Unraveling the link between major depression and Alzheimer's disease

The effects of prenatal stress in the APPswe/PS1dE9 mouse model of Alzheimer's disease

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The energy of the mind is the essence of life.

Aristotle

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List of abbreviations

AD: Alzheimer's disease

Aβ: amyloid-β

ACTH: adrenocorticotrophic hormone

APP: amyloid-β precursor protein

APOE: apolipoprotein E

BDNF: brain-derived neurotrophic factor

CRF: corticotrophin-releasing factor

DAB: 3,3'-diaminobenzidine

F-C: female control

F-PS: female prenatal stress

GAPD: glyceraldehyde 3-phosphate dehydrogenase

HPA: hypothalamic-pituitary-adrenal

IDE: insulin degrading enzyme

M-C: male control

MD: major depression

M-PS: male prenatal stress

NMDA: N-methyl-D-aspartate

PNRS: prenatal restraint stress

PS: prenatal stress

PS1/2: presenilin 1/2

SIPB: synaptophysin-immunopositive presynaptic bouton

SL: stratum lucidem

SM: stratum moleculare

SORL: sortilin-related receptor L

SSRI: serotonin-selective reuptake inhibitors

SR: stratum radiatum

TBS: Tris-buffered saline

TBS-T: Tris-buffered saline with 0.2% Triton-X100

Preface

It seems like just yesterday when I started my college career. And yet here I am, on the verge of graduating as a master in molecular life sciences. During the past five years I have come across a lot of interesting topics, but already very early I realized that it was the brain in particular that spiked my interest. It was for that reason that I opted for a senior internship in the field of neuroscience and I couldn't be more happy with the choice I made. The past eight months have been interesting and exciting. I got a chance to experience working on a real research project, to learn new lab techniques and to meet some interesting people.

During my internship I have realized that the road to scientific results can be very long, but I also learned that this is not a road you have to walk alone. Therefore I would like to take the opportunity to thank some people for their help and support in the past months.

First of all, I want to thank Annerieke Sierksma, for her daily guidance and support. Thank you for always making time to answer my questions and for all the help during our experiments in the lab. Also, thank you for reviewing my thesis all those times, your comments, tips and tricks were always very useful.

I also want to thank Dr. Jos Prickaerts and Dr. Daniel Van den Hove for giving me the opportunity to do this internship. It was nice to get a different point of view on my results and I have learned a lot from our discussions.

Next, thank you to my fellow interns in the student room. Ingrid, Romina, Sarina, Anouk, Wesley, Tahnee, Silke and Stephanie, it was fun to have you around. It was easy to get distracted with you next to me, but at the same time your laughter and silly comments cheered me up when I had a bad day.

Thank you to my friends at home as well. You were always there when I needed to be reminded that there exists a world outside the lab as well.

Last but not least, I want to thank my parents and my sister for supporting me during my studies and for always having faith in me.

Abstract

Alzheimer's disease (AD) is the most common form of dementia in the elderly. In 2006 the World Health Organization estimated that about 18 million people worldwide suffer from AD and this number is still increasing. On a molecular level AD is characterized by the extracellular deposition of Aβ proteins and intracellular aggregation of tau proteins, as well as severe neurodegeneration. Several brain areas are affected by the disease, but the hippocampus is one of the first regions were neuropathological problems occur. Risk factors for AD, such as age, gender and genes, e.g. APP and PS1, are already under investigation for several decades. A disorder that has received growing attention as a risk factor for AD is major depression (MD). MD is a common psychiatric disorder that is characterized by changes in mood and has a life-time prevalence of 13% in the European population. Several studies in humans have indicated that a history of MD increases the risk of developing AD, while animal studies show that a depressive-like phenotype increases neuropathology in AD mouse models. Using the APPswe/PS1dE9 mouse model of AD and prenatal stress (PS), a model for MD, the mechanisms underlying the link between MD and AD were investigated in the present study. We hypothesized that exposing APPswe/PS1dE9 mice to PS would increase AD-related neuropathology in the hippocampus, i.e. heightened plaque load and loss of plasticity, which could possibly explain the observed behavioral alterations. We see a clear distinction in plaque load between males and females, with females having a bigger plaque load then males. Moreover, we find that the cognitive problems in APPswe/PS1dE9 mice are sex-specific, with problems in male mice being attributed to changes in synaptic size, while in female mice plaque load seems to contribute more to cognitive deficits. Further, PS in early pregnancy reduces Aβ deposition and changes the HPA activity in response to stress after birth. In conclusion, PS affects the hippocampus differently in males and females, with changes in synapses being more pronounced in males, while there are more changes in Aβ load in females. Furthermore, we see that changes in behavior cannot be explained completely by changes plaque load or synaptic plasticity, thus other mechanisms, e.g. changes in brain-derived neurotrophic factor (BDNF), play a role as well.

De ziekte van Alzheimer (AD) is de meest voorkomende vorm van dementia bij ouderen. Volgens een schatting van de Wereldgezondheidsorganisatie leden in 2006 ongeveer 18 miljoen mensen wereldwijd aan AD en dit aantal neemt nog elke dag toe. Op moleculair niveau wordt AD gekarakteriseerd door een extracellulaire afzetting van Aβ proteïnen, een intracellulaire afzetting van tau proteïnen en ernstige neurodegeneratie. Verschillende gebieden in de hersenen worden aangetast tijdens het verloop van de ziekte, maar de hippocampus is één van de eerste gebieden waar neuropathologische problemen voorkomen. Er wordt al verschillende decennia onderzoek gedaan naar risicofactoren voor AD, zoals leeftijd, geslacht en verschillende genen, bijvoorbeeld APP en PS1. Een ziekte die steeds meer aandacht krijgt als mogelijke risicofactor voor AD is major depression (MD). MD is een veel voorkomende psychiatrische stoornis die gekarakteriseerd wordt door stemmingsveranderingen en een prevalentie van 13% heeft in de Europese bevolking. Verschillende humane studies hebben reeds aangetoond dat een geschiedenis van MD het risico op AD verhoogt. Verder hebben dierstudies aangetoond dat een depressief fenotype de neuropathologische symptomen in muismodellen van AD zal versterken. Door het APPswe/PS1dE9 AD muismodel te combineren met prenatale stress (PS), een diermodel voor MD, worden de mechanismen onderzocht die MD verbinden met AD. Onze hypothese is dat het blootstellen van APPswe/PS1dE9 muizen aan PS de AD-gerelateerde neuropathologie in de hippocampus, zoals Aβ afzetting en verlies van plasticiteit, versterkt. Onze resultaten geven een duidelijk verschil in plaque load aan tussen mannetjes en vrouwtjes, waarbij vrouwtjes een grotere plaque load hebben dan mannetjes. Verder blijkt dat de cognitieve problemen in APPswe/PS1dE9 muizen geslachtsafhankelijk zijn. Problemen in mannetjes lijken te worden veroorzaakt door veranderingen in synaps grootte, terwijl in vrouwtjes de Aβ afzetting een grotere rol lijkt te spelen. Bovendien zorgt blootstelling aan PS vroeg in de zwangerschap bij nakomenlingen voor een verlaging van Aβ afzetting en HPA-as activiteit in reactie op stress. In conclusie, PS beïnvloedt de hippocampus op een verschillende manier in mannetjes en vrouwtjes. In mannetjes spelen de veranderingen in synapsen een grotere rol, terwijl veranderingen in plaque load belangrijker zijn in vrouwtjes. Verder zien we dat de veranderingen in gedrag niet volledig verklaard kunnen worden door veranderingen in plaque load of synaptische plasticiteit, dus spelen andere mechanismen, bijvoorbeeld veranderingen in BDNF, ook een rol.

Introduction

1. Alzheimer's disease

1.1. Epidemiology

Alzheimer's disease (AD) is the most common form of dementia in the elderly. In 2006, the World Health Organization estimated that about 18 million people worldwide suffer from AD. The incidence of AD increases with age and due to a general ageing of the world's population, the number of AD patients is expected to increase to 34 million by 2025 (1). This increase will probably be most pronounced in developing countries, but also in the Netherlands an increase of 46% in the number of dementia cases is expected between 2005 and 2025 (2).

Clinically, AD starts with minor changes in mood and behavior and will progress to a state of disorientation, memory loss and impairment of speech. The patient will lose basic bodily functions and become immobile and mute. At present the AD therapies that are available are directed against improving symptoms. Memantine for example is a N-methyl-D-aspartate (NMDA) receptor antagonist that appears to have a beneficial effect on cognitive function in moderate to severe AD cases. Currently none of the available treatments is able to cure AD or stop the disease progression, hence the disease is always fatal; the patient will eventually die, usually from intercurrent pneumonia or other infections (3, 4).

1.2. Neuropathology

Neuropathologically AD is characterized by neuritic plaques. These plaques are mainly composed of an aggregated form of the peptide amyloid-β (Aβ), which is produced by proteolytic cleavage of the Aβ precursor protein (APP) by two proteases called β- and γ-secretase. APP is a protein that is thought to be important in wound repair as well as growth-stimulation. Proteolysis of this protein can happen via a non-amyloidogenic or amyloidogenic pathway (Fig. 1). In the non-amyloidogenic pathway APP is cleaved by α-secretases inside the Aβ sequence, generating a soluble N-terminal part of APP and a C-terminal fragment C83 anchored in the membrane. The C-terminal part can be processed further by γ-secretase. Alternatively, in the amyloidogenic pathway APP is cleaved by β-secretase at the N-terminal of the Aβ sequence leading to the formation of a N-terminal part of APP and a C-terminal part C99. Cleavage of the C99 intermediate at the C-terminal of the Aβ sequence by γ-secretase generates the amyloidogenic Aβ peptide. The cleavage site of γ-secretase is of critical importance. Depending on the site proteolysis by γ-secretase will produce different forms of Aβ, most commonly Aβ40 and Aβ42. The Aβ42 peptide has a greater tendency than Aβ40 to form fibrillary Aβ (5).

