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Biochemical Bases of Memory Disorders in Mouse and Rat Models

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DEDICATED TO

MY BELOVED PARENTS,

MY LOVELY HUSBAND

AND DAUGHTERS JWANA AND JULIA

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II. Acknowledgements

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III. Abstract

The determination of the biochemical basis for memory and learning is important for understanding the mechanisms that underlie neuronal and behavioral plasticity in health and disease conditions and to explore novel therapeutic strategies. The present work uses a mixed approach, combining pharmacological and genetic tools to investigate biochemical processes possibly involved in learning and memory. In the first set of experiments we focused on the role of amyloid beta (Aβ) dimer on learning and memory, attention and emotional processes. Aβ oligomers have been implicated as a key pathogenic factor in Alzheimer's disease, preceding the pathogenic Aβ soluble peptide aggregation. We investigated the impact of soluble Aβ dimer on behavior in wild-type and transgenic mice that express an Aβ-S8C mutation. In mice the Aβ-S8C mutation resulted in the generation of soluble synaptotoxic Aβ dimers (tgDimer). Interestingly, no significant monomer formation was observed. Aβ-S8C mutants showed no Aβ plaque formation, astrogliosis, and neuroinflammation. Animals were tested at the age of 7 and 12 months in a behavioral test battery that included the assessment of emotional behaviors as well as learning and memory formation. Also, hypothalamus-pituitary axis involvement was assessed by determining blood corticosterone levels after restraint stress. Additional tgDimer mutant and wild-type mice were used to determine post-mortem brain monoamine and acetylcholine neurotransmitters and metabolite concentrations via high-performance liquid chromatography (HPLC).

Findings from this study pointed to an Aβ dimer involvement in deficits related to emotion, attention, memory processes as well as motor- and reward-related learning. Additionally, Aβ dimer formation disturbed neurochemical homeostasis, by slowing serotonin turnover rate in the ventral striatum, amygdala and hippocampus.

Furthermore, age-related reductions of acetylcholine and dopamine levels were observed in the hippocampus of mice expressing Aβ dimers.

It has been hypothesized that neurosteroids play a role in the organization of behavior. In the second set of experiments we examined cognitive and emotional effects of intranasal (IN) applications of the endogenous neurosteroid pregnenolone (PREG) in rats. IN-PREG did not alter goal-oriented performance in the Morris water-maze (MWM). However, a promnestic effect was found in spontaneous object exploration tasks. Here, long-term memory, tested 48h after the sample trial, was facilitated in both novel object and object-location preference tests. Also, IN-PREG increased the resistance to behavioral extinction in the MWM during multiple non-reinforced extinction trials. IN-PREG treated animals spent more time searching in the former platform quadrant and crossed the former platform location more often than controls, reflecting either a higher resistance to extinction, a superior memory for the former location of the escape platform, or both. Also, IN-PREG treated animals reversed the amnestic effect of systemic anticholinergic scopolamine administration. Neither anxiolytic nor anxiogenic behavior was exhibited by IN-PREG treated rats, as compared to controls, in the elevated plus-maze task. Our findings suggest that IN-PREG can modulate learning and memory processes in rats. Importantly, the nasal route of administration may circumvent the adverse systemic effects of PREG. Hence, IN-PREG, in addition to conventional medication, holds a potential for the management and possible treatment of neurodegenerative health conditions that affect learning performance and memory processes.

IV. Zusammenfassung

Die Klärung der biochemischen Grundlagen lern- und gedächtnisassoziierter Prozesse stellt eine entscheidende Voraussetzung für ein genaueres Verständnis dabei beteiligter Mechanismen im Zusammenhang mit neuronaler und Verhaltensplastizität unter sowohl physiologischen, als insbesondere auch pathologischen Konditionen dar. Dieses wiederum ist Voraussetzung für die Entwicklung neuartiger therapeutischer Ansätze. Die vorliegende Arbeit bedient sich dabei eines kombinierten Ansatzes aus pharmakologischen und genetischen Ansätze um biochemische Grundlagen von lern- und gedächtnisassoziierten Prozessen näher zu betrachten. In einer ersten Reihe von Experimenten haben wir die Rolle von Amyloid-Beta-Dimer (Aβ) in Lern- und Gedächtnisprozessen, sowie auch Aufmerksamkeits- und emotionalen Funktionen betrachtet. Aβ-Oligomere werden als entscheidender pathogener Faktor in der Alzheimer'schen Krankheit betrachtet, die der pathologische Formierung von Aβ-Peptiden voraus gehen. Wir untersuchten dabei den Einfluss von löslichen Aβ-Dimeren auf Verhaltensparameter in Wildtypen und transgenen Mäusen, die eine Aβ-S8C Mutation expremieren. In diesem Modell führte die Aβ-S8C Mutation zu einem Aufkommen löslicher synaptotoxischer Aβ Dimere (tgDimer). Es wurden dabei keine Formierungen von Monomeren beobachtet. Aβ-S8C Mutanten zeigten keine Aβ Plague-Formierungen, Astrogliose oder inflammatorische Prozesse im Nervengewebe. Das Verhalten der Tiere wurde im Alter von sieben und zwölf Monaten mittels einer Testbatterie erfasst, die die Erhebung emotionsassoziierter Verhaltensweisen, sowie auch Lern- und Gedächtnisprozesse umfasste. Ferner wurde auch die Beteiligung der Hypothalamus-Nebennierenrinde-Achse über die Messung des Kortikosteron-Spiegels im Blut nach mechanischer Fixierung erhoben. In zusätzlichen Untersuchungen wurden post-mortem auch die intracerebralen Spiegel monoaminerger und acetylcholinerger

Neurotransmitter und deren Metabolite mittels Hochdruckflüssigkeitschromatographie erhoben (HPLC).

Die Ergebnisse dieser Studie deuten auf eine Beteiligung von Aβ-Dimeren hinsichtlich Einschränkungen von emotionalen, aufmerksamkeitsbezogenen, gedächtnisassoziierten, sowie auch motorischen Funktionen und dem Verstärkungslernen hin. Zusätzlich konnte gezeigt werden, dass die Formierung von Aβ-Dimeren sich ungünstig auf die neurochemische Homöostase auswirkt. So wurde eine Reduktion des Stoffwechsels von Serotonin im ventralen Striatum, der Amygdala und dem Hippocampus beobachtet. Desweiteren wurde auch ein altersassoziierter Rückgang der Konzentration an Acetylcholin und Dopamin im Hippocampus von Aβ-Dimere expremierenden Mäusen beobachtet.

Es wird angenommen, dass Neurosteroide eine wichtige Rolle in der Steuerung von Verhalten spielen. In einer zweiten Reihe von Experimenten haben wir die Effekte intranasaler Applikation (IN) des endogenen Neurosteroids Pregnenolon (PREG) auf kognitive und emotionale Funktionen in Ratten untersucht. IN-PREG wirkte sich dabei nicht auf zielgerichtetes Verhalten im Morris water-maze (MWM) aus. Ein promnestischer Effekt konnte allerdings mit Objektexplorationsaufgaben beobachtet werden. Dabei zeigte sich, dass die Langzeitgedächtnisbildung nach einem 48-stündigem Intervall positiv beeinflusst werden konnte. Dies betraf sowohl die Objekterkennung, als auch Informationen über die räumliche Position. Ferner wurde beobachtet, dass IN-PREG die Widerständigkeit gegenüber Extinktion im MWM über wiederholte Durchgänge der nicht-Verstärkung erhöht. IN-PREG-behandelte Tire verbrachten mehr Zeit mit der Suche in dem Quadranten, in dem sich vormals die Plattform befunden. Dies mag entweder auf eine höhere Widerständigkeit gegenüber Extinktionsdurchgängen

hinweisen, auf eine bessere Gedächtnisleistung oder aber auf beides. Desweiteren konnten positive Effekte auf die Gedächtnisbildung gegenüber systemischer anticholinerger Behandlung (Scopolamin) beobachet werden. Es wurden keine anxiogenen oder anxiolytischen Effekte von IN-PREG im elevated plus maze festgestellt. Unsere Beobachtungen deuten darauf hin, dass die Behandlung mit IN-PREG Lern- und Gedächtnisprozesse in Ratten beeinflussen kann. Es ist dabei hervorzuheben, dass die intranasale Verabreichung mögliche Nebenwirkungen einer systemischen Behandlung zu umgehen vermag. Folglich bietet die Erweiterung um eine IN-PREG Verabreichung ein Potential in der Behandlung neurodegenerativer Erkrankungen, die mit Beeinträchtigungen in Lern- und Gedächtnisprozessen einher gehen.

V. Abbreviations

Aβ	Amyloid beta peptide
Ach	acetylcholine
AD	Alzheimer's disease
APP	Amyloid precursor protein
BBB	blood-brain-barrier
Ch	Choline
CNS	central nervous system
CPP	conditioned place preference
DA	dopamin
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulfate
DOPAC	3,4-dihydroxyphenylacetic acid
EC	electrochemical detection
EPM	elevated plus maze
FST	force swimming test
5-HIAA	5-hydroxyindoleacetic acid
HPLC	high-performance liquid chromatography
5-HT	serotonin

5-HT1B	5-hydroxytryptamine receptor 1B
HVA	homovanillic acid
i.p.	intraperitoneal
IN	intranasal
LTP	long term potentiation
MID	multi infract dementia
min	minutes
MPTP	1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine
MTD	maximal trial duration
MWM	morris water maze
NMDA	*N*-methyl-D-aspartate
NOR	novel object recognition
OFT	open field test
OPP	object place preference
PREG	pregnenolone
PREGS	pregnenolone sulfate
RAM	radial arm maze
σ receptor	sigma receptors
Swe	swedish mutation (APP K670N/M671L)

Tg	transgenic
WT	wild type

1. Introduction

The determination of the biochemical foundations of learning and memory processes is one of the most ambitious and complex issues in neuroscience. One way of addressing this problem follows a reductionistic approach that scrutinizes the involvement of pathogenic entities or endogenous substances in the quest for the foundations of functional and dysfunctional forms of learning and memory. Combining behavioral analysis with neurotransmitter assessments allows the elucidation of molecular mechanisms that modulate learning and memory processes. In the present series of studies, pharmacologic and genetic manipulations were used to identify mechanisms that are involved in learning and memory performance. In Part I we investigated whether brain expression of amyloid beta (Aβ) dimer, a neurophatological hallmark of Alzheimer's disease (AD) has an impact on brain neurotransmitters levels and attentional, emotional and learning/memory processes. We used the transgenic tgDimer mouse model with Aβ-S8C mutation that expresses a high structurally stable amount of Aβ dimers with no other AD hallmarks emerging throughout their lifespan. Hence, this transgenic mouse allows investigating the mechanisms underlying the effects of chronic high levels of soluble Aβ dimer in pathological processes associate to AD.

In Part II, we investigated the effects of intranasal (IN) application of the neurosteroid pregnenolone (PREG) on learning and memory in rats. This endogenous neurosteroid is considered to be an intermediate substance in the neurosteroid biosynthesis

cascade, originating from cholesterol in neuronal tissues. Even though little is known about the activity of PREG as a neuro-active steroid by itself, former studies have provided evidence for its modulatory effect on several neurotransmitter systems associated with mood control, learning and memory processes (Ducharme et al., 2010; Flood et al., 1992; Zheng, 2009; Zorumski et al., 2013).

1.1. Part I. Amyloid beta dimers modulate emotional, attentional and learning / memory processes, and serotonin homeostasis

AD is a devastating neurodegenerative disease with increasing incidence and without a cure available. Recent literature has emphasized the role of soluble oligomeric Aβ as the primary driver of Alzheimer's disease (Cleary et al., 2005; Dickson et al., 1995; Haass and Selkoe, 2007; Krafft and Klein, 2010; Terry et al., 1991), presumably by promoting synaptotoxicity (Roselli et al., 2005; Shankar et al., 2007) and cognitive dysfunctions (Cleary et al., 2005; Maurice et al., 1996; McDonald et al., 1994; Nitta et al., 1994; Stéphan et al., 2001), even prior to neuronal loss (Cleary et al., 2005; Irizarry et al., 1997a, 1997b). Aβ peptide results from sequential steps of proteolytic processing of amyloid precursor protein (APP), a trans-membrane protein with large extracellular domains. APP undergoes sequential cleavages first by β-secretase cleaving enzyme, then by γ-secretase in order to generate Aβ peptides (O'Brien and Wong, 2011). Alternatively, APP cleavage can occur within the Aβ domain by the action of the

enzyme α-secretase, that generates soluble APPα and, therefore, prevents Aβ agglomeration (Choy et al., 2012).

Although extensive clinical and pre-clinical research has been performed, so far no effective therapies have been developed. This, coupled with an increase in AD prevalence, has led to a sharp escalation in the costs associated with patient care (Brodaty et al., 2011). There is an urgent need for a further understanding of the molecular mechanisms and pathways underlying AD pathology as a critical step towards intensified scrutiny of what should be targeted. Ideally, AD medication should ameliorate and perhaps restore neuronal and behavioral deficits in patients. In the last decade, soluble amyloid-beta has emerged as an important pathological factor in AD. It is highly synaptotoxic and very pervasive in the brain of AD patients (Klyubin et al., 2008; Mc Donald et al., 2010; Shankar et al., 2008). The formation of extracellular Aβ deposits was found to constitute a protective response, since it transforms toxic soluble Aβ oligomers into more stable aggregations (Cheng et al., 2007; Cohen et al., 2006; Treusch et al., 2009; Watson et al., 2005). However, despite contemporary failures in clinical trials targeting soluble Aβ, the Aβ–AD linkage is undeniable and supported by a large number of studies (Ferreira and Klein, 2011; Ondrejcak et al., 2010; Roychaudhuri et al., 2009; Sakono and Zako, 2010; Selkoe, 2001; Walsh and Selkoe, 2007) . The existing literature point out that the identification and

selective targeting of soluble and insoluble pools of Aβ peptides holds a significant potential for identifying a successful new therapeutic approach (Goure et al., 2014).

However, much of the current understanding of the synaptic Aβ is derived from *in vitro* studies, which use soluble Aβ concentrations greater than that exhibited by *in vivo*. Transgenic animal model have been criticized on issues such as creating proper Aβ *in vivo* concentrations, isoform variation of soluble Aβ, different Aβ deposit populations, and existent equilibrium between various multimer and conformer pools, as well as the presence of senile plaques and neurofibrillary tangles which always emerge at a certain point in the lifetime of the transgenic animals. Thus, so far, the examination of the biological effects of oligomers vs. plaques has been difficult because of their simultaneous occurrence.

Alternatively, exogenous intracerebral Aβ infusion has been performed to assess the impact of soluble Aβ oligomers on behavior and brain neurochemistry (Colaianna et al., 2010; Farzampour et al., 2016; Jean et al., 2015; Kincheski et al., 2017; Knight et al., 2016). However, these studies faced several limitations. The efficacy of the approach depends on the injection site and the specific peptide fragment injected. The various soluble Aβ isoforms, including Aβ(1-40), Aβ(1-42), Aβ(1-43), and many N-terminal truncated

isoforms vary in terms of neurotoxicity (Chambon et al., 2011; Goure et al., 2014). Also, the exogenous Aβ infusions, as performed so far, merely modeled the acute effect of soluble Aβ, thus being inappropriate to delineate the chronic effects of Aβ.

The transgenic Aβ-S8C (tgDimer) mouse expresses a high concentration of structurally stable Aβ dimers, but not monomers. No other AD hallmarks like insoluble amyloid plaques emerged at any time point across their lifespan. Thus, the molecular development of the tgDimer mouse was a major breakthrough in the study of Alzheimer's disease (Müller-Schiffmann et al., 2011). In order to selectively investigate the oligomerization and toxicity characteristics of structurally stable amyloid beta (Aβ) dimer, The Aβ polypeptide chain was linked by a disulfide bridge that was incorporated into the (N-terminus) by the introduction of cysteine amino acids (Cleary et al., 2005; Klyubin et al., 2005; Müller-Schiffmann et al., 2011). This resulted in neurotoxic Aβ dimer secretion, without affecting the trafficking and processing of the amyloid precursor protein (APP) by the cellular secretases (Müller-Schiffmann et al., 2011). Hence, this mouse model seems to mimics early stages of Alzheimer's disease and allows the investigation of mechanisms underlying the chronic effects of soluble Aβ dimers.

1.2. Part II. Promnestic effects of intranasally applied pregnenolone in rats

Neurosteroids refer to a subclass of endogenous steroids that can be synthesized in the central nervous system. Pregnenolone (PREG) is a naturally occurring endogenous steroid synthesized by the action of P450scc (Cholesterol Side-Chain Cleavage Enzyme) upon cholesterol. PREG is considered an intermediate substance in the neuroactive steroid biosynthesis pathway. It converts into different active metabolites, such as allopregnanolone and dehydroepiandrosterone (DHEA) (Hu et al., 2002; Sierra, 2004), but also into other steroidal hormones such as androgens, progesterone, and cortisol (Leão and Esteves, 2014). These active metabolites are capable of modulating neuronal activity (Baulieu et al., 2001; Porcu et al., 2009). Many studies have, however, demonstrated that, by itself, PREG is effective only as an active neurosteroid (Flood et al., 1992; Vallée et al., 1997; Zheng, 2009; Zorumski et al., 2013). Neuroactive steroids have been claimed to have specific roles in normal or pathological brain functions including motor skills, cognition, motivation, emotion, anxiety, depression, and traumatic brain injury (Dineley et al., 2002; Eser et al., 2008; Farr et al., 1995; Flood et al., 1992; Morley et al., 1984; Stein, 2001) . The decline of neuroactive steroids during aging and in AD is well established (Baulieu et al., 2001; Schumacher et al., 2003; Vallée et al., 2001; Weill-Engerer et al., 2002). Neuroactive steroids are considered to have a potential in the treatment for AD patients and corresponding clinical trials have been initiated

recently. The consequences of systemic PREG administration on different cognitive processes and mood regulation have been established by many of studies (Flood et al., 1992; Vallée et al., 1997; Zheng, 2009; Zorumski et al., 2013; Ducharme et al., 2010; Plescia et al., 2014). There is a risk of numerous steroid-related adverse effects such as irritability, anger, anxiety and weight gain with systemic PREG administration. Consequently, even if their use in ameliorating cognitive deficits in some neurodegeneration disorders is justified (Marx et al., 2009; Wong et al., 2012), their prophylactic administration is not recommended. Thus, a safer alternative with fewer side effects would be welcome. Beneficial effects of PREG applied via the intranasal (IN) route on active avoidance learning (due to its actions on neuronal activity and processing in different brain regions that are important for promoting learning and memory, including the hippocampus) have already been described (Ducharme et al., 2010). In this study we investigated the effects of IN PREG administration on various other aspects of learning and memory in rats using different behavioral paradigms.

2. Methods Overview

In the following, a brief description of the methods and experimental approaches used in this dissertation are given. For more details, the reader can refer to the methods sections of the published/submitted studies in the appendix.

2.1. Part I. Amyloid beta dimers modulate emotional, attentional and learning / memory processes, and serotonin homeostasis

2.1.1. Behavioral Paradigms

Morris Water Maze

The Morris water maze (MWM) task is based on rodents' ability to perform flexible goal-oriented learning (Morris, 1984). The hippocampus has been identified as a crucial brain structure for spatial learning and MWM-performance. When tested in MWM, animals with compromised hippocampal functions were impaired in spatial learning and memory compared to controls (Morris et al., 1982; Stewart et al., 2011). In the present experiment, tgDimer and WT controls were tested with an identical MWM protocol at 7 and 12 months of age. MWM testing started with one habituation trial (Fig.1) with 90-second duration without a platform in order to estimate swimming performance before the assessment of spatial learning in the hidden platform version of the task. Habituation was followed by 9 days of acquisition learning to escape onto the submerged platform (4 trials per day; 90 seconds inter trial interval) placed in the center

of a randomly chosen quadrant, which was maintained throughout the acquisition trials (Fig.1). At the beginning of an acquisition trial, the animal was placed in the pool, facing the wall at one of the four possible starting points. T animal was allowed to remain on the platform for 30 seconds. The day after the last acquisition day, a single "probe-trial" without a platform was run to test the animal's spatial memory for the platform location. To check for general sensory-motor abilities, the mice were tested in a "cued platform task" one day later, with a visible platform. The platform position was alternated for the cued trials and made visible by a metal cylinder painted with black and white stripes placed on the platform. The trial was completed when the mouse escaped to the cued platform or when the MTD had elapsed, after which the animal was guided to the platform (Bromley-Brits et al., 2011). Once on the platform, the animal was allowed to remain there for another 30 seconds.

The measures extracted for each acquisition trial were: time taken to escape to the submerged platform and the duration of thigmotactic swimming along the periphery of the maze via computer-based tracking software (Ethovision X® 8, Noldus, The Netherlands). In the probe trial, the time spent within the previously rewarded platform location and thigmotactic swimming was assessed. The time to reach the cued escape platform was scored in two trials.

Open-Field Test

Open field test (OFT) is a behavioral assay that relies on spontaneous behavior of rodents and was first applied by Walsh and Cummins, (1976). In this test, locomotor activity, the distance moved within the arena and the time spent in different parts of the arena are measured. The time spent and distance moved in the center and the corners/periphery of the open field are considered as indicators of unconditioned fear or anxiety (Prut and Belzung, 2003). The OFT was also used as a measure of selective and nonselective attention (Ruocco et al., 2014). The duration and frequency of rearings within the OFT were used as measures of attention (Aspide et al., 2000). A video-image analysis system (EthoVision X® 8.0, Noldus, Netherlands) was also used to analyze rearing, locomotion, and thigmotactic behavior.

Elevated Plus-Maze

A more reliable measure of fear/anxiety is the elevated plus-maze (EPM) (Denenberg, 1969; Ennaceur, 2014; Stanford, 2007;Ramos, 2008), a widely used paradigm to measure unconditioned anxiety-related and fear-related behaviors in rodents (Graeff et al., 1993). It is a one-trial task in which animals are placed into the center of a plus-shaped apparatus elevated above the floor, with 2 open and 2 walled arms, facing an open arm and their behavior is tracked for 5 minutes. The time spent and number of entries to the open and walled arms were assessed (Walf and Frye, 2007). In addition, more specific

ethological readouts reflecting anxiety and fearful behavior were measured. These readouts included "risk assessment" measures, that refer to a pattern of a defensive responses displayed in a potential aversive environment, that can be enhanced or reduced by anxiety modulating agents (Blanchard et al., 1990; Griebel et al., 1995). Risk assessment in the current study was represented by: a) "Stretched-attend postures" where the animal body remains in the closed arm, but the head and forepaws temporarily exit the closed arm, and b) "head dips" with animal bending its head beneath the open arm edge. According to conventional scores, increased anxiety is inferred from higher sojourn time in the walled arms. In terms of ethological readouts, increased anxiety is assumed if the frequency of risk assessment, stretched-attend postures, and head dips are increased.

Radial Arm Maze

The radial arm maze (RAM, eight-arm) paradigm has been employed to assess working memory (Olton and Feustle, 1981), as well as attention-related behaviors (Ruocco et al., 2014). To assess "non-reinforced exploration", the animals were allowed to explore the maze freely in the absence of reinforcement (non-baited arms) for 5 minutes. The behavioral parameters considered in this test were running speed, distance moved, and rearing duration (index for non-selective attention) (Ruocco et al., 2014). The number of

visited arms before the first repetition occurred was used as an index of selective spatial attention (SSA) (Ruocco et al., 2009, 2014, 2015).

Rotarod

This behavioral assay assesses motor function, coordination, balancing, as well as motor learning. It consists of a rotating drum which starts revolving at a constant speed of 24 revolutions per minute (rpm) or is continually accelerated from an initial low to a final high of up to 450 rpm within duration of 5 minutes (300 seconds). Each animal was placed on a rotating drum with a constant speed and a cutoff time of 5 min per trial. After 24 hours, the test was repeated with an accelerating drum over a period of 5 minutes in order to evaluate motor learning. For each day, 6 trials were administered, with inter-trial-intervals of 90 seconds. The latency of falling off was recorded. Animals remaining on the drum for more than 300 seconds per trial were removed, and the cut-off score of 300 seconds was recorded.

Forced Swimming Test

The forced Swimming test (FST) was developed to assess pharmacological effects of antidepressants in rodents (Porsolt et al., 1977). FST has subsequently become a popular depression-related or '*despair*' test. Despair is estimated via the duration of immobility and the animal's attempts to escape from the water (Petit-Demouliere et al., 2005). (However, FST's validity as a test for depression has been challenged (Chen et al., 2015;

de Kloet and Molendijk, 2016)). Within the first trial (pre-test trial), each animal was placed in a closed cylinder filled with water for 15 minutes. After a delay of 24 hours, the animals were again placed in the same cylinder for another 5 minutes (24-hour test trial). The animals' duration of swimming, immobility, and climbing behaviors were analyzed.

Restraint Stress and Hypothalamus–Pituitary–Adrenal (HPA)- Axis Alteration

The determination of hippocampus-HPA axis functionality and its interaction with spatial learning (Lathe, 2001; Roozendaal et al., 2001), and/or anxiety-related phenotypes (Braquehais et al., 2012) is essential for the adequate interpretation of the behavioral findings and validity of the animal model. In this experiment, after taking a baseline blood sample (S1), the animal was placed on a transparent restrainer and four other samples were taken over duration of 10 minutes. Ringer´s solution was subcutaneously injected into the animal to avoid hypovolemia. Afterward, samples were centrifuged and plasma was separated and then sent for biochemical corticosterone analysis.

2.1.2. Neurochemical Analysis

In order to explore whether behavioral phenotyping would influence brain neurotransmitter content, a group of experimentally naïve animals (tgDimers and WT controls at 7 and 12 months of age) were used as controls for animals that were

previously tested in the behavioral test battery in order to estimate the influence on the neurotransmitter balance.

The animals were anaesthetized with CO_2, decapitated, and relevant brain regions were separately dissected and stored at -80 °C until the time of analysis. By means of high-performance liquid chromatography with electrochemical detection (HPLC-EC), samples were analyzed for the following neurotransmitter content— DA and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC), serotonin (5-HT) and its metabolite 5-hydroxyindole acetic acid (5-HIAA), and acetylcholine and its metabolite choline (Ch). Neurotransmitter turnover rate was indexed by 5-HIAA/5-HT and DOPAC/DA ratios.

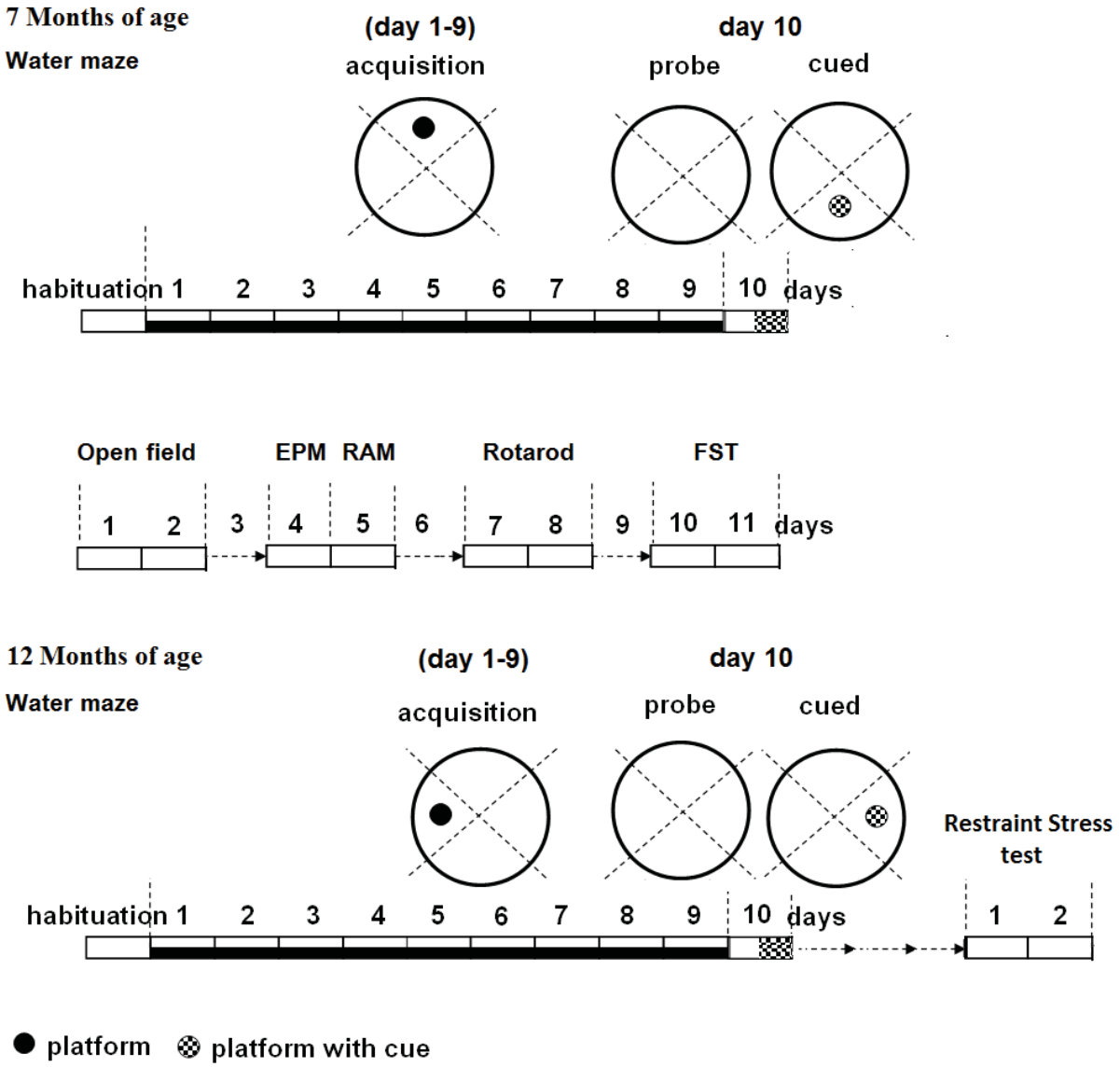


Figure 1: Schematic Experimental Design Sequence of Behavioral Characterization of tgDimer and C57BL/6N

Top Adult animals at 7 months (tgDimer and WT) begun with an MWM task, they were habituated for one day, followed by 9 acquisition days of learning to escape on a fixed location of the submerged PF (4 trials/day). This was followed by a single probe trial with a maze devoid of the PF. Thereafter, a cued MWM version with visible PF was run. MWM was followed by the EPM, the RAM, rotarod, and forced swimming (FS) tasks.

Bottom At 12 months of age, tgDimer and WT animals were habituated to the MWM for one day. This was followed by 9 days of training to escape to a stationary hidden PF location in acquisition days. The restraint stress test was conducted one week later. 6 blood

samples were collected over the course of 180 minutes for each animal in order to evaluate corticosterone levels.

