

Stress steroids as accelerators of Alzheimer's disease.

Effects of chronically elevated levels of allopregnanolone in transgenic AD models.

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Preface

I have long known and now further understand that scientific research is something extraordinary. It is to stand at the edge of knowledge, trying to make sense out of something not yet understood. It can be frustrating, challenging and utterly liberating.

I came into the scientific field of steroids because of my curiosity as to how these endogenous compounds control so many aspects of human life – from prenatal brain development and sexual differentiation via emotions and behaviours to the pathogenesis of various diseases and syndromes.

Chronic stress and Alzheimer's disease are two equally complex areas and with the field of steroids they are all entangled in an ocean of information and unanswered questions.

Standing in front of a great ocean can be rather intimidating. Still, by listening to the flow of the waves, by accepting one's own littleness and by savouring the beautiful greatness of the ocean a feeling of calm arises.

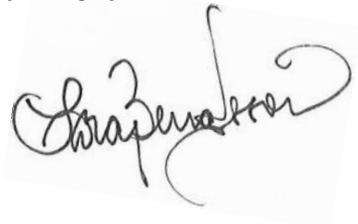
A handwritten signature in black ink, appearing to read 'Chapman', is written in a cursive style on a light-colored rectangular background.

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Abstract

Background Alzheimer's disease (AD) and dementia are devastating conditions not only for the affected patients but also for their families. The economical costs for the society are tremendous. Mid-life psychological stress, psychosocial stress and post-traumatic stress disorder cause cognitive dysfunction and lead to increased risk for dementia. However, the mechanisms behind stress-induced AD and dementia are not known. AD is characterized by solid amyloid plaques in the CNS. However, over the last decade it has been concluded that the levels of soluble beta-amyloid ($A\beta$) correlate to cognitive performance while plaques often do not. The soluble $A\beta$ accumulate intracellularly and disturb the synaptic function. Interestingly, the levels of intracellular $A\beta$ depend on neuronal activity. Previous studies have shown that decreased neuronal activity cause increased intracellular levels of $A\beta$ and cognitive decline. Stress steroids produced in the brain, e.g. allopregnanolone, enhance the activity of the GABAergic system, i.e. the main inhibitory system of the brain. Consequently, allopregnanolone affects neuronal activity. Therefore, it is possible that elevated levels of allopregnanolone (due to e.g. stress) cause increased intracellular levels of $A\beta$. This could be a mechanism behind stress-induced AD. The purpose of this thesis was to investigate if elevation of allopregnanolone is a possible link in the mechanism behind stress-induced AD by investigating the effects of chronically elevated levels of allopregnanolone in transgenic mouse models for AD.

Methods Swe/PS1 and Swe/Arc mice (transgenic models for AD) were treated chronically with elevated allopregnanolone levels, comparable to those at mild stress. After an interval of no treatment, the mice were tested for learning and memory performance in the Morris water maze. The brain tissue of the mice was then analyzed for disease markers, i.e. soluble and insoluble $A\beta_{40}$ and $A\beta_{42}$ using enzyme-linked immunosorbent assay, and amyloid plaques using immunohistochemistry and Congo red staining technique. The brain tissue was also analyzed for a marker of synaptic function, i.e. synaptophysin.

Results Chronic treatment of allopregnanolone caused impaired learning performance in both the Swe/PS1 and the Swe/Arc mouse models. The Swe/PS1 mice had increased levels of soluble $A\beta$ in both hippocampus and cortex. Interestingly, the levels of soluble $A\beta$ were unchanged in the Swe/Arc mice. Three months of allopregnanolone treatment in the Swe/PS1 mouse model caused decreased plaque size, predominantly in hippocampus. It may be concluded that chronic allopregnanolone elevation caused smaller but

more abundant congophilic plaques as both total plaque area and number of plaques were increased in mice with poor learning ability. Additional spots for accumulation of A β , predominantly the more toxic A β ₄₂, and thus additional starting points for plaque production could be a part of the mechanism behind stress-induced Alzheimer's disease.

Conclusions The conclusion of this thesis is that chronic elevation of allopregnanolon accelerated the development of Alzheimer's disease in the Swe/PS1 and the Swe/Arc transgenic mouse models. Allopregnanolone may be an important link in the mechanism behind stress-induced AD. However, further studies are required to grasp the extent of its pathological influence.

Populärvetenskaplig sammanfattning

Alzheimers sjukdom

Alzheimers sjukdom (AD) är en demenssjukdom som drabbar en stor del av befolkningen och framför allt äldre personer. Vida känt är att vid AD bildas amyloida plack i hjärnan samtidigt som hjärnan sakta bryts ner. Under de senaste åren har det dock blivit allt mer klarlagt att det inte i första hand är placken som orsakar nedbrytningen och ger symptom utan i stället förstadiet till plack: de amyloida proteinerna (beta-amyloider, A β). A β bildas och ansamlas inuti nervceller, stör nervcellernas funktioner och gör att hjärnans viktiga synapser bryts ner. Utan synapser fungerar inte signalvägarna i hjärnan och patienten får nedsatt kognitiv förmåga. Detta yttrar sig genom minnesförlust, svårigheter att kommunicera och personlighetsförändringar.

A β utsöndras från nervcellerna bland annat i samband med nervcellernas signalering mellan varandra. Alltså påverkas mängden intra- och extracellulärt A β av nervcellernas aktivitetsgrad. Denna aktivitetsgrad påverkas i sin tur av hjärnans generella excitation kontra inhibition.

Stress och allopregnanolon

Kronisk stress och depression, som är växande problem i samhället, har visats påverka kognitiv förmåga negativt och öka risken för demens och AD. Vid stress ökar nivåerna av stressteroider och en del av dessa produceras direkt i hjärnan och påverkar hjärnans funktion och aktivitet. En sådan stressteroid är allopregnanolon. Allopregnanolon är mycket potent och kan användas som anestesimedel. Det har sin effekt genom att stimulera det inhibitoriska GABA-systemet i hjärnan. Högre nivåer av allopregnanolon ger därmed en minskad aktivitet hos nervcellerna. Minskad nervcellsaktivitet har visats öka mängden intracellulär A β . En ökad mängd A β inuti cellerna stör cellernas funktion och viktiga synapser bryts ner.

Det har inte tidigare visats om just allopregnanolon kan påverka utvecklingen av AD och varför kronisk stress ökar risken för AD är ännu okänt. Vad som dock är känt är att kronisk användning av andra substanser som stimulerar GABA-systemet ökar risken för demens och/ eller AD hos människa. Dessa är t.ex. etanol och medroxy-progesteron acetat (MPA). Det har också visats att kronisk administration av barbiturater eller MPA stör den kognitiva förmågan hos råttor långt efter avslutad behandling och att behandling med benzodiazepiner (t.ex. Diazepam) accelererar sjukdomen i AD möss.

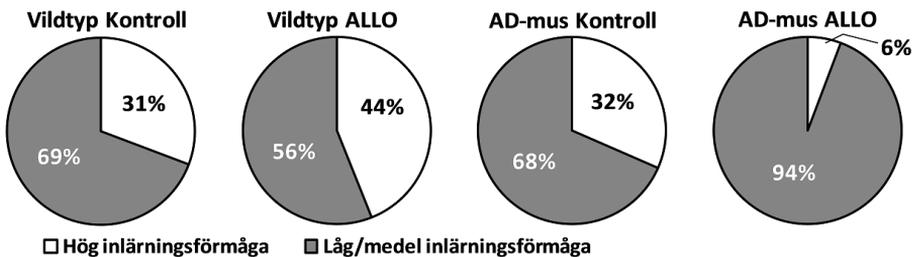
Det är dock inte känt ifall kroppsegna substanser som ökar vid stress kan ge samma effekt.

Metod

I forskning kring AD används transgena möss som uttrycker olika former av A β . Detta gör att de får en AD-liknande sjukdom, med amyloida plack och minnesstörningar. Med hjälp av dessa kan utvecklingen av AD studeras. En del forskning på AD kan göras på celler eller enbart proteiner, men för att studera kognitiv förmåga och hjärnans signalvägar krävs djurförsök. Möss från två olika transgena AD modeller, Swe/PS1 och Swe/Arc möss, behandlades kroniskt med milt förhöjda nivåer av allopregnanolon under olika tidsperioder. Efter ett uppehåll utan behandling testades mössens minne och inlärningsförmåga, varpå vävnadsprover insamlades. Hjärnan undersöktes för olika sjukdomsmarkörer, som plackmängd och mängden lösligt A β , men också för synaptisk funktion.

Resultat

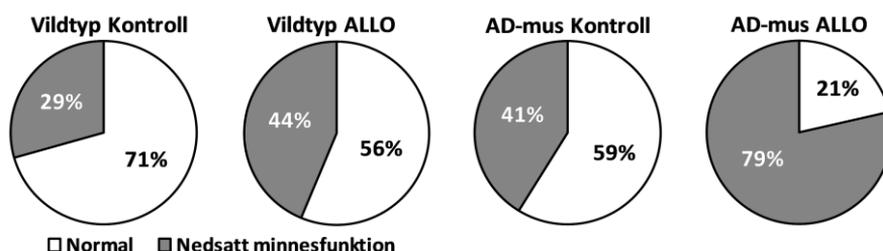
I denna avhandling har jag kunnat visa att kroniskt förhöjda nivåer av allopregnanolon accelererar sjukdomsutvecklingen i två olika transgena musmodeller för AD. Detta har identifierats i form av nedsatt kognitiv förmåga hos mössen, men också med ökade nivåer av A β .



Figur 1. Antal möss med hög respektive låg/medel inlärningsförmåga. Antalet möss med hög inlärningsförmåga var markant färre i gruppen AD-möss (här Swe/PS1) som behandlats kroniskt med allopregnanolone (ALLO).

Mössen studerades i ett test för sin förmåga att rumsligt orientera sig, i en s.k. Morris water maze. Detta är ett test där mössen simmar i en bassäng med en dold plattform, som de lär sig att lokalisera med hjälp av marke-

ringar i rummet. På detta sätt kan inlärningsförmågan studeras och också minnet av eventuellt befäst inläring. I båda modellerna, Swe/PS1 och Swe/Arc, gav kroniskt förhöjda allopregnanolonnivåer minskad inlärnings- och minnesförmåga. Påverkan på inlärningsförmåga var tydligast hos Swe/PS1-möss som behandlats under tre månader, där antalet möss med hög inlärningsförmåga var betydligt färre jämfört med kontroller (Figur 1). Hos Swe/PS1-mössen verkade framför allt hanar påverkas negativt av kroniskt förhöjda allopregnanolonnivåer. Markant förlorad minnesfunktion syntes tydligast hos Swe/Arc-mössen. Antalet möss med nedsatt minnesfunktion var klart fler efter bara en månads behandling med förhöjda allopregnanolonnivåer jämfört med kontroller (Figur 2). Både honor och hanar påverkades negativt, men allra tydligast syntes det hos honorna.

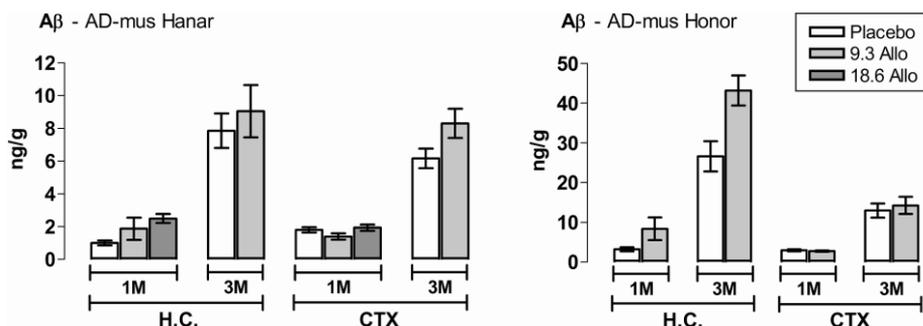


Figur 2. Antal möss med normal respektive nedsatt minnesfunktion. Antalet möss med nedsatt minnesfunktion var markant fler i gruppen AD-möss (här Swe/Arc) som behandlats kroniskt med allopregnanolone (ALLO).

Swe/PS1-mössen hade förhöjda nivåer av lösligt A β efter både en och tre månaders allopregnanolonbehandling (Figur 3). En månads behandling påverkade inte Swe/Arc-mössens A β -nivåer (tre månader testades inte). Lösligt A β motsvarar det A β som har möjlighet att störa den synaptiska funktionen eftersom det ännu inte aggregerats i amyloida plack. Det visade sig att ökad mängd lösligt A β korrelerade med försämrat minne hos Swe/PS1-mössen.

Mängden olösligt A β och antalet plack i hjärnan förändrades inte hos mössen i dessa studier. Dock förändrades utseendet av placken och således plackproduktionen av kroniskt förhöjda allopregnanolonnivåer. Det verkade också som om de AD-möss som behandlats med förhöjda allopregnanolonnivåer fick fler plack, men av mindre storlek. Detsamma gällde de möss som hade försämrad inlärningsförmåga. Dessutom verkade dessa möss ha färre

synapser eller sämre fungerande synapser i hjärnan. Med färre fungerande synapser kan nervcellerna i hjärnan inte fungera normalt, vilket kan leda till försämrat minne.



Figur 3. Mängden lösligt Aβ i AD möss (hanar respektive honor). Mängden Aβ var markant högre i framför allt hippocampus (H.C.) men också i cortex (CTX) bland de möss (här Swe/PS1) som behandlats kroniskt med allopregnanolon (doserna 9,3 och 18,6 Allo) jämfört med de som fått placebo. Behandlingen pågick i en (1M) respektive tre (3M) månader.

Slutsats

Slutsatsen utifrån de studier som ingår i denna avhandling är att kroniskt förhöjda nivåer av allopregnanolon, liknande de vid mild stress, påverkar utvecklingen av AD genom att accelerera dess förlopp. Detta kan vara ett led i varför kronisk stress i olika former orsakar kognitiva störningar och ökad risk för demens i människa. För att förstå sambanden i sjukdomsutvecklingen krävs ytterligare studier om allopregnanolon och AD.

List of original papers

This doctoral thesis is based on the following original papers, which in the text will be referred to by their Roman numerals.

- I. Chronically elevated allopregnanolone levels accelerate Alzheimer's disease in the transgenic A β PP_{Swe}PSEN1 Δ E9 mouse model.

Reprinted from Journal of Alzheimer's disease, 31: 71-84, 2012, Reprinted with permission from IOS Press.

- II. Brief but chronic increase in allopregnanolone cause accelerated AD pathology differently in two mouse models.

Reprinted from Current Alzheimer's Research, 10 (1): 38-47, 2013, Reprinted with permission from Bentham Science Publishing.

- III. Chronic allopregnanolone elevation cause altered plaque production in Swe/PS1 mice.

Manuscript.

Abbreviations

Aβ	Beta-amyloid (exists in various sub-types)
Aβ₄₀	Beta-amyloid 1-40 – sub-type consisting of amino acids 1-40
Aβ₄₂	Beta- amyloid 1-42 – sub-type consisting of amino acids 1-42
AD	Alzheimer’s disease
ALLO	Allopregnanolone
APP/AβPP	Amyloid precursor protein
Arc	Arctic mutation, see also E693G
CAA	Cerebral amyloid angiopathy
CNS	Central nervous system
ΔE9	Exon 9 deletion
E693G	The arctic mutation – amino acid number 693, glutamate, is replaced by glycine.
ELISA	Enzyme-linked immunosorbent assay
GABA	γ -amino butyric acid
GABA_A	γ -amino butyric acid (receptor) type A
IHC	Immunohistochemistry
K595N /M596L	The Swedish mutation – amino acid number 595, lysine, is replaced by asparagine and amino acid number 596, methionine, is replaced by leucine.
LTD	Long-term depression
LTP	Long-term potentiation
MWM	Morris water maze
PS1	Presenilin-1
RIA	Radio-labelled immunoassay
Swe	The Swedish mutation, see also K595N/M596L
Swe/Arc	The transgenic mouse model for AD which carries the gene for APP with the Swe and the Arc mutations.
Swe/PS1	The transgenic mouse model for AD which carries the gene for APP with the Swe mutation, and the gene for PS1 with Δ E9 mutation.

