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THÈSE

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Multidisciplinary analysis of biological effects of novel analogs of the neurosteroid allopregnanolone: evidence for a proliferative, neurogenic and neuroprotective action

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Моїм батькам

إلى والديّ

To my parents

A mes parents

ACKNOWLEDGEMENT

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ABREVIATIONS

$[Ca^{2+}]_i$	Intracellular calcium concentration
12 oxo-AP	12 oxo-allopregnanolone, Pregnane-12,20 dione 3-hydroxy (3α, 5α)
12 oxo-epiAP	12 oxo-epiallopregnanolone, Pregnane-12,20 dione 3-hydroxy (3β, 5α)
3α-HSD	3α-hydroxysteroid dehydrogenase
3β-HSD	3β-hydroxysteroid dehydrogenase
5α-DHP	5α-dihydroprogesterone
AD	Alzheimer´s disease
ADAM	A disintegrin and metalloprotease
AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
AF-1 and -2	Activation function 1 and 2
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
ANS	Analogs of allopregnanolone
aNSCs	Adult neural stem cells
AP	Allopregnanolone
Apaf-1	Apoptotic protease activating factor-1
APH-1	Anterior pharynx-defective-1
APOE	Apolipoprotein E
APP	Amyloid precursor protein
Ascl1	Achaete-scute homolog 1
ATP	Adenosine triphosphate
Aβ	Amyloid β-peptide
B	Bicuculline
BACE1	β-site APP cleaving enzyme 1
Bax	Bcl-2 associated X protein
BCA	Bicinchoninic acid
Bcl-2	B-cell Lymphoma 2
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BLBP	Brain lipid-binding protein

BMP	Bone morphogenetic protein
BrdU	5-bromo-2´-deoxyuridine
BSA	Bovine serum albumin
CA1 and CA3	Cornu Ammonis area 1 and 3
CalB	Calbindin
CaMK IV	Calcium/calmodulin-dependent protein kinase type IV
Caspase	Cysteine-dependent aspartate-directed proteases
CDK1	Cyclic dependent kinase 1
cDNA	Complementary DNA
CGC	Cerebellar granule cells
CNS	Central nervous system
CR	Calretinin
CREB1	Cyclin AMP-responsive element-binding protein
Ct	Threshold cycle
DAB	3,3′-diaminobenzidine
DBD	DNA-binding function
DCX	Doublecortin
DG	Dentate gyrus
DHEA	Dehydroepiandrosterone
DIV	Days *in vitro*
Dlx-2	Distal-less gene 2
DMEM	Dubelcco´s modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Nucleoside triphosphate
EC50	Half maximal effective concentration
EdU	5-ethynyl-2´-deoxyuridine
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
epiAP	Epiallopregnanolone, 3β-Hydroxy-5α-pregnan-20-one
ES	Embryonic stem
FAD	Familial Alzheimer disease
FADD	Fas-associated protein with death domain
FCS	Fetal calf serum

FGF2	Fibroblast growth factor 2
GABA	γ-Aminobutyric acid
GABA$_A$R	γ-Aminobutyric acid type A receptor
GCL	Granular cell layer
GFAP	Glial fibrillary acidic protein
H region	Hinge region
HBSS	Hanks Buffered salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HNSC	Human neural stem cells
HRP	Horse radish peroxidase
HS	Horse serum
Hu Aβ40, Aβ42	Human Aβ40, Aβ42
Iba1	Ionized calcium binding adaptor molecule 1
IL6	Interleukin 6
IPSC	Induced pluripotent stem cells
LV	Lateral wall of the lateral ventricle
LXR	liver X receptor
M	Mifepristone
MAPs	Microtubule-associated proteins
MCI	Mild cognitive impairment
MEM	Minimum essential medium
MMA	Methylazoxymethanol acetate
mPR	Membrane progesterone receptors
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger ribonucleic acid
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide
N	Nifedipine
NaN$_3$	Sodium azide
NDS	Normal donkey serum
NeuN	Neuronal nuclear antigen
NeuroD	Neurogenic differentiation factor
NF-κB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NFTs	Neurofibrillary tangles

NGF	Nerve growth factor
NGS	Normal goat serum
NHS	Normal horse serum
NMDA	N-methyl-D-aspartate
NRS	Normal rabbit serum
NSCs	Neural stem cells
nSR	Nuclear steroid receptors
NTg	Non-transgenic
O-allyl-AP	O-allyl-allopregnanolone, Pregnan-20 one 3-(2-propen-1-yloxy) (3 α, 5α)
O-allyl-epiAP	O-allyl-epiallopregnanolone, Pregnan-20 one 3-(2-propen-1-yloxy) (3β, 5α)
OB	Olfactory bulb
OD	Optical density
P	Provera, Medroxyprogesterone 17-acetate
P450c21	Cytochrome P450c21 or steroid 21-hydroxylase
P450scc	Cytochrome P450side-chain-cleavage
Pax6	Paired box protein 6
PB	Phosphate Buffer
PBS	Phosphate Buffered Saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PNS	Peripheral nervous system
PR	Progesterone receptors
PrP^C	Cellular prion protein
Prox1	Prospero-related homeobox gene 1
PSEN1 and 2	Presenilin 1 and Presenilin 2
PTSD	Post-traumatic stress disorder
PXR	Pregnane xenobiotic receptor
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Real-time reverse transcription polymerase chain reaction
RAGE	Receptor for advanced glycation end products
RFU	Relative fluorescence units

RMS	Rostral migratory stream
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
S12	Ribosomal protein S12
SDS	Sodium dodecyl sulfate
SEM	Standard error of the means
SGZ	Subgranular zone
Shh	Sonic hedgehog
siRNA	Small interfering ribonucleic acid
Sox2	SRY-related HMG-box gene 2
SSRIs	Selective serotonin reuptake inhibitors
StAR	Steroidogenic acute regulatory protein
SVZ	Subventricular zone
Tg	Transgenic
TH	Tyrosine hydroxylase
TMB	Tetramethylbenzidine
TNFα	Tumor necrosis factor alpha
TRIzol	Guanidinium thiocyanate-phenol-chloroform
TSPO	Translocator protein 18 kDa
Tuj-1	Class III beta-tubulin
VDLCC	Voltage-dependent L-type calcium channels
Wnt	Wingless
α7nAChR	α7 nicotinic acetylcholine receptor

INTRODUCTION

1. Neurodegenerative diseases

Neurodegenerative diseases are common disorders of the nervous system that affect millions of people worldwide and generate significant suffering for patients as well as medical and social costs of several billion euros per year.

Neurodegenerative diseases such as Alzheimer´s disease (AD), Parkinson´s disease, Huntington´s disease, amyotrophic lateral sclerosis, multiple sclerosis, frontotemporal dementia, spinocerebellar ataxia, motor neuron diseases, prion disease, peripheral neuropathic pain, dementia with Lewy bodies and many others present a common feature which is the deregulation of processes controlling the protection and survival of nerve cells, which leads to the degeneration of a part of the central nervous system (CNS) or peripheral nervous system (PNS) and neural death. In this thesis we will mainly focus on Alzheimer´s disease.

Neurological disorders caused by neurodegenerative diseases, depending on the brain region and the neuron type concerned, affect memory, cognition, language, perception and sensitivity to pain, locomotion and realization of motor actions. For example, loss of memory and cognitive functions are the hallmark of AD, whereas involuntary tremor and the loss of control of motor activities are the main symptoms of Parkinson's disease.

Aging is considered the primary risk factor for most neurodegenerative disorders. However, the cellular and molecular mechanisms involved in neurodegenerative diseases are still poorly understood, which explains the absence of effective treatment, despite the large number of affected people.

Interestingly, many forms of neurodegenerative diseases share a common pathological phenotype which is the accumulation of misfolded and aggregated proteins in the brain. Therefore, a lot of studies aim to better understand the processes which lead to generation of these protein aggregates in order to elaborate new therapeutic approaches (Ciechanover and Kwon, 2015).

2. Alzheimer´s disease

AD was first described in 1906 by *Alois Alzheimer*, a German psychiatrist and neuropathologist (Alzheimer et al., 1995), as a form of dementia that was subsequently named after him. His study was based on the observation of a 51-year-old female patient, *Augusta Deter*, who presented a progressive cognition impairment, disorientation, aphasia, hallucinations, mental confusion, and paranoid delusions (Maurer et al., 1997). From the post-mortem study of her brain, *Alois Alzheimer* was able to identify neuritic plaques and neurofibrillary tangles.

AD is the most common form of dementia and the first leading cause of death according to the *2015 Alzheimer's Disease Facts and Figures* (Alzheimer´s Association). It accounts for an estimated 70 % of cases of dementia and affects nearly 44 million people worldwide. Age is the main risk factor of AD, the incidence of AD increases rapidly with age: from 0.5 % among 65-70 years old to 15 % of individuals over the age of 80. With the continuous growth of the proportion of the older population due to medical progress, the number of patients is expected to increase to nearly 76 million by 2030 if breakthroughs in prevention or treatment are not achieved. Therefore, AD represents a major health concern and became a research priority worldwide.

AD typically progresses slowly in three clinical stages: preclinical, mild cognitive impairment (MCI) and dementia (Sperling et al., 2011). Changes in the brain related to Alzheimer's begin years before any symptoms of the disease.

AD leads to the progressive loss of mental and behavioral capabilities and of the ability to learn (Anand et al., 2014). The neuropathology of AD is characterized by abnormal protein deposits (Fig. 1): hyperphosphorylated tau proteins, which assemble into intraneuronal neurofibrillary tangles (NFTs) (Ballatore et al., 2007) and amyloid β-peptide (Aβ), which accumulates in the extracellular space of the brain as senile plaques (Glenner and Wong, 1984; Masters et al., 1985). The functional relationship between these two processes is heavily investigated (Ittner et al., 2010; Vossel et al., 2010).
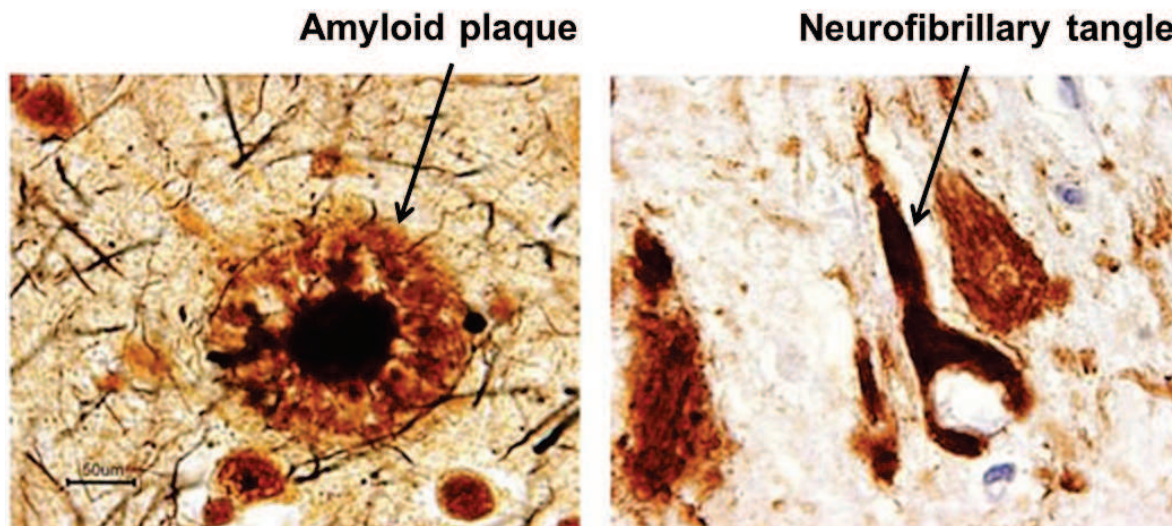
Figure 1: Amylpoid plaques and neurofibrillary tangles in a section from the hippocampus of an AD patient (Images taken from coloradodementia.org).

2.1. Neurofibrillary tangles

Neurofibrillary tangles are filamentous inclusions, consisting of paired helical filaments, which accumulate in the cell bodies and processes of neurons. These intraneuronal lesions are formed by highly phosphorylated forms of the microtubule-associated protein Tau (Fig. 1 and 2) (Goedert et al., 1989; Buée et al., 2000; Mandelkow and Mandelkow, 2012). The disequilibrium between the activities of protein kinases and phosphatases acting on tau leads to an early accumulation of phosphorylated tau proteins which later on form neurofibrillary tangles (Fig. 2). Tau protein, belonging to the family of microtubule-associated proteins (MAPs), is highly expressed in neurons. In physiological conditions, Tau is a soluble protein that participates in microtubule assembly and in their stabilization. Its activity is regulated by phosphorylation (Köpke et al., 1993). In pathological conditions, tau loses its solubility, forms filamentous structures and is abnormally phosphorylated on certain residues. As a consequence, Tau is no longer able to bind to microtubules, which leads to microtubule instability and axonal degeneration (Fig. 2) (Bramblett et al., 1993; Crespo-Biel et al., 2012).

Neurofibrillary tangles are not specific to AD. They were also identified in fronto-temporal dementia and in Parkinson´s disease (Poorkaj et al., 1998; Spillantini and Goedert, 1998).
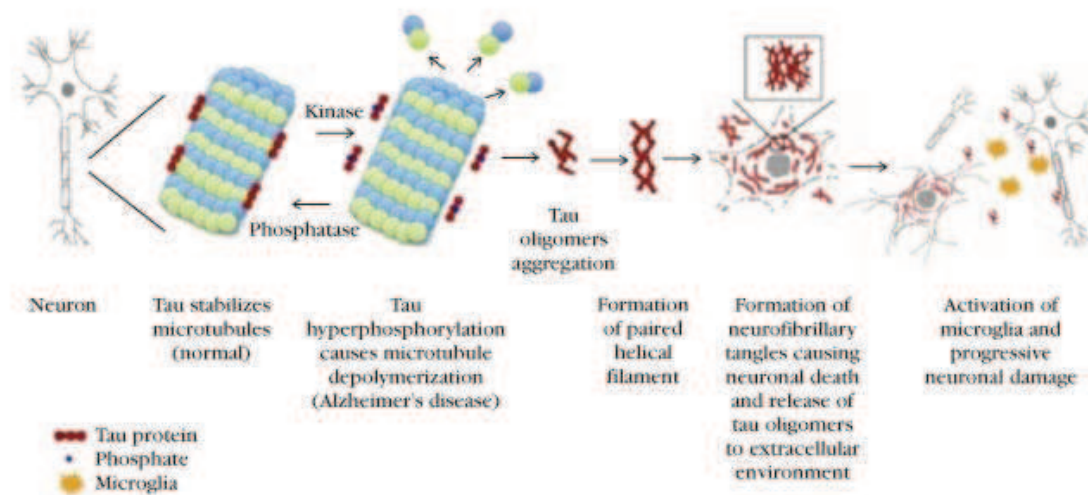
Figure 2: Formation of neurofibrillary tangles (NFTs) (Mokhtar et al., 2013).

2.2. Amyloid peptide

Amyloid β-peptide (Aβ) is the primary component of the senile plaques found in AD patient brain tissue (Masters et al., 1985). Aβ (4-kDa) is a proteolytic cleavage product of amyloid precursor protein (APP). APP, a type I transmembrane protein, can be cleaved by different proteolytic enzymes: α- β- and γ-secretases and processed through two different pathways: the amyloidogenic pathway and the non-amyloidogenic pathway (Eggert et al., 2004) (Fig. 3). Under physiological conditions, these two pathways are balanced as there is an equilibrium between the production of Aβ peptides and their clearance from the brain (Vetrivel and Thinakaran, 2006). In AD, there is a metabolic shift favoring the amyloidogenic cleavage of APP which, along with a reduction of Aβ clearance, leads to the accumulation of Aβ within the brain (Kunjathoor et al., 2004).

2.2.1. The non-amyloidogenic pathway

In the non-amyloidogenic pathway, the extracellular region of APP is cleaved by the metalloprotease α-secretase, releasing a soluble N-terminal fragment (sAPP-α) and a C-terminal fragment 83 (APP-CTF83) (Fig. 3). The sAPP-α domain is secreted into the extracellular space and is thought to have neuroprotective and neurotrophic effects (Furukawa et al., 1996; Kojro and Fahrenholz, 2005). The C83 fragment is subsequently cleaved by γ-secretase, releasing an APP intracellular domain (AICD) and a P3 fragment that is not toxic; in contrast it is believed to exert a neuroprotective

effect (Kojro and Fahrenholz, 2005; Dulin et al., 2008). Enzymes with α-secretase activity have been identified as members of the ADAM (A Disintegrin And Metalloprotease) family. The major members of the α-secretase family are ADAM9, ADAM10 and the tumor necrosis factor converting enzyme ADAM17 (Allinson et al., 2003; De Strooper, 2010).
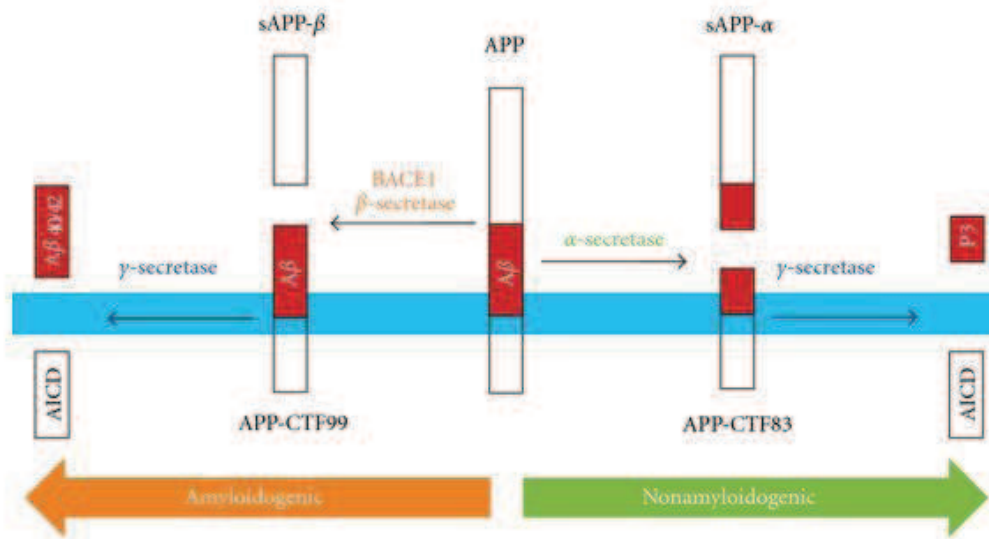
Figure 3: Amyloid precursor protein (APP) proteolytic pathways. The transmembrane protein APP (membrane indicated in blue) can be processed by two pathways, the nonamyloidogenic α-secretase pathway and the amyloidogenic β-secretase pathway (Wang et al., 2012).

2.2.2. The amyloidogenic pathway

The amyloidogenic pathway, the less abundant pathway for APP cleavage, leads to generation of Aβ peptides. In this pathway, β-secretase cleaves APP at the N-terminal boundary of the Aβ peptide domain and produces a smaller soluble N-terminal fragment (sAPP-β) and the C-terminal fragment 99 (APP-CTF99) (Fig. 3) (Haass, 2004). C99 fragment produces AICD and the full-length Aβ peptides upon the subsequent cleavage by γ-secretase. The length of Aβ peptides varies between 37 and 43 amino acids (Qi-Takahara et al., 2005). The biological functions of APPs-β, Aβ, and the AICD are not yet fully understood, although Aβ release has been shown to be associated with reduced synaptic activity and abnormal

neurotransmission (Kamenetz et al., 2003). The major Aβ species are Aβ40 and Aβ42 (Korczyn, 2008).

2.2.3. Enzymes implicated in proteolytic processing of APP

β-secretase activity is mainly due to the β-site APP cleaving enzyme 1 (BACE1), a type 1 transmembrane aspartic protease, that is related to pepsin and retroviral aspartic protease families (Hussain et al., 1999). BACE1 is the rate-limiting enzyme in the proteolytic processing of APP and is required for the production of Aβ. BACE1-processing in APP mutants can lead to a dramatic increase (Citron et al., 1992) or decrease (Jonsson et al., 2012) in amyloidogenic processing and to altered AD risks. γ-secretase has been identified as a multi-subunit aspartyl protease which is composed of presenilin 1 or 2 (PSEN1 or PSEN2) forming the catalytic core of γ-secretase, and three accessory proteins: nicastrin, anterior pharynx-defective 1 (APH-1) and presenilin enhancer 2 (PEN2) (De Strooper, 2003; Edbauer et al., 2003; Takasugi et al., 2003). These three proteins appear to be involved in the maturation and stability of the complex. γ-secretase is heterogeneous with respect to its protein composition and expression as six different functional γ-secretase complexes have been recognized which allow several cleavage possibilities of APP (Hébert et al., 2004; Shirotani et al., 2004).

2.2.4. The amyloid cascade hypothesis and its evolution

There are several Aβ peptide species, the most abundant ones produced by the amyloidogenic pathway are Aβ40 and Aβ42 (Korczyn, 2008). Aβ peptides are released as monomers which progressively self-aggregate into oligomers, protofibrils and fibrils, and finally are deposited and mature to amyloid plaques (Roberts et al., 1994). Aβ42, a hydrophobic peptide, has a strong tendency to aggregate and fibrilize which leads to the formation of amyloid fibrils and plaques (Jarrett et al., 1993a, b). Therefore, it is considered as a major cause of neurotoxicity and plays a crucial role in AD pathogenesis (Butterfield, 2002; Walsh and Selkoe, 2007; Yankner and Lu, 2009). Aβ42 was first described in familial AD forms (FAD) and an elevated ratio of Aβ42 to Aβ40 was detected in AD brains (Hardy and Selkoe, 2002). Interestingly, recent studies showed that Aβ oligomers, rather than plaques are the most toxic form of the Aβ peptide (Haass and Selkoe, 2007; Walsh and Selkoe, 2007).

The hypothesis that AD may arise from the accumulation of misfolded β-sheet proteins in a manner analogous to systemic amyloidoses was proposed after the isolation and identification of Aβ peptides in the brain of AD patients (Glenner and Wong, 1984). This hypothesis was supported by several studies; and in 1992, Hardy and Higgins named it the *amyloid cascade hypothesis*, which became the basis for work on AD pathogenesis (Hardy and Allsop, 1991; Hardy and Higgins, 1992; Tanzi and Bertram, 2005). The amyloid cascade hypothesis proposed that accumulation of β-amyloid peptides into neuritic and senile plaques in the brain, due to an imbalance between Aβ production and Aβ clearance, is the key pathogenic feature of AD; whereas the rest of the disease process, including the formation of neurofibrillary tangles is a consequence of this imbalance (Hardy and Selkoe, 2002). In the past few years, the amyloid cascade hypothesis has been modified because of a non-linear correlation between the number of amyloid plaques in the brain and the progression of AD pathology especially the degree of cognitive impairment (Games et al., 1995; Price et al., 2009). Results from recent studies now suggest a correlation between the concentration of soluble Aβ species and the degree of pathology (Giannakopoulos et al., 2003). Therefore, a modified amyloid cascade hypothesis has been proposed, suggesting that soluble Aβ oligomers are the major cause of synaptic dysfunction, neuron damage and memory impairment in early AD (Scheff and Price, 2006; Scheff et al., 2007; Selkoe, 2008).

