between condition and treatment in the hippocampal CA1 area (F(1,37)=7.52, p=0.009). ELS-APPswe/PS1dE9 mice treated with water displayed an increased plaque load, which was absent in APPswe/PS1dE9 animals treated with riluzole treatment (p<0.05) (Figure 3C). Plaque load did not correlate with cognitive decline (r=0.09, n=32, p=0.59) (Figure 3D).

4. Discussion

Previous studies have reported that early life stress can alter reversal learning, synaptic plasticity and amyloid levels in 12 months old APPswe/PS1dE9 mice. In the current study, we investigated whether riluzole, a modulator of glutamate levels, can rescue these effects. We found that ELS-induced impairments in synaptic plasticity, reversal learning and plaque load in APPswe/PS1dE9 mice...
can be rescued by prolonged riluzole treatment from post-weaning onward, likely by regulating EAAT2 expression.

Our current model for ELS has previously been shown to induce (age-related) impairments in spatial learning, memory processes (reviewed by\textsuperscript{68,69}) and synaptic plasticity\textsuperscript{70}. In addition, it has been shown that ELS aggravates AD-related neuropathology, including increased soluble A\textsubscript{β} levels, a higher plaque load, and impairs cognitive performance\textsuperscript{24,27,58}. In agreement, we found that ELS impaired synaptic plasticity in WT mice. In addition, LTP was impaired in APPswe/PS1dE9 mice which is in line with earlier studies showing impairments in synaptic plasticity in (transgenic) mouse models of AD\textsuperscript{28,71}. Moreover, ELS-exposure in APPswe/PS1dE9 mice further decreased synaptic plasticity, and even resulted in LTD. We then sought to investigate whether alterations in glutamatergic signalling might attenuate these effects by lifelong treatment with the glutamate modulator riluzole, given right after weaning. While riluzole did not affect LTP in Ctrl-reared wild type mice, it increased LTP in all other experimental groups, suggesting that the impairments resulting from both ELS and an APPswe/PS1dE9 background are indeed mediated by disturbances in glutamatergic signalling. Interestingly, riluzole treatment was most effective in APPswe/PS1dE9 mice. This effect was most pronounced in the first 10 minutes after stimulation, which could point to a different recovery of the presynaptic glutamate release between WT and APPswe/PS1dE9 mice after the 90 seconds of high frequency stimulation, and may have resulted in a depletion of synaptic vesicles. These effects of riluzole may be related to one of the many pathways associated to synaptic plasticity that are differentially regulated by AD\textsuperscript{52} and the exact nature of this interaction requires further investigation. Clearly, riluzole was able to prevent ELS and APPswe/PS1dE9-induced alterations in synaptic plasticity in 12 months old mice.

We have previously reported that ELS resulted in aberrantly increased LTP in older APPswe/PS1dE9 mice, which was paralleled by less specific memory formation on a fear conditioning task (Lesuis et al., submitted). Although these animals were recorded at different ages (6 vs. 12 months old), the opposing phenotypes are remarkable. Importantly, both excessively enhanced and decreased levels of LTP have been implicated in cognitive deficits\textsuperscript{72-74}, but future studies are required to investigate the age-dependent effects and the exact nature of ELS-induced effects on synaptic plasticity in APPswe/PS1dE9 mice.

LTP is an important cellular model for learning and memory\textsuperscript{75,76}, and functional brain abnormalities have been observed in humans decades before the development of other symptoms\textsuperscript{77,78}. We therefore tested whether ELS
Riluzole rescues ELS-induced deficits in APPswe/PS1dE9 mice

affected learning and memory in APPswe/PS1dE9 mice. Previously, we have reported that 12 month old ELS-APPswe/PS1dE9 mice are impaired in reversal learning in the Barnes maze. In line with these findings, we found at present that ELS exposure in APPswe/PS1dE9 mice did not alter acquisition learning, but impaired reversal learning. While riluzole slightly enhanced acquisition learning, it particularly prevented the deficits on reversal learning. Interestingly, riluzole treatment improved performance in both transgenic groups, as well as in the ELS-WT mice. Together, these observations indicate that in cognitively impaired animals, be it after ELS or due to an APPswe/PS1dE9 background, riluzole improves cognitive performance.

A possible mechanism via which the effect of riluzole may rescue both these impairments, could be through regulating EAAT2 expression, which is relevant for maintaining proper synaptic glutamate levels. EAAT2 regulates reuptake of glutamate outside the synaptic cleft, preventing excess glutamate from binding to extra-synaptic NMDA receptors, reducing synaptic efficiency and inducing LTD and excitotoxicity, and has been implicated in aging and various neurodegenerative diseases, including AD. Furthermore, EAAT2 haploinsufficiency aggravates cognitive impairments in an AD mouse model, while EAAT2 overexpression improves cognitive performance.

In line with this, we observed that EAAT2 immunoreactivity was significantly reduced with aging, while both ELS and an APPswe/PS1dE9 background further lowered EAAT2, which was strongest in APPswe/PS1dE9 mice exposed to ELS. Riluzole treatment strongly increased EAAT2 levels in the CA1 area of the hippocampus in all groups, irrespective of their genetic background or early life experience. Interestingly, EAAT2 expression correlated significantly with Barnes maze performance, indicating that EAAT2 is indeed relevant for memory formation. Increased immunoreactivity for EAAT2 was observed in the same region as where we observed decreases in synaptic plasticity in ELS-APPswe/PS1dE9 mice. In addition, others have previously observed increased spine clustering in the same area in riluzole-treated rats, which also correlated with cognitive performance, suggesting a potential mechanism by which riluzole can increase cognitive performance. However, in addition to regulating glutamate levels, the drug has additional pharmacological effects such as inhibiting Na+ channels. A possible contribution of these mechanisms to the present results cannot be ruled out.

Synaptic dysfunction is an important mechanism implicated in AD-related cognitive deficits and presenting as one of the first symptoms of AD. Amyloid-β (Aβ), one of the hallmarks of AD neuropathology, is closely related to glutamatergic dysregulation, since Aβ oligomers disrupt glutamate
uptake, reduce synaptic transmission, facilitate LTD and inhibit LTP\textsuperscript{83,84}. This is thought to occur through an excessive activation of extra-synaptic NMDA receptors\textsuperscript{83,85}, and a decrease in the expression of synaptic NMDA receptors\textsuperscript{86}. In parallel, neuronal activity, regulated by glutamatergic signalling increases the release of Aβ\textsuperscript{35}, possibly resulting in vicious cycle of neurotoxicity. In the current study, we find that plaque load was increased following ELS, an effect that was rescued by riluzole treatment. Likewise, we have previously shown that in APPswe/PS1dE9 mice soluble Aβ-40 and Aβ-42 levels are increased following ELS\textsuperscript{27}, although plaque load was not affected in this study. EAAT2 overexpression has previously been shown to decrease pathological markers in an AD mouse model\textsuperscript{79}, again supporting the hypothesis that improved regulation of glutamatergic signalling via enhanced EAAT2 uptake could potentially mitigate Aβ toxicity and worsen cognitive performance. This may suggest that normalising glutamate levels prevents Aβ pathology.

Taken together, the present results indicate that riluzole rescues deficits in reversal learning in 12 month old, ELS-exposed APPswe/PS1dE9 mice. The effects of riluzole are possibly mediated by alterations in synaptic plasticity that emerge already from a young age onwards (at least 6 months) since LTP deficits were completely rescued by riluzole supplementation. Future studies are required to investigate in more detail the critical time windows in which riluzole can prevent the ELS-induced impairments. Ultimately, reducing glutamatergic signalling could represent future therapeutic strategy for treating vulnerable individuals at risk of developing stress-aggravated AD, particularly in relation to adverse early life experiences.

5. Acknowledgements

We want to thank Chris Wijs for excellent caretaking of the mice and assistance with the riluzole treatment. This study was supported by Internationale Stichting Alzheimer Onderzoek (ISAO)/Alzheimer Nederland (grant: #12534 to HJK).
6. References


24. Hoeijmakers L, Ruigrok SR, Amelianichk A, Ivan...


35 Hardingham GE. Pro-survival signalling from the NMDA receptor: *Biochem Soc Trans* 2006; **34**:936–938.


37 Rusakov DA, Kullmann DM. Extrasynaptic glutamate diffusion in the hippocampus: ultrastructural constraints, uptake, and receptor activation. *J Neurosci* 1998; **18**:3158–70.


40 Furuta A, Rothstein JD, Martin LJ. Glutamate transporter protein subtypes are expressed differentially during rat CNS development. *J Neurosci* 1997; **17**:8363–75.


43 Potier B, Billard J-M, Rivière S, Sinet P-M, DENIS I, Champeil-Potokar G et al. Reduction in glutamate uptake is associated with extrasynaptic NMDA and metabotropic glutamate receptor activation at the hippocampal CA1 synapse of aged rats. *Aging Cell* 2010; **9**:722–735.


46 O’Connor RM, Pusceddu MM, Dinan TG, Cryan JF. Impact of early-life stress, on group III mGlu receptor levels in the rat hippocampus: Effects of ketamine, electroconvulsive shock therapy
Riluzole rescues ELS-induced deficits in APPswe/PS1dE9 mice


68. Walker C-D, Bath KC, Joels M, Korosi A, Larauche M, Lucassen PJ et al. Chronic early life stress induced by limited bedding and nesting (LBN) material in rodents: critical considerations of methodology, outcomes and translational...
69 Yam KY, Naninck EFG, Abbink MR, la Fleur SE, Schipper L, van den Beukel JC et al. Exposure
to chronic early-life stress lastingly alters the
adipose tissue, the leptin system and changes
the vulnerability to western-style diet later in
life in mice. Psychoneuroendocrinology 2017;
77 186–195.
70 Brunson KL, Kramár E, Lin B, Chen Y, Colgin
LL, Yanagihara TK et al. Mechanisms of late-
onset cognitive decline after early-life stress. J
71 Jacobsen JS, Wu C-C, Redwine JM, Comery TA,
Arias R, Bowlby M et al. Early-onset behavioral
and synaptic deficits in a mouse model of
Alzheimer’s disease. Proc Natl Acad Sci U S A
2006; 103: 5161–6.
72 Migaud M, Charlesworth P, Dempster M,
Webster LC, Watabe AM, Makinson M et al.
Enhanced long-term potentiation and impaired
learning in mice with mutant postsynaptic
www.nature.com/nature/journal/v396/n6710/
73 Willshaw D, Dayan P. Optimal plasticity from
matrix memories: What goes up must come
74 Hancock PJB, Smith LS, Phillips WA. A
biologically supported error-correcting learning
75 Malinow R, Malenka RC. AMPA receptor
trafficking and synaptic plasticity. Annu Rev
76 Kessels HW, Malinow R. Synaptic AMPA
receptor plasticity and behavior. Neuron 2009;
77 Sperling RA, LaViolette PS, O’Keefe K, O’Brien
J, Rentz DM, Pihlajamaki M et al. Amyloid
deposition is associated with impaired default
network function in older persons without
78 Reiman EM, Chen K, Alexander GE, Caselli RJ,
Bandy D, Osborne D et al. Functional brain
anomalies in young adults at genetic risk
for late-onset Alzheimer’s dementia. Proc Natl
79 Takahashi K, Kong Q, Lin Y, Stouffer N, Schulte
DA, Lai L et al. Restored glial glutamate
transporter EAAT2 function as a potential
therapeutic approach for Alzheimer’s disease. J
80 Bellingham MC. A review of the neural
mechanisms of action and clinical efficiency of
riluzole in treating amyotrophic lateral sclerosis:
What have we learned in the last decade? CNS
81 Selkoe DJ. Alzheimer’s disease is a synaptic
82 DeKosky ST, Scheff SW. Synapse loss in
frontal cortex biopsies in Alzheimer’s disease:
correlation with cognitive severity. Ann Neurol
1990; 27: 457–64.
83 Li S, Hong S, Shepardson NE, Walsh DM,
Shankar GM, Selkoe D. Soluble oligomers
of Amyloid β protein facilitate hippocampal
long-term depression by disrupting neuronal
84 Cheng L, Yin W-J, Zhang J-F, Qi J-S. Amyloid
beta-protein fragments 25-35 and 31-
35 potentiate long-term depression in
hippocampal CA1 region of rats in vivo. Synapse
85 Li S, Jin M, Koeglsperger T, Shepardson NE,
Shankar GM, Selkoe DJ. Soluble A[beta]
Oligomers Inhibit Long-Term Potentiation
through a Mechanism Involving Excessive
Activation of Extrasynaptic NR2B-Containing
86 Snyder EM, Nong Y, Almeida CG, Paul S, Moran
T, Choi EY et al. Regulation of NMDA receptor
Riluzole rescues ELS-induced deficits in APPswe/PS1dE9 mice
Early life stress impairs fear conditioning memory and synaptic plasticity; a potential role for GluN2B

Sylvie L. Lesuis, Paul J. Lucassen, Harm J. Krugers

Submitted
Abstract

Programming of the brain by early stress has been associated with alterations in structure and function of the hippocampus. Yet, the underlying molecular mechanisms remain largely elusive. In this study, we examined the effects of early life stress (ELS) – by housing mouse dams with limited nesting and bedding material from postnatal days 2-9 and examined in 6 month old offspring; 1) auditory fear conditioning, 2) expression of the hippocampal N-methyl-D-aspartate receptor (NMDA-R) subunits 2A and 2B (GluN2A, GluN2B), and expression of PSD-95 and synaptophysin, and 3) short- and long-term (LTP) synaptic plasticity. Given its critical role in NMDA receptor function and synaptic plasticity, we further examined the role of GluN2B in effects of ELS on synaptic plasticity and fear memory formation. We demonstrate that ELS impaired fear memory in 6 month old mice and decreased hippocampal LTP as well as the paired-pulse ratio (PPR). ELS also reduced hippocampal GluN2B expression. Interestingly, pharmacological blockade of GluN2B with the selective antagonist Ro25 6981 was less effective to reduce synaptic plasticity in ELS mice, and was also ineffective to impair memory retrieval in ELS mice. These studies suggest that ELS reduces hippocampal synaptic plasticity and fear memory formation and hampers GluN2B receptor function. As such, GluN2B may provide an important target for future strategies to prevent lasting ELS effects on cognition.

Keywords: memory, LTP, early life stress, NMDA, fear conditioning, HPA axis, paired-pulse ratio
1. Introduction

The “Developmental Origins of Health and Disease” (DOHaD) hypothesis postulates that perinatal environmental factors play an important role in determining the risk to develop pathology later in life\(^1\). Indeed, adversity early in life, such as experiencing emotional neglect, physical abuse or traumatic events, increases the risk for developing psychopathologies such as anxiety disorders and depression\(^2\)\(^–\)\(^5\) and cognitive dysfunction in later life\(^6\)\(^–\)\(^8\). However, the exact mechanisms that underlie the consequences of early life adversity for later brain function remain poorly understood.

Increasing evidence suggests that changes in hippocampal structure, neuronal networks and their functions may contribute to the early life stress-induced cognitive deficits. The hippocampus may be particularly sensitive since this area continues to develop into the postnatal period\(^9\),\(^10\). Indeed, human studies have shown that early life adversity is associated with a reduced hippocampal volume in adults\(^11\)\(^–\)\(^17\). In agreement, animal studies have confirmed that early life stress induces long-lasting structural and functional alterations in hippocampal neurons, such as a decreased hippocampal dendritic complexity and reduced synaptic density\(^18\)\(^–\)\(^24\). In addition, rat pups that received low amounts of maternal care early in life showed decreased dendritic complexity as well as lower expression of synaptic markers in the hippocampal CA1 and dentate gyrus at adulthood\(^20\),\(^25\),\(^26\). Also the number of hippocampal spines was found to be reduced in pups that received low compared to high amounts of maternal care\(^20\),\(^25\), and in mice that were exposed to chronic early life stress\(^27\).

Synaptic plasticity, which is an important substrate for memory formation\(^28\)\(^–\)\(^33\) is altered following reduced levels in maternal care and exposure to early life adversity\(^20\),\(^22\),\(^34\). Understanding how early life stress impacts synaptic plasticity could help to explain effects of early life stress on later-life cognitive impairments. As the composition of N-methyl-D-aspartate receptors (NMDARs) is crucial for the induction of LTP, and their properties may be altered by early life experiences\(^25\),\(^35\),\(^36\), NMDAR modifications could potentially be a link between effects of early life stress and cognitive impairment later in life. In this study we therefore examined whether early life stress (ELS) – induced by housing mice with limited nesting and bedding material from postnatal days 2-9\(^37\)\(^–\)\(^40\) – affects memory and long- as well as hippocampal short-term plasticity later in life. In addition, we tested whether GluN2B is involved in effects of ELS, given its role in synaptic plasticity, and learning and memory processes.
Chapter 7

2. Materials and Methods

2.1. Animals

All mice were kept under standard housing conditions (a 12/12h light schedule (lights on at 8 a.m.), temperature 20-22 °C, 40-60% humidity, standard chow and water were available ad libitum, and a radio provided background noise\(^37,39,41\)). Experimental procedures were conducted under the national Dutch law and European Union directives on animal experiments, and were approved by the animal welfare committee of the University of Amsterdam. To obtain experimental mice, two 10-week-old C57Bl/6J virgin females and one male mouse (Harlan Laboratories B.V., Venray, The Netherlands) were housed together for one week to allow mating. After another week of paired-housing, pregnant females were housed individually in a standard cage covered with a filter top and monitored daily for the birth of pups. When a litter was born before 10.00 a.m., the previous day was considered as the day of birth (postnatal day 0; PND 0), after which the early life paradigm was initiated at PND 2. At PND 21, mice were weaned and were housed with 2-5 same sex littermates per cage. All experimental mice were left undisturbed (except for cage cleaning once a week) until start of experimental procedures at 6 months.

2.2. Early life stress

At PND 2, litters were culled to 6 pups per litter, and dams and their litters were weighed and randomly assigned to the early life stress (ELS) or control condition, as described before\(^37-40\). Control dams were provided with a standard amount of sawdust bedding and nesting material (one square piece of cotton nesting material (5 x 5 cm; Tecnilab-BMI, Someren, the Netherlands)). The ELS dams were provided with a strongly reduced amount of sawdust bedding and half the nesting material (1/2 square piece of cotton nesting material (2.5 x 5 cm)), and a fine-gauge stainless steel mesh was placed 1 cm above the cage floor. Both control and ELS cages were left undisturbed until PND 9, after which all litters were weighed and placed in standard cages, with standard amounts of bedding and nesting material until weaning at PND 21.

2.3. Behavioural testing

In the present study we used male mice. At 5 months of age, i.e. one month prior to behavioural testing, mice were moved to a reversed day-night rhythm (lights on at 8.00 p.m.). One week prior to testing, mice were moved into the
testing room, where they were single housed and handled daily. Tests were conducted by an experimenter blind to the condition of the animals, in the dark, active phase of the animals between 1 and 5 p.m. in a testing room lit by two red spots (EGB, 25 Watt). During testing, mice were recorded by a camera connected to a computer with Ethovision software (version 13, Noldus, The Netherlands) and automatically scored by the software.

### 2.3.1. Fear conditioning
Mice were tested in a contextual and auditory fear-conditioning paradigm, a classic paradigm to assess fear memory. On day 1, mice were placed in a chamber which had a stainless steel grid floor connected to a shock generator. The chamber was cleaned with 1% acidic acid to create a recognisable odour trace and remove previous odours. Mice were allowed to explore the context for three minutes, after which a 30 seconds tone (76 dB, 2.8 kHz) was used. During the last 2 seconds of the tone, the mice received a single mild foot shock (0.4 mA). After this shock, the mice remained in the chamber for another 30 seconds. Twenty-four hours later, mice were reintroduced into the shock context for 3 minutes. One hour later, mice were placed in a novel context (round Plexiglas chamber cleaned with 20% EtOH), and after 3 minutes they were exposed to 30 second tone for 6 times, with 60 second intervals. Freezing behaviour of the animals was scored during every trial by an observer who was unaware of the experimental condition. Freezing was being defined as “no body movements except those related to breathing” and was expressed as % of total observation time.

### 2.3.2. Forced swim test
Seven days after fear conditioning, a forced swim test (FST) was conducted to elicit a stress response. Between 8 and 9 p.m. (start of the light phase) mice were placed in a cylinder with 1500 ml water from which they could not escape. After 6 minutes, mice were removed from the water and dried before being placed back in their home cage. Although the FST was merely used as a stressor, floating time and latency to float as percentage of total time in the cylinder were scored. However, no behavioural differences were observed, and these data are therefore not further presented/discussed.

### 2.4. Stress response
Blood samples were obtained by tail cut at 30 (“response CORT”) and 90 minutes (“recovery CORT”) after exposure to the FST. Blood was collected in EDTA coated tubes and analysed as described below (“Plasma corticosterone measurements”).

---

7
2.5. Sacrifice and organ collection

Mice were sacrificed one week after the last behavioural test by quick decapitation at the beginning of the light phase (8 p.m.). Blood sample were collected for basal CORT measurements, brains were dissected and the hippocampus was isolated from the right hemispheres and kept at -80 °C. Upon sacrifice, adrenal glands were removed and cleaned by removing fat tissue and weighted. Weights were presented as a percentage of body weight.

2.6. Plasma corticosterone measurements

Blood samples were collected in ice cold, EDTA-coated tubes (Sarstedt, Etten-leur, The Netherlands), placed on ice and centrifuged at 14,000 rpm for 15 minutes after which plasma was stored at -20 °C. Plasma CORT levels were measured using a commercially available radioimmunoassay kit (MP Biomedicals, Eindhoven, The Netherlands).

2.7. Western blot

To compare hippocampal protein levels between the groups, 6 month old mice were decapitated and hippocampi were dissected in saline on ice. Tissue was stored at −80 °C. For protein extraction, hippocampi were homogenised in RIPA buffer (150 mM NaCl, 1% Triton X100, 0.5% Sodium deoxycholate, 0.1% SDS at pH 7.6) using a small syringe. The samples were incubated on ice for 30 minutes and then centrifuged for 20 minutes at 16,000 rpm at 4 °C. Protein lysate was stored at −20 °C. For each sample the protein concentration was measured using a BCA Protein Assay (23225, Pierce (Thermo Fischer) The Netherlands). Samples containing between 10-30 μg protein in sample buffer were denaturised at 95 °C for 5 minutes. A polyacrylamide-SDS gel (Biorad, The Netherlands) was used for protein separation by electrophoresis. The proteins were transferred to a PVDF membrane (162-0177, Biorad, The Netherlands) in a Tris-glycine buffer. The membranes were cut for incubation with different antibodies. The membrane strips were then blocked in TBST containing 5% BSA for 1 hour. After blocking, blots were washed with TBST and incubated with primary antibodies at 4 °C overnight. Primary antibodies included MR (1:500, mouse; 1-18-1D5 Gomez-Sanchez), GR (1:500, rabbit, H-300, Santa Cruz), GluN2A (1:1000, mouse, MAB5216, EMD Millipore), GluN2B (1:1000, mouse, MAB5220, EMD Millipore), PSD-95 (1:3000, rabbit, D27E11, Cell Signalling), synaptophysin (1:3000, mouse, SY38, Abcam), α-tubulin (1:1000, mouse, 10D8, Santa Cruz) and GAPDH (1:3000, rabbit; 21185, Cell Signaling). After washing with TBS, blots were incubated with secondary antibodies for 2 hours at room
temperature (HRP conjugate, Biorad, The Netherlands). Blots were washed again and bands were visualised by chemiluminescence using an ECL Prime kit (RPN2232, Amersham, (GE Healthcare) The Netherlands). A Li-COR machine was used to measure the chemiluminescence. Optical density was determined in ImageJ. Measurements of the proteins of interest were corrected for total protein (GAPDH or α-tubulin band). Protein levels were calculated as the mean of three independent replicates.

2.8. Synaptic plasticity

2.8.1. Long-Term Potentiation (LTP)

Mice were sacrificed between 9 and 10 a.m. through quick decapitation for slice preparation. Immediately after decapitation, the brain was rapidly removed, and collected in ice-cold oxygenated (95% O₂/5% CO₂) solution containing (in mM): Cholinechloride (120), glucose (10), NaHCO₃ (25), MgSO₄ (6), KCl (3.5), NaH₂PO₄ (1.25), CaCl₂ (0.5). 350 µm thick coronal slices were cut using a microtome (Leica VT1000S). For recovery, slices were then incubated for 20 minutes in warm (32 °C) oxygenated standard artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl (120), KCl (3.5), MgSO₄ (1.3), NaH₂PO₄ (1.25), CaCl₂ (2.5), glucose (10), NaHCO₃ (25), after which the sections were maintained at room temperature. Sections containing the hippocampal CA1 area (bregma -2.0 mm to -3.2 mm) were placed in a recording chamber with a constant flow of oxygenated aCSF. Field excitatory postsynaptic potentials (fEPSPs) were recorded as described previously in the absence and presence of the GluN2B-NMDAR subunit antagonist Ro25 6981 (3 µM, Sigma) to assess a possible selective role of this particular subunit in these changes.

Field excitatory potentials (fEPSPs) were evoked using a stainless steel bipolar stimulation electrode (60 µm diameter, insulated except for the tip) positioned on the Schaffer collaterals and recorded through a glass electrode (2-5 MΩ impedance, filled with aCSF) positioned in the CA1 stratum radiatum. A stimulus-response curve was generated by gradually increasing the stimulus intensity to define a level that generated the half-maximal response that was used for the remainder of the experiment. Once the input-output curve for each recording was established, baseline synaptic transmission was monitored (0.03 Hz) during 10 minutes. When recordings were stable, a high frequency stimulation (10 Hz, 90 seconds) was applied. After the tetanus, the degree of potentiation was determined by recording the fEPSP every 30 seconds during 1 hour (0.03 Hz). The fEPSP magnitude was assessed by analysing the slope of the signal. The average baseline value was normalised to 100% and all values of the experiment were normalised to this baseline average.
2.8.2. Paired-pulse ratio
Paired-pulse facilitation was measured by determining the ratio of the evoked response to two subsequent stimuli at half maximal stimulus intensity. The second stimulus was compared to the first one, with varying inter-stimulus intervals between 20 and 500 ms (two trials at each interval) (adapted from47).

2.9. Drug treatment
The GluN2B antagonist Ro25 6981 maleate (Bioconnect, The Netherlands) was dissolved in 0.9% saline and injected i.p., 30 minutes prior to context retrieval, at a dose of 0 mg/kg, 3 mg/kg or 10 mg/kg (injection volume: 0.5 µl/gram body weight).