Figures & Tables

Fig. 1: Antal möss med hög respektive låg/medel inlärningsförmåga.

Fig. 2: Antal möss med normal respektive nedsatt minnesfunktion.

Fig. 3: Mängden lösligt A β i AD möss (hanar respektive honor).

Fig. 4: Human brain cross-sections. 2000-2012 © American Health Assistance Foundation.

Fig. 5: Amyloid plaques in human tissue. Re-printed with permission © Dr. D. P. Agamanolis (<http://neuropathology-web.org>).

Fig. 6: Steroid metabolism.

Fig. 7: The modified amyloid cascade hypothesis. Re-printed with permission © John Wiley and Sons [1].

Fig. 8: Study paradigm.

Fig. 9: The osmotic pump. Re-printed with permission © Durect Corporation.

Fig. 10: Elevated allopregnanolone levels during chronic treatment.

Fig. 11: The Morris water maze.

Fig. 12: The number of good learners decreased in the Swe/PS1 mice after chronic allopregnanolone treatment compared to vehicle.

Fig. 13: Chronic allopregnanolone treatment for three months increased path and latency to find the platform in male Swe/PS1 mice but not in wild-type mice.

Fig. 14: Decreased learning in Swe/Arc mice after chronic allopregnanolone elevation.

Fig. 15: Swe/Arc mice equalled the learning deficit in Swe/PS1 mice after chronic allopregnanolone elevation.

Fig. 16: The number of Swe/Arc mice with impaired memory increased after chronic allopregnanolone elevation.

Fig. 17: Swe/Arc mice equalled the memory impairment in Swe/PS1 mice after chronic allopregnanolone elevation.

Fig. 18: Possible increase in thigmotaxis in Swe/Arc mice to the level of that in Swe/PS1 mice.

Fig. 19: Learning performance.

Fig. 20: Thigmotaxis and swimming speed.

- Fig. 21:** Increased levels of soluble A β in Swe/PS1 mice after chronic allopregnanolone elevation.
- Fig. 22:** No effect on levels of soluble A β in Swe/Arc mice after chronic allopregnanolone elevation.
- Fig. 23:** Decreased congophilic plaque size after chronic allopregnanolone elevation.
- Fig. 24:** The natural brain pathology of male Swe/PS1 mice.
- Fig. 25:** The disturbed brain pathology of male allopregnanolone-treated non-learners.
- Fig. 26:** Endogenous allopregnanolone levels.
- Fig. 27:** Endogenous allopregnanolone levels at stress.
- Fig. 28:** Model of the proposed mechanism behind stress-induced AD.

Table 1: Breeding data.

Table 2: Final group compositions of investigated mice.

Table 3: Tests used for statistical analysis.

Table 4: The principle of the 3Rs.

Introduction

In the introduction to this thesis I describe the background to the various areas that are of importance for the foundation of the included studies and for the discussion of the presented results. I start with Alzheimer's disease (AD) in general, its clinical aspects and how it is affected by chronic stress. Further, I describe the GABA-system, how it is affected by neurosteroids and how these systems are linked to AD. A brief introduction on the relevant aspects of memory formation follows, as does an introduction of a few transgenic mouse models for AD. Finally, I give a short description of the current amyloid cascade hypothesis for the pathogenesis of AD.

Alzheimer's disease

Alzheimer's disease (AD) is a neurodegenerative disease, and the most common form of dementia among the elderly (2/3 of dementia cases) [2]. It is characterized by increasing cognitive dysfunction, synaptic loss, and brain atrophy [3]. AD is a devastating condition for those affected, both for the patients and their families. No actual treatment for AD exists at present, only symptom relief. Apart from being a severe condition, AD leads to great economical costs for society. As life expectancy increases so does the number of AD patients. The cause for AD is unknown. The majority of AD cases are sporadic, i.e. linked heredity cannot be identified. However, some factors have been shown to affect the onset of AD, and thus the rate of the disease development. One such factor is stress. Stress causes altered neurochemistry which affects many aspects of cognitive function and well-being. How stress affects the pathogenesis of AD is not known. With more knowledge gained on how stress affects the disease progression of AD, novel areas for research and treatment targeting may develop. If stress-induced dementia could be hindered and if the onset of AD could thus be postponed, then years of healthy living could be added to the lives of many patients and their families, as well as to the society in general.

Clinical development of AD

Symptoms

AD develops relatively slowly e.g. in comparison to vascular dementia [3]. Minor symptoms have often been present for a number of years before healthcare is sought. The initial stage of AD is characterized by short-term memory loss, fatigue, and communicative problems [2]. This may cause embarrassment and anxiety and the affected person may learn different tools to cope with or to hide the symptoms. Depression is also a common symptom, while long-term memory is usually preserved in the early stages. Amnesia and loss of spatial orientation occur at a later stage in the disease development and cause inability to deal with daily life. At this point the person affected by AD is in need of stability and encouragement, as well as general aid and supervision. The final stage of AD is characterized by confusion and severe amnesia. The affected is in demand of assistance to maintain basic requirements of daily living. Distinct personality changes are more common features of frontotemporal dementia but subtle changes often occur in AD. This may include increased agitation and egocentricity, decreased empathy, and impairment of emotional control.

Pathology

Brain atrophy is a main feature of AD, and it particularly affects the hippocampus and the temporal lobe [3] (Figure 4). Loss of neurons is seen in AD with hippocampus and neocortex being the most severely affected areas. Several neurotransmitter systems are affected, however selectively. Cholinergic neurons are affected early on and lost. Loss of neurons causes alterations in the neurochemistry of the brain leading to e.g. decline in cholinergic activity, which may underlie both cognitive and psychiatric symptoms.

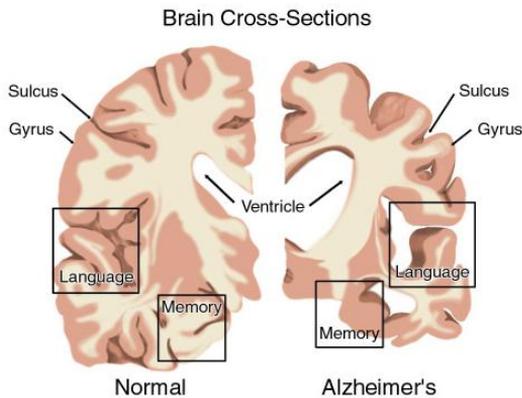


Figure 4. Human brain cross-sections. The figure shows typical pathological features of the cerebrum in late stage AD, i.e. atrophy of neocortex and hippocampus and enlarged ventricles, in comparison to normal status.

The most known histopathological feature of AD is the amyloid plaque. It was firstly described by Dr. Alois Alzheimer in 1907 [4]. Amyloid plaques predominantly consist of the aggregated protein beta-amyloid ($A\beta$) which originates from the amyloid precursor protein (APP). APP is a transmembranal protein, which is cleaved by secretases to form APP-fragments. One APP-fragment is $A\beta$ which is formed by activity of β - and γ -secretases. $A\beta$ is an amyloidogenic protein, meaning that it easily aggregates to form oligomers and fibrils leading to amyloid plaque production. The α -secretase cleaves APP within the $A\beta$ region. This activity leads to production of non-amyloidogenic fragments.

In this thesis, the APP-fragments of great interest are the A β polypeptides of 42 amino acids, i.e. A β ₁₋₄₂ (A β ₄₂), and 40 amino acids, i.e. A β ₁₋₄₀ (A β ₄₀). These A β peptides are highly amyloidogenic, especially A β ₄₂ [5]. They are produced along the secretory pathway and on the cell surface [6-8]. Soluble pools of A β , and again especially A β ₄₂, are neurotoxic [5]. They cause decreased synaptic function, synaptic loss and eventually neuronal death [9, 10]. Recent studies show that the soluble A β oligomers disturb synaptic function and correlate with symptom severity [11, 12]. A β ₄₂ form oligomers which are believed to be the major disturbers of cell function in AD [5]. With high enough A β concentration oligomers will be formed [13], eventually leading to plaque formation.

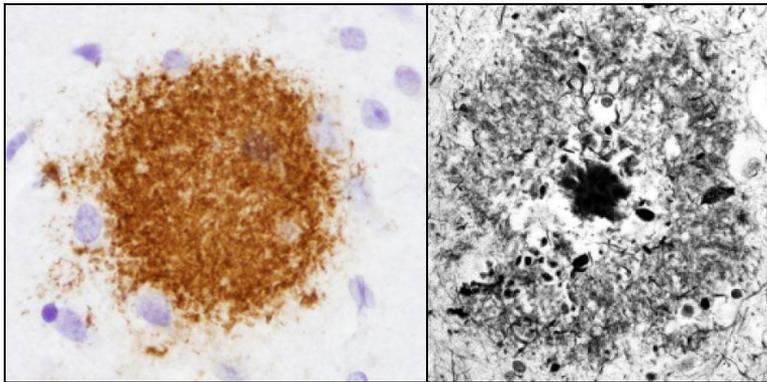


Figure 5. Amyloid plaques in human tissue. Images show A β ₄₂-specific immunostaining of a diffuse plaque (left) and silver staining of a neuritic plaque (right).

Two types of amyloid plaques are found in the AD brain: diffuse plaques and neuritic, also called dense-core, plaques (Figure 5). In the human brain, diffuse plaques mainly consist of A β ₄₂, while both A β ₄₂ and A β ₄₀ construct neuritic plaques. Diffuse plaques exist also in the healthy aged brain but they are more abundant in the AD brain. Neuritic plaques however exist almost exclusively in the AD brain. The A β of neuritic plaques has aggregated into fibrils and formed β -pleated sheets, which constructs the dense core of neuritic plaques [14]. While diffuse plaques have a homogenous morphology, the neuritic plaques consist of a dense core surrounded by a halo of plaque material (Figure 5). Neuritic plaques consist of swollen neurites and inflammatory factors, like reactive astrocytes and microglia while diffuse plaques do not. The different characteristics of the two plaque types and recent dis-

coveries indicate that they are formed along separate pathways [7]. Amyloid plaques also tend to cluster along vessels, causing cerebral amyloid angiopathy (CAA), which is not of focus in this thesis.

Hereditary AD

Most AD patients do not develop hereditary AD but sporadic. While it is unlikely that chronic stress can greatly affect the development of hereditary AD due to the dominance of the mutations, the development of sporadic AD may in comparison be more easily influenced by life style factors. Therefore, in the discussion on stress-induced AD it is logical to predominantly include the sporadic type. However, when studying the disease in animal models one benefits from using the hereditary type harboured by transgenic animal models and hereditary AD is thus briefly discussed in this thesis.

Hereditary AD has been identified in several families, along with the dominant autosomal mutation(s) causing the disease. These mutations occur in the genes for the proteins APP and/ or PSEN1 (see “Transgenic mouse models” for further information). To name a few: the so called Swedish mutation was found in a family in northern Sweden and other mutations have been named in similar fashion: e.g. the Arctic, the Dutch, the Italian, and the Iowa mutations. These dominant mutations cause AD with early onset, typically in the mid-forties.

Chronic stress & AD

Chronic stress is a multifaceted condition as it includes many forms, e.g. post-traumatic stress disorder or psychological and psychosocial stress sometimes followed by burnout syndrome. Although each condition is different they all affect long term cognitive function and some has even been found to increase the risk for dementia. Chronic burnout syndrome in humans affects cognition negatively [15]. Events of psychological stress in mid-life and psychosocial stress at work increase the risk for dementia [16, 17], as do post-traumatic stress disorder [18], and the loss of a parent during adolescence was shown to increase the risk for AD [19]. Perhaps ironically, the stress caused by caring for a spouse with AD may increase the risk for dementia [20]. In transgenic animal models, stress has been shown to cause impaired memory function [21-23]. Then, what is the link between chronic stress and dementia or AD? To answer this question one must search for answers within the brain.

The GABA system

GABA (γ -amino butyric acid) is a neurotransmitter active on the GABA receptor, which in most situations leads to hyperpolarisation of the neuron carrying the receptor. The GABA system is the main inhibitory system of the brain, with high GABAergic activity leading to low general neurotransmission. The GABA_A receptor is a heteropentameric receptor. Several subunit types have been discovered [24, 25], and the subunit composition of the receptor determines its sensitivity. The GABA_A receptor is a chloride channel and when activated it allows chloride to flow into the neuron, leading to hyperpolarisation. GABAergic neurons, i.e. neurons that secrete GABA, are relatively spared until the late stages of the disease development [26]. Interestingly, GABAergic inhibition may affect the early degeneration of other neurotransmitter systems in AD and thereby cause psychological symptoms [26, 27]. In late stage AD, the expression of GABA subunits is altered [28, 29], which may lead to altered sensitivity towards allopregnanolone [30].

GABA-active neurosteroids

Neurosteroids are synthesized in the CNS and adrenals independently of gonadal synthesis [31, 32]. They are produced at stress in parallel to other stress steroids such as cortisol in humans and corticosterone in rodents [32-35]. Originating from cholesterol, e.g. progesterone and cortisol are endogenously metabolized into GABA-active 3α -hydroxy- 5α -reduced neurosteroids, i.e. allopregnanolone and 5α -tetrahydrocortisol (Figure 6). In rodents, the GABA-active metabolite 3α -hydroxy- 5α -deoxycorticosterone (THDOC) is also produced (Figure 6). The GABA_A receptor is sensitive to neurosteroids, and differently so depending on the subunit composition of the receptor [36].