On the other hand, Braak and Braak demonstrated a clear correlation between the degree of neurofibrillary tangle pathology and cognitive impairment in AD patients (Braak and Braak, 1996). These observations have been proven by additional studies (Nelson et al., 2012; Jack and Holtzman, 2013). Moreover, intra-neuronal hyperphosphorylated Tau was found in brains of subjects with mild dementia, unaccompanied by β-amyloid pathology (Mazanetz and Fischer, 2007; Grinberg et al., 2009). Therefore an alternative hypothesis started to develop recently, suggesting that dysfunction in Tau homeostasis may be an early event in the physiopathology of AD, whereas Aβ overproduction and oxidative stress might be resulting consequences of this dysfunction in neuronal homeostasis.

2.2.5. Toxicity of amyloid beta oligomers

After the discovery that the most toxic form of the Aβ peptide are the Aβ oligomers (Haass and Selkoe, 2007), it has been shown that Aβ oligomers are produced by the

cooperative activity of both neurons and associated astrocytes (Dal Prà et al., 2014). Aβ oligomers induce toxic effects on synapses and mitochondria such as oxidative stress (Sultana et al., 2009) and tau hyperphosphorylation (De Felice et al., 2008). Oligomers are found both extracellularly and intracellularly and are capable of moving between the interior of the cell and the extracellular space (Gaspar et al., 2010). However, the exact mechanisms of amyloid oligomers formation and toxicity are still not clear. Several mechanisms have been proposed (Fig. 4) (Kayed and Lasagna-Reeves, 2013): (i) "Aβ receptors", a number of Aβ-binding proteins have been identified on the cell surface of neurons which could mediate Aβ-induced neurotoxicity (Fig. 4A). These proteins include the nerve growth factor (NGF) receptor, N-methyl-D-aspartate (NMDA) receptor, insulin receptor, Frizzled receptor and cellular prion protein (PrP^C) receptor (Costantini et al., 2005; Snyder et al., 2005; Magdesian et al., 2008; Zhao et al., 2008b; Laurén et al., 2009). (ii) Membrane permeabilization and Aβ-channels formation, which lead to disturbed homeostasis of calcium and other ions and subsequent promotion of free radical formation and Tau hyperphosphorylation (Fig. 4B) (Takashima et al., 1993; Yatin et al., 1998; Kagan et al., 2002; Kagan et al., 2004). (iii) Internalization and intracellular accumulation of Aβ by binding to several receptors such as scavenger receptor for advanced glycation end products (RAGE), α7 nicotinic acetylcholine receptor (α7nAChR) and apolipoprotein E receptor (APOE) (Fig. 4C) (Yan et al., 1996; Wang et al., 2000a; Bu et al., 2006). Intracellular accumulation of the oligomeric Aβ leads to proteasome impairment, mitochondrial dysfunction and disturbance of autophagy (Caspersen et al., 2005; Nixon et al., 2005; Mousnier et al., 2007).
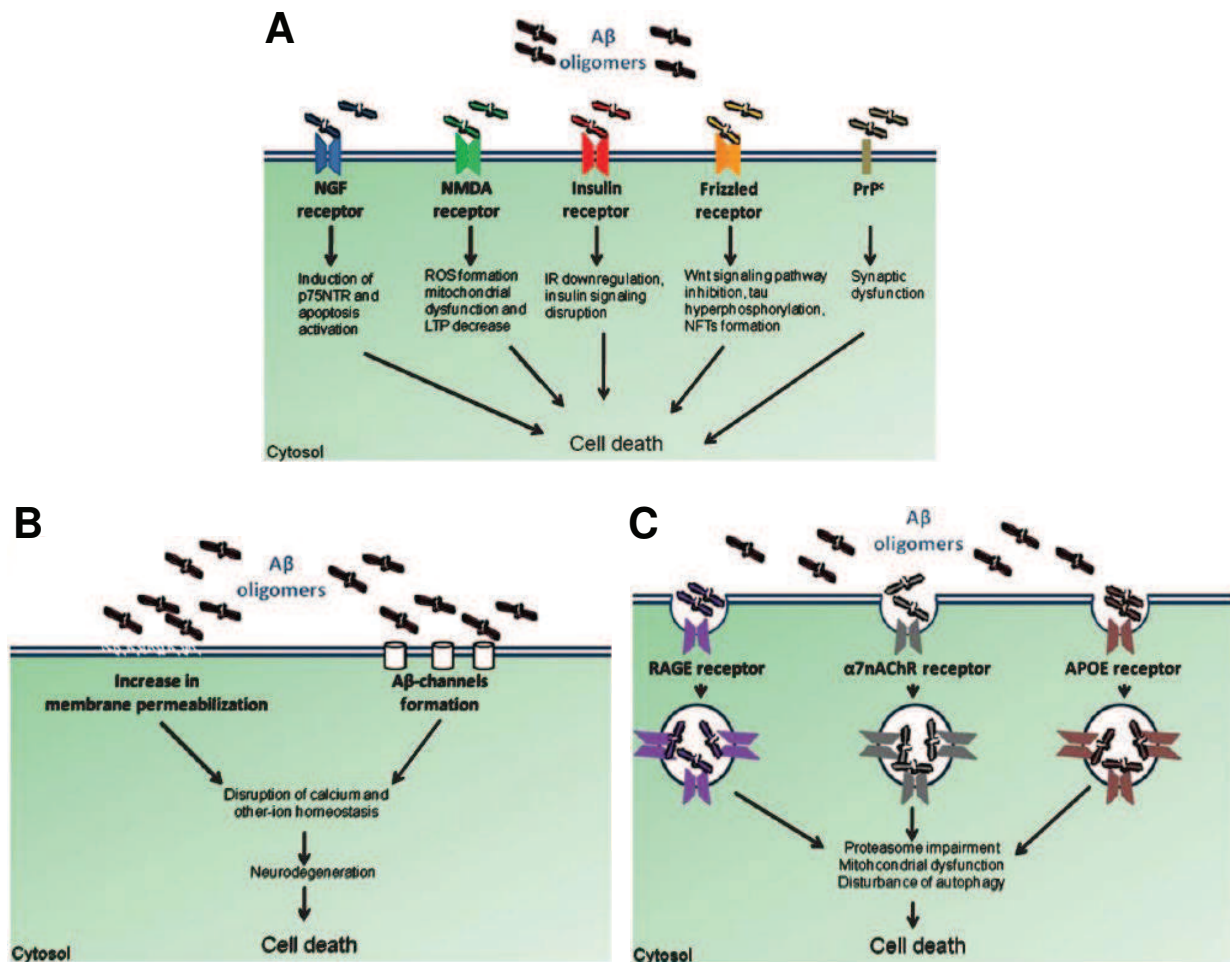
Figure 4: Proposed molecular mechanisms of amyloid beta oligomer toxicity. A) Aβ oligomers may bind to multiple receptors leading to the activation of various signaling pathways. B) Aβ oligomers insert into the membrane and subsequently form ion channels or pores which lead to neurodegenerative processes. C) The intracellular accumulation of Aβ oligomers and other aggregates causes many key pathological events of AD, including proteasome impairment, mitochondrial dysfunction and breakdown of many cellular processes (Kayed and Lasagna-Reeves, 2013).

2.2.6. Amyloid beta induced apoptotic pathways

Su and colleagues were the first to suggest that apoptosis is a physiopathological process involved in AD (Su et al., 1994). Since then, other studies have shown that increased production and accumulation of Aβ peptides induce neurotoxicity by activating apoptotic processes (LaFerla et al., 1995; Li et al., 1996; Cotman, 1998; Eckert et al., 2001). For example, exposure of PC12 cells and hippocampal neurons in culture to Aβ42 peptide activates two parallel apoptotic pathways which lead to cell death (Jordán et al., 1997; Troy et al., 2000). Aβ42 is able to activate the intrinsic

pathway of apoptosis (Fig. 5). Aβ42 downregulates bcl-2, a key anti-apoptotic protein, and upregulates bax, a protein known to promote apoptotic cell death induced by a decrease in mitochondrial membrane potential. Subsequently, cytochrome c is released by dysfunctional mitochondria. Cytochrome c, together with the adapter protein apoptotic protease-activating factor-1 (Apaf-1) and pro-caspase-9 forms an apoptosome (Shiozaki et al., 2002; Degli Esposti and Dive, 2003). The initiator caspase-9 becomes active and cleaves the effector pro-caspase-3 into its active form which initiates apoptosis leading to protein and DNA cleavage and cell death.

In addition, Aβ42 has been shown to have an effect on the extrinsic pathway of apoptosis (Fig. 5). Aβ42 may lead to the cross-linking and activation of death receptors, such as Fas receptor, resulting in activation of the initiator caspase-8 (Rohn et al., 2001). Caspase-8 then mediates the cleavage of the pro-caspase-3. Activated caspase-3 is the common, cell death initiating effector enzyme of both pathways.

Based on the strong evidence in favor of the amyloid cascade hypothesis and the toxic effect of Aβ oligomers, AD drug development was focused on molecules targeting Aβ generation and aggregation. Most drug candidates aimed to inhibit Aβ toxicity by reducing further Aβ aggregation and plaque formation. These drug candidates include secretase inhibitors to lower Aβ production from APP, Aβ aggregation inhibitors to inhibit Aβ oligomerization or fibrillization, Aβ degradation inducers to increase Aβ clearance, as well as active and passive Aβ immunotherapies designed to capture either soluble or aggregated Aβ, which then can be either degraded or cleared from the brain (Blennow et al., 2014).
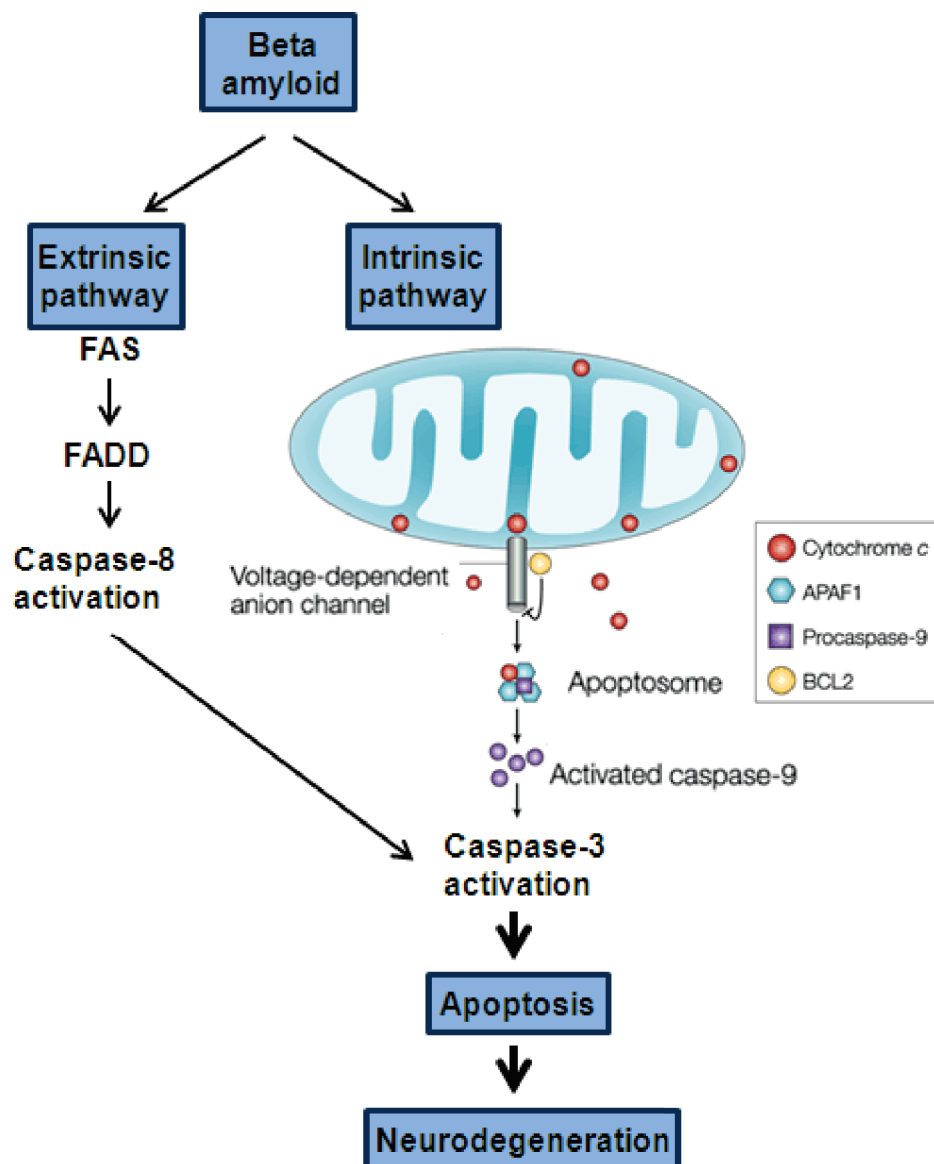
Figure 5: Aβ-induced apoptotic pathways. Aβ oligomers exert their toxic effects via two apoptotic pathways: the extrinsic and the intrinsic pathways. Both pathways lead to neuronal loss and neurodegeneration. Adapted from (Hersey and Zhang, 2001; Eckert et al., 2003).

It is important that therapeutic approaches aim at inhibiting disease progression but also at improving cognitive impairment due to neuronal loss. Therefore, in search of an effective treatment, it is essential to take into consideration two important aspects: (i) that of therapeutic neurogenesis which relies on the ability to stimulate the generation of new neurons in the nervous system to compensate for cell loss caused by neural damage, trauma, neurotoxic substances, oxidative stress, senescence or even brain aging; (ii) that of neuroprotection which aims to protect neurons against cell death and thus preserves their functions. Consequently, the exploitation of these

two aspects in the adult brain is a very promising avenue for research of new therapies against neurodegenerative diseases. Neurosteroids, endogenous steroidal compounds, have been found to positively influence both of these processes, and therefore, are promising therapeutic candidates. They are synthesized locally within the nervous system by both neurons and glial cells and have been shown to exert several effects in brain including neuroprotection (see parts 4 and 5, pages 33-50).

3. Adult neurogenesis in the rodent brain

Neurogenesis is defined as the process of generating functional neurons from stem and precursor cells. This process was believed to occur only during embryonic and perinatal stages of development in the mammalian brain (Ming and Song, 2005). In 1965, Altman and colleagues provided the first anatomical evidence for the presence of newly generated dentate granule cells in the postnatal rat hippocampus (Altman and Das, 1965). After several studies in different experimental models, it became accepted that mammalian brains retain the capacity to generate new neurons throughout life (Kempermann and Gage, 1999; Gross, 2000; Lie et al., 2004). In the mammalian CNS, active adult neurogenesis occurs in two specific "neurogenic" brain regions under normal conditions: (i) the subventricular zone (SVZ) lining the lateral ventricles, where new neurons are generated and then migrate via the rostral migratory stream (RMS) to the olfactory bulb (OB) to become interneurons and integrate into the neuronal circuit; (ii) the subgranular zone (SGZ) located in the dentate gyrus of the hippocampus, where new dentate granule cells are generated (Fig. 6). However, generation of new neurons was also observed in the hypothalamus (Kokoeva et al., 2005; Lee et al., 2012). It is still controversial whether neurogenesis occurs in neocortical areas (Bhardwaj et al., 2006; Zhao et al., 2008a). Knowledge regarding neurogenesis in the adult human brain is limited in comparison to that in rodents. However, it has been shown that new neurons are generated in the dentate gyrus of the hippocampus in the adult human brain in comparable numbers to those observed in rodents (Eriksson et al., 1998; Spalding et al., 2013). In the human olfactory system neurogenesis however, is extremely low even though quiescent neural stem cells and progenitor cells may also persist in the human SVZ (Sanai et al., 2004; Curtis et al., 2007). Recently, it has been reported that the human striatum is capable of generating striatal interneurons throughout life (Ernst et al.,

2014). Novel methods to measure levels of neurogenesis in the human brain, like the non-invasive magnetic resonance imaging technique, are needed to overcome the existing gap between animal and human research (Ho et al., 2013).
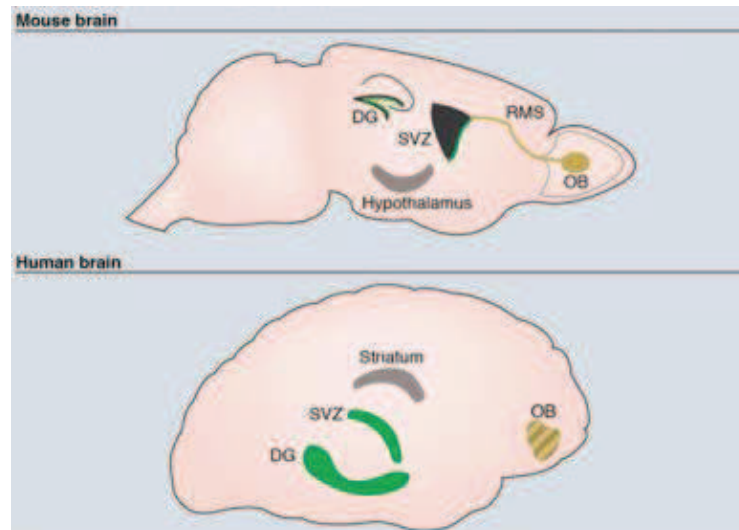
Figure 6: Schematic representation of neurogenic regions in the adult mammalian brain. The two major neurogenic regions: Subventricular zone (SVZ) and subgranular zone of the dentate gyrus (DG) in the adult mammalian brain are marked in green. Newly generated cells from the SVZ migrate via the rostral migratory stream (RMS) to the olfactory bulb (OB) to become interneurons. Neurogenesis in the human OB is relatively low. Additional sites of neurogenesis including the hypothalamus in the mouse brain and the striatum in the human brain are represented in gray (Braun and Jessberger, 2014).

3.1. Neural stem cells in the adult mammalian brain

Neural stem cells (NSCs) should fulfill two defined characteristics, the capacity for self-renewal through cell division and the capacity for multi-lineage differentiation (multipotency) into neurons, astrocytes and oligodendrocytes (Gage, 2000). Neural progenitors include all dividing cells with some capacity for differentiation.

Radial glia-like cells expressing the glial fibrillary acidic protein (GFAP) are the primary precursors of new neurons in the adult brain. In the SVZ-OB system, these radial glia-like cells are termed "Type B cells" while in SGZ of adult hippocampus, they are termed "Type 1-cells" (Alvarez-Buylla and Lim, 2004). In addition, non-radial stem cells exist in the adult SGZ. They are active neural stem cells expressing SRY-related HMG-box gene 2 (Sox2) and give rise to new neurons and glia in the adult SGZ (Suh et al., 2007).

3.2. The Subventricular Zone (SVZ)

The SVZ generates immature neuronal precursors called neuroblasts which migrate through the rostral migratory stream, to the olfactory bulb where they mature into local interneurons (Altman, 1969; Alvarez-Buylla et al., 1994; Lois and Alvarez-Buylla, 1994). The SVZ-OB represents an established system for studying neurogenesis and neuronal replacement as it includes the basic processes of NSCs maintenance, progenitor cell fate specialization, migration, differentiation and survival or death of newly born neurons. The SVZ in the adult mammalian brain contains four main cell types (B cells, A cells, C cells and ependymal cells), which are defined by morphology, ultrastructure and by the expression of various molecular markers (Doetsch et al., 1997) (Fig. 7).

- The type B cells, GFAP-expressing radial glia-like cells with ultrastructural characteristics typical of astrocytes, represent the quiescent NSCs within the SVZ neurogenic niche. They are slowly dividing cells (Doetsch et al., 1999) and give rise to highly proliferative intermediate precursor cells termed type C cells. B cells also express the intermediate filament proteins nestin and vimentin (Lendahl et al., 1990).

- The type C cells function as transit-amplifying progenitors intermediate between B and A cells. C cells are generated by asymmetric cell division of B cells and divide more rapidly (Doetsch, 2003a).They generate large numbers of neuroblasts, named type A cells. C cells express the progenitor cell marker nestin.

- Type A cells or migrating neuroblasts are typically organized as chains of cells throughout the SVZ, indicating that these neuronal precursors migrate closely associated to each other in the form of a chain aligned along the blood vessels (Lois et al., 1996). The immature neurons migrate through the RMS to the OB where they preferentially differentiate into olfactory granule interneurons, expressing calretinin (CR), calbindin (CalB) or tyrosine hydroxylase (TH) and periglomerular neurons expressing CR. They both integrate into local circuits (Alvarez-Buylla and Garcia-Verdugo, 2002; Carleton et al., 2003). Type A cells typically express the immature neuronal marker Tuj-1, doublecortin (DCX) and the differentiation factor Dlx-2.

- Type E cells are ciliated ependymal cells, building a single layer, which separates the above mentioned cells from the cerebrospinal liquid. They are essential for neuronal fate specification by providing inhibitors of gliogenesis.