2.10. Statistical analysis
Data were analysed using SPSS 22.0 (IBM software). All data are expressed as mean ± standard error of the mean (SEM). Data were considered statistically significant when p<0.05. Outliers were determined using a Grubb's test. Independent-samples t-tests were performed to compare between control and ELS groups. Appropriate corrections were applied when assumption of homogeneity of variance was not met. When the assumption of normality was not met, a Mann-Whitney test was conducted. A repeated measure ANOVA was performed to assess freezing behaviour during the different tones. Greenhouse-Geisser correction was applied when the assumption of sphericity was violated. To determine the effects of ELS on the degree of LTP, a repeated-measures ANOVA was performed using condition (control vs. ELS) as between-subject factor and slope of the pre- and post-stimulation fEPSP as the within-subject factor. We compared the baseline (-10 to 0 minutes) with total LTP (0 – 60 minutes after 10 Hz stimulation) and late LTP (50 – 60 minutes after 10 Hz stimulation). When significant, a post hoc Tukey test was performed to compare groups.

Table 1. Effects of ELS on body weight.

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>ELS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight gain PND 2-9 (g)</strong></td>
<td>3.32 ± 0.17 (12)</td>
<td>2.78 ± 0.15 (11)*</td>
</tr>
<tr>
<td><strong>Body weight PND 21 (g)</strong></td>
<td>8.54 ± 0.27 (12)</td>
<td>8.45 ± 0.17 (11)</td>
</tr>
<tr>
<td><strong>Body weight 6 months (g)</strong></td>
<td>29.4 ± 0.89 (12)</td>
<td>28.9 ± 0.55 (11)</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± S.E.M (n). * p<0.05.
3. Results

3.1. Early life stress paradigm

Housing litters in a cage with limited nesting and bedding material from PND 2-9 reduced body weight gain in the male early life stress (ELS) offspring when compared to control litters (t(21)=2.4, p=0.030) (Table 1). These findings are consistent with previous reports of the model, validating the effective application of the early stress paradigm. From PND 21 onwards, differences in body weight were absent between the experimental groups (PND 21: t(21)=0.27, p=0.784; 6 months: t(21)=0.47, p=0.64).

3.2. Fear conditioning

To assess emotional fear memory, mice were trained in an auditory fear-conditioning paradigm. Freezing behaviour was comparable between the

![Figure 1](image)

**Figure 1. Fear memory following ELS at adulthood.** A. No difference in freezing levels was present immediately after the foot shock. B. When placed back in the training context, ELS mice freeze less than Ctrl mice. C. In response to the tone, Ctrl mice freeze more than ELS mice. D. Also the average freezing percentage over the tones in lower in ELS mice. *: significant difference.
groups prior to the tone-foot shock combination (data not shown) and during the 30 seconds after the shock was received \((t(7)=1.81, \ p=0.11)\) (Figure 1A). When mice were placed back in the training context 24 hours later, ELS mice froze significantly less than Ctrl mice \((t(18)=2.33, \ p=0.03)\) (Figure 1B). When introduced in a novel, “safe” context one hour later, mice showed comparable low levels of freezing (“habituation”) (Figure 1C). In response to the tone, ELS animals overall froze less than Ctrl mice \((F(1,18)=8.59, \ p=0.01)\). Post hoc tests revealed that this was significant on tone 3, 4, and 6 (Figure 1C), and also the average freezing levels comparing all tones was lower in ELS mice relative to controls \((t(19)=2.3, \ p=0.03)\) (Figure 1D).

### 3.3. HPA axis signalling

To assess HPA axis responsiveness, corticosterone (CORT) levels were determined under basal conditions, and at 30 (“response levels”) and 90 minutes (“recovery levels”) after an acute stressor, i.e. after exposure to a forced swim test (Figure 2A). ELS did not affect basal CORT levels, nor the response or recovery of CORT levels following the stressor \((F(1,20)=0.47, \ p=0.50)\). However, the relative weight of the adrenal glands, which produce CORT, was significantly increased following ELS \((t(7)=3.42, \ p=0.01)\) (Figure 2B). Whereas hippocampal GR expression was not affected \((t(11)=1.49, \ p=0.17)\) (Figure 2C; see Supplementary Figure 1 for raw images of Western blots), MR levels were significantly reduced after ELS \((t(6)=8.25, \ p<0.001)\) (Figure 2D).

### 3.4. Synaptic proteins

Hippocampal GluN2A expression was not significantly affected by ELS \((t(8)=1.56, \ p=0.15)\), but GluN2B expression was lower after ELS \((t(16)=4.4, \ p=0.0004)\) (Figure 2E,F). No differences in PSD-95 and synaptophysin were found \((PSD-95: \ t(6)=0.03, \ p=0.9; \ synaptophysin: \ t(6)=1.2, \ p=0.27)\) (Figure 2G,H).

### 3.5. LTP and paired pulse facilitation

We first assessed the effect of ELS on baseline properties of the CA1 fEPSPs, based on input-output curves fitted with a Boltzmann equation (Table 2). ELS had no effect on the maximal slope of the fEPSP \((t(68)=0.87, \ p=0.39)\), nor on the half-maximum stimulus intensity \((t(68)=0.95, \ p=0.35)\) or the slope of the input-output curve \((t(68)=0; \ p=1.0)\). Using a 10 Hz, 90 seconds stimulation paradigm, LTP was found to be significantly reduced in ELS mice \((F(1,22)=6.68, \ p=0.017)\) (Figure 3A). Also during the last 10 minutes of recordings, when the signal had
GluN2B and early life stress

Figure 2. HPA axis activity after ELS measured at 6 months of age. 

A. Plasma CORT levels

B. Adrenal glands

C. GR

D. MR

E. GluN2A

F. GluN2B

G. PSD95

H. Synaptophysin

Figure 2. HPA axis activity after ELS measured at 6 months of age. 

A. HPA axis activity is not affected by ELS under basal condition, and 30 and 90 minutes after a stressor. 

B. Adrenal gland weight relative to total body weight increased following ELS. 

C. Hippocampal GR levels were comparable between Ctrl and ELS mice. 

D. MR expression was reduced following ELS exposure. 

E. GluN2A expression was not affected by ELS, but GluN2B was lower in ELS mice. 

F. GluN2A expression was not affected by ELS, but GluN2B was lower in ELS mice. 

G. PSD-95 and Synaptophysin. *: significant t-test.
Table 2. Basal field potential characteristics for hippocampal CA1 area.

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>ELS</th>
</tr>
</thead>
<tbody>
<tr>
<td>( R_{\text{max}} ) (mV/ms)</td>
<td>-0.31 ± 0.05</td>
<td>-0.26 ± 0.02</td>
</tr>
<tr>
<td>( I_h ) (µA)</td>
<td>2.24 ± 0.06</td>
<td>2.32 ± 0.06</td>
</tr>
<tr>
<td>S</td>
<td>0.16 ± 0.03</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>N (mice (slices))</td>
<td>9 (38)</td>
<td>6 (32)</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± S.E.M (n). Abbreviations used: maximal slope of the fEPSP \( (R_{\text{max}}) \), half-maximum stimulus intensity \( (I_h) \), and the slope of the input-output curve (slope factor S) in the CA1 area.

stabilised, ELS resulted in a reduction of LTP \( (t(22)=2.32, p=0.03) \) (Figure 3B). While we were able to induce significant LTP in control animals of 19.9% (mean slope in the interval 50-60 minutes post stimulation compared to baseline (100%): Ctrl: \( t(12)=3.70, p=0.003 \)), LTP could not be elicited to the same extent in ELS mice (4.6%, compared to baseline (100%): ELS: \( t(10)=1.38, p=0.20 \)) (Figure 3A,B).

![Figure 3A](image1)

**Figure 3.** Synaptic plasticity in the CA1 of the hippocampus. A. After a 10 Hz, 90 sec stimulation, LTP was significantly decreased following ELS, and B. also the average potentiation during the last 10 minutes was decreased. C. The paired-pulse ratio was lower in ELS mice than in Ctrl mice, with significant post hoc tests in the intervals from 50-200 ms. D. After 10 minutes of Ro25 6981 application, the 10 Hz 90 sec stimulation did not induce synaptic potentiation in either Ctrl or ELS mice, E. which was also reflected by the similar fEPSP during the last 10 minutes of recording. F. The relative change in the slope fEPSP following Ro25 6981 application was significantly bigger in Ctrl mice when compared to ELS mice.
In addition, we examined the paired-pulse ratio (PPR), a measure for short-term presynaptic plasticity, at different intervals ranging from 20 to 500 ms. Over the entire range between 20 to 500 ms, ELS mice displayed a significantly lower PPR than control animals (F(1,15)=5.44, p=0.03), and post hoc analysis showed that at all intervals until 100 ms ELS mice displayed a significantly lower PPR than control animals (20 ms: p=0.007; 40 ms: p=0.01; 60 ms: p=0.02; 80 ms: p=0.03; 100 ms: p=0.02; 150-500 ms: p>0.05) (Figure 3C).

3.6. GluN2B and synaptic plasticity

We next applied the GluN2B antagonist Ro25 6981 to the slices prior to the stimulation, in order to assess a potential role for GluN2B in effects of ELS on synaptic potentiation. Ro25 6981 had no effect on the properties of the slope fEPSP measured during baseline recording (F(1,22)=0.014, p=0.907) (Figure 3D). Following the 10 Hz, 90 seconds stimulation, both experimental groups showed no synaptic potentiation (F(1,22)=0.11, p=0.74) (Figure 3E). The relative difference in synaptic potentiation with and without Ro25 6981 was calculated by measuring the slope fEPSP (as % of baseline) with Ro25 6981 minus the slope without Ro25 6981 during the last 10 minutes of recording. This difference was significantly smaller in ELS mice compared to Ctrl mice (t(22)=2.42, p=0.02) (Figure 3F).

To extend our findings in vivo, we treated mice prior to the retrieval in a fear conditioning paradigm with 3 mg/kg or 10 mg/kg Ro25 6981 (or vehicle). There was a significant interaction effect between condition and treatment on freezing behaviour to the context (F(2,27)=6.59, p=0.005) (Figure 4A). Post hoc

![Figure 4. Functional effects of GluN2B blockage. A,B. Treatment with Ro25 6981 in vivo prior to retrieval decreased freezing levels to the context (A) and the cue (B) in a dose-dependent manner in Ctrl mice, while no effects of the treatment were observed in ELS mice.](image-url)
Chapter 7

analysis revealed that in Ctrl mice, 3 mg/kg and 10 mg/kg reduced freezing levels to the context, whereas no effects of Ro25 6981 treatment were observed in ELS mice (Figure 4A). In response to the cue, Ro25 6981 also resulted in an interaction effect (F(2,27)=4.57, p=0.02), whereby 10 mg/kg Ro25 6981 treatment resulted in reduced freezing in Ctrl, but not ELS mice (Figure 4B).

4. Discussion

In this study we investigated the effects of early life stress (ELS), induced by exposing dams and pups to limited nesting and bedding material from PND 2-9, on fear conditioning, hippocampal protein expression and hippocampal synaptic plasticity at 6 months of age. We demonstrate that fear memory was significantly impaired following ELS exposure. In parallel, both long term potentiation (LTP) and paired-pulse ratio were decreased in ELS animals. The expression of the GluN2B subunit, known to be important for LTP, was reduced after ELS. Interestingly, the application of the selective GluN2B antagonist Ro25 6981 revealed that ELS mice were also functionally less sensitive to modification of GluN2B function.

4.1. Effects of chronic early life stress

Previous studies have reported that exposing the dams and pups to limited nesting and bedding materials results in increased exits of the dam from the nest (i.e. fragmented maternal care) and a reduction in body weight gain of the pups between PND 2-9. In line with this, we report a similar reduction in body weight, confirming the effective application of stress in this model. At later ages, body weight was comparable between ELS and control mice as also reported by these studies. Previous studies have suggested that early life stress results in increased HPA axis reactivity and impaired negative feedback in response to stress. As a consequence, life-long, cumulative glucocorticoid exposure may be increased, providing a mechanism through which ELS-associated cognitive impairments could be mediated. Interestingly, in the current study, no differences were present in basal CORT levels, nor in the CORT response or in its recovery from stress-exposure. The absence of effects on corticosterone levels occurred despite the significant increase in adrenal gland weight following early life stress, which suggests the adrenal glands have been activated before for a prolonged period of time. Possibly, differences in CORT release at an earlier age may underlie this, or there are differences in CORT levels present at other time points than we currently investigated (e.g. differences in circadian rhythm that are revealed during the dark phase).
These possibilities may be supported by the decrease in hippocampal MR levels following ELS, which is in line with reports from other models of early life adversity (e.g. \textsuperscript{20,52}). In contrast to other studies using the presently applied model\textsuperscript{52,53} or other paradigms of early life adversity\textsuperscript{51,54,55}, we did not observe differences in hippocampal GR level expression at 6 months of age. This may point to strain or model-specific effects of early life stress on GR levels, but is consistent with our findings that also the recovery of the CORT levels in the same animals was comparable.

ELS reduced fear expression in a mild auditory fear-conditioning paradigm. In response to the context, Ctrl mice displayed higher levels of freezing than ELS mice, suggesting a reduced memory for the context in ELS mice. In addition, ELS mice also froze less in response to the tone in a neutral context. This was not observed following the first two tones, which may be caused by a ceiling effect, as freezing levels were high overall already. The finding that auditory fear conditioning was affected by ELS suggests that, in addition to the hippocampus, also the amygdala may be affected by ELS\textsuperscript{56}. Earlier studies have reported that ELS enhances freezing responses between the tones (i.e. in a potentially safe context) while the response to the tone itself was unaffected\textsuperscript{37}. Possibly, the fact that the animals were exposed to a fearful context just prior to exposure to the tones in our present study. This may have resulted in enhanced corticosterone levels already before tone exposure and could potentially affect subsequent freezing responses to the tone, which needs confirmation. The presently found effects are most likely not related to basolateral amygdala structure or basal synaptic transmission which was unaffected after ELS\textsuperscript{57,58} although effects of ELS on synaptic plasticity in the amygdala requires investigation. The presently applied 10 Hz stimulation paradigm induced LTP in the hippocampal CA1 area of control animals, while such synaptic potentiation was absent following ELS. Between PND 2-9, when ELS was applied, both glutamatergic and GABA-ergic transmission are still developing\textsuperscript{59–63}, and shape network activity. Exposure to early life adversity during this period may therefore lead to long lasting changes in synaptic plasticity and LTP\textsuperscript{19,35,64} that could underlie the deficits in learning and memory observed following ELS. For instance, chronic early life stress, introduced using a different stimulation paradigm, induced LTP deficits in the hippocampal CA\textsubscript{3}\textsuperscript{21,27} and CA\textsubscript{1} subregion\textsuperscript{19, 21} and offspring that received low amounts of maternal care did not show potentiation following a stimulation in the dorsal dentate gyrus\textsuperscript{25,65} or hippocampal CA\textsubscript{1}\textsuperscript{20,66}. Furthermore, maternal separation also impaired LTP in the prefrontal cortex\textsuperscript{67}, while maternal deprivation impaired LTP in the dentate gyrus\textsuperscript{22} and CA\textsubscript{1}\textsuperscript{64}. Together, these findings indicate that the early postnatal period is highly sensitive to disruptions like early stress that can have long-lasting consequences for the ability to induce LTP in adulthood.
Chapter 7

We found that not only LTP was affected, but also that short-term synaptic plasticity as measured by the paired pulse ratio (PPR) was affected by ELS. Several studies suggest that short-term plasticity, such as the PPR, plays an integral role in cognitive processing and memory. Although the effects of early life stress on the PPR have received relatively little attention, an impaired PPR in the CA1 of the hippocampus has been reported following exposure to an acute stressor, following exposure to inescapable foot shocks, or ex vivo after CORT application for minutes or hours. Our data suggest a strong reduction in the PPR following ELS. PPR is believed to depend on presynaptic mechanisms, such as an increased probability of neurotransmitter release and presynaptic Ca\(^{2+}\) mobilisation. Changes in these processes, possibly induced by ELS, may thus underlie the observed impairment in PPR. Although still debated, changes in presynaptic neurotransmitter release have been suggested to contribute to LTP induction and could thus possibly also contribute to the current ELS-induced impairment in LTP.

4.2. Role of the GluN2B

At present we found that ELS did not alter the expression of the synaptic proteins PSD-95 or synaptophysin in hippocampal homogenates. This is in line with data showing that ELS also failed to alter spine density in CA1, although a slight reduction was found in the CA3 subregion. In contrast, we found that the expression of the GluN2B subunit was decreased following ELS, while GluN2A expression was unaffected. NMDA receptors are heteromeric assemblies consisting of an GluN1 subunit and various GluN2 subunits, of which GluN2A and GluN2B are the major components in the hippocampus. During development, there is a switch in NMDAR composition. GluN2B is predominantly present in the early postnatal brain (notably coinciding with the time at which we applied ELS), and GluN2A increases during postnatal development. This process can be disturbed by early life stress, as maternal deprivation prevented the switch to a mature, GluN2A dominated NMDAR phenotype at PND 28-31. In addition, adult offspring from mothers who gave low levels of maternal care also showed higher GluN2B levels as well as higher GluN2A levels. Interestingly, by 8 weeks of age, the effects of maternal prenatal stress on GluN2B were found to be opposite, and hippocampal GluN2B-NMDAR subunit expression was reported to be reduced. This is in line with our current observation that chronic early life stress reduced GluN2B expression in 6 month old mice. The functional consequences of these GluN2B reductions are emphasised by the effects of blocking this subunit prior to LTP induction. Application of Ro25 6981, a selective GluN2B antagonist reduced LTP in Ctrl mice, but was relatively less effective in ELS mice. To further validate a role for
the GluN2B subunit after ELS, we examined whether Ro25 6981 differentially affected fear conditioning in ELS mice. Ctrl mice showed a dose-dependent impairment in memory retrieval following Ro25 6981 exposure. Interestingly, ELS mice were less sensitive to either an intermediate or high dose of Ro25 6981, and maintained the ability to retrieve the fear memory comparably to vehicle treated mice. This is, to the best of our knowledge, the first time that it has been demonstrated that ELS exposure results in reduced contribution of the GluN2B subunit to synaptic plasticity as well as memory formation. These findings thus support the hypothesis that the function of GluN2B is affected in adult mice that were previously exposed to ELS.

5. Conclusion

We report that ELS from PND 2-9 leads to persistent changes in fear conditioning and impairs short- and long-term synaptic plasticity at 6 months of age. This is associated with reduced hippocampal GluN2B expression and GluN2B function after ELS. A better understanding of the lasting consequences of early life adversity on behavioural and neurobiological parameters is crucial for understanding the sensitivity to develop psychopathology later in life. The present studies point to a potentially relevant role of GluN2B.

6. Acknowledgements

This study was supported by a grant from Internationale Stichting Alzheimer Onderzoek (ISAO)/Alzheimer Nederland (grant: #12534 to HJK)
Chapter 7

7. References

22. Oomen CA, Soeters H, Audureau N, Vermunt L, van Hasselt FN, Manders EMM et al. Severe early life stress hampers spatial learning and


47 Cazakoff BN, Howland JG. Acute stress disrupts paired pulse facilitation and long-term potentiation in rat dorsal hippocampus through activation of glucocorticoid receptors. Hippocampus 2010; 20. 1327–1331.
GluN2B and early life stress


8. Supplementary figures

Supplementary Figure 1. Typical examples of Western blots. Each lane represents a different animal. C: control mice; E: ELS mice; GR: glucocorticoid receptor; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; MR: mineralocorticoid receptor; NR2A: NMDA receptor subunit A; NR2B: NMDA receptor subunit B; PSD-95: post-synaptic density 95; α-tub: alpha-tubulin.

Figure continues on next page
GluN2B and early life stress

Supplementary Figure 1 (continued).

NR2B (~180 kDa)
PSD-95 (~95 kDa)
a-tub (~50 kDa)
Synaptophysin (~84 kDa)
Effects of corticosterone on mild auditory fear conditioning and extinction; role of sex and training paradigm

Sylvie L. Lesuis, Lisa A.E. Catsburg, Paul J. Lucassen, Harm J. Krugers

Learning & Memory
2018. 25:544-549
Abstract

Multiple lines of evidence suggest that glucocorticoid hormones enhance memory consolidation of fearful events. However, most of these studies involve male individuals. Since anxiety, fear and fear-associated disorders present differently in male and female subjects we investigated in mice whether male and female mice perform differently in a mild, auditory fear conditioning task and tested the modulatory role of glucocorticoid hormones. Using an auditory fear conditioning paradigm with different foot shock intensities (0.1 mA, 0.2 mA and 0.4 mA) and frequencies (1x or 3x), we find that intraperitoneal injections with corticosterone (2 mg/kg) immediately after training, altered freezing behaviour when repeated foot shocks were applied, and that the direction of the effects were opposite in male and female mice. Effects were independent of foot shock intensity. In male mice, corticosterone consistently increased freezing behaviour in response to the tone, whereas in female mice, corticosterone reduced freezing behaviour twenty-four hours after training. These effects were not related to the phase of the oestrous cycle. In addition, corticosterone enhanced extinction learning for all tones, in both male and female mice. These results emphasise that glucocorticoid hormones influence memory consolidation and retrieval, and underscore sex-specific effects of glucocorticoid hormones in modulating conditioned fear responses.

Keywords: sex, stress, corticosterone, fear conditioning, consolidation, retrieval, extinction, estrous
1. Introduction

Memories for fearful events are generally retained well\(^1,2\). Extensive evidence from human and animal studies have associated stress hormones like glucocorticoids (corticosterone in rodents; cortisol in humans) with altered memory formation\(^3\). Glucocorticoid hormones are produced by the adrenal glands and their release is increased during and after exposure to stress or emotional experiences\(^4\). These hormones readily cross the blood-brain barrier and bind to mineralocorticoid (MR) and glucocorticoid (GR) receptors present in the brain. Via genomic and non-genomic effects, they can influence neural function and memory formation\(^5,6\). Many studies have reported that corticosterone, as well as synthetic GR agonists, can alter cognitive functions and e.g. enhance memory consolidation\(^1,2\). While these behavioural effects after short term exposure are usually adaptive in nature, prolonged exposure to elevated glucocorticoid hormones may be deleterious and has been associated with stress-associated disorders\(^7\).

Many studies on the effects of glucocorticoid hormones have predominantly used male individuals to investigate their effects\(^8\), while memory formation under the influence of stress, as well as many anxiety-, stress and psychiatric disorders are present with distinct sex differences in humans. For instance, the lifetime prevalence of post-traumatic stress disorder (PTSD) is two times higher in women than in men\(^9\). Animal studies have reported striking differences in stress-responsiveness between sexes. For example, female rodents have higher basal corticosterone levels than males\(^10–15\), and a higher binding capacity for hippocampal GRs\(^16\). In addition, female rodents express less MRs\(^17\) as well as a lower binding of corticosterone to these receptors\(^18,19\). Interestingly, chronic stress in male rats downregulates GR immunoreactivity in the dentate gyrus and CA1 region of the hippocampus, but increases GR binding in CA1 in females\(^20,21\). In these studies, MR binding was increased in the CA3 region of female, but not in male rats.

Together, these studies suggest sex differences in the action of glucocorticoids. Indeed, sex differences are also present in cognitive performance. Male rodents have been reported to perform better than females in spatial memory tasks like the Morris water maze (e.g.\(^22\)). Yet, after acute and chronic stress, performance of male rats in spatial memory tasks was impaired\(^23–25\), whereas female animals improved their spatial memory abilities following stress\(^26\). Sex-differences have also been reported in fear-related memory\(^27\). However, whether glucocorticoid effects on memory consolidation in auditory fear conditioning paradigm differs between male and female animals, and how different aspects of the training paradigm contribute to
these effects, remains largely elusive. We therefore systematically investigated in mice how these hormones regulate fear memory formation in the context of varying training intensities, and whether effects were different between male and female mice.

Figure 1. Training and testing paradigm cued fear conditioning. A. Mice are trained in a fear conditioning paradigm with one or three 30 second tones, coupled with a 2 second foot shock of varying intensity (“training”). Immediately following training, mice were injected i.p. with corticosterone (2 mg/kg) or saline. Twenty-four hours later, mice were introduced in a novel environment, and re-exposed to the same 30 second tone for 6 times (“retrieval”). B. The effects of sex, foot shock intensity and foot shock frequency on freezing behaviour after re-exposure to a single tone at retrieval. All mice received a saline injection following training. A main effect was observed for foot shock intensity ($F(2,65)=40.89, p=0.001$), foot shock frequency ($F(1,65)=31.78, p=0.001$), and sex ($F(1,65)=14.01, p=0.001$), and an interaction effect was found between sex x intensity ($F(2,65)=3.31, p=0.05$), and between sex x frequency x intensity ($F(2,65)=4.18, p=0.02$).
2. Results

2.1. Effects of shock intensity and frequency on freezing behaviour in male and female mice

Male and female mice were trained in an auditory fear conditioning paradigm with varying foot shock intensities (0.1 mA, 0.2 mA or 0.4 mA) and frequencies (1x or 3x), and freezing behaviour to the tone was measured twenty-four hours later in a novel context by exposing mice to six tones (Figure 1A). Both the foot shock intensity and the frequency of foot shocks at training together determined freezing behaviour at retrieval (Figure 1B). Twenty-four hours after training, the three foot-shock paradigm increased freezing levels relative to a single foot shock of the same intensity (frequency effect: F(1,65)=31.78, p=0.001). Increasing the foot shock intensity also increased the freezing levels in response to a tone twenty-four hours later, also in both sexes (intensity effect: F(2,65)=40.89, p=0.001). Female mice overall displayed more freezing behaviour than male mice during the retrieval phase (sex effect: F(1,65)=14.01, p=0.001; sex x intensity interaction effect: F(2,65)=3.31, p=0.05). Following a three foot-shock training paradigm with an intensity of 0.2 mA, female mice displayed increased freezing behaviour when compared to males (post hoc: p<0.05). However, following a three foot-shock training paradigm with an intensity of 0.4 mA, male and female mice again displayed comparable freezing levels (post hoc: p>0.05).

2.2. Effects of corticosterone treatment on freezing behaviour

To investigate the effects of glucocorticoids, corticosterone or control saline injections were given immediately following the training. Corticosterone significantly affected freezing behaviour to the first tone, but differently in male and female mice, and depending on the frequency of the foot shock (treatment x sex x frequency: F(1,133)=10.46, p=0.002) (Figure 2A). Post hoc testing only revealed an effect of treatment in male mice following three foot shocks (p=0.049).

Corticosterone also significantly affected freezing behaviour to the subsequent tones, as measured by the average freezing over the six tones (Figure 2B, Supplementary Figure + Table 1). Corticosterone induced an overall effect on freezing behaviour to the tones during retrieval, although differently in male and female mice (sex*treatment effect: F(1,133)=17.21, p<0.001) (Figure
2B). These effects were irrespective of frequency or intensity of the training (treatment*frequency: F(1,133)=0.40, p=0.53; treatment*intensity: F(1,133)=0.80, p=0.45).