2.2. Part II. Promnestic effects of intranasally applied pregnenolone in rats

2.2.1. Spontaneous Object Exploration Tasks

These tasks are based on exploiting the natural tendencies of rodents to explore novel objects (Ennaceur and Delacour, 1988). Several spontaneous object exploration tasks have been developed (See Dere et al., 2007 for a review). In this dissertation, novel object recognition (NOR) and object place preference (OPP) tasks were used in order to evaluate rodent ability to recognize a novel object or whether a familiar object was moved to a novel position within a familiar open field. Adopting the classical design as introduced by Ennaceur and Delacour, (1988), both tests consisted of a two-trial procedure: a familiarization phase in which two equal objects are presented, and a testing phase in which the animal has to discriminate between novel and familiar components of the experimental configuration. It should be noted that spontaneous object exploration tasks do not require external motivation represented by reward and/or punishment. Only habituation to the test environment is required to minimize environmental neophobia that could confound object exploration (Gouveia and Hurst, 2017; Leger et al., 2013). During the test trial of novel object recognition, a familiar object is presented together with an unfamiliar one, upon which normal rodents spend more

time exploring the unfamiliar rather than the familiar object (Ennaceur, 2010). In the object place recognition task, a familiar object is displaced to a position that did not contain an object during the sample/familiarization phase. In this situation animals spend more time exploring the displaced object than the stationary one(Ennaceur et al., 1997). However, discrimination performance varies according to the delay period between the familiarization and the test phases, and the time of exploration of objects in the sample phase (Antunes and Biala, 2012; Ennaceur and Delacour, 1988).

In the present study, animals were presented with two objects placed within an open field. The rats were then allowed to explore the objects freely for 3 minutes in the familiarization phase (sample trial). After a 24-hour or 48-hour retention interval, the animals were again placed within the arena and were allowed to explore two objects. In the NOR task one of the familiar objects was replaced with an unfamiliar object, but the position where the objects were located were kept the same, while in the OPP task one familiar object was displaced to a location that was empty during the sample/familiarization phase.

Exploration of an object was defined as a physical contact with snout, vibrissae, or forepaws. Climbing on the object or passively sitting close to the object while not being oriented toward the object was not considered as object exploration. (Fig 2 depicts the protocols of spontaneous object exploration experiments).

2.2.2. Morris Water Maze

The Morris water maze (MWM) paradigm has been modified to investigate working and reference memory as well as various spatial navigation strategies (Bannerman et al., 2014; Brandeis et al., 1989; Dere et al., 2003; Dudchenko et al., 1997; Vorhees and Williams, 2006). In this experiment, two different MWM protocols were used for two batches of animals at 3-4 months of age (Fig 3 depict the protocols of MWM experiments).

In the first batch, animals were trained to escape to the hidden PF for 5 acquisition days (2 trials per acquisition day, 90 sec trial duration, 30 sec ITI, 30 sec on the PF). The acquisition days were followed by multiple "extinction (probe-trials)" days/sessions with multiple trials per day without a platform, instead of a single probe trial, in order to measure behavioral extinction of learned escape from water onto a submerged platform (Huston et al., 2009). The emotional alteration triggered by a negative reinforcement withdrawal, as well as the retention memory intensity indexed by conditioned place preference (CPP) for the former platform location was tracked (Schulz et al., 2004, 2007). Extinction trials were terminated after 90 sec. In the acquisition trials the time taken to escape onto the submerged platform was measured. In the probe trial, the time spent within the previously rewarded platform location and

swimming speed; in the cued version, the time to reach the cued escape platform was scored.

Intranasal dose was given 15 min prior to the 1st trial of each acquisition session from acquisition days (1-4), but not for the first cued version. Thereafter, IN was also applied 15 min prior to the 1st trial of the probe/extinction sessions from extinction days 7-14, and also prior to the second cued trial (Fig.3).

In the second batch, four acquisition sessions were done with a relocated PF position for each session, with the aim of testing flexibility of goal oriented behavior (4 trials per acquisition day, ITI 90 sec, time on the PF 30 sec, and MTD 90 sec). Four days after the last acquisition, another single acquisition session with four trials was applied, with a pre-trial single systemic injection of the amnesic agent scopolamine, in addition to intranasal administration.

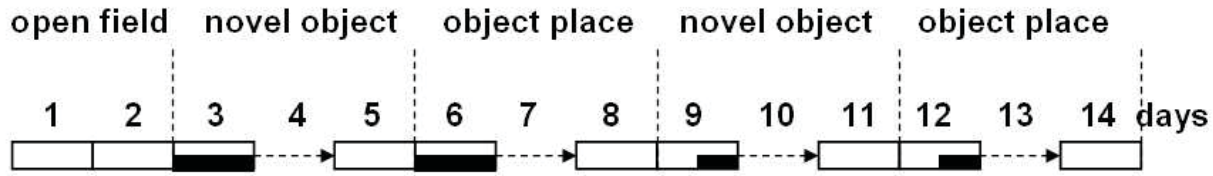
Intranasal dose was given 15 min prior to the 1st trial of each acquisition session. Also, a single scopolamine systemic dose was administered 60 min prior to the 1st trial of each acquisition session on acquisition day 15 (Fig.3).

2.2.3. Elevated Plus-Maze

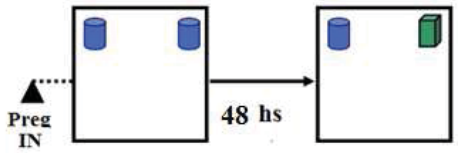
In this experiment, the EPM was conducted as a one-trial task in which each rat was placed in the center of the plus maze facing towards an open arm and allowed to

explore the entire apparatus freely for 5 minutes. The time spent and the number of entries into the open and walled arms was scored.

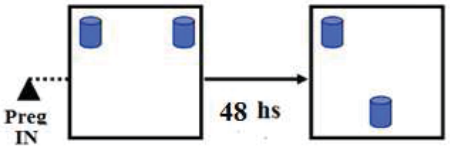
1st batch



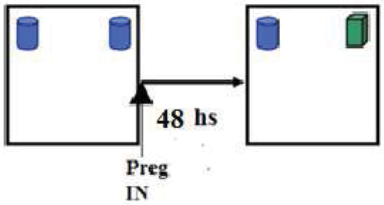
- **Novel object recognition task (pre-trial IN PREG – encoding)**



- **Novel object-place preference task (pre-trial IN PREG – encoding)**



- **Novel object recognition task (post-trial IN PREG– Consolidation)**



- **Novel object-place preference task (post-trial IN PREG– Consolidation)**

Figure 2: **Schematic design of spontaneous object exploration tasks**. Animals received prior- and post-familiarization trial intranasal administrations in novel object recognition and object place preference tasks, with a delay of 48 hours.

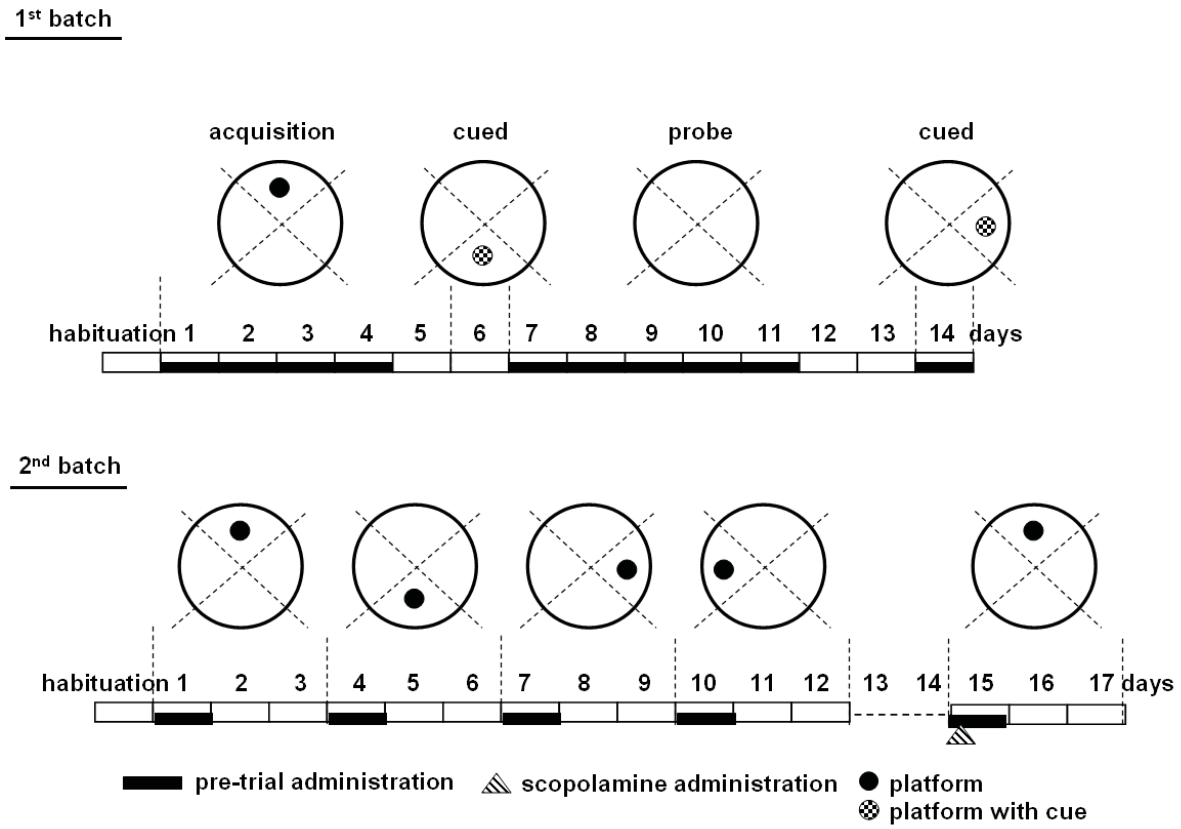


Figure 3: Schematic design of two Morris water maze experiments.
The first batch of animals was trained to escape onto the hidden platform (PF) during acquisition days (5 acquisition days; 4 trials/ day). This was followed by a cued version with PF visible. IN administration was given prior to the first trial from acquisition days 1-4, but not for the first cued version. Thereafter, the animals had five days probe/extinction sessions (days 7-11) with a daily IN administration prior to first trial. Then the second cued trial with IN administration was conducted. The second batch of animals received pre-trial IN-PREG administrations (on acquisition days 1, 4, 7, 10, and 15) with different platform locations on each day. Platform locations were however fixed within each acquisition day. Four trials per acquisition day were conducted. On day 15, with the platform relocated to a novel place, a single scopolamine injection was given prior to the first acquisition trial, in addition to the IN administration.

3. Results

For more details, the reader can refer to the results sections of the published/submitted studies in the appendix.

3.1. Part I. Amyloid beta dimers modulate emotional, attentional and learning/ memory processes, and serotonin homeostasis

Data from recent Alzheimer's disease literature points to a crucial role of soluble Aβ in early memory deficits and neuropsychiatric symptoms, rather than the insoluble/aggregated forms which accumulate into insoluble plaques (Rowan et al., 2005). The inexistent correlation between the degree of dementia and senile plaque pathology in addition to the synaptic dysfunction triggered by the existence of soluble Aβ forms, strongly supports a major pathogenic role for soluble but not packed forms of amyloid beta in Alzheimer's disease (Dickson et al., 1995; Hardy and Selkoe, 2002; Selkoe and Schenk, 2003; Terry et al., 1991). Using the tgDimer mice, we determined the isolated impact of soluble Aβ dimer over-expression on neurotransmitter homeostasis, cognitive and emotional function, that is not confounded by the co-expression of insoluble aggregates.

tgDimer mice showed deficits in learning to escape to the hidden PF location at both ages (7 and 12 months of age) when tested in the MWM. They exhibited longer latencies to reach the hidden PF as compared to the WT mice. Notably, this finding is consistent

with previous reports that have highlighted deficits in MWM acquisition and memory after intra-ventricular infusion of soluble Aβ (Faucher et al., 2016; Noshita et al., 2015; Shiao et al., 2017).

Furthermore, assessment of the degree of learning (slope over acquisition days) achieved across the first to the last acquisition day was evaluated. WT mice (at 7 and 12 months) and tgDimer mice (at 7 months only) exhibited a significant improvement across acquisition days (day 1 - 9), while the older tgDimer mice failed to do so. A more detailed analysis revealed that 7 months old tgDimer mice exhibited an improvement only during the second half of acquisition phase (from days 4 to 9) , but not across the first half of the acquisition stage (day 1 to 4).

In contrast to the controls, the mutant mice, when being retested at the age of 12 months, displayed no residual memory from the initial acquisition phase at the age of 7 months.

Besides, tgDimer mice exhibited poor spatial memory during the probe trial (with the platform being removed from the pool) at both ages tested (7 and 12 months). In order to assess a measure of the actual time spent with searching the platform location (spatial memory performance) during the spatial probe trial, that is not confounded by stress-related wall hugging or thigmothatic swimming in the perimeter of the pool, the time spent swimming close to the wall of the pool was subtracted from the total time spent in

the former platform quadrant. This provides a measure of the actual time spent searching for the platform location.

Mutant mice and WT controls at 12-months old were subjected to a restraint stress procedure with the aim of testing the responsiveness of the HPA-axis (Rabasa et al., 2011). Since no alteration of corticosterone levels were observed in aged tgDimer mice in comparison to the controls, we can exclude HPA axis dysregulation as a likely cause for disturbed memory processing (Lathe, 2001), at least in the tests that took place at 12 months of age.

In contrast to the spatial learning impairment found in the MWM, working memory performance in the RAM was found to be intact, indexed by comparable first error score between tgDimer and WT controls (Ruocco et al., 2015). This pattern of results suggests that spatial learning deficits of the mutants may not due to impaired working memory. According to O´Keefe et al. (1979), impaired working memory would affect the initial encoding of spatial relations between intra- and extra-maze cues, which is a prerequisite for the formation of a spatial/cognitive map.

Moreover, in both the RAM tests and behavioral assessment in the open field we observed a reduction of rearing duration. This suggests that the spatial learning deficit might be related to deregulation of attention and arousal processes (Morena et al., 2015; Prediger et al., 2005).

The tgDimer animals (7 months) did not differ from the WTs on conventional anxiety-related parameters in the EPM, namely, number of entries and time spent in the open vs. walled arms. Alternatively, the tgDimer animals exhibited higher frequency of "stretch-attend posture", indicating a greater level of anxiety. This finding is supported by a greater tendency toward thigmotactic swimming of tgDimer compared to WT in the probe trial (Gehring et al., 2015). According to the literature, contradictory findings were reported with regard to the impact of soluble Aβ on EPM behavior. Some authors report anxiogenic effects (Harkany et al., 2001; Pamplona et al., 2010) while others report null effects (Colaianna et al., 2010; Knight et al., 2016). However, it must be taken into account that in these studies only conventional readouts were analyzed.

In the FST, swimming duration was decreased with a trend towards increased immobility, indicating an increase of depression-related behavior (Porsolt et al., 1977). These results are in line with previous findings, showing an increase of immobility after intra-cerebro-ventricular injection of soluble Aβ (Colaianna et al., 2010).

Rotarod performance in adult tgDimer mice was impaired when tested with accelerated rotation but not under constant speed. Notably, swimming speeds in MWM, time to the PF in cued trials, horizontal activity in OF and RAM, as well as rotarod performance at constant speed were comparable between tgDimer and controls, suggesting that the

deficits found with accelerated rotarod may refer to deficient motor leaning than to general deficits in sensorimotor performance.

Post-mortem HPLC-analysis of the content of neurotransmitters revealed a significant decrease of hippocampal acetylcholine content in the tgDimer mice with ageing. This finding was in line with the expectation that hippocampal cholinergic neurons are highly influenced by soluble Aβ toxicity (Vaucher et al., 2001). WT controls exhibited an age-related elevation of dopamine (Everett, 1977), specifically in the hippocampal formation (Hernández et al., 2014). However, aged mutant mice showed no difference in DA levels compared to the adults. 5-HT turnover was decreased only in the hippocampus of aged tgDimer mice in comparison to the WT, while in the ventral striatum 5-HT turnover was decreased in both adult and aged tgDimer mice. In the amygdala, 5-HT turnover was only decreased in adult tgDimer mice.

In conclusion, the results suggest that over-expression of soluble Aβ dimer protein induces attentional, emotional and learning and memory deficits, together with neurotransmitter changes, in tgDimer mice. The tgDimer mouse can be viewed as a animal model of early AD, that is also characterized by over-expression of soluble Aβ and behavioral symptoms. Our neurochemical findings suggest that the behavioral consequences of soluble Aβ over-expression are possibly mediated by changes in the

brains serotonin system (for further details please refer to (Abdel-Hafiz et al., 2017, attached).

3.2. Part II. Promnestic effects of intranasally applied pregnenolone in rats

In this study, various behavioral tests were used to evaluate emotional and cognitive effects of intranasal PREG administration in healthy rats. Two PREG doses were incorporated into a lipophilic galenic matrix: the lower being 0.0187 mg/kg, and the higher being 0.373 mg/kg. A novel object preference test and a novel place preference tests were conducted (Fig. 2) to examine effects of IN PREG on memory. The higher dose of IN PREG enhanced long-term memory when given before the sample trial, but not when administered immediately after the sample trial in both novel object preference (NOR), and novel location preference (NLP) tasks compared to vehicle treated groups. In the novel object location task, the lower PREG dose improved memory encoding, but had no effect on memory consolidation (Habib et al., 2003; Izquierdo and McGaugh, 2000; McGaugh and Roozendaal, 2009). In Figure 2 a schematic representation of the protocol of both object exploration tasks is presented.

In the MWM, two protocols were used (Fig. 3). In the first, an acquisition phase with a stable PF location over 5 consecutive days was followed by an extinction phase with the platform removed. Following the same dose regiment used for the object exploration task, animals were divided into three groups: vehicle, 0.0187 mg/kg, and 0.373 mg/kg

dose groups. These three groups exhibited comparable learning performance during the acquisition stage. The inclination to swim into the previously rewarded quadrant in the extinction phase was taken as an indication of conditioned place preference (CPP) (Huston et al., 2009), which is considered as evidence for a stronger resistance to behavioral extinction (Schulz et al., 2007). When compared to the vehicle (VE)-treated group, the animals that received the lower dose of PREG spent more time searching in the former platform quadrant across extinction days 1 to 5. However, this effect was diminished upon discontinuing the IN treatment during extinction days 6 and 7. Thus, the increased resistance to extinction (enhancement in CPP) induced by the lower dose seems to be an acute effect of PREG that requires daily injections. Only the lower, but not the higher dose of PREG increased the resistance to behavioral extinction. It is possible that the extinction-resistance effect of the higher dose was masked by sensory-motor disturbance that compromised navigation in the pool.

The second MWM experiment was designed to evaluate whether IN PREG affected behavioral flexibility or the expression of adaptive strategies. This experiment was performed to exclude the possibility that PREG induces perseveration or behavioral rigidity. In this experiment the escape platform was re-located to a different quadrant to assess reversal learning (Dudchenko et al., 1997). Both IN PREG treated groups (0.0187 mg/kg, and 0.373 mg/kg) showed no significant difference in reversal learning as compared to VE-treated animals, suggesting that flexibility in behavioral

performance (adaptive behavior) was not affected by PREG. Therefore, the increased resistance to extinction that was observed after the lower dose of PREG in the first MWM experiment was not due to an inability to explore novel strategies or aiming to terminate the aversive stimulation. IN PREG treatments also counteracted the memory-impairing effects of scopolamine co-application (Klinkenberg and Blokland, 2010; Saucier et al., 1996). This finding indicates that IN PREG can antagonize the amnestic effects of anticholinergic agents (for further details please refer to (Abdel-Hafiz et al., 2016).

4. General Discussion

In the past decades a large number of studies have been published that aimed to elucidate the molecular mechanisms that underlie learning and memory processes. Such basic information is indispensable for the development of better medication for neurodegenerative diseases such as Alzheimer´s disease. However, despite great research efforts, the molecular basis of learning and memory are still not entirely clear and satisfactory pro-cognitive medication for treating, e.g. Alzheimer and advanced Parkinson patients, is still not available. In this context, the present study investigated the role of Aβ dimers on learning/memory and whether PREG could be a potential candidate for pro-cognitive medication.

4.1. The receptor level

It is well known that various PREG metabolites modulate neurotransmission in the brain. However, only a few studies have investigated the impact of PREG on neurotransmitters and its association with learning, memory and mood control. Both PREG and soluble Aβ have been shown to interact with N-methyl-D-aspartate (NMDA) and sigma receptors (Izzo et al., 2014; Nuwayhid and Werling, 2003; Urani et al., 2004; Zheng, 2009), and both have been shown to modulate Ach release by either direct or indirect ways (Jin et al., 2015; Mucke and Selkoe, 2012; Pedersen et al., 1996). The importance of Ach content is supported by our findings in part I and II of this dissertation, showing an age-related reduction in hippocampal Ach content by Aβ dimer chronic toxicity, and the antagonizing effect of IN PREG treatment on the memory-impairment triggered by systemic scopolamine administration. It is known that the hippocampus is among the first brain regions that exhibit neuronal loss and reductions in Ach levels in response to toxic forms of Aβ (Serrano-Pozo et al., 2011a; Van Hoesen et al., 1991;Chiba et al., 2008; Hoshi et al., 1997).

PREG administration by the intranasal, but not by the systemic route, differentially favors the hippocampus region (Ducharme et al., 2010), thus having a beneficial effect on hippocampus function possibly due to the modulation of intra-hippocampal neurotransmitter release that is important for learning and memory processes (Liu et

al., 2004; Maurice et al., 1994; Zheng, 2009). Together, these effects might also explain the neuroprotective properties of PREG against Aβ neurotoxicity (Akan et al., 2009; Gursoy et al., 2001).

Work by Ducharme et al., (2010), showed that titanium labeled IN PREG accumulates in certain brain regions, especially the hippocampus, rather than being equally distributed across the whole brain. This finding together with the high affinity of PREG for NMDA and σ receptors (Zheng, 2009), which are both known to be involved in learning and memory (Liu et al., 2004; Maurice et al., 1994; Tsai et al., 2009; Vianna et al., 2000), could explains the pro-mnestic action of PREG.

The facilitation of long-term memory after IN PREG administration might be the result of a direct interaction of PREG with hippocampal NMDA and/or σ receptors (Flood et al., 1992). Lesion and electrophysiological studies have shown that the hippocampal formation is known to play a critical role in novel object recognition tasks (Friedman et al., 2001; Habib et al., 2003; Hammond et al., 2004; Knight and Nakada, 1998) as well as in spatial learning and the preference for unfamiliar places (Stewart et al., 2011; Vogel-Ciernia and Wood, 2014). Given this wide range of hippocampal functions and the modulatory effect of PREG on intra-hippocampal neurotransmitters and receptors, the other behavioral effects of PREG, including the increased resistance to extinction, might also be explained by an effect on hippocampal functioning.

It has been reported that PREG stimulates hippocampal sigma receptors, which in turn can trigger hippocampal Ach release (Jin et al., 2015). This cascade of events might explain why PREG is able to antagonize the amnestic effect of the anti-cholinergic drug scopolamine. Scopolamine amnesic effects are well established in a variety of learning tasks such as the Morris water maze test (Klinkenberg and Blokland, 2010; Lee et al., 2015; Saucier et al., 1996), a standard behavioral task to evaluate hippocampal damage-related impairments (Bolhuis et al., 1994; Martin et al., 2005; Sutherland et al., 2001), Object recognition tasks (Dodart et al., 1997; Sambeth et al., 2007), in which intact hippocampus and para-hippocampus structures are required for novel object recognition memory (Hammond et al., 2004), and in the memory retrieval of inhibitory avoidance task (Botton et al., 2010), for which the integrity of the hippocampal cholinergic system is essential (Parfitt et al., 2012).

It is well known that Aβ impairs hippocampal-dependent learning and memory performance (Garcia-Osta and Alberini, 2009; Varghese et al., 2010). In the present study we showed that soluble Aβ dimers in the brain of tgDimer mice led to a depletion of hippocampal acetylcholine upon aging. This reduction, correlated with learning and memory impairments in tgDimer mice at 12 months of age. Cholinergic integrity is crucial for MWM performance (Saucier et al., 1996), as well as for non-selective attention (Warburton and Rusted, 1993) . Thus, the deficits in selective attention that

were observed in tgDimer mice might also be the consequence of a reduction in cholinergic neurotransmission due to Aβ neurotoxicity which also affects cortical cholinergic neurons (Perry and Hodges, 1999).

In the present study, tgDimer mice exhibited higher levels of anxiety in the EPM and more depression-like behaviors in the FST. In the MWM tgDimer mice exhibited increased thigmotactic swimming at both ages tested, regardless of whether a platform was present or not.

4.2. Soluble Aβ and pregnenolone

A link between soluble Aβ and neurosteroids has been proposed by many studies. Cholesterol (Chlr), a crucial component during neurosteroid biosynthesis, has been implicated in Aβ peptide biosynthesis. Chlr has been found to modify the amyloid precursor protein at certain cleavage sites (Kálmán and Janka, 2005; Ricciarelli et al., 2012). Cholesterol also facilitates plaque formation by decreasing the peptide aggregation threshold (Colell et al., 2009; Cossec et al., 2010; Di Scala et al., 2014). By contrast, the Aβ peptide affects cholesterol metabolism and thereby affects PREG and its sulfate ester derivate (Baulieu et al., 2001; Mellon, 2007). Indeed, in vitro testing of Aβ toxicity significantly increased PREG level and decreased cell viability (Calan et al., 2016).

Neurosteroids have been suggested to be crucial for neuronal survival (Charalampopoulos et al., 2008), and might be part of an adaptive mechanism against Aβ neurotoxicity (Guarneri et al., 2003). PREGS is synthesized from PREG. A simple sulferization process conversion can easily occur through a sulfo-transferase reaction in the cytoplasm (Herson et al., 2009; Vallée et al., 2003). Treatments with PREGS have secured the maturation and survival of newborn neurons in mouse models of Alzheimer's disease, which otherwise would show impaired neurogenesis (Xu et al., 2012). PREGS has also been shown to improve neurogenesis in the dentate gyrus (DG) of the hippocampus in adult and aged rodents (Mameli et al., 2005; Mayo et al., 2005). The hippocampus, is the first region to be affected by AD pathology (Serrano-Pozo et al., 2011; Van Hoesen et al., 1991) and by Aβ toxicity (Pooler et al., 2015).

Likewise, PREG exhibited a protective effect against Aβ-induced toxicity in neuronal cells within "*in vitro*" models (Akan et al., 2009; Cardounel et al., 1999; Gursoy et al., 2001; Mao and Barger, 1998). Taking together, it can be concluded that PREG and Aβ can antagonize each other mutually.

However, despite the existence of "*in vitro*" studies, limited data is available concerning "*in vivo*" studies investigating the link between PREG and Aβ in neurodegenerative diseases such as Alzheimer's disease.

5. Conclusions

The evidence provided in the present study using a battery of different tests demonstrates the beneficial effects of IN administration of PREG on cognitive and emotional behavior under healthy and disease conditions. Patients suffering from Alzheimer's disease exhibit Aβ mediated neurodegeneration concomitant with decreased PREG levels (Calan et al., 2016; Schumacher et al., 2003; Weill-Engerer et al., 2002). In vitro studies with mouse hippocampal cell lines have demonstrated a neuroprotective effect of pregnenolone on both Aβ- and glutamate-induced neurotoxicity (Gursoy et al., 2001).

IN application of PREG might be relevant as a treatment for cognitive and emotional symptoms in patients with early Alzheimer's disease, as it might prevent the progression of the disease at early stages by blocking Aβ toxicity, even before plaques have been generated and neurodegeneration is detectable.

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7. Original Research Articles

The published studies, on which the current dissertation is based,

7.1. Part I:

Publication # 1

Müller-Schiffmann, A., Herring, A., **Abdel-Hafiz, L.,** Chepkova, A.N., Schäble, S., Wedel, D., Horn, A.H.C., Sticht, H., de Souza Silva, M.A., Gottmann, K., et al. (2016). Amyloid-β dimers in the absence of plaque pathology impair learning and synaptic plasticity. **Brain** 139, 509–525.

Impact factor: 10.29

Contribution: Performed the behavioral studies, analyzed and helped to interpret the results

Publication # 2

Abdel-Hafiz, L., Müller-Schiffmann, A., Korth, C., Fazari, B., Nikolaus, S., Schäble, S., Herring, A., Keyvani, K., Lamounier-Zepter, V., Huston, J.P.,de Souza Silva, M.A. (2017). Abeta dimers modulate serotonin homeostasis, cognition- and affect related behaviors reminiscent of early Alzheimer's disease. Manuscript submitted for publication.

Impact factor: 5.15

Contribution: Performed the behavioral studies, analyzed and helped to interpret the results and write the manuscript.

7.2. Part II

Publication # 3

Abdel-Hafiz, L., Chao, O.Y., Huston, J.P., Nikolaus, S., Spieler, R.E., de Souza Silva, M.A., and Mattern, C. (2016). Promnestic effects of intranasally applied pregnenolone in rats. **Neurobiol. Learn. Mem**. 133, 185–195.

Impact factor: 3.54

Contribution: Performed the behavioral studies, analyzed and helped to interpret the results and write the manuscript

9. Declaration

Die hier vorgelegte Dissertation habe ich selbständig und nur unter Verwendung der angegebenen Literaturquellen angefertigt. Diese Arbeit wurde in der vorgelegten oder ähnlichen Form bei keiner anderen Institution eingereicht. Zudem erkläre Ich, dass Ich bisher keine erfolglosen Promotionsversuche unternommen habe.

Dusseldorf, den

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Amyloid-β dimers in the absence of plaque pathology impair learning and synaptic plasticity

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Despite amyloid plaques, consisting of insoluble, aggregated amyloid-β peptides, being a defining feature of Alzheimer's disease, their significance has been challenged due to controversial findings regarding the correlation of cognitive impairment in Alzheimer's disease with plaque load. The amyloid cascade hypothesis defines soluble amyloid-β oligomers, consisting of multiple amyloid-β monomers, as precursors of insoluble amyloid-β plaques. Dissecting the biological effects of single amyloid-β oligomers, for example of amyloid-β dimers, an abundant amyloid-β oligomer associated with clinical progression of Alzheimer's disease, has been difficult due to the inability to control the kinetics of amyloid-β multimerization. For investigating the biological effects of amyloid-β dimers, we stabilized amyloid-β dimers by an intermolecular disulphide bridge via a cysteine mutation in the amyloid-β peptide (Aβ-S8C) of the amyloid precursor protein. This construct was expressed as a recombinant protein in cells and in a novel transgenic mouse, termed tgDimer mouse. This mouse formed constant levels of highly synaptotoxic soluble amyloid-β dimers, but not monomers, amyloid-β plaques or insoluble amyloid-β during its lifespan. Accordingly, neither signs of neuroinflammation, tau hyperphosphorylation or cell death were observed. Nevertheless, these tgDimer mice did exhibit deficits in hippocampal long-term potentiation and age-related impairments in learning and memory, similar to what was observed in classical Alzheimer's disease mouse models. Although the amyloid-β dimers were unable to initiate the formation of insoluble amyloid-β aggregates in tgDimer mice, after crossbreeding tgDimer mice with the CRND8 mouse, an amyloid-β plaque generating mouse model, Aβ-S8C dimers were sequestered into amyloid-β plaques, suggesting that amyloid-β plaques incorporate neurotoxic amyloid-β dimers that by themselves are unable to self-assemble. Our results suggest that within the fine interplay between different amyloid-β species, amyloid-β dimer neurotoxic signalling, in the absence of amyloid-β plaque pathology, may be involved in causing early deficits in synaptic plasticity, learning and memory that accompany Alzheimer's disease.