Allopregnanolone

Allopregnanolone is one of the most potent GABA_A receptor active neurosteroids [37]. It is increased during stress, both via the adrenals and direct production in the brain [32, 38]. Allopregnanolone and THDOC have anaesthetic and anxiolytic properties by enhancing the effect of GABA on the GABA_A receptors [39, 40]. Via GABA enhancement, allopregnanolone and other metabolites affect the level of general neurotransmission in the brain [41]. Furthermore, 5α -tetrahydrocortisol enhances the effect of allopregnanolone on the GABA_A receptor [42], resulting in an added effect on the GABA_A receptor during stress when levels of both these steroids are elevated. In females, allopregnanolone is also increased during the progesterone peak of the menstrual cycle and during gestation [38, 43]. Late stage AD patients

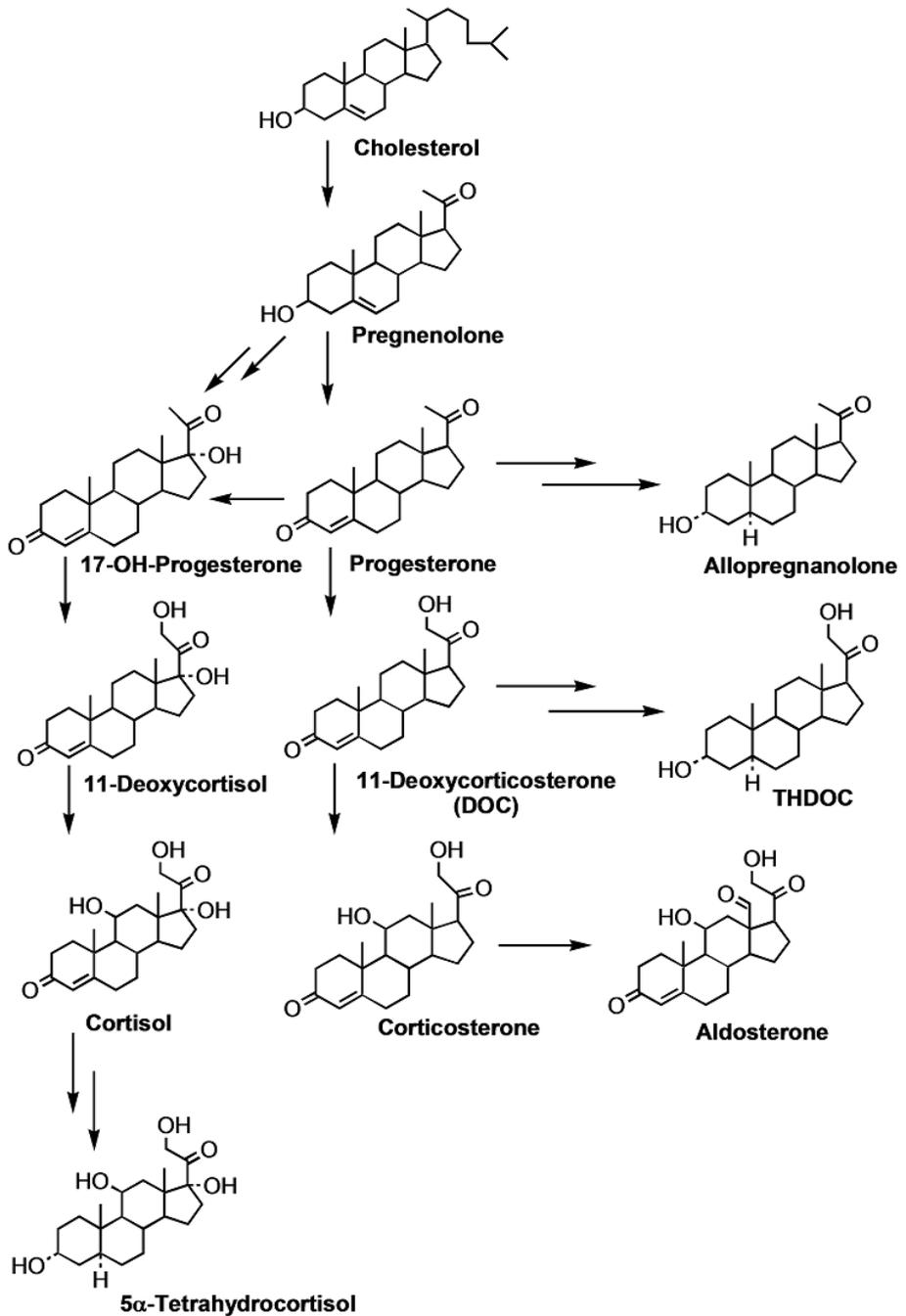


Figure 6. Steroid metabolism. Metabolism of cholesterol leads to production of GABA-active steroids, e.g. allopregnanolone and THDOC.

has decreased serum level of allopregnanolone compared to healthy controls [44, 45]. The levels of allopregnanolone in early stage AD patients have to my knowledge not been investigated.

An interesting aspect of allopregnanolone is its biphasic effect pattern [46]. High concentration of allopregnanolone is anxiolytic and sedative, while low concentrations are anxiogenic. A clinical aspect of this is the role of allopregnanolone in premenstrual syndrome and premenstrual dysphoric disorder [46]. In these situations, the individual's sensitivity to increasing levels of allopregnanolone (probably based on the GABA_A receptor subunit expression) determines the outcome. In AD patients, an altered expression of various GABA_A receptor subunits as been seen [28, 29], which may cause altered neurosteroid sensitivity [30]. Also, changes in allopregnanolone levels have been found in AD patients [44, 45]. Therefore, altered neurosteroid sensitivity may be a sign of long-term dysregulation of general neurotransmission. Is this dysregulation merely a late effect on the AD brain, or does it have an accelerating impact on the early disease development in AD patients? The answer to this question is not known.

Exogenous GABA-active compounds

Several exogenous compounds are positive GABA_A receptor modulators, including benzodiazepines, barbiturates, and ethanol. Long-term exposure to these caused persistent cognitive impairments and increased risk for dementia in humans [47, 48], and cognitive decline in rats [49]. Medroxyprogesterone acetate (MPA) is a synthetic progesterone-like compound. Similarly to allopregnanolone, MPA can induce anaesthesia with effect via the GABA_A receptor [37, 50, 51]. The Women's Health Initiative Memory Study has shown that long-term treatment with MPA + oestrogen doubled the risk for dementia [52]. Such effect was not seen in the oestrogen-treated group alone [53]. It was also shown that the increase in dementia cases was not due to ischemic complications [54, 55]. Furthermore, it has been shown that positive effects on cognition by oestrogen treatment in AD patients are suppressed by MPA [56], and MPA given to rats was shown to affect cognition negatively [57, 58]. These findings suggest that long-term exogenous modulation of the GABA_A receptor increases the risk for cognitive decline and AD.

Learning and memory

Memory is the function by which information is encoded, consolidated and retrieved. Generally, memory is divided into implicit and explicit memory. Implicit (or non-declarative) memory is based on learned activity, often

motor skills, e.g. riding a bike. Explicit (or declarative) memory involves facts and events that are consciously processed. Explicit memory can be further divided into semantic memory which handles learned facts, and episodic memory which handles information connected to an experienced context. Spatial and temporal memories are examples of episodic memory. The hippocampus is affected early in the disease development of AD leading to loss of episodic memory, i.e. reduced ability to consolidate new experiences. The spatial memory function is especially affected, leading to disorientation.

Synaptic plasticity in the hippocampus is vital for memory consolidation, and determined by neuronal long-term potentiation (LTP) contra long-term depression (LTD). Brief and strong activation of a neuron triggers LTP which enhances the neuron to fire more easily. LTD is the reverse, caused by long-term low impact activation and leads to a neuron that is more reluctant to fire. The hippocampus is especially crucial for the formation of spatial memory. Specialized place cells in hippocampus are triggered at recognized locations [59], and LTP in the hippocampus is required for spatial memory formation [60]. GABAergic inter-neurons balance the activity in hippocampus by fine-tuning inhibition and disinhibition, and this activity determines LTP contra LTD [61]. Therefore, as GABA-active steroids and exogenous compounds alter the activity of the GABAergic inter-neurons and thus LTP, shown in e.g. rat [62], it can be concluded that GABA-active compounds may also alter the memory function. Stress was shown to impair LTP and enhance LTD in hippocampus by affecting synaptic plasticity [63].

Other areas of the brain are involved in the memory process, with the hippocampus in the role of binding all the pieces of information together to form a memory [64]. Afferent neuronal pathways to the hippocampus originate in surrounding areas, e.g. the striatum which is important for planning and execution, the amygdala which is important for emotional involvement, and the parahippocampal cortex which is especially important for spatial memory. Efferent pathways lead back to these areas and to the neocortex.

Transgenic mouse models

As mentioned previously the mutations identified in hereditary AD can be used in different transgenic mouse models to study the pathogenesis of AD. These models display various aspects of the disease process. None of these models develop actual AD, but allows focus on specific mechanisms [65]. A few commonly used transgenic mouse models for AD are described below with focus on the two mouse models used in *Paper I, II* and *III*: i.e. the Swe/PS1 and the Swe/Arc models.

The Swe/PS1 mouse model

The Swe/PS1 mouse model harbours the human genes for APP with the Swedish mutation (Swe, K595N/M596L) and presenilin-1 (PS1) with exon 9 deletion ($\Delta E9$). Cleavage of APP with the Swe mutation increases the production of A β [66], while the $\Delta E9$ mutation shifts production towards formation of A β_{42} instead of A β_{40} [67, 68]. A β_{42} is more toxic than A β_{40} since it is more prone to form oligomers. The plaque pathology of both diffuse and neuritic plaques is evident early in the Swe/PS1 mouse and increases rapidly after 6 months of age, selectively in the cortex and the hippocampus [69]. The progression of CAA is slow in the Swe/PS1 mouse model, slower than in e.g. the Swe/Arc mouse model [69, 70]. The Swe/PS1 mouse model develops an AD-like disease at an early age, manifested by high levels of A β_{42} and cognitive deficits. The cognitive dysfunction emerges at around 6 months of age or later [71-74].

The Swe/Arc mouse model

The Swe/Arc mouse model is primarily exposed to A β_{40} , and only low levels of A β_{42} [70]. It carries the human gene for APP with the Swe and the Arctic mutation (Arc, E693G). The Swe mutation causes an increased production of A β [66]. The Arc mutation leads to a less hydrophilic A β , which is more prone to form oligomers and fibrils. It also seems to be less able to cross the blood-brain-barrier and clusters around vessels. CAA is therefore a feature of the Swe/Arc mouse model [70]. Apart from CAA, the A β_{40} is mostly associated to neuritic, i.e. dense-core, plaques. Diffuse plaques are not seen as frequently. The Swe/Arc mouse model displays a profound CAA from 7 months of age, with a dramatic increase between 9 to 15 months of age [70]. In previous studies the Swe/Arc mouse model performed with intact learning ability in the MWM at up to 9 months of age [70]. Memory performance was moderately changed around 6 months of age and definitely impaired at 9 months of age [70]. Parallel construction of another Swe/Arc mouse model harbouring the same mutations but with what appears to be a more aggressive phenotype [75]. This is not the Swe/Arc mouse model discussed in this thesis.

Other mouse models

One commonly used mouse model for investigation of AD is the Tg2576 model which has the Swe mutation only. Compared to the Swe/PS1 model it lacks the $\Delta E9$ mutation and thus have high levels of A β_{40} contra A β_{42} . Compared to the Swe/Arc model it lacks the Arc mutation and thus the altered hydrophilicity of A β . Therefore the Tg2576 model displays a milder and

more prolonged disease development than the Swe/PS1 [76] and the Swe/Arc. The development of plaques and CAA is slow. Initially only diffuse plaque can be identified while in aged mice also dense-core plaques can be found [76]. Other commonly used mouse models carry e.g. the Iowa or Dutch mutations. These mutations are very similar to the Arc mutation as they lead to altered hydrophilicity of A β and increased CAA [70, 77]. Another interesting AD model is the so called triple transgenic mouse (3xTg), which apart from the amyloid plaques develops neurofibrillary tangles [78, 79]. It was concluded that intraneuronal A β cause AD-related cognitive dysfunction and neurofibrillary tangles in the 3xTg mouse [78-80].

The (modified) amyloid cascade hypothesis

Amyloid plaques were the first biomarkers of AD, discovered by Alois Alzheimer in 1907 [4]. The plaques were thought to cause memory disturbance and brain atrophy, which was the basis for the original amyloid cascade hypothesis [81]. More recent discoveries have given reasons to modify the traditional ideas [1]. The modified amyloid cascade hypothesis does not focus on plaques alone, but on the events prior to plaque formation involving the intraneuronal pool of soluble A β monomers and oligomers (Figure 7). The soluble levels of A β correlate with synaptic function [5, 11, 12, 82, 83]. The accumulation of A β has also been described to directly cause AD symptoms [10], and correlates with disease progression [11, 12, 84]. Predominantly, it seems that the intraneuronal pool of soluble A β is responsible for synapse pathology [85-88]. Interestingly, the levels of intracellular A β are determined by synaptic activity [89, 90], and A β has been shown to be released in vesicles at depolarisation [91, 92]. By affecting the level of general neurotransmission via the GABA_A receptor with exogenous compounds, the flow of the amyloid cascade was altered in transgenic mouse models for AD. Chronic treatment with diazepam caused elevated levels of intracellular A β , and enhanced formation of neurotoxic oligomers, in turn leading to neuronal dysfunction and cognitive decline [93]. Treatment with picrotoxin, a GABA_A receptor inhibitor, rescued memory decline [94]. It may be that chronic stress can affect the amyloid cascade in a similar manner. Some endogenous stress steroids are positive modulators of the GABA_A receptor. This modulation can lead to reduced levels of neurotransmission, which in turn may lead to increased levels of intraneuronal A β , synaptic dysfunction, synaptic and neuronal loss, atrophy, and symptoms. Allopregnanolone is such a stress steroid.

The Modified β -Amyloid Cascade

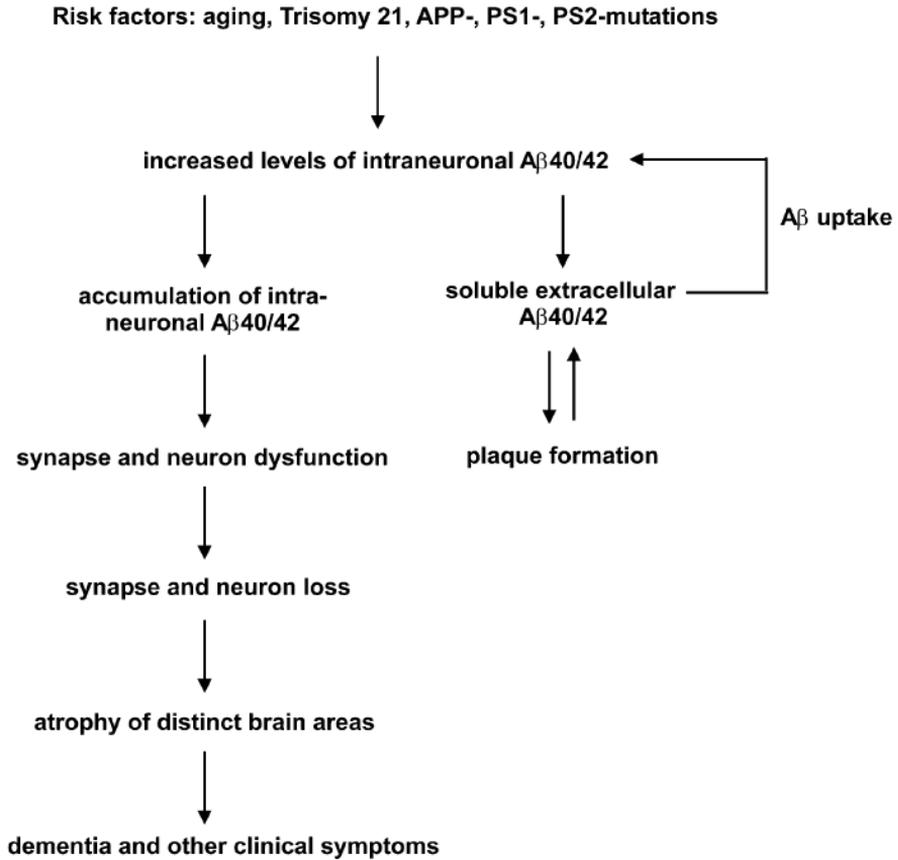


Figure 7. The modified amyloid cascade hypothesis.

Aims

The over-all aim of this thesis was to investigate the effects of the stress steroid allopregnanolone on the development of Alzheimer's disease in transgenic mouse models.