**Figure 2. The effect of corticosterone on freezing behaviour.** A. Freezing levels to the first tone. A significant interaction effect was observed between sex x frequency x treatment (F(1,133)=10.25, p=0.002), and corticosterone increased freezing only in male mice after a training paradigm with three foot shocks (p=0.049). B. Average freezing behaviour over the six tones. Corticosterone resulted in an overall effect on freezing behaviour to the tones during retrieval, although differently in male and female mice (sex*treatment effect: F(1,133)=17.21, p<0.001).
2.3. Effect of shock frequency (single vs. repeated foot shock) on corticosterone effects on freezing behaviour

After a single foot shock, the administration of corticosterone directly after training had no effect on the average freezing levels to the tones during the retrieval, regardless of the foot shock intensity or the sex of the animal, as indicated by comparable freezing levels to the tones (treatment effect: $F(1,61)=0.28$, $p=0.60$) (Figure 2B, Supplementary Figure + Table 1). When corticosterone was delivered after three foot shocks, it affected freezing at all shock intensities (treatment*frequency effect: $F(5,72)=7.85$, $p<0.001$) (Figure 2B), except for the 3 x 0.1 mA training paradigm, whereas no effect of corticosterone was observed in females (Figure 2A).

2.4. Role of sex in corticosterone-enhanced freezing behaviour

Interestingly, the effects of corticosterone on memory consolidation were sex-dependent. In male mice, corticosterone increased freezing to the tones at the 3 x 0.1 mA, 3 x 0.2 mA, and 3 x 0.4 mA training paradigms (Figure 2A). In female mice, corticosterone decreased freezing to the tones after 3 x 0.2 mA,
and 3 x 0.4 mA foot shocks, although no effect of corticosterone was observed at the 3 x 0.1 mA foot shock paradigm (Figure 2A).

2.5. Effect of oestrous cycle on corticosterone-induced freezing behaviour

No effect of oestrous cycle was observed on freezing behaviour, nor on the effects of corticosterone on freezing behaviour in female mice (main cycle effect: \( F(1,46)=1.13, p=0.29 \); treatment*cycle effect: \( F(1,46)=0.12, p=0.73 \) (data not shown).

2.6. Effect of corticosterone on extinction learning over the tones

The extinction of freezing responses after repeated tone-exposures, as measured by the difference in freezing level between tone 1 and tone 6, was different between the sexes. These effects also depended on training frequency and treatment (sex*frequency*treatment interaction effect: \( F(1,134)=4.8, p=0.03 \) (Figure 3). In male mice, corticosterone did not affect the extinction following a single foot shock. However, following three foot-shocks, corticosterone increased extinction over the tones in male mice, independent of foot shock intensity. In female mice, an effect of corticosterone treatment on extinction was observed following a single foot shock, independent of foot-shock intensity. Following three foot-shocks, corticosterone treatment no longer affected extinction levels.

3. Discussion

In this study we investigated whether male and female mice perform differently in a mild, auditory fear conditioning task with different foot shock intensities (0.1 mA, 0.2 mA, 0.4 mA) and frequencies (one or three times) and tested the modulatory role of glucocorticoids. We report that corticosterone treatment after training enhances freezing behaviour at retrieval in male mice, but reduces freezing in female mice. The effects of corticosterone treatment were only apparent after a three-times repeated foot shock paradigm, and not following a single foot shock, regardless of the foot shock intensity. Furthermore, corticosterone treatment increased extinction learning over the tones, in both male and female mice.
3.1. Sex differences

As expected, subjecting animals to higher foot shock intensities resulted in higher freezing levels. Likewise, exposure to three foot shocks at training also resulted in higher freezing levels at retrieval than training with a single foot shock of the same intensity. This illustrates that freezing behaviour at retrieval reflects the intensity of the learning experience at training, which could be an appropriate measure to assess the intensity of the memory, as suggested by previous studies. When comparable training parameters were applied, female mice always displayed higher freezing levels than male mice. Only after a three-times repeated foot shock of 0.4 mA did we not observe a difference between male and female mice. This may stem from a ceiling effect, as both sexes displayed relatively high freezing levels. For female mice, the freezing levels did not increase further between a three-times repeated foot shock of 0.2 mA and 0.4 mA, which may be attributed to a ceiling effect as well. For male mice, the increase in freezing between a three-times repeated foot shock of 0.2 mA and 0.4 mA was still substantial. These findings suggest that fear memory formation and consolidation might be different between the sexes. This could potentially be modulated by female sex hormone-dependent mechanisms (e.g. oestrogen and progesterone), that may influence plasticity-related associative fear memory. Indeed, a similar dimorphic pattern of corticosterone has been reported on a trace-conditioned eye blink response, and following a contextual training paradigm, females have also been reported to freeze more than males. However, in the current study, we did not observe any effect of oestrous cycle on freezing levels. Studies on sex differences following fear conditioning have been inconsistent, with studies reporting no effects of oestrous cycle on freezing behaviour, or decreased freezing levels in females. Although our study cannot explain the discrepancies between these studies, we speculate that they may arise from experimental variations within the fear-conditioning paradigm animal species or strain.

3.2. Corticosterone and freezing behaviour

Numerous studies have illustrated that glucocorticoids facilitate memory consolidation (e.g. ). Also in the current study, we find that corticosterone increases memory consolidation. The use of post-training administration of corticosterone, as opposed to corticosterone administration prior to training, suggests an effect on memory consolidation that is not confounded by possible effects on attentional, motivational or sensory-perceptual mechanisms, that
may have occurred when corticosterone treatment would have been given at the time of conditioning or testing. In both male and female mice, and regardless of foot shock intensity, corticosterone administration after training did not affect freezing levels at retrieval following a single foot shock. Yet, after three repeated foot shocks, corticosterone significantly increased freezing behaviour during retrieval. An exception was freezing behaviour in female mice after a three-times repeated foot shock of 0.1 mA. This effect can possibly be attributed to a floor effect, as freezing levels in control female mice were already very low. The difference between a single versus a repeated training paradigm is not merely due to the fact that a repeated foot shock induced overall higher freezing levels. For instance, freezing levels in females after a single 0.4 mA foot shock are higher than after 3 x 0.2 mA foot shock. Yet, corticosterone resulted in differences when the three-times repeated training paradigm is used. These results suggest that it is not the severity/adversity of the training paradigm per se that determines whether corticosterone alters freezing, but that it is the frequency with which the mouse is repeatedly exposed to the tone-foot shock that determines the effects of the hormone. Possibly, the learning component in a repeated foot shock paradigm is stronger than in a single foot shock paradigm, and such a paradigm may therefore be more susceptible to modulation by corticosterone. This notion is supported by the observation by Hui et al.\textsuperscript{43}, showing that corticosterone did not enhance freezing behaviour following an unpaired presentation of the tone and foot shock, or the tone or the shock alone, indicating that a learning process is critical for corticosterone to have an effect.

### 3.3. Effect of sex on corticosterone-induced freezing behaviour

Interestingly, the effects of corticosterone on freezing behaviour were opposite in male and female mice. In agreement with previous literature, showing that corticosterone selectively enhances memory in male rats\textsuperscript{43–45}, we found that corticosterone enhanced freezing behaviour in male mice. These results are consistent with previous findings indicating that corticosterone, as well as drugs that selectively activate GRs, enhance memory consolidation for several types of training, including discrimination learning, inhibitory avoidance, contextual fear conditioning, water-maze spatial training, and appetitive conditioning\textsuperscript{39,40,44–51}. On the other hand, we found that post-training treatment with corticosterone reduced auditory freezing in female mice, providing evidence that also the glucocorticoid effects on memory in these paradigms are sex-dependent.

In agreement with these findings, previous studies have demonstrated
that in females, corticosterone also impairs memory formation in a contextual fear conditioning paradigm and in spatial memory tasks\textsuperscript{52,53}. These differences may arise from interactions between HPA axis signalling and female sex-hormone dependent pathways (e.g. oestrogen and/or progesterone signalling). Alternatively, the effects of corticosterone have been reported to follow a Yerkes-Dodson or inverted-U shape dose response relationship, in which optimal enhancing effects on memory are seen at midrange doses, whereas high doses are less effective or may even impair memory\textsuperscript{5}. As female mice have both higher basal corticosterone levels and a stronger corticosterone release upon a stressor\textsuperscript{54}, the currently used dose of corticosterone (2 mg/kg) may not have been effective in enhancing memory in females.

Interestingly, the use of oral contraceptives has been shown to affect HPA axis responsiveness during stress exposure in females, resulting in a blunted cortisol response and a lack of stress-induced effects on memory\textsuperscript{55,56}. Aside from sex-specific differences in stress sensitivity or responsivity\textsuperscript{57}, in the human population, the effects of stress-induced glucocorticoid release on memory may therefore differ between men and women in part because of the high use of oral contraceptives by females. In future studies, the use of oral contraceptives will be important to take into consideration for proper interpretation of the results.

3.4. Corticosterone enhances extinction learning over the tones

Previous research has shown that corticosterone facilitates the extinction process\textsuperscript{42,58,59}. Our present study shows that corticosterone treatment immediately after training enhanced the extinction of freezing over the tones 24 hours later, when differences in corticosterone levels have already ceased to exist between the groups. The training paradigm and sex also played a role in this extinction, as in male mice, only a three-times repeated foot shock paradigm resulted in enhanced extinction, whereas in females a single foot shock paradigm enhanced extinction, irrespective of foot shock intensity. The current study cannot clarify the nature of this interaction between sex and foot shock frequency. However, both in male and female mice, corticosterone treatment after training enhanced extinction learning, in a comparable way in both sexes. The stronger extinction following corticosterone treatment corresponds to other studies showing facilitated extinction of fear behaviour after corticosterone treatment in fear conditioning\textsuperscript{60–62} or appetitive operant conditioning tasks\textsuperscript{63}.

Interestingly, the corticosterone-induced effects on memory strength
(enhancing memory in males while impairing it in females) appear to differ from the effects on extinction, as in both male and female mice, corticosterone enhanced extinction learning. This may indicate the involvement of different brain areas in the effects of corticosterone. Numerous studies have shown that auditory fear conditioning is largely dependent on amygdala activation, whereas prefrontal cortex-amygdala circuits are essential for fear extinction learning\textsuperscript{64,65}. This suggests that corticosterone may have different effects in amygdala and / or prefrontal cortex in male and female mice, which could contribute to the divergent nature of the hormone effect on memory consolidation and extinction.

4. Conclusion

The results reported here add to existing evidence that corticosteroid hormones influence memory consolidation. These findings emphasise sex-specific effects of corticosterone in a mild auditory fear condition paradigm. Furthermore, corticosterone enhanced extinction of fearful memories to the same extent in male and female mice. Together, these data suggest that fear memories may be better retained in male animals when compared to female animals. They further emphasise the importance of studying both males and females in stress-related phenotypes and warrant more studies into the mechanisms that underlie sex differences.

5. Materials and Methods

5.1. Mice and breeding

All mice were kept under standard housing conditions (temperature 20-22 °C, 40-60% humidity) Standard chow and water were available \textit{ad libitum}, and mice were housed on a 12/12 h light/dark schedule (lights on at 8 a.m.) and a radio provided background noise\textsuperscript{66}. All experimental procedures were conducted under the national law and European Union directive 2010/63/EU on animal experiments, and were approved by the animal welfare committee of the University of Amsterdam. Male and female C57Bl/6 mice were bred in house. After weaning, mice were housed with 2-5 same sex littermates per cage until the start of experiments.
5.2. Fear conditioning

Three month (± 2 weeks) old male and female mice were tested in an auditory fear conditioning paradigm. Two weeks prior to fear conditioning, mice were housed individually. All experimental procedures occurred in the morning between 09.00 a.m. and 11.00 a.m. During testing, the mice were recorded by a camera connected to a computer with Ethovision software (version 6.1, Noldus, The Netherlands). Mice were placed in a square chamber with black walls (W x L x H: 30 x 24 x 26 cm) which had a stainless steel grid floor connected to a shock generator, and which had been cleaned with 1% acidic acid to create a recognisable odour trace. Mice were allowed to explore the context for three minutes, after which once or 3 times, a 30-second tone was applied (2.8 kHz, 76 dB), coupled to a 2-second foot shock (0.1, 0.2 or 0.4 mA) during the last 2 seconds of the tone, with an inter-tone-interval of 60 seconds (the “training” phase) (see Figure 1A). After the last tone-foot shock pairing, the mice remained in the chamber for 30 seconds. Twenty-four hours later, mice were introduced in a novel, circular box (diameter: 35 cm, transparent walls, sawdust floor) cleaned with 25% EtOH. After 3 minutes, a 30-second tone was applied for 6 times, with an inter-tone-interval of 60 seconds. Freezing behaviour during every trial was scored by an observer blind to the experimental condition, with “freezing” being defined as “no body movements except those related to breathing” (68). 4 to 15 mice (7 on average) were used (see Supplementary table 1 for the number of mice per group).

5.3. Corticosterone treatment

Corticosterone (Sigma; 16 mg/ml dissolved in 99.9% EtOH and diluted 40x in saline) or vehicle (2.5% EtOH in saline) were injected intraperitoneally, immediately after the training (final dose: 2 mg/kg, injection volume: 5 µl/g body weight).

5.4. Oestrous cycle determination

20 µl of 0.9% saline was used to elute cells from the female’s vagina, which were spread on a glass slide and analysed directly after sampling by means of a light microscope with a 10x total magnification. Cycle stage of every female was assessed and classified as “oestrous” or “non-oestrous”, as described previously (69). Detection of the oestrous phase was performed after fear conditioning. Seventeen mice were in oestrous, and 53 mice were in non-oestrous.
5.5. Statistical analyses

Data were analysed using SPSS 22.0 (IBM software). All data are expressed as mean ± standard error of the mean (S.E.M.). Data were considered statistically significant when p<0.05. Outliers were determined using a Grubb's test. To determine the effects of treatment on freezing to the tones (Supplementary Figure 1, Supplementary Table 1), a two-way repeated measures ANOVA was performed using treatment (vehicle vs. CORT) as between-subject factors, and freezing behaviour to the different tones as the within-subject factor. A 2x3x2-way ANOVA was performed to assess the difference between groups accounting for sex, foot shock intensity and foot shock frequency. A 2x2x3x2-way ANOVA was performed to compare differences between groups accounting for treatment, sex, foot shock intensity and foot shock frequency.

6. Acknowledgements

This study was supported by Internationale Stichting Alzheimer Onderzoek (ISAO)/Alzheimer Nederland (grant: #12534 to HJK).
7. References


10. Figueiredo HF, Dolgas CM, Herman JP. Stress activation of cortex and hippocampus is modulated by sex and stage of estrus. *Endocrinology* 2002; **143**: 2534–2540.


Chapter 8

1999; \[ \beta \]
198


50  Roozendaal B, Nguyen BT, Power AE, McGaugh JL. Basolateral amygdala noradrenergic influence enables enhancement of memory consolidation induced by hippocampal...
The effects of sex and CORT on fear conditioning


64 Santini E, Ghe H, Ren K, Peña de Ortiz S, Quirk GJ. Consolidation of fear extinction requires protein synthesis in the medial prefrontal cortex. *J Neurosci* 2004; **24**: 5704–5710.


52 Snihur AWK, Hampson E, Cain DP. Estradiol and corticosterone independently impair spatial navigation in the Morris water maze in adult female rats. *Behav Brain Res* 2008; **187**: 56–66.


8. Supplementary data

Supplementary Figure 1. Freezing behaviour in response to the six tones during retrieval following saline (black) or corticosterone (red) treatment. Corticosterone was given immediately following auditory fear conditioning, and 24 hours later freezing behaviour to six consecutive tones was measured. *: main treatment effect.

*Figure continues on next page*
Effects of sex and CORT on fear conditioning

**Females**

<table>
<thead>
<tr>
<th></th>
<th>1 foot shock</th>
<th>3 foot shocks</th>
</tr>
</thead>
<tbody>
<tr>
<td>C.</td>
<td><img src="image" alt="Graph C" /></td>
<td><img src="image" alt="Graph D" /></td>
</tr>
<tr>
<td>D.</td>
<td><img src="image" alt="Graph G" /></td>
<td><img src="image" alt="Graph H" /></td>
</tr>
<tr>
<td>G.</td>
<td><img src="image" alt="Graph H" /></td>
<td><img src="image" alt="Graph L" /></td>
</tr>
</tbody>
</table>

Supplementary Figure 1. Freezing behaviour in response to the six tones during retrieval following saline (black) or corticosterone (red) treatment. Corticosterone was given immediately following auditory fear conditioning, and 24 hours later freezing behaviour to six consecutive tones was measured. *: main treatment effect.

Supplementary Figure 1 (continued).
Supplementary table 1. Statistical tests corresponding to the graphs in Supplementary Figure 1.

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tone: F (5, 55) = 3.30, p=0.01*</td>
<td>Tone: F (5, 50) = 3.00, p=0.02*</td>
</tr>
<tr>
<td></td>
<td>Treatment: F (1, 11) = 1.323, p=0.27</td>
<td>Treatment: F (1, 10) = 0.26, p=0.62</td>
</tr>
<tr>
<td></td>
<td>Saline group: N=6, corticosterone group: N=7</td>
<td>Saline group: N=6, corticosterone group: N=6</td>
</tr>
<tr>
<td>1x0.1 mA</td>
<td>Tone: F (5, 60) = 13.66, p&lt;0.001*</td>
<td>Tone: F (5, 40) = 19.57, p&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>Treatment: F (1, 12) = 7.68, p=0.02*</td>
<td>Treatment: F (1, 8) = 1.09, p=0.33</td>
</tr>
<tr>
<td></td>
<td>Saline group: N=7, corticosterone group: N=7</td>
<td>Saline group: N=5, corticosterone group: N=5</td>
</tr>
<tr>
<td>3x0.1 mA</td>
<td>Tone: F (5, 55) = 9.09, p&lt;0.001*</td>
<td>Tone: F (5, 40) = 15.91, p&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>Treatment: F (1, 11) = 1031, p=0.33</td>
<td>Treatment: F (1, 8) = 0.09, p=0.78</td>
</tr>
<tr>
<td></td>
<td>Saline group: N=6, corticosterone group: N=7</td>
<td>Saline group: N=5, corticosterone group: N=5</td>
</tr>
<tr>
<td>1x0.2 mA</td>
<td>Tone: F (5, 125) = 19.25, p&lt;0.001*</td>
<td>Tone: F (5, 50) = 6.77, p&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>Treatment: F (1, 25) = 11.08, p=0.01*</td>
<td>Treatment: F (1, 10) = 7.846, p&lt;0.01*</td>
</tr>
<tr>
<td></td>
<td>Saline group: N=12, corticosterone group: N=15</td>
<td>Saline group: N=6, corticosterone group: N=6</td>
</tr>
<tr>
<td>3x0.2 mA</td>
<td>Tone: F (5, 50) = 25.56, p&lt;0.001*</td>
<td>Tone: F (5, 50) = 3.38, p=0.01*</td>
</tr>
<tr>
<td></td>
<td>Treatment: F (1, 10) = 0.00, p=0.96</td>
<td>Treatment: F (1, 10) = 1.54, p=0.24</td>
</tr>
<tr>
<td></td>
<td>Saline group: N=6, corticosterone group: N=6</td>
<td>Saline group: N=6, corticosterone group: N=6</td>
</tr>
<tr>
<td>1x0.4 mA</td>
<td>Tone: F (5, 35) = 10.91, p&lt;0.001*</td>
<td>Tone: F (5, 55) = 3.18, p=0.01*</td>
</tr>
<tr>
<td></td>
<td>Treatment: F (1, 7) = 14.46, p=0.01*</td>
<td>Treatment: F (1, 11) = 7.45, p=0.02*</td>
</tr>
<tr>
<td></td>
<td>Saline group: N=4, corticosterone group: N=5</td>
<td>Saline group: N=7, corticosterone group: N=6</td>
</tr>
</tbody>
</table>

* indicates a significant main effect of either tone or treatment.
Effects of sex and CORT on fear conditioning
Glucocorticoids promote fear generalisation by increasing the size of memory-encoding neuronal ensembles

Sylvie L. Lesuis¹, Michel C. van den Oever², Nathalie Immerzeel¹, Rolinka J. van der Loo², Paul J. Lucassen¹, Harm J. Krugers¹

Submitted
Chapter 9

Abstract

Glucocorticoids help to remember threatening and stressful experiences. However, we show that high levels of these hormones after an aversive experience induces fear memory generalisation. This is accompanied by alterations in the electrophysiological properties and increase in size of neuronal ensembles in the dentate gyrus of the mouse hippocampus. Selective chemogenetic suppression of these ensembles prevents the expression of generalised fear memory induced by glucocorticoids.

Keywords: stress, memory, fear, generalisation, corticosterone, memory specificity, dentate gyrus.

1 Brain Plasticity Group, Swammerdam Institute for Life Sciences, Center for Neuroscience, University of Amsterdam, Amsterdam, The Netherlands
2 Department of Molecular and Cellular Neurobiology, Center for Neurogenomics and Cognitive Research (CNCR), Amsterdam Neuroscience, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands
1. Introduction

Stressful and emotionally arousing events are generally remembered very well. Enhanced memory retention clearly has adaptive and survival value, but only when memories remain specific for the arousing cue or context. If memory encoding and/or processing is aberrant, this can trigger inappropriately expressed, yet intrusive and vivid fear memories, even in safe situations and/or in the absence of predictive cues, such as seen in posttraumatic stress disorder (PTSD) or generalised anxiety disorders.

Glucocorticoid hormones (GCs) (cortisol in humans, corticosterone in rodents) are released from the adrenal cortex in response to stressful experiences. They enhance memory processing including response selection and memory consolidation by activating brain mineralocorticoid (MRs) and glucocorticoid receptors (GRs). Considering that stress can also induce memory impairments and fear generalisation in a time- and dose-dependent manner, we here tested whether GCs also modify specificity of a fear memory. Since recent studies demonstrate that a memory is encoded by small populations of cells (neuronal ensembles) that are activated during specific learning epochs, also known as ‘engram cells’, we examined whether GCs affect the formation of memory-encoding neuronal ensembles in the hippocampal dentate gyrus, and whether these engram cells are responsible for GC-induced fear generalisation.

We employed a specific experimental design to restrict fear responses to an appropriate predictor of an aversive stimulus. Mice were first exposed to an aversive situation (3 mild (0.2 mA) foot shocks that were paired with a discrete tone, in a specific context (conditioning context A)), immediately followed by saline or corticosterone (2 mg/kg) treatment (Figure 1a). This fear conditioning procedure increased plasma corticosterone levels that were even further increased and prolonged by post-training corticosterone administration. Plasma corticosterone levels returned to baseline levels at 5 hours after training (Supplementary Figure 1).

Exposure to context A, but not to the neutral context B, induced freezing behaviour in saline treated mice 24 hours after training (black bold line, Figure 1b). Exposing animals to the conditioned tone in context B evoked robust freezing, indicating that animals had learned the tone-shock association (Figure 1b). Corticosterone (dashed red lines) did not alter freezing in context A compared to control mice, but enhanced freezing behaviour in neutral context B, as well as to the tone in context B. Freezing in context B relative to context A was increased in corticosterone-treated mice (Figure 1c), while freezing to the
Corticosterone induces fear memory generalisation. 

**a** Schematic overview of the fear conditioning paradigm. 

**b** Following paired training, freezing in conditioning context A was comparable between experimental groups (t(7)=0.21, p=0.42). Freezing responses in the neutral context B (t(14)=4.05, p=0.001), and to the tone (t(14)=3.02, p=0.009) were enhanced after corticosterone treatment (black thin lines: individual data points of saline-treated mice; black thick line: average of all saline-treated mice; red dashed thin lines: individual data points of corticosterone-treated mice; red dashed thick line: average of all corticosterone-treated mice; grey area: session in context B). 

**c** Corticosterone-treated mice displayed more freezing behaviour in context B relative to context A (t(14)=2.84, p=0.01). 

**d** The ratio of freezing to the tone over baseline freezing in context B was not affected by corticosterone (t(14)=1.61, p=0.13). 

**e** Blocking the GR by RU486 treatment reduced corticosterone-induced memory strengthening and generalisation (treatment effect: F(2,19)=10.79, p<0.001; post hoc Tukey: saline vs. corticosterone: p=0.02; corticosterone + RU486 vs. RU486: p<0.001; saline vs. corticosterone + RU486: p=0.19).

Figure continues on next page.
Enhanced freezing in neutral context B was absent in mice that received corticosterone but did not undergo fear conditioning (Figure 1f-h), indicating that generalised freezing only occurs after an aversive experience. Together, this shows that increased corticosterone levels immediately after training elicit a generalised fear response in a neutral context, pointing to reduced specificity of the memory. Notably, the presence and predictability of the tone during training was required for this corticosterone-induced impairment in memory specificity, as following an unpaired training paradigm no effects of corticosterone were observed (Supplementary Figure 2).

To identify how corticosterone induced memory generalisation, we first examined neuronal activation in the DG, a region implicated in contextual memory and fear generalisation. We studied endogenous expression of the immediate early genes Arc and c-fos 90 minutes after training and corticosterone treatment, when both immediate early genes peak (Figure 2a). Corticosterone increased the number of c-fos+ cells, while Arc+ cell numbers were not affected (Figure 2b). Colocalisation of Arc+ cells with c-fos, which is induced in highly activated neurons, may indicate that within the Arc+ populations, the proportion of neurons with high activity patterns increased as a result of corticosterone treatment (Figure 2c). To determine whether corticosterone had a lasting effect on the size of the neuronal ensembles activated after fear conditioning, we used a transgenic mouse with destabilised
**Figure 2.**

| a. | Representative microscopic image showing Arc and c-fos expression 90 mins after training and corticosterone injection. Scale bar = 25 µm. | b. | Corticosterone treatment increased the number of c-fos⁺ cells in the DG (t(4)=3.44, p=0.03), while the number of Arc⁺ cells was unaltered (t(4)=0.85, p=0.44). c. Of the total Arc⁺ population, more cells became c-fos⁺ (t(4)=4.72, p=0.009). d. Representative microscopic image showing dVenus expression in saline and corticosterone-treated mice. Scale bar = 250 µm. e. The number of dVenus⁺ neurons in the dorsal DG was increased in corticosterone-treated mice in the DG 24 hours after conditioning (t(30)=4.92, p<0.001). f. Typical examples of mEPSC traces. g. The frequency of the mEPSCs was enhanced in dVenus⁺ neurons irrespective of treatment (interaction effect: F(1,13)=6.84, p=0.02). Corticosterone increased the mEPSC frequency in dVenus⁺ neurons (p=0.04). h. No effect of dVenus or treatment was observed in the mEPSC amplitude (main effect dVenus: F(1,13)=2.45, p=0.14). Figure continues on next page. |
Venus fluorescent protein (dVenus) expression under control of the Arc promotor (Arc::dVenus mice) (Figure 2d) to monitor activated neurons in the DG at 24 hours after conditioning. We found that the number of dVenus+ neurons in the dorsal DG were significantly increased in corticosterone-treated mice 24 hours after training (Figure 2e). This is in line with earlier findings that later waves in Arc induction after training are crucial in the perpetuation of fear memories. Together, these data indicate that enhanced corticosterone after training increased the size of activated DG neuronal ensembles. Since neural activation and excitability is relevant for the recruitment of neurons into a memory engram, we used whole-cell patch clamp recordings to investigate the effects of corticosterone on the physiological properties of dVenus+ neurons and non-activated (dVenus) neighbouring neurons after fear conditioning (Figure 2f). Irrespective of treatment, the frequency of mEPSCs was enhanced in dVenus+ neurons compared with dVenus+ neurons, but corticosterone significantly enhanced the mEPSC frequency in dVenus+ neurons (Figure 2g). Corticosterone did not affect the mEPSC amplitude in...
both cell populations (Figure 2h). Together with the increased population of c-fos+ neurons after corticosterone treatment (Figure 2b), the data suggest that corticosterone enhances overall activity of DG neurons, thereby biasing recruitment of neurons into the memory engram.