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Abbreviations: AMPA = L-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; EPSC = excitatory postsynaptic current; EPSP = excitatory postsynaptic potential; HFS = high frequency stimulation; LTP = long-term potentiation; NMDA = *N*-methyl D-aspartate

Introduction

Alzheimer's disease is the most prevalent neurodegenerative disease in humans. Amyloid-β-containing amyloid plaques and neurofibrillary tangles consisting of the microtubule associated protein tau (encoded by *MAPT*) in post-mortem neuropathological examinations, in conjunction with the clinical diagnosis of dementia, have been the three defining features for Alzheimer's disease for a century (Alzheimer, 1906).

While neurofibrillary tangles and amyloid-β plaque pathology are widely used for staging of Alzheimer's disease (Braak and Braak, 1991; Thal *et al.*, 2002), neurofibrillary tangles have been demonstrated to correlate better with cognitive deficits of patients with Alzheimer's disease than amyloid-β plaques (Nelson *et al.*, 2012). Similarly, *in vivo* neuroimaging technology with Pittsburgh compound B showed a lacking accuracy in identifying patients with mild cognitive impairment who would later develop Alzheimer's disease dementia (Zhang *et al.*, 2014).

Yet, the amyloid cascade theory (Hardy and Selkoe, 2002), founded on the solid genetic evidence of familial Alzheimer's disease, states that aberrant amyloid-β accumulation is the initial step of a cascade of events that leads to tau hyperphosphorylation, neuroinflammation and, ultimately, massive neuronal degeneration. The presence of amyloid-β oligomers, soluble multimers of amyloid-β monomers, rather than the occurrence of insoluble fibrillar amyloid-β that only poorly correlated with cognitive impairment (Terry *et al.*, 1991; Dickson *et al.*, 1995) is likely at the beginning of the amyloid-β cascade. Moreover, low-*n* oligomers like dimers are reasonably well correlating to synaptic loss and Alzheimer's disease (Klyubin *et al.*, 2008; Shankar *et al.*, 2008; Mc Donald *et al.*, 2010). Although unequivocal data on abundance and significance of amyloid-β oligomers *in vivo* are still limited, compelling *in vitro* studies have led some investigators to conclude that amyloid-β dimers, which have been detected to be highly abundant in brains of Alzheimer's disease mouse models and patients with Alzheimer's disease, are sufficient to account for neurotoxicity and initiating the Alzheimer's disease-typical cascade (Kawarabayashi *et al.*, 2004; Shankar *et al.*, 2008; Hashimoto *et al.*, 2012; Mc Donald *et al.*, 2010, 2015).

So far, the investigation of amyloid-β dimers and their elicited neuropathological, biochemical and behavioural effects *in vivo*, has been impossible owing to the equilibrium that exists between the different multimer and conformer pools of amyloid-β. The introduction of cysteines to generate new covalent intra- or intermolecular disulphide bonds has been successfully applied in numerous studies to increase the stability of proteins or to lock proteins in a specific conformation (Matsumura *et al.*, 1989; Shimaoka *et al.*, 2002), including the generation of synthetic amyloid-β dimers (Schmechel *et al.*, 2003; O'Nuallain *et al.*, 2010; Yamaguchi *et al.*, 2010). These synthetic amyloid-β dimers spontaneously formed protofibrillar structures at micromolar concentrations (Hu *et al.*, 2008; Yamaguchi *et al.*, 2010), of which one, the Aβ-S26C protofibril also turned out to be synaptotoxic (O'Nuallain *et al.*, 2010). Unfortunately, it is almost impossible to model single amyloid-β oligomer species with synthetic peptides in cell-free *in vitro* systems because of: (i) low folding reliability during bulk refolding; (ii) competition between specific oligomer assembly and unspecific aggregation; (iii) a steady state equilibrium between different amyloid-β multimers; and (iv) the usually unphysiologically high concentrations of amyloid-β in cell-free systems that do not reflect cellular amyloid-β assembly (Selkoe, 2008).

Soluble naturally secreted amyloid-β oligomers have been generated in a cell culture model, termed 7PA2, which expresses APP with the familial Indiana mutation APPV717F initially believed to secrete high amounts of amyloid-β dimers and trimers (Podlisny *et al.*, 1995). Recently, however, the nature of these cell-derived amyloid-β species has been questioned, as they may predominantly represent neurotoxic N-terminal elongated amyloid-β monomers, although low amounts of oligomers still have been detected (Welzel *et al.*, 2014). Previously, we demonstrated that stabilizing amyloid-β dimers by an intermolecular disulphide bridge within the N-terminus of amyloid-β (Aβ-S8C) led to the natural secretion of nanomolar amounts of synaptotoxic and stable amyloid-β dimers in a cell culture model, without influencing the trafficking or processing of APP by cellular secretases (Muller-Schiffmann *et al.*, 2011).

In the present study, we investigated the selective role of amyloid-β dimers and their signalling on brain pathology and behaviour *in vivo*, by expressing the Aβ-S8C containing APP transgene in a mouse, termed tgDimer mouse. We used the same expression vector that led to the generation of the well characterized APP23 Alzheimer's disease mouse

model, and used the same mouse strain (C57BL/6) (Sturchler-Pierrat *et al.*, 1997). In tgDimer mice, we detected high amounts of amyloid-β dimers, but no amyloid-β monomers in brain tissue of these mice. We demonstrate that amyloid-β dimers are unable to initiate the formation of insoluble amyloid-β or amyloid plaques, and can do so only when amyloid-β plaques are present from another source. Thus, the tgDimer mice for the first time allowed the dissection of specific effects mediated by the unique conformer of amyloid-β dimers in the absence of amyloid-β plaques. We observed no induction of tau hyperphosphorylation and neuroinflammation, but learning and memory deficits as well as impairment of synaptic plasticity suggesting that initial stages of cognitive decline in Alzheimer's disease are independent of amyloid-β plaque pathology.

Materials and methods

Antibodies

The IgG2b monoclonal antibody IC16 (Muller-Schiffmann *et al.*, 2010) recognizes the N-terminus of amyloid-β (amino acids 2–8) and was obtained from supernatants of IC16 hybridoma cells, that were cultured in CELLine two compartment bioreactors (Integra Biosciences). APP was visualized by the polyclonal CT15 antibody, which recognizes the C-terminal 15 amino acids of APP (Sisodia *et al.*, 1993). 4G8 recognizes residues 17–24 of amyloid-β and was obtained from Covance. 6F/3D, which is specific for amino acids 9–14 from amyloid-β (M0872) and a GFAP-specific antibody (Z0334) were purchased from Dako, an anti-Iba1 antibody (AP08912PU-N) was bought from Acris Antibodies and the total tau antibody HT7 (MN1000), as well as the anti-phospho-PHF-tau antibodies AT8 (MN1020) and AT180 (MN1040), from Thermo Scientific. In addition we used phospho-tau antibodies pS396 and pS404 from Life Technologies. Anti-actin (A2066) and anti-tubulin (T9026) antibodies were purchased from Sigma. The following secondary antibodies were used: goat anti-mouse-peroxidase, goat anti-rabbit-peroxidase (both from Thermo Scientific), goat anti-mouse IRDye 680RD and 800CW as well as goat anti-rabbit IRDye 680RD and 800CW (all from LI-COR). It is important to note that all antibodies that were used here for detection of amyloid-β are also capable of recognizing APP.

Animals

The Aβ-S8C mutation was introduced into the pTSC21-APP751swe vector (Sturchler-Pierrat *et al.*, 1997) by *in vitro* mutagenesis using the following oligonucleotides: Aβ-S8C-5'-3' gcagaattccgacatgactgcggatatgaagttcatcatcaaaaattggtg; Aβ-S8C 3'-5' caccaatttttgatgatgaacttcatatccgcagtcatgtcggaattctgc to yield the final pTSC21-APP751swe-S679C construct. After validation of the complete vector sequence the construct was used for microinjection into C57BL/6N embryos. The gene doses of three founder lines were quantified by quantitative polymerase chain reaction and the founder line exhibiting the highest dose was bred to homozygosity and termed tgDimer. For the seeding experiment tgDimer mice were crossed with CRND8 transgenic mice. CRND8 mice express APP with the familial Swedish and Indiana mutations and had been extensively characterized before (Chishti *et al.*, 2001). In the experiments described here offspring from the F1 generation were used that were heterozygous for both APP expression vectors: APP carrying the Swedish and Aβ-S8C mutation (from tgDimer) and APP carrying the Swedish and Indiana mutation (from CRND8). Brains from differently aged APP23 mice were used as control and obtained from Novartis.

Enzyme-linked immunosorbent assay

Amyloid-β_{40} and amlyloid-β_{42} were extracted from brain homogenates with 5 M guanidine/50 mM Tris HCl, pH 8.0 and quantified using the human/rat beta Amyloid-40 or -42 ELISA kit from Wako according to the manufacturer's recommendations. The kits used the BNT77 antibody [epitope amyloid-β (11–28)] for capturing and BA27 for detection of amyloid-β_{40} or BC05 for detection of amlyloid-β_{42}. The amyloid-β concentrations were calculated relative to the monomer concentration of the standard and are indicated as pmol/g (wet weight).

For quantification of total amyloid-β within the size exclusion chromatography fractions, 1 µg of IC16 in 100 µl phosphate-buffered saline (PBS) was immobilized per well of a 96-well Nunc® MaxiSorp® microtitre plate overnight. After washing the plate with PBS, the wells were blocked with PBS/1% bovine serum albumin (BSA) for 2 h at room temperature. Standards [synthetic amyloid-β_{40} (Sigma-Aldrich)] and samples were diluted in PBS/0.05% Tween 20 (PBST) + 1% BSA. After washing the wells with PBS, these dilutions were applied to the coated wells and incubated with agitation overnight at 4°C. The next day, the wells were washed four times with PBST and incubated with 50 µl of biotin labelled 4G8 (1:2500 in PBST/1% BSA) for 2 h at room temperature. The wells were again washed four times with PBST and bound 4G8-Biotin was detected with horseradish peroxidase-coupled streptavidin (Thermo Fisher Scientific) diluted 1:10 000 in PBST/1% BSA. After 1 h of incubation at room temperature and four washing steps with PBST bound enzyme activity was measured with 100 µl of OptEIA™ substrate solution (BD Biosciences). The enzyme activity was stopped with 100 µl of 25% H_2SO_4 and absorbance was read at 450 nm. Molar concentrations of Aβ-S8C dimers were calculated after quantifying amyloid-β relative to the monomer concentration of the standards used.

Western blot

For visualization of APP, Iba1, GFAP, and tau, 30 µg of 10% whole brain homogenates in 100 mM Tris pH 7.5, 140 mM NaCl, 3 mM KCl (TBS) containing Complete protease inhibitor cocktail (Roche) were separated on a NuPAGE® 4-12% Bis-Tris Gel (Life technologies), using NuPAGE® sample buffer either with addition of 2% (v/v) of β-mercaptoethanol or without, and blotted to a 0.2 µm nitrocellulose membrane. The membranes were blocked with 5% skimmed milk and incubated with either primary antibodies against APP (CT15: 1:3500), tau (MN1000, MN1020, MN1040, pS396 or pS404, each 1:1000), Iba1 (1:2000) or GFAP (1:2000) and actin (1:5000) or tubulin (1:5000). After incubation with

appropriate secondary antibodies, signals were quantified either by densitrometric analysis using ImageJ (NIH) or by applying the Odyssey infrared imaging system (LI-COR).

Immunoprecipitation of amyloid-β

Whole mouse brain hemispheres were homogenized to 10% w/v in TBS containing Complete protease inhibitor cocktail (Roche). Amyloid-β was immunoprecipitated from the samples by incubation with IC16-NHS-sepharose overnight at 4°C (Muller-Schiffmann *et al.*, 2011). After centrifugation, the beads were washed twice with PBS and amyloid-β was eluted with sodium dodecyl sulphate (SDS) sample buffer with or without reducing additives and boiled for 5 min. SDS-polyacrylamide gel electrophoresis (PAGE) and western blot were performed as described previously (Podlisny *et al.*, 1995). Conditioned medium from amyloid-β secreting 7PA2 cells (Podlisny *et al.*, 1995) served as control.

Four-step ultracentrifugation fractionation

Fractionation by 4-step ultracentrifugation was performed as described previously (Kawarabayashi *et al.*, 2001). Briefly, 300 µl of the homogenates were centrifuged at 100 000 *g* at for 1 h at 4°C. The supernatants were harvested and the pellets were resuspended in 300 µl TBS/1% Triton™ X-100 by sonication. After centrifugation at 100 000 *g* at 4°C for 1 h the supernatants were taken and the precipitates dissolved in 300 µl TBS/2% SDS. Following another round of centrifugation at 100 000 *g* at room temperature, the supernatants were harvested and the precipitates were finally dissolved in 300 µl of 70% formic acid before being centrifuged again for 1 h at 100 000 *g* at room temperature. The first three supernatants were diluted 20-fold in TBS and the formic acid fraction was neutralized in 20 volumes of 1 M Tris solution. Amyloid-β was immunoprecipitated by IC16-NHS-sepharose and visualized by western blot as described above.

Immunohistological staining

Amyloid-β (6F/3D), astrocytes (GFAP) and microglia (Iba1) staining was performed automatically in a TechMate instrument (Dako) on 5 µm sagittal brain sections. For amyloid-β staining, slices were pre-treated at room temperature for 3 min with 85% formic acid and then blocked with 3% H_2O_2 and a pre-blocking solution (Zytomed) for 5 min and 10 min at room temperature, respectively. Afterwards, sections were incubated for 30 min with the primary 6F/3D antibody (1:100) at room temperature, followed by a 20 min post-block incubation step (Zytomed) and subsequent incubation with a peroxidase-coupled polymer (Zytomed) for 30 min. Sections were visualized via incubation with 3,3'-diaminobenzidine (K5001, Dako) for 2 × 5 min. To highlight astrocytes, sections were pretreated with H_2O_2 and a pre-blocking solution as described above, followed by incubation with anti-GFAP antibody (1:4000) for 30 min at room temperature. Visualization was conducted by incubation with peroxidase polymer and 3,3' diaminobenzidene. Microglia were stained by pretreating sections with boiling citrate buffer (30 min, room temperature, pH 6.0), followed by permeabilization with 1% Triton for 20 min at room temperature. Then, sections were pre-blocked and incubated with Iba1-specific antibody (1:100) for 2 h at room temperature. Afterwards, sections were washed and incubated with anti-goat antibody (1:500, E0466, Dako) for 30 min at room temperature, followed by incubation with peroxidase polymer and 3,3' diaminobenzidine. Haematoxylin (1:25) served for counterstaining.

Behavioural testing

Animal experiments were performed in accordance with the German Animal Protection Law and were authorized by local authorities (LANUV NRW, Germany).

Mice

Seven-month-old male transgenic tgDimer mice ($n = 12$) and wild-type C57BL/6N control mice ($n = 9$) (weight 28–36 g) were bred and obtained from the Central Animal Laboratory at the University Hospital Essen. They were individually housed in translucent plastic cages (36.5-cm long, 20.7-cm wide, 14.0-cm high) under a reversed 12:12 h light-dark cycle (light off at 07:00 a.m.) and temperature controlled conditions (20 ± 2°C) with food and water administered *ad libitum*. They were allowed to adapt to the housing conditions for 10 days before the behavioural testing. Their health status (food and drinking behaviour, coat condition, bodily orifices) was monitored daily.

Water maze

The water maze consisted of a circular black pool 120 cm in diameter and 35 cm in height filled with 16.8 cm of water (20 ± 2°C). The water contained coffee whitener to enhance contrast for animal observation. The maze was divided into four equally sized virtual quadrants. The escape platform was a white circular platform (10.5 cm in diameter), located in the centre of one of these quadrants and submerged 2 cm below the water surface. Diffuse illumination by four LED lights (two focused to the cues, and two covered by a red transparent cover) around the maze provided a light density of ~19 lx at the upper edge, and 15 lx above the water surface. A video camera mounted above the pool was linked to a computer-based tracking (Ethovision XT 8, Noldus). Extra-maze cues were provided by the LED lights and different shaped figures placed on the wall.

Procedure

At the beginning of each trial the animal was placed into the pool, facing the wall at one of four possible starting points (north, south, east, west) along the perimeter of the maze. The order of start positions was randomized. One habituation trial of 90 s duration was run without a platform to assess swimming ability and motor control. This was followed by 9 days of acquisition by training the mice on the hidden platform place-learning task (four trials per day, 90 s intertrial interval). An acquisition trial was terminated when the animal escaped onto the hidden platform with all four paws or after 90 s had elapsed. In the latter case the animal was guided onto the platform by the experimenter. It was allowed to stay on the platform for 30 s and then removed from the maze and dried

under a red-light heating lamp between the trials. The acquisition trials were followed by a probe trial without a platform to assess retrieval of the previously learned platform. On the probe trial, the mice were removed from the pool after 90 s. Next, they were tested in the visible platform version (cued task) to check for general sensory-motor abilities (Morris *et al.*, 1982). This platform was cued via a 10.5 cm high cylinder, painted with black and white stripes. Four trials were averaged for each training day. In the probe trial, the time spent within the previously reinforced platform quadrant was assessed.

Statistical analysis

The data on acquisition and swimming speed in the water maze (the means of four trials per day) were subjected to repeated measures three-way ANOVAs with the factors Day, Genotype and Age. When appropriate this was followed by *post hoc t*-test to assess group differences. For the cued version of the water maze task, a *t*-test was carried out for between-group comparisons. The probe trial (as a measure of retention) assessed the time spent searching for the platform in the four quadrants of the maze. For this purpose the time engaged in thigmotactic swimming within the outer ring (width: 8 cm) along the maze wall was excluded. A two-way ANOVA with the factors Genotype and Age was followed by *post hoc t*-tests to compare the time spent in the former platform quadrant with time spent in the three non-reinforced quadrants, pooled. The *P*-values given are two-tailed and were considered significant if $\alpha \leqslant 0.05$. The software IBM SPSS Statistics 20.0 was used for all analyses.

Electrophysiological study on hippocampal slices

Experiments were performed on 400-µm thick hippocampal slices prepared from control and transgenic mice as described previously (Chepkova *et al.*, 2012). Slices were maintained in a submersion type recording chamber at 32°C and perfused at a flow rate of 2–2.5 ml/min with artificial CSF containing 125 mM NaCl, 1.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, 2.4 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM D-glucose and saturated with 95%O_2/ 5%CO_2 gas mixture. A glass recording electrode filled with artificial CSF was positioned in the stratum radiatum of area CA1 of the hippocampus to record field excitatory postsynaptic potentials (EPSPs) evoked by stimulation of the Schaffer collateral-commissural system. Bipolar stimulating electrodes were placed in the stratum radiatum at the CA2–CA1 border. After the initial testing of stimulus–response relationships, stimulus intensity was adjusted to induce field EPSPs with the amplitude of 30–50% of its value threshold for generation of population spikes. The standard experimental protocol included 20–30 min control recording with test stimuli applied every 30 s followed by high frequency stimulation (HFS) (two 1-s trains at 100 Hz and double intensity) and 90 min post-HFS recording. Signals were amplified, digitized at 10 kHz, and recorded on a PC using pClamp software (Molecular Devices). Ten consecutive responses (5 min recordings) were averaged off-line and the averaged field EPSP slopes were measured by straight line fitting. All measurements were normalized to the average slope value through the control period (baseline) and expressed in per cent of baseline. The magnitude of field EPSP potentiation was determined by averaging the first (initial potentiation) and the last (long-term potentiation, LTP) 20 min periods of post-HFS recordings. The data are presented as mean ± standard error of the mean (SEM). Statistical analysis of the data (one- and two-way ANOVA, *t*-test, chi-square and Fisher's exact tests) was performed by the GraphPad Prism 5 for Windows (GraphPad Software, San Diego, California, USA).

Isolation of Aβ-S8C dimers

Cell-secreted Aβ-S8C dimers were obtained from the supernatants of confluent CHO-APP-Aβ-S8C cells that were induced with 1 µg/ml doxycycline (Muller-Schiffmann *et al.*, 2011). The conditioned medium was harvested after 24 h and Aβ-S8C was immunoprecipitated with IC16 monoclonal antibody, which had been covalently coupled to NHS-sepharose (GE Healthcare). After washing with PBS, Aβ-S8C was eluted with 100 mM triethanolamine and neutralized with 100 mM Tris HCl, pH 8.0. In this manner, ~95% of Aβ-S8C was precipitated from the conditioned medium. Synthetic $Aβ_{42}$-S8C was obtained from JPT and dissolved to 1 mg/ml in hexafluoroisopropanol (HFIP). Ten micrograms of $Aβ_{42}$-S8C were evaporated, resolved in dimethyl sulphoxide (DMSO, final concentration: 100 µM) for 30 min at room temperature with agitation and oxidized to dimers in DMEM/F12 medium without FCS and phenol red (Gibco, life technologies) for 30 min at room temperature (final concentration: 2 µM). The cellular secreted and synthetic Aβ-S8C samples were then subjected to size exclusion chromatography using a HiLoad 16/60 Superdex 75 prep grade column (GE Healthcare) and eluted at 1 ml/min with 10 mM ammonium acetate pH 8.5. Calibration of the column was performed using Dextran blue (2000 kDa) and albumin (66 kDa; both from Sigma) as well as dimeric Aβ-1-16-S8C-GB1 (18.8 kDa) and monomeric Aβ-1-16-GB1 (9.4 kDa) that have been described in Dornieden *et al.* (2013) and were chosen to allow optimal comparison of the separated amyloid-β species. Fractions of 1 ml were collected. To prevent loss of the dimers by attaching to the surface of the vial, every second fraction was collected in vials, which already contained 500 µl of Neurobasal® medium supplemented with B27 (Gibco, Life Technologies), leading to a final concentration of ammonium acetate of 6.67 mM. Neighbouring fractions were eluted without medium and lyophilized fractions of the cellular secreted Aβ-S8C or 10 µl of the fractions obtained from the synthetic $Aβ_{42}$-S8C were analysed by Tricine-SDS-PAGE and western blot as described previously (Podlisny *et al.*, 1995). Fractions containing solely dimeric Aβ-S8C were pooled and quantified by ELISA. All amyloid-β samples were snap-frozen in liquid nitrogen and stored at −80°C prior to biological assessment.

Whole cell patch clamp

Cultures of primary cortical neurons were prepared from C57BL/6 J mouse foetuses at embryonic Day 18 (E18) as described previously (Mohrmann *et al.*, 2003). Neurons were grown in Neurobasal® A medium (Gibco, Life Technologies) supplemented with 2% B27, 100 U/ml penicillin, 100 µg/ml streptomycin, and GlutaMAX™ (Gibco, Life Technologies) on glass coverslips coated with 1 mg/ml poly-L-ornithine.

After 10 days *in vitro* the neurons were incubated with varying amounts of purified Aβ-S8C dimers with equivalent volumes of 33% Neurobasal A/B27, 6.67 mM ammonium acetate being used as a control. AMPA receptor-mediated miniature EPSCs were recorded from cells after 4 days of incubation. Using standard patch-clamp procedures, whole-cell recoding mode was established by monitoring capacitance changes using an oscilloscope. AMPA miniature EPSCs were isolated by addition of tetrododoxin to block action potentials, and by addition of gabazine and by the presence of Mg^{2+} (holding potential: −60 mV) to block GABAA receptors and NMDA receptors, respectively (Muller-Schiffmann *et al.*, 2011).

Results

Expression of high amounts of soluble dimeric amyloid-β in tgDimer mice

To investigate the isolated effects of amyloid-β dimers *in vivo*, we generated a transgenic mouse line, termed tgDimer mouse. In this mouse, the transgene for the full-length human APP751 includes the familial Swedish mutation (APP-K670N/M671L) for inducing a higher rate of amyloid-β processing (Cai *et al.*, 1993) and the amyloid-β dimer-stabilizing mutation (APP-S679C), here, for reasons of clarity termed Aβ-S8C. In the tgDimer mouse, transgenic mutant APP under the control of the neuron-specific *Thy1* promoter was ∼7-fold overexpressed in relation to endogenous APP of age-matched wild-type mice (Fig. 1A). Whereas processing of APP, as well as β- and α-secretase activity was not changed within tgDimer mice in comparison to APP23 mice (Fig. 1B), production of the APP intracellular domain from active membrane preparations of brain homogenates from tgDimer mice was reduced (Supplementary Fig. 1). We also observed significant amounts of dimeric APP and dimeric APP C-terminal fragments in addition to the monomeric forms in brain homogenates from tgDimer mice under non-reducing conditions (Fig. 1B). Interestingly, the tgDimer mice generated very high amounts of disulphide stabilized amyloid-β dimers that could be dissociated to monomers when applying reducing buffers (Supplementary Fig. 2), but not monomers or other SDS-stable low-*n* oligomers (trimers or tetramers) (Fig. 1C). Amyloid-β secreted from the 7PA2 cell line was initially used as a positive control for low-*n* oligomeric amyloid-β. However, the corresponding western blot signals of amyloid-β multimers are actually now thought to define N-terminal elongated amyloid-β monomer species, thus explaining the slightly different migration speed of the S8C-dimer and the 7PA2-derived amyloid-β species (Welzel *et al.*, 2014). We did not observe analogous signals to these elongated amyloid-β monomer species in tgDimer mice (Fig. 1C) suggesting that there are no N-terminally elongated amyloid-β species of significant amount generated in tgDimer mice.

Absence of insoluble amyloid-β aggregates and amyloid-β plaque pathology in tgDimer mice

Amyloid-β_{40} and amyloid-β_{42} levels in whole extracts of brain homogenates from tgDimer mice were quantified by ELISA in which the affinity of the capturing antibody towards amyloid-β was only slightly influenced by the S8C mutation, most likely due to a sterically limited binding of the antibodies to their epitope that resided in near proximity to the disulphide bridge of the dimer (Supplementary Fig. 3). The levels of amlyloid-β_{40} and amlyloid-β_{42} in the brain remained constant throughout the lifetime (as assayed at 3–24 months) of tgDimer mice (Fig. 2A) and were similar to those detected in young APP23 mice that had not yet deposited plaques (Maia *et al.*, 2013; Morales-Corraliza *et al.*, 2013). Also, the amlyloid-β_{42}/amlyloid-β_{total} ratio was not significantly changed throughout the lifespan, similar to wild-type mice (Supplementary Fig. 4), indicating no accumulation of amyloid-β and especially amyloid-β_{42}, in contrast to other Alzheimer's disease animal models (Sturchler-Pierrat *et al.*, 1997; Chishti *et al.*, 2001).

Consistent with the constant amyloid-β levels, no insoluble amyloid-β or amyloid-β plaques were detected in aged tgDimer mice after performing a sequence of biochemical extraction procedures (Fig. 2B and Supplementary Fig. 5) or immunohistochemistry (Fig. 2C). Of note, similar to reports of many other publications (for review see Wirths and Bayer, 2012) we observed intracellular immunoreactivity against the amyloid-β epitope in neocortex and hippocampus between 12 and 24 months of age (Fig. 2C). However, intraneuronal amyloid-β has been demonstrated to be common in human brain (Blair *et al.*, 2014) and not to be a predictor of brain amyloidosis (Wegiel *et al.*, 2007). Accordingly, after biochemical purification of brain extracts, in the tgDimer mouse, irrespective of their age and despite a high APP expression levels, we never saw amyloid-β immunoreactivity in the formic acid fraction of the tgDimer mouse that would correspond to amyloid-β deposited as amyloid (Roher *et al.*, 1993) (Fig. 2B). This contrasts with the results of the fractionation of brain homogenates of CRND8 mice (Fig. 2B) that display severe and early amyloid-β plaque pathology, here used as a technical positive control (Chishti *et al.*, 2001) and for example to another mouse model where no plaques, but insoluble amyloid-β was detected (Tomiyama *et al.*, 2010). Moreover, in contrast to tgDimer mice, free TBS-soluble amyloid-β was completely lacking in brain homogenates from CRND8 control mice, further demonstrating the high solubility of Aβ-S8C dimers. Of note, it has been shown that both in young mice of Alzheimer's disease models or wild-type mice, high amounts of amyloid-β are detected in the SDS-fraction (Kawarabayashi *et al.*, 2001; Hong *et al.*, 2011) indicating that Aβ-S8C dimers do not exhibit different solubility patterns.

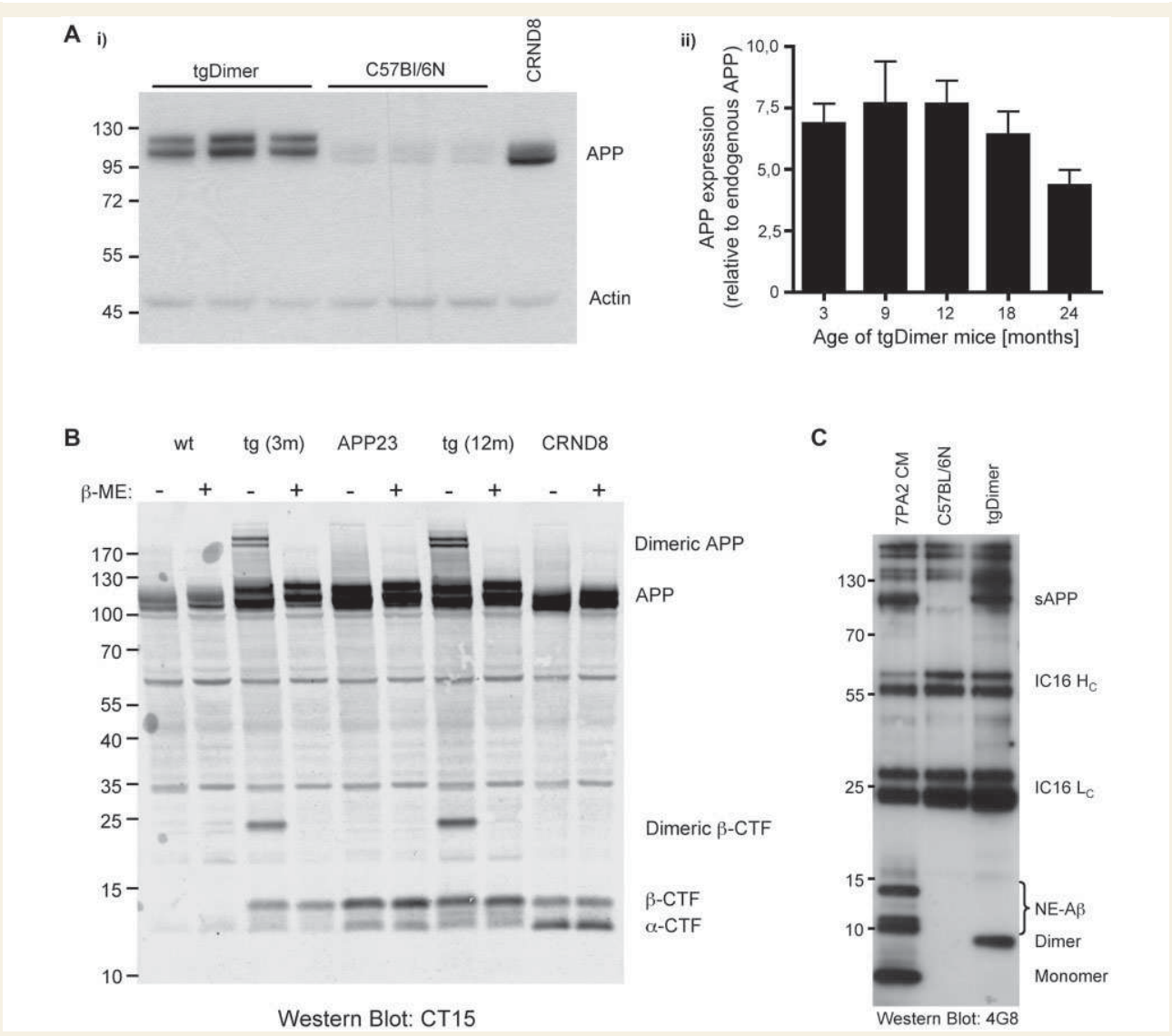

Figure 1 Generation of high levels of Aβ-S8C dimers from 7-fold overexpressed APP in tgDimer mice. (**A**) Expression levels of APP751swe-Aβ-S8C in tgDimer mice compared to endogenous mouse APP-levels. (**i**) Typical western blot (CT15) of 30 μg crude brain homogenates of 12-month-old tgDimer and C57BL/6N mice or a 7-month-old CRND8 control mouse under reducing conditions; (**ii**) densitometric analysis of APP levels in brains of ageing tgDimer mice in relation to endogenous APP expression in aged-matched wild-type C57BL6/6N mice. Mean + SEM; *n* = 3 for each age group. (**B**) Processing of APP751-Aβ-S8C. Western blot (CT15) of 30 μg crude brain homogenates from 3-month and 12-month-old tgDimer (tg) mice that were separated under non-reducing or reducing conditions. Brain homogenates of 3-month-old wild-type C57BL/6N (wt), APP23- and CRND8-mice served as control. (**C**) Exclusively dimeric Aβ-S8C but no monomeric amyloid-β was immunoprecipitated by IC16 from TBS/1% Triton™ X-100 extracts of 100 μl of brain homogenate of a 1-month-old tgDimer mouse (western blot: 4G8). As controls, immunoprecipitated amyloid-β from C57BL/6N mice or conditioned medium of 7PA2 cells are shown. The prominent signals detected in conditioned medium of 7PA2 cells likely represents N-terminal elongated amyloid-β monomers (NE-Aβ) (Welzel *et al.*, 2014). CTF = C-terminal fragment; ME = mercaptoethanol.