Studies in rodents have revealed that this dynamic neurogenic process generates thousands of neuroblasts per day; however, only a small fraction of immature neurons survive and functionally integrate into OB circuits (Carleton et al., 2003).
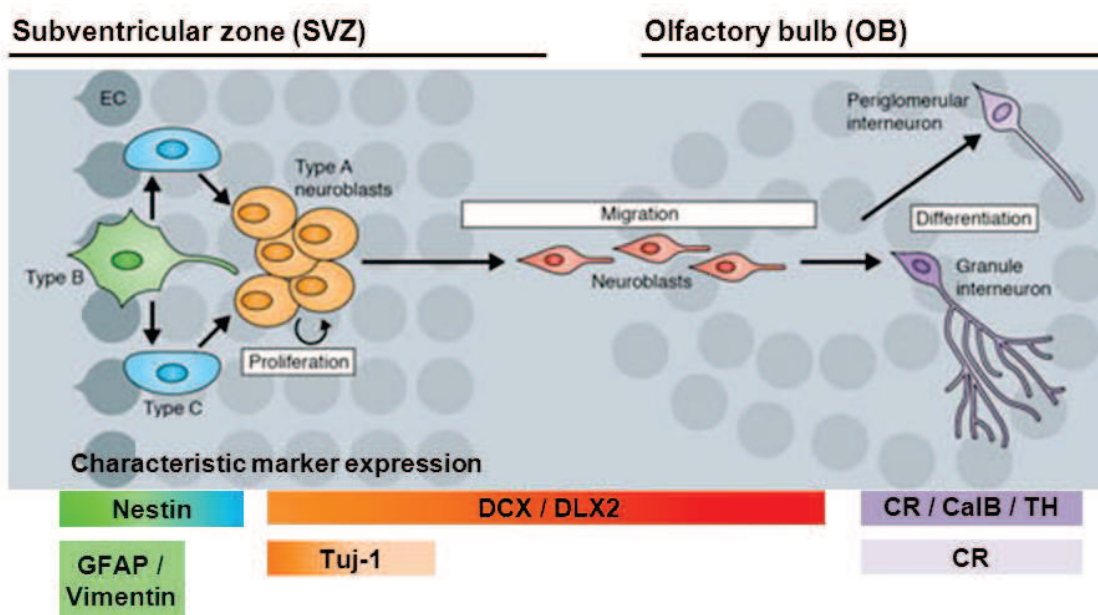
Figure 7: Adult neurogenesis in the subventricular zone of the lateral ventricle and olfactory bulb. Adult SVZ NSCs (type B) reside below the ependymal cell layer (EC) in the wall of the later ventricle. These cells give rise to rapidly dividing transit-amplifying progenitors (type C) that generate neuroblasts (type A). The newborn immature neurons migrate through the RMS towards the OB where neuroblasts preferentially differentiate into granule neurons and periglomerular neurons that integrate into OB neuronal circuits. Adapted from (Braun and Jessberger, 2014).

3.3. The Subgranular Zone (SGZ)

Neurogenesis in the SGZ of the dentate gyrus (DG) of the hippocampus has been extensively studied due to its possible implication in learning and memory (Kempermann et al., 2004a). In the adult SGZ, radial NSCs give rise to intermediate progenitors which in turn generate neuroblasts that migrate into the inner part of the granule cell layer and differentiate into dentate granule cells. Precursor cells in the SGZ seem to be different from the cells found in the SVZ (Seaberg and van der Kooy, 2002). Therefore, an alternative nomenclature, based on numbers, has been

proposed for the neurogenic cells located in the DG (Kempermann et al., 2004b) instead of the letters that had been originally used to describe the neurogenic cells within the SVZ, mentioned earlier. The SGZ in the adult mammalian brain contains three cell types (Type-1, Type-2 and type-3 cells) (Fig. 8) (Emsley et al., 2005).

- Type-1 cells, radial glia-like precursors, are quiescent neural stem cells expressing GFAP, nestin and the brain lipid-binding protein (BLBP).

- Type-2 cells, non-radial progenitors, are produced by type-1 cells. They are amplifying neural progenitors which lack glial features and express nestin and sox2 as markers. Type-2 cells can be divided into two subpopulations, both nestin-positive, but one being negative and the other one being positive for the immature neuronal marker DCX. They are named type-2a and type-2b, respectively (Kempermann et al., 2004b).

- Type-3 cells are slowly proliferating neuroblasts which express markers of the neuronal lineage such as DCX, NeuroD and Prox1. Immature neurons migrate into the granular layer and differentiate into mature granular neurons (expressing postmitotic neuronal markers such as Prospero-related homeobox gene 1 Prox1 and neuron-specific nuclear protein NeuN). Over a period of 3 weeks newborn granule neurons extend their dendrites towards the molecular layer of the DG and project axons through the hilus towards the CA3 region, and acquire their mature electrophysiological properties. The mature granule cells (expressing NeuN, Prox1 and calbindin) become functionally integrated into the hippocampus. A small proportion of progenitor cells differentiates into glial cells (Hastings and Gould, 1999; Markakis and Gage, 1999; van Praag et al., 2002; Zhao et al., 2006).

In the adult rodent brain, the majority of newborn cells die within the first days after birth (Sierra et al., 2010). The survival rate of newborn neurons that mature and integrate into the pre-existing circuit under normal condition is about 20 %.
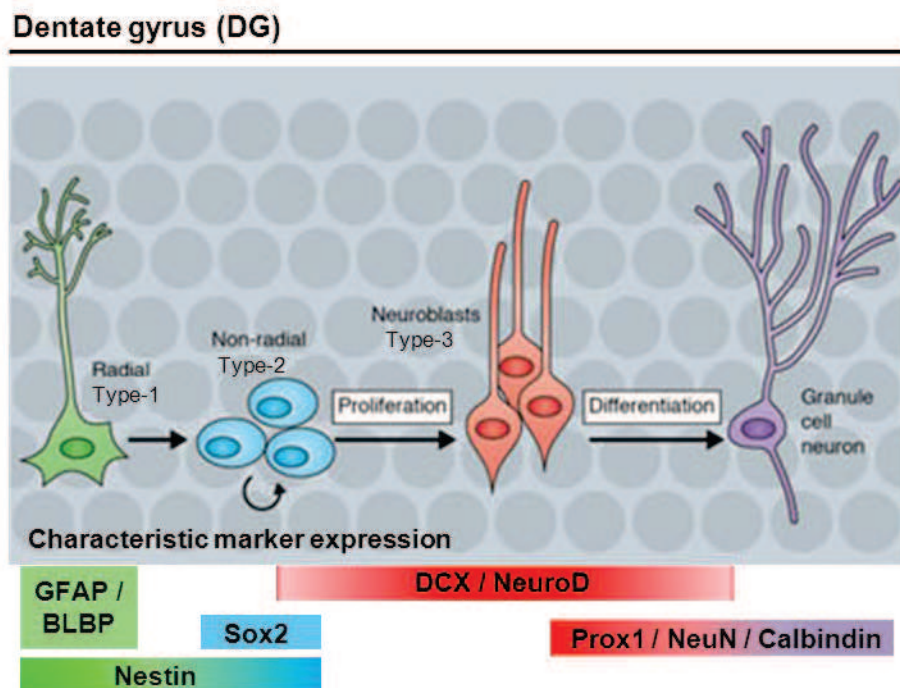
Figure 8: Adult neurogenesis in the subgranular zone of the dente gyrus of the hippocampus. Adult SGZ NSCs give rise to glutamatergic granule cells of the DG via a well characterized multistep process. Quiescent radial glia-like cells (type-1) enter the cell cycle and divide to produce transient amplifying non-radial glia cells (type-2). These cells form immature neurons (type-3) that integrate into the neural network. Adapted from (Braun and Jessberger, 2014).

Since the discovery of neurogenic areas in the adult brain, the molecular mechanisms that regulate the process of persistent formation of new neurons were intensively investigated. Each step of lineage progression is controlled by niche-derived and intrinsic mechanisms, which together ensure appropriate levels of proliferation of NSCs and progenitor cells, their correct differentiation, migration and integration of newborn cells. Sox2, NeuroD, Pax6, Prox1, Sp8, Ascl1 were identified as important transcriptional regulators of neurogenesis in the SVZ and/or SGZ (Zhao et al., 2008a). In addition, neurogenesis levels can be controlled by epigenetic mechanisms, for example through histone modifications or via small non-coding RNAs (Zhao et al., 2008a). Furthermore, neurotransmitters, growth factors and cytokines play a role in controlling NSC and progenitor cell activity and neuronal differentiation; such as γ-aminobutyric acid (GABA), glutamate, brain-derived neurotrophic factor (BDNF), epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2), wingless (Wnt) ligands, sonic hedgehog (Shh), bone morphogenetic proteins

(BMP), interleukin 6 (IL6) and tumor necrosis factor alpha (TNFα), among others (Zhao et al., 2008a).

3.4. Immunohistological markers for proliferation events, gliogenesis and neurogenesis in the adult hippocampus

Hippocampal neurogenesis is divided into different stages: proliferation, differentiation, migration, maturation and synaptic integration. This allows the monitoring of hippocampal neurogenesis in more detail, since the various developmental stages correlate with the expression of different markers.

3.4.1. Markers for neural stem cells

<u>SRY-related HMG-box gene 2 (Sox2)</u>

Sox2, a member of the Sox family, is a transcription factor that is essential for maintaining self-renewal, or pluripotency, of undifferentiated stem cells. Sox2 is expressed in the adult brain in non-radial stem cell-like cells (Ferri et al., 2004; Brazel et al., 2005). Sox2 is expressed mainly by type-2a cells and is rarely observed to be expressed by type -2b or type-3 cells (Steiner et al., 2006).

<u>Brain lipid-binding protein (BLBP)</u>

BLBP is a small nucleocytoplasmic protein expressed by adult radial glia-like cells (Pinto and Götz, 2007). It is expressed by type-1 cells in the DG (Brunne et al., 2010). Because BLBP is not co-expressed with the mature astrocytic marker S100beta nor with the markers of the neuronal lineage, DCX or NeuN, it represents a radial-glia-like progenitor marker (Brunne et al., 2010). BLBP positive radial glia-like cells can divide and thus are positive for Ki-67 (Hartfuss et al., 2001). Under specific circumstances, BLBP can also be expressed by astrocytes (Pinto and Götz, 2007).

3.4.2. Markers for proliferatively active cells

<u>Ki-67</u>

Ki-67, a nuclear protein, is necessary for cellular proliferation and is associated with ribosomal RNA transcription. Ki-67 is expressed during all active phases of the cell cycle (G_1, S, G_2, and mitosis) except the beginning of the G_1 phase and the resting phase G_0 (Zacchetti et al., 2003). It has a short half-life of about one hour. Ki-67 is

absent in quiescent cells and is not detectable during DNA repair processes. Ki-67 is intrinsically expressed and, therefore, does not exhibit side effects observed with nucleotide analogs that are used to identify dividing cells (Kee et al., 2002). For instance, application of 5-bromo-2´-deoxyuridine (BrdU) can cause cell stress and mutagenesis following incorporation. Therefore, Ki-67 is considered to be the preferred marker to monitor cell division.

Proliferating cell nuclear antigen (PCNA)

PCNA is a subunit of DNA polymerase-delta and is essential for DNA replication and the repair of DNA errors (Zacchetti et al., 2003). PCNA is highly expressed during G_1 and S-phases of the cell cycle, and its expression decreases in G_2 and M-phases (Linden et al., 1992). Since PCNA has a long half-life of about 8-20 hours, it can also be present in the early G_0 phase. PCNA is used as a proliferative marker for adult neurogenesis because of its involvement in DNA replication (Limke et al., 2003).

Both markers PCNA and Ki-67 can label proliferating cells. PCNA is expressed in all phases of the cell cycle including those where Ki-67 is not expressed; however, no significant difference has been found in cell numbers expressing those two markers in the DG of the hippocampus (Jinno, 2011).

3.4.3. Markers for the neuronal lineage

Neurogenic differentiation factor (NeuroD)

The basic helix-loop-helix protein NeuroD has been identified as a differentiation factor in diverse species, ranging from xenopus to humans (Lee et al., 1995; Tamimi et al., 1996). NeuroD has been shown to be important for the proper development of the DG (Miyata et al., 1999; Liu et al., 2000) and to be expressed in NSC progeny in the adult DG (Kawai et al., 2004). Therefore, NeuroD is used as a marker for the early stages of the neuronal lineage in the DG.

Neuronal migration marker doublecortin (DCX)

DCX is a microtubule-associated protein expressed by neuronal precursor cells and immature neurons in embryonic and adult cortical structures. It promotes microtubule polymerization and is present in migrating neuroblasts and young neurons (Francis et al., 1999; Gleeson et al., 1999). Neuronal precursor cells begin to express DCX while

still actively dividing, and their neuronal daughter cells continue to express DCX for 2-3 weeks as the cells mature into neurons. After 2 weeks, DCX expression starts to decrease and disappears at the time when these cells begin to express NeuN, a marker for mature neurons (Knoth et al., 2010). Therefore, DCX is used as a marker of immature neurons within the granule layer of the DG.

The differentiation factor NeuroD is used as an early neuronal marker during differentiation and migration stages of adult neurogenesis when cells are still mitotically active, while doublecortin identifies immature neurons during migration (mitotic) and targeting (postmitotic) stages.

3.4.4. Markers for the glial lineage

Glial fibrillary acidic protein (GFAP)

GFAP is an intermediate filament protein. It is widely used as a marker for mature astrocytes in the adult brain. However, as already mentioned, radial glia-like stem cells express GFAP in the neurogenic areas (Eckenhoff and Rakic, 1988; Maslov et al., 2004). Consequently, it is important to differentiate between GFAP as a marker for mature astrocytes and GFAP as a marker for glia-like stem cells. In the context of this project, GFAP was used as a marker for mature astrocytes whereas other markers were used for radial glia-like precursor cells as mentioned above.

The diverse markers, specific for either the neuronal or glial lineage, allow us to monitor the time course and fate of newly generated cells in detail. All available markers have various advantages and disadvantages; therefore it is important to carefully combine them to be useful to examine effects of experimental manipulations or in models of disease on neurogenesis.

3.5. Functional significance of adult neurogenesis

After the discovery of neurogenesis in the postnatal rat hippocampus, Altman (1976) postulated that newborn neurons are crucial for learning and memory. Since then, several studies investigated this hypothesis. To examine the role of new neurons in memory processes, studies have investigated the existence of a correlation between neurogenesis and memory performance. Different approaches were used to increase

or decrease neurogenesis. For example, physical activity or enriched environment was found to increase neurogenesis in rodents (Kempermann, 2011) and improve learning in hippocampus-dependent tests, such as spatial tasks (van Praag et al., 2005) and the object recognition task (Bruel-Jungerman et al., 2005). In contrast, stress and aging decreased neurogenesis and was often correlated with memory deficits in tasks depending on hippocampal function (Walter et al., 2011).

Two important behavioral capabilities, pattern separation and pattern completion, depend on information processing in the hippocampus. Several studies showed that pattern separation, which is the ability to distinguish similar stimuli and contexts, is mediated by the DG of the hippocampus (Hunsaker and Kesner, 2013). Pattern completion, on the other hand, which is the reinstatement of activity patterns correlated with complete contexts and association using only partial or degraded information, is mediated by the CA3 region of the hippocampus. Newborn granule neurons play a critical role in mediating pattern separation (Sahay et al., 2011b). Blocking neurogenesis by injecting anti-mitotic agents such as methylazoxymethanol acetate (MMA), irradiation with X-rays, or genetic techniques involving retroviral infection, showed that newborn neurons play a role in storage of long-term memory of hippocampal-dependent spatial information (Dupret et al., 2008). In addition, recent studies revealed that hippocampal neurogenesis plays an important role in spatial and object recognition memory (Jessberger et al., 2009), fear conditioning and synaptic plasticity (Saxe et al., 2006).

3.6. Adult neurogenesis during aging and neurodegenerative diseases

Several reports showed that adult neurogenesis is not stable in rodents. It is regulated by various physiological and molecular factors which up- or down-regulate neurogenesis by acting on neural stem and progenitor cell proliferation, differentiation and maturation (Ming and Song, 2011). For example, altered levels of neurotransmitter release, higher corticosteroid concentrations and accumulation of proteins that are associated with inflammation are able to inhibit neurogenesis (Cameron and McKay, 1999; Popa-Wagner et al., 2011; Villeda et al., 2011). However, the most potent physiological factor is aging. Adult hippocampal neurogenesis declines dramatically with age; in 21-month-old rats, the number of

newborn neurons in the DG is decreased by 90 % in comparison to rats of 6 months of age (Kuhn et al., 1996). Additional studies confirmed the pronounced age-related decrease of hippocampal neurogenesis in rodents, human and non-human primates (Gould et al., 1999; Leuner et al., 2007; Amrein et al., 2011). With aging a decrease in the number of dividing NSCs is also observed (Encinas et al., 2011).

Neurodegenerative disorders also negatively influence adult neurogenesis. Alterations in adult neurogenesis appear in different neurodegenerative diseases including AD, Parkinson's disease and Huntington's disease (Braun and Jessberger, 2014; Winner and Winkler, 2015).

In summary, two main effects on neurogenesis were detected in neurodegenerative diseases: (i) a reduction of neural stem and progenitor cell activity or impaired neuronal survival. All these deficits can lead to an overall reduction in the number of newborn cells; (ii) an alteration in the course of neurogenesis which can lead to aberrant maturation and consequently to abnormal integration of newborn neurons.

Adult neurogenesis in Alzheimer´s disease

Contradictory findings were reported with respect to the impact of AD pathology on adult neurogenesis. In a cohort of patients with AD, a post-mortem analysis showed that the expression and the number of cells expressing neurogenesis marker proteins (DCX, NeuroD) are increased in the DG of the hippocampus (Jin et al., 2004). These data suggested the existence of an intrinsic compensatory response, which however was not sufficient to balance the cell loss caused by the disease. More recent data using large cohorts of patients indicate that the number of NSCs (Sox2+) and new hippocampal neurons (DCX+) is reduced in AD patients. The increase observed in previous studies has been explained to result from glial and vasculature-associated changes (Boekhoorn et al., 2006; Crews et al., 2010). Some of the contradictory results could be explained by findings from a gene expression study, showing that AD neuropathology in the prefrontal cortex is preceded by changes in gene expression that point to increased synaptic activity and plasticity in human AD brains at different Braak stages (Bossers et al., 2010).

Studies in different mouse models of AD have reported changes in neurogenesis with very high variability depending on promoters, transgene expression, developing pathology at different ages and amount of overexpression/loss of the disease-

causing protein. Adult neurogenesis was altered in both directions, decreased and increased, in different AD transgenic models (Lazarov and Marr, 2010; Marlatt and Lucassen, 2010; Mu et al., 2010). It is difficult to compare the results of the different studies because of differences in experimental conditions, parameters evaluated (some studies focused only on proliferation, others on survival and maturation of new neurons) and the disease models (e.g. presence of extracellular amyloid-mediated or intracellular tau-mediated AD pathology). Despite this controversy, a large body of evidence suggests that high levels of amyloid peptide are deleterious for neurogenesis (Haughey et al., 2002). It is suggested that amyloid plaques may constitute a hostile environment for the development of new neurons either by themselves or by inducing inflammatory or oxidant mechanisms. Future studies must take into consideration the stage of disease and also the fact that neurogenesis is a process very sensitive to the extracellular environment and to the network activity in which new neurons integrate (Sun et al., 2009). Therefore, to be able to draw meaningful conclusions from AD preclinical models, a more systematic and comprehensive analysis appears to be a crucial requirement.

3.7. Contribution of neural stem/progenitor cells to brain repair

NSCs are characterized by their capacity for self-renewal and multipotency. They play important roles in CNS development, in the maintenance of cell number after injury and in disease as well as during natural cell turnover. For the treatment of nervous system disorders, NSC therapies have been developed in animal models and are starting to be converted into human patient clinical trials. NSCs that reside in the adult brain could offer an endogenous source of neurons and glia for the replacement of cells lost during the course of neurodegenerative diseases. For this to be successful, methods need to be established to enhance stem cell proliferation and cell migration towards lesioned tissue following injury or during neurodegenerative events. This could possibly be achieved through chemokine-directed migration towards lesions, although routes of delivery and proper neuronal differentiation within lesions remain complex issues to be solved (Li et al., 2012).

In addition, neural cells can be derived from embryonic stem cells (ES) or induced pluripotent stem cells (iPSCs) and transplanted into brain tissue (Jessberger et al., 2008; Niu et al., 2013). Cell replacement by NSC transplantation into the lesioned

brain has been shown to diminish pathological and functional deficits associated with diseases in the CNS (Yang and Wernig, 2013). Besides, NSCs may be useful not only to repair neuronal cell loss but also glial dysfunction, such as in the chronic demyelinating disease multiple sclerosis (Franklin and Ffrench-Constant, 2008; Jessberger et al., 2008). Stem cell-based therapies are currently being tested in clinical trials using fetal human NSC transplants to treat spinal cord injuries as well as the demyelinating disease Pelizaeus-Mertbacher (Gupta et al., 2012).

In AD mouse models, NSC grafts increased hippocampal synaptic density and improved cognitive functions (Blurton-Jones et al., 2009). A recent study revealed an increase in amyloid beta clearance and neuronal survival by using human mesenchymal stem cells that enhance autophagy in amyloid beta-treated neurons and mice (Shin et al., 2014). In addition, choline acetyltransferase positive neurons could be directly generated from mesenchymal stem cells. After transplantation, these cells could improve learning and memory capacity in AD animal models (Wu et al., 2007).

Despite many open questions as to the exact mechanism of how transplanted cells recover brain function and the ability of transplanted cells to migrate to the correct site and their proper differentiation and maturation into the desired cell type, NSCs are a promising approach in treating neurodegenerative disease.

4. Neurosteroids

4.1. Definition

In the 1980s, Baulieu and colleagues first reported the ability of neurons and glial cells to synthesize bioactive steroids *de novo* in the brain (Baulieu, 1991, 1997). They found that several steroids such as pregnenolone, dehydroepiandrosterone (DHEA) and their sulfated derivatives were present in higher concentrations in tissue from the nervous system (brain and peripheral nerve) than in the plasma and remained high even after adrenalectomy or gonadectomy (Corpéchot et al., 1981; Corpéchot et al., 1983). These steroids were named "neurosteroids" to differentiate them from steroids derived from the periphery (gonads, adrenals and placenta). Later on, several studies performed in different animal species confirmed the concept of neurosteroids (Baulieu, 1999; Mensah-Nyagan et al., 1999; Compagnone and Mellon, 2000; Schumacher et al., 2003).

A widely accepted definition of neurosteroids considers these molecules as endogenous steroidal compounds synthesized in neurons or glial cells of the CNS and PNS. Steroids to be considered as a neurosteroid must persist in substantial amounts in the nervous system after removal of peripheral steroidogenic glands (Melcangi et al., 2011a). Thus, the term neurosteroid refers to their site of production within the nervous system. According to this definition, progesterone would be called a steroid hormone when synthesized in an endocrine gland but would be regarded as a neurosteroid when it is produced in the brain (Schumacher et al., 2014). Other authors use the term neurosteroids to denominate reduced metabolites of the steroid hormones progesterone, deoxycorticosterone and testosterone like, for instance, allopregnanolone or allotetrahydrodeoxy-corticosterone, produced in the nervous system either *de novo* or by conversion of the hormone precursors (Reddy, 2010). By this definition the use of the term is restricted to derivatives of steroid hormones which, different from the parent steroid hormones, do not act via intracellular receptors regulating gene expression after DNA binding (Reddy, 2010). In other cases, the term neurosteroids is also used interchangeably with neuroactive steroids. The term "neuroactive steroid" refers to steroids which, independent of their origin, are capable of modifying neural activities. It includes all steroids that have an effect on nerve cells whether they are produced directly in the nervous system or by the endocrine glands or whether they are synthetic steroids with the ability to cross the blood-brain barrier after administration.

In summary, neurosteroids can be defined as steroid hormones or their metabolites that are produced in the nervous system and act as paracrine or autocrine factors that strongly influence system function and reactions to injury and disease.