Next, the prolonged detectability of dVenus was used to investigate whether neurons that were recruited during training, were re-activated during exposure to context A and B. Arc-dVenus mice treated with corticosterone showed similar levels of c-fos+ cells in the DG in context A, but an increase in c-fos+ cells in context B (Figure 2h,i). In parallel, corticosterone-treated mice showed increased colocalisation of c-fos and dVenus+ neurons (Figure 2j), indicating that corticosterone increased reactivation of DG neurons in a neutral context B.

We next investigated whether the neurons that were activated by training and corticosterone treatment were causally involved in the

Figure 3. Chemogenetic suppression of activated DG ensembles reduced corticosterone-induced generalisation of contextual fear. a Schematic representation of Fos promoter driven and 4OH T dependent conditional expression of hM4Di-mCherry. b Timeline of injection, viral transduction and behavioural testing paradigm. Freezing in context A and context B (+ tone) was assessed 24 h and 48 h after training, respectively.
Effects of glucocorticoids on neuronal fear ensembles

For this, we used a dual-virus variant of Targeted Recombination in Active Populations (TRAP), which enabled us to express an inhibitory Gi-DREADD (hM4Di) in dentate gyrus neurons under control of the Fos promoter in a Cre recombinase and 4-hydroxytamoxifen (4OHT) dependent manner (Figure 3a,b). After training plus corticosterone treatment, mice received 4OHT to allow expression of hM4Di-mCherry or mCherry alone (control) in c-Fos+ neurons in the dentate gyrus (Figure 3c,d). Groups did not differ in freezing behaviour during training (Supplementary Figure 3), nor when mice were re-exposed to context A (without CNO; Figure 3e). Thirty minutes before exposure to neutral context B, mice received CNO to suppress the activity of neurons that were tagged after training. Whereas corticosterone-treated control mice showed substantial freezing in context B, suppression of hM4Di+ neurons reduced freezing behaviour (Figure 3e) to a level that equalled control mice (comparable with Figure 1b). Importantly, freezing to the tone remained unaffected by CNO (Figure 3e), indicating that expression of generalised fear.

Figure 3 (continued). 

c. The centre of virus expression is depicted for all the animals in schematic coronal sections. d. Representative microscopic image showing the expression of hM4Di-tagged neurons. Scale bar = 125 µm. e. Freezing in context A did not differ between groups (t(15)=0.39, p=0.70). Silencing hM4Di-tagged neurons with CNO reduced freezing levels in the neutral context B (t(15)=4.97, p<0.001. Freezing to the tone was unaffected by silencing these neurons (t(15)=1.06, p=0.31). mCherry: n = 7; hM4Di-mCherry: n = 10. Data are means ± s.e.m. Statistical analysis was done with Student’s unpaired t-test. *: p<0.05; **: p<0.01; ***: p<0.001.
DG ensembles were not involved in auditory fear memory, but were necessary for the expression of generalised contextual fear memory.

Our present data show that corticosterone reduces memory specificity, in line with an earlier study demonstrating that corticosterone elicits memory impairments and inappropriate fear responses\(^4\). Memory specificity is related to the size (i.e. the number of neurons) of neuronal ensembles\(^1\). In line with this, we find that glucocorticoid effects on memory generalisation were accompanied by an increase in the number of activated DG neurons within the first 24 h after conditioning and an increased percentage of reactivated DG neurons upon memory expression in a safe context. Thus, the increase in neuronal ensemble size may result from a corticosterone-evoked increase in excitation of DG neurons through enhanced synaptic transmission, thereby promoting recruitment of additional cells into the fear memory trace. Alterations in the excitation of engram cells may therefore affect the size of a fear-encoding ensemble and induce fear generalisation. In line with this, we found that suppression of DG neuronal ensembles that were activated by fear conditioning and corticosterone prevented the expression of generalised contextual fear memory (Figure 3e).

The present study demonstrates that GCs reduce memory specificity, thereby promoting fear memory generalisation. We further show for the first time that GCs alter the size of distinct neuronal ensembles in the DG. Selectively suppressing these neuronal ensembles restores memory specificity. Understanding how glucocorticoids modify the delicate balance between memory strength and fear generalisation on the one hand and activity in neural ensembles on the other, may open novel avenues for the development of treatments for stress-related disorders such as PTSD.

2. Materials and Methods

2.1. Mice and breeding

WT mice (Harlan, The Netherlands) and Arc::dVenus mice (kindly provided by prof. dr. Steven Kushner, Erasmus University Rotterdam) that were backcrossed for more than 10 generations into C57BL/6J mice\(^7\), were used. Mice were maintained under standard housing conditions (temperature 20-22 °C, 40-60% humidity) on a 12 h light/dark cycle with standard chow and water available ad libitum. A radio provided background noise. All experiments were performed during the light phase, using male adult mice (postnatal weeks 8–12). Mice were individually housed for 14 days prior to the start of experiments. Experimenter
blinding was performed whenever possible. All experiments were conducted under the EU directive 2010/63/EU for animal experiments and were approved by the animal welfare committee of the University of Amsterdam.

2.2. Fear conditioning

Fear conditioning was performed using a standard fear conditioning chamber with a stainless steel grid floor connected to a shock generator. Mouse behaviour was recorded by a camera connected to a computer with Ethovision software (version 13.0, Noldus, The Netherlands), which automatically scored freezing behaviour. On the day of fear conditioning, mice receiving paired training were placed in the conditioning chamber for 180 s, followed by a series of three co-terminating presentations of a tone conditioned stimulus (CS) (30 s, 2.8 kHz, 82 dB) and a foot shock unconditioned stimulus (US) (2s, 0.2 mA). The inter-stimulus interval between tone-shock presentations was 60 s. In the unpaired training group, mice received the identical CS and US stimuli, but in an explicitly unpaired sequence. The first shock was delivered 180 s after introducing mice in the conditioning chamber, followed by 2 tones 30 s later, 20 s apart, followed 30 s later by a shock, and 30 s later another tone, followed 60 s later by the final shock. CS-evoked freezing was tested 24 hours after conditioning in the same (180 s), or a completely novel context (180 s baseline, 30 s tone). Naïve mice remained in their standard housing conditions until immediately prior to behavioural testing for context- or tone-evoked freezing.

2.3. Drug treatment

Corticosterone (Sigma) (16 mg/ml dissolved in 99.9% EtOH and diluted 40x in saline; final dose: 2 mg/kg, injection volume: 5 µl/g body weight) or both corticosterone and RU486 (Sigma) (40 mg/ml dissolved in 99.9% EtOH and diluted 20x in arachide oil; final dose: 10 mg/kg, injection volume 5 µl/g body weight) was injected intraperitoneally (i.p.) immediately following fear conditioning. The appropriate vehicle solution was administered to control mice accordingly.

2.4. Immunohistochemistry

Mice were sacrificed by decapitation, and brains were dissected and post-fixed in 4% paraformaldehyde in phosphate buffer (0.1 M PB, pH 7.4) at 4 °C. Left hemispheres were collected in light of the different Arc expression between the hemispheres, and were cryo-protected overnight in 30%
sucrose/0.1 M PB. Coronal sections of 40 µm, rostral to caudal, were collected using a freezing microtome and stored in antifreeze solution (30% ethylene glycol, 20% glycerol, 50% 0.05M PBS) at -20 °C until staining. All stainings were performed on parallel series from the same brains within a breeding cohort. Sections were incubated with Fab Fragments (1:200, AffiniPure Fab Fragment Goat anti-mouse IgG, Lot # 121337, Jackson ImmunoResearch) in case a mouse antibody was used. Sections were pre-incubated with a blocking mix (5% Normal Donkey Serum; 1% Bovine Serum Albumin) for 30 mins on a shaker at room temperature. They were then incubated with primary antibodies in blocking mix and stored overnight at 4°C after 1h at room temperature. The primary antibodies that were used were: Rabbit anti c-fos (sc-52, Lot # A3014, Santa Cruz Biotechnology, 1:1000); Mouse monoclonal ARC (sc-17839, Lot # J3116, Santa Cruz Biotechnology, 1:500); and Chicken anti-GFP (Lot # ab13970, Abcam, 1:750). Sections were washed with 0.1 M PBS (pH 7.4), and incubated with corresponding secondary antibodies in blocking mix for 2 h at room temperature. As secondary antibodies we used Alexa Fluor Dn anti-Rb 647 (Lot # 1626613, Life Technologies, 1:500); Alexa Fluor Gt anti-Ms 594 (Lot # 1107474, Life Technologies, 1:500); Alexa Fluor Gt anti-Ch 594 (Lot # A21468, Invitrogen, 1:500); and Alexa Fluor Gt anti-Ms 594 (Lot # 1920483, Invitrogen, 1:500). They were washed with 0.1 M PBS, placed in 0.01 M PB and mounted on slides. Vectashield with DAPI (Vectashield Mounting Medium with DAPI, H-1200, Vector Laboratories Inc.) was used for cover slipping.

Quantitative analysis of dVenus, arc, c-fos, and DAPI positive nuclei was performed on coronal sections of the dentate gyrus of the dorsal hippocampus (bregma -2.0 mm to -3.2 mm) across at least six sections per animal of matched anatomical levels along the rostro-caudal axis. Using a Nikon DS-Ri2 microscope, representative images of 10x magnification were systematically captured, and cells were manually counted. To assess colocalisation, cells (as assessed by DAPI+) were assigned to one of the following categories: 1) Arc-, c-fos-, 2) Arc+, c-fos-, 3) Arc-, c-fos+, 4) Arc+, c-fos+, and the portion of cells colocalising was calculated per animal.

2.5. Stereotactic microinjections

Before surgery, mice were pretreated with Temgesic (0.1 mg/kg) (RB Pharmaceuticals, UK), anesthesised with isoflurane, and placed in a stereotaxic frame. Lidocaine (2%, Sigma-Aldrich Chemie N.V., The Netherlands) was topically applied to the skull to provide local analgesia. Skin was retracted and holes were drilled in the skull above the dorsal hippocampus. AAV-Fos::CreERT2 (titer: 1.2 x 1012) and Cre-dependent AAV-hSyn:DIO-hM4Di and AAV-hSyn::DIO-
mCherry (titer: 5-6 x 10^{12}) were packaged as serotype 5 virus. A virus mixture of AAV5-Fos::CreERT2 and Cre-dependent AAV (ratio 1:500, AAV-Fos-CreERT2 was injected at a final titer of 2.4 x 10^9) was infused bilaterally in the dentate gyrus using microinjection glass needles (5 µl; flow rate: 0.1 µl/min; AP = -2.2; ML= ±1.2; DV= -2.2, relative to Bregma). After surgery, mice remained in their home cage for 3 weeks until the start of behavioural experiments.

2.6. 4-Hydroxytamoxifen (4OHT) treatment

4OHT (H6278, Sigma-Aldrich Chemie N.V., The Netherlands) (50 mg/ml 4OHT dissolved in DMSO (D8148, Sigma-Aldrich Chemie N.V., The Netherlands) and diluted 10x in saline containing 2% Tween80 (P1754, Sigma-Aldrich Chemie N.V., The Netherlands) and 10x in saline; final concentration: 2.5 mg/ml 4OHT, 5% DMSO and 1% Tween80 in saline) was injected 2 h after training in hM4Di-mCherry and control mice (final dose: 25 mg/kg).

2.7. Chemogenetic intervention

Clozapine N-oxide (CNO, BML-NS105, Enzo LifeSciences, Brussels) (dissolved in saline; final dose: 5 mg/kg, injection volume 10 µl/g body weight) was injected i.p. 30 min before a retrieval session in both mCherry and hM4Di-mCherry mice.

2.8. Electrophysiology

Mice were sacrificed through quick decapitation, followed by rapid removal of the brain in ice-cold oxygenated (95% O_2/5% CO_2) solution containing (in mM): Cholinechloride (120), glucose (10), NaHCO_3 (25), MgSO_4 (6), KCl (3.5), NaH_2PO_4 (1.25), CaCl_2 (0.5). Coronal slices (250 µm) were cut using a microtome (Leica VT1000S). For recovery, slices were incubated for 20 minutes in warm (32 °C) oxygenated standard artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl (120), KCl (3.5), MgSO_4 (1.3), NaH_2PO_4 (1.25), CaCl_2 (2.5), glucose (10), NaHCO_3 (25), after which the sections were maintained at room temperature (20 °C). Sections containing the dorsal hippocampal CA1 area (bregma -2.0 mm to -3.2 mm) of the left hippocampus were placed in a recording chamber with a constant flow of oxygenated aCSF.

Whole cell patch clamp recordings were made using an AXOPATCH 200B amplifier (Axon Instruments, USA), with electrodes from borosilicate glass (1.5 mm outer diameter, Hilgerberg, Malsfeld, Germany). The electrodes were pulled
on a Sutter (USA) micropipette puller. The pipette solution contained (in mM)
120 Cs methane sulfonate; CsCl (17.5); HEPES (10); BAPTA (5); Mg-ATP (2); Na-GTP
(0.5); QX-314 (10); pH 7.4, adjusted with CsOH; pipette resistance was between
3–6 MΩ. Under visual control (40X objective and 10X ocular magnification)
the electrode was directed towards a neuron with positive pressure. Once
sealed on the cell membrane (resistance above 1 GΩ) the membrane patch
under the electrode was ruptured by gentle suction and the cell was kept at
a holding potential of -70 mV. The liquid junction potential caused a shift of
no more than 10 mV, which was not compensated during mEPSCs recording.
Recordings with an uncompensated series resistance of <15 MΩ and <2.5
times of the pipette resistance with a shift of <20% during the recording, were
accepted for analysis. Data acquisition was performed with pClamp 8.2 and
analysed off-line with Clampfit 9.0. Miniature excitatory postsynaptic currents
(mEPSCs) were recorded at a holding potential of -70 mV. Tetrodotoxin (0.25
µM, Latoxan, Rosans, France) and bicuculline methobromide (20 µM, Biomol)
were added to the buffer to block action potential induced glutamate release
and GABAA receptor mediated miniature inhibitory postsynaptic currents
(mIPSCs), respectively. The events were identified as mEPSCs when the rise
time was faster than the decay time. mEPSCs were recorded for 5 min in each
cell.

2.9. Statistical analysis

Data were analysed using SPSS 22.0 (IBM software). All data are expressed as
mean ± standard error of the mean (SEM). Data were considered statistically
significant when p<0.05. Outliers were determined using a Grubb’s test.
Independent-samples t-tests were performed to assess differences between
Saline and CORT-treated mice. Appropriate corrections were applied when
assumption of homogeneity of variance was not met. When assumption of
normality was not met, Mann-Whitney test was conducted. A repeated measure
ANOVA was performed to assess freezing behaviour during the different
tones. Greenhouse-Geisser correction was applied when the assumption of
sphericity was violated.

3. Acknowledgements

The authors want to thank prof. dr. Steven Kushner (Erasmus Medical Center,
Rotterdam) for providing the Arc::dVenus mice, and dr. Priyanka Rao-Ruiz for
helpful discussions during the design of experiments.
4. Author contribution

SLL, MCvdO, and HJK contributed to conception and design of the study; SLL organised the database; SLL, RJvdL and NI conducted the experiments, SLL and NI performed the statistical analysis; SLL wrote the first draft of the manuscript; SLL, MCvdO, NI, PJL and HJK contributed to manuscript revision, read and approved the submitted version.
Chapter 9

5. References

6. Supplementary figures

Supplementary Figure 1: Plasma corticosterone levels measured at different time points after fear conditioning. Corticosterone injection results in increased circulating corticosterone levels 30 and 60 minutes after the fear conditioning. Data are means ± s.e.m.
Supplementary Figure 2: Unpaired or foreground conditioning (no tone present) does not result in differences in freezing after corticosterone injections. 

a. Schematic of the unpaired conditioning paradigm. 
b. Response of mice treated with saline or corticosterone following an unpaired fear conditioning paradigm to the context and tone. No difference in freezing levels was observed, nor in the ratio of freezing between context A over B, or between tone and cue freezing, as measures for memory specificity. n = 8 mice/group. Data are means ± s.e.m. Statistical analysis was done with Student’s unpaired t-test. Black thin lines: individual data points of saline-treated mice; black thick line: average of all saline-treated mice; red dashed thin lines: individual data points of corticosterone-treated mice; red dashed thick line: average of all corticosterone-treated mice.
Supplementary Figure 3. Freezing levels during training were comparable between mCherry and hM4Di-injected mice (F(1,15)=0.15, p=0.71). mCherry: n = 7; hM4Di-mCherry: n = 10. Data are means ± s.e.m. Statistical analysis was done with a repeated measures ANOVA.
General discussion

In this thesis, I investigated how stress, either chronically early in life, or acutely after learning experiences, affects learning and memory processes. I studied this in the adult and aging brain, and examined the underlying mechanisms. The first aim was to elucidate whether positive and/or negative early life experiences contribute to a different onset, or exacerbation of cognitive decline in relation to Alzheimer’s Disease (AD), and what the underlying mechanisms are. In the General Discussion – part 1, I will give a broader perspective on the current literature and discuss the role of early life experiences in modulating AD risk and progression, primarily from a preclinical perspective, and integrate our own findings presented in this thesis (Chapter 2 to 7). I finish with some outstanding questions that have arisen from our work and that of others.

A second question that I addressed in this thesis, is how glucocorticoid hormones determine memory strength and memory specificity, and what the underlying neuronal substrates are. Therefore, in the General Discussion – part 2, I discuss how neuronal ensembles, or engram cells that represent memory traces, are modified under the influence of stress hormones, and how this affects memory formation.
Parts of this discussion were published in adjusted form as:

Vulnerability and resilience to Alzheimer’s disease: Early life conditions modulate neuropathology and determine cognitive reserve


1. Early life experiences affect AD neuropathology and cognition

Several clinical studies have suggested an association between either stressful or enriching/stimulating experiences during childhood and the later risk to develop Alzheimer’s disease (AD). For example, more years of formal education have been associated with a lower risk to develop AD\(^1,2\). Also, early life ‘enrichment’ and more cognitive challenges and ‘stimulation’ were correlated with a later manifestation of AD symptoms\(^2-5\). In contrast, clinical literature further indicates that stressful events early in life are associated with a higher chance to develop AD\(^3,6\). While this association underscores the possible importance of the early postnatal period for AD symptomatology and neuropathology, the long time lag in between both hampers a deeper understanding of the underlying causes and possible mechanisms. To address this, animal models allow a more detailed investigation of these cause-and-effect relationships that may help to identify the underlying mechanisms by which e.g. environmental factors during the early life period can modify later AD symptoms and pathology. For example, the ability to model specific genetic risk factors for AD and the precise temporal control over the occurrence of life events make animal models highly suitable to study these mechanisms and the interactions between genes, the (early) environment and later AD risk.

1.1. Early life adversity and AD

As outlined in Chapter 1, genetically modified mice allow for modelling specific pathological features of AD, such as the expression of A\(\beta\) and tau at pathological levels. Many studies in transgenic mice have now demonstrated effects of early life experiences on later cognitive function. In the widely used APPswe/PS1dE9 mice, cognitive performance at an adult age was generally impaired when the mice had been exposed to prenatal or other forms of early life stress. For instance, exposing these mice to repeated, brief periods of restraint stress from embryonic day 1 to 7 resulted in impairments in object location memory at 6 months of age\(^7\). In addition, maternal separation attenuated spatial learning as tested in the Morris water maze task in 9 months old mice\(^8\). Furthermore, we have shown in this thesis that APPswe/PS1dE9 mice exposed to chronic early life stress from PND 2-9 displayed cognitive impairments one year later, specifically in the cognitive flexibility domain (Chapter 4)\(^9\). These latter effects were not caused by early life stress alone, since wild type mice exposed to early life stress were not impaired. Together, this suggests that early life stress may modify AD related processes and thereby accelerate and/or aggravate AD symptom development (Chapter 4)\(^9\).
These cognitive impairments are often accompanied by alterations in Aβ neuropathology. In middle-aged APPswe/PS1dE9 mice, we show that both plaque load and the soluble intracellular Aβ levels were increased following early life stress exposure (Chapter 4)⁸⁻¹⁰, although at 4 months of age, also a decrease in cell-associated Aβ has been reported after early life stress¹⁰. Interestingly, we observed the most striking differences in Aβ neuropathology in 6 month old APPswe/PS1dE9 mice, where early life stress induced a striking 6- to 7-fold increase in hippocampal soluble Aβ-40 levels, and even an 8- to 9-fold increase in Aβ-42 levels (Chapter 4). Although the relative increase in Aβ-42 levels after early life stress is much smaller at 12 months of age (1.2-fold increase), the overall Aβ-42 levels at 12 months are much higher. This results in a higher absolute difference in Aβ-42 pathology following early life stress, that also correlated with the deficits in cognitive flexibility observed at this age. Given the latter effect, it would be interesting to also measure soluble Aβ levels in the prefrontal cortex (PFC), as this brain region is strongly implicated in behavioural flexibility. Surprisingly, we did not observe a difference in hippocampal plaque load at any of the ages in this study (except in the subiculum at 6 months of age), suggesting a higher degradation and/or clearance of Aβ following early life stress. Also this needs to be further substantiated, e.g. by evaluating the expression of Aβ-degrading enzymes like neprilysin¹¹ and insulin-degrading enzyme (IDE)¹², and phagocytic uptake and degradation of Aβ (see also section 2.3).

Effects of early life adversity on later AD measures have been studied in other transgenic mouse models as well. For instance, in a model co-expressing mutant APP and tau (biAT-mice), chronic early life stress increased soluble Aβ levels already in four month old mice while it at the same time significantly reduced their life expectancy (Chapter 2)¹³. This illustrates that in a genetic background relevant for AD, additional exposure to early life stress can increase Aβ neuropathology prior to the onset of cognitive impairments and even affect life expectancy. However, as the cause of death has not been investigated extensively in this model, it remains unclear how ELS altered lifespan. Previous studies in the parental Tau.P301L line, which display hindbrain tauopathy, revealed an inverse relation between brainstem tauopathy and lifespan¹⁴,¹⁵. As the brainstem controls autonomous vital systems such as breathing, swallowing, and blood pressure, this may give a hint toward the cause of the premature death in Tau.P301L mice. However, the lifespan of biAT mice is even stronger reduced compared to Tau.P301L mice, suggesting that the additional and specific contribution of the APP.V717I mutant protein, possibly in combination with the tau mutation, is involved in the reduced survival of these ELS-exposed animals.
Alternatively, different transgenic AD mouse models expressing mutant forms of APP also die prematurely, possibly due to spontaneous seizures and abnormal epileptiform electroencephalography (EEG) activities\(^\text{16,17}\). Interestingly, ELS was also associated with a higher incidence of spontaneous seizures\(^\text{18-20}\) and enhanced excitability\(^\text{21,22}\). This may link ELS to the reduced survival in biAT animals possibly by potentiating epileptic seizures. Although these preclinical observations are consistent with the higher incidence of epileptic activity in aged AD patients as compared to non-demented elderly\(^\text{23}\) as also found with intracranial electrodes\(^\text{24,25}\), the timeline and mechanisms of human and rodent presentation of symptoms (neuropathology, cognitive decline, epileptic seizures) does not overlap, obscuring a direct translational value of our present findings.

The effects of stress early in life on both later cognition and AD-related neuropathology may not be specific for transgenic animals since also in wild type rodents, impairments in cognition occur following maternal separation that are accompanied by increased levels of A\(\beta\)-40 and -42, together resulting in an exacerbation of A\(\beta\) levels\(^\text{26}\), BACE expression\(^\text{27}\) and tau phosphorylation\(^\text{26,28-32}\). While in wild type animals, the A\(\beta\) monomers do not aggregate into neurofibrillary plaques, these findings do suggest that regardless of the genetic background of an animal, stress exposure, be it early or later in life, may promote APP processing towards the production of more amyloidogenic species, and may thereby modify the sensitivity to develop AD pathology later in life.

### 1.2. Early life enrichment and AD

Although less well studied, exposing mice to an enriched and ‘positive’ environment during the early life period may exert opposite effects on cognition and AD-related neuropathology when compared to conditions of early life stress (Chapter 3)\(^\text{13,33,34}\). For instance, neonatal handling (early handling, EH), twice daily from PND 1-21, which has been associated with enhancing levels of maternal care, prevented spatial cognitive deficits and emotional alterations at four months of age in 3xTgAD mice, an effect that was most pronounced in females\(^\text{33}\). Similarly, we found that daily handling from PND 2-9 prevented the cognitive impairments in APPswe/PS1dE9 mice at 11 months of age (Chapter 3)\(^\text{34}\). Interestingly, while deficits in hippocampus-dependent and PFC-dependent-memory performance were prevented by the neonatal handling procedure in this study, we found that amygdala-dependent memories were not affected in the same animals (Chapter 3)\(^\text{34}\). In line with this, EH reduced A\(\beta\) plaque load in the hippocampus, but not in the amygdala (Chapter 3)\(^\text{34}\).
In 4 month old biAT mice, neonatal handling reduced Aβ levels prior to the emergence of cognitive deficits, and notably, prolonged lifespan (Chapter 2)\textsuperscript{13}. Combined, these studies indicate that neonatal handling reduces or delays the incidence of AD-related pathology and cognitive impairments, with differential effects on hippocampal and amygdala function. This could potentially be attributed to a difference in developmental trajectories, since the hippocampus and amygdala display clear differences in postnatal growth and maturation\textsuperscript{35,36}. So far, it remains elusive what defines the optimal time window for installing lasting protective effects, an area of research that deserves more attention. In addition to the effects of positive stimuli during the early life period, there are other studies showing protective effects of environmental stimuli, such as housing mice in enriched environmental conditions or allowing them to exercise at adult or late age, on cognitive or neuropathological measures in different AD models (see e.g.\textsuperscript{37–43}).