Absence of endogenous tau-hyperphosphorylation and neuroinflammation in brains of tgDimer mice

The high amount of soluble amyloid-β dimers in tgDimer mice allowed the differentiation of neuropathological consequences triggered by either insoluble amyloid-β species (fibrils and plaques) or soluble amyloid-β dimers. We did not detect a significant change in phosphorylation of endogenous tau in biochemical analysis of brain homogenates (Fig. 3A and Supplementary Fig. 6A), nor in immunohistochemical staining (data not shown). We also found no signs of neuronal death and neuroinflammation with astrogliosis or microgliosis in the tgDimer mouse (Fig. 3B, C and

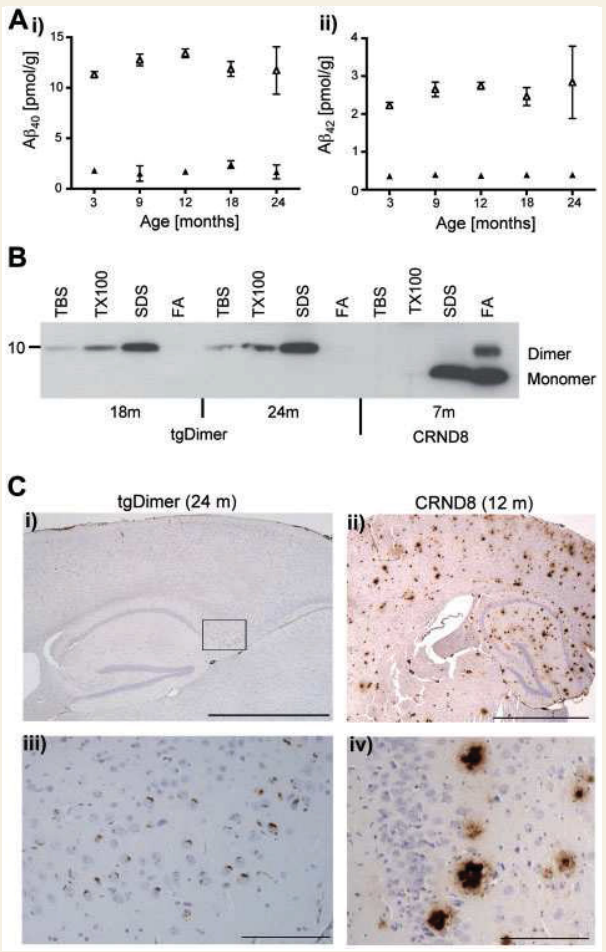

Figure 2 Absence of insoluble amyloid-β and amyloid-β plaque pathology in tgDimer mice. (**A**) ELISA quantification of total brain amyloid-β_{40} (**i**) and amyloid-β_{42} (**ii**) levels in tgDimer (open triangle) and age-matched wild-type C57BL/6N (filled triangle) mice (mean ± SEM; $n = 3$ for each age group). (**B**) Western blot of immunoprecipitated amyloid-β derived from a 4-step ultracentrifugation fractionation of brain homogenates of 18- and 24-month-old tgDimer or 7-month-old CRND8 mice (western blot: 4G8; representative image of three independent experiments). The fractions display free Tris-buffered saline soluble amyloid-β (TBS), membrane-bound soluble amyloid-β (TX100), protein-bound soluble amyloid-β (SDS) and insoluble amyloid-β (FA). (**C**) Absence of amyloid-β plaque pathology (**i**) and intracellular staining of an amyloid-β epitope (**iii**) in brain sections from 24-month-old tgDimer mice stained with 6F/3D [$n = 5$; Scale bars = 1 mm (*top row*) and 100 μm (*bottom row*)]. Brain sections of 12-month-old CRND8 mice, which show severe plaque pathology, were used as a control (**ii** and **iv**). FA = formic acid; SDS = sodium dodecyl sulphate; TX100 = Triton™ X-100.

Supplementary Fig. 6B), supporting the notion that neuroinflammatory responses and neuronal death are linked to the presence of amyloid-β species other than amyloid-β dimers, for example to those existing in amyloid-β plaques (Akiyama *et al.*, 2000).

Cognitive impairment in tgDimer mice

In Alzheimer's disease mouse models that develop amyloid-β plaques, learning and memory deficits have been demonstrated as evidence for aberrant amyloid-β pathology leading to cognitive deficits (Chen *et al.*, 2000; Chishti *et al.*, 2001). The fact that synaptic and behavioural deficits have also been observed in the absence of (Chen *et al.*, 2012), or prior to the appearance of plaques (Knobloch *et al.*, 2007; Hamilton *et al.*, 2010; Skaper, 2012), suggests that amyloid-β plaque toxicity may not be the critical or sole mechanism that underlies such deficits.

We tested the tgDimer mice in the Morris water maze, a standard behavioural task used to assess hippocampal dysfunction-related deficits in spatial learning and memory in Alzheimer's disease models (Morris *et al.*, 1982; Stewart *et al.*, 2011). We observed a deficit in learning to escape from the water maze in tgDimer mice that were tested at both 7 and 12 months of age (Fig. 4A). The results of three-way ANOVA revealed significant main effects of day (acquisition) [$F(8,240) = 22.14$ $P < 0.001$] and genotype [$F(1,30) = 28.00$, $P < 0.001$], a trend for a main effect of genotype interacting with age [$F(1,30) = 4.06$, $P = 0.053$], and a significant main effect of age interacting with day (acquisition) [$F(8,240) = 3.17$, $P = 0.002$]. *Post hoc t*-tests revealed significant differences in time to find the platform between adult tgDimer and C57BL/6N mice on Day 5 ($P = 0.002$), Day 6 ($P = 0.004$), Day 7 ($P = 0.029$) and Day 8 ($P = 0.024$) as well as between aged tgDimer and C57BL/6N mice on Day 3 ($P = 0.001$), Day 7 ($P = 0.006$), Day 8 ($P = 0.007$) and Day 9 ($P = 0.006$), indicating superior learning in the C57BL/6N mice. Unlike the C57BL/6N, at 12 months the transgenic mice showed virtually no savings from the learning level achieved at 7 months and did not exhibit a learning curve over the 9 days (36 trials) of testing (Fig. 4A). This poor performance of the tgDimer mice at 12 months retest could have hypothetically been influenced by the worse level achieved at the end of testing at 7 months, providing a retest advantage to the C57BL/6N mice. However, such an advantage cannot account for their failure to exhibit any improvement over the 36 trials. Additional analysis of the slope of the performance over trials confirmed that at 12 months of age the tgDimer mice did not display a significant improvement over trials (by paired sample *t*-test between first and last acquisition day $P > 0.05$) whereas they did so at 7 months ($P < 0.001$). The C57BL/6N mice displayed significant learning curves (slope) at both 7 and 12 months of age ($P < 0.001$ and $P = 0.016$, respectively). Thus, only the tgDimer mice displayed an ageing-related progression in the severity of cognitive decline, as indicated by failure to exhibit a trial-dependent improvement in performance at 12 months of age.

The tgDimer mice also failed to show significant savings (retention/memory) for the location of the hidden platform

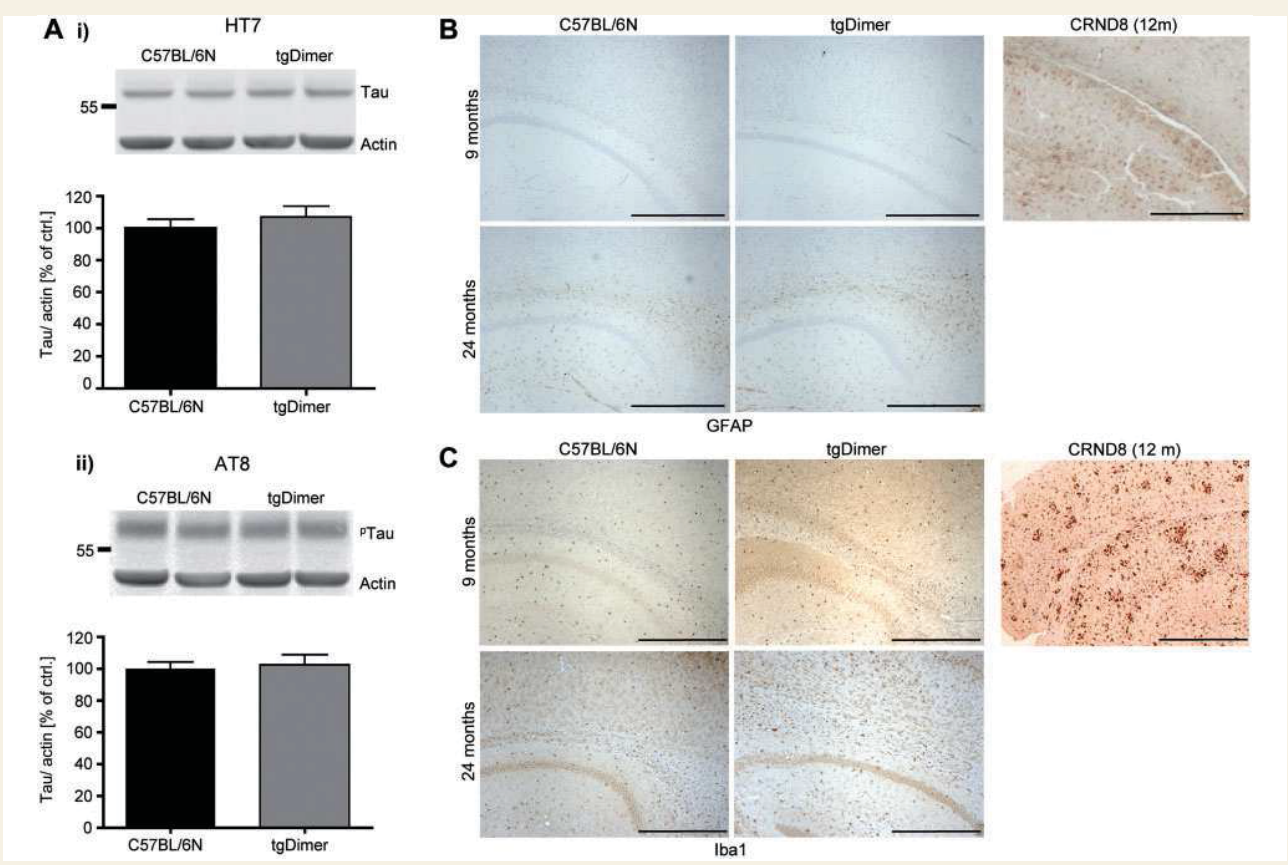

Figure 3 Pathological consequences in tgDimer mice. (**A**) Absence of hyperphosphorylated endogenous tau in tgDimer mice. *Top*: Western blots of 30 μg of crude brain homogenates from 13-month-old tgDimer or wild-type C57BL/6N mice stained with antibodies recognizing endogenous total tau (**i**, HT7) or tau that was phosphorylated at Ser202 and Thr205 (**ii**, AT8). Detection of actin was used as internal control (*bottom*). Densitometric analysis of tau signals were normalized to actin [mean + SEM; $n = 8$ (C57BL/6N), $n = 12$ (tgDimer)]. (**B** and **C**) Absence of neuroinflammation in tgDimer mice. Sagittal brain sections of adult (9 months) or old (24 months) tgDimer and C57BL/6 N mice were stained with markers for astroglia (GFAP) or microglia (Iba1). Cortical and hippocampal regions are shown. In comparison to age-matched wild-type mice no increased neuroinflammation was observed in tgDimer mice ($n = 3$, Scale bars = 500 μm). Brain sections of 12-month-old CRND8 mice served as controls (*right*).

in the probe trial at both ages (Fig. 4B). Unlike the C57BL/6N controls, they did not spend a significant amount of time in the former safe platform quadrant Q3, indicating poor retention. The two-way ANOVA for the results at 12 months revealed a significant main effect of genotype and quadrants for the aged 12-month-old mice [$F(1,17) = 4.68$, $P = 0.045$ and $F(1,17) = 9.52$, $P = 0.007$, respectively], as well as for the adult 7-month-old mice [genotype, $F(1,19) = 4.79$, $P = 0.041$, and quadrant, $F(1,19) = 14.36$, $P = 0.001$, respectively]. *Post hoc* paired sample *t*-test between Q3 versus pooled non-reinforced quadrants showed that only the C57BL/6N animals spent significantly more time in Q3 than in the pooled non-reinforced quadrants (C57BL/6N adult $P = 0.001$; aged $P = 0.029$). In fact, neither the adult nor aged tgDimer animals spent a significant amount of time in the former safe quadrant Q3.

These behavioural changes indicate deficient learning and memory in the tgDimer mouse. These deficits are unlikely to be a result of motoric or sensory deficits, as there were no significant differences between C57BL/6N and tgDimer mice in swimming speed and in success in escaping onto the platform when it was visible; also, neither speed of swimming nor escape onto a visible platform showed an age-dependent decline (Fig. 4A and C). These results demonstrate that hippocampus-related cognitive deficits occur in an animal that expresses amyloid-β dimers in the absence of plaques in the adult mouse and that these impairments are not compensated with age. Instead, some evidence indicates that they progress over age in this preparation.

tgDimer mice show impaired hippocampal synaptic plasticity

Synaptic plasticity is hypothesized to be a neurophysiological substrate for learning and memory. Hippocampal LTP has been found to be impaired in different transgenic

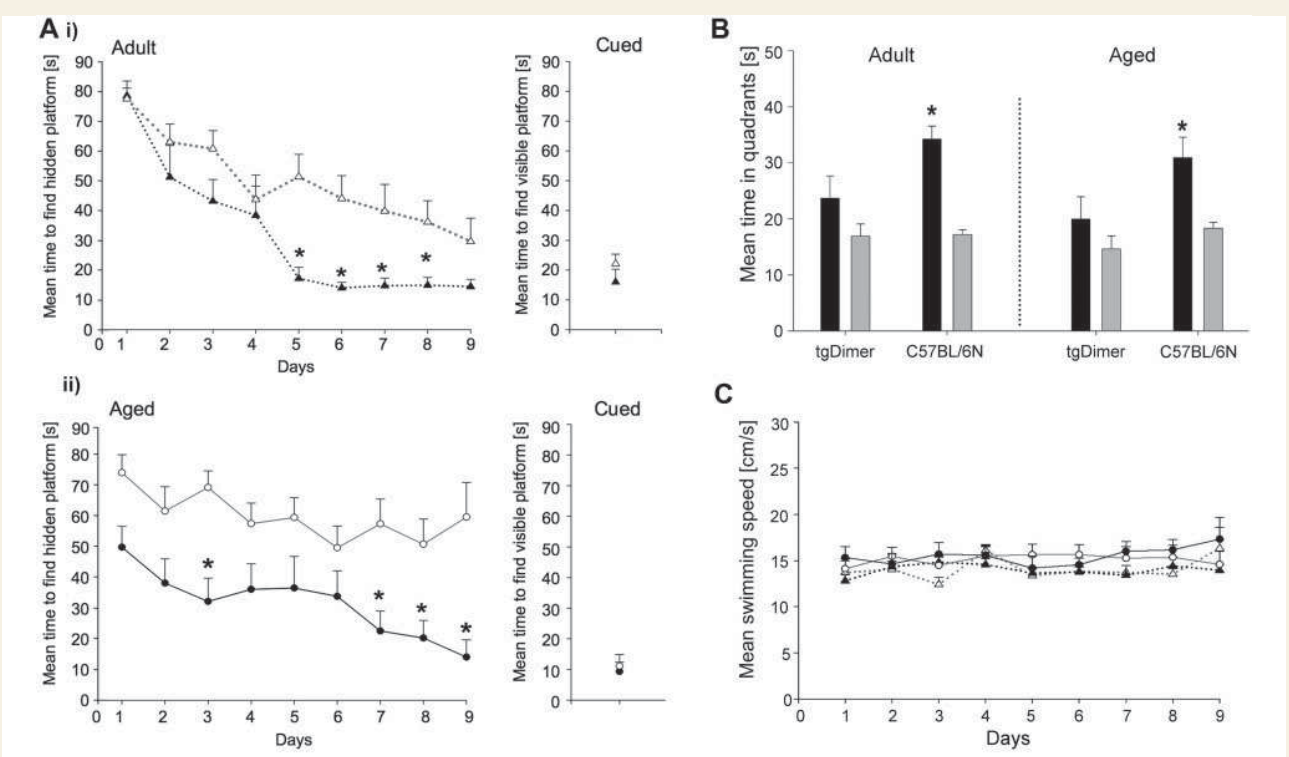

Figure 4 tgDimer mice display age-dependent cognitive deficits. (**A**) *Left*: Morris water maze testing of same mice at 7 (**i**) and 12 (**ii**) months. TgDimer (adult: $n = 12$, open triangle; aged, $n = 12$, open circle); C57BL/6N (adult: $n = 9$, filled triangle; aged, $n = 8$, filled circle). Each point represents the mean time to find the hidden platform, four trials per day, + SEM (*$P < 0.05$ C57BL/6N versus tgDimer). Note that unlike the C57BL/6N, at 12 months the tgDimer mice showed almost no savings from the level of performance attained at 7 months and did not exhibit a learning curve over the 9 days (36 trials) of testing. *Right*: One 'cued' trial with visible platform. Note that both groups exhibited efficient swimming to the visible platform. (**B**) Retention of memory was tested by a 90 s probe trial with the platform removed. Black bars represent the mean time occupancy + SEM in the former rewarded target quadrant grey bars represent total time spent in the three non-reinforced quadrants (*$P < 0.05$, significantly more time in reinforced versus non-reinforced quadrants). These data exclude the time spent in the outer ring of the pool, which consists mainly of thigmotactic swimming along the edge. (**C**) Swimming speed of the aged and adult tgDimer and C57BL/6N mice.

Alzheimer's disease models (Marchetti and Marie, 2011; Spires-Jones and Knafo, 2012) and been shown to be correlated with the degree of impairment in learning of the Morris water maze in the old rodent (Schulz *et al.*, 2002). We compared the characteristics of basal neurotransmission and plasticity in the Schaffer collateral-CA1 synaptic system of hippocampal slices from age-matched wild-type C57BL/6N and tgDimer mice. Analysis of input-output relationships using two-way ANOVA showed no significant difference in basal neurotransmission between wild-type and tgDimer mice at the ages of 6–8 months (adult) and 15–17 months (aged) (Fig. 5A). In the majority of hippocampal slices prepared from wild-type and tgDimer mice of the two ages, HFS of the synaptic input resulted in potentiation of evoked field EPSPs that either persisted (sustained LTP) or gradually decayed (transient LTP) towards the end of the 90-min observation period. There was no significant difference between genotypes and ages in the occurrence of sustained or transient LTP ($P > 0.05$ Fisher exact test, Table 1). The time course of the HFS-induced changes in CA1 postsynaptic responses (Fig. 5B) shows a significant deficit of synaptic plasticity in the tgDimer hippocampus: slices from adult and aged wild-type mice maintained the potentiation at the level of $150 \pm 8\%$ and $145 \pm 5\%$ of baseline, respectively, whereas in slices from age-matched tgDimer mice the potentiation decreased to $126 \pm 7\%$ and $117 \pm 3\%$ of baseline, respectively (the difference is significant at $P = 0.023$ for adult and $P < 0.0001$ for aged mice, unpaired *t*-test). Comparative analysis of characteristics of sustained and transient LTP showed that the decreased amount of potentiation in tgDimer mice is largely due to the lower magnitude of sustained LTP (Table 1). Besides, tgDimer mice showed more pronounced age-related decrease in the maintenance of sustained LTP: in slices from aged mice the amount of potentiation decreased by 16% (not significant) in wild-type and by 25% in tgDimer ($P = 0.0002$, unpaired *t*-test) compared to the initial level. The lower levels of the initial potentiation in the tgDimer slices (Fig. 5B and Table 1) and the more pronounced decline of potentiation with time suggest an impairment of both induction and maintenance mechanisms of hippocampal LTP in tgDimer mice.

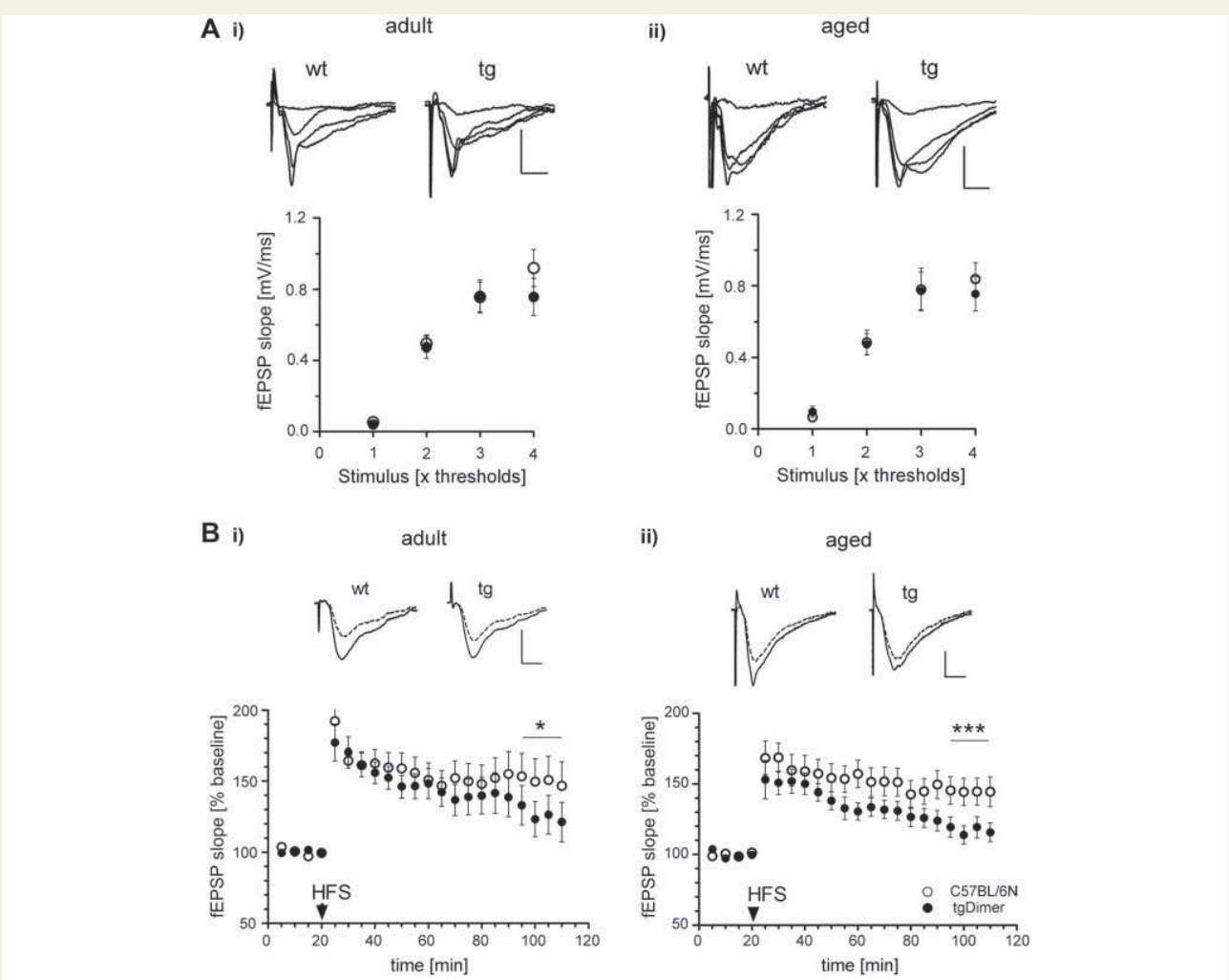

Figure 5 Impairment of LTP in the Schaffer collateral-CA1 synaptic system in tgDimer mice. The figure shows the characteristics of basal neurotransmission (**A**) and long-term potentiation (**B**) in the hippocampus of control (C57BL/6N, open circles) and tgDimer (tg, black circles) mice at the age of 6–8 (**i**) and 15–17 (**ii**) months. In **A**, the plots show the average stimulus–response relations in the correspondent age groups, representative examples of evoked field EPSPs at increasing stimulus intensities are shown in the *upper* part. The numbers on the *x*-axis mean the stimulus strength increasing several times compared to the threshold voltage. Each trace is an average of three responses to a given stimulus intensity. Calibrations: vertical −1 mV, horizontal −5 ms. The plots in **B** show the mean time course of hippocampal LTP in the two age groups. The events of high-frequency stimulation (HFS; two 1-s trains at 100 Hz) are marked by arrows. The upper parts show the representative examples of pre- (dotted lines) and 90 min post-HFS (solid lines) field EPSPs from the experiments summarized in the plots. Calibrations: vertical −0.5 mV, horizontal −5 ms. Mice: $n = 4$ per condition and slices: adult wild-type: $n = 29$; adult tgDimer: $n = 23$; aged wild-type (wt): $n = 23$, aged tgDimer: $n = 24$. The data are presented as mean ± SEM. A significant difference between wild-type and tgDimer mice in the potentiation magnitude within the last 20 min of recording is marked by asterisks: $*P = 0.023$, $***P < 0.0001$ (two-tailed Student's *t*-test).

Purified Aβ-S8C dimers are synaptotoxic in the picomolar range

Amyloid-β-induced impairment of synaptic functions has been attributed to the removal of AMPA receptors from synapses (Kamenetz *et al.*, 2003; Hsieh *et al.*, 2006). To validate the role of Aβ-S8C dimers in the impairment of LTP in the tgDimer mice we measured the decrease of AMPA receptor-mediated miniature EPSCs in primary mouse cortical neurons. We applied size exclusion chromatography to purify either naturally secreted Aβ-S8C dimers that were immunoprecipitated from conditioned medium of Aβ-S8C dimer-secreting cells, or oxidized synthetic $Aβ_{42}$-S8C dimers (Supplementary Fig. 7). Although naturally secreted Aβ-S8C dimers consist mainly of amyloid-$β_{40}$ and thus differed in their C-termini from the synthetic $Aβ_{42}$-S8C dimers, both amyloid-β dimer preparations mediated synaptotoxicity in the pM range and showed a significant decrease in both miniature EPSC frequency and amplitude (Fig. 6). Consistent with previous reports on different biological activities of synthetic versus secreted amyloid-β species (Jin *et al.*, 2011; Reed *et al.*, 2011), naturally secreted

Table 1 Sustained and transient LTP in wild-type and tgDimer mice

Mouse/slices tested (*n*)	Sustained LTP		Transient LTP	
	Initial	Late	Initial	Late
Adult wild-type *n* = 29	201 ± 7%	202 ± 9% *n* = 13	169 ± 6%	87 ± 3% *n* = 10
Adult tgDimer, *n* = 23	173 ± 4%**	171 ± 7%* *n* = 11	178 ± 9%	91 ± 5% *n* = 9
Aged wild-type *n* = 23	174 ± 7%##	158 ± 5%#### *n* = 16	165 ± 12%	98 ± 4% *n* = 4
Aged tgDimer *n* = 24	161 ± 6%	136 ± 3%***.#### *n* = 14	173 ± 11%	92 ± 4% *n* = 5

The table presents the average magnitudes of field EPSP slope potentiation in per cent of baseline (mean ± SEM) calculated for the periods 5–20 min (initial) and 75–90 min (late) after two 1-s trains of HFS delivered to the Schaffer collateral-commissural input. The terms 'adult' and 'aged' refer to male mice ($n = 4$ in each group) at the age of 6–8 months and 15–17 months, respectively. Sustained LTP was determined as a persistent enhancement of field EPSP slope observed for 90 min post-HFS. Transient LTP was characterized by a gradual decline of potentiated field EPSP to the baseline within 60–90 min post-HFS. According to the chi-square test the difference in the HFS outcome (sustained, transient or no LTP) in slices from wild-type and tgDimer mice is not significant. Asterisks mark significantly lower magnitudes of sustained LTP in tgDimer hippocampus compared to the age-matched wild-type: $^{*}P = 0.0201$; $^{**}P = 0.0014$; $^{***}P = 0.0004$, Student's *t*-test. Hash signs mark significant age-related decrease in the potentiation magnitude in the hippocampus of both wild-type and tgDimer mice: $^{\#\#}P = 0.0057$; $^{\#\#\#\#}P < 0.0001$, Student's *t*-test.