It is known that mutations found in AD patients often affect APP processing leading to overproduction of the toxic Aβ42 peptide, which will accumulate into neuritic plaques. The accumulation leads to the progressive degeneration of neurons, which is most pronounced in the frontal, temporal and parietal lobes of the brain. Aβ deposition is particularly high in the hippocampus of AD patients, which is also one of the first regions where neuronal cell death takes place in AD (4). The hippocampus is well known for its important role in memory and is involved in the cognitive processing of space and time (reviewed in (6)).

It is hypothesized that the accumulation of Aβ leads to another hallmark of AD, namely hyperphosphorylation of the protein tau. This causes a redistribution of the protein within the

neuron and a subsequent aggregation of the protein into neurofibrillary tangles. Tau normally binds tubulin to stabilize neuronal microtubules. In AD patients hyperphosphorylation of tau leads to an impaired microtubule function, resulting in neuronal dysfunction and cell death by breakdown of the neuronal cytoskeleton (3, 5).

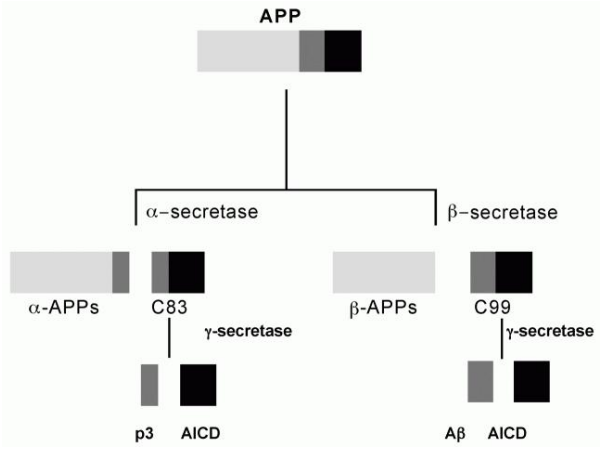

Figure 1. Schematic view of the Aβ precursor protein (APP) degradation pathway (7)

1.3. Risk factors

Several genes are thought to play a role in AD pathology, but these differ between the familial and the sporadic form of the disease. The first gene that was identified in relation to early onset (before 65 years of age) familial AD was the APP gene (8). Several missense mutations in this gene are linked to AD, but their causative mechanism is not yet understood. They might alter the metabolism of APP in favor of the neurotoxic Aβ42 peptide over the more soluble, rapidly cleared Aβ40 form (9). Other genes that are known to lead to the familial form of AD are the presenilin (PS1 and PS2) genes, which are thought to cause up to 80% of the familial AD cases. The proteins produced by these genes are subunits of the γ-secretase protein complex which is responsible for the cleavage of APP. The exact mechanism is not yet completely understood, but it is likely that mutations in PS1 and PS2 genes shift the cleavage site in APP and in that way elevate levels of Aβ42 (9, 10).

In these cases AD can be seen as an autosomal dominant disease resulting from a single gene defect, but the number of AD cases that is due to these mutations is less than 5% of total AD cases (10). While they account for the early onset familial form of AD, the sporadic form of AD has a multifactorial etiology with some genetic polymorphisms as predisposing factors. The ε4 allele of the apolipoprotein E (APOE) gene is one of the most well-known risk factors for the late onset sporadic form of AD. This particular allele is associated with a decreased age of onset and a lowered cognitive performance and the risk of AD increases when the number of allele copies increases (11, 12). Another gene that is known to be involved in the pathology of AD is the sortilin-related receptor gene SORL1. This protein binds directly to APP and acts as a sorting receptor, directing APP either to the endocytic or to the recycling pathway. It is suggested that SORL1 expression or function are causally linked to the pathogenesis of AD and that an underexpression of this protein will lead to an overexpression of Aβ and thus increase AD risk (13).

In addition several other genes, for example insulin degrading enzyme (IDE) and glyceraldehyde 3-phosphate dehydrogenase (GAPD), are also reported to influence AD risk (9, 14).
Furthermore, there are several epidemiological factors that are linked with an increased risk of developing AD. The incidence of AD increases strongly with age, ranging from 3% for individuals 65 to 74 years old to 47% for individuals over 85 years old (3). Gender also affects the incidence of AD, although the reason for this remains uncertain. Hebert and colleagues reported that women and men have the same risk of AD. However, because women live longer, more will develop AD and they will survive longer with the disease (15). A meta-analysis reported that women do have a significantly higher risk for developing AD than men. They suggest that this could either be the result of a sex-APOE genotype interaction or that hormonal changes in postmenopausal women in association with other factors account for this increased risk (16). Moreover, another study shows that the association between AD pathology and clinical symptoms of AD in women is more than six times larger than in men (17).

2. Major depression

2.1. Epidemiology

A disorder that received growing attention as a possible risk factor for developing AD is major depression (MD). In Europe, a life-time prevalence of approximately 13% was reported for this disease (18). MD is a common psychiatric disorder that is characterized by alterations in mood and that causes symptoms ranging from loss of appetite and weight changes to insomnia, cognitive problems and thoughts of death and suicide (19). On average, an episode of MD lasts about two years, but without proper treatment, the disease will reoccur in 50% of the cases (19). Women are at greater risk to develop MD than men; studies report that the likelihood to develop MD is two times bigger in women than in men (20, 21). There is a study proposing that women are more prone to develop MD because they respond stronger to severe stressful life events, which are known to be related to MD, when compared to men (21). The neurobiological basis for this difference remains largely unknown. It has been suggested that gender differences in hormonal status may play a role, or that the difference may be related to sexual dimorphism in some of the brain areas that are important in MD, such as the hippocampus, hypothalamus or amygdala (20). However, these hypotheses need further investigation.

2.2. Molecular mechanisms

There are several hypotheses about the molecular biology of MD (reviewed in (22)). One of the best studied hypotheses of depression is the monoamine hypothesis. This hypothesis states that MD is caused by a decreased concentration of neurotransmitters serotonin, norepinephrin and dopamine in synapses of the brain. This hypothesis gave rise to a first generation of antidepressants, the trycyclic agents and monoamine oxidase inhibitors. These medicines work by inhibiting the neuronal reuptake of serotonin or norepinephrine and inhibiting monoamine degradation by monoamine oxidases, respectively. This results in an increase in the amount of synaptic monoamines, which is thought to produce positive neuroplastic changes. Because the exact molecular mechanisms of MD are still unknown, very limited progress has since been made in the development of new antidepressant medication. A second generation of antidepressant

therapies, such as SSRIs (serotonin-selective reuptake inhibitors), has been developed. However, these newer therapies have the same mechanism of action as the first generation antidepressants, only with fewer side effects (20, 22).

Another well studied hypothesis is the corticosteroid hypothesis, which says that hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis is the primary mechanism in depression. In normal physiological conditions this system is activated in response to stress (Fig. 2). Cells of the hypothalamus produce corticotrophin-releasing factor (CRF), which stimulates cells of the pituitary to produce adrenocorticotrophic hormone (ACTH). This ACTH will in turn activate the adrenal glands to produce glucocorticoids (cortisol in humans). Hyperactivity of the HPA axis will cause an increased production of cortisol, which is indeed observed in MD. Normal levels of cortisol activate hippocampal cells, which will inhibit CRF production in the hypothalamus and thus inhibit further cortisol production. However, a chronically elevated level of cortisol is thought to be toxic to hippocampal neurons and in this way hampers cognition. Indeed, a reduction in hippocampal volume is seen in persons suffering from depression (23). Further, as the hippocampus inhibits HPA activity in normal conditions, damage to hippocampal neurons will create a vicious circle of constant HPA hyperactivity (20).

Some also suggest that MD is caused by a decrease in neurogenesis and neuroplasticity in the limbic system (20). A smaller hippocampus is often seen in patients with MD (24). This can be caused by a decreased brain-derived neurotrophic factor (BDNF) concentration in this area of the brain (25). It is known that acute as well as chronic stress decreases BDNF concentration in the hippocampus of rodents (26). However, direct evidence that links hippocampal BDNF to depression is still limited.

Up until now, it remains unclear which of these abnormalities are the primary cause of MD and which are secondary to another initiating cause.

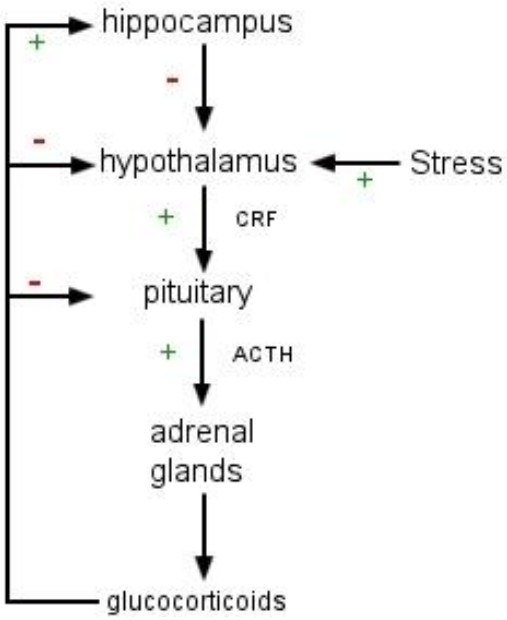

Figure 2. Schematic view of the hypothalamus-pituitary-adrenal (HPA) axis and its inhibition by the hippocampus

2.3. Animal models

Aside from limited knowledge about the molecular mechanisms underlying MD there is also a lack of knowledge about the genetic causes of the disease, which makes it difficult to study MD in laboratory animals. At this moment, a much used animal model to study MD in rodents is the use of prenatal stress (PS). The use of this model is based on the fact that a higher incidence of behavioral and neurobiological abnormalities is seen in adults who were exposed to PS. For example, a study that compared subjects that were prenatally exposed to maternal stress in the form of a major earthquake to matched controls concluded that exposed subject had increased levels of severe depression and overall depressive symptoms compared to controls (27). In animal studies PS has been associated with depressive-like behavior as well as several neurobiological changes. For example, rats exposed to prenatal restraint stress (PNRS) show a highly responsive HPA axis and changes in the monoamine content and turnover in several brain areas (28). Furthermore, several other studies have shown that PS is associated with increased depressive-like behavior and that depression-related effects of PS can be decreased by treating animals with antidepressants (29, 30).