1.3. Role of the PFC

Most AD rodent studies have investigated changes in the cognitive domain as important readout parameter, with a strong focus on spatial memory and hippocampal neuropathology. Considerably less attention has been given to cognitive deficits caused by AD-pathology in other brain regions. For instance, both the observed deficits in behavioural flexibility following chronic early life stress (Chapter 4)\textsuperscript{9}, and the improvement in working memory performance that occur following neonatal handling (Chapter 3)\textsuperscript{34} hint towards modifications in PFC function. The PFC could be a particularly relevant brain area in relation to ELS since it develops strongly in the first postnatal week (comparable to the hippocampus), exceeding the developmental window of most other brain structures\textsuperscript{36,44}. In wild type mice, early life stress has already been shown to hamper dendritic development and spine density in the PFC, and maternal deprivation has been reported to affect PFC volume\textsuperscript{45}, further indicating the relevance to investigate this brain region in more detail in relation to early life stress and AD outcome\textsuperscript{46}.

1.4. Role of tau pathology

In addition, tau pathology has so far received less attention with respect to early life programming. Tau pathology is potentially relevant given that its expression correlates well with cognitive deficits in Alzheimer’s disease, more so than amyloidoic levels. Also, the expression of tau protein and its phosphorylation is strongly developmentally regulated\textsuperscript{47–49}, is sensitive to stress\textsuperscript{45,46}, and tau
phosphorylation has been associated with cognitive decline\textsuperscript{52,53}. In addition, reducing tau levels per se is protective in specific disease conditions (Cheng 2014) including amyloid induced cognitive deficits\textsuperscript{54–57}.

There is a substantial amount of literature on the effects of adult stress or glucocorticoid exposure on various tau and phosphorylation measures in different tau and AD mouse models, often studied from a cognitive perspective or in relation to structural plasticity, neurogenesis and sex\textsuperscript{32,50,51,58–64}. Tau pathology, which is generally represented by intracellular neurofibrillary tangles\textsuperscript{65}, is enhanced by stress-exposure\textsuperscript{66} and hyperphosphorylated tau, as precursor to intracellular tangle formation, was e.g. found to accumulate after exposure to a stressor\textsuperscript{60,67–70}.

In transgenic mice, so far only few studies have evaluated tau levels following early life experiences. We found that both early life stress and neonatal handling do not strongly affect later tau pathology in biAT mice (Chapter 2)\textsuperscript{13}, although this could possibly be attributed to the relatively young age at which we evaluated tau pathology. Others have found improved cognition and reduced tau pathology following maternal dexamethasone, a synthetic glucocorticoid receptor (GR) agonist, treatment\textsuperscript{61}. Although early environmental factors have been shown to affect tau hyper-phosphorylation in wild type rodents\textsuperscript{26,64,71}, further studies, especially in more advanced tau pathology models, are required before any conclusions can be drawn with regard to the possible consequences of early life experiences for later tau pathology.

1.5. Conclusion

In conclusion, there is substantial evidence from rodent studies that the perinatal environment determines later brain vulnerability or resilience against cognitive impairments and Aβ neuropathology later in life. Early life adversity generally worsens cognitive performance and aggravates Aβ neuropathology, whereas more positive experiences like early life enrichment can delay these cognitive deficits, at least for some behavioural domains, and attenuate Aβ neuropathology.

2. Early life environment, Aβ and tau neuropathology

To further understand the association between early life experiences and the vulnerability or resilience to develop AD, it is important to understand how early life experiences can alter AD pathology. The steady-state levels of Aβ depend
on a balance between APP processing, the rate of amyloid production and clearance of the peptide from the brain\textsuperscript{72}. Several lines of evidence indicate that early life experiences could affect these processes. Likewise, also tau hyper-phosphorylation can be potentiated by factors induced early in life.

### 2.1. Hypothalamus-pituitary-adrenal (HPA)-axis

As discussed in Chapter 1, the early postnatal environment is a strong determinant of HPA axis activity, thereby regulating later-life sensitivity to stressors\textsuperscript{73} and circulating levels of glucocorticoid hormones (cortisol in humans, corticosterone in rodents). Positive early life experiences generally dampen HPA axis reactivity, resulting in lower CRH and glucocorticoid levels in response to a stressor, whereas early life adversity generally increases HPA axis reactivity\textsuperscript{73,74}. As a consequence, the subsequent, cumulative exposure to glucocorticoids and/or CRH in adult animals is often persistently enhanced by early life stress exposure\textsuperscript{74}.

Human studies have shown that at the early stages of AD, basal levels of circulating cortisol are often elevated\textsuperscript{75–78}. AD and dementia patients also show a failure to suppress their endogenous cortisol after administration of the synthetic glucocorticoid dexamethasone\textsuperscript{79–81}, indicating a dysfunction in the feedback of the HPA axis. Elevated basal cerebrospinal fluid (CSF) cortisol levels were specifically found in MCI patients who later developed AD, but not in MCI patients with other underlying neuropathologies\textsuperscript{82}. Moreover, higher baseline CSF cortisol levels were associated with a faster clinical worsening and cognitive decline in MCI patients once they were developing AD\textsuperscript{82}. However, HPA dysfunction does not seem to worsen any further as the disease progresses\textsuperscript{83,84}, suggesting that early life induced alterations in HPA axis function may in particular accelerate the onset of AD pathogenesis.

Rodent studies further indicate that repeated stress exposure or pharmacological treatment with (synthetic) glucocorticoids can induce pathological processing of both A\textsubscript{β} and tau in 3xTg-AD mice\textsuperscript{67} or wild type rats\textsuperscript{85}. In addition, using the GR antagonist mifepristone (RU486), we were able to reverse the ELS-induced impairments in cognitive flexibility as well as reduce A\textsubscript{β}-40 and A\textsubscript{β}-42 levels. These effects were most prominent in ELS-APPswe/PS1dE9 mice. This finding provides support for the notion that changes in HPA axis activity contribute to the accelerated cognitive decline in ELS-APPswe/PS1dE9 mice. In addition, a reduction in APP-derived C99 and C83 fragments was reported in 3xTg-AD mice after mifepristone treatment indicating effects on the amyloid processing pathway\textsuperscript{86}. Since modulation
of glucocorticoid levels or GR activation can modify Aβ neuropathology\textsuperscript{67,87,88}, this points towards a causal involvement of glucocorticoids in the initial development, or later promotion of AD neuropathology, rather than that the alterations in glucocorticoids observed in AD may result as a consequence of disease progression.

Notably, early life stress also increased the expression of BACE1 in adult APPswe/PS1dE9 mice (Chapter 4)\textsuperscript{9}, and in wild type mice\textsuperscript{26,89,90}. Enhanced BACE1 expression following early life or adult stress exposure can be a direct effect of altered glucocorticoid signalling, as BACE1 contains glucocorticoid binding sites\textsuperscript{91}. Indeed, mifepristone treatment, reducing corticosterone levels, also reduced BACE1 expression (Chapter 4)\textsuperscript{9}. Thus, altered BACE1 expression could be a mechanism through which glucocorticoid signalling modifies AD outcome.

Following chronic early life stress, wild type animals show decreased corticosterone levels in response to an acute stressor, whereas APPswe/PS1dE9 mice exposed to the same paradigm, but not control-reared AD mice, displayed elevated corticosterone levels (Chapter 4)\textsuperscript{9}. This suggests that AD neuropathology by itself can also affect HPA axis functioning. In order to determine whether alterations in HPA axis activity indeed precede or determine the enhanced AD neuropathology, HPA axis functioning should be evaluated at a younger age, and e.g. selectively modulated experimentally prior to the manifestation of ELS-induced alterations in AD neuropathology.

Increased glucocorticoid exposure most likely cannot fully account for all neuropathological changes observed following early life experiences. In Chapter 3 we observed an increase in stress and recovery levels of corticosterone in early handled APPswe/PS1dE9 mice, which were accompanied by a rescue in cognitive deficits and a reduction in plaque load\textsuperscript{34}. These observations suggest that other factors arising early in life, beside corticosterone signalling, contribute to the resilience to develop AD (see section 3 for potential mechanisms).

Although less extensively described in recent literature, chronic stress or glucocorticoid exposure also induce abnormal hyper-phosphorylation of tau in wild type\textsuperscript{29} (see also paragraph 1.4) and 3xTg-AD mice\textsuperscript{67}. Glucocorticoids e.g. potentiate the ability of centrally infused Aβ to induce hyper-phosphorylation of tau epitopes associated with AD\textsuperscript{29}, suggesting that also tau pathology is affected by HPA axis-related mechanisms. Although speculative, this could be a mechanism by which early life experiences, via alterations in HPA axis activity, could modulate the development of tau pathology in later stages\textsuperscript{92,93}. Together, these studies indicate that alterations in glucocorticoid signalling,
which are both altered by early life experiences, can potentially promote AD pathology. Modulating these systems may thus directly affect pathological markers like Aβ production and tau hyper-phosphorylation. However, further research is needed to study the exact underlying mechanisms and causality, in particular with respect to tau pathology.

### 2.2 Blood–brain barrier integrity

The total amounts of Aβ in the brain are controlled by a steady-state, homeostatic balance of production and removal. In humans, approximately 25% of Aβ is cleared from the brain via the blood-brain barrier (BBB). Post-mortem studies have shown that BBB integrity declines with age, and might therefore be involved in the onset of dementia. Both acute and chronic activation of the stress system may compromise the permeability of the blood-brain barrier. Restraint stress in rodents induces damage to the capillary brain endothelial cells and alters expression of the tight-junction proteins occludin, claudin-5, and glucose transporter-1 in brain capillaries, pointing to impaired BBB functioning.

In particular, low density lipoprotein receptor-related protein 1 (LRP1) and the receptor for advanced glycation end products (RAGE) seem to play central roles in transport of Aβ over the BBB. RAGE is an important transporter in regulating the influx of circulating Aβ into the brain. Corticosteroid treatment increases the expression of RAGE which can thereby promote transport of Aβ into the brain. In addition, LRP1 regulates the efflux of brain-derived Aβ into the circulation. Interestingly, LRP1 has glucocorticoid receptor binding sites and is, at least in macrophages, regulated by glucocorticoids. Dysfunction of LRP1 following prolonged high exposure of glucocorticoids could therefore result in a less efficient transport of Aβ from the brain and result in increased Aβ levels in the brain. Although further experimental validation is required, particularly with regard to how early life experiences regulate BBB integrity and permeability, (early) stress could potentially influence Aβ clearance from the brain by altering BBB permeability.

### 2.3. Neuroinflammation

In addition, the neuro-inflammatory response is involved in Aβ clearance. Microglia e.g. bind Aβ oligomers and fibrils and clear Aβ from the brain through the secretion of Aβ-degrading enzymes like neprilysin, insulin-degrading enzyme (IDE), and through the phagocytic uptake and active degradation of Aβ. Both IDE and neprilysin activities are reduced in AD, and, interestingly,
are further inhibited by glucocorticoids\textsuperscript{103}. In response to Aβ oligomers, microglia induce an acute inflammatory response, to aid clearance and restore homeostasis\textsuperscript{104-106}.

However, when Aβ levels accumulate over time, the physiological functions of microglia, such as synaptic remodelling, may be compromised and lead to a chronic neuro-inflammatory response\textsuperscript{107}. The progressive microglial activation, elevated pro-inflammatory cytokine levels and morphological changes of microglia together may result in functional and structural neuronal alterations that ultimately can promote neuronal degeneration\textsuperscript{107}. Adverse early life experiences alter the number, morphology, phagocytic activity and gene expression of microglia in the developing hippocampus, which extends into the juvenile period (reviewed by\textsuperscript{108-110}), and also affects their neuroinflammatory response to amyloid in APPswe/PS1dE9 mice in an age-dependent manner\textsuperscript{10}. These changes are associated with abnormalities in developmental processes mediated by microglia, including synaptogenesis, synaptic pruning, axonal growth, and myelination (reviewed by\textsuperscript{110,111}) and are thought to make them more responsive to subsequent inflammatory challenges like Aβ (microglial 'priming')\textsuperscript{109,112-114}. Conversely, neonatal handling programs the expression of the anti-inflammatory cytokine IL-10 early in development by decreasing its methylation within microglia and attenuating glial activation\textsuperscript{115}. Whether the enhanced Aβ pathology after early life stress reduces the microglial response, or whether early life programming causes alterations in microglial activation, which in turn may modulate Aβ neuropathology, requires further investigation.

Together, impairments in glial functioning and/or in the inflammatory response to Aβ, possibly modulated or “primed” by early life experiences, could lead to the altered Aβ phagocytic capacity or clearance, and hence an altered Aβ burden with increasing age. Further studies are required to examine whether positive early life experiences increase AD resilience via the modulation of neuro-inflammatory responses and whether early life events can program microglia directly or indirectly.

3. **Modulation of cognitive and brain reserve by early life experiences**

Besides their direct role in AD neuropathology and related cognitive decline via regulating Aβ and tau, early life experiences may also modify the brain's ability to cope with the pathological burden of AD. For instance, a healthier, more active and more flexible brain may have a higher capacity to “circumvent” or delay effects of an insult and hence cope in a better way with the challenges
posed by the AD pathology\textsuperscript{16}. This concept has been termed “brain” or “cognitive reserve”, and has been introduced to explain individual variation in vulnerability and resilience for age-related cognitive decline (see Box 1). These concepts stem from findings that brain pathology (such as plaque load) is an unreliable predictor of human cognitive performance per se given that with a comparable pathological load, some patients perform better than others in cognitive tasks\textsuperscript{1}. This could be a secondary mechanism, in addition to the mechanisms described above, through which early life experiences determine behavioural AD outcome.

3.1 Early life experiences, brain and cognitive reserve

The hypothesis that early life experiences influence brain or cognitive reserve, and may either protect against or aggravate the clinical consequences of AD neuropathology, comes from several epidemiological studies. For instance, individuals with less than 8 years of formal education had a 2.2 times higher risk of developing dementia than individuals with more than 8 years of education. Moreover, participants with low socio-economic status were at 2.25 times greater risk of developing dementia than those with high lifetime occupational attainment\textsuperscript{17}, and the occurrence of a parental death between age 0-18 is associated with a higher incidence of AD\textsuperscript{6}. Conversely, higher social economic family status reduced the risk of dementia lastingly\textsuperscript{3}, while both the number of years of formal education\textsuperscript{2}, and higher school grades appear to protect against dementia, even in the absence of later-life educational or occupational stimulation\textsuperscript{4}, while early life linguistic ability is a strong predictor for later-life cognitive performance and being raised in a bilingual family e.g. protects against AD\textsuperscript{5,118}. Furthermore, elderly people participating in frequent leisure activities expressed a 38\% lower risk of developing dementia\textsuperscript{119}.

Such associations between early life environmental factors and AD indicate that cognitive stimulation at an age at which the brain is still in development may contribute to the building of cognitive reserve and thereby reduce the risk for later AD, while disturbances like stress or trauma during early life can be detrimental for building cognitive reserve. This is consistent with observations in animal studies demonstrating the existence of specific “critical periods” during early life\textsuperscript{20,121} when disturbances in neuroplasticity can have a long-lasting impact on brain function. Overall, these studies indicate that educational and possibly also specific occupational experiences may create a reserve that could delay consequences of AD pathology for behavioural symptoms (Figure 1). This does not exclude the important contribution of the genetic background nor of familial and societal factors that may also promote
a higher educational and occupational stimulation and thereby a better coping with pathological load in later ages.

Despite the support for this theory from epidemiological studies, more
controlled studies aimed to determine whether (early) environmental factors
can actually help build AD resilience are thus far limited. In particular, the
question remains open what the molecular and cellular substrates are that
mediate such effects of life experiences, especially those occurring early in life,
on cognitive reserve and clinical AD outcome.

3.2. Animal research on early life experiences and cognitive reserve

Although attractive as a concept, it is currently unknown which brain
mechanisms underlie brain and cognitive reserve. To address this, animal
studies are required to investigate whether and how a brain and cognitive
reserve can be installed, and what the underlying molecular and cellular
substrates are.
One possible mechanism that could underlie cognitive reserve is the ability/capacity to withstand and/or compensate for dysfunction in one brain circuit by recruiting associated, unaffected brain circuitries. This would allow for switching between cognitive strategies and for using alternative and/or additional brain networks, to e.g. better cope with the gradual accumulation of Aβ pathology. For instance, Granger et al.\textsuperscript{122} observed that male and female mice overexpressing the human APP transgene exhibited a similar neuropathological load, while females displayed earlier cognitive impairments than males. Assuming that task performance was not influenced by sex per se, this suggests that males could compensate for Aβ-associated impairments, and that they did so by alternating navigational search strategies, which would have required different brain circuits and adopting alternative spatial search strategies in the Morris water maze task. In contrast, females failed to efficiently switch from systematic to spatial learning strategies, potentially indicating a weaker cognitive reserve\textsuperscript{122}. A similar inability to adopt alternative behavioural strategies has been observed after early life stress. Prenatal and postnatal stress in rodents has been reported to bias navigation strategies towards more rigid, inflexible striatum-based learning strategies even under low stress conditions\textsuperscript{123-125}. This suggests that (early life) stress may decrease cognitive flexibility and can modify the ability to activate different brain areas, which can be interpreted as a reduction in cognitive reserve. Indeed, we show that APPswe/PS1dE9 mice exposed to chronic early life stress specifically show impaired behavioural flexibility in the Barnes maze (while initial acquisition is not worsened by early life stress) (\textit{Chapter 4})\textsuperscript{9}. More extensive studies are required, also examining the underlying circuitry, to better understand how cognitive reserve and flexibility contribute to early life stress-induced exacerbation of AD symptomatology.

### 3.3. Early life experiences and brain reserve

More research has been conducted in animal models assessing how early life experiences can affect components that contribute to brain reserve, the neural substrates of brain functioning. This involves synaptic plasticity and proteins that regulate synaptic function, that all determine plasticity of the brain and may render the brain more or less susceptible for AD related pathological changes.

#### 3.3.1. Synaptic plasticity

Disturbances in long-term potentiation (LTP) have been implicated in the early manifestation of AD\textsuperscript{126,127}. Several \textit{in vitro} and \textit{in vivo} studies have shown that Aβ oligomers trigger synaptic dysfunction (e.g.\textsuperscript{128}) by weakening synapses,
Box 2. Reflections on the limited nesting and bedding materials (“chronic early life”) stress model.

The value of any animal model relies strongly on the reproducibility of its main readouts, both within and across different laboratories. Nowadays, reproducibility is an important consideration in many animal studies, which can be compromised by subtle variations in experimental conditions and/or by low power. In particular, stress-related experiments are very sensitive to mild disturbances and variations in its procedures.

At PND 9, immediately following the early life stress procedure, outcomes of the limited nesting and bedding model seem to be robust, with comparable reports of increased CORT levels and reductions in body weight gain, that were also consistently found in all our studies (reduced body weight gain: Chapter 2, 4-7; CORT levels: unpublished observations). However, in all the studies described in this thesis, we found no effects on body weight at PND 21, and age at which some studies did report differences (e.g. 245).

At adulthood, ELS results in enhanced anxiety-like behaviour (although not found by, or by us (unpublished observation)) and impaired spatial memory. While some studies report effects of ELS on basal plasma corticosterone levels at adulthood, several studies have not found these increases. Also the effects of the model on cued fear conditioning are variable, as e.g. reported differences in the identification of “safe” periods (i.e. no tone present), while we specifically show differences in their response during the tone (Chapter 5 and 7).

In line with this, even within our own studies, we observed some contradictions in the outcomes of the model. For instance, in Chapter 5 we report aberrant, strongly increased LTP in ELS-APPswe/PS1dE9 mice, but in Chapter 6, we find LTD in the same mice. Interestingly, this was both associated with a worsened cognitive performance. Importantly, these studies analyse LTP at different ages (Chapter 5: ± 12 months old; Chapter 6 and 7: ± 6 months old), although preliminary unpublished data from the mice described in Chapter 5 at 6 months of age show a similar increase in LTP in ELS-APPswe/PS1dE9 mice. In WT mice LTP was impaired following early life stress in Chapters 6 and 7, but not in Chapter 5, which emphasises the need to investigate the role of age.
impairing LTP and by reducing the density of dendritic spines\textsuperscript{127,129–134}. Under conditions where LTP induction is already challenged, e.g. following early life stress exposure\textsuperscript{74,135,144,136–143}, the effects of Aβ on synapses and plasticity can be aggravated, thereby accelerating the onset of cognitive impairments. In contrast, enhanced LTP after early life enrichment can potentially alleviate the effects of Aβ and delay the onset of cognitive impairments. As Aβ specifically targets synapses and disrupts synaptic signalling pathways, a larger or smaller dendritic tree and/or of spine density could provide a structural substrate that could modulate effects of Aβ exposure, and hence make specific neurons more or less vulnerable to Aβ-induced dysfunction.

We have also shown that following early life stress in wild type mice, it is no longer possible to induce LTP in the CA1 of 6 month old mice \textit{(Chapter 6)}.
supporting previous findings that early life stress impairs LTP. In APPswe/PS1dE9 mice, ELS even resulted in long-term depression (LTD) (Chapter 6), which could possibly mediate the impairments in spatial memory that we observed in this study. Counterintuitively, we found that hippocampal synaptic plasticity in 1 year old APPswe/PS1dE9 mice following early life stress was in fact enhanced (Chapter 5), although this was still accompanied by decreased spatial flexibility in the Barnes Maze (Chapter 4) and diminished specificity of fear memory (Chapter 5). Although enhanced synaptic plasticity is usually associated with improvements in cognitive performance, others have also shown that networks composed of synapses that exhibit aberrant synaptic potentiation, rather than synapses in which synaptic strength is bidirectionally controlled, decrease their storage capacity and increase errors, thereby failing to adequately store memories. This awaits further study (see also Box 2 for further considerations of the use of this early life stress model).

Together, these studies indicate that early life experiences alter synaptic plasticity, which may influence the adult brain’s capacity to ‘circumvent’ and cope with AD-associated insults, thereby prolonging the period of healthy cognitive performance despite ongoing Aβ neuropathology.

### 3.3.2. N-methyl-D-aspartate (NMDA) receptors

A potential molecular mediator of altered synaptic plasticity is the expression or activation of the glutamatergic N-methyl-D-aspartate (NMDA) receptors. In Chapter 5, Chapter 6 and Chapter 7, we investigated the expression and/or function of NMDA receptor subunits following early life stress and/or in relation to an APPswe/PS1dE9 transgenic background. During development, there is a switch in NMDA-R composition, with GluN2B being predominantly present in the early postnatal brain. Over time, the number of GluN2A subunits grow, and after 2 weeks they outnumber the GluN2B. This process can be disturbed by early life stress, as maternal deprivation delays the switch to a mature, predominantly GluN2A containing NMDA-R phenotype at PND 28-31. Interestingly, by 8 weeks of age, the effects of maternal separation stress on the GluN2B-GluN2A switch were reversed to less GluN2B expression in the hippocampus. In line with this, we find in Chapter 7 reduced GluN2B subunit expression following ELS in 6 month old wild type mice. In addition, we show that fEPSP synaptic plasticity measures of 12 month old ELS-APPswe/PS1dE9 mice are largely insensitive to application of the GluN2B antagonist Ro25 6981 (Chapter 5). This suggests that possible alterations in NMDAR expression or composition following ELS have functional consequences for the ability to elicit synaptic plasticity in APPswe/PS1dE9 mice.
Figure 2. Model of how the pathways of early life stress and AD may both result in excess extra-synaptic glutamate, activating extrasynaptic NMDA-R and promoting pathways inducing LTD, and how treatment with the glutamate modulator riluzole can counteract both of its effects. **A.** In young, healthy mice, there is a moderate release of glutamate, resulting in a balance between synaptic and extra-synaptic activation of NDMA-Rs. **B.** AD is accompanied by increased release of glutamate, resulting in higher activation of extra-synaptic NMDA-Rs, promoting the induction of LTD over LTP. **C.** ELS exposure in AD mice lowers the expression of EAAT2 on glial cells, reducing the reuptake of extra-synaptic glutamate and further promoting the activation of extra-synaptic NMDA-Rs. **D.** Lifelong riluzole treatment decreases the release of glutamate and increases its reuptake by glial cells through EAAT2, preventing the activation of extra-synaptic NMDA-Rs. This results in the induction of LTP.
Interestingly, life-long treatment with the glutamate modulator riluzole increased synaptic plasticity as well prevented the cognitive deficits induced by early life stress in APPswe/PS1dE9 mice (Chapter 6). Riluzole specifically decreases the activation of extra-synaptic NMDA-Rs by enhancing the expression of EAAT2 on glial cells\[^{151}\], preventing excess glutamate from binding to these NMDA-Rs. Whereas synaptic NMDA activity is critical for LTP and memory formation, extra-synaptic NMDA activation is associated with the induction of long-term depression (LTD) and excitotoxicity\[^{152}\]. The expression of glutamate transporters, including EAAT2, decrease following early life stress\[^{153}\] and in AD\[^{154,155}\]. As such, early life stress and AD share a common pathway through which they can induce altered glutamatergic signalling, promoting the induction of long-term depression and activating pro-apoptotic pathways (see Figure 2 for a model of how early life stress and AD, via altered glutamatergic signalling, may exert additive effects in the induction of LTD and excitotoxicity).

How the effects observed after riluzole treatment (Figure 2; Chapter 6) relates to the decreased GluN2B expression after early life stress at 6 months (Chapter 7)\[^{156}\] remains to be investigated in more detail. In our study, GluN2B expression was analysed in whole cell homogenates, whereas important differences are present in the expression of synaptic versus extra-synaptic GluN2B expression\[^{157}\]. Alternatively, compensatory mechanisms could have been activated to compensate for the reduction in GluN2B expression, for instance by recruiting higher levels of other NMDA-R-subunits. Indeed, the unresponsiveness of early life stress mice to Ro25 6981 treatment (a selective GluN2B-R antagonist) during a fear conditioning experiment supports this notion, as early life stress animals were able to learn the association between a tone and a foot shock in this paradigm, whereas control mice treated with Ro25 6981 were unable to do so (Chapter 7)\[^{156}\].