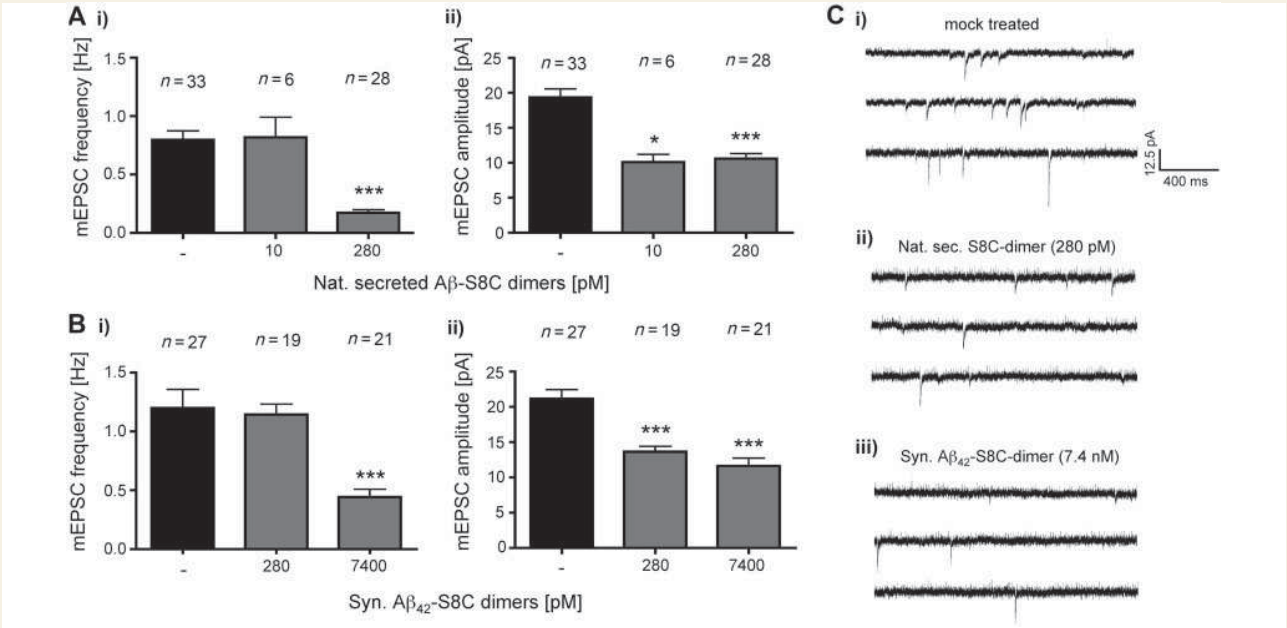

Figure 6 Purified Aβ-S8C dimers are highly synaptotoxic at picomolar concentrations. Miniature EPSCs were recorded after incubation of the cells for 4 days with isolated natural secreted (Nat. sec.) Aβ-S8C dimers (**A**) and synthetic (Syn.) $A\beta_{42}$-S8C dimers (**B**). Both amyloid-β dimer preparations mediated synaptotoxicity in the pM range and showed a significant decrease in both miniature EPSC frequency (**i**) and amplitude (**ii**). Error bars indicate standard deviation, ($^{*}P < 0.05$, $^{***}P < 0.001$ by Kruskal-Wallis tests with Dunn's *post hoc* tests). (**C**) Representative recordings of miniature EPSCs derived from individual neurons that were mock treated (**i**) or incubated with 280 pM of isolated natural secreted (**ii**) or 7.4 nM of synthetic (**iii**) Aβ-S8C dimers.

Aβ-S8C dimers of the same amount were~30-fold more synaptotoxic than the fully synthetic counterpart.

Aβ-S8C dimers can be sequestered by amyloid-β plaques

It has been hypothesized that insoluble amyloid-β plaques are an immediate cellular defence against amyloid-β oligomer toxicity (Cheng *et al.*, 2007; Treusch *et al.*, 2009). To determine whether our paradigmatic Aβ-S8C dimers are able to integrate into existing amyloid-β plaques, we performed a genetic seeding experiment where we crossed tgDimer mice into CRND8 mice that develop amyloid-β plaques starting at 3 months of age (Chishti *et al.*, 2001). These double-transgenic mice generate both Aβ-S8C dimers as well as aggregation-prone wild-type human amyloid-β, but they did not develop differently

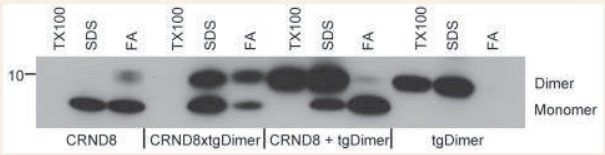

Figure 7 Amyloid-β dimers can be sequestered by amyloid-β plaques. Fractionation of TX100-soluble, SDS-soluble and formic acid-extractable insoluble amyloid-β derived from 20 µl of 6-month-old CRND8 or CRND8xtgDimer double transgenic mice, or control. As a control, 20 µl of CRND8 brain homogenate was spiked with 300 µl of tgDimer brain homogenate (CRND8+ tgDimer) immediately prior to fractionation. The double transgenic CRND8 × tgDimer mouse showed strong signals of dimeric amyloid-β in the formic acid (FA) fraction whereas no significant increase of amyloid-β dimers were detected in the formic acid fraction of the spiked control in comparison to CRND8 alone. Western blot: 4G8; representative image of three independent experiments.

from either parent and did not show obvious clinical symptoms at the age of 6 months. In this double transgenic mouse line, Aβ-S8C dimers were detected in the insoluble formic acid fraction, co-purifying with insoluble wild-type amyloid-β and, thus were indeed pulled down along with genuinely insoluble material. A control fractionation of brain homogenate of tgDimer mouse that was spiked with CRND8 brain homogenate immediately before fractionation was devoid of dimeric Aβ-S8C in the formic acid fraction, demonstrating that Aβ-S8C dimers were not artificially co-purified by insoluble wild-type amyloid-β (Fig. 7). We did not detect any Triton™ X-100-soluble Aβ-S8C dimers in the double transgenic mice, indicating an efficient sequestration of soluble Aβ-S8C dimers into the amyloid-β plaques. This finding demonstrates that Aβ-S8C dimers can principally be incorporated into amyloid-β plaques but cannot itself initiate plaque formation in the absence of insoluble amyloid-β seeds.

Discussion

Our results show that the tgDimer mouse, which expresses a single, distinct amyloid-β-conformation, the amyloid-β dimer, exhibits impairments in learning and memory, as well as in neuroplasticity, all this in the absence of insoluble amyloid-β or amyloid-β plaque deposition. Behavioural deficits and changes in neuroplasticity have also been reported prior to the appearance of plaques in other Alzheimer's disease mouse models, indicating that toxicity mediated by insoluble amyloid-β may not be the critical or sole mechanism that underlies such deficits in Alzheimer's disease (Knobloch *et al.*, 2007; Hamilton *et al.*, 2010; Chen *et al.*, 2012; Skaper, 2012). Our results therefore suggest that Aβ-S8C dimers may play a causal role in the cognitive deficits preceding the onset of significant amyloid-β plaque accumulation.

In terms of the level and cellular specificity of transgene expression, the tgDimer mouse is comparable to the well characterized APP23 Alzheimer's disease mouse model (Sturchler-Pierrat *et al.*, 1997), as both of these models use the C57BL/6 background to express APP751 including the Swedish mutation driven by the neuron-specific *Thy1* promoter. Both models yield a ~7-fold overexpression of APP and generate comparable levels of soluble amyloid-β. Despite these similarities, the APP23 mouse displays a spectrum of Alzheimer's disease-like neuropathology including amyloid-β plaque deposition at ~6 months of age, followed by massive gliosis and incipient hyperphosphorylation of tau. In contrast, all of these symptoms, which are comparable to later stages of Alzheimer's disease in humans, are absent in tgDimer mice. The mutation introduced to stabilize the amyloid-β dimer neutralizes the amyloid-β plaque promoting effect of the Swedish mutation. This remarkable difference allows us to put major neuropathological hallmarks of Alzheimer's disease pathology into a causal relationship: our results suggest that neuroinflammation and tau hyperphosphorylation are likely to be triggered by soluble or insoluble higher structured amyloid-β assemblies, which are not displayed by our mouse model, rather than amyloid-β dimers. A potential caveat could be that endogenous mouse tau in tgDimer mice is not a target of dimeric human amyloid-β whereas human tau could well be, as tau pathology, like tau tangles, has never been observed in an single transgenic APP-overexpressing mouse model for Alzheimer's disease with endogenous tau (Morrissette *et al.*, 2009).

The high amount of dimeric Aβ-S8C in the brain homogenates of tgDimer mice was surprising. In accordance with our cell culture model of Aβ-S8C (Muller-Schiffmann *et al.*, 2011) and another work expressing APP with a cysteine mutant at position 28 of amyloid-β *in vitro* (Scheuermann *et al.*, 2001), we observed dimerization already at the level of APP. Formation of APP dimers early after protein biogenesis suggests that Aβ-S8C dimers may be cleaved from APP as a preformed dimer thus, explaining the high amounts of dimeric β-C-terminal fragment and Aβ-S8C dimers measured in the absence of monomers. Early dimerization of APP in our model may also prevent non-specific oxidation with other cellular factors via the free cysteine. This is also evidenced by the clear absence of any non-specific western blot signals under non-reducing conditions in comparison to APP23 (Fig. 1B). The dimers purified from the Aβ-S8C cell culture supernatant were highly synaptotoxic, therefore likely accounting for the effects observed in our LTP and behavioural analysis. However, in tgDimer mice, as in every other Alzheimer's disease model that overexpresses APP, we cannot completely exclude potential effects due to a high abundance of APP or its derivatives, of which some, like the β-C-terminal fragment, APP intracellular domain and caspase cleaved APP fragments, also have been attributed to contribute to toxicity (Yankner *et al.*, 1989; Kinoshita *et al.*, 2002; Lu *et al.*, 2003).

The synaptotoxic potential of the stabilized amyloid-β dimers is obvious in the tgDimer mice through their exhibition of age-related impairments of learning and memory (Fig. 4), which may be associated with a synaptic plasticity deficit. Alterations in hippocampal LTP ranging from no change to a dramatic impairment were reported in previous studies on different mouse models of Alzheimer's disease (Marchetti and Marie, 2011; Spires-Jones and Knafo, 2012). The neurotoxic effects of the Aβ-S8C dimers not only in the hippocampus, but also in the cortex and ascending monoaminergic systems responsible for cortical arousal, could be a source of the progressive neural damage resulting in age-related behavioural/neural deficits. This reflects the situation in APP23 mice, in which age-dependent cognitive decline has been observed already in mice younger than 6 months, before the onset of plaque pathology (Van Dam *et al.*, 2003). We propose that the state of pathology in the tgDimer mouse resembles in many key aspects, an early stage of Alzheimer's disease or mild cognitive impairment, at which point the classical Alzheimer's disease neuropathology has not yet developed and synaptotoxic amyloid-β low-*n* oligomers are biochemically (see Fig. 2B, TBS fraction), but not yet microscopically detectable (Selkoe, 2008).

Purified Aβ-S8C dimers, whether naturally secreted or synthetic, were extremely synaptotoxic in the picomolar range (Fig. 6), clearly confirming that they had been folded into a bioactive conformation and thus reached at least a similar level of activity to synthetic Aβ-S26C dimer preparations published before (Hu *et al.*, 2008; Shankar *et al.*, 2008; O'Nuallain *et al.*, 2010; O'Malley *et al.*, 2014). However, toxicity of S26C dimers has been reported to be dependent on formation of non-fibrillar, Thioflavin T-positive aggregates that were generated in concentrations $>2.5\,\mu M$ (O'Nuallain *et al.*, 2010). Size exclusion chromatography isolation of Aβ-S8C dimers yielded concentrations in the low nanomolar range, far below the reported critical concentration for Aβ-S26C dimers. Thus, it is unlikely that fibrillar aggregates of Aβ-S8C dimers are the underlying toxic entity. However, this does not exclude the possible existence of soluble meta-stable higher structured oligomers that were formed by building blocks of dimeric amyloid-β.

Of note, synthetic $A\beta_{42}$-S8C dimers were 30-fold less neurotoxic when directly compared to equimolar amounts of the cell-derived secreted Aβ-S8C dimers. This has been observed before with wild-type amyloid-β oligomers (Jin *et al.*, 2011; Reed *et al.*, 2011) and most likely reflects N- or C-terminal modifications within amyloid-β that are dependent on eukaryotic cellular factors and are, therefore, absent in synthetic amyloid-β preparations (Saido *et al.*, 1996). Although we were not able to detect them, small amounts of N-terminal elongated synaptotoxic amyloid-β monomers (Welzel *et al.*, 2014) may have co-migrated and co-eluted from size exclusion chromatography together with the Aβ-S8C dimers and contributed to the synaptotoxic effect. The high synaptotoxic potency of both preparations suggest that the main toxic effects of the amyloid-β dimers are not determined by the C-terminus of the amyloid-β species that formed the dimer, since in contrast to the $A\beta_{42}$-S8C dimer preparations cellular secreted amyloid-β consisted mainly of amyloid-β_{40} (Supplementary Fig. 8). Interestingly, the lowest amounts of amyloid-β dimer preparations were sufficient to affect the amplitude of AMPA-mediated miniature EPSCs, which indicates changes in postsynaptic functions, such as AMPA receptor synaptic density (Lisman *et al.*, 2007). AMPA receptors, which are important for hippocampal synaptic plasticity, have previously been shown to be affected by soluble naturally-secreted amyloid-β oligomers (Shankar *et al.*, 2008). Furthermore, endocytosis of synaptic AMPA receptors by amyloid-β has been linked to synaptic depression and dendritic spine loss (Hsieh *et al.*, 2006). Amyloid-β dimers may therefore represent a trigger for this effect.

The strong synaptotoxicity of the Aβ-S8C dimers is likely a direct consequence of the conformational transition state-stabilizing disulphide bond within the N-terminus of amyloid-β. This domain of amyloid-β plays a crucial role in oligomer/fibril formation and execution of toxic functions, as demonstrated by the fact that both post-translational modifications and naturally occurring mutations within the N-terminal domain increase oligomerization propensity and toxicity (Schlenzig *et al.*, 2009; Ono *et al.*, 2010; Kumar *et al.*, 2012). Antibodies that target the N-terminus, but not those targeting the C-terminus, of amyloid-β were shown to reduce amyloid-β plaque load, but also to avert synaptotoxicity mediated by synthetic amyloid-β oligomers (Bard *et al.*, 2000; Zago *et al.*, 2012). Recently, an intermolecular β-sheet structure has been attributed to the N-termini of amyloid-β oligomers, but not fibrils, and has been shown to be critical in the impairment of LTP (Pan *et al.*, 2011; Haupt *et al.*, 2012). This structural feature of synaptotoxic amyloid-β oligomers may be mimicked by the covalent stabilization caused by the N-terminal cysteine of Aβ-S8C dimers. Therefore, Aβ-S8C dimers may particularly be interesting for screening of drugs targeting the synaptotoxic N-terminal domain of oligomers *in vitro* and *in vivo*.

The mutation of serine to cysteine at codon 8 of amyloid-β was selected purposefully after extensive molecular modelling with the aim of conserving a structure resembling that of the wild-type dimer as closely as possible, while also stabilizing its half life time dramatically (Supplementary Fig. 9; and Horn and Sticht, 2010; Muller-Schiffmann *et al.*, 2011). Introduction of disulphide-bonded cysteine 8 into the experimentally determined structure of the mature fibril (Lu *et al.*, 2013) revealed only very minor steric clashes thus offering no satisfactory explanation for the complete absence of fibrils. An alternative explanation would be that the S8C mutation might inhibit fibrillation itself. There exists considerable experimental (Stravalaci *et al.*, 2011; Lam *et al.*, 2013) and computational (Nguyen *et al.*, 2007; Rojas *et al.*, 2010) evidence that fibril growth occurs via the addition of flexible monomers to an amyloid-β seed via a two-step 'dock-lock' process. Here, in a rapid first step the monomer docks to a

preformed oligomer/fibril seed. During the slower lock phase the monomer undergoes conformational changes by forming a β-strand that is in registry with the oligomer/fibril. This mechanism cannot occur in the tgDimer mice due to the absence of monomeric amyloid-β and fibrillar seeds (Figs 1C and 7). Moreover, molecular modelling indicates that seed nucleation itself is disfavoured in tgDimer mice, as the disulphide-bonded dimer adopts a rather rigid conformation that is distinct from both the flexible monomer and the U-shaped oligomer (Horn and Sticht, 2010; Muller-Schiffmann *et al.*, 2011). Thus, nucleation into higher order oligomer seeds would require the simultaneous docking of two Aβ-S8C dimers that, at this time, adopt an elongation competent conformation, which is highly unlikely due to the low physiological concentrations of the amyloid-β dimers (Supplementary Fig. 10A). Consequently, the Aβ-S8C dimer cannot efficiently generate plaque seeds, providing a plausible explanation for the absence of fibrillar oligomers and plaques in tgDimer mice.

Our findings demonstrate the difference between being able to form the initial building block (or seed) for plaques themselves and being able to be incorporated into such existing building blocks. Our modelling data demonstrate that Aβ-S8C dimers can be stabilized in elongation competent fibrillar structures, without reducing their stability (Supplementary Fig. 10B). Specifically, our genetic seeding experiment confirmed that an environment that allows amyloid-β plaque formation (the CRND8 mouse) and, therefore, provides amyloid-β seeds, can facilitate the sequestration of toxic amyloid-β dimers into insoluble amyloid assemblies, despite their ability to seed plaques alone (Fig. 7). This is in line with findings showing that amyloid-β dimers were more abundant in insoluble than in soluble fractions of brain homogenates of patients with Alzheimer's disease (McLean *et al.*, 1999). Therefore, our data feed to recent suggestions that sequestration and accumulation of oligomers into amyloid inclusion bodies and extracellular plaques may at least initially serve protective functions in neurodegenerative diseases by shifting the equilibrium away from the toxic oligomers (Cohen *et al.*, 2006; Cheng *et al.*, 2007; Treusch *et al.*, 2009).

So far, our Aβ-S8C dimer model is the only *in vitro* and *in vivo* model available that reliably produces high amounts of exclusively soluble amyloid-β oligomers (i.e. specifically dimers). Our work suggests that amyloid-β dimers in the absence of plaques may play a causal role in the learning and memory deficits and the LTP changes commonly described in plaque-developing Alzheimer's disease models and sometimes seen prior to the appearance of plaques. Moreover, amyloid-β dimers do not form seeds. However, we show that they can be incorporated into amyloid-β plaques. Our data thus strongly support the significance of amyloid-β dimers as a valid target for therapeutic intervention as previously proposed (Hefti *et al.*, 2013).

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Supplementary material

Supplementary material is available at *Brain* online.

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Abeta dimers modulate serotonin homeostasis, cognition- and affect-related behaviors reminiscent of early Alzheimer's disease

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Abstract

Background: We used a mouse model (tgDimer mouse) for early Alzheimer's disease (AD) that expresses exclusively soluble Abeta dimers, and therefore is devoid of Abeta plaques, astrogliosis and neuroinflammation, to dissect the selective effects of this Abeta oligomer on neurotransmitter levels and behaviors in 7- and 12-month old animals.

Methods: The mice were subjected to the Morris water maze, tests of motor activity, attention, anxiety, habituation learning, working memory, and depression-related behaviors. Levels of acetylcholine, dopamine, and serotonin were measured in neostriatum, ventral striatum, prefrontal cortex, hippocampus, amygdala and entorhinal cortex by high-performance liquid chromatography.

Results: The tgDimer mice exhibited disturbances in neurotransmitter homeostasis, including an inhibition of the serotonin turnover rate in the ventral striatum and an age-related depletion in hippocampal acetylcholine. They also showed anxiety-related behavior in the elevated plus maze, and despair-related behavior in the forced swimming test. Cognitive deficits included impaired memory in the Morris water maze and non-selective attention deficits. Deficient motor learning was found in the rotarod test. Stress-test results indicated an intact HPA axis, based on corticosterone levels.

Conclusions: The results demonstrate the selective role of Abeta dimers modulating the neurotransmitters serotonin and acetylcholine, as likely happens in early stages of AD, leading to cognitive and depression / anxiety-like phenotypes. For these phenotypes, therefore, neither Abeta plaques nor astrogliosis or neuroinflammation is responsible, but Abeta dimers, that thus contribute to regulating neurotransmitter homeostasis. The tgDimer mouse, thus, allows a more specific description of behavioral phenotypes in early AD where frequently anxiety/depression-related syndromes occur.

Introduction

Early diagnosis of AD is a goal of high priority, since it is believed that potential pharmacotherapies could act more efficiently before the massive structural damage that occurs once Aβ plaques are formed, and eventually even prevent it. The early stages of AD, however, are not well explored. About 30% of individuals with mild cognitive impairment (MCI) convert to early stages of AD (1), but prediction rates are low. Cognitive deficiency correlates higher with synaptic density than with neuronal loss. This is supported by the findings that Aβ promotes axonal pruning (2,3) and disrupts cognitive functions in the absence of neuronal loss (4–8). As a consequence, soluble Aβ oligomers rather than Aβ plaques are increasingly believed to be responsible for the progressive neuronal degeneration in AD (4,9–12). Especially Aβ dimers have been shown to be synaptotoxic and highly prevalent in the brain tissue of AD patients (13–15).

Depression and anxiety are risk factors for AD, and occur as part of the early symptoms of AD. A recent meta-analysis concluded a prevalence of depression in about 30% of patients with AD (16), another study reported an equally high prevalence of depression and anxiety symptoms in MCI patients (17). The mechanisms of how AD pathophysiology is linked to depression or anxiety are unknown, but one possibility is that Aβ oligomers mutually interact with various neurotransmitter systems. Whether, in turn, neurotransmitter systems feedback on Aβ oligomer expression and toxicity is unclear. There is substantial evidence for the involvement of N-methyl-D-aspartate (NMDA) and glutamatergic metabotropic receptors in mediating the synaptic binding and neurotoxicity of soluble Aβ (18–20), which may account for the early episodic memory deficits in AD (21). On the other hand, Aβ deposition has been linked to the massive loss of cholinergic neuron in the basal forebrain and other brain areas (22). Accordingly, cholinergic deficits are considered to be the major pathological characteristic of AD and also represent the main target for current available pharmacological treatment (18,23,24).

Soluble Aβ oligomers are possibly the culprits for both the early cognitive (MCI) and affective (depression, anxiety) symptoms. Until recently it was not possible to investigate *in vivo* the selective roles of the Aβ dimer through the lifespan of the host, due to the existing kinetic equilibrium of different multimers and the occurrence of different forms of amyloid deposits. While studies on intracerebral Aβ-infusion have provided evidence of behavioral actions of soluble Aβ (25–28), there is no study on transgenic animal models, which has been successful in specifically assessing the impact of the Aβ dimer on neurochemical, cognitive and emotional behavioral parameters.

The development of the transgenic Aβ-S8C mouse (tgDimer mouse) enabled the selected study of Aβ oligomers in early AD (29). In order to stabilize Aβ dimers, two Aβ monomers were linked by a disulfide bridge that was incorporated into the amyloid precursor protein (APP) by the introduction of cysteine amino acid at position 8 of the Aβ domain (corresponding to APP751-S679C). This led to the natural secretion of neurotoxic Aβ dimer, which did not influence the trafficking and processing of the APP by cellular secretases (29). The Aβ-S8C mutation in the tgDimer mouse resulted in a high concentration of Aβ dimers, but not of monomers. Moreover, no insoluble amyloid species or plaques were generated over its lifespan. This allowed distinguishing between Aβ-specific effects and other neuropathological alterations such as amyloid plaques, cerebral amyloid angiopathy and neurofibrillary tangles, as associated with other transgenic models (30). The tgDimer mouse was found to exhibit deficits in learning and memory as well as hippocampal long-term potentiation comparable to findings in classical AD mice. These findings suggest that soluble Aβ dimers, *per se*, are able to induce neurotoxicity and aberrant synaptic signaling as well as to impair cognitive functions in the absence of plaque pathology or elicited effects such as neuroinflamation of astrogliosis (31).

The rationale of the present study was the behavioral, and neurochemical characterization of the Aβ-S8C mutation in adult (7 months old) and aged (12 months old) tgDimer mice in comparison to age matched wildtype (WT) C57BL/6N controls. The mice were subjected to the Morris water maze (MWM) test, assessing navigational learning and retrieval. Furthermore, the 7 months old mice underwent tests of motor activity, attention, emotionality, and habituation learning in the open field, anxiety- and fear-related behaviors in the elevated plus-maze (EPM), working memory and attention-related behaviors in the radial arm maze (RAM), depression-related behaviors in the forced swimming test (FST) and motor coordination and learning on the rotarod. Furthermore, in the 12 months old animals' blood corticosterone levels were determined after restraint stress test in order to assess hypothalamus-pituitary axis (HPA) functionality . In further batches of adult and aged tgDimer mice and WT controls, levels of acetylcholine (ACh), dopamine (DA), serotonin (5-HT) were measured in neostriatum, ventral striatum, prefrontal cortex, hippocampus, amygdala and entorhinal cortex via high-performance liquid chromatography (HPLC).

Material & Methods

Subjects. Twelve homozygous male tgDimer mice and eight WT C57BL/6N controls were tested behaviorally at 7 and 12 months of age. At age of 7 months, they were tested in the

MWM, open field, EPM, RAM, FST and on the rotarod. At age of 12 months, they were again tested in the MWM. Corticosterone levels in blood were assessed after restraint stress. For *post mortem* analysis of neurotransmitter levels, 19 further male tgDimer (7 months old: n = 10; 12 months old: n = 9) and 21 WT C57BL/6N (7 months old: n = 12; 12 months old: n = 9) were employed. They were obtained from the Central Animal Laboratory of the University Hospital Essen, Germany and housed individually at 20 ± 2°C, under a reversed 12:12 h light-dark cycle (light off at 07:00 a.m.) and humidity (60% ± 2°C) with food and water *ad libidum*. Experiments were carried out in accordance with the German Animal Protection Law and approved by the local authority (Landesamt für Natur-, Umwelt- und Verbraucherschutz, North Rhine-Westphalia).

Neurochemistry. From both hemispheres, neostriatum, ventral striatum, prefrontal cortex, hippocampus, amygdala and entorhinal cortex were dissected and levels of ACh, DA and 5-HT as well as the DA metabolite dihydroxyphenylacetic acid (DOPAC) and 5-HT metabolite 5-hydroxyindole acetic acid (5-HIAA) were determined by high-performance liquid chromatography with electrochemical detection (HPLC-EC) (see Supplementary Information for details)

Behavioral testing

The sequence of tests administered to the animals is depicted in figure 1. In all experiments the behaviors were recorded via camera and DVD recorder and subjected to *post hoc* analysis, using tracking software (Ethovision X® 8, Noldus, Netherlands) (Details of behavioral and statistical methods can be found under Supplementary Information).

Seven-months old mice

Morris water maze (MWM). The tgDimer WT mice underwent the following procedure: On day 1, they were habituated to the pool devoid of the platform for 90 sec. On 9 subsequent days, they were trained to escape onto a stationary hidden platform (4 trials/day). They were tested 24 h later with a probe trial without a platform present as a test for memory of the location of the rewarded platform, followed by two trials with a visible platform (cued platform task). At 12 months of age they were again tested with an identical protocol. Behaviors evaluated were: time to platform, swimming speed and time spent in thigmotaxis (along the wall)

Open field.

The animals were allowed to explore the open field arena for 5 min. General activity was assessed by the following parameters: (a) distance moved within the field, (b) duration and frequency of rearing.

Elevated plus maze (EPM). The EPM is used to measure anxiety- and fear-related behaviors (32). The animals were placed onto the central platform and were allowed to explore for 5 min. Behaviors assessed were: (a) time spent on the open and closed arms and the central platform, (b) "risk assessment" indexed by the stretch-attend posture (head and forepaws exit the closed arm, whereas the body remains in arm), (c) head dips on the open arms, and (d) distance moved.

Eight-arm radial maze (RAM). The eight-arm RAM is employed to assess working memory, and attention-related behaviors (33). A procedure involving non-baited arms was used to assess ''non-reinforced exploration''. The animals were placed on the center platform and allowed 5 min of exploration. Behavioral parameters assessed: (a) velocity, (b) distance moved, (c) duration of rearing (as a measure of non-selective attention, (33), and (d) the number of arms visited before the first repetition (first error), as a measure of selective spatial attention (SSA) and working memory (34).

Rotarod. Rotarod performance was determined over 2 days with 6 trials (inter-trial-interval: 90 sec) per session. On day 1, mice were placed on the drum, rotating at constant speed of 24 rpm with a cut-off time of 5 min. On day 2, 6 trials were conducted with speed accelerating up to 450 rpm over 5 min. The time until the mouse fell off was recorded.

Forced swimming test (FST). The FST has been employed to assess depression-related behavior (''learned despair''), as indicated by duration of immobility and attempts to escape from the water (35). Behaviors analyzed were the duration of swimming, immobility (lack of motion except for movements to keep head above water and climbing (attempts to escape by movement of forepaws on the wall).

12-months old mice

Morris water maze (MWM). The procedure was identical to the MWM experiment conducted on the same mice at age 7 months.

Restraint stress and corticosterone analysis. 12-months old tgDimer mice and WT controls were subjected to a restraint stress procedure to test for responsiveness of the HPA-axis (36). Blood samples were taken by incision in distal part of the tail. After a baseline blood sample (S1), the animal was placed into a restrainer (Harvard Apparatus) for 10 minutes and a second sample (S2) was taken. Additional samples were taken after 60 (S3), 120 (S4) and 180 minutes (S5). Blood was collected in 300 µl EDTA-coated vials (Microvette® CB 300 Sarstedt®). Corticosterone plasma concentrations were analyzed with ELISA (IBL International, Hamburg, Germany).

Results

Neurochemistry

Acetylcholine

For hippocampal ACh levels, the one-way ANOVA revealed a significant main effect of *age* ($F_{1,37}$=5.571, p=0.024), but not of *genotype* (p>0.05; Fig. 2A). Accordingly, *post hoc* t-test for independent samples showed significantly less hippocampal ACh in aged relative to adult tgDimer mice (p=0.006) but not in the WT controls (p>0.05). For entorhinal cortex (EC), one-way ANOVA did not reveal a significant main effect for *age* and *genotype* (p>0.05). However, *post hoc* t-test indicated significantly less ACh in the aged tgDimer relative to the aged WT controls (p=0.033; Fig. 2B), No significant differences in ACh concentration were found in the other investigated brain regions (p > 0.05).

Serotonin and 5-HIAA

No significant differences in 5-HT and 5-HIAA levels were found between tgDimer and WT mice in any of the investigated brain regions (p> 0.05).

For the hippocampal 5-HIAA/5-HT turnover rate, the one-way ANOVA revealed a significant main effect of *genotype* ($F_{1,36}$=9.670, p=0.004; Fig.2C). Accordingly, the *post hoc* t-test showed a significantly smaller 5-HIAA/5-HT ratio in 12-months old tgDimer mice relative to WT controls (p = 0.003).

In the ventral striatum, a significant main effect of *genotype* ($F_{1,17}$=10.830, p=0.005) was found for the 5-HIAA/5-HT turnover rate (Fig.2D). Post hoc t-tests revealed a significantly

smaller ratio in the tgDimer mice at both, 12 months (p=0.046) and 7 months of age (p=0.018).

In the amygdala (Fig.2E), the ANOVA revealed a significant main effect of *genotype* ($F_{1,17}$=7.313, p=0.016) and the *post hoc* t-test showed a significantly higher 5-HIAA/5-HT ratio in 7-months old controls relative to tgDimer (p=0.040), and a trend for significance in the 12-months old WT relative to tgDimer (p=0.072).

No significant difference was observed in any of the other investigated brain regions.

Dopamine and DOPAC

For hippocampal DA levels, the one-way ANOVA revealed a significant main effect of *age* ($F_{1,29}$=7.102, p=0.013). Accordingly, the *post hoc* t-test showed that DA levels were significantly higher in the hippocampus of aged relative to the adult WT, but not the tgDimer mice (p=0.022, Fig. 2F). No significant differences in DA concentrations were found in the other brain regions (p > 0.05).

No significant differences between groups in DOPAC levels and DOPAC/DA ratio were found in the investigated brain regions.