Several forms of stress (summarized in (31)) are used in the PS model, but by far the most used method of applying stress is by using PNRS. In this method the pregnant animal is placed in a plastic cylinder for a certain amount of time, which varies between different studies, and is unable to turn around or escape. From previous studies it is known that PNRS leads to a dysregulation of the serotonin system and the HPA axis in both rats and mice (28, 32). In most cases PNRS is applied in the last weeks of pregnancy, however, a study published in 2008 hypothesizes that the effect of stress may be different when applied at different time points during pregnancy. This study shows that sex-specific effects of PNRS on affect and cognition are more pronounced when stress is applied during early gestation, compared to mid and late gestation (32).

3. Linking major depression and Alzheimer's disease

3.1 Evidence from literature

More and more evidence suggests that MD can act as a risk factor for dementia in general, and AD in particular. In 2006, a meta-analysis (33) was conducted of 20 case-control and cohort studies, that had investigated the relation between a history of depression and AD. This meta-analysis concluded that people with a history of depression were more likely to be diagnosed with AD later in life and thus confirmed that MD is a risk factor for AD. In two postmortem studies it was indicated that patients diagnosed with AD, who have a history of MD, not only show a faster cognitive decline, but also exhibit a higher plaque load and more advanced stages of neurofibrillary tangles in the hippocampus than AD patients without a history of MD (34, 35). Further, AD and MD have several histopathological and neurobiological features in common. Neurodegeneration, for example, is an important characteristic for both MD and AD. As mentioned above, BDNF signaling is thought to play a role in the neurobiology of MD (25). In AD a decrease in BDNF protein levels and BDNF mRNA has been observed as well (36, 37).

Aside from human studies, several animal studies have indicated a possible link between MD and AD. It was demonstrated that chronic immobilization stress accelerates cognitive impairment and increases extracellular amyloid deposition and neurodegeneration in the APP_{V717I}-CT100 transgenic

mouse model of AD (38). In addition, it has been reported that exposing the Tg2576 mouse model of AD to chronic isolation or restraint stress, does not only lead to MD-associated neurobiological symptoms, but also to an increase in AD-related neuropathological symptoms (39, 40). Another studie in Tg2576 mice also showed that PS leads to an increase in cognitive decline (41).

3.2 Aim of the study

In this study, the APPswe/PS1dE9 mouse model is used as a model for AD. This mouse model expresses a mutant form of the human APP gene and of the human PS1 gene, both of which cause the mice to develop Aβ deposition typical of AD at six months of age (42). To study the link between MD and AD, we combined this mouse strain with the PNRS model. Preliminary data from our lab show that exposure to PNRS leads to a decreased spatial memory in male APPswe/PS1dE9 offspring, while spatial memory increases in the female APPswe/PS1dE9 offspring after exposure to PNRS. Further, female offspring also shows more depressive-like behavior in response to PNRS (data not published).

Which mechanisms underly these behavioural alterations remains to be elucidated. We hypothesize that exposing APPswe/PS1dE9 mice to PNRS will increase AD-related neuropathology in the hippocampus, i.e. heightened plaque load and loss of plasticity, which can possibly explain the observed behavioural alterations.

Materials and methods

1. Animal model

In this study we used the APPswe/PS1dE9 mouse model as a model for AD. This mouse model expresses a mutated form of the human APP gene and of the human PS1 gene, both of which cause the mice to develop Aβ deposition typical of AD at six months of age (42). To study the link between MD and AD, we combined this mouse strain with the PS model, which is an established developmental model for MD in rodents (29, 30). Approval from the animal ethical committee (DEC) was obtained.

During the first week of pregnancy, pregnant APPswe/PS1dE9 mice (dams) were exposed to restraint stress by being placed in transparent cylinders with five mililiters of water for 45 minutes, three times a day, whilst being exposed to bright light. Dams that were kept undisturbed in their cages during this period were used as controls. After weaning, offspring was housed per two and divided over four groups: control males (M-C), control females (F-C), PS-exposed males (M-PS) and PS-exposed females (F-PS). At three months of age, all animals were housed individually as a result of severe aggressive behavior among the male offspring. At six months of age, half of the offspring underwent behavioral testing to examine affective behavior and cognitive performance. Affective behavior was determined using the elevated zero maze and the forced swim test. To study cognitive performance, the object location task and the y-maze were used. The other half of the offspring was left undisturbed in their cages. Blood was collected from the tested animals via saphenous vein puncture under basal, stressful and recovery conditions for corticosterone measurements.

2. Tissue preparation

Three days after blood collection, the tested animals were sacrified by intracardial perfusion with Somogyi fixation under deep pentobarbital anaesthesia. The brains were removed and cryo-protected by immersion in 15% sucrose in Tris-buffered saline (TBS) at 4°C overnight. They were quickly frozen using CO_2 and embedded in Tissue-Tek embedding medium (Sakura Finetek, Alphen aan den Rijn, the Netherlands). The embedded brains were cut free floating into ten series of 30 µm thick coronal sections using a CM 3050 cryostat (Leica, Wetzlar, Germany). During cutting, the first series of sections was mounted onto glass slides to check the quality of the sections. This series was stored at -20°C until analysis, while the other nine series were stored at -80°C.

The undisturbed animals were sacrificed by quick decapitation after which their brains were removed and microdissected. The hippocampus and frontal cortex of both hemispheres were isolated from the rest of the brain and all three parts were snap frozen in liquid nitrogen. The hippocampi and frontal cortices were homogenized using a Beat Beater (Biospec products, Bartlesville, USA) for 2 x 30 seconds in 1 ml ice-cold lysis buffer (NaCl, Tris-HCl, Igepal, glycerol and sodiumorthovanadate). Brain homogenates were stored at -80°C until further analysis.

3. Nissl staining

A Nissl staining was performed on the series of sections mounted onto glass slides during cutting. Sections were thawed and air-dried for 30 minutes. The sections were washed 20 minutes in sodiumacetate buffer with acetic acid. Next the sections were put in a defatting solution (2%

Triton-X100, 100% ethanol and milliQ water) for 20 minutes before washing them in sodiumacetate buffer with acetic acid again. Sections were stained by incubating them 24 minutes in cresyl violetacetate in sodiumacetate buffer with acetic acid. After staining the sections underwent a series of wash and dehydration steps. First the sections were washed 3 x 1 minute in sodiumacetate buffer with acetic acid and next they were washed with 100% ethanol for 30 seconds. Then they were washed 2 x 5 minutes in isopropanol and finally 2 x 5 minutes in xyleen. After washing the sections were coverslipped with Depex (Klinipath BV, Duiven, the Netherlands) and stored at room temperature until further analysis.

4. Aβ immunostaining

For the Aβ staining, sections were thawed in TBS and divided over two cups per animal to ensure proper staining. The sections were washed 3 x 10 minutes in TBS-T (TBS with 0.2% Triton-X100), TBS and TBS-T respectively, before incubating them overnight with the anti-Aβ 1-17 antibody (1:1200, Sigma, Zwijndrecht, the Netherlands) in TBS-T at 4°C. After overnight incubation the sections were washed 3 x 10 minutes in TBS-T, TBS and TBS-T, respectively, and incubated for 2 hours with Alexa 594 (donkey anti-mouse, 1:200, Breda, The Netherlands) in TBS-T at room temperature. Afterwards the sections are washed 3 x 10 minutes in TBS-T, TBS and TBS-T respectively, and incubated for 10 minutes with Thioflavine S (0.0075%) in TBS-T at room temperature. Next, the sections were first washed 5 minutes in 70% ethanol and then 10 minutes in TBS, before incubating them for 30 minutes with Hoechst (1:500, Sigma, Zwijndrecht, the Netherlands) in TBS at room temperature. After washing the sections 3 x 10 minutes in TBS, the sections were mounted onto slides with 80% glycerol in TBS as mounting medium and stored in the dark at 4°C until analysis.