The specific aims included to investigate how chronically elevated levels of allopregnanolone affect:

- The **learning and memory performance** of the transgenic Swe/PS1 and the Swe/Arc mouse models.
- The **distribution of A β** between the soluble and the insoluble phase in the transgenic Swe/PS1 and the Swe/Arc mouse models.
- The levels of **histological markers** of Alzheimer's disease in the transgenic Swe/PS1 mouse model.
- The **synaptic function** in the transgenic Swe/PS1 mouse model, by investigating the levels of synaptophysin.
- The **correlations** between the different parameters listed above.

Materials & Methods

In this section I briefly describe the various materials, methods and techniques used to investigate the presented aims.

Transgenic mice

Transgenic mice of the Swe/PS1 (*Paper I, II, and III*) and the Swe/Arc (*Paper II*) models were used to study the development of AD and the effect of chronically elevated allopregnanolone levels on the development of AD. The individual mice were obtained from in-house breeding.

Breeding

Strategies for breeding were chosen based on the manual from the Jackson Laboratories, USA (Breeding Strategies for Maintaining Colonies of Laboratory Mice, 2009). Breeding couples were transgenic male mice (Swe/PS1 or Swe/Arc) and wild-type female mice (C57Bl/6J). The Swe/PS1 males and wild-type females for initial breeding were purchased from the Jackson Laboratories, USA. The Swe/Arc male mice were donated by Professor Nitsch and mated with wild-type females from in-house breeding. Therefore, the Swe/Arc mice used in *Paper II* had at least 50% identical background compared to the Swe/PS1 mice, which is beneficial when comparing characteristics of the two AD models. However, the pilot studies including aged Swe/Arc mice were performed using aged animals that arrived

	<u>Swe/PS1</u>	<u>Swe/Arc</u>	<u>Wild-type</u>
Litter size (no. of pups/ litter \pm S.D.)	5.8 \pm 2.3	5.9 \pm 2.4	6.8 \pm 2.1
No. of litters/ couple (mean \pm S.D.)	2.0 \pm 1.4	1.8 \pm 1.0	2.2 \pm 0.8
No. of weeks as couple (mean \pm S.D.)	18.1 \pm 9.1	18.8 \pm 8.8	15.9 \pm 4.1
Genotype (% AD pups/ litter)	45%	42%	n.a.
Sex (% females/ litter)	47%	53%	51%
Premature deaths (% of total)	12.8%	0%	1.7%

Table 1. Breeding data. The data is based on 161 litters and 960 pups of the Swe/PS1 (116 litters), Swe/Arc (24 litters), and pure C57BL/6J (21 litters) breeding. Premature deaths were deaths after the age of 3 weeks and before the age of 12 months.

directly from Professor Nitsch and these mice were on a hybrid background of C57Bl/6J and DBA/2. The breeding of Swe/PS1, Swe/Arc, and wild-type mice resulted in expected number of pups (The Jackson Laboratory) (Table 1). The Swe/PS1 suffered some premature deaths (after the age of 3 weeks

and before the age of 12 months), which was expected (the Jackson Laboratories, USA). The Swe/Arc did not suffer any premature deaths.

Subjects & genotyping

The final group compositions of the investigated mice for each study respectively are given in Table 2. To identify and to confirm preservation of the genotype all animals were genotyped with PCR at weaning and at termination.

Genotype	Treatment	Dose (nmol/h)	Paper I	Paper II	Paper III*
			n (F+M)	n (F+M)	n (F+M)
Swe/Arc	Vehicle	-	-	17 (8+9)	-
Swe/Arc	Allopregnanolone	9.3	-	14 (7+7)	-
Wild-type	Vehicle	-	-	17 (10+7)	-
Wild-type	Allopregnanolone	9.3	-	16 (9+7)	-
Swe/PS1	-	-	-	16 (8+8)	-
Swe/PS1	Vehicle	-	19 (9+10)	28 (12+16)	16 (6+10)
Swe/PS1	Allopregnanolone	4.7	18 (7+11)	-	-
Swe/PS1	Allopregnanolone	9.3	17 (7+10)	33 (16+7)	14 (4+10)
Swe/PS1	Allopregnanolone	18.6	-	7 (0+7)	-
Wild-type	-	-	-	19 (10+9)	-
Wild-type	Vehicle	-	13 (6+7)	35 (18+17)	-
Wild-type	Allopregnanolone	4.7	12 (6+6)	-	-
Wild-type	Allopregnanolone	9.3	13 (7+6)	27 (17+10)	-
Wild-type	Allopregnanolone	18.6	-	7 (0+7)	-

Table 2. Final group compositions of investigated mice. Swe/Arc and Swe/PS1 mice were used with wild-type siblings for each mouse model respectively (separated by vertical lines). The mice were untreated or treated chronically with physiological levels of allopregnanolone or vehicle for 4 weeks (Paper II) and 12 weeks (Paper I and III) respectively from the age of 10 weeks. The number of animals per group is stated in total (n), and for each sex respectively (F+M). *The same individuals as in Paper I.

Study paradigm

The aim of these studies (Paper I, II, and III) was to investigate the effect of chronically elevated levels of allopregnanolone on the development of AD at an early stage of the disease development. However, during adolescence the

levels of steroids and the sensitivity towards these can vary greatly compared to that of adult mice [95]. As mice sexually mature at 5-8 weeks of age, 10 weeks of age was selected as the starting point for treatment. The length of the treatment period was selected to correspond to a substantial time period of a mouse's life (of approximately 2 years) and a wash-out period of four weeks was allowed from end of treatment until start of behavioural testing. This was done to ensure that the long-term, in-direct effects of the chronically elevated levels of allopregnanolone were studied and not the direct effects of the treatment [96]. This led to the study paradigm presented in Figure 8.

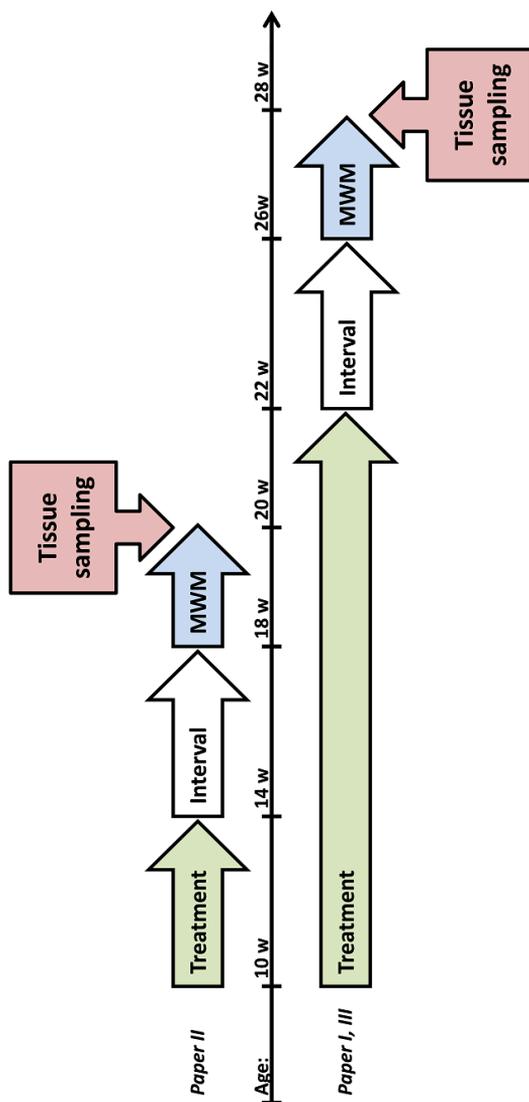


Figure 8. Study paradigm.

Chronic elevation of allopregnanolone

The exposure to chronically elevated levels of allopregnanolone (*Paper I, II, and III*) was achieved by treatment using osmotic pumps (Figure 9).

Osmotic pumps were filled with the desired substance (here allopregnanolone) and sealed with the flow moderator. When the pump has been inserted subcutaneously, water from the extracellular space slowly flows across the external semi permeable membrane and fills the space underneath containing the osmotic agent. As water fills this space the substance inside is pushed out from the pump via the delivery portal.

The osmotic pump of the used model secretes the allopregnanolone solution evenly over four weeks (on average 0.11 μl solution per hour).

The pumps were exchanged monthly, and at the end of the treatment the final pump was removed. The achieved allopregnanolone levels were chosen to match endogenous levels of allopregnanolone during mild stress.

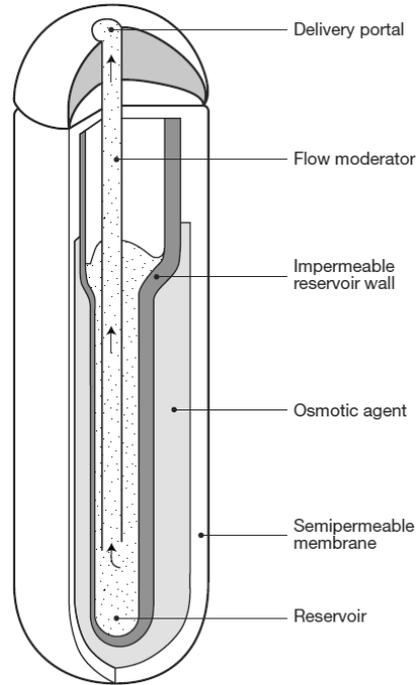


Figure 9. The osmotic pump.

Pharmacokinetic study

The chronic treatment performed in the reported studies (*Paper I, II and III*) was designed to simulate levels of allopregnanolone achieved at mild stress. By using osmotic pumps, allopregnanolone was evenly secreted during the treatment period, and the brain levels of allopregnanolone were mildly, but significantly increased (Figure 10, *Paper I*). There were no differences between male and female mice in the base-line levels, but the increase during treatment was larger in females. This was probably due to a smaller body size.

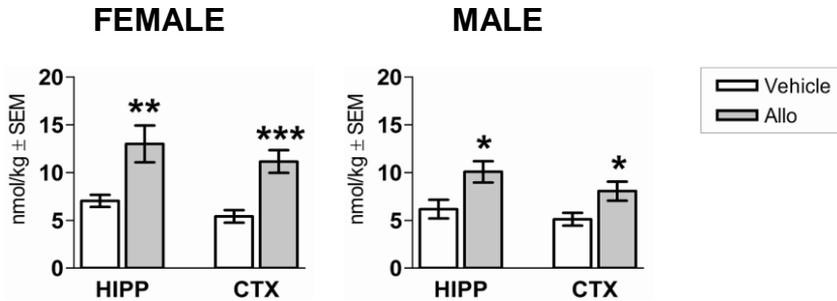


Figure 10. Increased allopregnanolone levels during chronic treatment. The figures show the allopregnanolone levels in hippocampus (HIPP) and cortex (CTX) in wild-type mice. The mice were treated chronically with allopregnanolone ($n = 20$ females + 20 males) or vehicle ($n = 10 + 10$) for 2-4 weeks from the age of 10 weeks. Tissues were collected during treatment. *** $p < 0.001$, ** $p < 0.01$ * $p < 0.05$, vs. vehicle.

Morris water maze

The Morris water maze (MWM) was used in *Paper I* and *II* to examine learning and memory performance. It is a task aimed at assessing the ability to learn the spatial position of a hidden platform in a pool of water using visual cues around the pool (Figure 11) [97]. Several different set-ups of MWM are used, with standard pool sizes ranging from 50-200 cm in diameter [98]. The aim of the studies was to investigate an early stage in the disease development, and therefore a larger pool was chosen to create a more difficult task.

Specific parameters

The pool diameter was 150 cm, which corresponds to a relatively large pool. The platform was 10 cm in diameter and submerged 3-4 mm under the surface of the water. Mice easily become hypothermic, and it is important to keep sufficient water temperature (24 °C in our set-up), and to not permit to long swims. Therefore, after each swim the mice were gently dried, placed in a heated chamber for a few minutes, and then returned to their home cage.

Mice are generally very good swimmers, however they prefer to avoid it and the introduction of the MWM is a stressful event. The mice were therefore habituated to the swimming procedure by two 60-second swims with an

interval of 24 hours prior to the learning phase. No platform was positioned in the pool during the habituation.

The learning phase stretched over six days with four swims each day at 10-minute intervals. The starting point for each daily swim was a different compass direction in a randomized order, and the mice were put into the water facing the rim of the pool. The platform was positioned in the centre of the goal quadrant throughout the learning phase. Each swim lasted until the mouse climbed onto the platform or until 120 seconds had elapsed, whichever occurred the soonest. The mouse was then allowed to sit on the platform for 15 seconds to familiarize itself with the area. If the mouse had not found the platform after 120 seconds, it was gently placed on it. A probe trial was performed on the day after the last learning phase session. Each mouse was then allowed to swim for 60 seconds in the pool with no platform. The probe trial is considered a test of memory function.

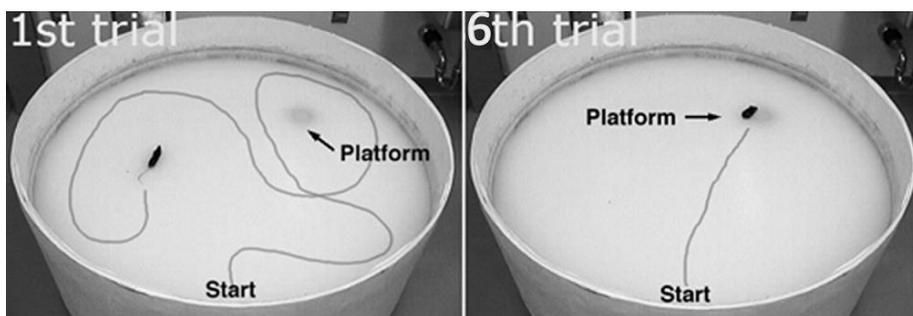


Figure 11. The Morris water maze. On the 1st trial (first day of the learning phase) the mouse searches for a way to escape the water. On the 6th trial (6th and last day of the learning phase) a normal and healthy mouse will have learnt the existence of the hidden platform, memorized its location, and will swim directly to it. Clearly visible cues (not shown here) around the pool aid the mouse in its spatial orientation.

The behaviours of the mice were recorded using HVS Image 2020 Plus (HVS Image Ltd, UK). Parameters analysed during the learning phase were path, latency, swimming speed, floating, thigmotaxis (% time spent swimming along the rim of the pool), Gallagher measure (GM; average distance to platform), and for the probe trial (memory function) they were %-time spent and %-path travelled in the goal quadrant.

Resident/ Intruder stress model

To achieve a mild chronic stress response in Swe/PS1 and wild-type mice (*pilot study*), the previously reported Resident/ Intruder stress model (R/I) was used [99]. This method is based on the strong territorial instinct in male mice. A resident mouse was once daily (five days/week for four weeks) exposed to an intruder in its cage. The mice were allowed direct interaction for 10 minutes (or shorter in case of fighting) and spent one hour in the resident's cage but separated by a Plexiglas plate with drilled holes for smell and sound interaction.

Forced swim test

To provoke an acute stress response (*pilot study*) a protocol based on the forced swim test was used. Forced swim test has been shown to increase corticosterone levels in mice [100]. The mice were placed in a pool of water (with no platform) for 60 seconds before being rescued and allowed to dry in a heating chamber for a few minutes. The mice were then allowed to rest for 30 minutes before decapitation in order to catch the peak allopregnanolone level in brain after acute stress [32]. The mice in the pilot study had not been exposed to a swimming task previously.