3.3.3. Repressor element-1 silencing transcription factor (REST)

The disturbed developmental switch to increased GluN2B expression, as described in section 3.3.2, has been suggested to be mediated by an impaired activity of the transcriptional repressor element-1 silencing transcription factor (REST) in the hippocampus following early adversity\[^{150}\]. REST is a genesilencing factor expressed during development that inactivates neuronal genes important for synaptic functioning, among which the gene encoding for GluN2B, and is essential for the experience-dependent fine-tuning of gene expression involved in synaptic activity and plasticity\[^{158,159}\]. The composition of the NMDA receptor is of particular relevance as Aβ acts specifically via the GluN2B subunit, effecting a switch in subunit composition from GluN2B to GluN2A\[^{160}\]. REST has been found to be present during normal aging of
cortical and hippocampal cells, but to be lost in both MCI and AD. Also, REST switches off genes promoting cell death while promoting the expression of various genes involved in the protection against stress. Cognitively healthy elderly people indeed show increased REST levels compared to cognitively impaired elderly, together suggesting REST can be neuroprotective. This makes REST an interesting candidate that could link early life experiences to later resilience to AD. However, whether changes in REST expression following early life experiences persist into aging is unknown and remains to be further investigated.

3.3.4. Early Growth Response Protein 1 (EGR1)
Another candidate to mediate effects of early life experiences on AD vulnerability/resilience is EGR1 (also commonly referred to as Zif268, NGFI-A or KROX-24), a transcription factor critically involved in processes underlying neuronal activity, from neurotransmission and synaptic plasticity to higher order processes such as learning and memory, and to the response to emotional stress and reward. EGR1 expression is induced in neurons by activity-dependent synaptic plasticity upon learning. Both the complete absence and the heterozygous deletion of EGR1 is associated with impaired LTP maintenance over longer periods of time. In contrast, EGR1 overexpression enhances LTP. There is also extensive evidence that EGR1 expression is sensitive to natural environmental stimuli, such as learning tasks, and learning-related increases in EGR1 expression have been reported in many paradigms and brain structures.

EGR1 is expressed at low levels during the postnatal period. Over a period of about 2 weeks (for the hippocampus), expression levels slowly increase to reach adult levels. Interestingly, neonatal handling increased EGR1 mRNA and protein levels, while postnatal restraint stress downregulated EGR1. Furthermore, early life stress induces rapid alterations in the acetylation of histones H3 and H4, which correlate with the expression of EGR1, and stress-induced activation of the GR itself also regulates EGR1 expression. This highlights a role for EGR1 as an experience-dependent mediator of the adaptation to different early environments. It is tempting to speculate that the altered expression of EGR1, usually measured acutely after the early life period, may be a starting point for a lasting dendritic and synaptic reorganisation following these experiences.

EGR1 expression is of particular interest in shaping brain reserve in AD, as it is upregulated during the non-symptomatic stages of AD, but not in symptomatic stages in humans and is also downregulated in cognitively impaired aged mice. The effects of EGR1 may counteract Aβ...
mediated synaptotoxicity; in patients that do show AD-pathology but do not have cognitive decline (Braak stages II-III) EGR1 may be upregulated to increase synaptic plasticity as an attempt to compensate for Aβ-induced neuropathology. After a certain threshold has been reached, EGR1 is likely no longer able to compensate sufficiently and may no longer cope with the synaptotoxic consequences of Aβ, and cognitive impairment associated with the symptomatic stage of AD is thought to commence. Lower initial levels of EGR1 following early life adversity could thus result in a lower capacity to counteract, or cope with, Aβ neurotoxicity, and an earlier display of cognitive impairment, whereas higher baseline EGR1 expression following positive early life experiences would allow the brain to counteract Aβ neurotoxicity for a longer period of time. More recently, EGR1 has also been implicated as a driving factor of AD neuropathology and cognitive decline, since hippocampal EGR1 inhibition was shown to reduce tau phosphorylation, lower Aβ pathology and improve cognition in 3xTG-AD mice. Since EGR1 inhibition was also shown to activate BACE1 activity, this calls for further studies into the role of (early life) modulation of EGR1 and its implication in cognitive impairment and AD neuropathology.

3.3.5. Activity regulated cytoskeleton-associated protein (Arc)
Several potential target genes of EGR1 have been implicated in AD vulnerability, among which the immediate-early gene Arc (also commonly referred to as Arg3.1), which is activated upon EGR1 expression. Arc is critical for memory consolidation and is abundantly expressed in dendrites, the postsynaptic density, and the nucleus. Glutamatergic neurons in the brain express Arc in response to an increase in synaptic activity in relation to a range of behavioural and learning paradigms and appear altered in AD (models). Arc is implicated in the homeostatic scaling of synaptic strength by selectively lowering the levels of AMPA-receptors that contain subunit GluA3. GluA3-containing AMPA-receptors, in contrast to those containing subunit GluA1, traffic to synapses in a manner that is independent of neuronal activity. Thus, while active synapses are enriched for GluA1, synapses that are deprived of input are enriched for GluA3. Interestingly, the presence of GluA3 is required for Aβ to mediate synaptic and memory deficits, suggesting that Arc expression may render synapses resistant to Aβ. Besides this protective role, Arc may also contribute to the pathogenesis of AD by regulating the neuronal production of Aβ.

Arc expression is regulated via activation of GRs, the expression of which is affected by early life experiences. Indeed, life-long Arc expression can be determined early in life, and Arc mRNA expression was e.g. strongly reduced in aged rats with a history of maternal separation. Furthermore,
Arc expression is reduced with aging per se in wild type animals\(^6\), possibly underlying impairments in cognitive performance with older age, and particularly in AD. For example, following learning experiences, Arc expression was lower in the neocortex of AD transgenic mice, indicating an impairment in neuronal encoding and network activation\(^{200}\). Increased levels of A\(\beta\) in transgenic mice expressing human APP (hAPP) resulted in impaired Arc expression and hyperexcitable networks and the subsequent development of seizures\(^{17,201}\). This suggests that increasing Arc levels prior to the development of AD neuropathology, e.g. through positive early life experiences, could possibly protect for a longer period of time, and also against the cognitive impairments that accompany AD neuropathology.

### 3.4. Conclusion early life environment and cognitive/brain reserve

Together, the findings presented in this thesis and in current literature highlight that programming of neuronal function and structure by early life experiences may determine aspects of brain and cognitive reserve. The induction of LTP is decreased/weakened following early life adversity or early stress, whereas a positive early life environment enhances/increases brain plasticity. The installation of such alterations occurs prior to disease onset and can possibly modify brain function at many levels. Thereby, such changes may determine the extent of reserve that the brain encompasses, which could determine its ability to later cope with further insults, including a.o. the emergence of amyloid-\(\beta\) and tau changes and other aspects of AD neuropathology.

Experimental evidence for this hypothesis is thus far limited however, and very few studies have addressed effects of early life experiences on the abovementioned parameters in genetic AD models. Whether the molecular changes in e.g. REST, EGR1, and Arc expression following early life experiences indeed persist throughout the life span of an animal and can thus actually affect the rate of aging, remains to be further investigated. A correct interpretation of the functional implications of the stress- or AD-induced up- or downregulation of some of these markers or processes underlying effects of early life experiences on cognitive reserve is further complex. The magnitude and direction of these neurochemical changes depend on a variety of factors, including the type and severity of the stressor, the age of the animal during stress exposure and the age, sex and species of the animal used, as well as the brain area and cell types studied. Further research is therefore needed to answer the question whether the stress-induced up- or downregulation of a given process is beneficial or detrimental for neuronal and synaptic plasticity, and whether this may then mediate the potential to adapt brain and behaviour to a stressful or AD-related
micro environment before any clinical application of any of these targets can be implemented. In particular, carefully controlled, well-timed and region-specific interventions on these targets in animal models should be performed.
Figure 3. Schematic model depicting how early life experiences could modulate later AD vulnerability or resilience. Early life experiences directly modulate AD pathogenic pathways by e.g. altering tau phosphorylation and the production and/or clearance of Aβ, resulting in a higher pathological load. Secondly, early life experiences may in a more indirect manner determine the establishment of a cognitive and/or brain reserve, yielding the brain more vulnerable to pathological insults later in life. Combined, these two pathways can mediate the effects of early life experiences on the vulnerability or resilience of the brain to AD.

Before we can causally link them to AD resilience, let alone consider them as a target for human interventions.
4. Conclusion

4.1. Lessons from animal models of AD

In animal models for AD, early life experiences can have a profound impact on aging and survival, later cognitive function and the development of AD specific neuropathological features. These effects are two-sided: directly by altering disease-modifying factors, and indirectly by affecting the brain’s ability to cope with these insults (see Figure 3 for a schematic overview). Although not addressed in sufficient detail yet, animal models for early life stress are particularly suitable to identify the so far unknown key molecular and cellular mechanisms that may underlie brain and cognitive reserve and the correlations between specific early life experiences and later AD risk (see Box 3 for an overview of some of the outstanding questions in this respect).

4.2. Clinical implications

Identification of the factors that are causally related to AD resilience could be pivotal in individual risk assessment and determining disease vulnerability for aged individuals and MCI patients. In addition, they might aid the future development of early environmental and/or pharmacological interventions aimed to increase AD resilience (see Box 4 for an overview of the remaining outstanding questions and). However, we warrant caution in the (over) interpretation of the available preclinical findings and their relevance for the clinic since the fundamental basis of the described targets and their causal relevance to AD is not yet fully understood. Also, the gap between preclinical and clinical studies can be vast. To bridge this gap, clinical validation of the concepts identified in rodent studies may yield insight towards relevance for patients. In particular, existing longitudinal cohort studies like the Dutch Hunger Winter or the ABCD cohorts, could help to identify whether early stress affects AD related parameters, and from there, help to identify critical time windows during which cognitive reserve is most effectively established. Alternatively, this could be further extended by implementing ‘signatures’ or biomarkers of early life stress, as is now done for adult stress exposure based on e.g. hair cortisol measurements\textsuperscript{202}.

One of the few interventions that has been shown to be successful in rodent studies at older ages and after a relatively short treatment, while also being FDA approved, is targeting the action of glucocorticoid hormones\textsuperscript{9,86}. A small clinical trial in AD patients and old macaque monkeys reported improvements in cognition after treatment with mifepristone (GR antagonist)\textsuperscript{203,204}, although...
1. What is the role of the PFC in ELS-induced cognitive deficits?
The effects of early life stress have predominantly been investigated in relation to hippocampal functioning. However, many behaviours that are affected by early life stress also depend on PFC functioning, and a more extensive understanding of this brain area would therefore be pivotal in understanding the full consequences of early life stress.

2. Do alterations in HPA axis activity precede alterations in AD neuropathology?
Although different studies have shown that HPA axis activity is altered in AD models, it is unclear whether these changes are a consequence of AD neuropathology, or more causally, steer AD pathogenesis. Investigating HPA axis reactivity and circulating glucocorticoid levels at different ages prior to the presence of AD neuropathology will unravel whether and how these changes are the cause or consequence of AD.

3. What is the role for tau hyper-phosphorylation in stress-induced acceleration of AD development?
While the association between (early life) stress and tau is probable, it has so far received little experimental validation with regard to AD mouse models.

4. Are NMDAR expression and composition involved in altered glutamatergic signalling after ELS, during aging and in AD?
How is synaptic and extrasynaptic NMDAR expression and subunit composition affected by stress, AD, and aging? Is glutamatergic signalling is increased? Does this result in excess glutamate and activation of extrasynaptic NMDARs, and in the activation of pro-apoptotic pathways?

5. Does early adversity affect the cognitive reserve?
There are ample indications that early life stress affects brain reserve. However, whether and how early life adversity affects cognitive reserve remains to be determined in more detail. To this end, an operational, translational definition of how cognitive reserve is determined neurobiologically is imperative to converge findings from rodents and humans. The critical question is what the underlying neuronal networks, connections and synaptic properties are that mediate this cognitive reserve. Behaviourally, it will be important to understand whether (and how) early life adversity affects learning strategies and behavioural flexibility in AD mouse models, also measures of cognitive reserve.
the time window was short and sample size small. Furthermore, AD patients with the highest baseline cortisol levels benefited most from a mifepristone intervention and showed persistent memory improvements up to 8 weeks after discontinuation of the treatment. This could therefore potentially present a promising strategy to further explore, specifically in relation to AD cases with a stress history.

In conclusion, the mechanisms that are identified in preclinical studies and their verification in clinical studies will hopefully benefit the identification and stratification of specific populations with higher vulnerability to develop AD, as well as aid in the selection of putative targets. Ultimately, this may promote the development of an early and targeted treatment approach during the many decades between the early life environment and clinical AD presentation.

**Box 4. Outstanding questions for clinical research**

1. **What are critical time windows for development of AD pathology?**
   Can critical time windows be identified in humans during which stress modifies AD risk? What are the critical periods for early stress exposure in humans, and can interventions during those periods indeed prevent later effects on AD changes?

2. **Do early life experiences affect AD in humans?**
   Rodent studies indicate a strong relationship between early life experiences and the development of AD pathology. It remains elusive, however, whether such associations also exist in humans. Can human longitudinal cohort studies confirm the associations found in preclinical studies between early life experiences, AD vulnerability / resilience and brain function?

3. **Modifying vulnerability for AD pathology?**
   It will not only be important to understand whether and how effects of early life adversity can be overcome, but in general the field needs to consider whether and how strategies can be developed, optimised and implemented to increase the resistance for developing and delaying AD pathology. Based on fundamental studies, this may involve strategies involving e.g. cognitive stimulation and nutrition. Within the framework of the early life period and cognitive reserve, what are the most relevant substrates regarding AD vulnerability that allow to be studied and manipulated in both humans and rodent models?
Positive and negative early life experiences differentially modulate long term survival and amyloid protein levels.
General Discussion – part 2

In the final part of this thesis (Chapter 8 and 9), I investigated how stress hormones modify the consolidation of fear memories. Stressful or threatening events are usually remembered very well, an effects that is attributed, among others, to the enhanced release of glucocorticoids (cortisol in humans; corticosterone in rodents) upon a stressful encounter\(^205\). In Chapter 8, I systematically tested different fear conditioning paradigms with varying foot shock frequency and intensity, to evaluate the effects of artificially increasing the corticosterone levels after training on fear memory. Indeed, I find that glucocorticoid exposure following fear conditioning enhances the strength of this memory, but only in male mice. In females, corticosterone treatment actually weakened fear memory. The underlying mechanisms remain elusive. Effects of corticosterone follow a Yerkes-Dodson, or ‘inverted-U shape’ dose-response relationship, in which optimal enhancing effects on memory are seen at midrange doses, whereas higher or lower doses are less effective or may even impair memory\(^206\). Since female mice have both higher basal corticosterone levels and a stronger corticosterone release upon exposure to a stressor\(^207\), possibly the dosage that is required in males mice to enhance memory, may have impairing effects on female memory formation.

While the enhanced retention of information following a stressful or threatening event may have important adaptive value for future survival of an organism, such events can also trigger inappropriately expressed, yet intrusive and vivid memories, which can result in a pathological state, such as also seen in e.g. posttraumatic stress disorder (PTSD)\(^208\). Such anxious states are characterised by re-experiencing the fear response also in safe situations, notably in the absence of any predictive cues, and thus in an a-specific and/or generalised manner.

Using the most optimal fear-conditioning paradigm (as determined in Chapter 8) to observe effects of corticosterone on memory, in Chapter 9 I investigated in more detail the effects of corticosterone on fear memory in male mice. We found that artificially raising corticosterone levels directly after fear learning resulted in a more generalised fear response. A delicate balance between memory specificity and generalisation is essential to enable efficient encoding of highly salient, stress-related information. Although some level of generalisation is adaptive as it allows a learned response to transfer to other relevant situations and stimuli, the overgeneralisation of a-specific fear responses can be debilitating. Indeed, stressful learning processes contribute to fear sensitisation, resulting in exaggerated startle responses to trauma-unrelated stimuli\(^209\). This impairment is very similar to that observed in PTSD
patients in which contextual peri-traumatic cues are often forgotten, whereas salient but irrelevant ones are strongly remembered. These salient cues, and others more or less similar to them can then induce a strong fear response in contexts different from the traumatic one\textsuperscript{208,210}. Interestingly, noradrenaline, another key mediator of the stress response, has been shown to contribute to memory specificity\textsuperscript{211}. Possibly, a closely governed balance between the release of noradrenaline and glucocorticoids is essential to the formation of reliable memories, and we speculate that an imbalance in either could contribute to memory generalisation and PTSD-like symptoms.

It is noteworthy to mention that the effects of corticosterone on memory strength and specificity were only observed when during the training, foot shocks were preceded by a tone. When either no tone was present, or it was present but not predictive for a foot shock, corticosterone did not modulate memory formation of the foot shock, indicating a critical role of (brain regions}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{brain_circuitry.png}
\caption{Brain circuitry involved in fear memory. Contextual information from the hippocampus and tone information from the amygdala converge to determine the conditioned response after a fearful event. Although our current work has mostly focussed on the role of the hippocampus, as evident from this scheme, there is a central role for the amygdala in the integration of the information from the context and the tone, that ultimately determines the conditioned response. We propose a critical role for the amygdala in the expression of fear memory generalisation that should be studied further. CS: conditioned stimulus, US: unconditioned stimulus, CR: conditioned response, LA: lateral amygdala, BLA: basolateral amygdala, HC: hippocampus.}
\end{figure}
mediating the effects of) the tone. Although the focus of our current study was on the dentate gyrus of the hippocampus (given its important role in pattern separation and the contextual aspect of memories) these findings also point towards a regulatory role of the amygdala, which determines the cue-associated component of memory. It would therefore also be very relevant to study in more detail the role of the amygdala in the effects of corticosterone on memory (Figure 1), and whether e.g. inhibiting neuronal ensembles in the amygdala upon retrieval would also prevent the contextual memory generalisation.

**Outstanding question 1: What is the role of the amygdala in the corticosterone-induced effects on memory specificity and strength?**

Secondly, we studied how these generalised memories were stored in the brain. Until recently, most studies focused on the effects of glucocorticoids in defined, entire, brain areas, such as the amygdala and the hippocampus, but there is strong experimental support for the existence of more sparsely distributed network of neurons, coined 'engram cells' by Richard Semon in 1921. They have recently become a prominent focus in the field of how memories are stored in the brain. These neuronal ensembles, consisting of 10-30% of the eligible neurons, are both necessary and sufficient to recall a fear memory. Moreover, a recent study by Ryan et al. showed that these cells undergo lasting cellular changes following memory acquisition, and are subject to both synaptic strengthening and structural plasticity. These cells that were active during memory consolidation or retrieval are usually defined by the expression of immediate-early genes (IEGs) such as the activity-regulated cytoskeleton-associated protein Arc or c-fos.

In Chapter 9, I visualised IEG expression in the dentate gyrus, the main area for contextual memory, to assess whether our observed generalisation of context is accompanied by a change in the number of neurons that is activated upon memory acquisition or retrieval. Most neurons in this area can express multiple IEGs, but the extent to which their expression patterns overlap in response to learning remains elusive. For this reason, we assessed expression of both c-fos and Arc in response to memory acquisition and found that these expression patterns were differentially affected by corticosterone treatment: both show increased expression levels but do so at a different time. Different IEGs exhibit different properties, showing variations in function, the degree of neuronal activity that is required to induce expression and in their spatiotemporal dynamics of expression. C-fos and Arc transcription in response to a learning experience follows a dynamic, wave-like pattern over a prolonged period of time. This generates the possibility that second
or even third ‘waves’ of c-fos and Arc transcription take place several hours after their initial transcription, which is thought to correspond to the time-window for memory consolidation. Our corticosterone treatment increased c-fos expression possibly during its first transcriptional ‘wave’, whereas Arc expression was only affected in its subsequent waves.

This raises the question of how corticosterone affects the time window in which memories are consolidated. It is generally accepted that the window for memory (re)consolidation is approximately 6 hours\textsuperscript{222,225,226}. One possibility is that corticosterone prolongs the time window during which neurons can be incorporated into this network, in line with our observation that also more neurons are indeed recruited into the memory ensemble.

**Outstanding question 2: How does corticosterone affect the time window in which memories are consolidated?**

Computational studies propose that fear memory strength is related to the synaptic strength within the underlying neuronal ensemble, whereas fear
specificity is related to the size (i.e. the number of neurons) in this ensemble\textsuperscript{216,227}. Memory specificity is then thought to be determined by the size of the memory ensemble encoding the event\textsuperscript{227}. We find that corticosterone recruits more neurons into the memory trace in the dentate gyrus, and thereby contributes to a more generalised representation of such a memory (see Figure 2 for a model).

It has been generally accepted that a coordinated activation of the neurons within a memory ensemble underlies memory retrieval, but the network dynamics that underlie memory acquisition still trigger some debate. One hypothesis is that learning induces hippocampal oscillations in excitation that induce a reactivation of the initially activated neuronal populations and thereby drive memory consolidation\textsuperscript{228}. The occurrence of these oscillations most likely depends on the activity of inhibitory interneurons that have been previously implicated in the suppression of neurons that are not being allocated to a memory ensemble\textsuperscript{214,216,229}. Interneurons can create alternations of inhibition and disinhibition that regulate activity of excitatory neurons in such a way that these are synchronously activated and reactivated. Mizunuma et al.\textsuperscript{230} showed subsequent reactivations of \textit{Arc} expressing neurons that were congruent to spontaneous excitation in the hippocampus after novel context exposure. This nicely fits in with our current observations regarding \textit{c-fos} and \textit{Arc} expression patterns (Chapter 9), as well as with the decrease in parvalbumin expression between 30 and 90 minutes after fear memory acquisition (unpublished observation). It is relevant to examine if corticosterone affects this balance between excitation and inhibition, as a potential secondary mechanism through which stress hormones could modify the size of the memory engram.

**Outstanding questions 3: How does corticosterone determine the balance between excitation and inhibition, and do changes in this balance contribute to engram size and memory specificity?**

Our studies 1) provide evidence that stress hormones contribute to memory strengthening, but also to impairments in memory specificity in rodents, which resembles PTSD-like memory impairments and 2) identify the dentate gyrus as a key cellular circuits involved in PTSD symptoms. Understanding the molecular mechanisms through which glucocorticoids can modify neural activation and neural ensembles in the dentate gyrus could therefore open new avenues for innovative treatments for PTSD.
Chapter 10

References


Stress
Running exercise
β
Mechanical


116 Swaab DF. Brain aging and Alzheimer’s disease, &quot;wear and tear&quot; versus &quot;use it or lose it&quot;? Neurobiol Aging; 12, 317–24.
123 Sutherland RJ, McDonald RJ, Savage DD. Prenatal exposure to moderate levels of ethanol can have long-lasting effects on hippocampal synaptic plasticity in adult offspring. Hippocampus 1997; 7: 232–8.
Maternal stress produces learning mechanisms of late-β2004; APP processing

Chapter 10

Champagne D, Bagot R, van Hasselt F, Nguyen H-B, Bagot RC, Diorio J, Wong TP,


Shankar GM, Bloodgood BL, Townsend M, Walsh DM, Selkoe DJ, Sabatini BL. Natural oligomers of the Alzheimer amyloid-β protein induce reversible synapse loss by modulating an NMDA-type glutamate receptor...


Age and Synaptic and NRSF-dependent 


8885–8895.


174. Maddox SA, Monsey MS, Schafe GE. Early


190 Morin J-P, Díaz-Cintrá S, Bermúdez-Rattoni F, Delint-Ramírez I. Decreased levels of NMDA but not AMPA receptors in the lipid-raft fraction of 3xTg-AD model of Alzheimer’s disease: Relation to Arc/Arg3.1 protein expression. Neurochem Int 2016; 100: 159–163.


194 Béïque J-C, Na Y, Kuhl D, Worley PF, Huganir RL. Arc-dependent synapse-specific homeostatic
216 Liu X, Ramirez S, Pang PT, Puryear CB,


221 Minatohara K, Akiyoshi M, Okuno H. Role of immediate-early genes in synaptic plasticity and neuronal ensembles underlying the memory trace. Front Mol Neurosci 2016; 8: 78.


243 Yam KY, Naninck EFG, Abbink MR, la Fleur SE,
Schipper L, van den Beukel JC et al. Exposure to chronic early-life stress lastingly alters the adipose tissue, the leptin system and changes the vulnerability to western-style diet later in life in mice. *Psychoneuroendocrinology* 2017; 77: 186–195.


245 Kanatsou S, Kuil LE, Arp J, Oitzl MS, Harris AP, Seckl JR et al. Overexpression of mineralocorticoid receptors does not affect memory and anxiety-like behavior in female mice. Article type: Received on: Accepted on: Frontiers website link: Citation: Overexpression of mineralocorticoid receptors does not affect memo. *Front Cell Neurosci* 2015; 11.


Positive and negative early life experiences differentially modulate long term survival and amyloid protein levels.
English summary

Exposure to stressful experiences, either early or later in life, can have a strong impact on learning and memory in adult and ageing individuals. Early life experiences in particular have been implicated in determining the vulnerability and resilience for cognitive decline, for instance when the brain is already vulnerable, such as seen in Alzheimer’s disease (AD). The first aim of this thesis was to study the effects of experiences early in life (albeit positive or negative) on aging- or AD-related cognitive decline, and to better understand the underlying mechanisms. I particularly focused on the role of the hypothalamus-pituitary-adrenal (HPA)-axis, and on the expression and functionality of glutamate receptors in this process. The second aim of this thesis was to investigate why stressful memories are retained so well. Recent studies suggest that only a subset of neurons is required for any memory trace (‘engram cells’). Using novel genetic and molecular approaches, these engram cells were visualised, characterised and manipulated, to unveil effects of glucocorticoids on memory formation.

In Chapter 1, I reviewed the effects of early life experiences on later behaviour and functional plasticity of the brain. I discussed the evidence that early life experiences long-lastingly alter HPA axis (re-)activity, thereby shaping behaviour and brain function during adult life and aging. Finally, I reviewed data supporting the hypothesis that early life experiences, either positive or negative, can alter the vulnerability to develop AD, and I investigated elements of that hypothesis in chapters 2, 3, 4, 5 and 6.

In Chapter 2, I showed that manipulating the amount of maternal care that pups receive can have profound effects on the lifespan and pathological markers in mice with transgenic overexpression of APP and tau (biAT mice), the main neuropathological features of AD. Increased levels of maternal care (“early handling”) was induced by separating the dam and her pups for fifteen minutes per day during the first week after birth (postnatal day 2-9). Upon reuniting the dam and her pups, the dam intensifies licking and grooming behaviour towards the pups, which results in an ‘enriched environment’ for the pups. Remarkably, early handling resulted in a longer lifespan and lower β-amyloid (Aβ) levels later in life. On the other hand, reduced levels of maternal care (“early life stress”), induced by placing the litter under impoverished housing conditions, shortened the lifespan and increased Aβ levels later in life (i.e., all at an early stage of the disease).