5. Synaptophysin immunostaining

The sections were thawed in TBS and divided over two cups per animal. The sections were washed 3 x 10 minutes in TBS (0.05M, pH 7.6) and incubated with 0.3% H_2O_2 for 30 minutes at room temperature to block endogenous peroxidases. After incubation the sections were washed 3 x 10 minutes in TBS-T (0.2% Triton X-100), TBS and TBS-T, respectively. Next, the sections were incubated with 5% normal donkey serum in TBS-T for 30 minutes at room temperature to block aspecific binding of the primary antibody. After incubating in the blocking solution, sections were incubated overnight with the mouse anti-synaptophysin antibody (1:200, Millipore, Amsterdam, the Netherlands) in TBS-T with 0.5% normal donkey serum at room temperature. The next day, the sections were washed 3 x 10 minutes in TBS-T, TBS or TBS-T respectively. After washing, the sections were incubated for two hours with biotinylated donkey anti-mouse antibody (1:100, Bio-connect BV, Huissen, the Netherlands) in TBS-T with 0.5% normal donkey serum. Following washing 3 x 10 minutes in TBS-T, TBS and TBS-T respectively, the sections were incubated for 90 minutes with the VECTASTAIN ABC kit (Vector laboratories, Burlingame, CA, USA) at room temperature. After incubation the sections were washed 3 x 10 minutes in Tris-HCl (0.05M, pH 7.6) and incubated for 10 minutes with 3,3'-diaminobenzidine (DAB) diluted 1:1 in Tris-HCl and H_2O_2. After incubation with DAB the sections were washed 3 x 10 minutes in TBS, mounted on slides and dried overnight. On the last day sections were dehydrated in a dehydration series of 2 x 50%

ethanol, 2 x 70% ethanol, 3 x 96% ethanol, 2 x 100% ethanol and 2 x ultraclear, 3 minutes in each solution. After dehydration, the sections were coverslipped with Pertex (Klinipath BV, Duiven, the Netherlands) and stored at room temperature until further analysis.

6. Analysis of hippocampal volume

Stereo Investigator software (version 8.26, MBF Bioscience, Williston, VT, USA) was used for volume analysis. Unilateral hippocampus volumes were determined by drawing the outlines of the total hippocampus between Bregma -1.22 mm and -3.64 mm (43) in Nissl-stained sections. The delineations were made using a 4x objective (numerical aperture [NA]=0.16). Volume of the stratum lucidem of CA3 (SL) was determined between Bregma -1.34 mm and -3.64 mm in synaptophysin-immunostaind sections. Delineations were drawn using a 10x objective (NA=0.40) and perfected using a 20x objective (oil, NA=0.80). All delineations were performed on a stereology workstation consisting of a modified BX50 bright field microscope (Olympus, Tokyo, Japan), Olympus UPlanApo objectives (Olympus, Tokyo, Japan), a three-axis high-accuracy computer-controled stepping motor specimen stage for automatic sampling (4x4 Grid Encoded Stage; Ludl Electronics, Hawthorne, NY, USA), a linear z-axis position encoder (Ludl), a MBF-CX9000 CCD color camera (1.200x1.800 pixels; CX9000; MBF Bioscience) and controling software (MBF Bioscience). The actual volumes were calculated from the projection area measurements and the average section thickness (30µm) using Cavelieri's principle (44). In this analysis the control groups (M-C and F-C) consisted of 5 animals and the PS groups (M-PS and F-PS) of 6 animals.

7. Stereological analysis of plaque load in the hippocampus

All Aβ immunostained sections containing hippocampal tissue, ranging from Bregma -1.06 mm to -3.88 mm, were analyzed unilaterally using Stereo Investigator. Per animal an average of 9 sections was analyzed, depending on the individual rostro-caudal extension of the hippocampus and the quality of the sections. Delineations of the total hippocampus were made using a 4x objective (NA=0.16) and perfected using a 20x objective (oil, NA=0.80). Within the delineation the plaque area was measured with the Area Fraction Fractionator probe of the Stereo Investigator software using a 40x objective (oil, NA=1.00). All measurements were performed using a modified Olympus BX50 fluorescence microscope (Olympus, Tokyo, Japan) on the stereology working station that was also used for the analysis of hippocampal volume. The plaque area was compared to the total hippocampal area of that respective section to get an estimate of the plaque load. During statistical analysis a distinction was made between the dorsal, middle and ventral part of the hippocampus. The part of the hippocampus ranging from Bregma -1.34 mm to -2.30 mm was considered the dorsal part, the middle part ranged from -2.30 mm to -2.92 mm and the ventral part of the hippocampus ranged from -2.92 mm to -3.88 mm. In this analysis all groups (M-C, M-PS, F-C and F-PS) consisted of 5 animals.

8. Analysis of synaptophysin-immunopositive presynaptic boutons

All synaptophysin immunostained sections containing hippocampal tissue, ranging from Bregma -1.34 mm to -3.64 mm, were analyzed unilaterally. Per animal an average of 8 sections was analyzed, depending on the individual rostro-caudal extension of the hippocampus. Three

subregions within the hippocampus were assessed, i.e. stratum radiatum of CA1 (SR), stratum lucidem of CA3 (SL) and stratum moleculare of the dentate gyrus (SM). Within these regions, three randomly selected sites were evalutated (Fig. 3). The density of the synaptophysin-immunopositive presynaptic boutons (SIPBs) was estimated as previously described (45). In short, a photo was taken of the chosen areas using a Olympus BH-2 microscope equipped with a CDD video camera (Paes, Zoeterwoude, The Netherlands). In these photos SIPBs were detected by CellP, an image analysis system, slightly modified for detection of grayscale punctae (AnalySI-pro, Münster, Germany). First, in every image the region of interest was defined. Next, shading error correction was performed before measurements to correct for irregularities in illumination of the microscopic fields. Following shading error correction, a differential contrast enhancement filter (DCE) was applied to selectively enhance weak differences in contrast. Lastly, the detection threshold was tested and kept at the same level for all samples. All measurements were performed on a single focal plane and cell bodies, blood vessels and artifacts, e.g. Aβ plaques, were excluded from the analysis. Particles smaller than 0.025 μm² were considered noise and not taken into account in the analysis. From these data the mean SIPB density per μm² was calculated. Furthermore, the frequency distribution was generated for each picture in order to calculate the mean SIPB densities per μm² within the following ranges: 0.025-0.2 μm², 0.2-0.3 μm², 0.3-0.5 μm², 0.5-2 μm² and bigger than 2 μm². SIPB density was evaluated in the total hippocampus, as well as the dorsal (Bregma: -1.34 to -2.30 mm), middle (-2.30 to -2.92 mm) and ventral (-2.92 to -3.64 mm) part of the hippocampus separately. In this analysis the male groups (M-C and M-PS) consisted of 5 animals, while the female groups (F-C and F-PS) consisted of 6 animals.

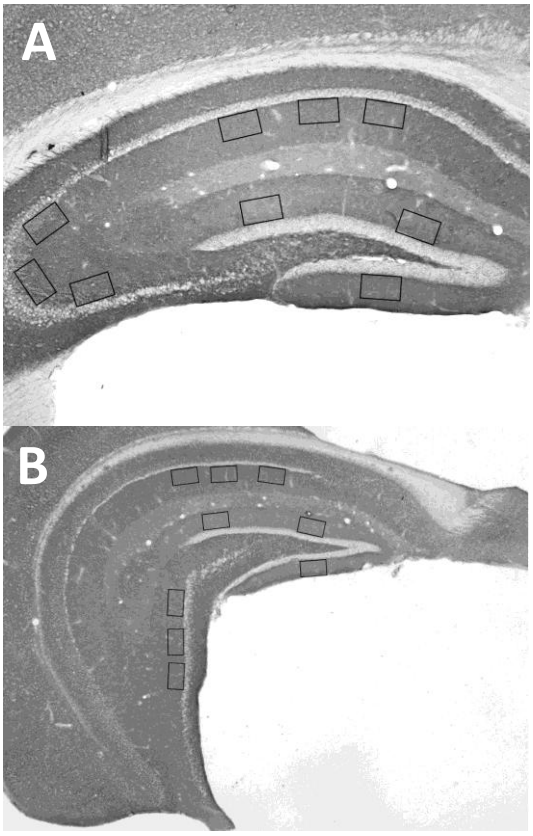

Figure 3. Representative photos of the hippocampus before (A, 4x) and after (B, 2x) SL of CA3 splits in two parts. Squares indicate the areas where photos for quantitative SIPB analysis were taken within SM, SL and SR.

9. Corticosterone radioimmunoassay

To collect serum, blood samples from basal, stress and recovery conditions were centrifuged at 5000 rpm for 10 minutes at 4°C. After centrifugation serum was transferred into eppendorf tubes at 4°C and stored at -80°C until further use.

For the corticosterone radioimmunoassay, an ImmuChem™ Double Antibody Corticosterone ^{125}I RIA Kit for rats and mice (MP Biomedicals, Orangeburg, NY, USA) was used. The assay was performed according to the manufacturer's instructions. Briefly, for each sample 5 µl serum was diluted in steroid diluents (1:2000). Of this dilution 50 µl was transferred to a glass test tube and 50 µl corticosterone-^{125}I was added to each tube. Next 100 µl anti-corticosterone was added to all tubes, after which tubes were vortexed and incubated at room temperature for 2 hours. After incubation 250 µl precipitant solution was added to all tubes. Tubes were vortexed thoroughly and centrifuged at 2300 rpm for 15 minutes. After centrifugation supernatant was aspirated and precipitate was counted in a gamma counter.

10. Statical analysis

All statistics were carried out using SPSS software (version 17.0.2, SPSS Inc., Chicago, USA). For statistical analysis of hippocampal volume, plaque load, SIPB number and SIPB density a two-way ANOVA was performed with volumes, plaque load, percentage of SIPBs and SIPB density as the dependent variables, respectively, and sex and treatment as fixed factors. When significant differences were found in either sex, treatment or the interaction between both (sex*treatment), one-way ANOVA was performed comparing control mice to PS mice of the same sex.

In case of corticosterone concentrations values were ln-transformed to obtain normally distributed residuals. A two-way ANOVA was performed with concentration as the dependent variable and sex and treatment as fixed factors, followed by a one-way ANOVA comparing control mice to PS mice of the same sex if significant differences were found. Further, a repeated measures analysis was performed on corticosterone concentration with sex and treatment as independent variables.

Statistical significance was established at $p<0.05$ for two-way ANOVA and repeated measures analysis and at $p<0.025$ for one-way ANOVA.