Dissection of mouse brain

The brain of each mouse was collected and rapidly dissected on ice under microscope. The right hemisphere was kept intact for histological analysis (*Paper III*) and the left hemisphere was further dissected into hippocampus and cortex (*Paper I and II*). For the pilot study of endogenous allopregnanolone levels the left hemisphere was dissected into hippocampus, frontal cortex and posterior cortex (including occipital, parietal and temporal cortex) or kept intact.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) is a method to quantify compounds recognized by anti-bodies. Here, it was used to quantify A β (*Paper I and II*), with commercially available ELISA kits specific for A β ₄₀ and A β ₄₂ respectively. Manual homogenization in standard Tris-HCl buffer allowed collection of the soluble A β fraction. The tissues were further dissolved in Guanidinium-Tris-HCl buffer. Guanidinium denatures proteins and allowed collection of the insoluble A β fraction. Each fraction respectively was analyzed with ELISA.

Congo red staining

The Congo red chemicals react with amyloid fibrils which produce a red staining of the plaque. Since diffuse plaques do not contain β -sheet structures they are not stained by Congo red. Dense-core plaques on the contrary have a solid core of amyloid β -sheets and are therefore detected by this method. In *Paper III* the brain tissue sections were Congo red stained, and counter stained with Mayer's haematoxylin in order to visualize structures in the tissue. Congophilic plaque load (area and number per measured tissue area) was manually quantified in hippocampus, frontal and posterior cortex (including occipital and parietal cortex).

Immunohistochemistry

Immunohistochemistry (IHC) is a method to stain a certain protein in a fixed tissue by using an anti-body raised against that specific protein. The location of the protein in question can be investigated. To some extent this method of staining can also be used for quantification of the protein. In *Paper III* the brain tissues from Swe/PS1 mice were analyzed using IHC for $A\beta_{42}$ -specific plaques and for synaptophysin.

A β_{42} -specific plaques

$A\beta_{42}$ is associated to both diffuse and dense-core plaques. Therefore, $A\beta_{42}$ -specific IHC detects both plaque types in contrast to the Congo red staining which mainly stains dense-core plaques. It was therefore expected to yield another result than that from the Congo red staining technique. The $A\beta_{42}$ -specific IHC was detected with fluorescence and the visible plaques were manually quantified.

Synaptophysin

Synaptophysin is a synaptic glycoprotein involved in the function of synaptic vesicles. It is used as a marker for synaptic function, with increasing levels indicating higher synaptic function which correlates with cognitive function [10, 101, 102]. The IHC staining to detect synaptophysin was visualized with DAB peroxidase kit and the grey-scale intensity of the staining was semi-automatically quantified.

Celite chromatography – Radio-labelled immunoassay

Celite chromatography is a method for sample purification after which the compound of interest can be quantified using radio-labelled immunoassay

(RIA). These methods were used to quantify allopregnanolone in brain tissue (*Paper I* and *pilot studies*) and in plasma (*Paper I*). Prior to Celite chromatography purification, lipophilic compounds were extracted from the plasma samples in diethyl ether and from the brain tissue samples in ethanol. The diethyl ether and ethanol respectively were then further purified with Celite chromatography. The fraction containing allopregnanolone was collected and quantified with RIA using a polyclonal rabbit antibody [103]. This is a highly specific method which allows analysis of allopregnanolone separated from other endogenous steroids.

Statistical analysis

When performing statistical analysis one assumes that no difference exists between the compared groups, i.e. the null hypothesis. The statistical analysis aims to determine how great the risk is of incorrectly rejecting the null hypothesis, e.g. incorrectly stating to have an effect by treatment. This is a type 1 error and the risk is quantified in the p -value. The type 2 error is the failure to reject a true null hypothesis, e.g. incorrectly stating to have no effect by treatment. In order to avoid the type 1 and type 2 errors, the appropriate statistical tests must carefully be selected. Which statistical analysis to perform, and to present, can be an everlasting discussion. There are few absolute rights and wrongs, and many opinions. The tests for statistical analysis that were chosen to perform and to present are commented on and listed in Table 3.

Test:

Description:	Applicable studies:
Mann-Whitney U test	
This is a non-parametric test for comparison of two independent groups. Non-parametric analysis is appropriate for data that is not normally distributed and/or has small group sizes. Some of these data are normally distributed and some are not. However, all data were collected from relatively small groups and non-parametric analysis is preferred.	<i>Paper I</i> <i>Paper II</i> <i>Paper III</i> <i>Pilot study</i> – pharmacokinetics <i>Pilot study</i> – endogenous levels
Pearson's correlation coefficient, r	
A measure of linear dependence between two variables. It is highly reliable for use on normally distributed data. For other data sets it can be used, but with caution.	<i>Paper I</i> <i>Paper III</i>
2- and 3-way ANOVA	
Repeated measures analysis of variance. This analysis is used for several dependant values. The dependency refers to values that for each individual include several points of measure which depend upon each other. Included is also a test of between subjects effect to compare independent groups.	<i>Paper I</i> <i>Pilot study</i> – MWM set-up
Ryan-Einot-Gabriel-Welsch multiple range test (REGW-test)	
A non-parametric alternative as ad hoc test to follow the 2-way ANOVA. This is test of between subjects effect, i.e. it compares independent groups.	<i>Pilot study</i> – MWM set-up
Fischer's exact test	
A test that is used in contingency tables. It is preferable when sample sizes are small. Noted in comparison is that the more commonly used Chi ² -analysis requires large sample sizes and would not be appropriate in these studies.	<i>Paper I</i> <i>Paper II</i>

Table 3. Tests used for statistical analysis.

Results & Discussion

The main conclusion from *Paper I, II, and III* is that chronic elevation of allopregnanolone accelerated the disease development in transgenic AD mice. This was concluded as the Swe/PS1 mice responded with impaired learning and memory performance and increased levels of soluble A β . The Swe/Arc mice responded with impaired learning and memory while their levels of soluble A β were un-affected. It was also found that chronic elevation of allopregnanolone levels disturbed the *natural* plaque production. Furthermore, the learning and memory dysfunctions seen in the transgenic AD mice were not identified in the wild-type mice.

Further in the Results & Discussion I present the results more in depth, and discuss these findings in contrast to previously reported data.

Cognitive performance

Cognitive function is a term that includes many processes, e.g. memory and learning, attention, and decision making. In the MWM the mice use several areas of the brain in combination [98], and use different strategies to perform the task. In order to find their way in a set space they make spatial decisions based on available cues [97]. The motif for learning is the unlikable situation of being in water. By remembering where the platform is positioned in relation to the available cues, the time spent in the water can be minimized.

Learning

Chronic elevation of allopregnanolone was found to cause impaired learning performance in transgenic AD mice (*Paper I* and *II*). Among Swe/PS1 mice the number of good learners was decreased after three months of chronic allopregnanolone elevation (Figure 12, *Paper I*). The greatest effect on learning was seen among male mice alone with increased latency and path to find the platform (Figure 13). The learning impairment was not unexpected, as chronic treatment with other GABA_A receptor active compounds have led to cognitive decline in AD mouse models [93, 94]. However, it has not previously been shown that the endogenous allopregnanolone can give rise to similar effects. The wild-type mice were un-affected by chronic allopregnanolone treatment in terms of learning and memory. This was however unexpected since negative effects on cognition has been seen also in wild-type animals [49, 57, 58], and in humans [47].

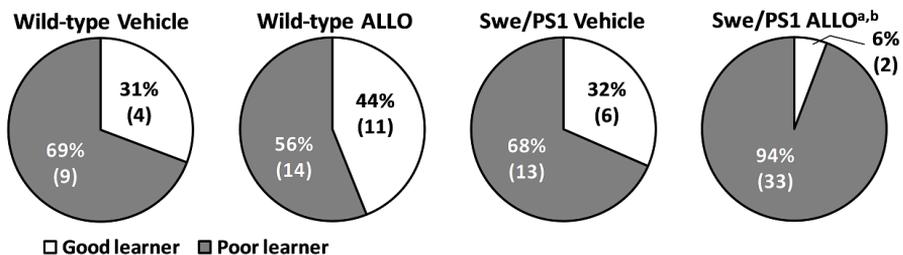


Figure 12. The number of good learners decreased in the Swe/PS1 mice after chronic allopregnanolone treatment compared to vehicle. The figure shows the percentages of (number of) good and poor learners within each group, including Swe/PS1 and wild-type mice. The mice were treated chronically with allopregnanolone (ALLO) or vehicle for 12 weeks from the age of 10 weeks. **a)** $p \leq 0.01$ vs. Swe/PS1 Vehicle. **b)** $p < 0.001$ vs. Wild-type ALLO.

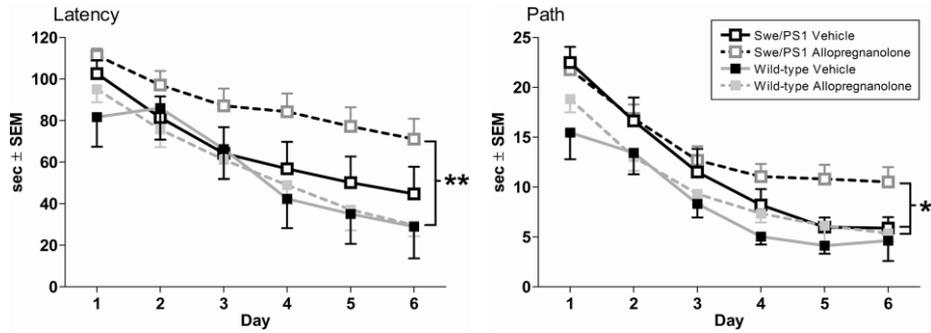


Figure 13. Chronic allopregnanolone treatment for three months increased path and latency to find the platform in male Swe/PS1 mice but not in wild-type mice. The figure shows learning curves (i.e. path or latency to find the platform) for male Swe/PS1 mice in the MWM, day 1-6. The mice were treated chronically with allopregnanolone or vehicle during 12 weeks, from the age of 10 weeks. Data is shown as mean \pm SEM. * $p < 0.05$ Swe/PS1 Allopregnanolone vs. Swe/PS1 Vehicle on Day 4-6, and $p < 0.01$ vs. Wild-type Allopregnanolone on Day 1-6. ** $p < 0.01$ Swe/PS1 Allopregnanolone vs. Wild-type Allopregnanolone on Day 1-6.

Chronic allopregnanolone elevation of one month only did not obviously impair the learning function of the Swe/PS1 mice (*Paper II*). It is possible that a minor change was seen in the male Swe/PS1 mice, while the females seemed rather improved by the treatment. While the Swe/PS1 mice showed little effect on cognition after one month's treatment and major effect after three month's treatment, the Swe/Arc mice appeared more sensitive. The Swe/Arc mice had impaired cognitive performance after only one month of elevated allopregnanolone levels with increased path to find the platform on the last day of the learning phase (Figure 14, *Paper II*). The wild-type mice did not show impaired learning after chronic allopregnanolone treatment.

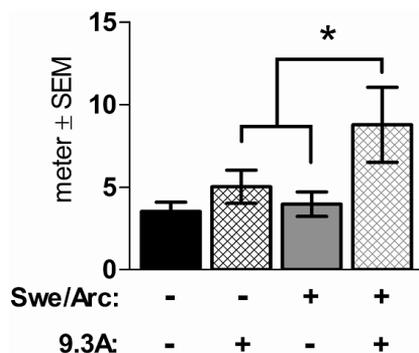
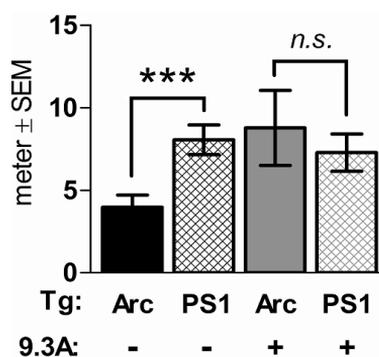


Figure 14. Decreased learning in Swe/Arc mice after chronic allopregnanolone elevation. The figure shows path to find the platform of Swe/Arc and wild-type mice in the MWM on the last day of the learning phase. The mice were treated chronically with allopregnanolone (9.3A) or vehicle during 4 weeks, from the age of 10 weeks. Data is shown as mean \pm SEM. * $p < 0.05$.

It is clear that the transgenic genotypes of Swe/Arc and Swe/PS1 mice lead to unequal predispositions for MWM performance. When given vehicle the two models performed differently in the MWM. The Swe/PS1 mice clearly had learning dysfunctions at this age (both at 20 and 28 weeks of age) while the Swe/Arc mice did not. The differences in learning ability between Swe/PS1 and Swe/Arc mice were shown in e.g. the learning performance, where the Swe/PS1 mice had significantly longer path to reach the platform on the final day of training compared to the Swe/Arc (Figure 15). After chronic allopregnanolone treatment of one month the Swe/Arc mice were severely affected compare to vehicle, and performed as poorly as the Swe/PS1 mice (of both treatment groups). The direct comparison between Swe/PS1 and Swe/Arc has to my knowledge not been reported previously. It would appear that the Swe/PS1 is a more aggressive model of AD than the Swe/Arc.

Figure 15. Swe/Arc mice equalled the learning deficit in Swe/PS1 mice after chronic allopregnanolone elevation.

The figure shows path to find the platform of Swe/Arc and Swe/PS1 mice in the MWM on the last day of the learning phase, and a comparison of genotypes. The mice were given vehicle or 9.3 nmol/h allopregnanolone (9.3A) during 4 weeks, from the age of 10 weeks. Data is shown as mean \pm SEM. *** $p < 0.001$, n.s. $p > 0.05$.



Memory

Impaired memory function in transgenic AD mice after chronic elevation of allopregnanolone was identified in *Paper I* and *II*. The impairments were especially obvious in the Swe/Arc mice (*Paper II*). The path in the goal quadrant was significantly reduced and the number of mice with impaired memory increased after chronic allopregnanolone treatment (Figure 16). In the Swe/PS1 mice given one month of treatment (*Paper II*) the male mice showed signs of impaired memory while the females did not. However, after three months of treatment both male and female Swe/PS1 mice had impaired memory function (*Paper I*).

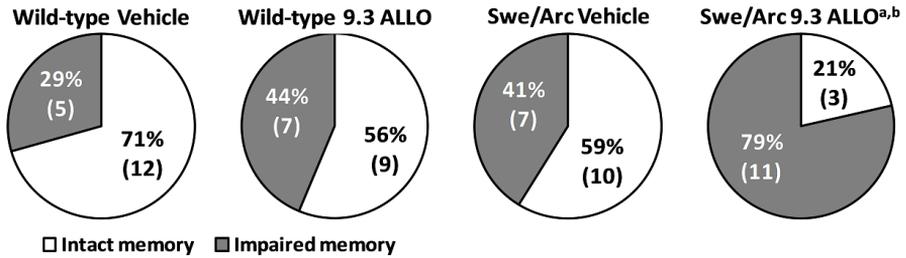
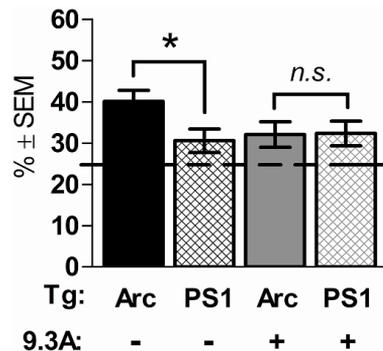


Figure 16. The number of Swe/Arc mice with impaired memory increased after chronic allopregnanolone elevation. The figure shows the percentages of (number of) mice with intact and impaired memory within each group, including Swe/Arc and wild-type mice. The mice were treated chronically with allopregnanolone (9.3 ALLO) or vehicle for 4 weeks from the age of 10 weeks. **a)** $p < 0.01$ vs. Wild-type 9.3 ALLO. **b)** $p < 0.05$ vs. Swe/Arc Vehicle.