4.2. Biosynthesis

Neurosteroid biosynthesis (neurosteroidogenesis) in the nervous system of vertebrates has been described in detail. It occurs in the CNS or PNS either *de novo* from cholesterol or from the metabolism of circulating steroid precursors (Fig. 9) (McEwen, 1994; Mensah-Nyagan et al., 1994; Mensah-Nyagan et al., 1996; Mensah-Nyagan et al., 1998; Mensah-Nyagan et al., 1999; Tsutsui et al., 1999; Compagnone and Mellon, 2000).
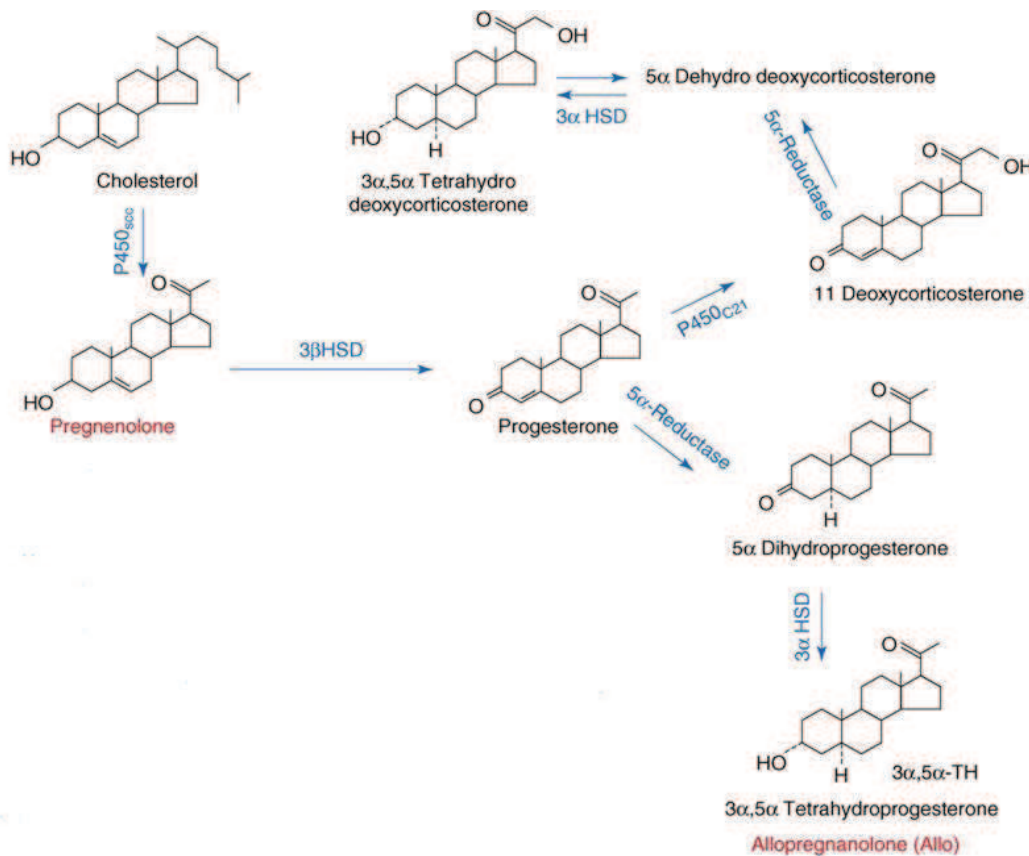
Figure 9: Biosynthetic pathways for neurosteroids, exemplified by progesterone-related steroids. Adapted from (Charalampopoulos et al., 2008). Abbreviations: P450scc, cytochrome P450side-chain-cleavage; P450c21, cytochrome P450c21 or steroid 21-hydroxylase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 3α-HSD, 3α-hydroxysteroid dehydrogenase.

Pregnenolone, the common precursor molecule for the synthesis of steroids, is produced from cholesterol (Fig. 9) in mitochondria as the rate-limiting step in the synthesis of all steroids. It is then converted to steroid hormones containing an oxidized ring A, as exemplified for progesterone in Figure 9. Enzymes required for steroid hormone synthesis, like 3β-hydroxysteroid dehydrogenase (3β-HSD) in the case of progesterone, have been localized in the nervous system (Dupont et al., 1992; Mensah-Nyagan et al., 1994; Guennoun et al., 1995). The locally synthesised hormones can act as neurosteroids via the classical genomic mechanisms. Alternatively, the hormones can be converted in two steps to neurosteroids with a reduced ring A by the sequential action of the enzymes 5α-reductase and 3α-hydroxysteroid dehydrogenase (3α-HSD) as shown for allopregnanolone and tetrahydrodeoxycorticosterone (Fig. 9). These two enzymes have been shown to be expressed by neurons and glia (Melcangi et al., 1993; Melcangi et al., 1994). A series

of other neurosteroids exist which also contain a reduced ring A and lack the ketone on carbon 3. A common characteristic of these steroids is that they do not interact with the classical steroid receptors but function via the activation of membrane receptors or channels.

4.3. Mechanisms of action

Steroid hormones acting as neurosteroids classically exert their biological actions in the nervous system through nuclear steroid receptors (nSRs) which are ligand-activated transcription factors that regulate gene expression. Five major groups of nSRs were identified and characterized: glucocorticoid receptors, estrogen receptors, androgen receptors, progestin receptors and mineralocorticoid receptors (Beato and Klug, 2000). A new nSR was identified recently, pregnane xenobiotic receptor (PXR), acting as a homeostatic regulator involved in neurosteroidogenesis of allopregnanolone in the brain (Frye et al., 2014a, b, c). The classical steroid receptors are functionally composed of three main domains: a hormone-independent activation function 1 domain (AF-1 domain), a DNA-binding function (DBD domain) and a hormone-dependent activation function 2 domain (AF-2 domain). These functional regions are divided into six structural subdomains (Fig. 10).
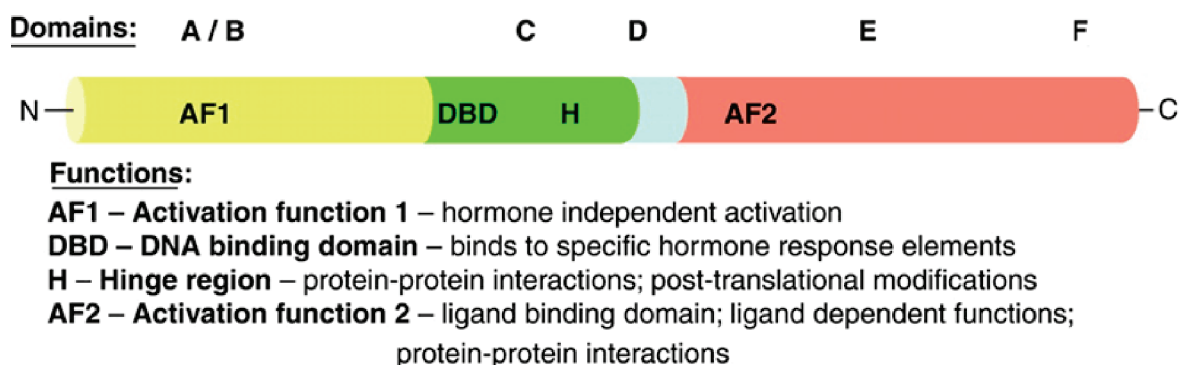
Figure 10: General structure of nuclear hormone receptors. Steroid receptors differ in details, but are generally composed of multiple structural subdomains (A-F), and functional domains (in colors) (Brisken and O'Malley, 2010).

The inactive receptor protein is located in the cytoplasm. Upon ligand binding it dimerizes and translocates to the nucleus where it binds to specific DNA sequences, steroid response elements, to regulate the transcription of steroid-sensitive genes. Besides the classical mechanism of action, cytoplasmic steroid receptors, e.g. the

progesterone receptor (PR), can also be translocated to the cell membrane to interact with different intracellular signaling pathways (Schumacher et al., 2014). In the nervous system, PR have often been found on axons and dendrites and in the vicinity of synapses, indicating that this non-genomic mode of action may be of particular relevance in the nervous system. Finally, there is a lot of evidence that progesterone effects can be mediated by completely different mechanisms following the conversion of steroid hormones into their reduced metabolite allopregnanolone as indicated by the persistence of effects in PR knockout mutants.

Neurosteroids representing reduced metabolites of hormone steroids like allopregnanolone or sulfated steroids like pregnenolone sulfate do not bind to the cytoplasmic steroid receptor to directly influence gene transcription. In contrast to the genomic steroid action, which develops relatively slowly (several minutes to hours and days), non-genomic steroid effects are principally characterized by their insensitivity to inhibitors of transcription and protein synthesis, and by their rapid onset of action (within seconds to minutes). They were first detected in 1941 by Selye, who showed that intraperitoneal injection of progesterone induced a quick anesthetic effect in rats after a few minutes (Selye, 1941). It was also shown that the administration of aldosterone causes cardiovascular effects within 5 minutes (Klein and Henk, 1963). Such a fast action of neurosteroids is not compatible with a genomic mechanism but rather suggests a membrane effect. Since then, numerous studies have shown that neurosteroids control neuronal excitability and activity through the modulation of neurotransmitter receptors such as the $GABA_A$ receptors, NMDA receptors and sigma receptors (Mellon and Griffin, 2002; Belelli and Lambert, 2005). As neurosteroids are synthesized in the nervous system, they thus can act in a paracrine or autocrine manner on those receptors to modulate nerve cell activities (Mensah-Nyagan et al., 2008).

5. Allopregnanolone (AP)

5.1. Biosynthesis

All neurosteroids are derived from cholesterol, which is a hydrophilic molecule and therefore not able to enter into mitochondria by simple diffusion. The protein complex "transduceosome" is responsible for the translocation of cholesterol to the inner

membrane of mitochondria. This complex is composed of the translocase 18 kDa (TSPO) formerly known as the peripheral benzodiazepine receptor and the steroidogenic acute regulatory protein StAR (Rupprecht et al., 2010). Inside the mitochondria, cholesterol is converted to pregnenolone by the enzyme P450scc (Liu et al., 2007). Then, pregnenolone is reduced to progesterone by 3β-hydroxysteroid dehydrogenase either in the mitochondria or in endoplasmic reticulum membranes depending on the steroidogenic cell type. Progesterone is metabolized to 5α-dihydroprogesterone (5α-DHP) by the enzyme 5α-reductase. Both progesterone and 5α-DHP bind to the classical intracellular PR (Fig. 11), which regulate gene transcription within the cell nucleus or interact with kinases and components of intracellular signaling pathways within extra-nuclear compartments. Then, 5α-DHP is converted to allopregnanolone (AP) by 3α-hydroxysteroid dehydrogenase (Mellon, 2007). AP is not able to bind to PR because of the hydroxyl group at carbon 3, but is a potent positive allosteric modulator of GABA$_A$ receptors (Belelli and Lambert, 2005). The last step of AP synthesis is reversible allowing AP to be converted back to 5α-DHP (Fig. 11) which is a potent agonist at the nuclear PR. This is a mechanism by which AP could regulate gene transcription via PR.
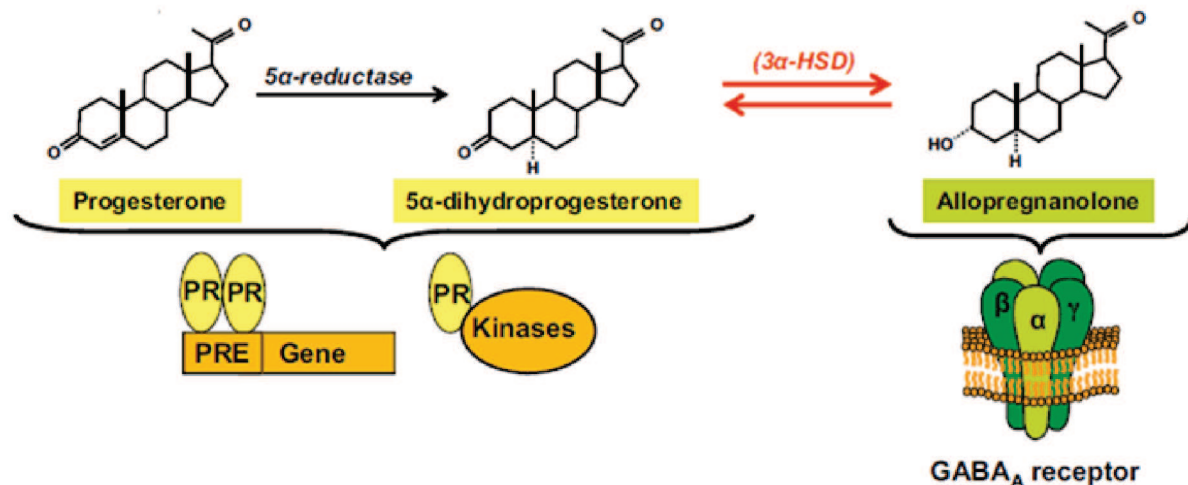
Figure 11: Biosynthetic pathway of allopregnanolone. Adapted from (Schumacher et al., 2014).

5.2. Mechanisms of action

The reduced metabolite of progesterone, AP, was one of the first neurosteroids that were shown to modulate neuronal excitability by interaction with the $GABA_A$ receptor (Majewska et al., 1986; Callachan et al., 1987). AP is a potent positive allosteric modulator of $GABA_A$ receptors ($GABA_A$Rs) and enhances GABAergic inhibition by increasing frequency and duration of chloride channel opening leading to chloride influx thereby modulating GABAergic inhibition within the brain (Reddy, 2003; Belelli and Lambert, 2005). APs action on the $GABA_A$R is concentration dependent. At low nanomolar concentrations, AP enhances the action of the natural ligand GABA at $GABA_A$Rs, while at higher concentrations (micromolar range) AP directly activates the $GABA_A$R channel complex (Callachan et al., 1987; Puia et al., 1990) .

$GABA_A$Rs, principal mediators of the fast inhibitory transmission within the CNS, are members of the Cys-loop family of ligand-gated ion channels (Schofield et al., 1987) and are pentameric proteins consisting of five subunits (Farrar et al., 1999). There are 19 subunits known to participate in forming functional receptor complexes: α_{1-6}, β_{1-3}, γ_{1-3}, δ, ϵ, π, θ and ρ_{1-3} (Barnard et al., 1998). Most $GABA_A$Rs contain α-, β-, and γ-subunits, in a stoichiometry of $2\alpha:2\beta:1\gamma$ (Fig. 12). The most common subtype within the CNS (60%) is the $\alpha_1\beta_2\gamma_2$ isoform (Möhler et al., 2002; Möhler et al., 2004). The γ-subunit can be substituted by a δ-subunit. The subunit composition influences receptor sensitivity to GABA, channel kinetics and desensitization, subcellular localization and pharmacological properties. For example, the δ-subunit is a highly sensitive target for neurosteroids (Lambert et al., 2009). $GABA_A$R modulation by neurosteroids is enantioselective and is partially dependent upon the receptor subunit composition. Channel opening of $GABA_A$Rs is enhanced by benzodiazepines, barbiturates, anesthetics as well as neurosteroids, whereas it is blocked by antagonists such as bicuculline and picrotoxin (Belelli and Lambert, 2005).
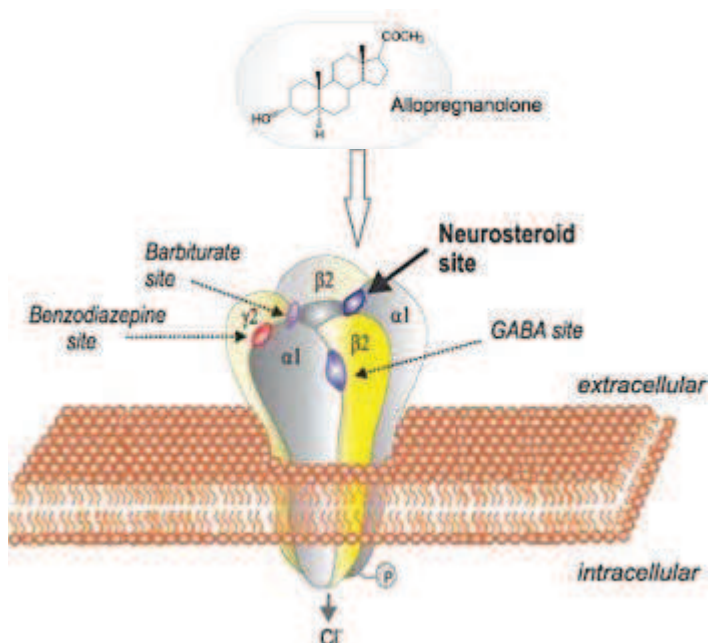
Figure 12: The GABA_A receptor and its various binding sites. The GABA_AR has several binding sites for modulatory substances such as GABA, benzodiazepines, barbiturates and neurosteroids. Adapted from (Reddy, 2010).

AP binds to the GABA_AR at sites distinct from those binding GABA, benzodiazepines, barbiturates and ethanol (Fig. 12) (Hosie et al., 2006). In fact, AP regulates the activity of the GABA_AR through two discrete binding sites located within the transmembrane domains which mediate potentiation and direct activation effects, respectively. The potentiation effect is mediated by a cavity formed by the α-subunit transmembrane domains. The direct activation of the GABA_AR by AP is mediated by interfacial residues between α- and β-subunits, and is enhanced by steroid binding to the potentiation site (Hosie et al., 2006). Both sites are highly conserved in all the subtypes of GABA_ARs.

Binding of AP results in a prolonged opening time of the chloride ion channel that leads to enhanced inhibitory transmission. Synaptic GABA_ARs mediate rapid phasic inhibition of postsynaptic currents, while the activation of extra-synaptic GABA_ARs results in tonic inhibition (Belelli and Lambert, 2005; Farrant and Nusser, 2005). Phasic inhibition occurs when high levels of GABA, rapidly activate post-synaptic GABA_ARs resulting in a transient inhibitory response. Tonic inhibition is observed when constant low levels of GABA activate extra-synaptic GABA_ARs and generate persistent inhibition of neuronal excitability. AP potentiates both phasic and tonic inhibition by modulating synaptic and extra-synaptic GABA_ARs (Belelli and Lambert, 2005; Farrant and Nusser, 2005). As AP levels in the brain vary greatly, its effects on

neuronal excitability are dependent on the brain region. Within the dentate gyrus of the hippocampus, e.g., AP is actively metabolized and GABAergic synapses have low sensitivity to this neurosteroid. In contrast, within the CA1 region of the hippocampus, AP metabolism is less active and neurons respond strongly to low concentrations of this neurosteroid. These findings suggest a crucial role for local steroid metabolism in modulating $GABA_AR$-mediated inhibition in a regionally dependent manner (Belelli and Herd, 2003).

The structure-activity relationship of neurosteroid actions interacting with the $GABA_AR$ has been studied widely (Laubach et al., 1955; Gyermek et al., 1968; Gyermek and Soyka, 1975; Sear, 1997). Several analogs of AP were synthesized to study this relationship (Akk et al., 2009). These studies revealed functionally important structural characteristics: the geometry between rings A/B; a hydrogen-bond donator in C3 position; a hydrogen-bond acceptor in C20 position and/or a flexible bond at the C17 position (Purdy et al., 1990; Zorumski et al., 2000). The α-configuration at C3 and the β-configuration at C17 are important for the binding of AP to a variety of proteins by means of hydrogen bonding with polar or charged residues (Brzozowski et al., 1997; Grishkovskaya et al., 2000). It has also been reported that replacing the hydrogen of hydroxyl with methyl decreases potency because the ability of the steroid to donate a hydrogen bond was eliminated (Upasani et al., 1997).

The proliferation-promoting effect of AP has been shown to be mediated via $GABA_ARs$ (Fig. 13) (Brinton, 2013). GABA is typically an inhibitory neurotransmitter, but in neural progenitor cells GABA becomes excitatory because of SLC12A2 cotransporter expression which results in a higher intracellular concentration of chloride (Cl^-) in comparison to the extracellular concentration. This results in an efflux of Cl^- in developing neurons, accompanied by membrane depolarization (Cherubini et al., 1990; Perrot-Sinal et al., 2003). Subsequently, voltage-dependent L-type calcium channels (VDLCC) are activated (Fig. 13) (Dayanithi and Tapia-Arancibia, 1996; van den Pol, 2004). Calcium influx activates Ca^{2+}-dependent kinase, CaMK IV, which in turn phosphorylates and activates the transcription factor cyclin AMP-responsive element-binding protein (CREB1) and subsequently regulates expression of cell cycle genes. Thus, AP treatment leads to increased expression of genes which promote mitosis and decreased expression of proteins which repress the cell cycle (Fig. 13)

(Wang et al., 2005). Stimulation of proliferation induced by AP is abolished by blocking GABA$_A$Rs or VDLCC (Wang et al., 2005; Wang and Brinton, 2008).
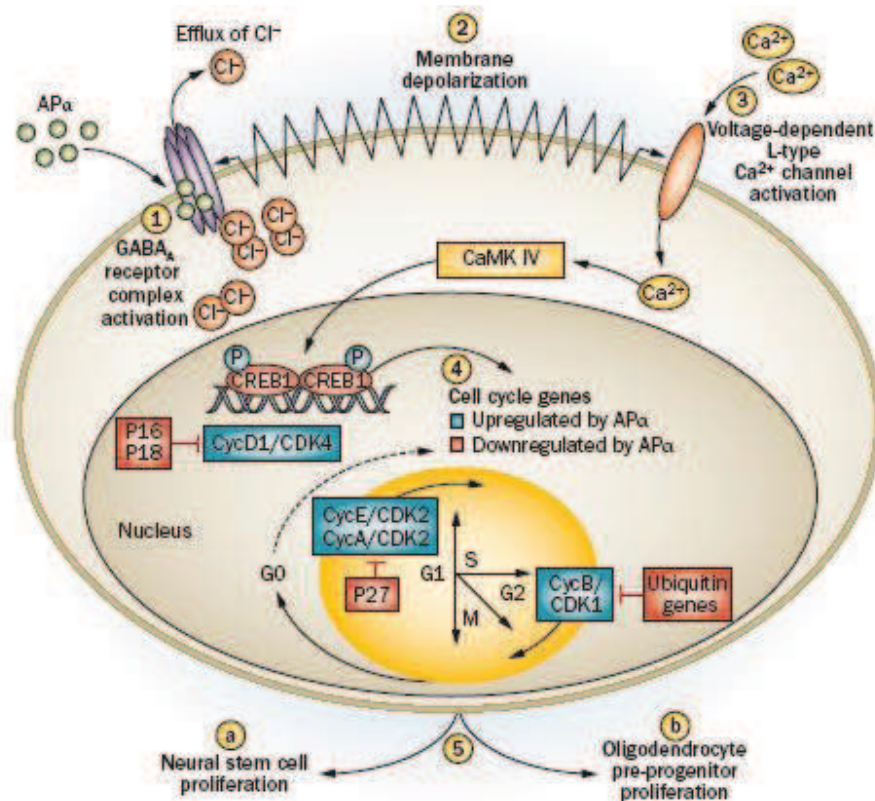
Figure 13: Model for the mechanism of AP-induced stimulation of proliferation in neural stem cells. In mature neurons, AP modulates the GABA$_A$R to increase chloride influx thereby hyperpolarizing the neuronal membrane and decreasing neuron excitability. In progenitors and immature neurons the transmembrane Cl$^-$ concentration gradient is reversed leading to an efflux of Cl$^-$ through the GABA$_A$R channels, depolarization of the membrane and opening of VDLCC. Ca^{2+}-induced intracellular signaling can thus influence gene expression. It has been shown to increase expression of genes that promote mitosis and to decrease expression of proteins that repress the cell cycle (Brinton, 2013).