Results

1. Prenatal stress decreases plaque load in the dorsal part of the hippocampus in female mice

To test the effect of PS on plaque load in the hippocampus of APPswe/PS1dE9 mice, measurements of plaque load (Fig. 4) were carried out in the total hippocampus, as well as in the dorsal and ventral part of the hippocampus separately.

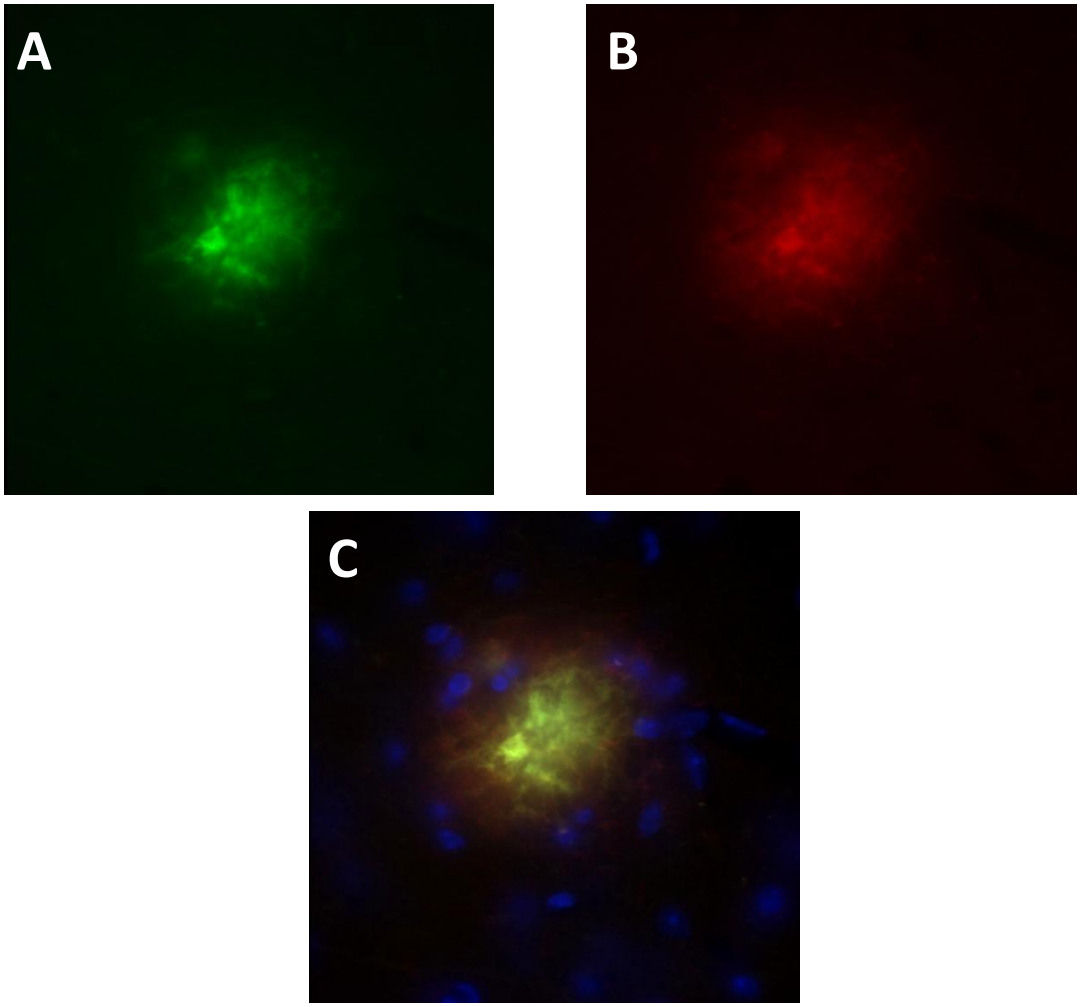

Figure 4. Example of an Aβ plaque after staining. A: staining with Thioflavine S. B: staining with anti Aβ 1-17 antibody. C: merge photo of Thioflavine S, anti Aβ 1-17 and Hoechst staining.

There was a significant effect of sex ($F(3, 16)=17.038$, $p<0.01$) on plaque load in the entire hippocampus, with females having a bigger plaque load then males (Fig. 5A). This difference was also observed when the dorsal ($F(3, 16)=5.763$, $p<0.05$), middle ($F(3, 14)= 6.816$, $p<0.05$) and ventral ($F(3, 15)=11.763$, $p<0.01$) part of the hippocampus were analyzed separately (Fig. 5B-D).
There was no significant effect of treatment on plaque load in the entire hippocampus or in the middle part of the hippocampus. In the dorsal part a trend towards a significant effect of treatment ($F(1, 8)=0.249$, $p<0.05$) was seen in females, with prenatally stressed females having a lower plaque load compared to control females (Fig. 5B). In the ventral part a decrease in plaque load

was seen in PS males compared to control males, but this effect did not reach significance (Fig. 5D).

Correlation analyses indicated a moderate negative correlation between the results of the object location task at 1 hour and plaque load in the entire hippocampus (Pearson's=-0.658, $p<0.01$) and in the ventral part of the hippocampus (Pearson's=-0.620, $p<0.01$). No correlations were observed with the results of the spatial variant of the y-maze, the second test of cognitive performance. There were also no correlations observed with tests of affective behavior, i.e. elevated zero maze and forced swim test (data not shown).

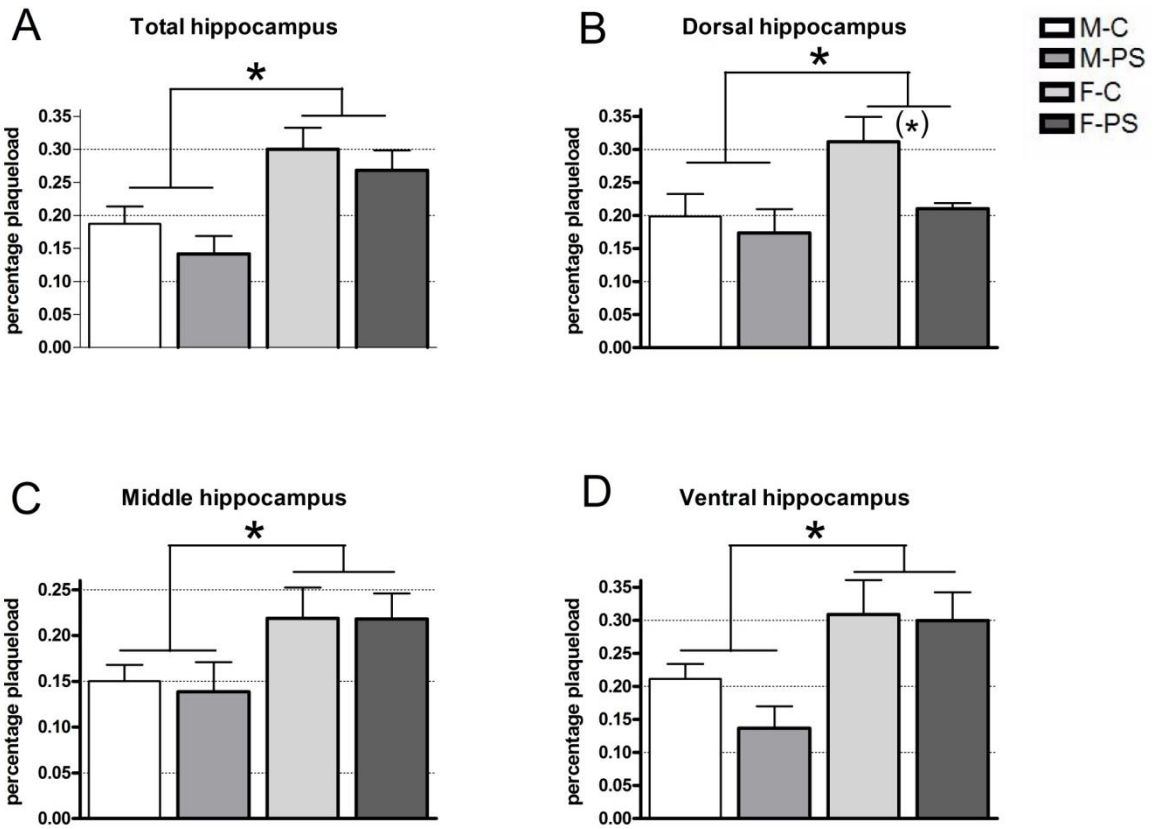

Figure 5. Results of the plaque load analysis in the hippocampus. Graphs show mean and SEM of the amount of plaques, expressed in percentage of the total hippocampal area. Results are shown for the total hippocampus (A), the dorsal (B), middle (C) and ventral (D) part of the hippocampus. The different bars represent the different groups: from left to right, M-C, M-PS, F-C and F-PS. Plaque load differs in males and females in total hippocampus as well as the dorsal and ventral part. In the dorsal part of the hippocampus a trend towards a PS effect in females is seen. *: $p<0.05$; (*): $0.025<p<0.05$.

2. Prenatal stress does not affect volume of the total hippocampus or stratum lucidem of APPswe/PS1dE9 mice

To test for a possible effect of treatment on volume, volume calculations were done for the entire hippocampus and stratum lucidem.

Statistical analysis indicated that there was no effect of treatment, sex or sex-treatment interaction on the volume of the entire hippocampus or the volume of the stratum lucidem (Fig. 6).