Morris water maze

Over the acquisition days in the Morris water maze, the aged tgDimer mice exhibited significantly more thigmotactic swimming than the WT (t-test; p=0.020), and took significantly longer to find the hidden platform (two way ANOVA; table1, Fig.3). The adult tgDimer mice exhibited enhanced thigmotactic swimming concurrently with deficient learning only in the second half of the acquisition session (days 5 - 9). During the first half (days 1 - 4), they performed comparable to the WT, both in latency to escape to the hidden platform and in thigmotactic swimming. A detailed analysis showed a comparable performance of adult WT and tgDimer mice in learning to escape to the hidden platform, with both groups showing a significant degree of learning (controls: p=0.003, tgDimer: p=0.029, paired sample t-test, between days 1 - 4). However, the tgDimer failed to show significant learning over the rest of the acquisition days (4 to 9) (controls: p=0.023, tgDimer: p>0.05). At 12 months of age the tgDimer (p>0.05), unlike the WT (p=0.016), failed to show significant learning over the whole 9 days of acquisition. Note, that the mean thigmotactic swimming ratio represents the time spent in the thigmotaxis region divided by the total time in the arena. Also, during the probe trial, the time spent within the thigmotaxis area in adult and aged tgDimer mice was significantly longer relative to their WT controls (p=0.003 and p<0.001, respectively). There were no significant differences between groups in swimming speed and in latency to reach the platform on the cued platform trials.

Open field

In the tgDimer mice, duration of rearing behavior was decreased relative to WT controls (t-test, p=0.017; Fig.4). Two-way ANOVAS for repeated measures revealed significant main effects of *time* (30sec time bins during the 5 min-trial ($F_{9,171}$=6.089, p<0.001) and *genotype* ($F_{9,171}$=7.923, p=0.003) as well as an interaction ($F_{1,19}$=10.911, p=0.004). *Post hoc* between-group comparisons revealed that rearing was decreased in tgDimer mice relative to WT controls in numerous time bins (90, 120, 180, 240 and 300 sec, p<0.05), indicating that this group may have had a non-selective attention deficit (37). Horizontal locomotor activity (distance moved) in the open field was not significantly different between groups (p>0.05).

Elevated plus maze

The analysis of time spent in the open arms, closed arms and center in the EPM failed to reveal significant differences between groups (table in Fig.6). However, a more detailed analysis of risk assessment revealed that the tgDimer animals exhibited more stretch/attend postures than the WT controls (p=0.004, t-test). This indicates that under a potential threat (as suggested by the elevated, open area), tgDimer mice show an increase in anxiety-related behavior.

Radial arm maze

For the duration of rearing, the two-way ANOVA for repeated measures revealed main effects of *time* ($F_{1,18}$=14.468, p<0.001) and *genotype* ($F_{1,18}$=5.443, p=0.031). Post hoc t-test for revealed significantly less time engaged in rearing behavior by the tgDimer than the WT controls (min 3: p=0.032, min 5: p=0.011; Fig. 4). There were no significant differences between groups in horizontal locomotor activity (velocity, distance moved) in the RAM revealed (p>0.05). The number of arms visited before the first repetition (first error) was not significantly difference between tgDimer and WT mice (Fig.4; p>0.05).

Rotarod

Two-way ANOVAs for repeated measures for time spent on the rotarod, when rotating with constant speed, showed a main effect of *trial* ($F_{5,95}$=24.407, p<0.001), but not *genotype* ($F_{1,19}$=1.535, p>0.05). Over the 6 trials, the tgDimer, as well as WT mice, exhibited an increase of time spent on the rotarod rotating with constant speed with no significant between-group difference in performance (p>0.05; Fig.6).

The two-way ANOVA for the time spent on the rotarod, when rotating with accelerating speed, revealed a significant main effect of *genotype* ($F_{1,19}$=6.037, p=0.024), but not of *trial* ($F_{5,95}$=0.719, p>0.05), nor an *interaction* ($F_{5,95}$=0.379, p>0.05). *Post hoc* comparisons revealed significantly impaired performance of tgDimer relative to WT mice (trial 1: p=0.030, trial 5: p=0.022).

Forced swimming test

The 7-months old tgDimer mice exhibited significantly less swimming behavior (t-test, p=0.029), and a trend towards more immobility (p=0.075) relative to the WT controls in the 24h test trial (Fig.7). Both groups exhibited a significant decrease in duration of swimming from the pre-test trial to the 24h test trial (p=0.009 for tgDimer mice and p<0.001 for WT controls). Likewise, both groups showed a significant decrease in duration of immobility from the pre-test trial to the 24h test trial (t-test, p<0.001 for tgDimer mice and WT controls). Climbing behavior was not significantly different between groups (p>0.05).

Restraint stress and corticosterone analysis

The results of the corticosterone analysis after restraint stress in 12-months old mice are presented in Fig.6. The two-way ANOVA showed a main effect of *trial* ($F_{4,64}$=16.478, p<0.001), but not of *genotype* and *interaction* (p>0.05).

Discussion

In this study, the tgDimer mouse was analyzed for changes in neurochemistry and behavior. In this model, Aβ dimers, but not monomers, other oligomeric species or insoluble Aβ are expressed, and neither astrogliosis nor neuroinflammation occurs (30). The exclusive expression of Aβ dimers in the absence of any other neuropathology allows the assessment of the biological and behavioral changes caused by the abundance of this conformer. In the tgDimer mouse, we found deficits in 5-HT turnover in the HC, ventral striatum, and amygdala, decreased ACh levels in the HC, as well as deficits in memory, non-selective attention and motor learning. These mice also demonstrated increased anxiety- and despair-related behaviors.

Neurochemistry

Although the absolute values of 5-HT and 5-HIAA were unchanged in the tgDimer mouse vs. control (Fig.2), a significant reduction of the 5-HIAA/5-HT ratio was observed in the

hippocampus, ventral striatum, and amygdala of tgDimer mice, indicating 5-HT dysfunction. 5-HT homeostasis is regulated by a presynaptic inhibitory action of 5-HT_{1B} autoreceptors (38,39). Previous findings provided evidence for the direct involvement of 5-HT_{1B} autoreceptors in the regulation of 5-HT turnover (40,41). 5HT synthesis may be secondary to the turnover (40). 5-HT_{1B} receptors are located pre-synaptically on serotonergic and post-synaptically on non-serotonergic neurons, acting as auto-and hetero-receptors, respectively (42–44). Thus, our data suggest the hypothesis that the Aβ dimers lead to a sensitization of 5-HT_{1B} receptors in the hippocampus, ventral striatum and amygdala. An Aβ-induced depletion of 5-HT may be excluded as a reason for the observed reductions in 5-HT turnover since neither 5-HT nor 5-HIAA levels differed between tgDimer mice and controls. Correspondingly, intraventricular injection of toxic peptide Aβ 25-35 reduced the 5-HT turnover rate in rats (45).

Hippocampal ACh did not significantly differ between tgDimer mice and WT controls at both ages. However, a significant age-related decline was detected in the hippocampus of aged compared to adult tgDimer mice, and in tgDimer relative to WT in EC at 12-months of age. Both hippocampus and EC are highly interconnected structures important for memory formation (46,47) and are the earliest regions prone to AD pathology (48,49) and Aβ toxicity (50).

Our finding of an age-related elevation in DA levels in the hippocampus of WT controls supports earlier similar findings in rodents (51,52). This elevation in DA was impeded in the tgDimer mice and may be related to the toxicity of intraventricular Aβ injection on DA levels as previously reported (45,53).

Cognition

The Morris Water Maze is widely used to assess hippocampal damage and spatial memory deficits (54,55). The tgDimer at both ages were impaired in both acquisition and retention in the MWM (30). In the present study the increased thigmotactic swimming at both ages of tgDimer relative to WT controls during the acquisition trials, indicated a higher effort to escape the maze in favor of active searching for the hidden platform location. This may reflect an inhibition of reward-motivated behavior by a greater level of escape/despair behavior, rather than a working memory deficit. Intact working memory was also indicated by the comparable performance of the tgDimer and WT in the RAM first error score. 5-HT_{1B} receptors have a crucial role in depression (56), and 5-HT_{1B} receptor sensitization was found to inhibit reward-related behavior (57–59). On the other hand, the deficiency in learning and

retention capabilities of the tgDimer mice may be related to the diminished hippocampal ACh level found in this study. Cholinergic integrity is essential for MWM performance, and the deterioration of MWM performance by anticholinergic agents (e.g. scopolamine) is well established (60,61). It is also known that hippocampal cholinergic neurons are sensitive to Aβ toxicity (62).

The adult tgDimer mice exhibited less rearing in the open field and in the RAM, indicating deficient non-selective attention (63,64). Attention deficits are one of the main elements of cognitive decline in AD and abnormal cortical cholinergic transmission may underlie the attentional dysfunction in AD (65). Impairments in orienting ‘’non-selective’’ and/or visiospatial ‘’selective’’ attention are common in dementia patients (66) and may be ameliorated by cholinergic treatment (67). Notably, the tgDimer mice showed intact working-memory in the reward-free version of the RAM, suggesting that the cognitive deficits in this mouse are limited to reward-governed learning/memory and to attention-related processes.

We found no significant differences between the tgDimer and WT mice in corticosterone levels in response to restraint stress over the course of 180 min’s of testing. This suggests an intact HPA axis response of the tgDimer mice and, therefore, permits to exclude HPA axis dysregulation as a cause for disturbed memory processing (68) .

On the rotarod, the tgDimer were likely deficient in motor learning, rather than in general motor coordination and fatigue resistance. They exhibited significantly less time to fall off the rod under accelerated speed, but comparable performance with the WT controls under constant speed, in open-field horizontal activity and in swimming speed in the water maze. Brain dopamine is crucial for motor learning (69,70), and the deficiency in dopamine triggered by soluble Aβ in the present study may relate to the deficit found in motor learning. Adult rodents with APP mutation also showed rotarod performance comparable to WT controls (71–73). However, a study in transgenic mice revealed deficient motor abilities, accompanied by intraneuronal Aβ accumulation, followed by the axonal degeneration of motor neurons prior to plaque deposition (74).

Depression- and anxiety-related behaviors

In the EPM task, risk assessment in the tgDimer mice was augmented, indicating an increase of anxiety-related behavior. Similarly, tgDimer mice displayed a trend towards increased thigmotactic swimming in MWM, probe trial (Fig. 5), which also may be interpreted to reflect anxiety-related behavior (75). Thus, in the present study, soluble Aβ dimers in the

tgDimer mice had an anxiogenic impact in both the EPM and the probe trial of the MWM task. In the FST, the tgDimer mice exhibited reduced swimming and a trend towards increased immobility, suggestive of behavioral despair (76). These results are in line with previous findings, showing an increase of immobility after intracerebroventricular injection of soluble Aβ (25). Similarly, in the APPswe/PS1 mouse model of AD, which is characterized by increased levels of soluble A*β* (77), increased immobility was found in the FST (78).

In summary, the present findings demonstrate a neurochemical and behavioral syndrome caused by an overabundance of Abeta dimers in the absence of Abeta plaques, astrogliosis or neuroinflammation, reminiscent of early stages of AD, when no neuropathological hallmarks are present. This syndrome consists of deficits in water maze learning and retrieval, non-selective attention, and motor learning, as well as depression- and anxiety-related behaviors and inhibition of reward-related behavior. This set of phenotypes may, be considered a signature of behavioral changes caused by Abeta dimers and reveal potential mechanisms of action. For example, these phenotypes may be explained in part by effects of the Aβ dimer on 5-HTergic and cholinergic neurons. these results also point to a physiological role of Aβ dimers in modulating the function of 5-Htergic and cholinergic neurons, and the neurotransmitter homeostasis of serotonin and acetylcholine. Our findings also provide a rationale for pharmacotherapeutic interventions targeting acetylcholine and serotonin functions and homeostasis in early AD.

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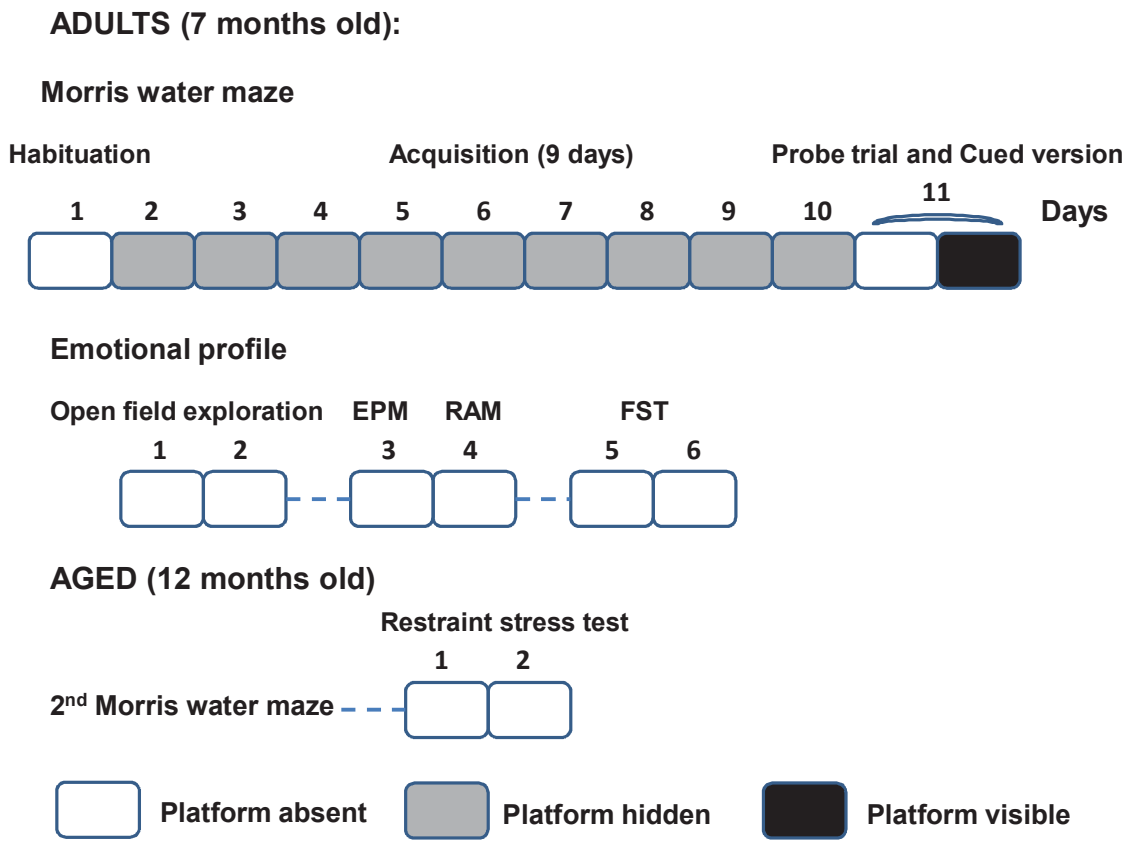

Figure 1: Sequence of behavioral characterization of tgDimer and C57BL/6N control mice. The adult animals were habituated to the Morris water maze (MWM) for 1 day in the pool devoid of the platform, followed by 9 days of training to escape onto a hidden platform (4 trials/day), followed by a single probe trial with the platform absent and a cued trial with the platform visible (data have been previously published) (30). Subsequent behavioral testing included the open field, elevated plus maze (EPM), radial arm maze (RAM), rotarod

and forced swimming test (FST). The aged animals underwent a second test in the MWM according to the same protocol. One week later, restraint stress was administered and 6 blood samples were collected over the course of 180 min's for corticosterone analysis.

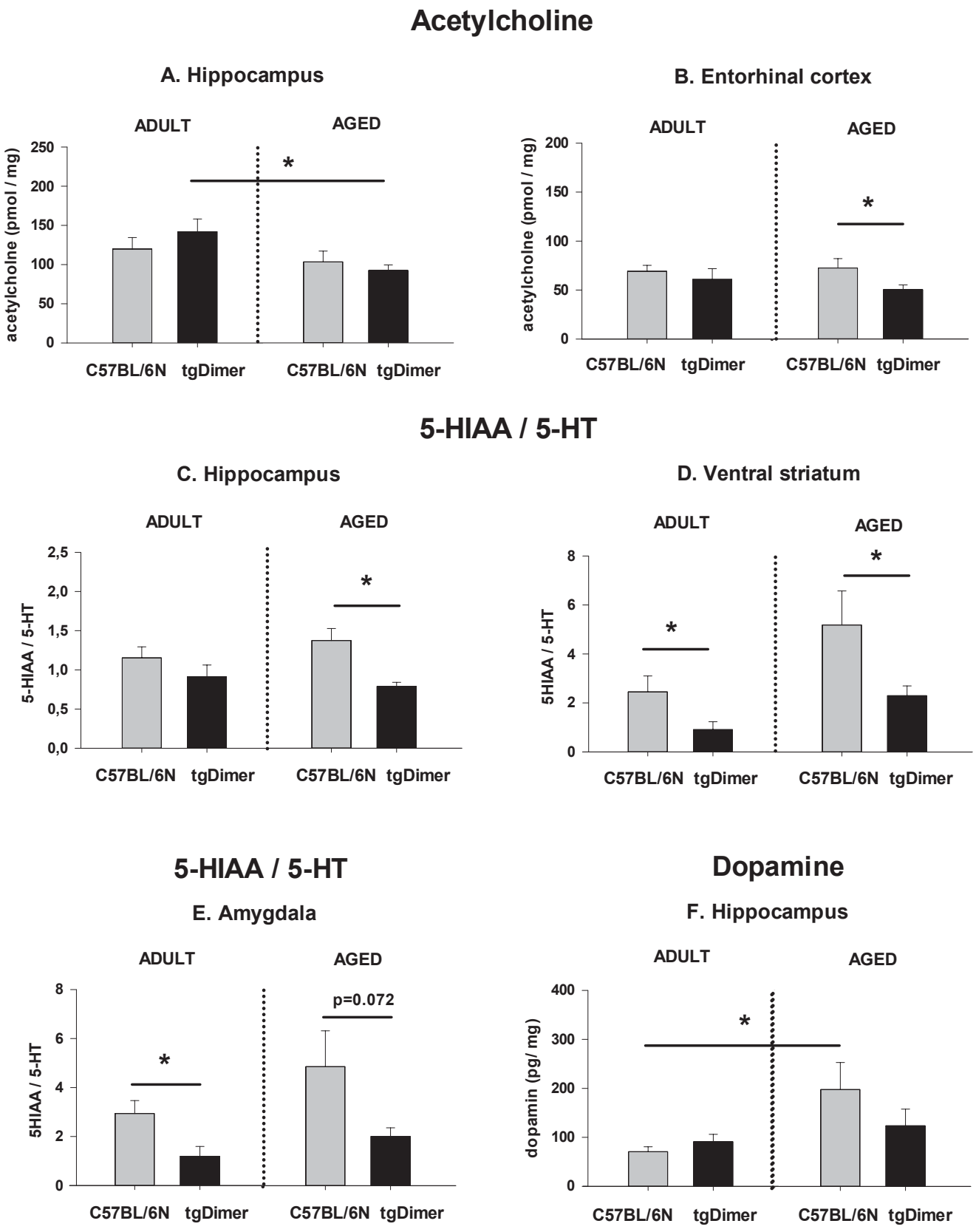

Figure 2: Neurochemical evaluation in different brain areas. **(A)** A significant age-related decrease in ACh levels in the hippocampus of the tgDimer mice, but not in wildtype (WT,

C57BL/6N) controls. **(B)** In the entorhinal cortex ACh levels were lower in the aged tgDimer mice than in the controls. **(C)** 5-HIAA/5-HT turnover in the hippocampus was significantly lower in aged tgDimer mice than in WT controls. **(D)** The 5-HIAA/5-HT ratio was significantly lower in the ventral striatum of tgDimer mice relative to WT controls. Adult and aged tgDimer mice had a significantly lower 5-HIAA/5-HT ratio compared to WT controls. **(E)** A significantly lower 5-HIAA/5-HT ratio was found in the amygdala of adult tgDimer mice compared to controls, and a trend towards a significantly lower ratio in aged tgDimer mice. **(F)** Significantly more DA was found in the hippocampus of aged WT controls compared to adults, but not in the tgDimer mice. (* = $p < 0.05$; post-hoc t-tests).

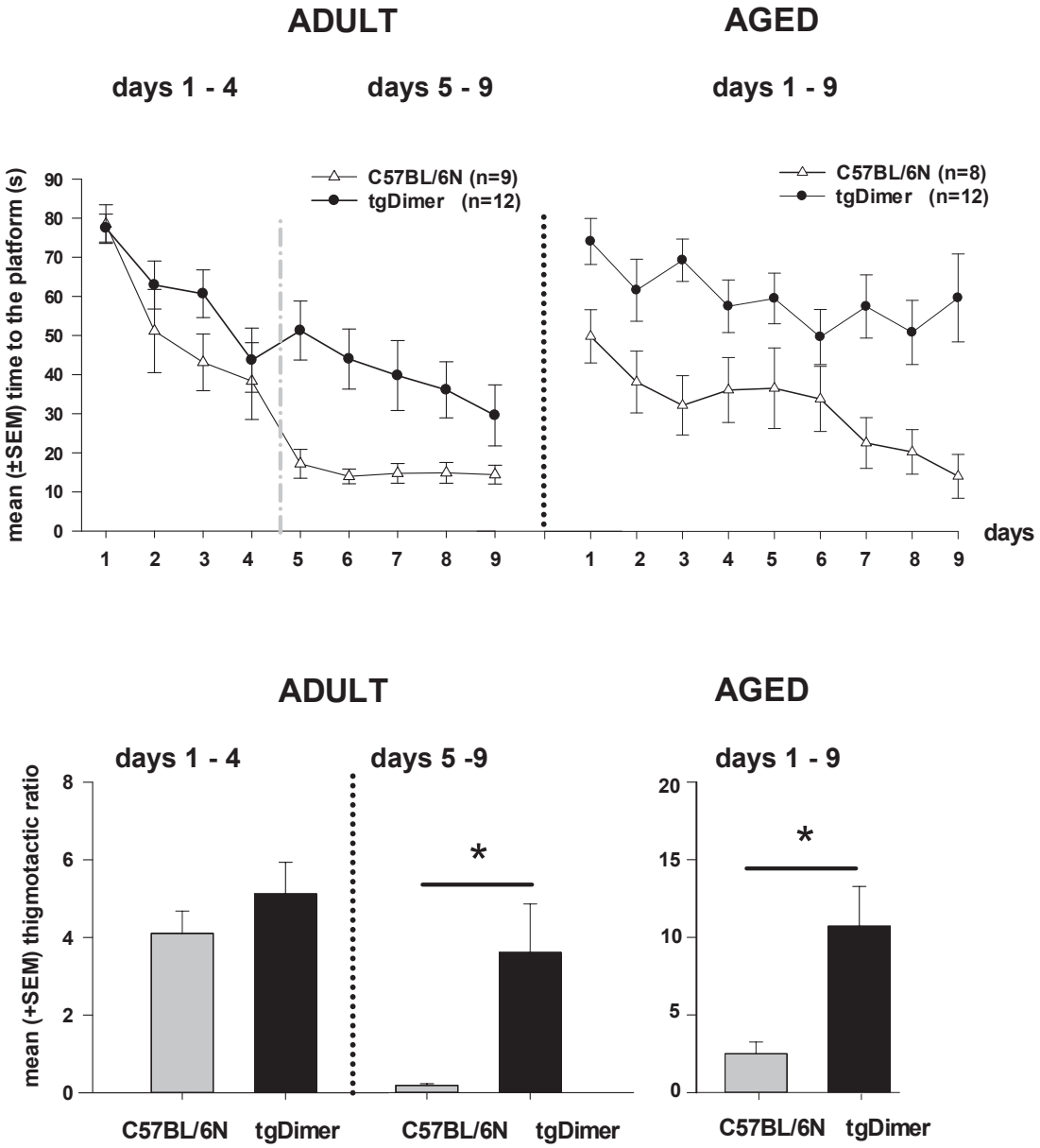

Table 1

Acquisition, Latency to escape onto the hidden platform:			
2-ways ANOVA/main factor:	**Adult (Acquisition days 1-4)**	**Adult (Acquisition days 5-9)**	**Aged (Acquisition days 1-9)**
Acquisition days	$F_{3,57}$=14.457, p<0.001	$F_{4,76}$=2.812, p=0.031	$F_{8,88}$=3.930, p=0.001
Genotype	$F_{1,19}$=1.285, p>0.05	$F_{1,19}$=9.610, p=0.006	$F_{1,11}$=29.304, p<0.001
Interaction	$F_{3,57}$=0.967, p>0.05	$F_{4,76}$=1.879, p>0.05	$F_{8,88}$=0.788, p>0.05
Acquisition, Thigmotaxis behavior:			
Independent sample t-test:	p>0.05	p= 0.029	p= 0.020

Figure 3: Top: Morris water maze testing at ages 7 (adult) and 12 (aged) months over 9 days, 4 trials per day ($p \leq 0.05$ WT vs tgDimer). Bottom: Thigmotactic swimming. At 7 months age the tgDimer and WT performed comparably in time to find hidden platform and

in duration of thigmotactic swimming (given as ratio) during days 1 – 4. However, over the course of acquisition days 5 - 9 the tgDimer were deficient in maze learning and exhibited more thigmotactic swimming. Likewise, the aged tgDimer exhibited significantly deficient learning together with more thigmotactic swimming compared to the WT (*$P \leq 0.05$) (see also Table 1).

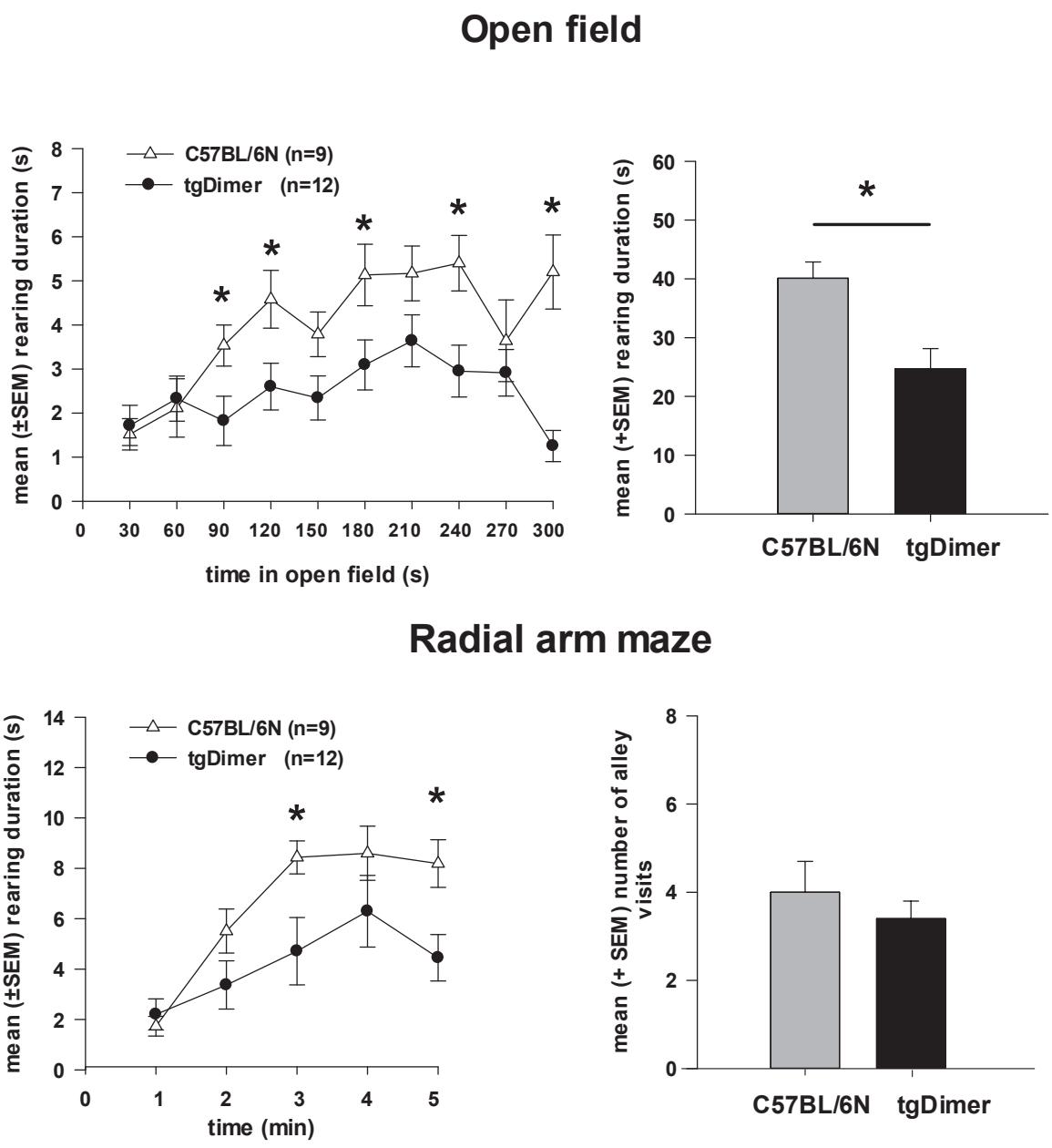

Figure 4: Duration of rearing in the open field (top) and radial-arm maze (RAM) (bottom, left) in tgDimer mice and WT controls. Bottom, right**:** first error score, selective special attention in the RAM (*$P < 0.05$).

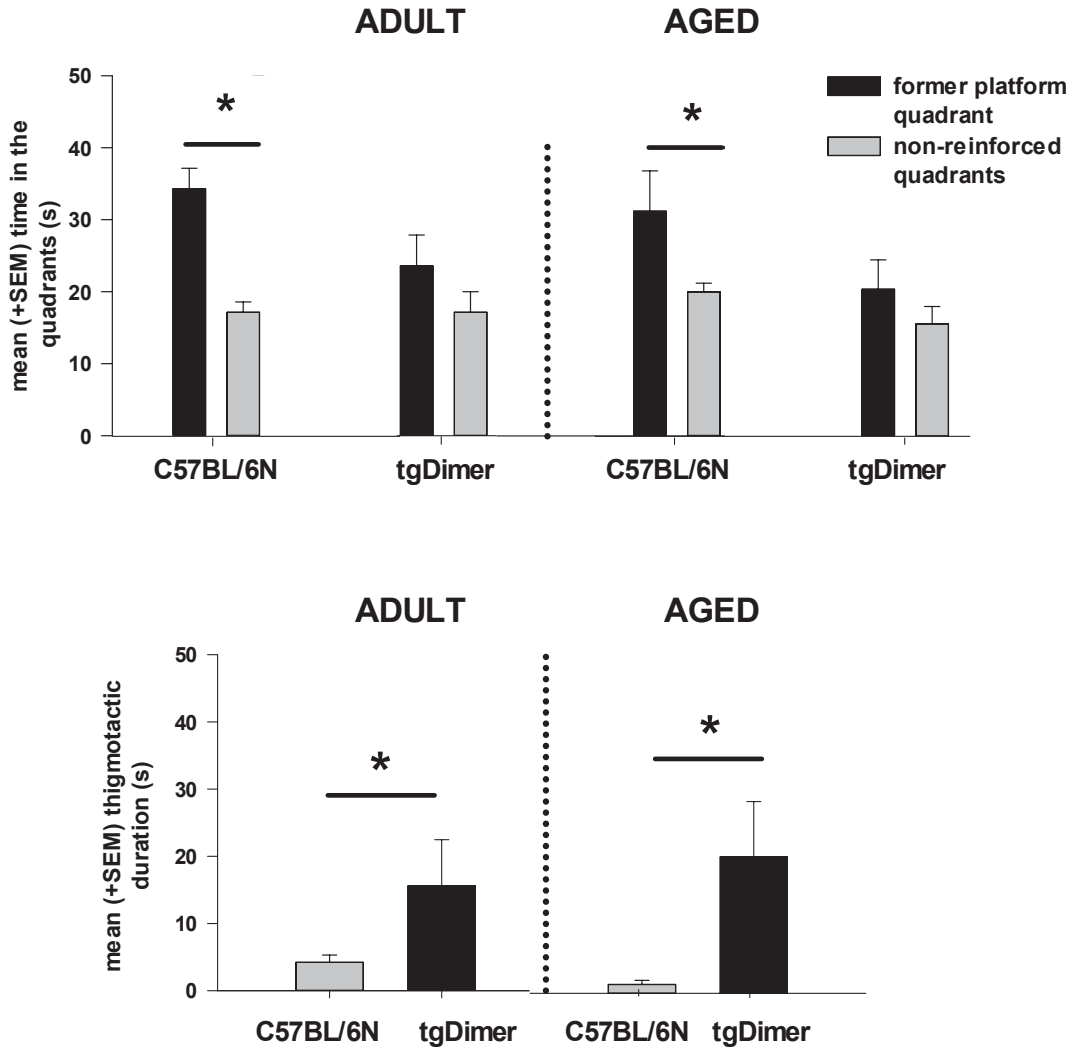

Figure 5: Probe trial with the platform absent (memory test). Top: Time spent in the former rewarded quadrant vs total time spent in the non-reinforced region (the three quadrants other than the former rewarded quadrant). Unlike the tgDimer mice, the WT spent more time in the reinforced vs the non-reinforced quadrants. Bottom: Duration of thigmotactic swimming (sec) in 7- and 12-months old mice during the probe trial**.** The tgDimer mice spent significantly more time swimming along the maze wall compared to WT mice. (*$p < 0.05$).