In addition, AP may protect neuronal networks against excitotoxic damage by modulating GABA$_A$Rs that exert an effect on synaptic release and activity of glycine, a major inhibitory neurotransmitter (Chesnoy-Marchais, 2009; Chesnoy-Marchais 2013). Moreover, some studies postulated that APs potent peripheral analgesic effect may be mediated in part by effects on T-type calcium channels and glycine membrane receptors (Pathirathna et al., 2005; Mitchell et al., 2007).

Recently, another target of AP, the pregnane-X-receptor (PXR) has been identified. AP acting via this receptor exerts a neuroprotective effect in a mouse model of

Nieman-Pick type C disease (Langmade et al., 2006). PXR acts as a ligand-activated transcription factor and regulates the expression of genes involved in xenobiotic detoxification and apoptosis (Orans et al., 2005).

AP can also bind to membrane progesterone receptors (mPRs) which are different from classical intracellular PR. mPRs are proteins with seven transmembrane domains and might be directly coupled to pertussis-sensitive G proteins and inhibit adenylyl cyclase activity (Zhu et al., 2003; Thomas, 2008). Thomas and Pang (2012) showed that AP is a potent mPRα agonist in the immortalized hypothalamic neuronal cell line GT1-7. AP acts as an agonist when binding to mPRδ and other mPRs, by activating second messengers and decreasing starvation-induced cell death and apoptosis in mPRδ-transfected cells and in hippocampal neuronal cells. This makes mPRδ a potential mediator of non-classical anti-apoptotic actions of neurosteroids in the CNS (Pang et al., 2013). mPRs are extensively and abundantly distributed in rat, mouse and human brain and spinal cord which would allow them to mediate the neuroprotective actions of AP (Labombarda et al., 2010; Meffre et al., 2013; Pang et al., 2013).

5.3. Role of allopregnanolone in psychiatric disorders

AP regulates many different physiological processes in the CNS. It has anxiolytic, analgesic, anesthetic, antidepressant and sedative properties by activating GABA$_A$Rs (Brinton, 1994; Liu et al., 2002). Clinical trials showed that reduction of AP in plasma, serum and cerebrospinal fluid might be linked to several affective disorders including major depression (Romeo et al., 1998; Uzunova et al., 1998; Schüle et al., 2006), anxiety disorders (Ströhle et al., 2002; Rupprecht et al., 2009), postpartum depression (Bloch et al., 2000; Nappi et al., 2001; Frye, 2011), posttraumatic stress disorder (Rasmusson et al., 2006), negative symptoms in schizophrenia (Marx et al., 2009; Marx et al., 2011), or impulsive aggression (Nelson and Pinna, 2011). These data suggested that downregulation of AP in the brain might be a risk factor for the development of psychiatric disorders. Studies using animal models and also trials in patients strongly suggest that AP has a therapeutic potential for treating these affective disorders. Some data also revealed a relationship between AP and bipolar disorder. In bipolar patients during depressive episodes, a reduction in AP levels were observed (Hardoy et al., 2006). In contrast, Brambilla and colleagues observed

elevated plasma AP levels in patients with panic disorder (Brambilla et al., 2003). The fact that AP levels are low during depressive episodes and high during panic attacks can be explained by the hypothesis that AP may exert a protective stabilizer role, acting like an endogenous mechanism of control.

AP has been widely studied in posttraumatic stress disorder (PTSD). Socially isolated mice were used as a mouse model for PTSD as they reproduce the behavioral deficits and symptoms observed in human anxiety disorders and PTSD (Uzunov et al., 1996) demonstrated that the decrease observed in AP levels is linked to the etiology of the disorder. In particular, the aggressive behavior of mice was strongly related to reduced AP levels in corticolimbic regions (Pinna et al., 2003). Another study showed that PTSD and anxiety disorders were improved by AP administration (Pibiri et al., 2008). Moreover, the action of some antidepressant agents, in particular selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine, paroxetine, sertraline, restored AP brain levels in depression or other affective disorders. In addition, SSRIs, by increasing AP levels, were able to attenuate behavioral deficits displayed by socially isolated mice (Pinna et al., 2003; Pinna et al., 2006). Preclinical studies have also shown that acute administration of certain antipsychotic drugs increased levels of AP in the cerebral cortex which by enhancing GABAergic neurotransmission may explain their antipsychotic action in schizophrenia (Marx et al., 2006b).

However, AP possesses some pharmacokinetic limitations that restrict its therapeutic use: (i) AP has a low bioavailability due to rapid inactivation by glucuronidation and sulphate conjugation and (ii) AP may undergo oxidation to the ketone at the 3α-hydroxyl group rendering it inactive. Therefore, many studies aimed at developing new synthetic neurosteroids to improve the therapeutic potential (Reddy and Kulkarni, 2000; Reddy, 2010). Three synthetic neurosteroids have been investigated with respect to their therapeutic potential (ganaxolone, alphaxalone, and minaxolone). Ganaxolone is already used in clinical trials for epilepsy (Reddy, 2010); however, until now synthetic neurosteroids could not be established for the treatment of anxiety disorders or depression (Nothdurfter et al., 2012).

5.4. Neuroprotective effects of allopregnanolone in neurodegenerative disorders

AP has a major influence on CNS activity and is crucial for growth and survival of neurons and glial cells (Wang et al., 2005; Melcangi et al., 2008). AP treatment has been shown to exert a protective effect after traumatic brain injury by reducing edema, inflammation and apoptosis. AP treatment decreased expression of the pro-apoptotic proteins caspase-3 and Bax, decreased mitochondrial cytochrome c release and apoptotic DNA fragmentation as well as the size of the astrocytic glial scar at the site of a lesion (Djebaili et al., 2004; Djebaili et al., 2005; Sayeed et al., 2009). In an animal model of excitotoxicity, female rats injected with kainic acid, AP diminished reactive gliosis in the hippocampus (Ciriza et al., 2004). Similar effects were observed in hypoxic cultures of perinatal cerebral cortex and hippocampus (Kruse et al., 2009; Bossers et al., 2010). In organotypic spinal cord cultures subjected to injury, AP reduced membrane damage and prevented neuronal death (Labombarda et al., 2013). In animal models of acute ischemic stroke, AP reduced cerebral infarction volume, improved blood brain barrier integrity as well as memory and learning (Sayeed et al., 2006). In a MPTP-lesioned mouse model of Parkinson´s disease, AP prevented the loss of tyrosine hydroxylase-positive cells in the substantia nigra and improved motor performance (Adeosun et al., 2012). In Niemann-Pick type C disease, AP retarded the beginning of neurological symptoms, enhanced Purkinje and granule cell survival in the cerebellum, decreased cholesterol accumulation, reduced inflammation and stimulated myelination (Griffin et al., 2004; Liao et al., 2009). In experimental autoimmune encephalomyelitis, a model of multiple sclerosis, AP blocked aggravation of the immune response by decreasing the immunoreactivity of several markers in the lumbar spinal cord such as monocytoid cell marker and ionized calcium-binding adapter molecule 1 (Noorbakhsh et al., 2011). In the 3xTgAD mouse model of AD, AP stimulated neurogenesis and oligodendrogenesis, decreased neuroinflammation and amyloid beta levels while upregulating markers of white matter regeneration and cholesterol homeostasis (Chen et al., 2011). Therefore AP has emerged as a potential therapeutic candidate against AD as detailed below. AP exerts protective effects also in the PNS. It has been show to enhance sciatic nerve conduction velocity and mRNA levels of myelin proteins in experimental models of peripheral diabetic neuropathy (Leonelli et al.,

2007). AP is considered as a promising candidate for the treatment of chemotherapy-induced peripheral neuropathy as detailed below (Patte-Mensah et al., 2014) because of its ability to repress neuropathic symptoms (allodynia/hyperalgesia) induced by antineoplastic drugs such as vincristine and oxaliplatin in experimental rat models of peripheral neuropathic pain (Meyer et al., 2010, Meyer et al., 2011).

5.5. Allopregnanolone as a therapeutic candidate for the treatment of neuropathic pain

AP exerts an antinociceptive effect in several animal and human models (Nadeson and Goodchild, 2000, 2001; Gambhir et al., 2002) and generates peripheral analgesia via activation of GABA$_A$Rs and possibly also by inhibiting T-type calcium channels (Pathirathna et al., 2005). Pain transmission is controlled in the spinal cord dorsal horn which contains binding sites for AP (Patte-Mensah et al., 2006; Mensah-Nyagan et al., 2008; Mensah-Nyagan et al., 2009). Data from our lab also revealed the presence and activity of enzymes involved in AP synthesis in the nervous structures controlling nociception (Patte-Mensah et al., 2003; Patte-Mensah et al., 2004a; Patte-Mensah et al., 2004b; Patte-Mensah et al., 2005; Mensah-Nyagan et al., 2008; Meyer et al., 2008; Mensah-Nyagan et al., 2009). Importantly, endogenous AP could be demonstrated to be involved in the control of sciatic nerve injury-induced neuropathic pain (Mensah-Nyagan et al., 2008; Mensah-Nyagan et al., 2009; Schaeffer et al., 2010). Pharmacological inhibition or inactivation of the AP synthesizing enzyme 3α-HSD by siRNA led to an increase in pain perception by reducing thermal and mechanical sensitivity thresholds (Meyer et al., 2008; Patte-Mensah et al., 2010).

Neuropathic pain is a major unwanted side-effect of cancer chemotherapy with antineoplastic drugs such as vincristine and oxaliplatin (Antoine and Camdessanché, 2007; Moore and Pinkerton, 2009). Hyperalgesia and allodynia are the essential neuropathic pain symptoms caused by these two drugs (Antoine and Camdessanché, 2007). Recent studies from our lab demonstrated that AP suppressed allodynia and hyperalgesia that were evoked by vincristine and oxaliplatin when applied either before (prophylactic) or after (corrective) treatment with the cytostatic drugs (Meyer et al., 2010; Meyer et al., 2011). Based on these

results, AP was suggested as a promising candidate for an effective therapeutic intervention in anticancer drug-induced painful neuropathy.

5.6. Allopregnanolone as a regenerative therapeutic in Alzheimer´s disease

5.6.1. Allopregnanolone promotes neurogenesis *in vitro*

AP has been identified as a proliferative factor in the nervous system. It stimulates proliferation of neural stem and precursor cells as well as oligodendrocyte progenitors (Wang et al., 2005; Wang et al., 2010; Schumacher et al., 2012). In addition, in the PNS, AP promotes proliferation, recovery from spinal injury and remyelination of peripheral nerve axons (Melcangi et al., 2011b; Schumacher et al., 2012).

During granule cell neurogenesis in the neonatal cerebellum of the rodent, increased 3α-HSD enzyme activity is paralleled by enhanced synthesis of progesterone and its metabolite AP (Ukena et al., 1999). Therefore, Keller and colleagues (2004) studied the role of AP on cerebellar granule cells (CGC) neurogenesis. They observed that AP is able to increase proliferation of immature CGC in cultures prepared from 6- to 8-day-old rats (Keller et al., 2004). This effect was blocked by bicuculline (a competitive $GABA_AR$ antagonist), picrotoxin (a non-competitive $GABA_AR$ antagonist) and nifedipine (VGLCC antagonist).

Another study discovered that AP induces neurite regression of hippocampal neurons in culture (Brinton, 1994). This effect was explained by the fact that cultures treated with AP contained an increased number of mitotically active cells. In a subsequent study, the same lab demonstrated that AP also enhanced proliferation and generation of neurons in embryonic (E18) rat hippocampal cultures (Wang et al., 2005). The majority of cells in these cultures represent neuronal precursors. Treatment with AP increased the number of proliferating neuronal progenitors (BrdU incorporation) with a biphasic dose-response curve, with high doses repressing proliferation. The effect was stereospecific as AP stereoisomers, epiallopregnanolone, epipregnanolone and 5 α-pregnan-3β-ol were without effect. AP treatment could be shown to upregulate gene expression levels of activators of cell-cycle progression (cyclins, cyclin-dependent kinases CDKs, cell proliferation marker

PCNA) and to simultaneously downregulate the expression of cell-cycle inhibitors (CDK inhibitors p16 and p18) (Wang et al., 2005). The VGLCC antagonist nifedipine completely abolished the AP effect. The authors concluded that calcium influx through voltage-gated channels was evoked in the immature neurons by $GABA_AR$-mediated membrane depolarization. This hypothesis was verified in an electrophysiological study (Wang and Brinton, 2008) showing that AP-induced rise of intracellular Ca^{2+} was inhibited by nifedepine and by the $GABA_AR$ antogonists picrotoxin and bicuculline. Taken together, these results indicated that AP-induced $[Ca^{2+}]_i$ rise requires activation of $GABA_ARs$ and L-type calcium channels in rat hippocampal neuron cultures. This AP-induced rise in $[Ca^{2+}]_i$ might be the signaling initiation mechanism for AP-induced neuroprogenitor cell proliferation and cell cycle gene expression. Similar findings were described in fetal rat hypothalamic neurons (Dayanithi and Tapia-Arancibia, 1996), suggesting that the AP-induced rise in $[Ca^{2+}]_i$ is a general effect in developing neurons.

AP increased the formation of neurospheres by NSCs from both embryonic rat brain and adult brain derived from the DG of 10-week-old mice. In the same study, AP was shown to induce an increase in proliferation of human neural stem cells (HNSC) derived from the cerebral cortex (Wang et al., 2005). In agreement with results using rat neuroprogenitor cells, AP-induced proliferation in HNSC was dose depend with a biphasic response. The proliferation-promoting effect of AP in HNSC was much greater than that in rat hippocampal neural progenitors.

Together, these findings indicate that AP can promote proliferation of neural progenitor cells of diverse phenotypes derived from multiple sites.

5.6.2. Allopregnanolone reverses neurogenic and cognitive deficits in aged and Alzheimer´s disease mutant mice

In the aged and AD brain, a decline of neurogenic activity in the SGZ and SVZ region has been observed (Kuhn et al., 1996). This is paralleled by reduced AP levels in plasma and brain in aged humans and AD patients (Genazzani et al., 1998; Weill-Engerer et al., 2002; Marx et al., 2006a) suggesting that low AP levels might play a role in AD etiology. Consequently, AP treatment was proposed as a strategy for promoting endogenous NSC proliferation and neurogenesis in the adult brain in the context of neurodegenerative diseases or following CNS injury. Due to its low

molecular weight and its hydrophobic properties, peripherally applied AP can easily penetrate the blood brain barrier and induce its beneficial effects in the CNS.

The potential of AP to promote neurogenesis has been investigated during aging in normal mice as well as in a mouse model of AD, the 3xTgAD mouse (Wang et al., 2010; Singh et al., 2012). The triple transgenic mice carry mutations in the genes for tau, the amyloid precursor protein and for presenilin 1 and develop an age-dependent AD neuropathology, including formation of amyloid beta plaques and neurofibrillary tangles, and exhibit early deficits in learning and memory (Oddo et al., 2003a; Oddo et al., 2003b; LaFerla et al., 2007).

In adult male mice aged between 3 and 12 months, AP did not show any effect, neither on neural progenitor cell survival and proliferation nor on cognitive function (Wang et al., 2010; Singh et al., 2012). This was explained by the existence of a robust and maximally active neurogenic system in these animals. However, in 15-month-old mice, which exhibited a significantly reduced level of neurogenesis and impaired cognitive functions, AP increased the survival of proliferating cells (BrdU-positive) as well as the learning ability of these animals (Singh et al., 2012).

In the 3xTgAD mouse mutants, the efficacy of AP to promote neurogenesis in the hippocampal SGZ and to restore learning and memory deficits depended on the age of the animals and the state of AD pathology. A reduction of neurogenic activity was already detectable at an age of 3 months, prior to the appearance of morphological indicators of AD in these mice (Wang et al., 2010). AP administration increased, in a dose-dependent manner, the number of proliferating progenitor cells and restored SGZ proliferation to the level observed in age-matched non-transgenic mice. These data were supported by an increased expression of the proliferative markers, PCNA (proliferating cell nuclear antigen) and CDK1 (cyclic dependent kinase 1) in AP-treated 3xTgAD mice. The newly formed neuroprogenitor cells generated following AP treatment acquired a neuronal phenotype (Wang et al., 2010).

It has been shown that there is a strong correlation between the extent of adult neurogenesis and particular hippocampus-dependent cognitive functions (Deng et al., 2010; Aimone et al., 2011). Spatial and temporal learning and memory seem to be dependent on the generation of new neurons in the DG. In addition to neurogenic deficits, 3-month-old 3xTgAD mice exhibited deficits in learning and memory. AP treatment in these mice increased learning and memory performance to a level similar to non-transgenic animals. A correlational analysis demonstrated a linear

relationship between the number of proliferating cells and enhanced memory performance in AP-treated 3xTgAD mice.

Analysis of the effects of AP in older 3xTgAD mice revealed that the beneficial action of the neurosteroid was preserved in animals that displayed mild to moderate intracellular Aβ burden (Singh et al., 2012) but was no longer detectable in 12-month-old mice with severe Aβ accumulation and formation of extracellular Aβ plaques. These data indicated that in this mouse model, AP has therapeutic potential only during initial stages of disease-development, but becomes ineffective at later stages of the disease when extracellular amyloid plaques have been formed.

Studies conducted by Chen and colleagues (2011) demonstrated the importance of the temporal regimen of administration of AP to achieve the maximal therapeutic effect of this compound: one administration per week for 6 months proved to be optimal, as opposed to once per month or every other day treatment for 3 months. Initiation of treatment at a pre-pathology stage was more efficient as opposed to starting when animals already showed an advanced pathology. From these results in the animal model, it was concluded that in humans, AP would be most potent in delaying progression of AD when brains still have neurogenic and myelination capacity. Thus, patients diagnosed with mild cognitive impairment and patients with early stage familial AD could be the populations to be targeted in future clinical trials.

Translation of preclinical AP studies to clinical practice will have to take into consideration several crucial elements that will determine the effective potential of AP as a therapeutic agent such as dosing regimen, formulation, route of administration, gender, age and progression of disease. Clinical human trials showed that AP is a safe, blood brain barrier penetrant molecule (van Broekhoven et al., 2007; Kask et al., 2008; Kask et al., 2009). Due to the beneficial regenerative and neuroprotective effects of AP, a phase I clinical trial of AP in treating AD is ongoing (ClinicalTrials.gov Identifier: NCT02221622).

6. PhD project

6.1. Hypothesis

A wealth of studies has demonstrated that neurosteroids, and AP in particular, exerts a variety of beneficial effects in the context of neurodegenerative diseases and other CNS or PNS disorders. First clinical trials are under way to assess their therapeutic potential e.g. in patients with neuropathic pain, traumatic brain injury or AD. In the clinical situation, however, the multiple cellular effects of AP might create undesired side effects. For instance, in AD both neuroprotection by AP against Aβ toxicity as well as the stimulation of neural progenitor cell proliferation to compensate for cell loss would be therapeutically meaningful. However, when exploiting the anti-nociceptive effect of AP for the treatment of neuropathic symptoms caused by cancer therapy, the proliferation-promoting activity would be contra-indicated in the presence of residual cancer cells. The diversity of the AP effects is likely to be related to the different molecular mechanisms which have been shown to mediate neurosteroid activities. Therefore, we hypothesized that it might be possible to synthesize AP derivatives of higher specificity with respect to neuroprotective versus proliferation-stimulating activities.

Another fact that hinders the therapeutic use of AP is its low bioavailability which is due to its rapid inactivation through glucuronidation or sulfation at the 3α-hydoxyl group. Derivatization of AP at the hydroxyl group which prevents its inactivation and elimination could therefore provide neurosteroid analogs with higher biological efficacy. Moreover, the absence of a free hydroxyl group at position 3 would also prevent re-oxidation of AP to 5α-dihydroprogesterone which would be able to exert additional non-AP specific genomic effects via binding to the nuclear steroid receptor. Thus, it was the aim of a collaborative behavioral and neurobiological project to examine the efficacy and specificity of a set of novel AP analogs with respect to their neurogenesis-promoting and neuroprotective activities as well as their protective effects against aging-related and AD-associated cognitive deficits.

The novel AP analogs (ANS) were synthesized by the lab of Dr. M. Miesch (University of Strasbourg) as described previously (Patent number *PCT/FR2012/05616; WO 2012/127176 A1; US 2014/0058079 A1*). The compounds tested included four analogs: pregnane-12,20 dione 3-hydroxy (3α, 5α) or **12 oxo-AP**; pregnane-12,20 dione 3-hydroxy (3β, 5α) or **12 oxo-epiAP**; pregnan-20 one 3-

(2-propen-1-yloxy) (3α, 5α) or **O-allyl-AP**; pregnan-20 one 3-(2-propen-1-yloxy) (3β, 5α) or **O-allyl-epiAP**.

6.2. Objectives

Studying these novel AP analogs (ANS) is the focus of a multidisciplinary research project of which the main objectives of my thesis work were:

1- To examine effects of ANS on adult neurogenesis in the rodent brain, more specifically, examine the action of ANS on proliferation and differentiation of adult neural stem cells (aNSCs) in cultures derived from the subventricular zone of the mouse brain.

2- To assess the capacity of ANS to protect aNSCs against apoptosis induced by amyloid beta peptide 1-42 (Aβ42) which is involved in the etiology of AD.

3- To examine if the effects of ANS on aNSCs can be confirmed in hippocampal neurogenesis that has been shown to play an important role in memory formation and learning.

4- To investigate effects of ANS against the age-dependent decline of neurogenic activity *in vivo* and to correlate them with the results of cognitive tests obtained by our collaborators.

5- To test the ability of ANS to attenuate the decrease of neurogenic activity in a transgenic mouse model (Tg2576) reproducing symptoms of AD.

6.3. Experimental models

To reach our objectives, we have used aNSC cultures, which are an *in vitro* model for adult neurogenesis as these cells reproduce the characteristics of NSCs by their capacity to proliferate, self-renew, and generate multipotent progeny (Reynolds and Weiss, 1992; Richards et al., 1992; Weiss et al., 1996; Rietze and Reynolds, 2006; Young et al., 2006; Young et al., 2007). In addition, we used primary hippocampal cell cultures from early postnatal (P2) rat and mouse hippocampus. These two culture systems were used to evaluate effects of ANS on cell proliferation and differentiation.