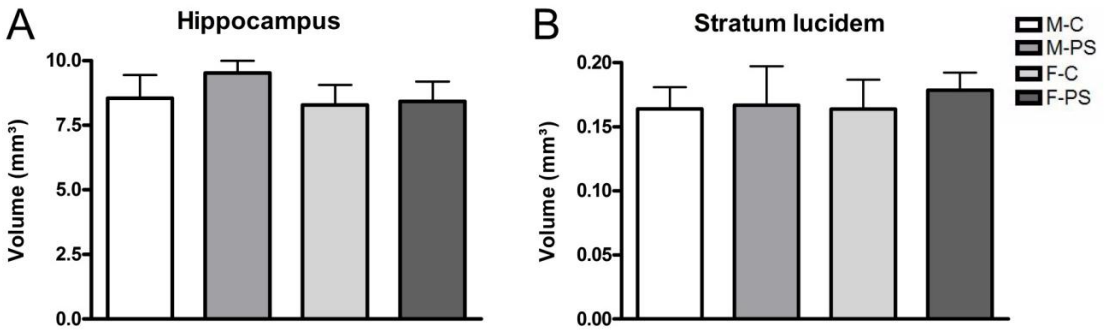

Figure 6. Results of the volume analysis of total hippocampus (A) and stratum lucidem (B). Graphs show mean and SEM of the measured volumes. The different bars represent the different groups: from left to right, M-C, M-PS, F-C and F-PS. No significant differences in volume were found.

3. Exposure to prenatal stress does not affect total SIPB density in the hippocampus

To investigate the effect of treatment on synaptic plasticity in the hippocampus of APPswe/PS1dE9 mice, SIPB density was evaluated for SM, SL and SR in the entire hippocampus, as well as in the dorsal, middle and ventral part of the hippocampus separately.

There was no effect of sex in any of the regions in the total, dorsal or ventral hippocampus (Fig. 7A, B and D). In the middle part of the hippocampus a significant effect of sex ($F(3, 17)=5.132$, $p<0.05$) was seen in SL, with females having a higher SIPB density than males (Fig. 7C). Sex did not have an effect in the other regions of the middle part of the hippocampus. Treatment and sex-treatment interaction did not affect SIPB density in any part of the hippocampus (Fig. 7).

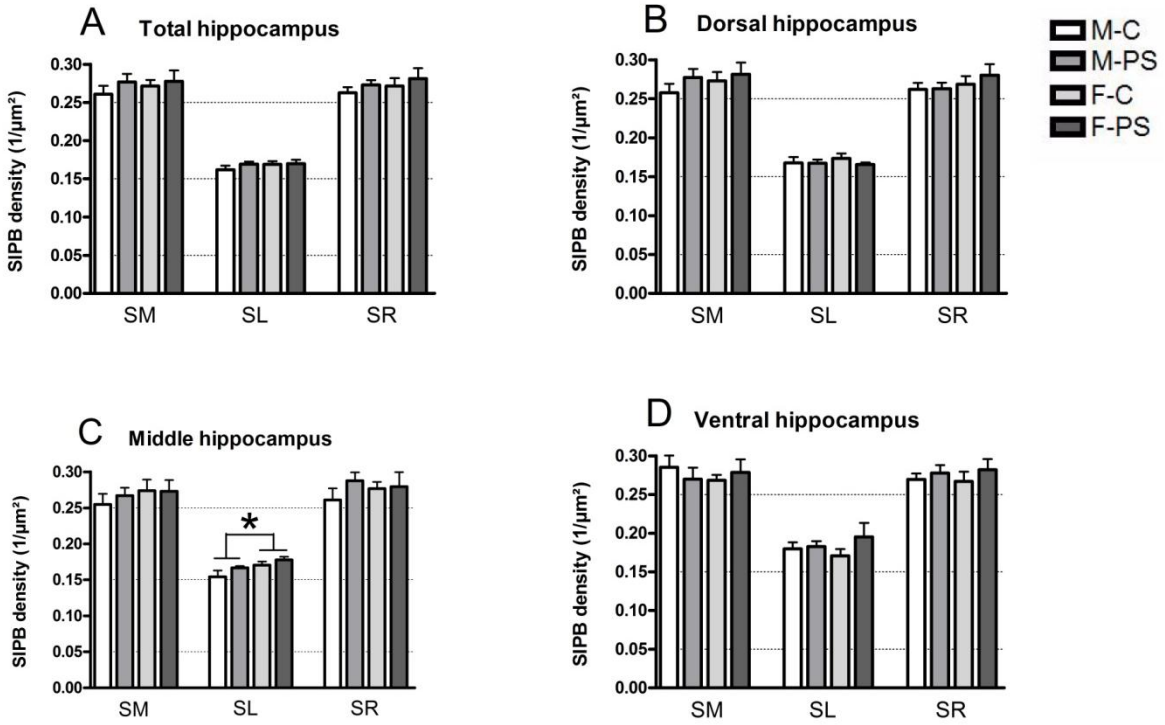

Figure 7. Results of SIPB density analysis in the hippocampus. Graphs show mean and SEM of SIPB density. Results are shown for the total hippocampus (A), dorsal (B), middle (C) and ventral (D) part of the hippocampus. Density was measured in three regions: SM of gyrus dentatus, SL of CA3 and SR of CA1. In each region the different bars represent the different groups: from left to right, M-C, M-PS, F-C and F-PS. SIPB density differs between males and females in SL in the middle part of the hippocampus. *: $p<0.05$.

4. SIPB density and particle number are altered after exposure to prenatal stress

4.1 Higher density of SIPBs with size 0.5-2 µm² in the SL of male mice

To look at the possible changes in SIPB size, all SIPB particles were subdivided in five classes according to their size: 0.025-0.2 µm², 0.2-0.3 µm², 0.3-0.5 µm², 0.5-2 µm² and >2 µm². SIPB density was then calculated for each class separately within the SM, SL and SR.

In SL treatment significantly ($F(1, 8)=11.759$, $p<0.01$) affected SIPB density in the 0.5-2 µm² class in males. Prenatally stressed males had a significantly higher SIPB density in this class compared to control males (Fig. 8D). In the class consisting of SIPBs >2 µm² a significant sex-treatment interaction effect ($F(3, 18)=10.547$, $p<0.05$) on SIPB density was seen in SL. While PS increased SIPB density in males, it decreased SIPB density in females (Fig. 8E). Further analysis comparing control and PS animals from the same gender revealed that treatment did not significantly affect any gender, but a trend ($F(1, 8)=5.966$, $p<0.05$) towards a significant effect of treatment was seen in males (Fig. 8E).

Treatment did not affect SIPB density in the SL in any of the other classes, nor did it affect SIPB density in SM or SR. Except for the one described above, no sex-treatment interaction effect was seen. Sex did not affect SIPB density (Fig. 8).

4.2 Exposure to prenatal stress alters SIPB particle percentages in female mice

Besides looking at the differences in SIPB density in a certain class, the relative proportion of each class was also assesed, by calculating what percentage of the total SIPB number a certain class occupied within the SM, SL and SR.

In SM a significant effect of treatment was ($F(3, 18)=6.413$, $p<0.05$) found in the 0.3-0.5 µm² class. Further analysis indicated that prenatally stressed females had a significantly lower percentage of SIPBs in this class compared to control females ($F(1, 10)=10.531$, $p<0.01$, fig. 8C). No effects of treatment were seen in any of the other classes and sex and sex-treatment interaction did not have an effect in SM.

In SL statistical analysis revealed a significant sex-treatment interaction effect in both the 0.025-0.2 µm² ($F(3, 18)=6.412$, $p<0.05$) and the >2 µm² class ($F(3, 18)=9.465$, $p<0.01$). Further analysis did not reveal an effect of treatment in any gender. However, a trend towards a significant effect ($F(1, 10)=5.162$, $p<0.05$) of treatment was seen in female mice in the >2 µm² class, with a higher percentage of SIPBs in control females compared to PS females (Fig. 8E). No other effects of sex-treatment or treatment were seen in SL. Sex did not affect SIPB size in this region.

In SR an effect of treatment ($F(3, 18)=5.895$, $p<0.05$) was seen in the 0.025-0.2 µm² class. Further analysis showed that this effect was not significant in any gender, but in females a trend towards a significant effect of treatment ($F(1, 10)=6.763$, $p<0.05$) was seen, with PS females having a higher percentage of SIPBs in this class compared to control females (Fig. 8A). Treatment also significantly affected SIPB number in the 0.5-2 µm² class ($F(3, 18)=8.437$, $p<0.01$) in SR. Again, further analysis revealed that females accounted for this difference, with PS females having a lower SIPB percentage compared to control females ($F(1, 8)=7.529$, $p<0.025$, fig. 8D). Treatment did not have any other effects in SR and neither sex or sex-treatment interaction had an effect on SIPB size in this region.