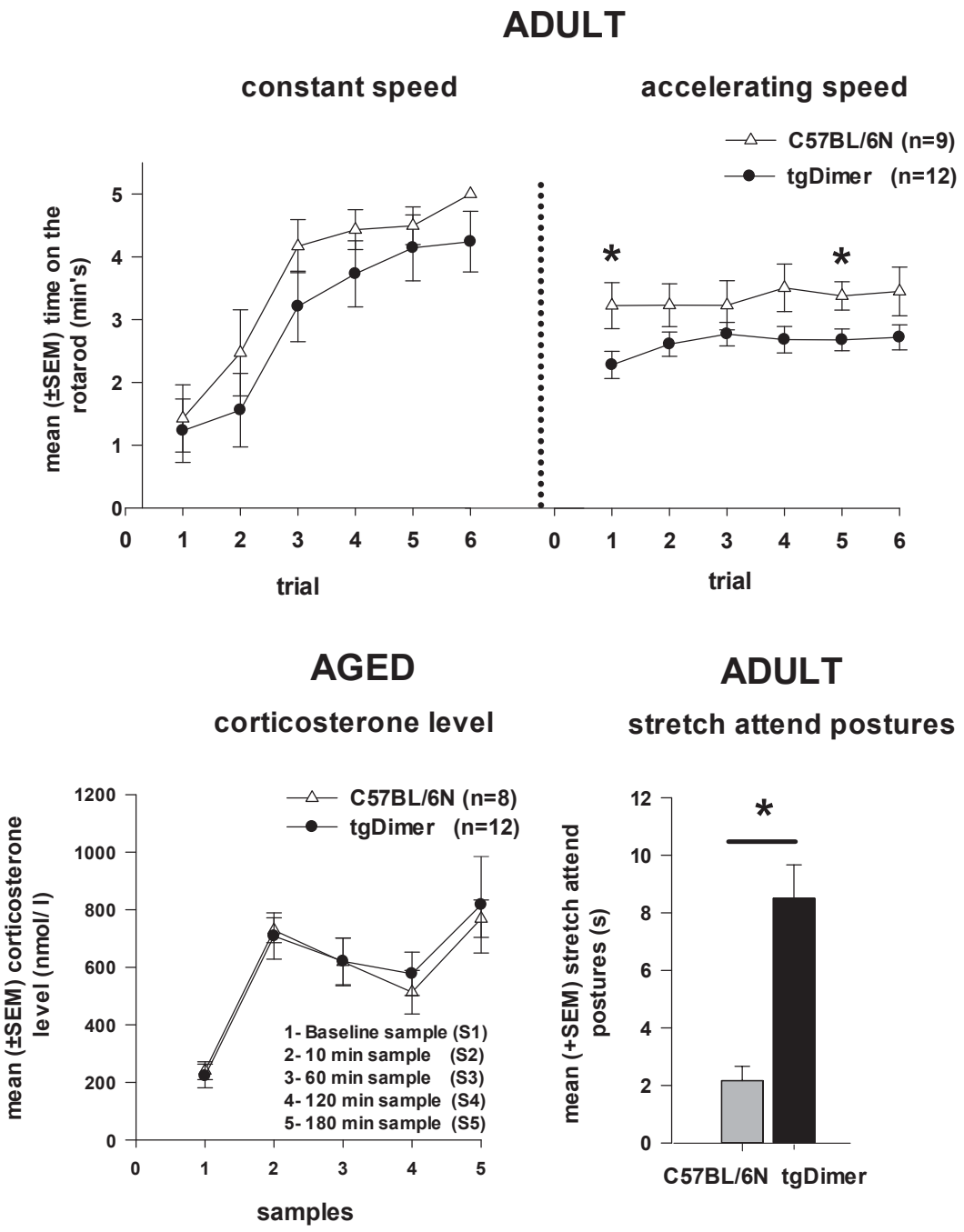

Table 2: elevated plus maze

duration (sec)	C57BL/6N - adult	tgDimer- adult
open arm	68.44 ± 10.48	66.42 ± 10.43
closed arm	181.89 ± 7.51	175.67 ± 5.52
center region	44.33 ± 6.14	47.25 ± 5.80

.

Figure 6: Top: Rotarod performance of 7-month old tgDimer mice and WT controls at constant and accelerating speed. Mean time (± SEM; min) the mice remained on the rotating rod, with a cut-off of 5 min per trial (*P < 0.05). Bottom, left: Corticosterone levels in the blood of aged (12 months old) tgDimer mice and WT controls in baseline and 10, 60, 120 and 180 min after restraint stress. Bottom, right: Elevated plus-maze behavior. Mean frequency of stretch/attend posture (±SEM) in 7 months old tgDimer mice and WT controls. Table 2**:** Time spent in different regions of the elevated plus maze.

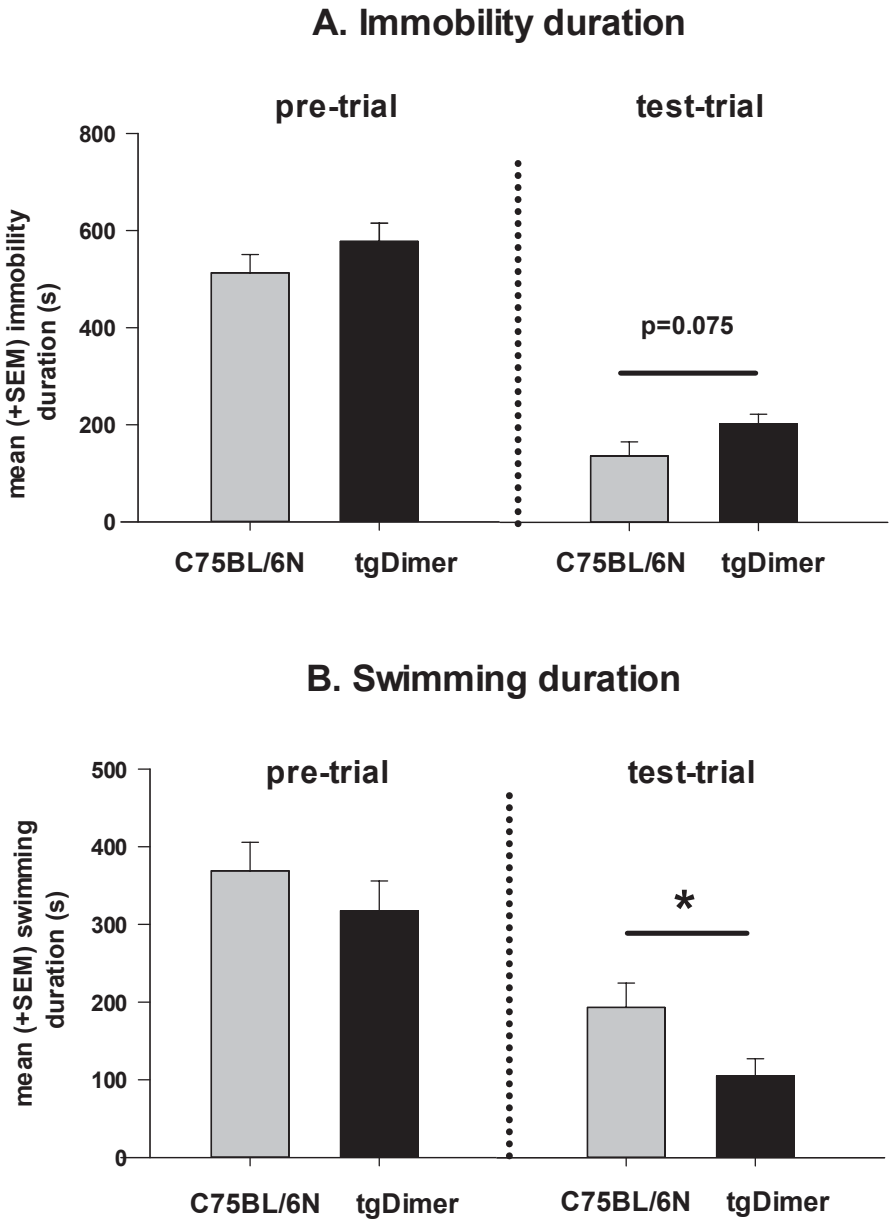

Figure 7: Forced swimming test. Duration of immobility (top) and swimming behavior (bottom) of 7 month old tgDimer and WT mice. The tgDimer mice exhibited significantly less swimming behavior (p=0.029) and a trend towards more immobility (p=0.075) than the wildtype mice in the 24h test trial.

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Promnestic effects of intranasally applied pregnenolone in rats

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ABSTRACT

The neurosteroid pregnenolone (PREG) has been shown to have memory-enhancing and anti-depressant action. The present study addresses the question of whether intranasally applied pregnenolone (IN-PREG) also has promnestic properties in the rat. We examined the effects of IN-PREG at doses of 0.187 and 0.373 mg/kg on memory for objects and their location on learning and retention of escape in a water maze, and on behavior on the elevated plus maze. The main findings were: (a) Pre-trial, but not post-trial, administration of IN-PREG facilitated long-term memory in a novel object-preference test and a novel object-location preference test when tested 48 h after dosing. (b) Over the duration of 5 days of extinction trials, after learning to escape onto a hidden platform in a water maze, the animals treated with IN-PREG spent more time in searching for the absent platform, indicating either, or both, superior memory for the former position of the escape platform, or a higher resistance to extinction. (c) Administration of the anticholinergic, scopolamine, disrupted learning to escape from the water maze in the vehicle-treated group. The IN-PREG treated groups exhibited superior escape learning in comparison with vehicle controls, indicating that the treatment countered the scopolamine effect. IN-PREG treatment had no influence on behaviors on the elevated plus maze. Our results demonstrate that IN-PREG is behaviorally active with cognitive enhancing properties comparable to those known from studies employing systemic PREG administration.

1. Introduction

The intranasal (IN) route of administration allows the targeting of therapeutics by bypassing the blood-brain barrier (BBB), thereby preventing first-pass metabolism and minimizing peripheral side effects (e.g., Banks, Niehoff, & Mattern, 2009; de Souza Silva, Topic, Huston, & Mattern, 2008b; Dhuria, Hanson, & Frey, 2010; Hanson & Frey, 2008; Quintana, Guastella, Westlye, & Andreassen, 2016; Wong & Zuo, 2010). Numerous agents delivered via the nasal route have been shown to have therapeutic effects in rodents, nonhuman primates and humans. For example, IN delivery of L-3,4-dihydroxyphenylalanine alleviated parkinsonian symptoms in rats (Chao et al., 2012) and IN administration of insulin improved memory in healthy subjects and in patients with Alzheimer disease (Benedict et al., 2004; Craft et al., 2012; Reger et al., 2008).

Pregnenolone (PREG) is an endogenous steroid hormone. Brain astrocytes and neurons express cytochrome P450 cholesterol side-chain cleavage enzyme (CYP450scc), which converts cholesterol to PREG, which in turn may be converted to the pathway DHEA, testosterone and the 2 estrogens. It may also be converted to the pathway progesterone, cortisol, progesterone, aldosterone. Pregnenolone sulfate (PREG-S) is synthesized from PREG, but also from cholesterol sulfate (CS) by CYP11A1 and therefore, different pathways for PREG and PREG-S are discussed (Harteneck, 2013). With regard to PREG's effects in the brain, is it still considered the inactive precursor of neuroactive steroid hormones, such as PREG-S. There is, however, some evidence that the former also acts as a neurosteroid itself. PREG modulates several neurotransmitter systems, e.g. the N-methyl-D-aspartate (NMDA) and sigma receptors, and has been associated with the control of mood, learning and memory processes (Flood, Morley, & Roberts, 1992; Vallée et al., 1997; Zheng, 2009; Zorumski, Paul, Izumi, Covey, & Mennerick, 2013). It is a precursor to steroids such as allopreg-

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nanolone (Allop), dehydroepiandrosterone and progesterone, which have also been implicated in emotional and cognitive performance (Barros, Tufik, & Andersen, 2015; Vallée et al., 1997; Zorumski et al., 2013) and been shown to have behavioral and neurochemical action when administered intranasally, such as testosterone (de Souza Silva, Mattern, Topic, Buddenberg, & Huston, 2009) and progesterone (de Souza Silva, Topic, Huston, & Mattern, 2008a)

PREG-S is considered as an excitatory neuroactive steroid, since it positively influences glutamatergic NMDA receptors and negatively modulates γ-aminobutyric acid type A ($GABA_A$) receptors in the CNS (Gibbs, Russek, & Farb, 2006). PREG and its sulfate esters have been indicated to have memory-enhancing and antidepressant effects (Akwa, Ladurelle, Covey, & Baulieu, 2001; Brown et al., 2014; Flood et al., 1992; Marx et al., 2006). For example, early studies demonstrated that intracerebroventricular injection of PREG-S in rodents compensated scopolamine-induced learning deficits in visual discrimination (Meziane, Mathis, Ungerer, & Paul, 1996) and improved spatial memory together with increased acetylcholine release in the hippocampus (Darnaudéry, Koehl, Piazza, Moal, & Mayo, 2000). Morris water maze learning was found to be correlated with hippocampal levels of PREG-S (Vallée et al., 1997) and its systemic administration enhanced visual-spatial performance and correlated with hippocampal and perirhinal cortical neuronal activities in rats (Plescia et al., 2014).

PREG levels in rodents were increased by the administration of the antidepressant fluoxetine (Marx et al., 2006), and PREG was suggested to alleviate depressive symptoms in patients with bipolar depression (Brown et al., 2014). Thus, consistent with the findings of decreased PREG levels in patients with major depression (George et al., 1994) and Alzheimer's disease (Schumacher et al., 2003; Weill-Engerer et al., 2002), PREG is likely to play a role in regulating mood as well as mnemonic mechanisms.

Ducharme et al. (2010) compared IV with IN administration of PREG in mice and found that the distribution of tritium-labeled PREG favored some brain regions, e.g. the olfactory bulb, hippocampus and hypothalamus, and facilitated active avoidance learning. The aim of the present study was to more thoroughly assess the effects of intranasally administered pregnenolone in multiple learning and memory tasks in rats. We hypothesized that IN-PREG would facilitate memory encoding and/or consolidation in two novelty preference paradigms after a delay of 48 h. We also investigated its effects on Morris water maze learning. We then assessed its influence on extinction of learned water-maze escape behavior, with the hypothesis that IN-PREG should increase memory for the site of the missing escape platform and thus, increase resistance to extinction of searching behavior for the platform. We also examined IN-PREG's influence on scopolamine-induced memory impairment in the water maze, a pharmacological model that is characterized by learning and memory deficits resulting from the cholinergic dysfunction incurred by scopolamine, an antagonist of muscarinic acetylcholine receptors (Klinkenberg & Blokland, 2010; Saucier, Hargreaves, Boon, Vanderwolf, & Cain, 1996). Finally, we tested the action of IN-PREG on anxiety-like behaviors in the elevated plus maze.

2. Materials and methods

2.1. Subjects

A total of 50 adult male Wistar rats 3–4 months of age and weighing between 260 and 335 g, were obtained from the Tierversuchsanlage, University of Düsseldorf, Germany. They were grouped five per cage (60 × 38 × 20 cm) and housed under a reversed light–dark cycle (light off from 07:00–19:00 hs), with free access to food and water. The experiments were in accordance with the European Communities Council Directive (86/609/EEC) and approved by the German Animal Protection Law Authorities – LANUV Nordrhein-Westfalen.

2.2. Drugs

Pregnenolone (PREG; Bayer HealthCare Pharmaceuticals), mixed in a proprietary lipid-based gel formulation for IN administration, was provided by M et P Pharma AG, Emmetten, Switzerland. Two different concentrations were used in all the experiments: 5.6 mg/mL and 11.2 mg/mL. Vehicle (lipid-based gel) was applied as a control. For each administration, 5 μL of the formulation was slowly applied into each nostril via a Transferpettor pipette (BRAND GMBH + CO KG, Wertheim, Germany). Given that the animals had an average weight of 300 g, the doses used were 0.187 mg/kg and 0.373 mg/kg, or a total of 0.056 mg and 0.112 mg per animal. The two doses used are based on pilot studies demonstrating them to have promnestic effects in tests of object recognition. Scopolamine (Sigma–Aldrich, Germany) was dissolved in phosphate-buffered saline (PBS) at the dose of 0.75 mg/kg and applied subcutaneously (Schaeble, Huston, Barros, Tomaz, & de Souza Silva, 2012).

2.3. Apparatus

An **open field** made of gray polyvinyl chloride (60 × 60 × 30 cm) was used for the spontaneous object exploration tests. The apparatus was located in a sound attenuating room and a camera connected to a screen and video recorder, mounted 2 m above the field, provided signals to tracking software (Ethovision X® 8, Noldus, Netherlands). One white circle (diameter 21 cm) and one rectangle with black-white stripes (30 × 30 cm) were pasted on the walls as spatial cues. A LED light was used for illumination (∼ 6 lx at the center and 4 lx in the corners). For the spontaneous object exploration tests, four different sets of objects made of porcelain were used (each 18–34.5 high and 8–12 cm in diameter and with adequate weight, >1 kg, so that the animals could not displace them). Pilot studies were made to ensure that the animals had no particular preference for any of the objects.

An **Elevated Plus Maze** (EPM) was used to assess anxiety-related behaviors. The EPM was elevated 50 cm high and composed a central platform (10 × 10 cm) which connected with four arms (each 50 × 10 cm) into the shape of a cross. Two of the arms were walled (38.5 cm high), while the other two were without walls. The two types of arms were placed opposite to each other (luminous density ∼14.8 lx on the closed arms, ∼4.6 lx on the open arms and ∼8.4 lx on the center). A camera was mounted 1.5 m above the apparatus and connected to a DVD-recorder for recording behaviors.

A **Morris Water Maze** (black, diameter: 180 cm) apparatus was used to gauge spatial learning and memory (Morris, 1984). The pool was filled with water to 30 cm (24 ± 2 °C) and contained a black escape platform (diameter: 10 cm). The platform was set 2 cm below the water surface. Four LED lights around the pool provided light intensity of approximately 2.75–3.00 lx. A camera mounted centrally above the maze was connected to a DVD recorder and to the tracking software (Ethovision X® 8, Noldus, Netherlands). Images on the walls varying in shape and color served as distal extra-maze cues.

2.4. Experimental procedures

Two batches of 25 animals each were used. Each batch included a vehicle group (n = 9), a PREG 0.056 mg group (n = 8) and a PREG

0.112 mg group (n = 8). The *first batch* of animals was subjected to open field habituation (10 min per day for two consecutive days), followed by novel object preference (NOP) and object location preference (OLP) tests, with each object exploration test being conducted twice (Fig. 1). The animals were subjected to both, pre- and post-trial administration of PREG in tests of novel object preference and object location preference (Fig. 1). The order of testing and drug administration was as follows: *Pre-trial, NOP*: Three groups of animals received either intranasal vehicle (n = 9), IN-PREG 0.056 mg (n = 8) or IN-PREG 0.112 mg (n = 8) 10 min before the sample trial, followed by the test trial given 48 h later. *Pre-trial, OLP*: One day later, the same procedure was repeated to test for OLP. *Post-trial, NOP*: One day after the OLP test, the NOP test was repeated, except that intranasal administration was immediately after the sample trial. They were tested 48 h later. *Post-trial, OLP*: One day later, a second OLP test was administered using post-trial administration. One day later, the same animals received daily administrations of PREG and were tested in the Morris water maze (details described below). The animals of the *second batch* were tested in the elevated plus maze and then in the Morris water maze (details described below). Behavioral tests were conducted between 10:00 and 16:00 h. Ethanol (70%) was used to clean the apparatus after each trial, except the water maze.

2.5. Effects of pre-trial vs. post-trial IN-PREG on memory encoding and consolidation of memory for object and place

Pre-trial administration of drugs is considered likely to modulate performance by influencing associative processes and attention to the task, whereas post-trial application is considered to have a likely influence on processing of short-term memory or hypothetically, its "consolidation" into a more permanent state (McGaugh, 2000). For the following object preference tests, the exploration time for each object used in the test trial was recorded by an experimenter who was blind to the design.

2.5.1. Novel-object preference (NOP) test

Rodents tend to spend more time exploring a novel object than an old one. This indicates that they habituated to the old object and, thus, remembered it (Ennaceur & Delacour, 1988). This test consists of one sample trial and one test trial, separated by a 48 h interval. For purpose of habituation to the environment, each animal was placed into the empty open field for 10 min for two consecutive days. One day after habituation, two identical objects were placed at two out of four possible corners in the arena. The animal was placed into the center of the open field and allowed to explore freely for 4 min. After the 48 h delay interval, one of these familiar objects was replaced by a novel object and the animal was placed back into the arena and again allowed to explore for 4 min. The positions used for placing the objects were identical in the two trials. This test was conducted for pre-trial and post-trial administration tests. The delay between sample trial and test trial was chosen on the basis of data indicating that normal untreated animals were deficient in novel-object preference and object-location preference tests after 24 h (Dere, Huston, & de Souza Silva, 2007; Ennaceur & Meliani, 1992).

2.5.2. Object-location preference (OLP) test

Rodents show memory for place by spending more time in exploring a displaced object than a stationary one (Ennaceur, Neave, & Aggleton, 1997). This test also employed one sample trial and one test trial, separated by a 48 h delay. One day after the novel object preference test, the animal was placed into the center of the arena containing two identical objects placed at two corners

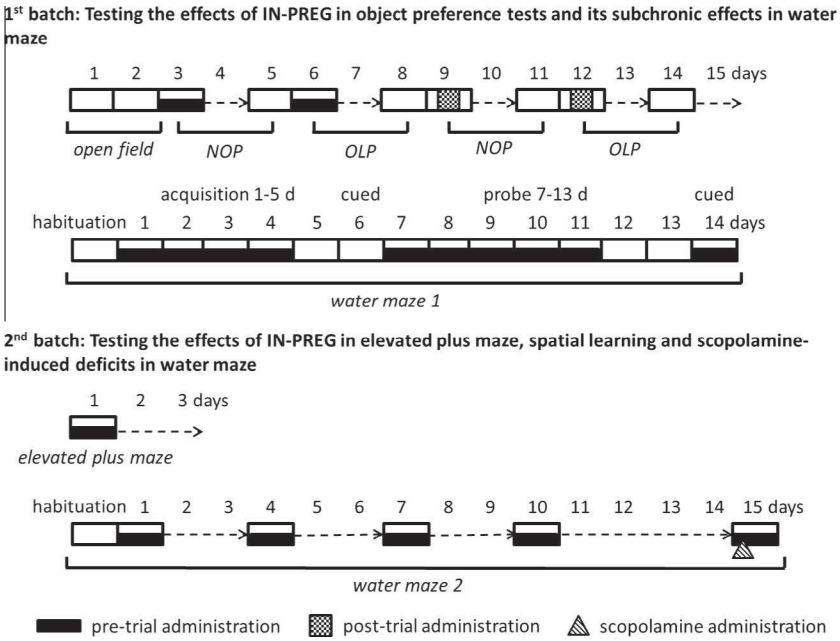

Fig. 1. Experimental procedure for the two batches of animals. Batch 1: The animals, they were habituated to the open field for two consecutive days. One day later, they received a series of object preference tests in the following order: novel object preference (NOP), object location preference (OLP), NOP and OLP, with the inter-trial-interval of 48 h. Intranasally applied pregnenolone (IN-PREG) was administrated prior to the sample trial of NOP and OLP at day 3 and 6, respectively. It was also administrated immediately after the sample trial of NOP and OLP at day 9 and 12, respectively. One day after the final OLP test, the water maze 1 experiment was begun. In water maze experiment 1, the animals were trained to find a hidden platform during days 1–5, followed by two cued trials on day 6 and probe/extinction sessions (containing no platform) from day 7 to 13. One day later, two cued trials were again applied. Intranasal administration was administered 10 min prior to the 1st of the two daily trials over four days of acquisition (1–4 days), five days of probe/extinction sessions (7–11 days) and the last cued trials (14 day). Batch 2: IN-PREG was administrated 15 min prior to the elevated plus maze test. Two days later, the water maze experiment 2 was commenced: Four trials were administered on each acquisition day (days 1, 4, 7, 10 and 15). The location of the platform was different during each acquisition session, but was fixed within a session. Intranasal administration was given 15 min prior to the 1st of the four trials of each acquisition day. A single systemic administration of scopolamine was given 60 min prior to the 1st of the four trials on day 15.

and allowed to freely explore for four minutes. In the test trial, the animal was again placed into the arena with one of the familiar objects located at the same old location, while the other one was moved to a novel location. Four minutes were allowed for the animal to explore freely.

2.6. Elevated plus maze

For this test, each animal received an intranasal administration and 15 min later was placed into the center of the plus-maze, facing one of the two open arms. They were tested over a 5-min period and the following measures were taken: number of entries into and time spent in the open arms, enclosed arms, and the central area of the apparatus. Ethological parameters, including so-called "risk-assessment" were also measured, including the duration of head dips on the open arms (animal's head beneath the edge of the arm) and body stretching (staying in the closed arms, while stretching the body and using forepaws and head to explore).

2.7. Water maze (WM)

2.7.1. Habituation trial

One day before conducting the water maze tests below, a 90 s habituation trial was given in the pool devoid of the platform. Vehicle was administered to all animals prior to the trial (10 min in experiment 1; 15 min in experiment 2). On all trials the animal was placed into the pool facing the wall at one of the four possible starting points, randomly chosen (north, east, south or west). After each trial, the animal was dried under a red-light heating lamp before being returned to the home cage. The inter-trial interval was 90 s between all trials.

2.8. WM - Experiment 1

2.8.1. Acquisition testing

Acquisition testing was performed over five consecutive days (2 trials per day) with a stationary hidden platform. The rat was trained to escape onto the submerged platform placed in the center of a randomly chosen quadrant of the water maze, whereby this position was maintained over the trials. The acquisition trial was terminated when the animal escaped onto the platform or 90 s had elapsed. In the latter case the experimenter guided and placed the animal onto the platform. Each animal was allowed to stay on the platform for 30 s.

2.8.2. Cued trials

One day after the last acquisition trial, two cued trials were given with a visible platform to test for possible sensorimotor deficits in finding the goal (Morris, 1984). A metal cylinder (height: 25 cm; width: 2 cm) painted with black and white stripes was attached to the platform. The position of the platform was different on each trial. A trial was completed when the animal escaped onto the cued platform, or 90 s had elapsed, after which the animal was guided to the platform.

2.8.3. Probe/extinction trials

On the next day, probe/extinction sessions (2 trials/day) without a platform were administered for seven days. These trials lasted 90 s before the animal was removed from the maze. One day after the last extinction session, another two *cued trials* were administered, with the procedure being identical to the one above. The following parameters were measured by computer-based tracking software (Ethovision X® 8, Noldus, Netherlands): time spent within the previously rewarded platform quadrant, time engaged in thigmotactic swimming along the edge of the pool, swimming speed, distance moved, and immobility (lack of motion except for movements to keep the head above the water, an index of behavioral despair (Schulz, Topic, de Souza Silva, & Huston, 2004).

2.8.4. Pre-trial administration

Either vehicle, n = 9; PREG 0.056 mg, n = 8; PREG 0.112 mg, n = 8 was administered 10 min prior to the 1st of the daily two trials (a) over four days of acquisition, (b) the first five days of extinction and (c) the cued trials on day 14 (Fig. 1: upper). The administrations were given according to the previous group assignment.

2.9. WM - Experiment 2

The animals received four acquisition sessions with a hidden platform. Each session consisted of 4 trials/day. Within each 4-trial acquisition day, the location of the platform was fixed in one quadrant, but was relocated to another quadrant on the subsequent acquisition day. The interval between each session was 48 h. Intranasal PREG was given 15 min prior to the first trial of each 4-trial acquisition session on days 1, 4, 7 and 10 (Fig. 1: bottom).

2.9.1. Scopolamine administration

Four days after the final acquisition session, a new 4-trial acquisition session was begun, lasting one day. Each animal was injected subcutaneously with scopolamine 60 min prior to the 1st of the 4 acquisition trials (Fig. 1: bottom). They also received intranasal administrations 15 min before the first trial according to their group assignment.

2.10. Statistics

Two ways ANOVA with the between factor "group" and within factor "objects" were applied to analyze the object exploration tests. Also, two-way repeated measures ANOVAs were conducted for analysis of water maze data with the between-factor "group" and within factors "day", "trial" and "treatment". Post-hoc Fisher's LSD tests were applied for between-group comparisons when appropriate. For the results of the cued water maze and elevated plus maze tests, one-way ANOVAs were used. Paired *t*-tests were used to compare performance between trials within each group in the water maze studies. The significance level was set as $\alpha < 0.05$. The software IBM SPSS Statistics 20.0 was used for all analyses.

3. Results

3.1. Intranasal pregnenolone facilitated long-term memory for an object and its location

3.1.1. Novel object preference

3.1.1.1. Pre-trial administration. A mixed two-way ANOVA was conducted. There was a significant effect of "object" ($F(1,22) = 10.787$, $p = 0.003$), but not of "group" and "object × group" interaction ($p > 0.05$) when the administration was given pre-trial in the NOP test. Since an "object" effect was found, we further tested whether the exploration times of the old and novel object differed within each group. The group that received 0.112 mg PREG exhibited novel object preference when tested 48 h later ($p = 0.0235$; paired *t*-test), while the other two groups did not ($ps > 0.05$; Fig. 2). No group difference was found in the analysis of the total exploration time during the test trial ($p > 0.05$; one-way ANOVA).