The proliferation-promoting effects of ANS were evaluated using BrdU/EdU incorporation assays, thymidine analogs, which label cells in the S-phase of the cell cycle. Cells were co-stained for different cellular markers to characterize their

phenotype and examine the effects of the compounds on differentiation. All these markers were identified immunocytochemically.

For the induction of cell death by apoptosis we have treated aNSCs with the amyloid beta peptide 1-42 (Aβ42). The excess production of Aβ42 is one of the most important pathophysiological hallmarks of AD (LaFerla et al., 1995; Li et al., 1996; Cotman, 1998; Eckert et al., 2001). Using this model system, we examined the protective potential of the analogs against Aβ42-evoked toxicity.

The ability of selected analogs to ameliorate the aging-related decline in neurogenesis during normal brain aging was examined in 21-month-old C57BL/6J male mice. Tg2576 mice on the other hand were used as an AD mouse model to investigate the ability of analogs to improve cognitive deficits seen in these animals. Tg2576 mice overexpress a mutant form of amyloid precursor protein (APP) and develop amyloid plaques and progressive cognitive deficits. Morphological studies were performed on both experimental models to determine the number of stem cells, newborn cells and neurons in one of the neurogenic areas, the subgranular zone of the dentate gyrus in the hippocampus by using specific cellular markers.

MATERIALS AND METHODS

1. *In vitro* experiments

1.1. Adult neural stem cell cultures (Neurosphere culture)

Adult neurogenesis occurs in the subventricular zone (SVZ) of the lateral walls of the lateral ventricles (Alvarez-Buylla and Garcia-Verdugo, 2002). *In vitro*, adult neural stem cells (aNSCs) from the adult SVZ have been defined by their capacity to self-renew, proliferate and generate multipotent progeny (Reynolds and Weiss, 1992; Richards et al., 1992; Weiss et al., 1996; Rietze and Reynolds, 2006; Young et al., 2006; Young et al., 2007). aNSCs grow forming clusters that have been named neurospheres. This *in vitro* model for adult neurogenesis has been used to evaluate cell proliferation, self-renewal, and differentiation potential. aNSCs are grown in a defined serum-free medium in the presence of mitogenic growth factors (EGF and bFGF) on a non-adhesive substrate (Fig. 14). NSCs and neural progenitors start to proliferate in response to the mitogens after about 24 hours in culture, forming small clusters of cells by two to three days (Fig. 15). The clusters continue to grow in size with time. By approximately day six, the cell clusters, called neurospheres, typically measure 100 - 200 µm in diameter (Svendsen et al., 1998), and are composed of approximately 10000 - 100000 cells. At this point, the neurospheres have to be passaged, to prevent the cell clusters from growing too large, which can lead to necrosis as a result of a lack of oxygen and compromised nutrient exchange at the center of the neurospheres. By repeating dissociation for multiple passages, NSCs present in the culture will self-renew and produce a large number of progeny, resulting in a relatively consistent arithmetic increase in total cell number over time.

NSCs and neural progenitors can be induced to differentiate by removing the mitogens and plating dissociated cells on an adhesive substrate, such as laminin, in the presence of a low serum containing medium. After several days, all of the NSCs and their progeny will differentiate into the three main cell types found in the CNS: neurons, astrocytes and oligodendrocytes (Fig. 14).

Neurosphere cultures are an excellent method to obtain and expand aNSC-derivatives, to investigate their properties in different conditions and to provide a source of cells for replacement or neuroprotection upon injury. Neurospheres can be dissociated and cultured for many passages (up to 60). However, when they are re-

cultured for more than 10 passages, their biological properties may change (Morshead et al., 2002) and cells acquire, in some cases, tumor-like phenotypes and progressive chromosomal instability (Vukicevic et al., 2010). Thus, low passage number neurospheres (< 8 passages) were used for the experimental work.
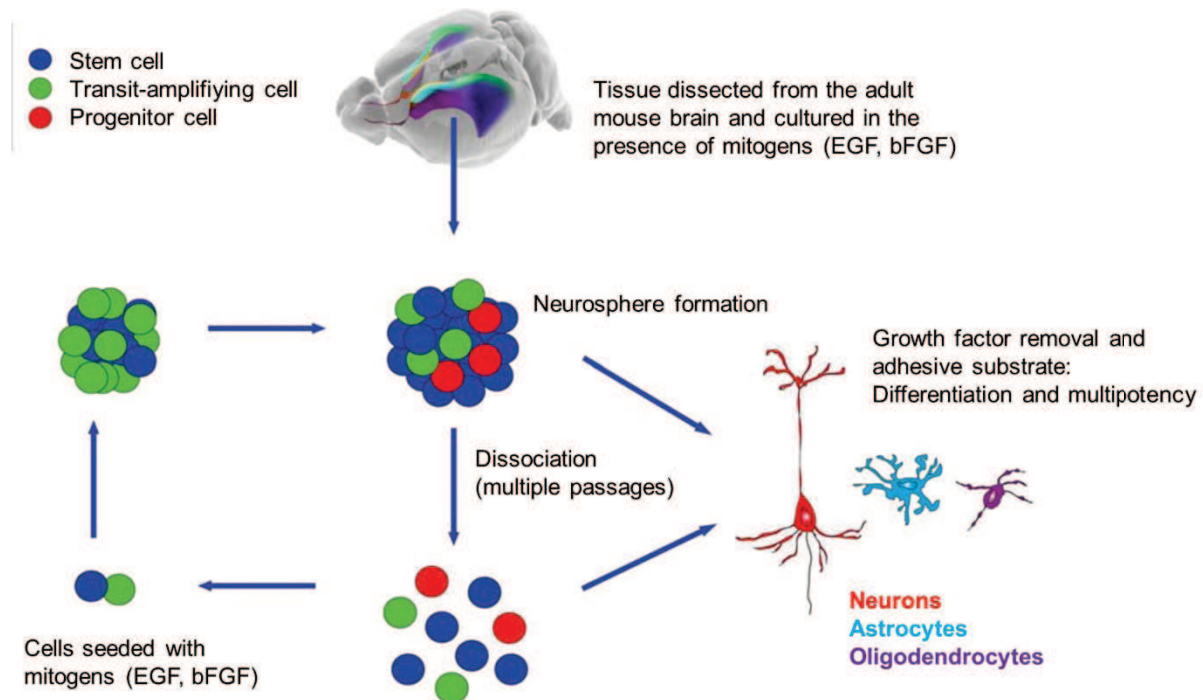
Figure 14: Schematic representation of the *in vitro* neurosphere culture system. NSCs are isolated from the mouse SVZ, which is dissected, then mechanically and enzymatically dissociated to yield a single cell suspension, and finally seeded in the presence of mitogens, such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). Adapted from (Gil-Perotín et al., 2013).
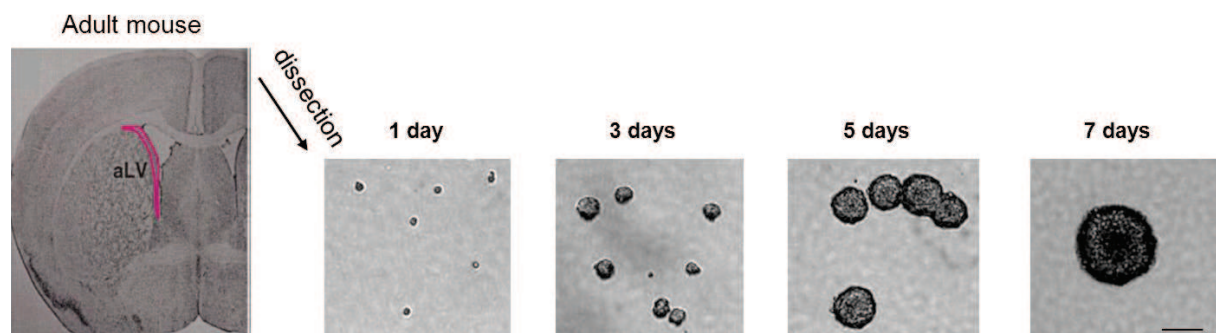
Figure 15: *In vitro* adult neural stem cell cultures. The SVZ from the lateral wall of the lateral ventricle (aLV) of an adult mouse is dissected and dissociated to yield a single cell suspension. NSCs and neural progenitors start to proliferate in response to the mitogens after about 24 hours in culture, forming small clusters of cells by two to three days. The clusters, called neurospheres, continue to grow in size with time and they have to be passaged after 5 to 7 days. Scale bar = 100 μm.

1.2. Primary hippocampal cell cultures

Primary dissociated neuronal cultures are widely used as a research tool to investigate pathological mechanisms associated with e.g. neurodegenerative disease.

Primary hippocampal cell cultures are obtained from early postnatal (P2) rat and mouse hippocampus. Following enzymatic and mechanical dissociation, cells are grown on a poly-D-Lysine-coated glass substrate. Primary hippocampal cell cultures form a mixed neuronal-glial cell culture. Neuronal cells develop extensive axonal and dendritic arbors over time, express neuronal and synaptic markers, and form functional synaptic connections (Beaudoin et al., 2012) (Fig. 16). They therefore can be used to examine multiple properties of neurons at the individual cell and single synapse level.
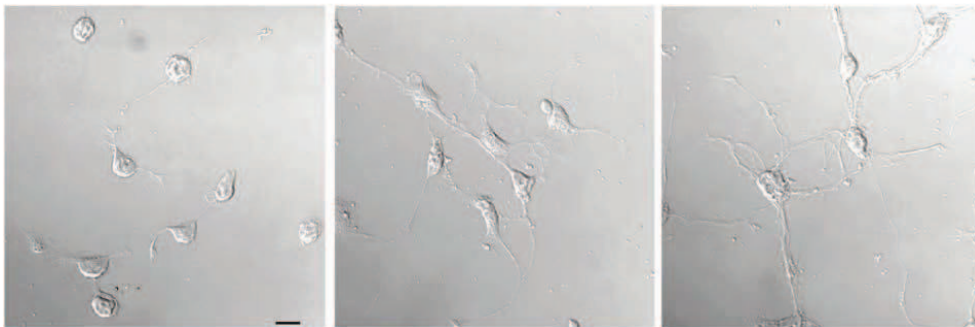
Figure 16: Primary hippocampal cell cultures from postnatal mice. Images of hippocampal cells at different stages of culture: DIV 0 (Days *in vitro*) (a), DIV 1 (b) and DIV 2 (c). Scale bar = 10 µm. Adapted from (Beaudoin et al., 2012).

1.3. Human neural stem cells (HNSC 100)

The HNSC 100 cell line originates from retrovirally transduced (v-myc) growth factor-expanded human neural progenitors of diencephalic and telencephalic regions of a 10 weeks gestational age, aborted human Caucasian embryo. HNSC 100 cells stably express nestin, grow as neurospheres in the presence of EGF and bFGF and readily and spontaneously differentiate upon mitogen withdrawal into all three fundamental CNS lineages (Villa et al., 2000).

1.4. Material

1.4.1. Antibodies

Primary and secondary antibodies employed for immunocytochemical staining are summarized in Table 1.

Primary antibody	Source	Dilution	Provider
BrdU	Rat monoclonal	1:10000	Abcam, Cambridge, UK
Nestin	Mouse monoclonal	1:1000	Santa Cruz, Heidelberg, Germany
GFAP	Rabbit polyclonal	1:2000	Dako, Hamburg, Germany
GFAP	Mouse monoclonal	1:600	Santa Cruz, Heidelberg, Germany
Tuj-1	Mouse monoclonal	1:1000	R&D Systems, Wiesbaden, Germany
DCX	Goat polyclonal	1:500	Santa Cruz, Heidelberg, Germany
Secondary antibody	**Source**	**Dilution**	**Provider**
Alexa Fluor 488 anti-rat IgG	donkey	1:300	Life Technologies, Darmstadt, Germany
Alexa Fluor 488 anti-rabbit IgG	donkey	1:300	Life Technologies, Darmstadt, Germany
Cy3 anti-mouse IgG	donkey	1:800	Jackson ImmunoResearch Europe, Newmarket, UK
Cy3 anti-goat IgG	donkey	1:800	Jackson ImmunoResearch Europe, Newmarket, UK

Table 1: Antibodies used in the immunocytochemistry.

1.4.2. RNA oligonucleotides

The oligonucleotides used for qRT-PCR were purchased from Eurofins Genomics, Ebersberg, Germany. Primer sequences are shown in Table 2.

Gene	Sens	5'-3' sequence
S12	Forward	GGCATAGCTGCTGGAGGTGTAA
	Reverse	GGGCTTGGCGCTTGTCTAA
Tuj-1	Forward	GCGCATCAGCGTATACTACAA
	Reverse	TTCCAAGTCCACCAGAATGG
GFAP	Forward	ATTGCTGGAGGGCGAAGAA
	Reverse	CGGATCTGGAGGTTGGAGAA
Bax	Forward	CGGCGAATTGGAGATGAACT
	Reverse	TGCCATCAGCAAACATGTCA
Bcl-2	Forward	AACCGGGAGATCGTGATGAA
	Reverse	GCATCTCCAGCATCCCACTC

Table 2: Oligonucleotide sequences.

1.4.3. Reagents, chemicals and material

Analogs of allopregnanolone were synthesized and provided by the Laboratoire de Chimie Organique Synthétique, UMR 7177, Institut de Chimie de l´Université de Strasbourg as part of a collaborative project funded by INTERREG IV. Synthesis of these compounds is detailed in the patent number *PCT/FR2012/05616, WO 2012/127176 A1* and *US 2014/0058079 A1*. The compounds and their nomenclature are listed in Table 3. Their chemical structures are shown in Figure 17. Stock solutions of neurosteroids, prepared in cell culture grade dimethyl sulfoxide (DMSO), are as follows: 12 oxo-AP 100 mM; AP, 12 oxo-epiAP and O-allyl-AP 50 mM; epiAP 10 mM and O-allyl-epiAP 5 mM.

Other chemicals, kits and devices used are listed in table 4.

Monomeric Aβ42 was prepared by dissolving Aβ42 peptide on ice with occasional vortexing in sterile Phosphate Buffered Saline (PBS) at a concentration of 100 µM (451 µg/ml). Single use aliquots were stored at -80 °C.

Neurosteroids	Provider
Allopregnanolone (AP) 3α-Hydroxy-5α-pregnan-20-one	Tocris Bioscience, Wiesbaden-Nordenstadt, Germany and Santa Cruz Biotechnology, Heidelberg, Germany
Epiallopregnanolone (epiAP) 3β-Hydroxy-5α-pregnan-20-one	Sigma-Aldrich, Taufkirchen, Germany
12 oxo-epiallopregnanolone (12 oxo-epiAP) Pregnane-12,20 dione 3-hydroxy (3α, 5α)	Laboratoire de Chimie Organique Synthétique, UMR 7177, Institut de Chimie de l´Université de Strasbourg, 1 rue Blaise Pascal, 67008 Strasbourg, France (Patent number *PCT/FR2012/05616; WO 2012/127176 A1; US 2014/0058079 A1*)
O-allyl-epiallopregnanolone (O-allyl-epiAP) Pregnan-20 one 3-(2-propen-1-yloxy) (3β, 5α)	
12-oxo-allopregnanolone (12 oxo-AP) Pregnane-12,20 dione 3-hydroxy (3β, 5α)	
O-allyl-allopregnanolone (O-allyl-AP) Pregnan-20 one 3-(2-propen-1-yloxy) (3α, 5α)	

Table 3: Neurosteroids used in this study.
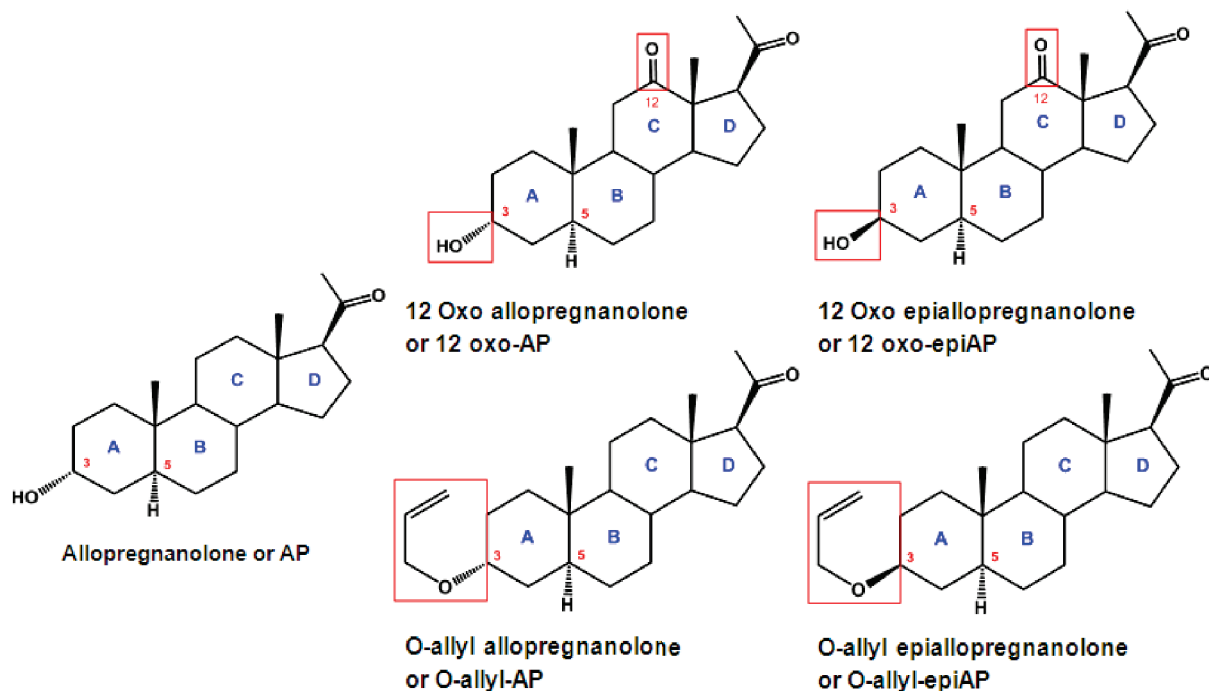
Figure 17: Chemical structures of allopregnanolone analogs investigated in this study.

Chemicals	Provider
Neurobasal A medium	Life Technologies, Darmstadt, Germany
DMEM (Dubelcco´s modified eagle medium)	Life Technologies, Darmstadt, Germany
HBSS (Hanks Buffered salt solution)	Life Technologies, Darmstadt, Germany
Trypsin	Sigma, München, Germany
BSA (Bovine serum albumin)	Sigma, München, Germany
Accutase	Sigma, München, Germany
Penicillin / streptomycin	Life Technologies, Darmstadt, Germany
L-glutamine	Life Technologies, Darmstadt, Germany
GlutaMax	Life Technologies, Darmstadt, Germany
FCS (Fetal Calf Serum)	Biochrome, Berlin, Germany
Laminin	Life Technologies, Darmstadt, Germany
Poly-D-lysine	Sigma, Taufkirchen, Germany
MTT (Thiazolyl blue tetrazolium bromide)	Sigma, Taufkirchen, Germany
TRIzol reagent	Life Technologies, Darmstadt, Germany
DMSO (Dimethyl sulfoxide)	Applichem, Darmstadt, Germany
B-27 supplement	Life Technologies, Darmstadt, Germany
EGF, recombinant, murine	Peprotech, Hamburg, Germany
bFGF, recombinant, human	Peprotech, Hamburg, Germany
Aβ42 peptide	Innovagen, Lund, Sweden
PFA (Paraformaldehyde)	Carl Roth, Karlsruhe, Germany
BrdU (5-Bromo-2'-deoxyuridine)	Sigma, Taufkirchen, Germany
Nifedipine	Tocris Bioscience, Wiesbaden-Nordenstadt, Germany
Bicuculline methiodide	Tocris Bioscience, Wiesbaden-Nordenstadt, Germany
Mifepristone	Sigma-Aldrich, Taufkirchen, Germany
Provera (Medroxyprogesterone 17-acetate)	Sigma-Aldrich, Taufkirchen, Germany
Protease inhibitor cocktail (AEBSF)	Sigma Aldrich, Taufkirchen, Germany
Kits	**Provider**
Verso cDNA Synthesis Kit	Abgene, Thermo Scientific, St-Leon-Rot, Germany
SYBR Green Fluorescein mix	Abgene, Thermo Scientific, St-Leon-

	Rot, Germany
Click-iT EdU imaging kit	Life Technologies, Darmstadt, Germany
EdU-click 488	baseclick GmbH, Tutzing, Germany
Cell Event Caspase-3/7	Life Technologies, Darmstadt, Germany
Cell proliferation ELISA BrdU	Roche Diagnostics, Mannheim, Germany
Human Aβ40 ELISA	Invitrogen, Camarillo, USA
Human Aβ42 ELISA	Invitrogen, Camarillo, USA
Pierce BCA Protein Assay	Pierce Biotechnology, Thermo Scientific, Rockford, USA
Device	**Provider**
ELISA Reader Fluostar Optima	BMG-Labtech, Ortenberg, Germany
Nanodrop 1000	peQLab Biotechnologie, Erlangen, Germany
Thermocycler	Biometra, Göttingen, Germany
MyiQ Single Color Real-Time PCR Detection System,	BioRad, München, Germany
Fluorescent microscope BX60	Olympus, Hamburg, Germany
With DCF 350 FX Camera	Leica, Wetzlar, Germany

Table 4: Chemicals, kits and devices.