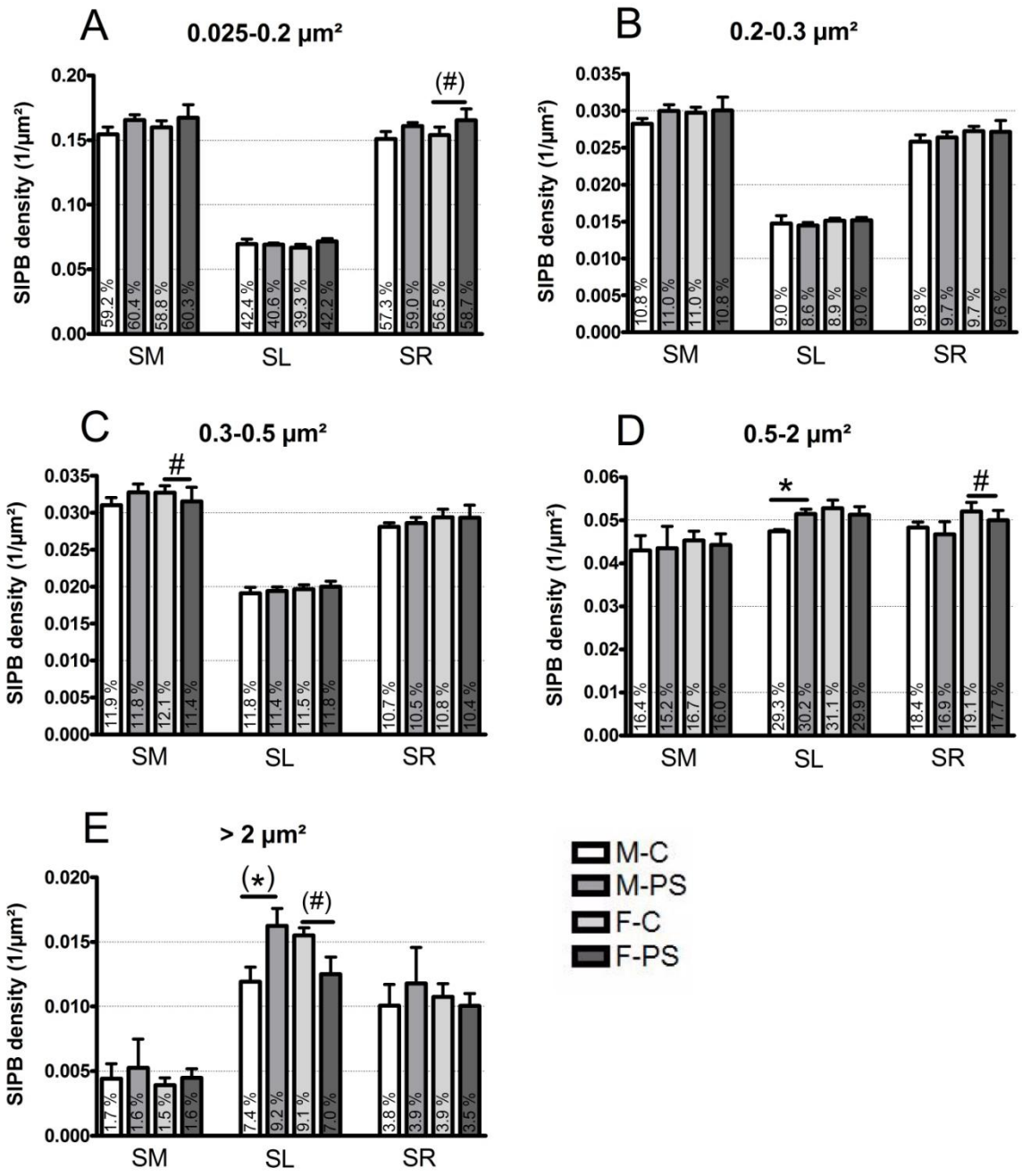

Figure 8. Results of SIPB density analysis in the hippocampus according to size. Graphs show mean and SEM of SIPB densities. Results are shown for the five classes: 0.025-0.2 µm² (A), 0.2-0.3 µm² (B), 0.3-0.5 µm² (C), 0.5-2 µm² (D) and larger than 2 µm² (E). SIPB density was measured SM, SL and SR for all five classes. In each region the different bars represent the different groups: from left to right, M-C, M-PS, F-C and F-PS. Percentages in the bars represent the number of SIPBs compared to the total number of SIPBs in this groups in this region. PS significantly affects SIPB density in SL in males in the class containing SIPBs ranging 0.5-2 µm². SIPB number is significantly affected by PS in females in SM in the 0.3-05 µm² class and in SR in the 0.5-2 µm² class. SIPB density: *: $p<0.025$; (*): $0.025<p<0.05$. SIPB number: #: $p<0.025$; (#): $0.025<p<0.05$.

4.3 Prenatal stress does not alter SIPB density in dorsal and ventral part of the hippocampus separately

As seen in the plaque load analysis, it is possible that effects in the dorsal hippocampus are dampened by a lack of effect in the ventral hippocampus and vice versa. We therefore analyzed the SIPB density in the dorsal and ventral part of the hippocampus separately.

The differences that were seen in the total hippocampus in the >2μm² class were also present in the dorsal and ventral hippocampus, although they did not reach significance. In the dorsal part of the hippocampus a significant sex-treatment interaction effect ($F(3, 18)=6.338$, $p<0.01$) on SIPB density in SL was seen in the 0.5-2 μm² class. While SIPB density decreased in females in this class as a result of PS, it increased in males. Further statistical analysis revealed a trend towards a significant effect of treatment ($F(1,10)=5.161$, $p<0.05$) on SIPB density in females, with prenatally stressed females having a lower SIPB density compared to control females. No other interaction effects or effects of treatment were seen, nor did sex have an effect in the dorsal part of the hippocampus. No effects on SIPB density were found in the ventral part of the hippocampus (data not shown).

5. Prenatal stress does not affect corticosterone concentration

A radioimmunoassay was used to investigate possible differences in corticosterone concentration. Corticosterone levels were measured in basal, stress and recovery conditions and then compared between the different groups.

In both basal and stress conditions a significant effect of sex ($F(3, 49)=11.363$, $p<0.01$ and $F(3, 45)=159.204$, $p<0.01$, respectively) on corticosterone concentration was seen, with females having a higher concentration compared to males (Fig. 9). This effect of sex was no longer significant in recovery conditions, but a significant sex-treatment interaction effect ($F(3, 35)=4.877$, $p<0.05$) was seen in this condition. PS elevated plasma corticosterone levels in both males and females when compared to controls, although this effect did not reach significance in any gender.

When comparing the three different conditions, effects were seen between the subjects as well as within the subjects. Both between and within the subjects a significant sex-condition interaction effect was seen ($F(3, 33)=3.488$, $p<0.05$ and $F(3, 33)=3.846$, $p<0.05$, respectively). Within the subjects a sex-treatment-condition interaction effect ($F(3, 33)=3.210$, $p<0.05$) was also seen.

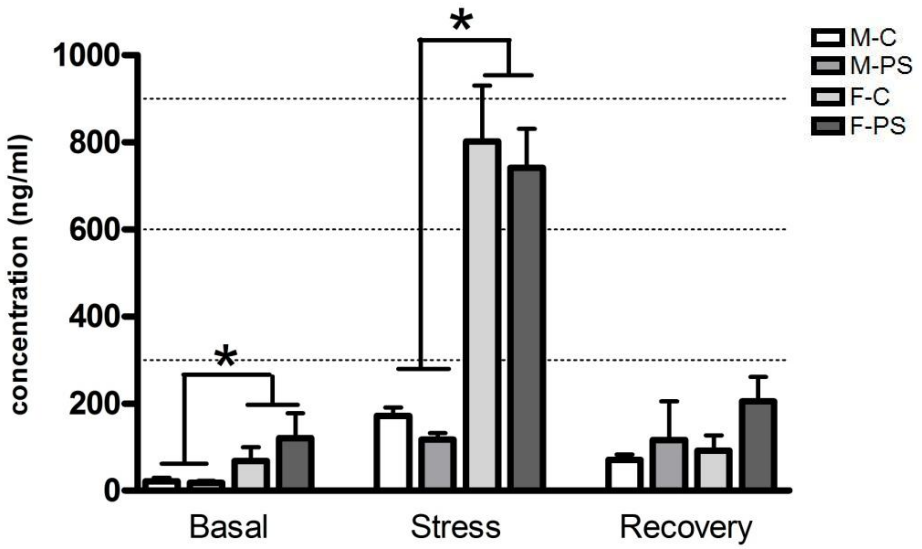

Figure 9. Results of corticosterone radioimmunoassay. Graphs show mean and SEM of corticosterone concentration. Corticosterone concentration was measured in basal, stress and recovery conditions. In each condition the different bars represent the different groups: from left to right, M-C, M-PS, F-C and F-PS. Females have higher corticosterone concentrations than males in both basal and stress conditions. *: $p<0.005$.

Discussion

Accumulating evidence from both human and animal studies suggests that MD can act as a risk factor for dementia in general, and AD in particular (33, 35, 39). Preliminary data showed changes in cognitive abilities in APPswe/PS1dE9 mice after exposure to PS. We initially hypothesized that these changes in cognition might be explained by a heightened plaque load or loss of plasticity in the hippocampus induced by changes in HPA axis activity after exposure to PS.

Contrary to our expectations, exposure to PS tended to lead to a decrease in plaque load in the total hippocampus of APPswe/PS1dE9 mice. More specifically, female mice that are exposed to PS showed a decreased plaque load in the dorsal part of the hippocampus in combination with an improved cognitive performance when compared to female controls. The observed decreased plaque load in combination with a better cognitive performance is in line with the well-known amyloid hypothesis, however, the results of the plaque load analysis do not explain the changes in cognition in prenatally stressed male mice in the object location task, nor do they explain the decrease in depressive-like behavior after PS in female mice in the forced swim test.

It is difficult to find a solid connection between the results of our plaque load analysis and the preliminary behavioral data. Our findings in males do not support the amyloid hypothesis of AD, however, it is not the first time that such findings are reported. In female APPswe/PS1dE9 mice it was shown that envirionmental enrichment improves cognitive abilities and at the same time leads to an increased Aβ load (46). Interestingly, it was found that in male APPswe/PS1dE9 mice environmental enrichment lowers Aβ burden (47). This contradiction between male and female mice points towards an important role of sex in the development of Aβ plaques. Thus, even though deposition of Aβ is the most known and most studied neuropathological feature of AD, it seems that its contribution to the cognitive problems seen in APPswe/PS1dE9 mice is sex-dependent and that females are more prone to the consequences of amyloid plaque formation than males.