The two mouse models displayed different base-line conditions also in the MWM probe trial. The vehicle-treated Swe/PS1 mice swam a significantly smaller part of its path in the goal quadrant, i.e. showed poorer memory performance, compared to the Swe/Arc mice (Figure 17). After chronic allopregnanolone treatment for one month the Swe/Arc mice were affected and had as poor memory as the Swe/PS1 mice. The Swe/PS1 mice were not further impaired by the treatment. Any differences in starting conditions in the probe trial are of course linked to the performance during the learning period. Since the Swe/PS1 mice did not learn as well as the Swe/Arc, they were not likely to remember as much.

Figure 17. Swe/Arc mice equalled the memory impairment in Swe/PS1 mice after chronic allopregnanolone elevation. The figure shows path in the goal quadrant of Swe/Arc and Swe/PS1 mice in the MWM on the last day of the training phase, and a comparison of genotypes. The mice were given vehicle or 9.3 nmol/h allopregnanolone (9.3A) during 4 weeks, from the age of 10 weeks. Data is shown as mean \pm SEM. * $p < 0.05$, n.s. $p > 0.05$.



Swimming behaviour

The vehicle-treated Swe/PS1 mice showed high levels of thigmotaxis, in both *Paper I* and *II*, and after chronic allopregnanolone treatment they had increased path and latency but unchanged levels of thigmotaxis. The vehicle-treated Swe/Arc mice had low levels of thigmotaxis. The allopregnanolone-treated Swe/Arc mice had somewhat higher levels, but they were still considered low and not significant increased. However, allopregnanolone-treated Swe/Arc mice nearly equalled the Swe/PS1 mice in level of thigmotaxis, which indicates that the Swe/Arc mice indeed had increased thigmotaxis after allopregnanolone treatment (Figure 18). Thigmotaxis is thought to be caused by anxiety [104], and the thigmotaxis in general was reduced over the course of the learning phase (data not shown). As discussed in *Paper I* the Swe/PS1 mice over-all had higher levels of thigmotaxis compared to wild-type mice and the levels were not increased in parallel to the observed learning impairment compared to vehicle-treated mice. It is therefore unlikely that thigmotaxis and increased anxiety was the cause of learning impairment in the Swe/PS1 mice after chronic allopregnanolone elevation.

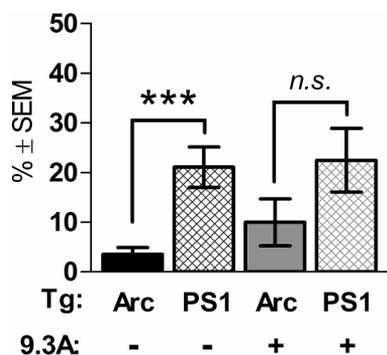


Figure 18. Possible increase in thigmotaxis in Swe/Arc mice to the level of that in Swe/PS1 mice. The figure shows the portion of time with thigmotaxis behaviour of Swe/Arc and Swe/PS1 mice in the MWM on the last day of the learning phase, and a comparison of genotypes. The mice were given vehicle or 9.3 nmol/h allopregnanolone (9.3A) during 4 weeks, from the age of 10 weeks. Data is shown as mean ± SEM. *** $p < 0.001$, n.s. $p > 0.05$.

Swe/Arc mice had significantly higher swim speed than the Swe/PS1 mice independent of treatment (data not shown). In cases where the swimming speed is different between the compared groups, the path travelled to find the platform can be more appropriate to investigate than the latency. Overall levels of floating were low (< 15%) and no differences in the amount of floating were found between the groups (data not shown).

Set-up of MWM

Several pilot studies were done in order to optimize the method and to establish a desired level of difficulty in the task. In summary it was found that the chosen MWM set-up enabled wild-type mice to satisfactorily learn the task with good memory performance with four days of training (Figure 19, data for memory performance not shown). The task was however still difficult enough to enable identification of any performance improvements. This was concluded as e.g. some individuals showed the task possible to complete in less than 10 seconds (compared to the mean of 31 seconds on the last day of the learning phase). When aged Swe/PS1 mice were introduced it was found that they required additional days of training to satisfactorily learn the task with reasonable memory performance, as they showed significantly poorer learning compared to both wild-type and Swe/Arc mice (Figure 19). Aged Swe/Arc mice performed at the same level as wild-type mice. Interestingly, the Swe/PS1 mice given allopregnanolone treatment showed equal or possibly even poorer learning compared to aged Swe/PS1 mice.

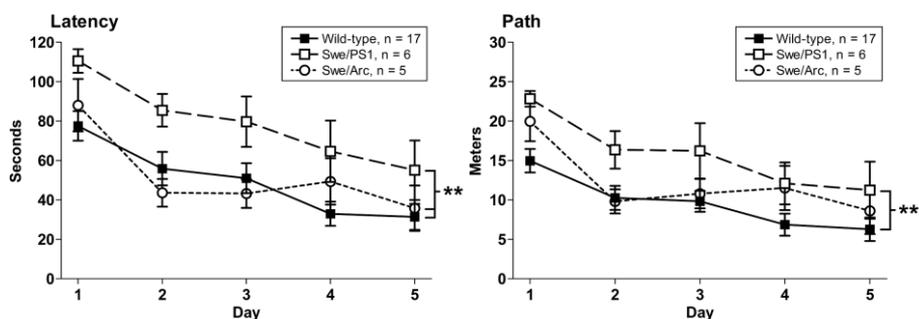


Figure 19. Learning performance. Aged un-treated mice were used to optimize the MWM method (un-published data). Swe/PS1 mice had significantly longer latency/ path to reach the platform, compared to both wild-type and compared to Swe/Arc mice (latency only). Wild-type female (n = 8) and male (n = 9) mice of 12 months, Swe/PS1 female (n = 3) and male (n = 3) mice of 12 months, and Swe/Arc male (n = 5) mice of 12-22 months of age were used. Data is shown as mean \pm SEM. ** $F_{\text{Latency}}(2, 25) = 5.172$; $p = 0.013$, and $F_{\text{Path}}(2, 25) = 5.493$; $p = 0.011$ respectively.

The aged Swe/PS1 mice had higher levels of thigmotaxis compared to the Swe/Arc and the wild-type mice (Figure 20). High level of thigmotaxis appears to be a phenotype of the Swe/PS1 mice and not of the Swe/Arc or wild-type mice. Increased thigmotaxis could be a sign of increased anxiety, which

would disturb the learning process. Interestingly, the chronic allopregnanolone treatment affected learning ability in Swe/PS1 mice but not the level of thigmotaxis (*Paper II*), and the Swe/Arc mice showed no obvious effect on thigmotaxis after allopregnanolone treatment. Therefore, increased anxiety is not likely the factor disrupting learning after chronic allopregnanolone treatment. No differences in floating were found between the genotypes (data not shown). The aged Swe/Arc mice differed from both wild-type and Swe/PS1 mice with higher swimming speed (Figure 20).

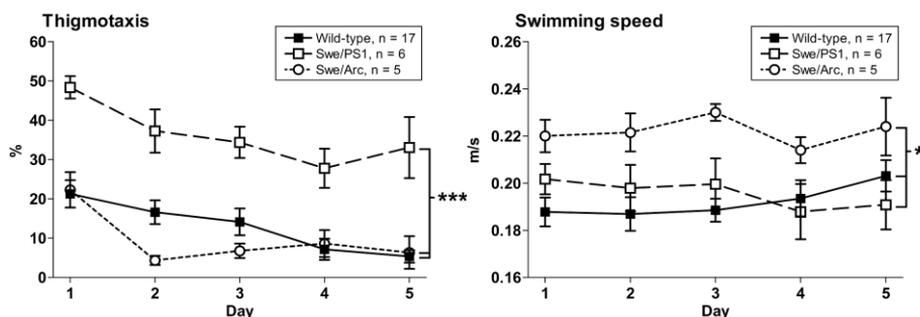


Figure 20. Thigmotaxis and swimming speed. Aged un-treated mice were used to optimize the MWM method (un-published data). Swe/PS1 mice performed with significantly more thigmotaxis, compared to both wild-type and Swe/Arc mice. Swe/Arc mice had significantly higher swimming speed, compared to both wild-type and Swe/PS1 mice. Data is shown as mean \pm S.E.M. See Fig. 19 for group compositions. *** $F(2, 25) = 17.302$; $p < 0.001$ and * $F(2, 25) = 4.710$; $p = 0.018$.

Some researchers prefer to opacify the water, because of the risk that the platform may be visible to the mice when swimming in clear water. The mice are however unlikely to see the platform unless they are actually on top of it. This is due to light reflexion on the water surface, the proximity of the mice to the surface, and the positioning of the mouse in the water (with most of the body submerged and head facing upwards). Still, an additional pilot study was carried out to investigate possible differences in water opacified with milk powder compared to clear. In summary, no statistical differences were found in task performance or behaviour when wild-type mice completed the MWM in opaque water and the same in clear water (data not shown). However, observational findings gave reason to believe that the mice were more troubled in the opaque water than in the clear water. They seemed to be deeper submerged into the opaque water when swimming compared to the clear water. A larger part of the body was soaked, and the

swimming seemed more strugglesome in the opaque water. In addition, the mice seemed to require longer recovery time. As no statistical differences in performance or behaviour were found and as the task was easier to perform with clear water (for both man and mouse), clear water was chosen for the experimental studies of *Paper I* and *II*.

Conclusions

The conclusions drawn based on the MWM performances discussed above is that chronic elevation of allopregnanolone levels impaired learning and memory in two different transgenic mouse models for AD, i.e. both Swe/PS1 and Swe/Arc mice, but with different onset of action. The Swe/Arc mouse model seems to be less affected by the disorder but to be more vulnerable to allopregnanolone influence. This result was seen long after the end of treatment and is therefore regarded as permanent damage. It is also concluded that this impairment in learning and memory is a sign of accelerated disease development as the effects were not seen in wild-type mice.

A β & amyloid plaques

A β is the main pathological marker of AD in Swe/PS1 and Swe/Arc mice with increasing levels with disease progression. Analysis of amyloid plaque load can to some extent be used as diagnostic tool but it has been shown to poorly correlate to symptom severity. Quantification of soluble A β has proven a much more reliable tool for this purpose. However, as AD progresses in transgenic AD mice both the levels of A β and the amyloid plaques increases, and can be used to determine the disease severity at a certain time point. The Swe/PS1 mice treated for three months were analyzed for levels of insoluble and soluble A β_{42} and A β_{40} , congophilic plaque load, and A β_{42} -specific plaque count. The Swe/PS1 mice treated for one month and the Swe/Arc mice were analyzed for soluble A β_{42} and A β_{40} .

Soluble A β

The levels of soluble A β correlated well with cognitive performance in transgenic mouse models for AD, as declining performance follow increasing A β levels [105]. Chronic allopregnanolone elevation led to increased levels of soluble A β in the Swe/PS1 mice (Figure 21, *Paper I* and *II*), which suggests that the disease development was accelerated in the allopregnanolone-treated mice. This was most evident in the hippocampus, and the increase was more substantial in female mice than in male. The levels of A β were in general higher in females than in males, which has been reported previously in transgenic mouse models [106, 107]. The reason for this is however un-

known. Interestingly, while the levels of $A\beta_{42}$ in hippocampus and cortex and $A\beta_{40}$ in hippocampus were two to four times higher in females than males the levels of $A\beta_{40}$ in cortex was equal between the sexes.

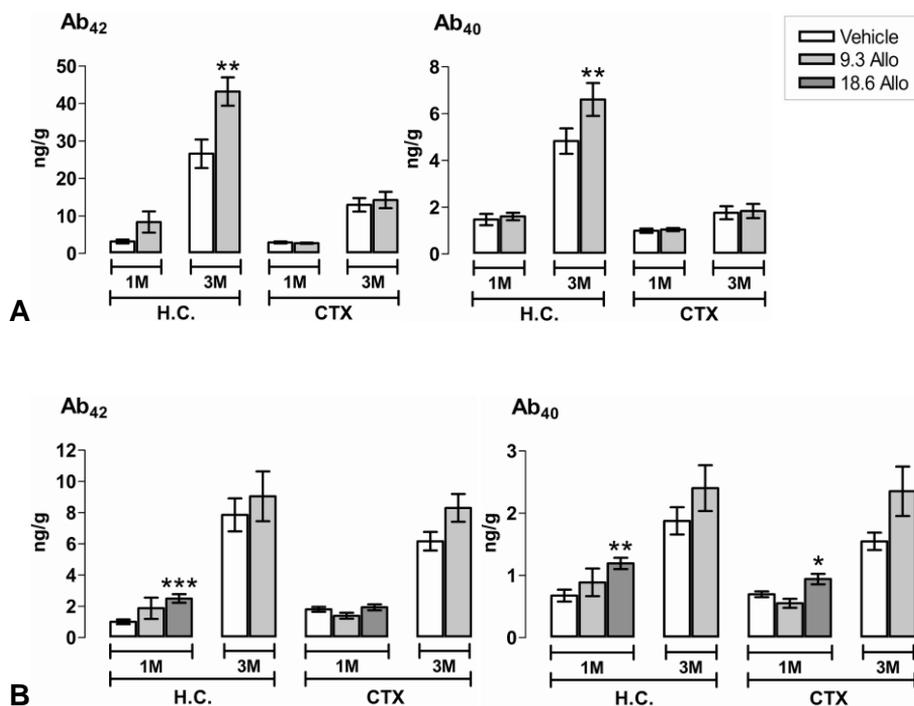


Figure 21. Increased levels of soluble $A\beta$ in Swe/PS1 mice after chronic allopregnanolone elevation. The figures show levels of soluble $A\beta_{42}$ and $A\beta_{40}$ in hippocampus (H.C.) and cortex (CTX) in female (A) and male (B) Swe/PS1 mice, respectively. The mice were treated with allopregnanolone (9.3 nmol/h; 9.3 Allo or 18.6 nmol/h; 18.6 Allo) or vehicle for 4 weeks (1M) and 12 weeks (3M) from the age of 10 weeks. The data are shown as mean per g wet tissue \pm S.E.M. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ vs. Vehicle.

In the Swe/Arc mice, the levels of soluble $A\beta$ were not affected by chronic allopregnanolone treatment for one month (Figure 22). Female and male Swe/Arc mice had equal levels of $A\beta$ in hippocampus. However, the female Swe/Arc mice had higher levels of soluble $A\beta_{42}$ and $A\beta_{40}$ in cortex. The levels of $A\beta_{42}$ in the Swe/Arc mice were lower compared to the Swe/PS1 mice. This is to be expected since the PS1 mutation of the Swe/PS1 mice leads to increased levels of $A\beta_{42}$ contra $A\beta_{40}$. However, as the levels of $A\beta_{40}$ were equal or moderately lower in the Swe/Arc mice compared to the Swe/PS1, the total

levels of soluble A β were significantly lower in the Swe/Arc mice. This indicates that the disease progress is more aggressive in the Swe/PS1 model compared to the Swe/Arc.