1.5. Cell culture

1.5.1. Preparation of primary neurosphere cultures

Neurosphere cultures were prepared from the SVZ of adult male mice (6-8 weeks of age, C57BL/6) and maintained in **complete culture medium**, composed of:

- Neurobasal A medium
- B27 2%
- GlutaMax 2 mM
- L-glutamine 1 mM
- Penicillin / Streptomycin both at 100 µg/ml
- Recombinant murine epidermal growth factor (EGF) 20 ng/ml
- Recombinant human basic fibroblast growth factor (bFGF) 10 ng/ml

Adult mice were decapitated and brains were dissected and placed in a dish containing cold Hanks Buffered Salt Solution supplemented with 10 mM HEPES-

Buffer. Once all blood vessels and meninges have been removed, cerebral hemispheres were separated. Both hemispheres were then cut rostrally and caudally of the anterior commissure, obtaining a coronal section at approximately Bregma + 0.4 mm (Fig. 18) (Paxinos and Watson, 1998). The SVZ from the lateral wall of the lateral ventricle was then microdissected, washed with fresh HBSS and minced with a fine scalpel blade followed by incubation at 37°C for 10 min. HBSS was then replaced with 0.25 % trypsin in HBSS and incubation continued for 20 min at 37°C. Following centrifugation (200 g, 5 min) and washing in Neurobasal A with 1 % bovine serum albumin (BSA), cells were dissociated by trituration with a fire polished Pasteur pipette. Dissociated cells were centrifuged and resuspended in complete culture medium (see above). The cell suspension from one animal was seeded in 5 ml of medium in a T-25 culture flask (Greiner Bio-One, Frickenhausen, Germany) and cultures maintained at 37°C / 5 % CO_2. Primary neurospheres formed within 5-7 days and were passaged by enzymatic dissociation with Accutase. After trituration, cells were counted in a hemocytometer (Marienfield Superior) and seeded at a density of 12500 cells/ml in complete culture medium in T-25 flasks (2000 cells/cm^2). The cells obtained after dissociation of primary neurospheres yielded secondary neurospheres within 5-7 days. Passaging could be repeated several times every 5-7 days. For the experiments presented in the thesis low passage number neurospheres (< 8 passages) were used. Animal experiments were in accordance with German federal law and were approved by the local animal care committee (Regierungspräsidium Freiburg, Freiburg, Germany).
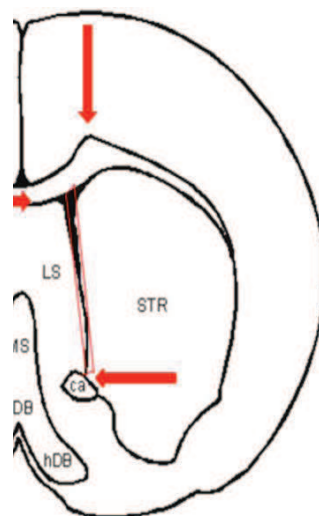
Figure 18: Coronal section through the adult mouse brain. The red arrows indicate the incision sites. Adapted from (Paxinos and Watson, 1998).

1.5.2. Passaging of neurosphere cultures

Neurosphere cultures were routinely passaged every 5-7 days by enzymatic treatment using accutase which detaches and separates the cells without damaging them. The culture medium with floating neurospheres was collected from the culture flask into a tube of appropriate size and centrifuged at 200 g for 3 min. The supernatant was discarded; the pellet was resuspended in 350 µl accutase and centrifuged again at 200 g for 3 min. The supernatant was discarded again; and the pellet was resuspended in 150 µl fresh accutase, briefly mixed, incubated at 37°C for 10 minutes in a thermoshaker and then centrifuged at 200 g for 3 min. The supernatant was discarded, cells were triturated gently with 150 µl of Neurobasal A medium containing 2 mM GlutaMax, 1 mM L-glutamine, penicillin / streptomycin (both at 100 µg/ml) and left to settle for 3 min. Then, 150 µl of the supernatant were transferred to a new tube containing 700 µl of fresh neurobasal A medium. The trituration procedure was repeated with an additional 150 µl of medium. Total cell number was determined using 10 µl of the cell suspension and a hemocytometer. Cells were seeded in culture flasks at a density of 12500 cells/ml in Neurobasal A, with an addition of B27 supplement (2 %), 20 ng/ml EGF, and 10 ng/ bFGF.

1.5.3. Proliferation and differentiation of aNSC cultures

Cell proliferation of neurosphere cells was determined by measuring the incorporation of the thymidine analog EdU (5-ethynyl-2´-deoxyuridine) to label cells in the S-phase. Dissociated neurosphere cells were plated at a density of 5×10^4 cells per well in 24-well plates in the presence of 2% B27, 1 ng/ml EGF and 0.5 ng/ml bFGF on glass coverslips which had been coated with 0.1 mg/ml poly-D-Lysine. After 2 days, the medium was changed and neurosteroids were added to 500 nM (final DMSO concentration 0.2 %). After an additional day, EdU (1 µM; Click-iT EdU Imaging Kit) was added for 2 hours before fixing the cells with 4 % paraformaldehyde (PFA) in 0.1 M phosphate buffer pH 7.4 (PB) for 20 min. Incorporated EdU was visualized by a click reaction using Click-iT EdU imaging kit (Life Technologies).

Differentiation of aNSCs was initiated by seeding cells at a density of 5×10^4 cells per well in 24-well plates on coverslips coated with 0.1 mg/ml poly-D-Lysine and 0.625 µg/cm^2 laminin in low serum containing medium (1 % FCS) without mitogens for 5 days.

1.5.4. Isolation, culturing and proliferation of primary hippocampal cell cultures

Primary cultures of dissociated hippocampal cells were prepared from the brains of P2 rats (Sprague Dawley) and mice (C57BL/6). Animals were euthanized by decapitation; skin was gently dissected and the skull was cut open by making an incision at the base of the brain. Then the brain was transferred to a 35-mm dish containing **preparation medium** (Fig. 19), composed of:

- Minimum essential medium (MEM) Eagle's with Earle's BSS
- GlutaMax 2 mM
- pH = 7.35
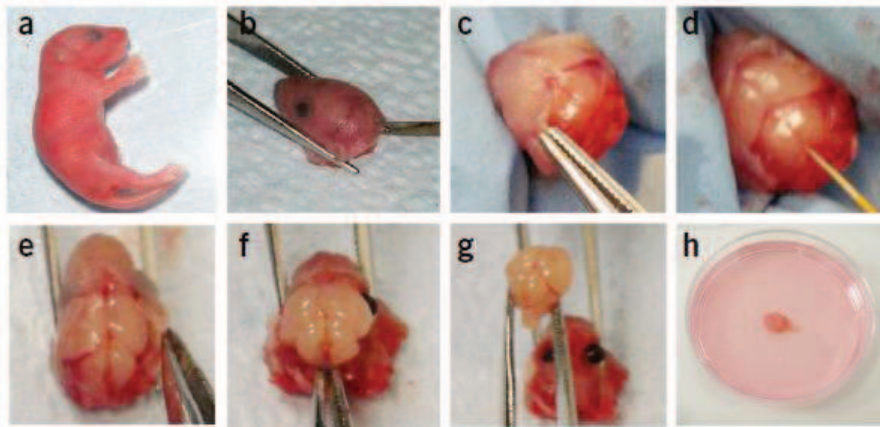
Figure 19: Illustration of the technique to remove brains from P2 mice. (a,b) Decapitate pups and remove the head. By using fine scissors, make a midline incision of the skin in the caudal part of the skull. (c) Extend the incision anteriorly. (d) Make a small incision at the base of the skull and follow along the midline. (e) Separate the two halves of the skull to reveal the brain. (f) Gently place the forceps underneath the brain and lift it from the skull. (g,h) Gently remove the intact brain (g) and quickly place it into dissection medium (h) (Beaudoin et al., 2012).

The two halves of the brain were separated by making a sagittal cut along the midline and the cerebellum was discarded. Under a dissecting microscope, midbrain and thalamic tissue were gently removed to leave an intact hemisphere containing the cortex and hippocampus. Afterwards, by using forceps, the anterior cortex was pinned down and another pair of forceps was used to pick and grab the meninges carefully and gently peel them off. The meninges should be removed completely so that they do not contribute any non-neuronal cells to the culture. At this stage, the hippocampus can be identified by its C-shaped structure and opacity, which differ

from the neighboring cortical tissue. The cortex can also be dissected out and processed similarly for cortical cultures. By using forceps or fine scissors, the hippocampus was isolated carefully and collected in a 35-mm dish containing fresh preparation medium (Fig. 20).
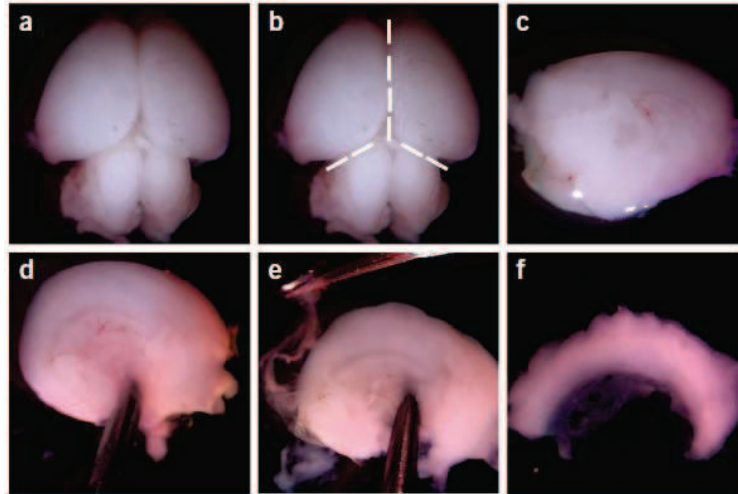
Figure 20: Steps for dissection of the hippocampus from the intact brain. (a) Place the brain dorsal side up in dissection medium. (b) Separate the hindbrain region and make a sagittal incision to separate the two hemispheres. (c) Place each hemisphere with the medial side facing up and remove any noncortical forebrain tissue. (d) Hold the hemisphere in place using forceps; use caution so as not to damage the hippocampus. (e) Remove the meningeal tissue with another pair of forceps. (f) Dissect out the hippocampus and the cortex (Beaudoin et al., 2012).

The isolated and minced hippocampal tissue was incubated with 0.25 % trypsin in HBSS for 15 min at 37°C. Afterwards, cells are centrifuged at 100 g for 5 min. Then, cells are removed and placed in a new tube to which 1 ml of **dissociation medium** was added:

- Hanks Buffered salt solution
- BSA 3 mg/ml
- HEPES-Buffer 10 mM
- $MgSO_4$ 3 mg/ml
- DNA-sel 0.025 %
- Trypsin inhibitor 0.4 mg/ml

Cells were triturated through a series of fire-polished Pasteur pipettes; 1 ml of warm fresh medium was added and the cell suspension was centrifuged at 20 g for 5 min. The supernatant containing the cells is re-centrifuged at 160 g for 5 min. The

supernatant is discarded and the pellet is resuspended in 2 ml of **plating medium** (DMEM with 1 g/l of glucose, 10% FCS and GlutaMax 2 mM). Total cell number was determined by counting 10 µl of the cell suspension in a hemocytometer. Dissociated cells were then plated on poly-D-lysine-coated coverslips in 48-well plates at a density of 4×10^4 cells/well. Cells were grown in **maintenance medium** containing:

- Neurobasal A medium
- B27 2%
- GlutaMax 1 mM
- Penicillin / Streptomycin both at 100 µg/ml

Cultures were maintained in a humidified atmosphere at 37°C / 8 % CO_2. After 24 hours, steroids were added to 500 nM / 0.2 % DMSO for another 24 hours. Then, 1 µM of BrdU or EdU was added for 2 hours before fixing the cells with 4 % PFA. Incorporated nucleotide analogs were visualized either by immunocytochemistry (BrdU) or by the click reaction using a Click-iT EdU imaging kit (EdU).

1.5.5. Human neural stem cell cultures

A seed stock cryovial was removed from -150°C and thawed very rapidly in a 37°C water bath. The thawed cells suspension (0.5 ml) was then diluted with 5 ml pre-warmed growth medium, followed by centrifugation at 200 g for 3 min. The supernatant was discarded and the cells were resuspended in fresh growth medium and seeded into 25 cm^2 flasks at high density to optimize recovery and cultured at 37°C / 5 % CO_2. **Growth medium** is composed of:

- DMEM/F12 (Invitrogen)
- HEPES-Buffer 5mM
- Glucose 6 g/l
- AlbuMAX-I 0.5 %
- Penicillin / Streptomycin both at 100 µg/ml
- Nonessential amino acids 1 %

supplemented with N2-supplement 2%, EGF and bFGF both at 20 ng/ml.

HNSCs were passaged using the standard cell dissociation protocol for neurosphere cultures described above. Cells were cultured in growth medium described above and were passaged once every 4-5 days.

1.6. MTT cell viability assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) assay is based on the conversion of MTT into formazan crystals by living cells as a result of their mitochondrial activity. Since for most cell populations the total mitochondrial activity is related to the number of viable cells, this assay is broadly used to measure cell proliferation or the *in vitro* cytotoxic effects of drugs. Therefore, this colorimetric assay was used to quantitatively measure cell survival, proliferation and viability. NADPH-dependent cellular oxidoreductase enzymes (mitochondrial succinate dehydrogenases) reduce the tetrazolium salt MTT to a purple, insoluble formazan reaction product. A lysis buffer (10% SDS, 0.01 M HCl) is added to dissolve the insoluble product. Absorbance of the resulting lysate is then measured spectrophotometrically, using an ELISA-reader, yielding absorbance values (595 nm) as a function of the concentration of converted dye (Fig. 21).

Effects of neurosteroids on cell growth of aNSCs

Dissociated neurospheres were seeded at a density of 1500 cells (in 50 µl) per well into 96-well plates in complete medium. Neurosteroids (AP, O-allyl-epiAP and O-allyl-AP) were diluted in complete medium (50 µl) and administrated directly to the cells at concentrations ranging from 0 to 10000 nM with 0.2 % as a final concentration of DMSO. Cells in the presence of neurosteroids were incubated for 5 days at 37°C / 5 % CO_2.

Effect of monomeric Aβ42 on aNSCs viability

Dissociated neurospheres were seeded at 8000 cells (in 100 µl) per well into 96-well plates in complete medium. After 8 h incubation, cells were exposed to Aβ42 peptide (5 µl) at various concentrations (0, 0.625, 1.25, 2.5, 5 and 10 µg/ml) for 3 days to determine effective Aβ42 doses inducing a significant decrease in cell viability.

In all experiments described above, 10 µl of MTT solution (5 mg/ml in PBS) was added to each well at the end of the incubation followed by incubation for 4 h at 37°C / 5 % CO_2. Then 100 µl of lysis solution were added to each well and incubation continued overnight to dissolve the precipitate formed. The absorbance of the resulting colored solution which is proportional to the number of living cells was

measured at a wavelength of 595 nm with an ELISA-reader. Reference measurements were made at a wavelength of 690 nm. Absorbance values of medium without cells were subtracted (Blank). Values measured in control conditions (absence of neurosteroids or absence of Aβ42) were arbitrarily set to 100 %. Values for percentage of cell survival were obtained by using the following calculation:

$$\text{Cell survival (\%)} = \frac{Absorbance\ sample - Absorbance\ medium}{Absorbance\ untreated\ cells - Absorbance\ medium} \times 100$$
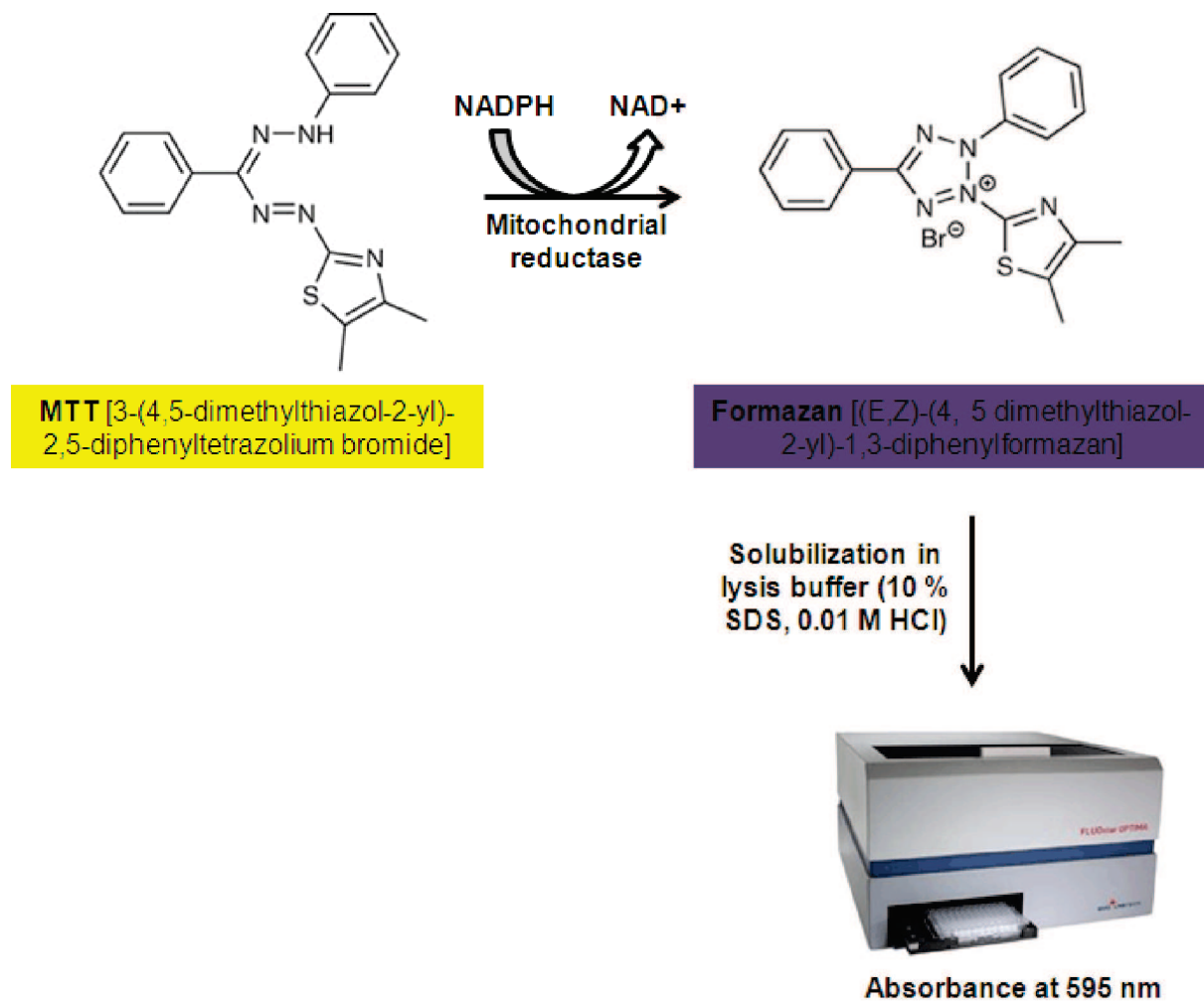
Figure 21: MTT assay principle. The yellow MTT tetrazole is reduced to insoluble purple formazan in the mitochondria of living cell. After solubilization in lysis buffer, absorbance at 595 nm is measured which is proportional to the number of viable cells. Adapted from (Wendt, 2014).

1.7. Immunocytochemistry

1.7.1. Coating of coverslips

Acid-cleaned and sterilized round coverslips (12 mm or 8 mm diameter) were placed in the wells of 24-well or 48-well plates respectively, and coated with 400 µl of 0.1 mg/ml poly-D-Lysine overnight. Before seeding cells, the coverslips were washed three times with sterile water to ensure complete removal of cytotoxic poly-D-lysine.

1.7.2. Cell fixation

Medium was completely removed and cells were fixed with 4 % PFA in 0.1 M phosphate buffer pH 7.4 for 20 min at room temperature (RT). Following removal of PFA, 0.1 M PB containing 0.02 % NaN_3 was added and plates were kept at 4°C until further processing.

1.7.3. Immunocytochemical staining (ICC)

The principle of the indirect immunofluorescence labelling technique is to incubate cells with a first antibody that binds to the target molecule or specific antigen. Bound primary antibody is detected by a secondary antibody coupled to a fluorescence dye for visualization (Fig. 22). The secondary antibody recognizes antibodies of the host animal species used to obtain the primary antibody. Multiple primary antibodies, as well as the secondary antibodies, can be incubated together being careful not to use primary antibodies made in the same animal species.
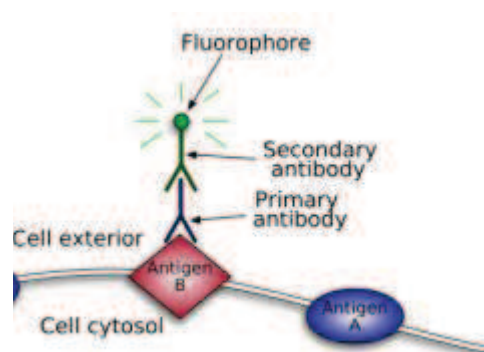
Figure 22: Principle of the indirect immunofluorescence labelling technique (Image taken from med.unsw.edu.au/cellbiology).

Following fixation coverslips were washed three times with 0.1 M PB, pH 7.4. For anti-BrdU staining, DNA was denatured with 2 N HCl at 37 °C for 15 min. Unspecific binding sites were blocked by incubation for 60 min at RT in blocking solution (9 % normal horse serum, 1 % NDS, 0.3 % Triton X-100 and 0.02 % NaN_3 in 0.1 M PB) followed by an overnight incubation in primary antibody at 4 °C in PB containing 10 % of blocking solution. The primary antibodies were diluted according to table 1. After washing with PB three times for 10 min, coverslips were incubated for 2 hours at RT in the dark with the appropriate secondary antibodies coupled to Alexa AF488 or Cys3 diluted according to table 1 in PB containing 1 % of horse serum and 1 % of an appropriate normal serum. Following secondary antibody incubation, coverslips were washed 3 times with PB for 10 min. DNA staining was performed with Hoechst 33342 (Life Technologies, Darmstadt, Germany) at a final concentration of 5 µg/ml for 30 min at RT protected from light followed by washing with PB (3 x 10 min). The coverslips were air-dried and mounted with fluorescent mounting medium (Immu-Mount, Thermo Scientific Shandon, Schwerte, Germany) on microscope slides (Langenbrinck, Emmendingen, Germany). Specimens were examined on an Olympus BX60 epifluorescent microscope with a Leica DFC 350 FX camera. Images were documented using IrfanView software (version 4.36, Vienna University, Austria).