Further, we must keep in mind that, although amyloid deposition is a key diagnostic feature of AD, it has been suggested that these plaques are a consequence rather than a cause of the disease. Some even suggest that extracellular Aβ deposition is a cellular defense mechanism, protecting the intracellular environment of the cells against detrimental Aβ species (48). Thus, our results in males might be explained by a switch from extracellular to intracellular Aβ deposition, which might damage the intracellular environment of the cells and cause problems in cognition. However, this cannot be completely confirmed in this study, since we did not measure intracellular Aβ.

Aside from a different response to plaque load, female mice also showed a higher plaque load when compared to male mice. The effect of sex on plaque load that is seen in the present study was expected, since it is known from previous research that sex plays an important role in the Aβ pathology in APPswe/PS1dE9 mice. A study published in 2003 already showed that Aβ40 and Aβ42 levels as well as the number of amyloid plaques are higher in female APPswe/PS1dE9 mice compared to age-matched male mice (49).

Interestingly, when looking at the effect of a depressive-like phenotype on Aβ load, our results contradict what has been described in literature. It has been shown that AD patients with a history of MD show both a faster cognitive decline and a worse neuropathology, e.g. a higher plaque load, when compared to AD patients without a history of MD (34). Furthermore, in both the APP_{V7171}-

CT100 transgenic mouse model and the Tg2576 mouse model of AD exposure to chronic stress is known to lead to not only an increase in cognitive decline, but also increased Aβ deposition and neurodegeneration (38, 41). To date, we are the first ones to combine the APPswe/PS1dE9 mouse model of AD with the PS model of depression and the precise role of PS in lowering Aβ deposition remains to be elucidated. We propose that exposure to PS early in pregnancy might put an unknown preconditioning mechanism into motion, causing prenatally stressed animals to be less likely to develop Aβ deposits. Perhaps applying PS at a different time point of gestation can have a differential effect on plaque load. However, further research is needed to confirm this suggestion.

Aside from plaque deposition, changes in neuroplasticity are another important feature of AD, and have been suggested to play a key role in the cognitive problems seen in AD. In AD patients synaptic density is known to be decreased and changes in the number of presynaptic boutons are reported to correlate better to cognitive changes than Aβ pathology (50-52). Further, it is shown that both memory problems and synaptic deficits are seen long before Aβ deposition in the Tg2576 AD mouse model (53).

With regard to plasticity in the hippocampus, no changes in volume of the total hippocampus or stratum lucidem were seen in the present study. Total SIPB density also did not change after exposure to PS. Nevertheless, in PS male mice an increase in the density of large SIPBs, with a concomitant decrease in smaller SIPBs, is seen in the stratum lucidem.

The lack of volume changes in the present study is not an unexpected result, since neuronal loss has not yet been reported as a key feature of this particular mouse model. Other mouse models of AD often also lack neuronal cell death as a neuropathological hallmark. For example, when looking in the CA1 region of the hippocampus both PSAPP and Tg2576 mice show an age-related increase in Aβ deposition, but no significant neuronal loss is seen with increasing age (54, 55).

Interestingly, our data indicate that while cognition in female APPswe/PS1dE9 mice seems to be influenced more by amyloid deposition, in male mice the shift from smaller to bigger SIPBs might account for their cognitive problems. In both AD patients and animal models of AD synaptic hypertrophy has been documented before. For example, a study in AD patients found a decrease in synapse number while synapse size increased, when comparing them to normal adults. This process was also seen in physiological aging, but it was far more severe in AD (56). In a study in Tg2576 mice a maintained synaptic density was seen during aging, while an age-related decrease was seen in non-transgenic controls in the neocortex as well as in the hippocampus (57). These data suggest that the changing synapse morphology might be an adaptive response, in which the increasing size of the synaptic terminals acts as a compensatory change to a decreasing number of synapses. Thus, it is possible that the observed synaptic swelling not only is responsible for the cognitive problems in male APPswe/PS1dE9 mice, but also is a prelude to a decrease in synaptic density when the disease progresses.

Indeed, we must take into account the animal model that was used here and the age at which we examined it. In a study by West and colleagues (58) synaptic contact number and size were investigated in CA1 of APPswe/PS1dE9 mice at the age of 12 months. No age-related changes in synapse size were seen, but they did find an increase in the number of synaptic contacts. A suggested explanation for this finding is that APP, which is present in abundance in the APPswe/PS1dE9 mouse model and is thought to be synaptotrophic, may be responsible for the

observed increase in synapse contact number. An alternative explanation given in this study is that the observed shift in relative distribution of the differentially sized SIPBs represents a temporary compensatory response to synapse dysfunction which will eventually break down and result in synapse loss at a later age. The animals used in our study were six months of age at the time of death and it is possible that the disease had not progressed far enough for an alteration in synaptic density to be observed.
Another possible explanation for the lack of changes in total SIPB density is the applied form of PS. It is expected that both PS and aging increase AD pathology. According to the study by West (58) synaptic density increases with age in the APPswe/PS1dE9 mouse model. However, in our study no changes in SIPB density are seen after exposure to PS, which might indicate that the applied form of PS is not detrimental enough to cause changes in synaptic density at this age.
In summary, our findings again point towards an important role of sex in the pathology of AD, as not only plaque deposition, but also the synaptic changes seem to be sex-dependent.

Like with plaque load and synaptic density, we also observed a difference between males and females in corticosterone response in APPswe/PS1dE9 mice. This result is not surprising, since it is well-known that males and females react differently to stressful situations. Consistent with the present data, in a study in DBA/2 mice it was reported that female mice not only had higher basal corticosterone concentrations, but also a higher response to stress (59).
In the present study we see that PS reduces the activity of the HPA axis in stressful conditions, while the return to basal corticosterone levels in the recovery condition is delayed. From literature it is known that exposure to stress hormones during life increases Aβ pathology in AD mouse models. For example, it has been shown in Tg2576 mice that isolation stress increases corticosterone levels together with increases in Aβ deposition (39). Another study, in 3xTg-AD mice, showed that glucocorticoid administration increases Aβ deposition (60). Interestingly, in Wistar rats, it was shown that PS also causes HPA axis hyperactivity. Corticosterone levels were more increased after exposure to stress in PS offspring compared to control offspring (61). This study contradicts the results from the present study, however, we must take into account the age at which corticosterone concentration was measured and the time at which the PS was applied. In a preliminary study in our lab (data not published) it was shown that five month old rats exposed to PS show an increase in basal corticosterone concentration and an inadequate response to stress when compared to controls, while at three months of age no differences were seen between control and PS rats at baseline, whereas stress-induced corticosterone levels were higher in PS males.. Therefore, it is possible that our results are influenced by the age of the animals and would have been different at another age.
Furthermore, in our study PS was applied during the first week of pregnancy, while in the reported study pregnant rats were stressed during the last week of pregnancy. Therefore, as stated above, we propose that exposure to PS in early pregnancy might cause an unknown preconditioning mechanism to take place. This preconditioning mechanism might lessen the activation of the HPA axis in response to stress after birth and simultaneously slow down Aβ deposition. We suggest that the underlying mechanism might be a predictive adaptive response. This means that the developing organism will use developmentally plastic processes, e.g. epigenetic changes or changes in homeostatic control mechanisms, to set its postnatal phenotype to one that it predicts

will give an optimal chance of survival to reproduce when reaching adulthood (62). Indeed, it is reported that epigenetic changes in response to the fetal environment can influence the choice of exon usage in the glucocorticoid receptor gene, in that way altering the HPA axis activity throughout life (63). However, further research is needed to completely understand the effect of PS on the stress response later in life.

Conclusion and synthesis

1. Future research

In the present study it seems that different mechanisms contribute to AD pathology in males and females and further research is needed to elucidate the contribution of sex to AD neuropathology in APPswe/PS1dE9 mice.

Further, it would also be of interest to look at a possible effect of age or genotype. In the current study we only took into account transgenic animals that were 6 months of age at the time of death. We suggest that in a next study mice of different ages, as well as both transgenic and wild-type animals should be studied. In that way it would be possible to investigate whether age-related changes take place in APPswe/PS1dE9 mice, for example in synaptic density. In the current set-up, one cannot distinguish between the effects of PS by itself and the interaction effect of PS and AD mutations. By using wild-type animals as well as transgenic animals, the effects of PS and genotype can be investigated separately as well as interactively.

Lastly, it is of great interest to further investigate the preconditioning effect that seems to take place in APPswe/PS1dE9 mice after exposure to PS in early pregnancy. Not only the underlying mechanisms that cause this preconditioning have to be studied, but also the time frame during pregnancy and/or the neonatal period in which this effect takes place.

2. General conclusion

We hypothesized that exposing APPswe/PS1dE9 mice to PS would increase the AD-related neuropathology in the brain, which could possibly explain the behavioral changes observed in our preliminary study. We expected that PS would lead to a heightened plaque load and a loss of plasticity in the hippocampus of APPswe/PS1dE9 mice. However, contrary to our hypothesis, PS did not only decrease the plaque load, but also seemed to change the HPA activity in response to stress after birth. Further, we do see changes in synaptic plasticity in the hippocampus, but these changes are only present in males.

In summary, it can be said that exposure to PS in early pregnancy is thought to work as a sort of preconditioning mechanism, reducing the stress response later in life and in that way slowing down Aβ deposition. Further, the cognitive problems seen in APPswe/PS1dE9 mice seem to be sex-dependent. The cognitive deficits in male mice can probably be attributed to changes in synaptic size, while in females Aβ deposition seems to have a more important role in cognitive function. Thus, we can conclude that AD pathology is sex-dependent, although further research is needed to elucidate the exact underlying mechanisms.

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