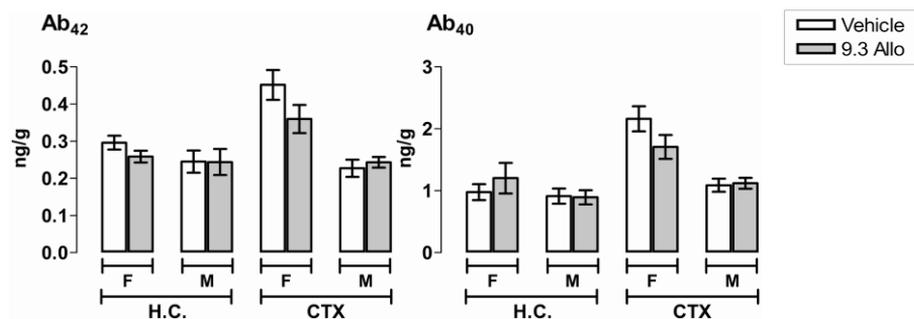


Figure 22. No effect on levels of soluble A β in Swe/Arc mice after chronic allopregnanolone elevation. The figures show levels of soluble A β_{42} and A β_{40} in hippocampus (H.C.) and cortex (CTX) in female (F) and male (M) Swe/Arc mice, respectively. The mice were treated with allopregnanolone (9.3 nmol/h; 9.3 Allo) or vehicle for 4 weeks from the age of 10 weeks. The data are shown as mean per g wet tissue \pm S.E.M.

Insoluble A β

No differences in the quantified levels of insoluble A β_{42} or A β_{40} were found in the Swe/PS1 mice after three months of chronic increase in allopregnanolone levels compared to vehicle (data not shown, *Paper I*), and the identified levels were comparable to those reported elsewhere [108]. As in the case of soluble A β , the female mice had two to three times higher levels of insoluble A β in all areas compared to the male mice, but the levels of insoluble A β_{40} in cortex, which were equal between the sexes.

Plaque load

There were no significant differences in congophilic plaque area or plaque count after chronic allopregnanolone treatment compared to vehicle (data not shown, *Paper III*). However, the levels of congophilic plaques, both area and count, were increased in poor learners. Congruently with the data for soluble and insoluble A β , the plaque load in hippocampus and cortex was higher in females than in males (data not shown, *Paper II*). However, this was only seen among the vehicle-treated mice. Among allopregnanolone-

treated mice the plaque load was equal between females and males. This indicates that the male and female mice were contrarily affected.

Plaque size

By dividing the total congophilic plaque area by plaque count the average plaque size was achieved (*Paper III*). In both male and female mice the congophilic plaque size was decreased in hippocampus (Figure 23). As neither plaque area nor plaque count was significantly altered after allopregnanolone treatment it is likely that both factors were important for the change in size. This would lead to more abundant but smaller plaques after chronic allopregnanolone treatment compared to vehicle. Previously, it was discussed that increased levels of soluble A β may lead to decreased plaque load. This could be the phenomenon seen here as the levels of soluble A β indeed were increased after chronic allopregnanolone treatment.

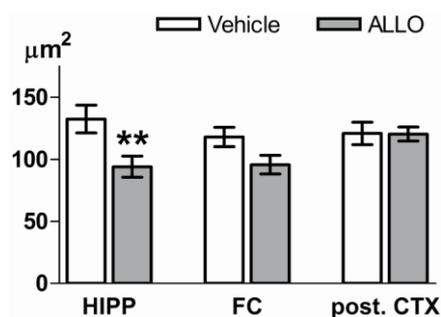


Figure 23. Decreased congophilic plaque size after chronic allopregnanolone elevation. The figure shows congophilic plaque size in hippocampus (HIPP, frontal cortex (FC), and posterior cortex (post. CTX; incl. occipital and parietal cortex) in Swe/PS1 mice. The mice were treated with allopregnanolone (9.3 nmol/h; ALLO) or vehicle for 12 weeks from the age of 10 weeks. The data are shown as mean \pm S.E.M. ** $p < 0.01$ vs. Vehicle.

A β_{42} -specific plaque count

No significant effects on the A β_{42} -specific plaque counts were identified after allopregnanolone treatment compared to vehicle in Swe/PS1 mice (data not shown). The levels were not statistically different between male and female mice and between learners and non-learners but appeared to be elevated among non-learners.

Conclusions

Signs of advanced pathology were identified in the Swe/PS1 mice as the levels of soluble A β were increased after chronic elevation of allopregnanolone levels. The levels of insoluble A β , the A β_{42} -specific plaque count

and the amyloid plaque load were unchanged. However, the distribution of plaques was changed with smaller but possibly more abundant plaques. This could be a sign of increased induction of plaque production leading to a higher number of affected neurons.

Synaptic function

Synaptophysin is a synaptic glycoprotein involved in the function of synaptic vesicles. While its exact function is unknown, knock-out mice lacking synaptophysin showed impaired learning in the MWM [109]. Synaptophysin is thus used as a marker for quantification of healthy synapses and for investigation of synaptic function. In AD patients, A β accumulation leads to synaptic dysfunction, which in turns lead to cognitive disturbance [10, 102]. Increasing levels of intraneuronal A β in transgenic AD mice decreases the levels of synaptophysin [82, 101].

Levels of synaptophysin

As an acceleration of AD in the Swe/PS1 mice (and the Swe/Arc mice) had been found, further investigation was performed to determine if the cognitive impairment was caused by decreased synaptic function. Therefore, the levels of synaptophysin were quantified in the Swe/PS1 mice treated with chronic allopregnanolone elevation for three months. The levels of synaptophysin appeared to be decreased after chronic allopregnanolone elevation compared to vehicle and also among non-learners compared to good learners (*Paper III*). As synaptic function is required for memory function and allopregnanolone-treated mice were more often non-learners one would expect that they would have decreased levels of synaptophysin. However, these decreases were not statistically significant and it could not be confirmed that the learning and memory impairments identified in the allopregnanolone-treated transgenic mice were caused by synaptic failure. It is possible that the levels of synaptophysin were indeed decreased after allopregnanolone treatment. However, the six week interval that was allowed after the end of treatment prior to tissue collection may have allowed synaptic recovery leading to restored levels of synaptophysin and loss of inter-group differences.

Conclusions

The levels of synaptophysin were not obviously altered after chronic elevation of allopregnanolone levels, but appeared to be decreased.

Correlations

In the Swe/PS1 mice, increased hippocampal levels of soluble and insoluble A β correlated to poorer memory performance (*Paper I*). This was expected, as A β levels have been shown to correlate with synaptic loss and cognitive decline previously [10, 11, 93]. The main conclusion from *Paper III* is that chronic allopregnanolone treatment caused disturbed brain pathology in cortex in male Swe/PS1 non-learners (Figure 24 and 25). The *natural* brain pathology of the male Swe/PS1 mice included positive correlations between the levels of insoluble A β , congophilic plaques and A β ₄₂-specific plaques. This was identified in the group of vehicle-treated male mice and also in the smaller group of vehicle-treated male learners. However, these pathologic markers did not correlate with synaptic function, and not with learning and memory performance in the group of vehicle-treated males, regardless of learning ability. In the brain pathology of the group of allopregnanolone-treated male non-learners, the levels of insoluble A β , congophilic plaques and A β ₄₂-specific plaques did not correlate. Interestingly, it was found that the levels of synaptophysin and insoluble A β became important factors correlating to learning performance. Higher levels of insoluble A β correlated to lower levels of synaptophysin. Unexpectedly, as synaptophysin is a marker for synaptic function, lower levels of synaptophysin correlated to better learning in the group of allopregnanolone-treated non-learners. However, the levels of synaptophysin within the group were somewhat lower compared to vehicle-treated, and therefore the correlation is unlikely the explanation for the differences in learning performance between allopregnanolone-treated and vehicle-treated. It rather shows signs of disturbed brain pathology. To my knowledge, these changes have not been identified previously.

Conclusions

The conclusion drawn from the above discussion regarding correlations is that an imbalance occurs in allopregnanolone-treated non-learners. The natural correlations between different forms of A β are lost while MWM performance, synaptic function and insoluble A β become interdependent.

Endogenous allopregnanolone levels

Late stage AD patients have decreased levels of allopregnanolone [44, 45], and altered expression of selected GABA_A receptor subunits compared to healthy controls [28, 29, 110]. Therefore, it is of great interest to investigate if these alterations also occur in transgenic AD mice. A minor pilot study was performed to quantify the endogenous levels in young and aged, female and male, wild-type and Swe/PS1 mice respectively.

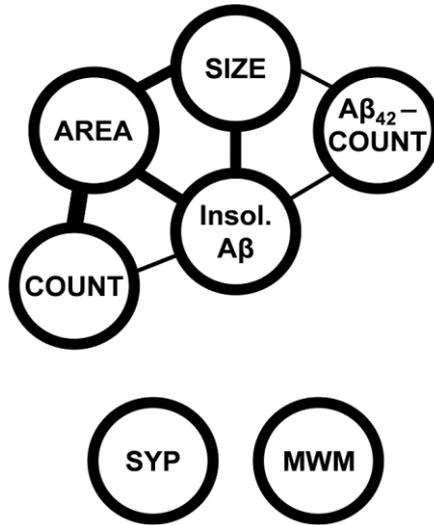


Figure 24. The natural brain pathology of male Swe/PS1 mice. Congophilic plaque load (AREA, COUNT, and SIZE) positively correlates to Aβ₄₂-specific plaque COUNT, and levels of insoluble Aβ₄₂ and Aβ₄₀ (Insol. Aβ). Levels of synaptophysin (SYP) and MWM performance do not correlate with any parameter. Lines show correlations, with thicker lines indicating higher statistical significance.

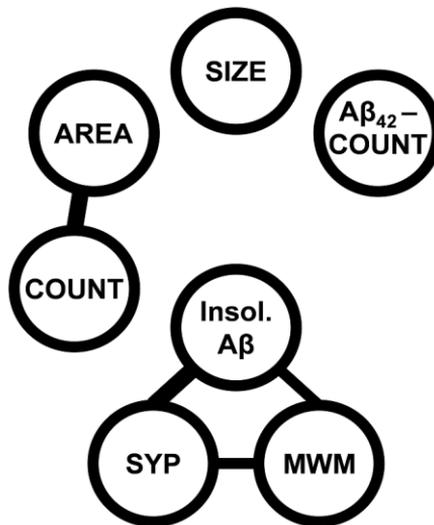


Figure 25. The disturbed brain pathology of male allopregnanolone-treated non-learners. MWM performance, levels of synaptophysin, and levels of insoluble Aβ₄₂ and Aβ₄₀ (Insol. Aβ) correlate with each other, but not with congophilic plaque load (AREA, COUNT, and SIZE) and Aβ₄₂-specific plaque COUNT. Lines show correlations, with thicker lines indicating higher statistical significance.

Base-line levels

It was found that aged mice have lower levels of allopregnanolone, regardless of genotype (Figure 26). This was seen in both males and females respectively (data not shown). The levels were over-all equal in Swe/PS1 mice compared to wild-type (data not shown), and equal or somewhat higher compared to previously reported brain levels [111-114]. The levels of allopregnanolone were higher in hippocampus compared to frontal cortex in all groups. Interestingly, aged Swe/PS1 females had higher levels compared to aged wild-type females in frontal cortex (data not shown). This difference was not seen in males alone but was still visible in the pooled groups in Figure 26. This would indicate that the Swe/PS1 mice have higher levels of allopregnanolone than wild-type mice at a stage of fully developed AD. This does not parallel to what has been found in AD patients [44, 45], but indicates an endogenous dysregulation of allopregnanolone.

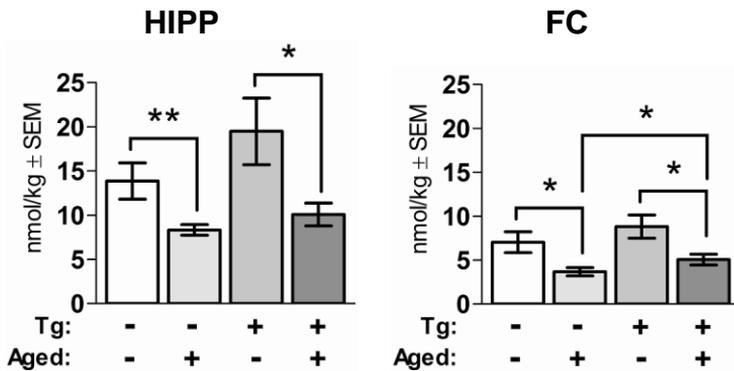


Figure 26. Endogenous allopregnanolone levels. The figures show endogenous allopregnanolone levels in hippocampus (HIPP) and frontal cortex (FC) in young (20 weeks) and aged (36 weeks), wild-type and Swe/PS1 mice (Tg) respectively. Group compositions from the left: Young wild-type ($n_{HIPP}=15$, $n_{FC}=14$), aged wild-type ($n_{HIPP}=13$, $n_{FC}=12$), young Swe/PS1 ($n_{HIPP}=11$, $n_{FC}=11$), and aged Swe/PS1 ($n_{HIPP}=12$, $n_{FC}=22$). ** $p < 0.01$, * $p < 0.05$.

Stress response

Previous reports show that allopregnanolone levels are increased at acute stress [32, 35]. Furthermore, it was shown that chronic stress may decrease allopregnanolone levels at base-line but increase the response to acute stress [34]. The stress response in transgenic AD mice has not been completely

investigated regarding allopregnanolone levels. Therefore, a pilot study was performed with the purpose of investigating the effect of stress on allopregnanolone levels in male Swe/PS1 mice compared to wild-type mice. It was found that the mice had increased levels of allopregnanolone after a period of chronic stress (Figure 27), with similar responses in wild-type and Swe/PS1 mice. This was seen after a mild chronic stress of daily intruder interactions using the R/I stress model. The increase in allopregnanolone levels caused by mild chronic stress was in similar magnitude compared to the levels achieved by the allopregnanolone treatment described in this thesis. Interestingly, after acute stress in chronically stressed mice the Swe/PS1 mice responded with even higher levels of allopregnanolone while the wild-type mice had unchanged levels. This would indicate that the presence of A β in the Swe/PS1 mice affected the stress-response system. Interestingly, AD patients were more sensitive to an HPA (hypothalamic-pituitary-adrenocortical axis) stimulation test leading to higher levels of cortisol compared to controls [44, 115]. This indicates that AD patients have affected stress-response system. The presented data suggests that transgenic AD mice have affected stress-response system, similar to that of AD patients.

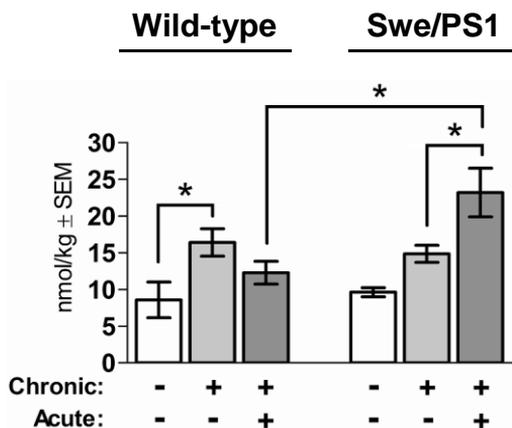


Figure 27. Endogenous allopregnanolone levels at stress. The figures show the endogenous allopregnanolone levels in whole brain hemisphere in young, male wild-type (WT) and Swe/PS1 (AD) mice respectively. Group compositions from the left: unstressed ($n_{WT}=4$, $n_{AD}=2$), chronically stressed ($n_{WT}=12$, $n_{AD}=11$), chronically and acutely stressed ($n_{WT}=10$, $n_{AD}=3$). * $p < 0.05$.

Conclusions

The main conclusions drawn from the discussions above is that the baseline allopregnanolone levels decrease with age in both Swe/PS1 and wild-type mice, but may be somewhat higher in the aged female Swe/PS1 mice compared to the wild-types. Furthermore, the acute stress response is altered after a period of chronic stress in Swe/PS1 mice compared to wild-type. This indicates a dysregulation of the stress response system due to the presence of A β .