3.1.1.2. Post-trial administration. When the administrations were applied *post-trial* after the sample trial, there were no significant

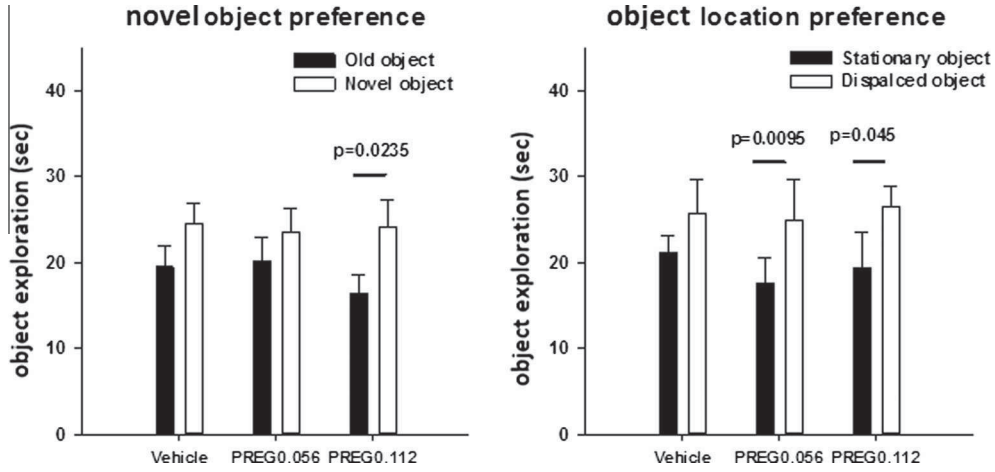

Fig. 2. Pre-trial intranasal administration of pregnenolone (PREG) improved novel object recognition and object location when tested 48 h later. In the novel object preference test, the PREG 0.112 mg group explored the novel object more than the old one, while the other two groups did not. In the object location preference test, the PREG 0.056 mg and 0.112 mg groups explored the displaced object more than the stationary one, while the vehicle-treated group did not. Values are presented as mean + S.E.M.

effects of "object", "group" and their interaction ($p > 0.05$), and neither of the three groups exhibited novel object preference ($ps > 0.05$; data not shown). There was also no significant difference in total exploration time ($p > 0.05$).

3.1.2. Object location preference

3.1.2.1. Pre-trial administration. A mixed two-way ANOVA was applied. A significant effect of "object" ($F(1,22) = 9.829$, $p = 0.005$), but not of "group" and "object × group" interaction ($p > 0.05$), was found. Given the significant effect of "object", the exploration time for stationary and displaced objects was compared within each group. The groups administered 0.056 mg and 0.0112 mg PREG exhibited object location memory 48 h later ($p = 0.0095$, $p = 0.045$, respectively; paired *t*-tests), whereas the vehicle pre-trial treated group did not ($p > 0.05$; Fig. 2). There was no significant group difference in total exploration time ($p > 0.05$; one-way ANOVA).

3.1.2.2. Post-trial administration. No significant effects of "object", "group" and their interaction were found ($p > 0.05$). Likewise, none of the three groups exhibited memory for object location ($ps > 0.05$; data not shown). No group difference was found in total times for object exploration during the test trial ($p > 0.05$).

3.2. No effect of Intranasal pregnenolone on anxiety-related behaviors

Analyses of the elevated plus-maze results, revealed no significant group differences for the measured parameters, including time spent on the open and closed arms and center region of the maze and number of head dips on open arms and stretchings (staying in closed arm, while stretching body and using forepaws and head to explore) ($ps > 0.05$; one-way ANOVAs). Details are presented in Table 1.

3.3. Water maze experiment 1: Intranasal pregnenolone increased time in previously rewarded platform quadrant during the probe/extinction session

3.3.1. Acquisition session

A repeated two-way ANOVA with the between "group" and within "day" factors was conducted. There was a significant effect of "day" ($F(3,66) = 19.258$, $p < 0.001$) but not of "group" and "group × trial" interaction ($p > 0.05$) in the analysis of the time to find the platform over the four acquisition days. The subsequent one-way ANOVAs showed no significant group difference at any day ($p > 0.05$). Thus, daily intranasal administrations over four days did not influence learning in the water maze (Fig. 3A). There was also no group difference on day 5 (when drug administration was withheld) as tested by one-way ANOVA ($p > 0.05$; Fig. 3A).

Table 1
Behaviors on the elevated plus maze. Mean (±sem) time spent (seconds, s) on the open and closed arms and center region of the elevated-plus maze (EPM) and duration of head dips on open arms (head beneath edge of arm) and of body stretching (staying in closed arm, while stretching body and using forepaws and head to explore). Entries into the open or closed arms (counts) are also listed. There were no significant group differences. Testing sessions lasted 5 min and were begun 15 min after intranasal administration of vehicle or pregnenolone.

Time spent in/entries in	Vehicle	PREG 0.056 mg	PREG 0.112 mg
Open arm (s)	253.48 ± 28.49	264.97 ± 46.31	256.06 ± 42.77
Open arm (counts)	24.78 ± 7.46	17.37 ± 11.92	21.80 ± 9.27
Closed arm (s)	9.63 ± 12.65	15.37 ± 32.62	17.38 ± 29.79
Closed arm (counts)	3.56 ± 2.92	3.88 ± 7.14	4.16 ± 5.45
Center region (s)	36.92 ± 18.68	19.68 ± 15.42	26.61 ± 14.03
Head dips (s)	22.30 ± 17.45	8.18 ± 4.65	19.17 ± 18.82
Body stretching (s)	36.53 ± 6.99	30.10 ± 11.11	29.64 ± 14.10

3.3.2. Cued trials

No significant group difference was found in the cued version of the water-maze test on day 6 (drug free; $p > 0.05$; one-way ANOVA; Fig. 3B).

3.3.3. Probe/extinction session

3.3.3.1. IN-Preg treatment. Two-way ANOVA showed that over the five administration days there was a significant "group" ($F(2, 22) = 4.227$, $p = 0.028$) effect, but no "day" and "group × day" effect ($p > 0.05$). Since a main effect of "group" was found, post-hoc LSD test were applied: these indicated that the 0.056 mg PREG group spent significantly more time in the previously rewarded platform quadrant than the vehicle ($p = 0.036$) and 0.112 mg PREG ($p = 0.012$) groups (Fig. 3C).

3.3.3.2. IN-Preg discontinued. Two-way ANOVA carried out on the last two extinction days, when IN-Preg was no longer administered, did not reveal significant main effects for "group", "day" and its interaction "group × day" ($p > 0.05$).

3.3.3.3. Visible platform trial. No significant group difference was found in the cued version at day 14 (under drug administration;

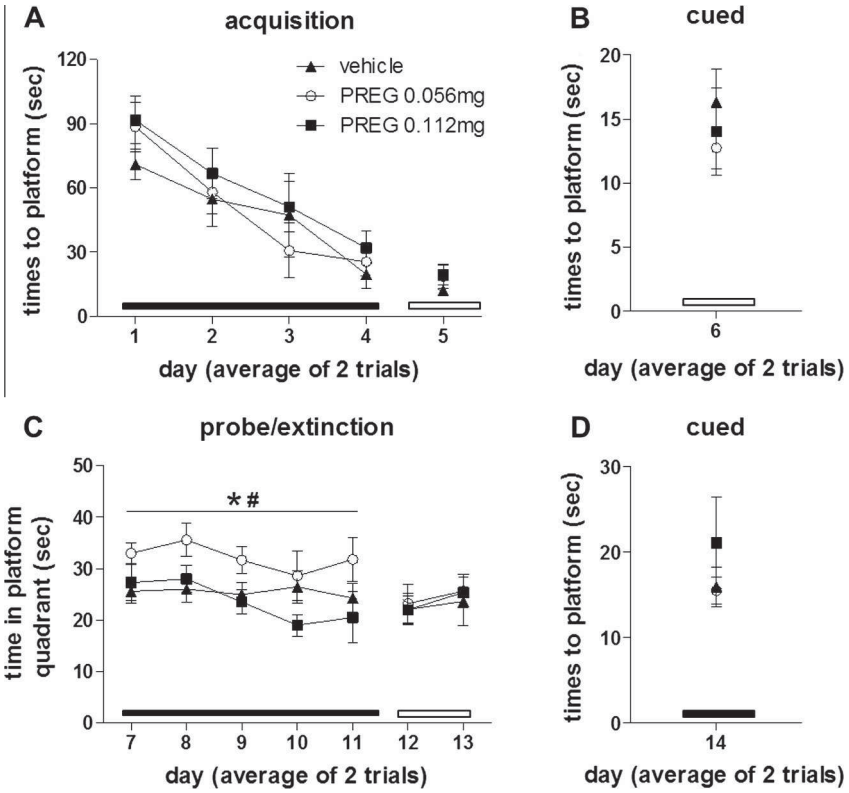

Fig. 3. Water maze experiment 1: Effects of subchronic intranasal pregnenolone (PREG) on time to reach the escape platform during acquisition and time spent in the platform quadrant during probe/extinction trials in the water maze. (A) PREG did not influence learning during the acquisition sessions. (B) During the visible platform (cued) trials, there was no significant difference between groups on drug-free day 6. (C) The daily treated PREG 0.056 mg group spent significantly more time in the previously rewarded platform quadrant during the five days of probe/extinction sessions ($^{*}p < 0.05$ compared to the vehicle group; $^{\#}p < 0.05$ compared to the PREG 0.112 mg group). There was no longer a significant difference between groups in the following two drug-free days (12 & 13). (D) No group difference was found in the final cued session when treatment was again administrated. Solid and empty lines within each panel indicate periods with and without drug administration, respectively. Values are presented as mean ± S.E.M.

$p > 0.05$; one-way ANOVA; Fig. 3D). Thus IN-PREG did not influence sensory-motor abilities in the escape response.

3.3.4. Immobility during probe/extinction

Over the five administration days, two-way ANOVA showed a significant effect of "day" ($F(4,88) = 17.573$, $p < 0.001$), but not "group" and "group × day" effects ($p > 0.05$) on duration of immobility. There were also no effects of "group", "day" and "group × day" in the analysis of the last two non-administration days ($p > 0.05$; Fig. 4A). Thus, the duration of immobility progressively increased over extinction trials in all groups, and intranasal PREG did not significantly influence this variable.

3.3.5. Distance moved and swimming velocity

There were significant effects of "day" ($F(4,88) = 41.393$, $p < 0.001$) for swimming velocity (cm/s; Fig. 4B) and distance swum ($F(4,88) = 41.964$, $p < 0.001$; Fig. 4C), but not of "group" and "group × day" interaction ($p > 0.05$) as analyzed by two-way ANOVAs over the five administration days. No effects of "group", "day" and "group × day" interaction were found for the two drug-free days ($p > 0.05$). Hence, administration of PREG influenced neither the distance swum, nor the speed of swimming during the extinction trials.

3.3.6. Thigmotaxic swimming

Two-way ANOVA showed a significant effect of "day" ($F(4,84) = 3.776$, $p = 0.007$), but not of "group" and "group × day" interaction ($p > 0.05$) over the five administration days for thigmotactic swimming. Two-way ANOVA showed no significant effects of "group", "day" and "group × day" on the two drug-free days ($p > 0.05$; Fig. 4D). Thus, PREG treatment had no influence on amount of swimming exhibited near the rim of the pool.

3.4. Water maze experiment 2: Intranasal pregnenolone ameliorated scopolamine-induced deficits in water maze performance

3.4.1. Acquisition

There was a significant effect of "day" ($F(3,66) = 52.581$, $p < 0.001$), but not of "group" and their interaction ($p > 0.05$) in the analysis of water-maze learning over trials during the four acquisition days (with 2-day interval between sessions; two-way ANOVA; Fig. 5A). Thus, as found with the first batch of animals, IN-PREG administration did not influence water-maze escape learning.

After administration of scopolamine, there was a significant effect of "trial" ($F(3,66) = 3.789$, $p = 0.014$), but not of "group" and "group × trial" ($p > 0.05$; two-way ANOVA). Since a significant "trial" effect was found, paired *t*-tests were used to compare performance between the first and second trials in order to assess whether or not significant learning occurred. Both, the PREG 0.056 mg and 0.112 mg-treated groups showed a significant decrease in time to find the platform from the first to the second trial ($t(7) = 2.506$, $p = 0.041$; $t(7) = 3.181$, $p = 0.015$, respectively), while no significant improvement was found in the vehicle group ($p > 0.05$; Fig. 5B).

Both IN-PREG groups reached the platform faster than the vehicle group on the second trial (Fig. 4B). These groups did not differ significantly and the small group sizes suggested a lack of power.

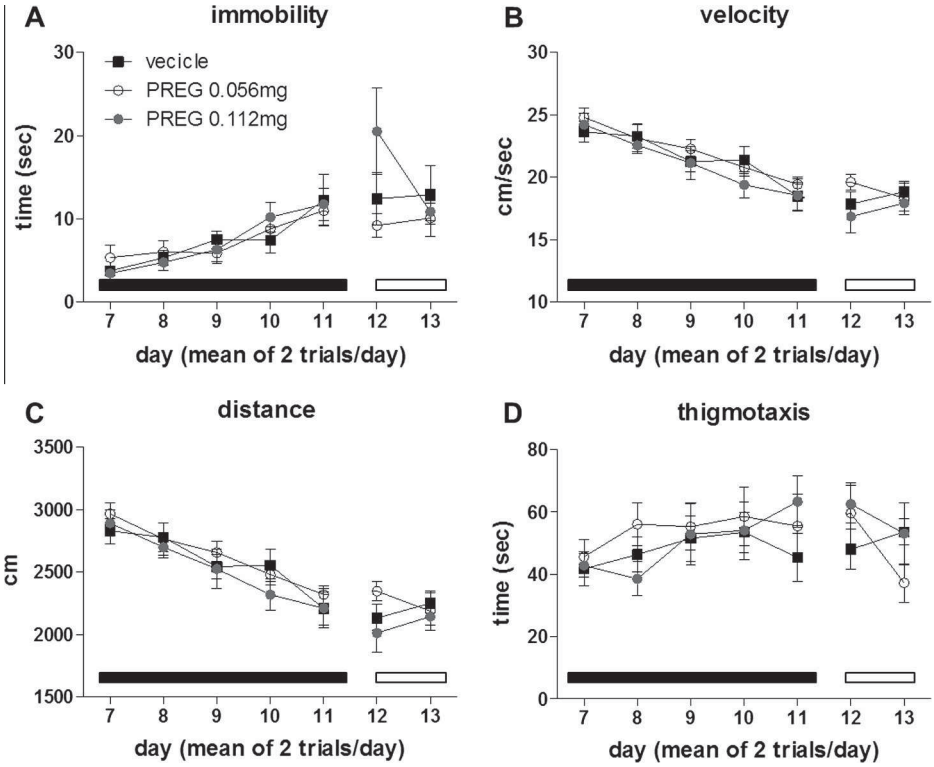

Fig. 4. Water maze experiment 1. Effects of subchronic intranasal pregnenolone (PREG) during probe/extinction sessions in the water maze. (A) immobility, (B) velocity of swimming, (C) distance swum and (D) duration of thigmotactic swimming during probe/extinction testing in water maze. Solid and empty lines within each panel indicate periods with (days 7–11)) and without (days 12–13) drug administration, respectively. No significant group differences were found during the PREG administration and non-administration days. Values are presented as mean ± S.E.M.

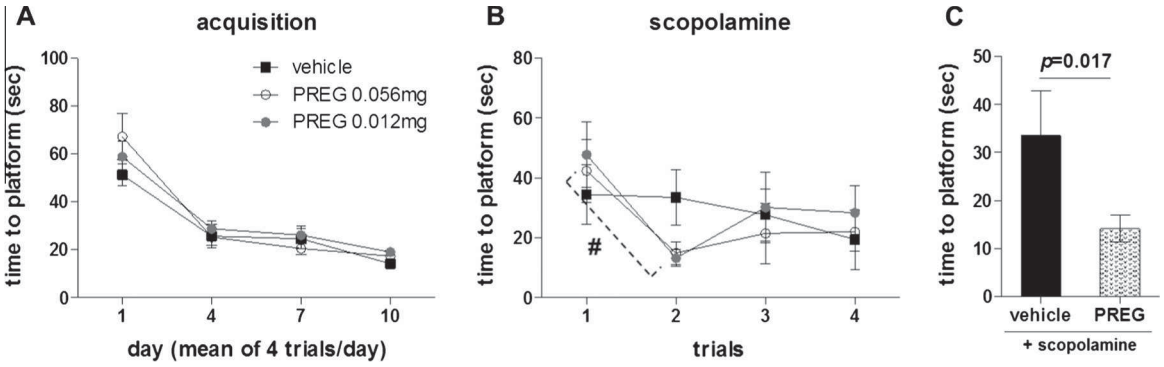

Fig. 5. Water maze experiment 2. Effects of intranasal pregnenolone (PREG) on time to find hidden platform. (A) Daily PREG treatment did not significantly influence learning over four acquisition days (4 trials/day). (B) After systemic injection of scopolamine, the two PREG-treated groups exhibited significantly improved learning from trial 1 to trial 2 ($^{\#}p < 0.05$), whereas the vehicle group did not. (C) The pooled PREG groups required significantly less time to find the escape platform compared to the vehicle group during the second trial, when under the influence of scopolamine. Values are presented as mean ± S.E.M.

Therefore, we pooled the two IN-PREG-treated groups and found a main effect of "trial" ($F(3,69) = 2.628$, $p = 0.05$), but not of "group" and "group × trial" ($p > 0.05$). One-way ANOVAs revealed that the PREG group found the platform significantly quicker than the vehicle group during the second trial ($F(1,23) = 6.578$, $p = 0.017$; Fig. 5C). Taken together, the results indicate that the IN-PREG treatment ameliorated learning deficits incurred by scopolamine in the water maze.

4. Discussion

We examined the effects of intranasal PREG on memory for objects and their location, on learning and retention of escape onto safety in a water maze and on behavior on the elevated plus maze. The main findings are: (a) Pre-trial, but not post-trial, administration of PREG facilitated long-term memory in novel-object preference and novel place-preference tests when tested 48 h after exposure. (b) After learning to escape onto a hidden platform in a water maze, extinction (probe) trials were administered. During the extinction trials, animals treated with IN-PREG spent more time in searching for the absent platform, expressing either superior memory for the former position of the escape platform and/or a higher resistance to extinction. (c) IN-PREG countered the disruptive effect of the anticholinergic scopolamine on learning to escape from the water maze. (d) IN-PREG did not influence rate of learning in the water maze. (e) No evidence was found for an

influence of IN-PREG on anxiety-related behaviors on the elevated plus maze.

There is increasing interest in the nasal route of application as an alternative to systemic injection as a means of targeting the CNS (Banks et al., 2009; Dhuria et al., 2010; Hanson & Frey, 2008; Quintana et al., 2016). Particularly the IN administration of steroids may be safer than the conventional methods of application (Banks et al., 2009). Previous studies have shown that IN-PREG might be more effective than substances with narrower therapeutic windows in the treatment of memory deficits (Ducharme et al., 2010), since it rapidly bypasses the BBB and increases concentrations in the CNS (Dhuria et al., 2010; Quintana et al., 2016). Thus, as demonstrated by the present study, IN-PREG can be an effective method to deliver this substance to the brain and to incur behavioral actions.

Administration of IN-PREG pre-, but not post-trial, strengthened long-term memory in both, a novel-object preference and a novel-location preference test, when tested after 48 h. The effectiveness of pre-trial, but failure of post-trial application suggests that this substance exerted its promnestic action by influencing processes related to the acquisition of information - its encoding - rather than having a selective influence on the consolidation of information (see Izquierdo and McGaugh (2000) and McGaugh and Roozendaal (2009) for interpretations of pre- vs post-trial drug applications on learning and memory). Pre-trial effects on performance could also be a result of drug action on other variables that may influence acquisition, such as attentiveness to the task and level of arousal. The behavioral action found is in keeping with the many findings of memory facilitation reported with systemic, intracranial and IN application of PREG or PREG-S (Akwa et al., 2001; Darnaudéry et al., 2000; Ducharme et al., 2010; Flood et al., 1992; Meziane et al., 1996; Plescia et al., 2014; Vallée et al., 1997).

In the water maze, escape onto the hidden platform can be viewed in terms of negative reinforcement (i.e., reward based on the cessation of a punishing stimulus) that leads to maze learning, whereby the probe/extinction testing assesses the degree of conditioned place preference (CPP) for a particular quadrant (Huston, Schulz, & Topic, 2009). PREG administered during the acquisition trials did not facilitate rate of learning to find the escape platform (all groups progressively improved comparably in performance over trials and days). However, when presented during the probe/extinction trials - with the escape platform absent - PREG 0.056 mg led to an increase in the time spent in the previously rewarded quadrant over all 5 extinction days. Such an increase in resistance to extinction can be considered an instance of an enhanced CPP (Schulz, Buddenberg, & Huston, 2007; Schulz, Huston, Buddenberg, & Topic, 2007). As this enhancement appeared already on the first day of extinction, it may be that the treatment administered during acquisition led to an enhanced spatial long-term memory expressed as stronger preference for the previously rewarded quadrant during extinction. Such an interpretation would be in line with our other findings of long-term enhancement of object and place memory by pre-trial IN-PREG.

On the other hand, since the increase in resistance to extinction disappeared when drug administration was discontinued on the next two days, the enhancing effect could be attributed solely to the daily treatment over the five days of extinction testing. In this case, the action of the PREG could be a result of enhancement of memory retrieval processes. Or, it could be a result of a "motivational" effect, i.e., increase in the motivation to continue seeking the goal in the previously rewarded place. However, the disappearance of effect during drug withdrawal could also be a result of a performance decrement due to state-dependent learning. An increase in time spent in the previously located platform area could also be a result of animals becoming "inflexible" in their search for alternative save areas. This explanation is unlikely to pertain to the present results, since in water-maze exp. 2, the treated animals, as well as controls, performed comparably and progressively improved in escape learning when the platform was re-located between each session. If IN-PREG had induced "inflexible" behavior, the treated animals should also have spent more time in the previously rewarded platform area during each session, especially in the first trial.

The amnesic effects of scopolamine are well established and have been demonstrated in the Morris water maze (Klinkenberg & Blokland, 2010; Saucier et al., 1996). The IN-PREG-treated groups pre-treated with scopolamine required less time to find the hidden platform than the scopolamine + vehicle rats. Note that this effect was evident after a fresh exposure to a platform relocated to a novel place. Whereas performance was comparable across groups on trial one, when the platform was found by chance, the deficit appeared on trial two, indicating lack of learning in the scopolamine treated group, but not the scopolamine + PREG treated group; both doses of PREG were effective in preventing this impairment. The similar performance on trial one precludes the possibility of initial group differences in sensorimotor performance due to scopolamine.

These results are in agreement with the previous reports of amelioration of scopolamine-induced memory/learning deficits by PREG-S (Meziane et al., 1996; Urani, Privat, & Maurice, 1998; Vallée et al., 2001). It is possible that PREG interacts with the activity of cholinergic systems, since application of PREG-S induced acetylcholine release in hippocampal and cortical areas (Darnaudéry, Koehl, Pallarés, Moal, & Mayo, 1998; Darnaudéry, Pallarés, Pizza, Le Moal, & Mayo, 2002; Darnaudéry et al., 2000; Pallarés, Darnaudéry, Day, Le Moal, & Mayo, 1998; Vallée et al., 1997). The promnestic actions of IN-PREG may refer to a direct steroid modulatory effect on cholinergic neurons, since PREG was also shown to have memory-enhancing effects when locally injected into the medial septum or nucleus basalis magnocellularis, the main sources of cholinergic innervation to the forebrain (Darnaudéry et al., 2002; Pallarés et al., 1998), although deleterious effects were also reported (Nanfaro, Cabrera, Bazzocchini, Laconi, & Yunes, 2010).

The main findings here are unlikely to be confounded by attentional, sensorimotor, or anxiety-related factors, since all of the animals exhibited comparable performance during water maze extinction in swimming speed, and time to find the cued platform. We also found that IN-PREG had neither anxiolytic-like nor anxiogenic-like effects in the elevated plus-maze. Since PREG-S and Allop are considered to play key roles in controlling behaviors on the elevated plus-maze (Akwa, Purdy, Koob, & Britton, 1999; Mòdol, Darbra, & Pallarés, 2011; Serra et al., 2000), we expected IN-PREG to also influence anxiety-related behaviors in this maze. The lack of any such action could be due to the unique features of nasal administration, such as rapid delivery of substances into the CNS and their distribution in the brain that favors certain regions over others, as is the case with IN-PREG (Ducharme et al., 2010). Thus, some effects of IN-PREG may be expected to differ from those obtained via systemic application. Alternatively, the behavioral action of IN-PREG could be more prominent in deficit models than in non-deficit models. Such an interpretation is in line with the positive effects of IN-PREG on time-delayed memory, on resistance to extinction and on a scopolamine-induced learning deficit, but not on the elevated plus maze.

Pregnenolone has been proposed to be involved in the processing of emotions, particularly in depression-related behaviors (Zorumski et al., 2013). PREG levels are lower in major depressives than in controls (George et al., 1994) and fluoxetine administration can increase PREG levels (Marx et al., 2006). Administration of PREG in patients with bipolar depression was also shown to have

beneficial effects (Brown et al., 2014). Thus, the superior performance by the PREG treated group during the extinction/probe trials in water maze (Fig. 3C) could be a synergic effect of (1) PREG interacting with the hippocampal NMDA and/or sigma receptors, leading to memory facilitation (see above) and (2) the increased PREG levels alleviating the depression-like state that accompanies extinction of water-maze learning; i.e., the withdrawal of an expected reward (Huston et al., 2009). However, given that we did not find effects of IN-PREG on measures of immobility during the extinction trials, an interpretation of its effects in terms of anti-depressive action is tenuous. We also found that IN-PREG had neither anxiolytic-like nor anxiogenic-like effects in the elevated plus-maze. Although the present discussion is framed in terms of PREG, all of the results may also be attributable to action of its derivatives, e.g. Allop, dehydroepiandrosterone and progesterone, which have also been linked to emotional behaviors and memory processing (Barros et al., 2015; Vallée et al., 1997; Zorumski et al., 2013).

PREG interacts with the NMDA and sigma receptors (Zheng, 2009), engaging neurotransmitter systems known to modulate the processing of learning and memory, especially in the hippocampus (Liu et al., 2004; Maurice et al., 1994; Tsai et al., 2009; Vianna et al., 2000). Given that the distribution of tritium-labeled IN-PREG also differentially favored the hippocampus (Ducharme et al., 2010), the long-term memory facilitation induced by IN-PREG may be due to the interaction between PREG and the NMDA and/or Sigma receptors in this region. Flood et al. (1992) found that post-training intracerebroventricular administration of PREG and PREG-S improved retention of avoidance learning. Melchior and Ritzmann (1996) and Ducharme et al. (2010) confirmed this result. Maurice, Su, and Privat (1998) attributed promnestic effects of PREG to its interaction with the Sgma-1 receptor, which seems to be involved in learning and memory, response to stress and depression, psychostimulant-induced sensitization, vulnerability to addiction and pain perception (Hayashi & Su, 2004; Maurice, 2004). Maurice, Phan, Urani, and Guillemain (2001) suggested that the Sigma-1 receptor is not necessarily involved in normal learning and memory processes, but rather exerts promnestic action when neurotransmission systems appear deficient. Sigma-1 receptors are potent modulators of acetylcholine release, and agonists have recently attracted much attention as potential therapeutic drugs for cognitive and affective disorders (e.g. Jin, Fang, Zhao, & Liu, 2015). Vallée et al. (2014) found that PREG interacts with the type-1 cannabinoid receptor, raising the possibility that this receptor may also have a mechanistic role in the present findings. More studies are required to elucidate the relationship between IN-PREG action and associated neurotransmitter systems, e.g. NMDA, sigma and type-1 cannabinoid receptors.

Given the larger number studies indicating PREG-S as having promnestic effects, it would have been interesting to compare the influence of IN-PREG and IN-PREG-S. Problems in such a comparison would likely result from the different application forms and galenical formulations used: *For one*, the blood-brain-barrier is a major hurdle for molecules. Theoretically PREG should cross the BBB more efficiently than PREG-S since it is neutral and lipophilic (permitting rapid permeation across cell membranes), whereas PREG-S is negatively charged due to its sulfate group. It should be emphasized that nasal application is the only application form where, in addition to the BBB pathway, two additional ones are available for brain targeting - the "olfactory" and the "trigeminal" nerve pathways. Accordingly, it was shown that results obtained with intravenous application of PREG cannot be compared to results obtained with its nasal application. The uptake by 11 brain regions of tritium-labeled PREG was assessed after intranasal and intravenous (IV) application. With intranasal administration, brain levels of PREG did not vary over time (2–120 min), whereas brain levels were higher early (10 min or less) after IV administration. With IV administration, uptake by brain regions did not vary, whereas the olfactory bulb, hippocampus, and hypothalamus had high uptake rates after IN administration, and PREG was more resistant to degradation after IN administration. *Secondly*, in order to apply PREG or PREG-S, it must be incorporated into a galenical formulation (matrix). PREG has a solubility in water of 0.0136 mg/mL only (i.e., it is insoluble). PREG-S is commercially available as "PREG-S sodium salt (CAS 1852-38-6)" which is soluble in water at 1.93 mg/mL. Converting PREG into the sulfate form obviously changes the solubility of the molecule in lipids and blood. In terms of pharmacokinetics and pharmacodynamics, this is quite an intervention since this modification also changes the partition-coefficient of the molecule which estimates the distribution of a drug within the body: hydrophobic drugs like PREG are preferentially distributed to hydrophobic compartments such as the lipid bilayers of cells while hydrophilic drugs such as the PREG-S are preferentially found in aqueous compartments such as the blood. Thus, for the distinct physical-chemical properties of PREG and PREG-S, a direct comparison of effects in the same matrix would be interesting but would require a complex galenical formulation which has not been described so far. The nasal gel tested in the present publication is lipophilic and without modification not suitable for the incorporation of PREG-S.

5. Conclusion

Our results have shown that the administration of Pregnenolone by the nasal route enhanced long-term memory for an object and its location, compensated scopolamine-induced water-maze learning deficits and provide evidence for improved spatially instated long-term memory as tested by water-maze probe/extinction trials. These results confirm an important role for PREG in the control of emotional behaviors and cognitive functions, either as an active neurosteroid modulating other neurotransmitter systems, or as a precursor to its downstream steroids. Most importantly, the results indicate that the nasal application of PREG has promnestic action in several tests of learning and memory, comparable to those obtained by other routes of administration in the rodent. These results suggest the prospect of employing the nasal route for medicinal application of PREG also for the human as an alternative method with non-invasive, rapid and direct delivery of substance to the CNS, for example, as a possible promnestic agent in patients with Alzheimer's disease who exhibit decreased levels of PREG (Schumacher et al., 2003; Weill-Engerer et al., 2002).

Conflict of interest statement

C.M. is an employee of M et P Pharma AG.

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9. Declaration

Die hier vorgelegte Dissertation habe ich selbständig und nur unter Verwendung der angegebenen Literaturquellen angefertigt. Diese Arbeit wurde in der vorgelegten oder ähnlichen Form bei keiner anderen Institution eingereicht. Zudem erkläre Ich, dass Ich bisher keine erfolglosen Promotionsversuche unternommen habe.

Dusseldorf, den; 21.02.2018