Using a different genetic mouse model for amyloid-β-associated neuropathology (APPswe/PS1dE9 mice), I validated in Chapter 3 in more detail
the early handling mouse model that resulted in enhanced levels of maternal care. Early handling reduced hippocampal plaque pathology, while plaque load in the amygdala remained unaffected. Importantly, when adult APPswe/PS1dE9 mice were tested in spatial, hippocampus-dependent cognitive tasks, early handling prevented the APPswe/PS1dE9-induced cognitive impairments. This was not the case in amygdala-dependent memory tests.

In Chapter 4, I investigated the role of the HPA axis in the effects of early life adversity on AD-related changes. Early life stress increased Aβ neuropathology from adult age (6 months) onwards, ultimately resulting in cognitive impairments at older age (12 months), in particular in the domain of cognitive flexibility. HPA-axis responsiveness was increased in these mice, which negatively correlated with cognitive performance. Interestingly, a brief, 3-day treatment with mifepristone (which targets glucocorticoid receptors), at an age at which cognitive impairments were already present, was found to reverse the early life stress-induced impairments in cognitive flexibility and Aβ neuropathology. This highlights the important role of glucocorticoid hormones in the development of AD neuropathology and symptomatology.

While declarative memory is often impaired in AD, emotionality is frequently enhanced in AD patients. In Chapter 5, I report that APPswe/PS1dE9 mice exposed to early life stress show an enhanced responsiveness to fearful cues, but also to non-fearful cues. I next investigated whether this was associated with alterations in hippocampal synaptic plasticity, i.e. the cellular substrate of learning and memory, in one year old mice. I found that APPswe/PS1dE9 mice exposed to early life stress also showed an atypical, enhanced form of synaptic plasticity, that coincided with a reduced sensitivity to a GluN2B receptor antagonist. These findings may point to a central role for the NMDA receptor in mediating effects of early life stress on adult plasticity.

To further explore the underlying mechanisms of altered synaptic plasticity and cognitive deficits, in Chapter 6, I treated APPswe/PS1dE9 mice with the glutamate modulator riluzole throughout their life. While riluzole prevented the impairments in synaptic plasticity from an early age onwards, it even improved cognitive performance in older APPswe/PS1dE9 mice that were exposed to early life stress. The effects of early life stress, aging and AD, and the rescue by riluzole, were accompanied by changes in the expression of the excitatory amino acid transporter 2, which is important for the synaptic reuptake of glutamate. These findings point towards an important role for glutamatergic signalling in early life stress-induced cognitive impairments in AD.
In order to better understand how early life stress determines learning and memory processes in aging and AD, I addressed in Chapter 7 how early life stress affects short-term and long-term synaptic plasticity in wild type mice. I found that early life stress impaired both these forms of synaptic plasticity. In addition, early life stress reduced the expression of the NMDA receptor subunit 2B in the hippocampus. Blocking the NMDA receptor subunit 2B had no effect on either memory performance, or on synaptic plasticity in mice exposed to early life stress, while this subunit was critically important for these processes in control mice, suggesting that the effects of early life stress may be mediated, in part, by the NMDA receptor subunit 2B.

In the final part of this thesis, I investigated how acute exposure to stress hormones affects learning and memory. In Chapter 8, I showed that the administration of corticosterone immediately after auditory fear conditioning enhanced memory consolidation. Using different auditory fear conditioning paradigms, application of corticosterone was found to have opposite effects on conditioned fear memories in male and female mice. Whereas corticosterone increased memory in male mice, the hormone reduced conditioned fear memory in female mice. Interestingly, corticosterone increased extinction learning in both male and female mice. Together, this indicates that fear memory retention is differently affected by corticosterone in male and female mice.

These findings are expanded on in Chapter 9 where I investigated the effects of a brief corticosterone treatment on memory specificity in male mice. Corticosterone decreases the accuracy of the memory of the event, since these mice displayed generalised fear when they were placed in a safe environment. Using transgenic reporter mice that allow the investigation of cells expressing the immediate-early-gene Arc, it was possible to molecularly and electrophysiologically characterise subpopulations of neurons in the hippocampal dentate gyrus in relation to a memory trace. Corticosterone administration to trained animals increased the number of Arc-positive neurons in the dentate gyrus, and these cells are also activated following the retrieval of the memory. Arc-positive neurons are more active than Arc-negative neurons, yet corticosterone specifically changes the activity in Arc-negative neurons. When we subsequently inhibited these dentate neurons specifically using DREADD technology, this prevented the generalisation of fear induced by corticosterone, while memory for the tone was left unaffected. This illustrates that the training-induced recruitment of a subset of dentate gyrus neurons underlies the generalisation of fear by corticosterone.
In Chapter 10, I summarise the main outcomes of this thesis, and discussed them in a broader perspective. First, I discussed how early life experiences can program brain (or neuronal) structure and function in a lasting manner, and how this may impact cognition and neuropathology in an AD background. I speculate that besides a direct modulation of AD neuropathology by the HPA axis, early life experiences may also shape the brain or cognitive 'reserve' early on, thereby rendering some individuals more resilient or vulnerable to AD-associated impairments than others. Secondly, I discussed how glucocorticoid hormones influence memory strength and memory specificity, and in particular address the role of memory engram cells. Finally, I formulated some remaining outstanding questions that may help move the field of (early life) stress and memory formation ahead.
Sylvie L. Lesuis, Paul J. Lucassen, Harm J. Krugers

General Introduction

Positive and negative early life experiences differentially modulate long term survival and amyloid protein levels.

Nederlandse samenvatting

(Dutch summary)
Nederlandse samenvatting

Blootstelling aan stress kan een sterke invloed hebben op leer- en geheugenprocessen. Dit is reeds bekend in volwassen en oudere individuen, maar met name stressvolle gebeurtenissen in het vroege leven blijken ook een belangrijke rol te spelen bij (het versnellen van) cognitieve achteruitgang. Dit is met name relevant als het brein al extra kwetsbaar is, zoals het geval is bij de ziekte van Alzheimer.

Het eerste doel van dit proefschrift was om te bestuderen hoe vroege levenservaringen (positief dan wel negatief) veroudering en Alzheimer-gerelateerde veranderingen beïnvloeden, wat ik in hoofdstuk 2, 3 en 4 in muismodellen voor deze ziekte onderzoek.

Een tweede doel was om een beter beeld te krijgen van de onderliggende mechanismen die ten grondslag liggen aan de effecten van stress tijdens het vroege leven op zowel de latere cognitie, veroudering als de ziekte van Alzheimer.

Ten derde bestudeer ik hoe acute blootstelling aan stress hormonen de vorming van geheugen direct kunnen beïnvloeden, en welke mechanismen daaraan ten grondslag kunnen liggen.

In Hoofdstuk 1 geef ik een overzicht van de effecten van vroege levenservaringen op later gedrag en plasticiteit in het brein. Ik bediscussieer de evidentie in de literatuur dat vroege levenservaringen de activiteit van de stress- of HPA as permanent kunnen veranderen, en blijvende gevolgen kan hebben voor gedrag en hersenfunctie tijdens het volwassen leven en bij veroudering. Ten slotte geef ik een overzicht van de data die de hypothese ondersteunt dat vroege levenservaringen (positief danwel negatief) de kwetsbaarheid voor de ziekte van Alzheimer kan beïnvloeden, waarvan ik bepaalde factoren verder onderzoek in de hoofdstukken 2, 3, 4, 5 en 6.

In Hoofdstuk 2 laat ik zien dat de hoeveelheid zorg die een moedermuis aan haar pups besteed, hun latere leven kan beïnvloeden. Door die zorg bijvoorbeeld te veranderen, bleken er sterke gevolgen zijn voor de levensverwachting en pathologische veranderingen in de hersenen van zgn. transgene ‘Alzheimer muizen’ met een overproductie van amyloid-β (Aβ) en tau (biAT muizen), de belangrijkste neuropathologische kenmerken van de ziekte van Alzheimer, in hun hersenen. Verbeterde moederzorg (‘early handling’) ontstaat als de moeder en haar pups tijdens de eerste week na de geboorte (postnatale dag 2-9) dagelijks voor 15 minuten van elkaar worden gescheiden. Bij de hereniging
van de moeder en haar pups, laat de moeder dan veel intensievere zorg zien, wat ertoe leidt dat de pups verder in een “verrijkte omgeving” opgroeien. Opmerkelijk genoeg resulteerde deze vroege verrijking er ook toe dat de pups langer bleven leven en minder Aβ in hun brein hadden. Daarnaast heb ik ook verminderde moederzorg geïnduceerd (“vroge levensstress”) door de moeder en haar pups te huisvesten onder verarmde omstandigheden met een klein nest en geen zaagsel in de kooi bv, wat ertoe leidde dat deze muizen een lagere levensverwachting en meer Aβ in het brein hadden.

Door gebruik te maken van een ander klassiek Alzheimer muismodel, de APPswe/PS1dE9 muis die Aβ-pathologie in het brein ontwikkelt, heb ik in **Hoofdstuk 3** verder onderzoek gedaan naar de effecten van early handling op Alzheimer-gerelateerde factoren. Early handling zorgde voor vermindering van de plaque pathologie in de hippocampus, terwijl de plaque pathologie in een ander hersengebied, de amygdala, niet beïnvloed werd op volwassen leeftijd. Toen deze muizen vervolgens getest werden in ruimtelijke, hippocampus-afhankelijke cognitieve taken, bleek door early handling, de cognitieve achteruitgang volledig voorkomen te kunnen worden. Dit was niet het geval bij geheugentaken die afhankelijk waren van de amygdala.

In **Hoofdstuk 4** heb ik onderzocht wat de rol is van de HPA as bij de effecten van vroege stress op Alzheimer gerelateerde achteruitgang. Vroege levensstress zorgt voor verhoogde Aβ neuropathologie, wat al zichtbaar op volwassen leeftijd (6 maanden), en dit leidt ook tot een versterkte cognitieve achteruitgang, met name cognitieve flexibiliteit, op oudere leeftijd (12 maanden), met name cognitieve flexibiliteit. De HPA as activiteit was verhoogd in deze muizen, en negatief gecorreleerd met hun cognitieve prestaties. Vervolgens hebben we laten zien dat al een korte, 3-daagse behandeling met de stof mifepristone (wat de stresshormoon receptor GR blokkeert), de cognitieve problemen en verhoogde Aβ neuropathologie in deze muis, geheel kan tegengaan. Dit benadrukt de belangrijke rol die stress hormonen zoals glucocorticoid mogelijk spelen bij de ontwikkeling van Alzheimer neuropathologie en symptomen.

Hoewel het declaratieve geheugen vaak afneemt bij de ziekte van Alzheimer, wordt emotionaliteit versterkt. In **Hoofdstuk 5** laat ik zien dat APPswe/PS1dE9 muizen die blootgesteld zijn aan vroege stress inderdaad een versterkte angstreactie laten zien bij cues die een angstige gebeurtenis aankondigen, maar ook in reactie op cues die niet verbonden zijn met een eerdere stressvolle gebeurtenis. Ik heb onderzocht of dit gerelateerd was aan veranderingen in synaptische plasticiteit, het cellulaire substraat voor leren en geheugen, in de hippocampus van muizen die 1 jaar oud zijn. Hier heb
ik laten zien dat APPswe/PS1dE9 muizen die waren blootgesteld aan vroege levensstress, een atypische en juist versterkte synaptische plasticiteit laten zien, terwijl de gevoeligheid voor een GluN2B receptor antagonist afneemt. Deze bevindingen suggereren een centrale rol van de NMDA-receptor bij de effecten van vroege levenservaringen op volwassen plasticiteit.

Om verder te onderzoeken wat de onderliggende mechanismen zijn van de veranderde synaptische plasticiteit en cognitieve achteruitgang, heb ik in Hoofdstuk 6, APPswe/PS1dE9 muizen gedurende hun hele leven behandeld met de glutamaat-modulator riluzole. Riluzole behandeling voorkwam de nadelige gevolgen op synaptische plasticiteit al vanaf een vroege leeftijd, en verbeterde zelfs de cognitieve vaardigheden van oude muizen die blootgesteld waren aan vroege levensstress. De effecten van vroege levensstress, veroudering en de ziekte van Alzheimer, en de verbeteringen van riluzole, gingen gepaard met een verandering in de expressie van de excitatory amino acid transporter 2, welke belangrijk is voor de heropname van glutamaat bij de synapse. Dit suggereert een belangrijke rol voor glutamaterge transmissie in de effecten van vroege levensstress op de ziekte van Alzheimer.

Om beter te begrijpen hoe vroege stress leren en geheugen beïnvloedt tijdens de veroudering en de ziekte van Alzheimer, heb ik in Hoofdstuk 7 onderzocht hoe vroege stress de korte- en lange-termijn synaptische plasticiteit (LTP) beïnvloedt in wild type dieren. Vroege stress bleek beide vormen van plasticiteit te verminderen. Daarnaast nam ook de expressie van de NMDA-receptor subunit 2B (GluN2B) in de hippocampus af. Het blokkeren van de GluN2B had echter geen effect op geheugen en synaptische plasticiteit in muizen die waren blootgesteld aan vroege levensstress, terwijl de GluN2B essentieel was voor deze processen in controle muizen. Dit wijst erop dat de GluN2B een belangrijke mediator kan zijn van de effecten van vroege levensstress tijdens de rest van het leven.

In het laatste gedeelte van deze thesis onderzocht ik hoe de acute blootstelling aan stresshormonen leren en geheugen kan beïnvloeden. In Hoofdstuk 8 toonden we aan dat corticosteron blootstelling direct na een angstvolle ervaring, het vastleggen van het geheugenspoor kan beïnvloeden. Door gebruik te maken van verschillende parameters voor dit zgn. conditioneringsexperiment, konden we laten zien dat corticosteron tegenovergestelde effecten heeft op het geheugen in mannelijke vergeleken met vrouwelijke muizen. Hoewel het stresshormoon de geheugenvorming in mannen versterkte, zorgde het hormoon in vrouwelijke muizen voor een verzwakking van het geheugen. Het was opvallend dat corticosteron de extinctie van het geheugen wel in beide geslachten versterkte. Dit onderzoek
lauw daarmee zien dat geheugenvorming anders beïnvloed wordt door stresshormonen in mannelijke en vrouwelijke muizen.

Deze bevindingen zijn verder uitgebreid in Hoofdstuk 9, waar ik de effecten van corticosteron blootstelling op geheugenspecificiteit in mannelijke muizen heb bestudeerd. Corticosteron zorgt ervoor dat de precisie van het geheugen afneemt, wat zorgt voor een gegeneraliseerde angstresponse, zelfs wanneer de muizen in een veilige omgeving geplaatst worden. Door gebruik te maken van transgene reportermuizen kunnen we cellen onderzoeken die de immediate-early-gene Arc tot expressie brengen. Hierdoor was het mogelijk om juist die cellen die verantwoordelijk zijn voor dit geheugenspoor, zowel moleculair als ook elektrofysiologisch te kunnen karakteriseren in de gyrus dentatus van de hippocampus. Corticosteron zorgt voor een verhoging van het aantal Arc-positieve cellen in de gyrus dentatus van getrainde dieren. Daarnaast zorgt corticosteron ervoor dat de Arc-negatieve cellen actiever worden, wat er mogelijk voor zorgt dat deze cellen sneller opgenomen worden in het geheugenspoor. Toen we vervolgens m.b.v. DREADD-technologie selectief alleen deze neuronen afremden, konden de effecten op door corticosteron veroorzaakte angst generalisatie voorkomen worden. De geheugenvorming werd verder niet beïnvloed. Dit toont aan dat de rekrutering van nieuwe cellen in de gyrus dentatus een essentiële rol spelen bij de generalisatie van angst door corticosteron.

In Hoofdstuk 10 vat ik de belangrijkste bevindingen van deze thesis samen, en bespreek ik ze in een bredere context. Ik bediscussieer hoe vroege levenservaringen het brein langdurig kunnen 'programmeren', en welke gevolgen dit heeft voor leren en geheugen en neuropathologie in modellen voor de ziekte van Alzheimer. Vervolgens speculeer ik dat, naast een directe rol van vroege levenservaringen via de HPA as, vroege levenservaringen ook cruciaal zijn in het ontstaan van een cognitieve reserve, een conceptueel begrip wat gebruikt wordt om te begrijpen waardoor sommige individuen meer weerstand hebben tegen de ziekte van Alzheimer dan anderen. Ten tweede bespreek ik hoe glucocorticoid hormonen geheugensterkte en –specificiteit beïnvloeden, met een speciale focus op de rol van de zgn. 'Engram' cellen. Ten slotte heb ik een aantal belangrijke, nog openstaande vragen geformuleerd, welke het veld van (vroege) stress en geheugen verder zouden kunnen helpen.
Positive and negative early life experiences differentially modulate long term survival and amyloid protein levels.
Samenvatting voor niet-ingewijden

In het dagelijks leven ervaren we regelmatig stress. Deze stress is vaak goed voor je; we hebben het nodig om adequaat te kunnen reageren op situaties die onze aandacht nodig hebben, om ons aan te passen aan onze omgeving, en om te overleven (“acute, adaptieve stress”). Als stress echter te heftig is, of te lang aanhoudt, kan deze goede stress omslaan in slechte stress (“schadelijke stress”). Langdurige stress ondermijnt het geheugen, het vermogen om te plannen en beslissingen te nemen en om onze emoties te controleren. Dit kan verregaande gevolgen hebben, waaronder een toegenomen risico op angststoornissen, posttraumatische stresssyndroom, burn-out, of depressie, en het kan zelfs de ziekte van Alzheimer verergeren.

Acute, adaptieve stress

Wat gebeurt er eigenlijk tijdens stress? Als er iets spannends, naars of inspannends plaatsvindt, stuurt je brein een signaal naar je bijnieren om de stresshormonen adrenaline en cortisol aan te maken. Hierdoor gaan je hartslag, bloeddruk en ademhaling omhoog, waardoor je alert wordt en je je beter kunt concentreren op je omgeving. Dit helpt om goed met de ontstane situatie om te gaan. Zo onthoud je beter wat er in die periode gebeurt, waardoor je er de volgende keer beter op voorbereid bent. Dit kan bijvoorbeeld handig zijn als je betrokken bent bij een (bijna) verkeersongeval, waarbij je de details rondom het ongeval vaak beter onthoudt dan van een willekeurig ander moment dat je deelnam aan het verkeer. Hierdoor herinner je je elke keer als je in dezelfde of een vergelijkbare situatie bent dat dit gevaarlijk is en er dus extra waakzaamheid geboden is. Hopelijk voorkomt dit dan dat je nogmaals in een vergelijkbare gevaarlijke situatie terecht komt. Na afloop van de stressvolle omstandigheden nemen de toegenomen stresshormonen weer af, waardoor de stressreactie beëindigd wordt.

Schadelijke stress

Wanneer de stressvolle gebeurtenissen elkaar snel opvolgen, of als de stressvolle gebeurtenis té intens is, kan het voorkomen dat je brein het niet lukt om die stresshormonen weer naar rustniveau terug te brengen, waardoor het stresssysteem ontspoort, het lichaam in een staat van paraatheid blijft en mensen gevoelig worden voor nieuwe stressvolle...
geburenissen ("chronische stress"). Dit kan bijvoorbeeld gebeuren bij militairen die uitgezonden worden en daarbij in aanraking komen met stressvolle situaties. Dit kan in sommige gevallen leiden tot een posttraumatische stress syndroom (PTSS), waarbij het geheugen niet adequaat/precies werkt. De herinnering aan de stressvolle gebeurtenis kan in zo'n geval ook opgeroepen worden in situaties die wel veilig zijn, zoals bijvoorbeeld een ballon die uit elkaar klap op een feestje. Deze verminderde precisie van het geheugen – er wordt immers een herinnering opgeroepen die niet hoort bij de omgeving, of context, waarin men zich bevindt – kan vergaande gevolgen hebben voor hoe iemand kan functioneren.

Eén van de vragen die ik heb geprobeerd te beantwoorden in dit proefschrift, is wat er precies gebeurt met de cellen in het brein waardoor de sterkte van het geheugen toeneemt, maar de precisie van het geheugen afneemt bij stressvolle gebeurtenissen. Om dit te onderzoeken hebben we gebruik gemaakt van de nieuwste technieken en speciale muizen, die cellen in hun hersenen hebben die groen worden zodra ze actief betrokken worden bij het herinneren van een gebeurtenis. Als gevolg van stress zijn er meer groene cellen aanwezig in het brein van deze muizen. Mogelijk worden er als gevolg van stress téveel cellen betrokken bij een geheugen, waardoor de herinnering ook geactiveerd wordt in situaties die totaal afwijken van de stressvolle, gevaarlijke situatie.

**Stress tijdens de kinderjaren**

Hoewel iedereen weleens last heeft van stress, zijn sommige mensen gevoeliger voor de negatieve gevolgen van stress dan anderen. Naast erfelijke factoren, kan dit ook komen door ervaringen tijdens de kindertijd. De kindertijd is een hele gevoelige periode waarin de "blauwdruk" van het brein voor de rest van het leven gemaakt wordt. In deze periode ontwikkelt het brein zich heel snel, waardoor verstoringen tijdens deze periode verstrekende gevolgen kunnen hebben voor hoe het brein werkt op latere leeftijd. Zo zijn er onderzoeken gedaan naar geadopteerde kinderen die de eerste paar jaar van hun leven zijn opgegroeid in Roemeense kindertehuizen. Eten was meestal aanwezig, maar er was vaak sprake van verwaarlozing, zoals een gebrek aan troosten, knuffelen en andere aandacht bij de (zeer jonge) kinderen, waardoor ze onder veel stress zijn opgegroeid. Onderzoeken hebben laten zien dat deze kinderen vaak minder intelligent zijn dan leeftijdsgenoten, impulsiever zijn, aandachtsproblemen hebben, en sociale gedragsproblemen hebben. Dit gaat gepaard met veranderde
activiteit in het brein. Ook dichter bij huis komen dit soort problemen voor. Zo heeft Nederlands onderzoek onder pubers laten zien dat kinderen die voor hun vijfde verjaardag een of meerdere ingrijpende gebeurtenissen mee hadden gemaakt (zoals een scheiding, ongeluk of ziekenhuisopname), minder hersencellen (“grijze stof”) hadden dan pubers die zo iets niet hadden meegemaakt. Het gevolg is een minder flexibel brein, wat later sterker reageert op nieuwe stressvolle gebeurtenissen, en dat kan weer gevolgen hebben voor de mentale gezondheid.

De gevoeligheid van het brein tijdens de vroege kinderjaren werkt twee kanten op. Kinderen die in een hele veilige, stimulerende omgeving opgroeien, zijn over het algemeen beter bestand tegen stress op latere leeftijd, en zijn minder gevoelig voor stress-gerelateerde aandoeningen.

**Dieronderzoek**

Toch blijft het erg moeilijk om dit soort processen tot in detail te bestuderen in mensen. Hoewel je door gebruik te maken van een MRI-scanner kan kijken naar de grootte en vorm van verschillende hersengebieden, is het niet mogelijk om afzonderlijke cellen te bestuderen in mensen, laat staan dat we een beeld kunnen krijgen van de stoffen in die cellen die een belangrijke bijdrage leveren aan het functioneren van afzonderlijke cellen en hersengebieden. Daarnaast zou dit soort onderzoek in mensen erg lang duren, omdat er bijvoorbeeld wel 40 jaar kan zitten tussen de kindertijd en het moment dat stress-gerelateerde aandoeningen zich openbaren. Daarom wordt er voor dit soort onderzoek veel gebruik gemaakt van dieren. Dieronderzoek heeft bijvoorbeeld laten zien dat muizenbaby’s die opgroeien in een kale omgeving met minder moederzorg, op latere leeftijd meer stresshormonen produceren en meer problemen hebben met hun geheugen. De uitlopers van hersencellen in bepaalde hersengebieden die belangrijk zijn voor geheugen worden korter. Hersencellen kunnen hierdoor minder goed contact maken met elkaar, waardoor de hersenen minder goed werken. Deze gevolgen kunnen het gehele leven merkbaar blijven. In mensen hadden we dit niet kunnen onderzoeken.

**Ziekte van Alzheimer**

Een voorbeeld van een ziekte waarbij stress een rol kan spelen is de ziekte van Alzheimer. We weten al dat mensen die veel stress ervaren op middelbare leeftijd een grotere kans hebben om later Alzheimer te krijgen. Het zou dan ook kunnen dat stress tijdens de kinderjaren de veranderingen in de
hersen en gedrag in relatie tot de ziekte van Alzheimer kan verergeren. Dit hebben wij onder andere onderzocht in andere speciale muizen, die verschijnselen van de ziekte van Alzheimer vertonen op latere leeftijd. Deze muizen maken het eiwit amyloid-bêta aan, wat klonten vormt in het brein, daardoor het functioneren van de hersenen bemoeilijkt en onder andere voor geheugenverlies zorgt, vergelijkbaar met wat er gebeurt in het brein van patiënten met de ziekte van Alzheimer. Stress op jonge leeftijd heeft dit proces bij deze muizen verergerd. Vervolgens hebben we muizen behandeld met een stofje dat de werking van stresshormonen verminderde, wat er verrassend genoeg voor zorgde dat de klontering van het amyloid-bêta eiwit en de geheugenproblemen voorkomen konden worden. Door deze speciale muizenbaby’s op te laten groeien in een “warm nest” met extra veel moederzorg, was het juist mogelijk het geheugenverlies in deze muizen tegen te gaan.

Geheugenverlies bij de ziekte van Alzheimer wordt onder andere veroorzaakt doordat cellen minder goed met elkaar kunnen communiceren of zelfs doodgaan. Een van de stoffen die zorgt voor deze communicatie, glutamaat, is zowel bij de ziekte van Alzheimer alsook bij muizen die veel hebben stress ervaren verstoord. Door de glutamaathuishouding weer te herstellen, kunnen zowel de effecten van stress vroeg in het leven, alsook de nadelige gevolgen van de ziekte van Alzheimer worden tegengegaan. Als gevolg hiervan verbetert de communicatie tussen cellen en wordt het geheugen beter.