Main conclusions

The main conclusions drawn from this thesis are as follows.

Chronically elevated allopregnanolone levels

- ✓ Cause **impaired learning and memory** performance in the Swe/PS1 and the Swe/Arc mouse models.
- ✓ Cause **increased levels of soluble A β** in the Swe/PS1 mouse model and not in the Swe/Arc.
- ✓ Cause **no obvious effect on plaque load and levels of insoluble A β** in the Swe/PS1 mouse model.
- ✓ Cause **altered plaque pattern** in the Swe/PS1 mouse model.
- ✓ Cause **disturbed correlations** between pathological markers of AD in the Swe/PS1 mouse model.
- ✓ **Increase the importance of insoluble A β levels for the synaptic function**, or vice versa, in the Swe/PS1 mouse model.

In addition,

- ✓ The **Swe/PS1 mouse model** displays a **more aggressive** AD progression than the Swe/Arc model.
- ✓ The **Swe/Arc mouse model** appears **more cognitively vulnerable** to the influence of elevated allopregnanolone levels than the Swe/PS1 model.

Based on the above, the major outcome of this thesis is that

Chronically elevated levels of allopregnanolone accelerate the disease progression in transgenic AD models.

General discussion & Implications

In this final section I discuss the main conclusions, drawn based on the findings presented in this thesis, and possible interpretations by presenting a hypothesis for the mechanism behind stress-induced AD. I discuss another, previously stated hypothesis for the effect of allopregnanolone on the development of AD as well as some strengths and weaknesses of *Paper I, II* and *III*. Finally, I discuss the clinical relevance of the presented findings.

The hypothesis of the mechanism behind stress-induced AD

In this thesis it has been shown that chronically elevated levels of allopregnanolone increased the levels of soluble A β , impaired learning and memory function and disrupted the *natural* relationships between pathological markers in transgenic mouse models for AD. This was not unexpected as allopregnanolone is a GABA_A receptor active stress steroid. Treatment with other GABA_A receptor active compounds had congruent effects in other studies [93, 94]. The modified amyloid cascade hypothesis focuses on the events prior to plaque formation, i.e. the intraneuronal pool of soluble A β monomers and oligomers. Disturbances in the amyloid cascade can be caused by altered levels of neuronal activity, i.e. neurotransmission [89, 90], as A β is released to the extracellular space in vesicles at depolarisation [91, 92].

Based on the above, the hypothesis of the mechanism behind stress-induced AD is presented as follows and described in Figure 28. In an unstressed state of mind, the GABAergic activity is set at a certain level which allows a certain level of general neurotransmission. The A β is along neurotransmission secreted to the extracellular space leading to lower intracellular concentrations. Lower intracellular concentrations of A β decreases the probability for production of A β -oligomers. At stress, the levels of allopregnanolone are increased, both via the adrenals and from production directly in the brain. Since allopregnanolone affects the general neurotransmission and intracellular concentration of A β depends on the level of neurotransmission, increased allopregnanolone levels may cause increased intracellular levels of soluble A β . In consequence, increased production of toxic A β oligomers may follow. This in turn leads to synaptic dysfunction and cognitive symptoms. This may be the scenario of the increased cognitive pathology identified in this thesis.

Another hypothesis for AD & allopregnanolone

Another hypothesis for how allopregnanolone can affect the development of AD has been proposed [116]. This hypothesis is one stating beneficial effects of allopregnanolone, as it can have regenerative effects on neural progenitor cells. Firstly, it was shown that allopregnanolone promoted neural progenitor cells in vitro [117]. Secondly, the effects in transgenic AD mice were investigated. It was found that developing neurons are alternatively affected by allopregnanolone compared to grown neurons, and that a one-time injection of allopregnanolone reversed the neurogenic and cognitive deficits in the 3xTg AD mouse model [118, 119]. Furthermore, it was shown

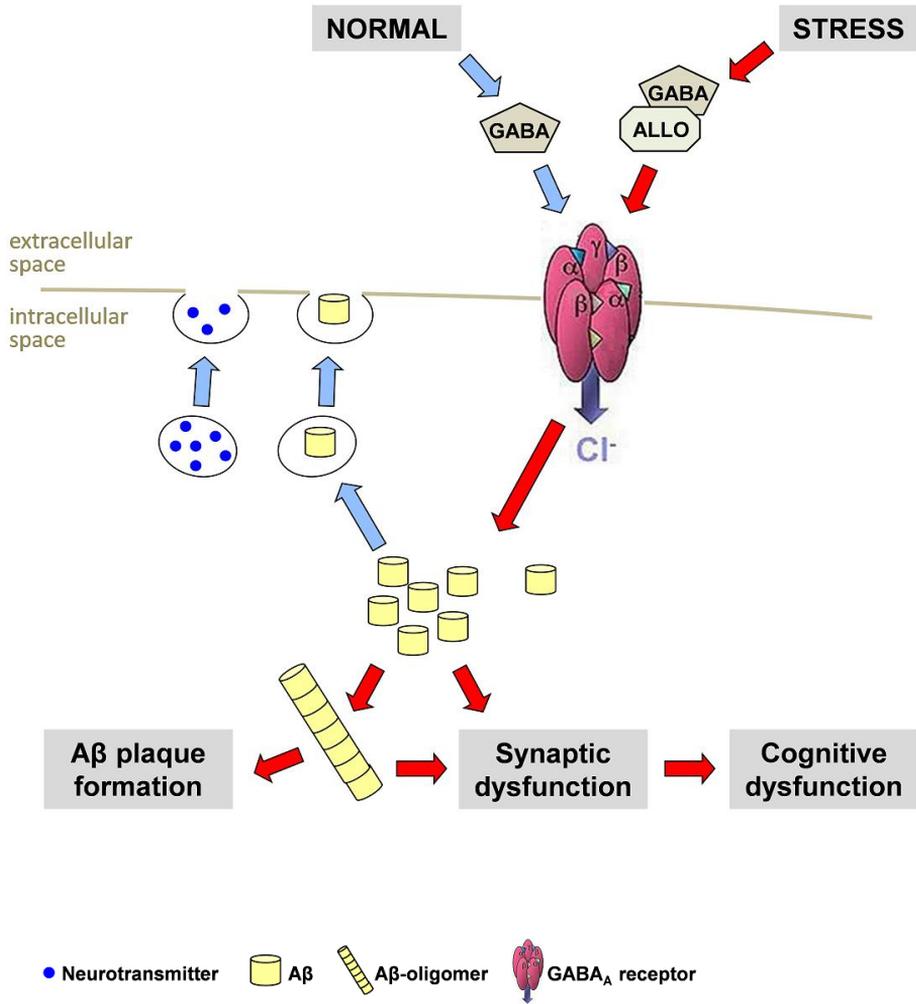


Figure 28. Model of the proposed mechanism behind stress-induced AD.

that one weekly injection of allopregnanolone led to proliferation of neural progenitor cells in hippocampus [120], and improved cognition in the 3xTg mice [121]. This mechanism is altogether a different aspect than that of chronic elevation of allopregnanolone. Interestingly, the positive effect of a weekly allopregnanolone injection was not seen with three weekly injections [120], which instead impaired neurogenesis compared to vehicle. This implies that the paradigm of three injections per week leads to chronic elevation of allopregnanolone, similar to the treatments described in this thesis. Possibly, this can lead towards an additional hypothesis of how chronically elevated levels of allopregnanolone accelerates AD in transgenic mice, i.e. via impairment of neurogenesis.

Strengths and limitations

Interpretations

In the discussed studies (*Paper I, II, and III*) the animals were chronically treated for one and three months respectively after which an interval of four weeks was allowed before the introduction of the MWM. With two weeks of MWM procedures, this led to an interval of six weeks before the tissues were sampled. This paradigm was used to ensure that the long-term effects and the indirect effects of the chronically elevated levels of allopregnanolone was studied and not the direct effects of the treatment [96]. However, it is possible that such a long interval allowed recovery of any disturbances caused by treatment. In such case, it is likely that the reported effects were even greater just after the end of treatment. One should also consider if withdrawal effects after treatment could have caused the presented results. While this could lead to an adjusted hypothesis, the reported effects would still remain.

In the current studies, the increased levels of A β and the impaired learning and memory function after chronic allopregnanolone treatment does not seem to occur in the same animals. Although both findings are signs of increased pathology in the animals, they are not obviously correlated. Furthermore, it is unlikely that soluble A β levels correspond to intracellular levels alone but rather the combined intra- and extracellular levels. Therefore, it cannot be confirmed that chronically increased allopregnanolone levels affect the intracellular A β levels. Intra-neuronal levels of A β have been found to cause synaptic dysfunction and cognitive decline [10, 85]. Still, soluble levels also correlate to disease severity [11, 12], and thus of relevance. Furthermore, it is possible that the reported effects were not due to changes in the distribution between intra- and extracellular compartments, but increased production of A β . While this is unlikely since the levels of insoluble

A β were unchanged, further analysis of e.g. the C-terminal fragment of APP could confirm or dismiss that possibility.

Well functioning synapses and thus high levels of synaptophysin are presumed to enable good learning performance. However, new synapses are created as new memories are consolidated. As the MWM is a task of learning and memory this ought to happen during the process, which probably leads to increased levels of synaptophysin. Good learners might therefore have higher levels of synaptophysin than poor learners after the MWM procedure, and not before. While this would say that chronic allopregnanolone treatment does not directly cause decreased levels of synaptophysin, it would also say that it leads to disruption of (an) other factor(s) important for learning, which in turn would lead to a predisposition of learning dysfunction. From the results of the included studies it is not possible to conclude which scenario is true.

Intra-group variability

In these studies it was found that some individuals were affected while others were not, which led to large variability within the groups. Large variability can hide effects by treatment. However, in the case of allopregnanolone this variability is of special interest. As the effect of allopregnanolone is biphasic with increasing levels, and as the sensitivity to allopregnanolone can vary, the effects of chronically altered allopregnanolone levels could possibly differ greatly between individuals. The conclusion is that in some cases the group sizes were too small to be further split into e.g. responders and non-responders. Still, statistically significant differences were found, but it is possible that other effects were not discovered.

Relevance of the chosen transgenic models

Both the Swe/PS1 and the Swe/Arc mouse model were affected by chronically elevated levels of allopregnanolone, by acceleration of the disease development. Therefore, it can be concluded that both models were relevant for the aimed investigations. It can also be concluded that they responded somewhat differently. This was mainly seen in different responses by female and male mice. Female and male Swe/Arc mice showed equal levels of soluble A β and performed equally in the MWM, after allopregnanolone and vehicle treatment respectively. Female and male Swe/PS1 mice were different and responded differently to treatment. They had not equal levels of A β , their MWM performance was somewhat un-equal in the vehicle-treated group, and most importantly they responded differently to allopregnanolone

treatment. Sex differences have been reported before [106, 107], but the reasons behind these variations are unknown.

It was also evident that the Swe/PS1 mouse model develops cognitive impairment at an earlier age than the Swe/Arc. This has importance for which stage of the disease development that is aimed to investigate. The aim of this thesis was to investigate the effects of allopregnanolone treatment at an early stage, prior to cognitive symptoms. As the Swe/PS1 develops AD more aggressively at an early age and as it is beneficial to include adult mice only, it could be that the Swe/Arc mouse model is more relevant than the Swe/PS1 for this purpose using the described study regime.

One could argue that the use of transgenic mouse models that develop hereditary AD, rather than spontaneous AD, is of poor use when you want to study in fact spontaneous AD. It is of course important to remember this when discussing the results from these studies. Still, one has to assume that the pathogenesis of the two forms of AD is similar (until if/when proven otherwise). Therefore, studies of this kind ought to be relevant. However, it would also be of interest to investigate the effect of chronically elevated levels of allopregnanolone in a model for sporadic AD. As rodents do not develop AD naturally, such models are not as easily available. Still, it is strengthening that similar results have been achieved in two separate mouse models with different pathology. Unfortunately, the Swe/Arc model was not as thoroughly investigated as the Swe/PS1 model. It would be of interest to study the effect of chronic allopregnanolone treatment on the plaque production, levels of insoluble A β , and synaptic function in the Swe/Arc mice as well.

Ethical considerations

All studies included in this thesis have been approved by the animal ethics committee in Umea, Sweden, and all experiments were conducted according to the general guidelines by the Swedish Board of Agriculture (Jordbruksverket).

When conducting research including animal experiment one is to consider “the principle of the 3Rs”, including the topics *Replace*, *Reduce* and *Refine*. In Table 4 follows further description of “the 3Rs”, and how it was taken into consideration within the scope of this thesis.

Replacement	
If possible one is to replace the use of animals (living vertebrates) in experimental research.	The experiments included in this thesis were performed to investigate behavioural aspects, and also histological aspects. Therefore, the use of an animal model is required. The alternatives of cultured cells, donated organs, and lower species like invertebrates would not be sufficient for this purpose.
Reduction	
One is to reduce the number of animals used in experimental research to a minimum.	The aim was to keep the number of animals at a minimum. Appropriate group sizes were estimated based on the knowledge gained from previous experiments within the research team, from the published literature, and from pilot studies. It is always a narrow line as to how many animals are the minimum but still enough. If the group sizes are not large enough the whole experiment is unnecessarily done and the number of animals certainly not reduced. I believe the group sizes in these studies were sufficient in most, but unfortunately to small in some cases.
Refinement	
One is to improve one's scientific procedures to minimize the suffering of the animals.	<ul style="list-style-type: none"> • All operators handling the animals had sufficient training for the task. • Animals lived in groups with siblings as far as possible and also separated in cases of bullying. The animals were provided with food and water <i>ad libitum</i> and also nesting materials. Daily monitoring ensured the well-being of the animals. • Animals were handled to become habituated to novel situations and tasks. • Pilot studies were performed with small group sizes to refine the methods prior to the use of larger groups. • Proper use of anaesthetics and pain-relief ensured minimal suffering.

Table 4. The principle of the 3Rs. Descriptions and taken considerations.

Clinical relevance

In this thesis I have discussed evidence pointing towards enhanced development of AD in transgenic mice due to chronic exposure to elevated allopregnanolone levels, corresponding to that of mild stress. One can question if the present findings has any relevance for human AD development. Chronic stress seems to be an important risk factor for dementia development and/ or cognitive dysfunction [15, 16, 18], and allopregnanolone levels are increased during stress [32-35]. Several exogenous compounds are active on the GABA_A receptor, e.g. benzodiazepines, MPA, ethanol, and are frequently used in long-term clinical treatment or abuse. Negative effects on

cognition was persistent after long-term benzodiazepine treatment [47], and 5 years of treatment with MPA doubled the frequency of dementia in postmenopausal women [52]. Interestingly, MPA not only have similar effect on the GABA_A receptor as allopregnanolone but MPA treatment also increases the brain levels of allopregnanolone [122]. Of course, mice and humans are not the same species. However, there are many similarities between the two and while results on mice cannot be directly transferred to humans they can still indicate a possible connection. The degree of disease acceleration observed in this thesis was severe as otherwise cognitively intact AD mice suffered loss of memory function. In human AD, this could be the difference between living self-sufficiently at home and living with the requirements of professional care.

The presented findings indicate that dysregulation of endogenous neurosteroids is an important factor behind stress-induced AD. This is a novel approach to the dementia research. Therefore, further studies are required to expand our understanding regarding allopregnanolone and other GABA_A receptor active stress and sex steroids as links in the mechanism behind stress-induced AD.

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