Kort samengevat heb ik in dit proefschrift laten zien dat stress hele verschillende effecten kan hebben. Adaptieve stress zorgt ervoor dat je gebeurtenissen beter onthoudt, maar wanneer dit te lang duurt of te heftig wordt dan kan dit veranderen in schadelijke stress. Het geheugen wordt dan minder precies, waardoor bijvoorbeeld veilige situaties niet meer goed herkend worden. Ook kan langdurige stress, zeker als het gebeurt tijdens de kwetsbare periode na de geboorte, de gevolgen van de ziekte van Alzheimer verergeren, terwijl opgroeien in een “warm nest” er juist voor zorgt dat je minder gevoelig bent voor deze ziekte. Ik heb een aantal aanknopingspunten gevonden die mogelijk de effecten van stress op de ziekte van Alzheimer kunnen voorkomen of verminderen, hoewel dit nog wel uitgebreid onderzocht moet worden in mensen.
Positive and negative early life experiences differentially modulate long term survival and amyloid protein levels.
Sometimes
you can't make it
on your own

U2
Acknowledgements

De laatste regels van dit proefschrift wil ik wijden aan al de mensen die me hebben geholpen en gesteund tijdens het tot stand komen van dit proefschrift.

Ten eerste wil ik mijn promotor Harm bedanken. Vanaf het moment dat ik als masterstudent (veel te laat) binnen kwam om te vragen of je echt geen stageplek voor me had, tot aan het moment dat je me een PhD positie aanbood, wat nu, 6 jaar verder, heeft geleid tot dit boekje, heb je altijd je vertrouwen in mij laten blijken. Je enthousiasme en eeuwige optimisme werken aanstekelijk, en hebben me steeds opnieuw gestimuleerd verder te denken. “Even kort data bespreken” duurde geregeld de halve middag, maar leidde vaak wel tot leuke discussies, en ook de avonden lang dissecter en in de stallen en de tripjes naar Leuven zal ik niet snel vergeten. Ik wil je daarom heel erg bedanken voor de fijne samenwerking van de afgelopen jaren. Bedankt ook voor het vertrouwen en de flexibiliteit die je me gaf om zelf invulling te geven aan mijn projecten, waar ik veel van geleerd heb en die me de mogelijkheid hebben gegeven echt mijn eigen stempel op dit werk te drukken. Ook wil ik je bedanken voor de vrijheid om mijn werk zelf in te delen, waardoor ik altijd probleemloos mijn persoonlijke leven heb kunnen combineren met mijn werk.

Paul, ook jou wil ik hartelijk bedanken voor al je advies, tijd, vertrouwen en wijsdelen die je de afgelopen jaren hebt gedeeld. Ook afspraken met jou duurde nooit “maar even”, en gingen ook zelden over het onderwerp wat ik van tevoren bedacht had, maar na afloop was ik altijd weer een flink aantal mooie anekdotes en nieuwe inzichten rijker, waaronder de mooie uitspraak “Never overestimate your audience”, die me nog lang bij zal blijven. Zeker op het einde heb je uitgebreid de tijd genomen om mij van advies te voorzien over mijn vervolgcarrière, en ook al had ik duidelijk aangegeven liever binnen Europa te blijven voor mijn volgende stap, de suggesties voor overseas bleven binnenkomen, wat uiteindelijk ook heeft geholpen bij mijn definitieve keuze om naar Canada te gaan. Dank daarvoor.

Mijn commissie wil ik ontzettend bedanken voor hun tijd en expertise.

Paranimfen, een PhD doe je nooit alleen, en zonder jullie twee had ik het zeker niet gered. Jullie hebben me door alle moeilijke momenten heen geholpen, maar zeker ook de goede momenten met me gevierd.

Mara, niet vaak kom je iemand tegen die zo like-minded is op veel vlakken. Je eerlijke, directe, no BS oordeel over alles heb ik heel erg kunnen waarderen. Als ik ergens mee zat wist je vaak al voordat ik het zelf had aangegeven dat het
“echt even tijd was voor koffie”, en als er iets gebeurde (goed of slecht) was jij de eerste tegen wie ik het wilde vertellen. Jij was het vaak die mij een spiegel voorhield en in de goede richting duwde als ik ergens niet uitkwam. Maar wat heb jij ook een ontwikkeling doorgemaakt de afgelopen jaren zeg! Ik vind het zo mooi om jou te hebben zien groeien van een onzeker en voorzichtig meisje (in het lab dan, daarbuiten kon jij als geen ander je mannetje wel staan :P), naar een sterke volwassen vrouw die staat voor haar eigen visie en belangen! Ik hoop dat je dat zelf ook inziet. Ik ben jaloers op jouw briljante plan om heerlijk 2 maanden door Australië te gaan reizen, hoewel ik het wel heel jammer vind dat je er in mijn laatste maanden niet bent. Gelukkig kon ik mijn verdediging zo plannen dat jij net weer terug bent, want ik had me geen verdediging kunnen inbeelden waarbij jij niet aan mij zijde zou staan! Ik ga jou en je prachtige uitspraken missen (don’t worry, ik heb een heel mooi lijstje van jouw pareltjes die we bewaren voor jouw verdediging)!

Lieve Kit, wat ben ik jou gaan waarderen tijdens de afgelopen jaren. De “maximaal-10-minuten-koffie-pauze-beloofd-ik-zet-een-timer!” die altijd een uur duurde, je bizarre slechte plan-vermogen (hoewel je daar zelf denk ik nog steeds anders over denkt), je chaotische, lange verhalen met veel te veel detail die niemand kon volgen, je leercurve die alle kanten op ging, je koken met stokjes, je gekke gewoontes en bijgeloven (iets met twee eieren), je heilige overtuiging dat in Badhoevedorp wonen een goed idee was, je idiote plan voor een anti-body farm, je slechte nerd-science grappen en je flauwe gevoel voor humor (Lianne die een stekker niet in een stopcontact krijgt is blijkbaar hilarisch) en zo kan ik nog uren doorgaan. We hebben samen veel mooie wetenschap gedaan, nog mooiere tripjes gemaakt naar Italië, Bilbao, en San Diego, en vele, vele uren gepraat. Hoewel je soms ook onuitstaanbaar was in je koppigheid of overtuiging dat er NU pauze gehouden moest worden (desnoods in ons kantoor terwijl iedereen wilde werken), kan ik me een PhD zonder jou niet voorstellen. Zoals je het zelf verwoordt kom ik binnenkort weer bij jou in de buurt wonen (dat Badhoevedorp heeft echt jouw gevoel van “dichtbij” verpest), maar ondanks de afstand die er nu tussen ons is, is het ons heel goed gelukt om contact te houden, en ik hoop dat we dat vol blijven houden!

Ook ALLE andere leden van de Lucassen groep (nu en in het verleden) zijn onmisbaar geweest voor het voltooien van mijn PhD. Wetenschap is leuk, maar het wordt pas echt leuk door de mensen waarmee je het doet. En jullie hebben allemaal op je eigen manier mij verder geholpen in deze periode; door te helpen in het lab, overwinningen te vieren, door opbeurende woorden, of juist een luisterend oor als het eens tegen zat. Vooral de bizarre uren in december 2017, gecombineerd met de (bijna) dagelijkse frustratie-borrels die
we daarna in de Common Room deden zal ik niet snel vergeten!

**Gideon**, jij was altijd mijn eerste go-to persoon als ik ergens vragen over had of niet wist hoe ik het moest aanpakken. Op al mijn lab-gerelateerde vragen had jij direct een antwoord, en meer dan eens heb je me behoed voor grote fouten, maar ook (of juist) daarbuiten had je vaak helder advies over hoe ik zaken aan moest pakken. Het gaat wennen worden om niet meer dagelijks toegang te hebben tot zo'n fijne sparrings-partner!

Vanaf het eerste moment dat ik jou leerde kennen, **Pascal**, was het duidelijk dat jij een echte levensgenieter was, die altijd wel in was voor een borrel, uitgebreid diner of feestje. Op je best was je tijdens onze congresvakantie in Milaan en Florence, waarbij jij ons wel even liet zien hoe je je als een echte Italiaan hoort te gedragen, de lekkerste restaurants wist te vinden en ons rondleidde in allerlei Italiaanse steden. Ook ons tripje naar Melt en de vele festivals in Amsterdam waren altijd een groot succes! We zijn nu samen aan het post-doccen op de UvA, waar we samenwerken aan hele leuke wetenschap, maar gelukkig heeft dat ons niet veel serieuzer gemaakt en is er nog geregeld tijd voor een borrel of feestje!

Het is ons gelukt **Lianne**! Tijdens een groot gedeelte van onze carrière werden wij als één gezien, en nu hebben we ook dit traject alletwee afgerond. Van de vele koffiepauzes tijdens onze stage en samen bier drinken en stage lopen in München, tot daarna weer samen starten aan een PhD op de UvA en ons dubbele bezoek aan San Diego en de mooie roadtrip ervoor, of de vele avonden bierdrinken in de Polder; we hebben mooie dingen meegemaakt! Hoewel we vele uren samen op de bank in de Common Room of bij Sjeel met een kopje koffie hebben doorgebracht, ging het daarbij schrikbarend vaak over werk, nieuwe plannen, of uitdagingen in het lab. Helaas werkten wij als sport-partners wat minder goed, want vaker wel dan niet eindigden onze sportplannen op het terras van de Oerknal in plaats van in de sportschool. Hoewel onze wegen zich inmiddels helaas van elkaar gescheiden hebben, was het een hele leuke tijd samen!

**Kitty**, op het moment dat ik dit schrijf sta jij op het punt van een hele grote nieuwe stap in jouw leven, eentje die ook maar weer laat zien hoe relatief en onbelangrijk alles wat we hier doen eigenlijk is. Heel veel geluk met je kleine meid, geniet ervan! Hoewel zij vanaf nu natuurlijk altijd op één zal staan hoop ik dat het je lukt om (uiteindelijk) je motivatie en passie voor wetenschap weer op te pakken, want dat past zo goed bij je! **Silvie**, mijn naamgenootje ☺, veel leuke momenten hebben we meegemaakt, tijdens borrels, op de ONWAR, en op andere feestjes (“zoete witte wijn ♫”). Ga daarmee door, juist ook nu je
Addenda

in de tweede helft van je PhD bent aanbeland, dat houdt het leuk! **Janssen**, the “newbie” of the group! Your candid enthusiasm was inspiring! I really enjoyed having you as a part of our group, from the beginning onwards you were always up for a nice chat in the lab, a beer after work or a nice party! Don’t worry so much about everything, don’t be too strict on yourself, and start trusting yourself!

Dan de oudgedienden van de Lucassen groep, waar ik ook vele mooie herinneringen aan heb. **Eva**, gelukkig ben jij nog regelmatig op kantoor, waar jouw verfrissende, positieve kijk op dingen altijd naar een oplossing leidt. **Hui!** Thanks for all your help throughout the years (especially with all my ephys attempts), you were an incredibly nice colleague. It’s been a lot of fun working with you, and I’ll really miss your (on/over the edge) jokes. **Marijn**, toen ik begon was jij al vele jaren bezig op het Science Park. In het begin schrok ik soms een beetje van jouw cynische houding en kritische blik, maar door de jaren heen ben ik hem steeds beter gaan begrijpen en waarderen. De barbecues op jouw dakterras waren ook altijd een groot succes, en je hebt me laten zien dat een PhD ook anders kan, met vele dutjes, feestjes (fistpumps) en uitslapen (vast ook afgewisseld met momenten van hard werken).

**Aniko** and **Carlos**, I want to thank you as well, for all the constructive comments that I received from you throughout the years, and the advice, both with regard to my research as well as for my career.

**Lieve Wendy**, hoewel we uiteindelijk maar kort echt samengewerkt hebben, heb jij wel een hele belangrijke rol gespeeld aan het begin van mijn wetenschappelijke carrière. Als masterstudent twijfelde ik heel sterk welke kant ik op wilde; ik vond wetenschap wel leuk, maar al dat labwerk zag ik niet zitten. Tijdens mijn stage nam je altijd tijd om mij dingen te leren en uit te leggen, betrok je mij in jouw eigen onderzoek, en hebben we samen hele leuke projecten gedaan, ook al was ik officieel helemaal jouw student niet. In korte tijd heb je mij zoveel geleerd en me laten zien hoe leuk ook juist het praktische gedeelte van de wetenschap is, dat jij mij ertoe geïnspireerd hebt om door te gaan in de wetenschap en een PhD te gaan doen. Dank daarvoor!

**Lieve Beryl**, **Nicole**, **Amber**, **Melissa**, **Judith**, **Lisa**, **Nathan**, **David**, **Eleni** en **Nathalie**, allemaal hebben jullie op je eigen manier bijgedragen aan dit proefschrift. Veel van het werk wat hierin beschreven is, is eigenlijk door jullie uitgevoerd, en ik heb veel geluk gehad met zulke enthousiaste en getalenteerde studenten. Dank jullie wel voor de vele dagen (en avonden, en weekenden...) dissecteren, snijden, mounten, kleuren, microscopen, cellen tellen en analyseren. Deze thesis had er heel anders uitgezien zonder jullie
hulp, en ik wens jullie het beste toe met jullie toekomst, binnen of buiten de wetenschap!

Ook wil ik alle anderen bedanken die de afgelopen jaren onderdeel zijn geweest van de Lucassen groep, als PhD student, postdoc of stagiair. Er zijn ontelbare momenten geweest in het lab, of ’s avonds met een borrel, diner of feestje die ik niet snel zal vergeten. Veel van die momenten heb ik ook gedeeld met mijn andere collega’s van het SILS-CNS van de Smidt, Wadman, Kessels, and Pennartz groepen tijdens de maandelijkse borrel, de CNS battles (hoewel we daar nou niet bepaald in uitblonken) of tussendoor. Bedankt dat jullie het SILS zo’n leuke werkomgeving hebben gemaakt!

In het bijzonder ook bedankt aan Lianne. Vanaf het begin van mijn studententijd hebben wij samen opgetrokken, eerst als ActieCie, later gewoon als vrienden en daarna ook als collega’s. Jij hebt mij zien (en geholpen) ontwikkelen van verlegen student naar beginnend PhD, naar de plek waar we nu alletwee staan. Regelmatig bespreken we alle perikelen van ons onderzoek, gedoe met studenten, en onze PIs, wat mij altijd weer relativering geeft. Ik vond onze wetenschappelijke kruisbestuiving ook heel leuk, met de les Ephys 101 (van jou) en Pipetteren 101 (van mij) om wat meer van elkaars wereld te snappen. Jij bent nu ook bezig met de laatste (zware) loodjes, maar ik weet zeker dat je het kan!

Lieve Babette, Anouk, en Merel, al vanaf het begin van onze studententijd vormden wij een team. Eerst als bestuur van Congo, maar eigenlijk vooral als vrienden. Onze vakanties naar bijvoorbeeld Winterberg, Marrakesh, Lissabon of Bristol, en de ontelbare lunches, koffietjes, etentjes en avondjes uit waren altijd de perfecte afwisseling om even goed te ontspannen. Babette, onze wekelijkse lunch- en koffiedates zijn echt iets waar ik naar uitkijk (hoewel ik niet zeker weet of je dat voor mij doet of om Alexia te zien). Je gezelligheid, relativeringsvermogen en onbevooroordeelde luisteren kan ik altijd heel erg waarderen, evenals je onuitputtelijke kennis over bier overigens. Anouk, het lukt ons nooit om lang in hetzelfde land te zijn. Nadat je al halve studententijd in het buitenland had doorgebracht, vond jij het ook nog eens nodig om jouw PhD in Bristol te gaan doen, en net nu je bijna klaar ben en (hopelijk!) terugkomt, vertrek ik naar Canada. Ondanks de afstand kan ik altijd bij jou terecht voor de belangrijke dingen in mijn leven (zoals die keer dat ik je zaterdagochtend om 7 uur had gebeld om iets belangrijks te vertellen, en jij al meteen wist wat het was). Heel veel succes met het afronden van jouw PhD! Merel, ook jij bedankt voor de urendurende lunches met goede gesprekken en nog betere koffie, in allemaal gekke vega en vegan restaurantjes in Amsterdam en ver daarbuiten. Helaas is de afstand tussen ons nu ook wat groter geworden (en straks met
Toronto zeker), maar we verzinnen er wel iets op!

Lieve Opa en Oma, ook al hebben jullie mijn promotietraject niet echt meer bewust meegekregen, ik wil jullie toch bedanken voor alle steun van de afgelopen jaren, en voor alle interesse in wat ik aan het uitspoken ben. Hoewel jullie niet altijd snapten waar ik mee bezig was (Opa: “onbestaanbaar, een muis heeft beslist geen hersens”, en Oma: “Ik heb laatst een muis gevange in onze muizenvaal, zal ik hem voor je bewaren dan heb je weer een extra muis om onderzoek op te doen”), zijn jullie altijd extreem betrokken geweest bij mijn doen en laten. Dat ik weet hoe trots jullie zijn doet me veel goed.

Lieve familie, vaak hebben jullie onbegrijpelijke verhalen over muizen en papers moeten aanhoren, maar altijd waren jullie geïnteresseerd in hoe het ging. Adrie en Marianne, wat ben ik jullie dankbaar voor alle steun die jullie mij hebben gegeven tijdens mijn studie en promotie. Al lang voordat ik aan deze PhD begon hebben jullie mij geleerd vertrouwen te hebben in mijzelf, het beste in mezelf naar boven te halen, en nooit “nee” te accepteren, wat me veel heeft geholpen tijdens deze grote uitdaging. Reem, dank voor al je feedback, ik denk dat jij degene ben die het meest van dit boekje gelezen heeft... En ook al twijfel je zelf soms zoveel dat je niet weet wat je in een restaurant zal bestellen, je kon mij altijd van goed advies voorzien. Gelukkig ben jij naar de "goede kant" overgestapt (mr. Lease-auto), maar ik hoop alsnog dat je je plannen om in het buitenland te werken niet laat varen (Canada schijnt erg mooi te zijn 😊). Op naar jouw eerste publicatie! Laura, de afgelopen jaren heb je ons regelmatig meegesleept naar de prachtigste vakantieoorden, en hoewel ik in het begin altijd twijfelde of ik wel mee kon gaan (“geen tijd, te druk”), lukte het je altijd weer om het zo aantrekkelijk te maken dat ik geen nee kon zeggen, en dat was maar goed ook. Ik vind het zoo leuk dat wij nu in dezelfde fase van ons leven zitten, en dat ik dat nu met jou kan delen!

Lieve kleine Alexia, ook al ben je nu nog te klein om dit te lezen, ook jij hebt zeker je stempel op dit werk gedrukt. Meer dan wie ook heb jij me geleerd het perspectief te zien van waar ik mee bezig ben. Je hebt me geleerd dat mijn tijd waardevol en kostbaar is, en dat ik kritisch moet nadenken waaraan ik het besteed. Maar het meest van alles heb je me laten zien hoeveel geluk er schuilt in de kleine dingen, en ik hoop zo dat het lukt de komende jaren dat onbevangen geluk vast te houden.

Lieve Martin, hoewel je mij vaak voor gek hebt verklaard voor mijn, in jouw ogen dan, bizarre werkethos en drive om wetenschap te doen, bleef je me altijd voor 100% steunen. Als ik’s avonds veel te laat, moe en hongerig thuis kwam, was je er altijd met een luisterend oor (en vaak ook een lekker maaltje). Je heb zelfs
een keer een hele avond mee zitten cellen analyseren toen ik de voorbereiding voor één of andere belangrijke meeting weer eens helemaal verkeerd had ingeschat (zoals helaas wel vaker voorkwam). Jouw onvoorwaardelijke steun heeft me door dit proces heen gesleept, je stimuleerde me als ik er geen zin meer in had, maar vooral: remde me af als ik weer eens alle perspectief uit het oog verloren was. Het is altijd "jij en ik tegen de wereld" geweest, en dat hebben de afgelopen jaren alleen maar meer bevestigd. Ik ben zo trots op de ontwikkeling die jij de afgelopen jaren hebt doorgemaakt, en zeker sinds we Alexia hebben. Bedankt voor alles, ik hou van je.

Ik hoop dat het duidelijk is dat de totstandkoming van dit boekje teamwork is geweest, en hoop dat ik niemand ben vergeten te bedanken.
Addenda

PhD portfolio

(Inter)national presentations

2018 Lab meeting prof. dr. Sheena Josselyn: “Glucocorticoids induce generalised fear by increasing the size of memory-encoding neuronal ensembles”. Oral presentation.
Lab meeting prof. dr. Stephen Maren: “Glucocorticoids determine memory strength and neuronal allocation to a hippocampal engram”. Oral presentation.
Federation of European Neuroscience Societies (FENS) Forum 2018: “Memory generalisation following stressful events can be traced back to the dentate gyrus”. Poster presentation.
Stress meeting Utrecht (lab prof. dr. Marian Joëls): “Corticosterone induces memory generalisation; a role for the dentate gyrus”. Oral presentation.
Dutch Neuroscience meeting 2018: “The neuronal underpinning of stress-induced memory generalisation”. Oral presentation, session chair “In search of the memory engram”.


Speaker in the Masterclass led by prof. dr. Sheena Josselyn (SickKids Hospital, Toronto, Canada): “Neuronal allocation to a hippocampal engram by glucocorticoid hormones”. Oral presentation.


2016

Society for Neuroscience meeting 2016: “Early life stress enhances amyloid pathology and aggravates cognitive decline; rescue by short blockade of glucocorticoid receptors at mid-age”. Poster presentation.

Speaker in the Masterclass led by Bruce McEwen (The Rockefeller University, New York): “Early life stress accelerates amyloid pathology and cognitive decline in an Alzheimer mouse model; rescue by briefly blocking GRs”. Oral presentation.


Speaker in the Masterclass led by Helen Scharfman (NYU School of Medicine, New York): “Early life stress and the development of Alzheimer’s disease”. Oral presentation.

Lunteren, The Netherlands.


Dutch Neuroscience meeting 2015: “Positive and negative early life experiences modulate survival and amyloid protein levels in a mouse model for Alzheimer’s Disease”. Poster presentation.

Amsterdam, The Netherlands.


Lunteren, The Netherlands.

Alzheimer’s Disease Workshop: Alzheimer’s Disease Research from Different Perspectives, "Early Life Stress and Alzheimer’s Disease". Data pitch.

San Diego, U.S.A.


Lunteren, The Netherlands.

Grants & awards:

2018 Travel grant from the Committee of Higher Education and Training (CHET) from the FENS for the attendance of the Japan Neuroscience meeting 2018 in Kobe, Japan

2018 Travel Grant for Young Researchers from the Genootschap ter bevordering van Natuur-, Genees-, en Heelkunde (GNGH) to attend the FENS 2018, Berlin, Germany

2017 Travel grant for attending the EBBS meeting 2017 in Bilbao, Spain

2016 Travel grant from Alzheimer Nederland for attending the Society for Neuroscience meeting 2016 in San Diego, U.S.A.

Best poster prize, the EBBS-EMCCS joint meeting 2016
2013  Travel grant for the attendance of the Alzheimer’s Disease Workshop in San Diego, U.S.A., November 2013, ISAO
   Sponsored attendance of the Endo-Neuro-Psychomeeting
   Personal grant Saal van Zwanenburg fonds
   Personal grant Hendrik Muller fonds

2012  Travel grant Erasmus program
   Travel grant Amsterdam Universiteitsfonds

Professional society memberships:

2018  Japan Neuroscience Society
2017-current  European Brain and Behaviour Society (EBBS)
2016  Society for Neuroscience (SfN)
2016-current  Dutch NeuroFederation
2016-current  Federation of European Neuroscience Societies

Public outreach/academic activities:

2011 – 2013  Master Committee, organising various activities for master students in Biological and Biomedical Sciences.
   Student association C.O.N.G.O. Amsterdam.
2009  Organising committee National Biology Student Congress. LOBS (“Dutch National Biology Students Association”).
2008 – 2010  Board member (2y) of the student association for Biology, Biomedical Sciences, and Psychobiology.
   C.O.N.G.O.

Conference organisation:

2018  Session organiser and chair at DN Meeting Lunteren.
2017  Organising committee SILS-CNS research day, University of Amsterdam.
2015  Organising committee yearly retreat of the Graduate School Neurosciences Amsterdam Rotterdam (ONWAR).
Addenda

Peer-reviewed publications


**Book chapters**


**Submitted manuscripts**

**Lesuis, S.L.,** van den Oever, M.C., Immerzeel, N., Lucassen, P.J., Krugers, H.J. *Glucocorticoids induce generalised fear by increasing the size of memory-encoding neuronal ensembles.*

**Lesuis, S.L.,** Lucassen, P.J., Krugers, H.J. *Treatment with the glutamate modulator riluzole prevents early life stress-induced cognitive deficits and impairments in synaptic plasticity in APPswe/PS1dE9 mice.*

**Lesuis, S.L.,** Lucassen, P.J., Krugers, H.J. *Early life stress impairs fear conditioning memory and synaptic plasticity; a potential role for GluN2B.*

**Lesuis, S.L.,** Lucassen, P.J., Krugers, H.J. *Early life stress amplifies fear responses and hippocampal synaptic potentiation in the APPswe/PS1dE9 Alzheimer mouse model.*
About the author

Sylvie Lisa Lesuis was born on the 21st of May, 1989, in Rotterdam, The Netherlands. She grew up in this town and obtained her pre-university degree in 2007 at the Wolfert van Borselen Bilingual (English-Dutch) secondary school, where she also obtained her International Baccalaureate Certificate English A1 higher level. After this, she moved to Amsterdam to obtain her propaedeutics in Social Sciences (2009), and her bachelor’s degree in Psychobiology (2010) at the University of Amsterdam (UvA). She proceeded her education at the UvA within the Master programme Biomedical Sciences – Neuroscience, with the specialisation Psychopharmacology and Pathophysiology, from which she graduated cum laude in 2013.

As part of her Master’s training, Sylvie performed several research internships. At the Brain Plasticity Group (SILS-UvA), under the supervision of dr. Harm Krugers and dr. Erik Manders, she investigated the effects of stress hormones on hippocampal spines. She wrote her literature thesis about the consequences of prenatal exposure to selective serotonin reuptake inhibitors (SSRI) and/or maternal depression on brain development and behaviour of children. Sylvie continued to study the effects of stress during her second internship in the group of dr. Mathias Schmidt at the Max Planck Institute for Psychiatry in Munich, Germany. Here, she investigated how stress early in life programs the brain to cope with future challenges. This resulted in her first scientific publication.

In November 2013, Sylvie continued her academic career as a PhD student at the Swammerdam Institute for Life Sciences (UvA) under the guidance of dr. Harm Krugers and prof. dr. Paul Lucassen. In this project, she focussed on whether and how early life experiences determine the brain’s vulnerability to Alzheimer’s disease. Simultaneously, Sylvie has been investigating how stress hormones enhance memory formation, and what the underlying neuronal profile is of memory traces. The findings of the various studies that Sylvie conducted are presented in this thesis, have been presented at multiple national and international meetings, and have been published in peer-reviewed journals.

Sylvie is currently working as a postdoctoral researcher in the group of prof. dr. Onno Meijer, and she will soon continue her academic career as a postdoctoral researcher in the laboratory of prof. dr. Sheena Josselyn at the Sick Kids Hospital in Toronto, Canada.