1.7.4. Identification of proliferating cells by incorporation of BrdU and EdU

In some experiments, the analysis of cell proliferation was performed by adding 5-bromo-2'-deoxyuridine (BrdU) to the cells and by visualizing its incorporation with an anti-BrdU-antibody. Quantification was performed by counting BrdU-immunoreactive cells. For immunocytochemical detection of incorporated BrdU, a denaturation step is required which hydrolyses DNA. As DNA denaturation affects nuclear counterstaining with the Hoechst dye, unstained cells are difficult to discern. Therefore, we used the Click-iT EdU method which is not based on detection via antibodies and does not require DNA denaturation. Instead, Click-iT EdU is based on a click reaction, a copper-catalyzed covalent reaction between an alkyne group (coupled to EdU) and an azide group (coupled to the Alexa Fluor dye) (Fig. 23). Standard aldehyde-based fixation and detergent permeabilization are sufficient for the click-iT detection reagent to gain access to the DNA.
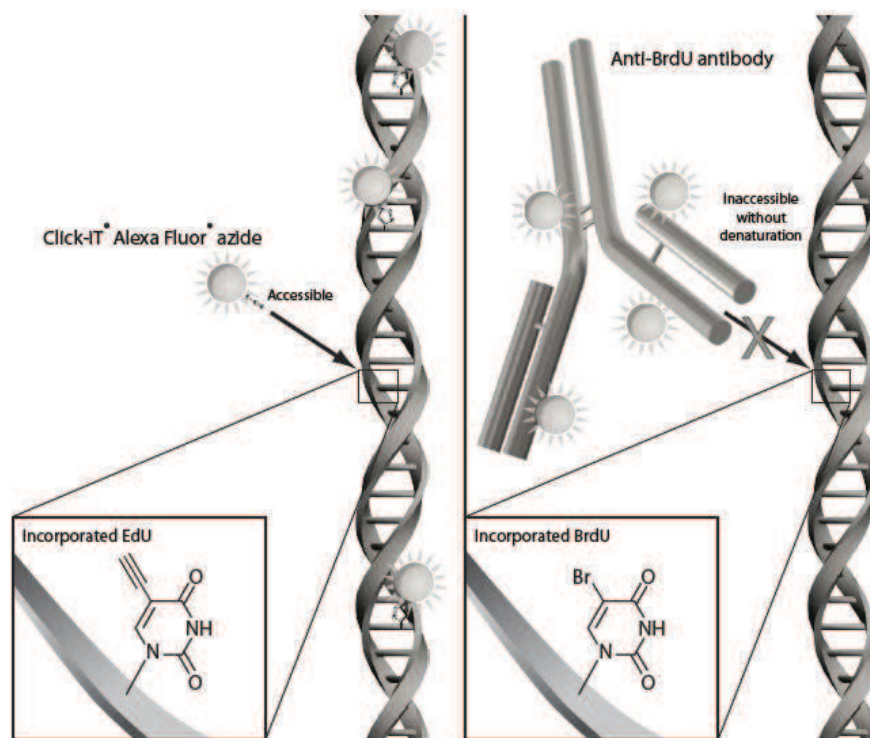
Figure 23: Detection of incorporated EdU with the Alexa Fluor® azide versus incorporated BrdU with an anti-BrdU antibody. The small size of the Alexa Fluor® azide eliminates the need to denature the DNA for the EdU detection reagent to gain access to the nucleotide (Click-iT EdU Imaging Kit, Invitrogen).

Fixed cells were washed twice with 3 % BSA in PBS for 10 min and permeabilized with 0.5 % Triton X-100 in PBS for 20 min at RT. After washing the coverslips twice with 3% BSA in PBS for 10 min, the **Click-iT reaction cocktail** was prepared according to the manufacturer's suggestions (Table 5).

Reaction components	Volume (µl)
1x Click-iT reaction buffer	515
$CuSO_4$	24
Alexa Fluor azide	1.5
Reaction buffer additive	60

Table 5: Click-iT reaction cocktail.

The Click-iT reaction cocktail must be used within 15 min of preparation and the ingredients must be added in the order listed in the table 5; otherwise the reaction will not proceed optimally. The Click-iT reaction cocktail (30 µl) was added to the coverslips and incubated for 30 min at RT protected from light.

After washing three times with 3 % BSA in PBS for 10 min, unspecific biding sites were blocked by incubation for 15 min at RT in blocking solution (9 % NHS, 1 % NDS, 0.3 % Triton X-100 and 0.02 % NaN_3 in 0.1 M PB) followed by antibody labelling of the samples and mounting as described above.

1.7.5. Caspase-3/7 assay, quantification of apoptotic cells by cell counting

The CellEvent[TM] Caspase-3/7 green detection reagent assay was used to investigate the toxic effect of Aβ42 peptide on aNSC cultures. This assay uses a fluorogenic substrate for activated caspases 3 and 7. The reagent consists of a four amino acid peptide (DEVD) conjugated to a nucleic acid binding dye. It is a cell-permeant substrate and intrinsically non-fluorescent, because the DEVD inhibits the ability of the dye to bind to DNA. After activation of caspase-3 or caspase-7 in apoptotic cells, the DEVD peptide is cleaved, enabling the dye to bind to DNA and produce a bright fluorescent signal.

Dissociated neurosphere cells were plated at a density of 2 x 10^4 cells per well in 24-well plates on poly-D-Lysine coated coverslips in complete medium. After 24 hours, Aβ42 was added to a final concentration of 2.5 and 5 µg/ml. After another 24 hours, caspase-3/7 substrate to a concentration of 5 µM was added and cells were incubated for 30 min at 37°C followed by fixation with 4 % PFA, washing with 0.1M PB and mounting for fluorescence microscopy as described above. Hoechst 33342 stained the nuclei in blue and apoptotic cells with activated caspase-3/7 showed bright green nuclei.

1.8. Caspase-3/7 assay, quantification by fluorometric measurement

Fluorometric measurement of caspase-3/7 activity was used to quantify the extent of apoptotic cell death. Dissociated neurosphere cells were cultured in suspension at a density of 10^4 cells per well in 96-well plates in complete medium. To test the protective effect of analogs of allopregnanolone against Aβ42 toxicity, two different experimental setups were used. In the first, cells were pretreated with neurosteroids (500 nM) for 1 h before applying Aβ42 to 1.25 µg/ml for 2 days (Fig. 24A). In the second, cells were first incubated with 1.25 µg/ml Aβ42 for 16 hours, before the treatment with neurosteroids (500 nM). After two additional days of incubation,

cultures were evaluated for caspase-3/7 activity (Fig. 24B). For both setups caspase-3/7 substrate (5 µM) was added at 3 DIV and fluorescence was measured using an ELISA-reader (excitation: 485 nm, emission: 510 nm), 30 min after substrate addition.
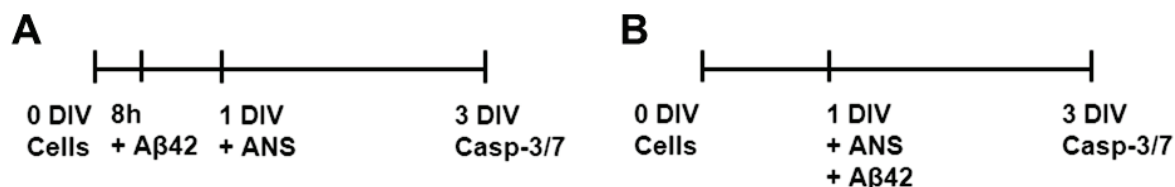
Figure 24: Experimental setups used for quantification of the protective effect of analogs of allopregnanolone by fluorometric measurement of caspase-3/7 activity: Prophylactic strategy (A) and Corrective strategy (B).

1.9. BrdU cell proliferation ELISA

A colorimetric immunoassay was used for the quantification of cell proliferation based on the measurement of BrdU incorporation during DNA synthesis in proliferating cells.

Dissociated HNSC cells were seeded at a density of 750 cells (in 50 µl) per well into 96-well plates, coated with 0.1 mg/ml poly-D-Lysine. Neurosteroids (AP and O-allyl-AP) were diluted (50 µl) and administered directly to the cells at concentrations ranging from 0 to 5000 nM. Cultures were incubated for 3 days at 37 °C / 5 % CO_2. Then, BrdU was added to 1 µM (in 10 µl; BrdU ELISA kit, Roche) and cells were reincubated for additional 5 h. Labelling medium was removed and 200 µl per well of FixDenat solution from the kit was added to denature and fix the cells followed by incubation for 30 min at RT. After removing FixDenat solution, 100 µl of peroxidase coupled anti-BrdU antibody was applied for 45 min at RT. Antibody conjugate was removed followed by rinsing the cells three times with 200 µl of washing solution. Finally, 100 µl of substrate solution (tetramethyl-benzidine + H_2O_2) was applied and the plate was incubated for 15 min at RT in the dark. The colored product was quantified spectrophotometrically with the ELISA-reader at 450 nm and a reference wavelength of 690 nm.

1.10. Real-time reverse transcription polymerase chain reaction (qRT-PCR)

1.10.1. RNA extraction

RNA isolation with TRIzol (guanidinium thiocyanate-phenol-chloroform) maintains RNA integrity during cell lysis, while at the same time disrupting and breaking down cell components. After solubilization, the addition of chloroform and centrifugation causes phase separation, where protein is extracted to the organic phase, DNA resolves at the interface, and RNA remains in the aqueous phase (Fig. 25). After transferring the aqueous phase, RNA can be recovered by precipitation with isopropanol.
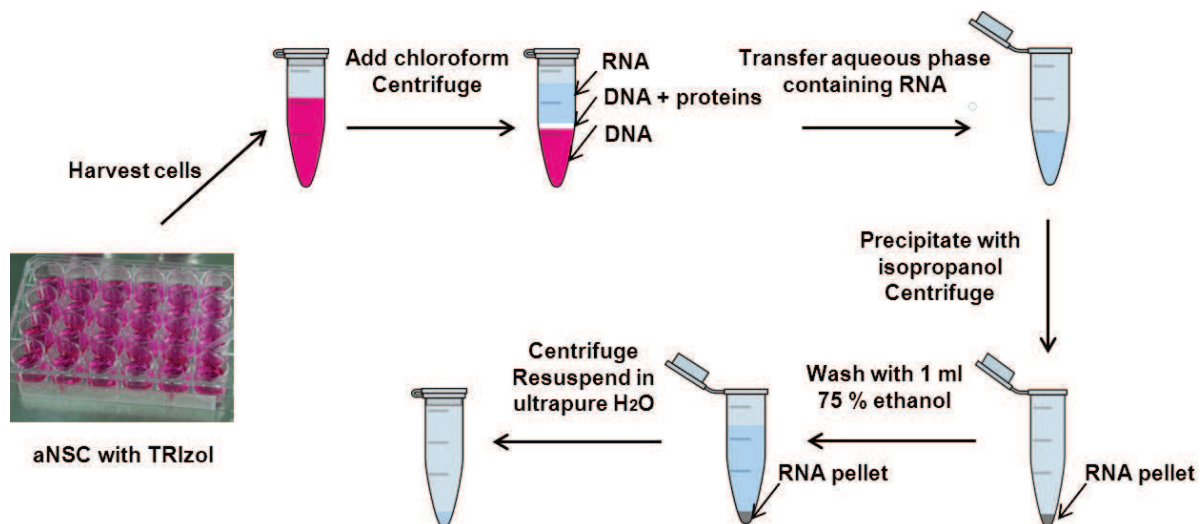
Figure 25: Schematic representation of the major steps for TRIzol RNA extraction. Adapted from (Wendt, 2014).

Dissociated neurosphere cells were plated at a density of 5×10^4 cells per well in 24-well plates on coverslips coated with 0.1 mg/ml poly-D-Lysine in Neurobasal A medium in the presence of B27 2 %, EGF1 ng/ml and bFGF 0.5 ng/ml. Cells were treated with 500 nM / 0.2 % DMSO of neurosteroids (AP, 12 oxo-AP and O-allyl-AP) for 3 days at 37°C / 5 % CO_2. Afterwards, medium was removed and cells were lysed in 1 ml TRIzol. At this stage samples could be frozen at -20° C until further purification.

For RNA isolation, the frozen samples were thawed and 200 μl of chloroform was added, vortexed for 15 seconds and incubated for 5 min at RT. Subsequently, lysates were centrifuged at 12000 g for 20 min at 6°C. The aqueous phase (400 μl) was

transferred to a new Eppendorf tube containing 2 µl glycogen (20 µg/ml; Peqlab) followed by addition of 400 µl ice cold isopropanol (v/v). The samples were left to precipitate for 10 min at RT. Then, they were vortexed briefly and centrifuged at 12000 g for 20 min at 6 °C. The supernatant was discarded and the isolated RNA pellets were washed with 1 ml ethanol 75 % and centrifuged at 12000 g for 20 min at 6 °C. The supernatant was carefully removed and the pellet air-dried at RT. The pellet was then dissolved in 20 µl ultrapure water and incubated at 55 °C for 10 min.

1.10.2. RNA concentration measurement and quality determination

The quality of the isolated RNA was determined by optical density (OD) measurements at two wavelengths 260 nm and 280 nm (Nanodrop; Peqlab). The RNA concentration was determined using Beer-Lambert law. Knowing that 1 $OD_{260\,nm}$ corresponds to 40 ng/µl of total RNA, the concentration in ng/µl is determined using the following equation: $[RNA] = OD_{260\,nm} \times 40$. RNA has its maximal absorbance at 260 nm while aromatic amino acids have their maximal absorbance at 280 nm. Consequently, the quality of RNA can be determined by the absorbance ratio $OD_{260\,nm}$ / $OD_{280\,nm}$ which should be greater than 1.7 for pure RNA.

1.10.3. Reverse transcription

Reverse Transcription is a process, in which single-stranded RNA is reverse transcribed into complementary DNA (cDNA) using reverse transcriptase. The resulting cDNA is used as the template for amplification by quantitative polymerase chain reaction (qPCR).

For reverse transcription, the Verso cDNA Synthesis Kit (Thermo Scientific) was used, which contains a reverse transcriptase with significantly attenuated RNase H activity.

To 11 µl RNA solution containing a maximum of 1 µg RNA, 1 µl of Anchored Oligo dT primer (500 ng/ml) was added and incubated for 10 min at 70 °C. The reaction was stopped by placing the samples immediately on ice. Then, for each sample, a reaction mix containing dNTPs, reverse transcriptase and buffer was prepared as follows:

5x cDNA synthesis buffer	4 µl
dNTP Mix (5 mM)	2 µl
Reverse Transcription Enhancer	1 µl

Verso Enzyme Mix   1 µl

The reaction mix (8 µl) was added to each sample containing 12 µl of RNA, nuclease-free water and Oligo dT primer. Samples were incubated in a thermocycler (Biometra, Göttingen, Germany) with the following reaction protocol:

Steps	Temperature (°C)	Duration (min)
1	25	10
2	42	40
3	95	2
4	10	1
5	15	Pause

Reverse transcription products were stored at - 20°C.

1.10.4.   Real-time reverse transcription polymerase chain reaction

In traditional PCR, the amplified product or amplicon is detected at the final phase or end-point of the PCR reaction using agarose gels. In contrast, real-time PCR has the ability to monitor the progress of the PCR as it occurs (in real time). Data is collected throughout the PCR process, rather than at the end of the PCR. In real-time PCR, quantification is based on the point in time during cycling when the amount of amplified product reaches a certain threshold (threshold cycle) rather than on the amount of target accumulated after a fixed number of cycles.

In our experiments, quantitative PCR was performed using SYBR Green (ABgene, Germany). Forward and reverse primer sequences used are shown in table 2. After amplification, melting curves of the RT-PCR products were acquired to demonstrate target specificity.

qPCR was performed with a MyiQ Cycler (MyiQ Single Color Real-Time PCR Detection System, BioRad) using the following cycling protocol:

Cycling step	Temperature (°C)	Time (min:sec)	Number of cycles
Initial denaturation	95	0:30	1
Polymerase activation	95	15:00	1
Denaturation	95	0:15	50
Annealing+Extension	61	1:00	50

The reaction was followed by a melt curve program to confirm the specificity of amplification:

Cycling step	Temperature (℃)	Time (min:sec)	Number of cycles
Denaturation	95	1:00	1
Starting temperature	60	1:00	1
Melting curve	60, 0.5 increments	0:10	70

The results were analyzed using iQ™5 system software (BioRad 2.0 Version) using raw data by the method described in (Zhao and Fernald, 2005). The algorithm determines the efficiency of DNA amplification for each primer. As efficiencies show slight variation between different primers, they were taken into account when calculating final Ct values for the housekeeping gene (S12) and the gene of interest. For quantification, corrected Ct values were used to determine expression levels of the gene of interest as fold S12 by application of the delta Ct method [calculated as $2^{\Delta Ct}$ with ΔCt = Ct (S12) - Ct (gene of interest)].

The obtained values were used to compare differences in gene expression levels under different culture conditions.

All samples were analyzed in duplicates (technical replicates).

1.11. Cell counting and statistical analysis

Cell counting was performed by counting cells in 30 fields of view per coverslip at 40x magnification.

All statistical analyses were performed using GraphPad Prism (version 6) using one-way ANOVA, followed by Dunnet´s or Bonferroni´s multiple comparison test. Significance is indicated as follows: *[#]$p<0.05$, **[##]$p<0.01$; ***[###]$p<0.001$; **** $p<0.0001$.

2. *In vivo* experiments

2.1. Animals

All the *in vivo* studies including animal housing, training and behavioral tests were performed by the Laboratoire de Neurosciences Cognitives et Adaptatives, Université de Strasbourg, UMR 7237 Centre National de la Recherche Scientifique, Strasbourg,

France under the supervision of Dr. Chantal Mathis. All experimental procedures were conducted in conformity with the institutional guidelines (council directive 87/848, October 19, 1987, Ministère de l'agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale; National Institutes of Health publication, 86–23, revised 1985). The project was approved by the local ethics committee CREMEAS (AL/14/21/02/13).

For our experiments, we used C57BL/6J male mice (Janvier, Le Genest Saint Isle, France) and Tg2576 (Tg) female mice and their wild-type female littermates (NTg; Taconic Europe A/S, Danemark). These transgenic mice overexpress the Swedish mutated form of the human amyloid precursor protein APP (double mutation Lys670-Asn, Met671-Leu [K670N, M671L] of human APP (1-695)) under the control of a promoter of the hamster prion protein (Hsiao et al., 1996). Mice were housed individually in Makrolon cages (16 x 32 x 14 cm) under controlled temperature (23 ± 1 °C) and a 12/12 hours light/dark cycle (lights on at 7:00 am). Food and water were available ad libitum and paper towels were provided for nesting. Each animal was regularly weighed and manipulated to become familiar with the experimenter.

O-allyl-AP-treated aged mice:

For this cohort, 6 young (7 month old) and 24 aged mice (21 month old) were used. They were injected intraperitoneally with 0.3 % hydroxypropyl cellulose, used as vehicle, and with three doses of O-allyl-AP in 0.3% hydroxypropyl cellulose (1 mg/kg, 2 mg/kg and 4 mg/kg) three times per week for 4 weeks (300 µl injection volume). There were 5 groups with 6 animals per group:

- Young 7 month old vehicle-treated mice
- Aged 21 month old vehicle-treated mice
- Aged 21 month old 1 mg/kg O-allyl-AP-treated mice
- Aged 21 month old 2 mg/kg O-allyl-AP-treated mice
- Aged 21 month old 4 mg/kg O-allyl-AP-treated mice

AD mouse model experiments:

For each treatment, 24 Tg2576 mice and 6 NTg were used. Animals were injected intraperitoneally with 3 doses of O-allyl-AP or O-allyl-epiAP (1, 2 and 4 mg/kg or 2, 4 and 8 mg/kg respectively) and vehicle 3 times per week for 4 weeks (300 µl injection volume). At the beginning of treatment, the O-allyl-epiAP mouse cohort was 9 months

old, while the O-allyl-AP mouse cohort was 10 months old. There were 5 groups with 6 animals per group for each treatment:

- NTg vehicle-treated mice
- Tg2576 vehicle-treated mice
- Tg2576 dose 1-treated mice
- Tg2576 dose 2-treated mice
- Tg2576 dose 3-treated mice

After 4 weeks of drug-treatment, the following behavioral tests were performed: pattern separation (aged mice) and object location (Tg2576 mice) (Sahay et al., 2011b; Sahay et al., 2011a; Yassine et al., 2013). At the end of the behavioral studies, brains of half of the animals per group were fixed by perfusion for morphological analysis while brains of the other half of the animals were frozen for biochemical and molecular analysis.

2.2. Anesthesia and perfusion of the animals

Animals were anesthetized by intraperitoneal injection of 300 µl of a mixture of ketamine (2.5 %), xylazine (0.12 %) and acepromazine (0.025 %) in 0.9 % NaCl. Intracardiac perfusion was then carried out by perfusing 20 ml of 0.9 % NaCl solution buffered with 10 mM phosphate buffer, pH 7.4, followed by approximately 80-100 ml of ice-cold paraformaldehyde solution (4 % PFA in 0.1 M phosphate buffer). Following perfusion brains were removed from the cranium, placed in the same fixative at 4 ℃ for 24 h and then transferred to phosphate buffer (0.1 M) with 0.05 % NaN$_3$ and stored at 4 ℃.

2.3. Brain freezing

Animals were decapitated following cervical dislocation. Brains were dissected, cerebellum and olfactory bulb were removed and hemispheres were separated, frozen on dry ice and stored at -80 ℃.

2.4. Morphological analysis

2.4.1. Vibratome sectioning

Fixed brains were embedded in 5 % agarose and cut on a vibratome (LEICA VT1000 S) in 0.1 M phosphate buffer pH 7.4. Coronal sections (50 µm) were collected in 8 series in 12-well plates in 0.1 M PB, 0.05 % NaN_3, pH 7.4.

2.4.2. Immunohistochemistry (IHC)

Immunohistochemical staining was performed using detection of bound primary antibodies by biotinilated secondary antibodies and avidin-peroxidase using nickel-cobalt intensified 3,3′-diaminobenzidine (DAB) as a peroxidase substrate.

After washing the free-floating sections with 0.1 M PB, they were placed in methanol (100 %) containing 0.3 % for 20 min at RT in order to inhibit the activity of endogenous peroxidases. Sections were washed 3 times with 0.1 M PB followed by incubation in blocking solution for 1 hour at RT to saturate non-specific binding sites. All blocking solutions contained 1 % Triton-100 plus 0.02 % NaN_3. The composition of the blocking solution depends on the secondary antibody used (Table 6):

Secondary antibody	Blocking solution
Goat anti-rabbit	10 % NGS
Rabbit anti-goat	5 % NRS + 5 % HS
Horse anti-mouse	10 % HS

Table 6: Blocking solutions used in immunohistochemistry.

The sections are incubated overnight with the primary antibody (Table 7) diluted in a solution of 9 parts 0.1 M PB plus 1 part blocking solution. The next day, after washing the sections 3 times with 0.1 M PB, they were incubated for 1 hour at RT in the presence of the biotinylated secondary antibody (Vector, 1:200 in PB), then 30 min in a solution of the avidin-biotinylated peroxidase complex following the manufacturer's recommendations (Vector, Elite-kit). After washing 3 times with 0.1 M PB, sections were incubated with the DAB substrate solution.

Preparation of DAB solutions A and B:

A: 7.8 ml PB + 0.2 ml of 25 mg/ml DAB + 50 µl of 1 % $Ni(NH_4SO_4)_2$ + 60 µl of 1 % $CoCl_2$.

B: 1 ml H_2O + 3.3 µl of 30 % H_2O_2.

First, sections were equilibrated with solution A (500 µl) and then the reaction was started by adding 5 µl of solution B. Color development was controlled microscopically as the development time of the reaction depends on the abundance of the antigen to be detected (Table 7). The reaction was stopped by three washes in 0.1 PB. After several additional washes, sections were mounted on gelatinized slides and dried at RT for 24 h.

The following day, sections were dehydrated in a graded series of ethanol starting with H_2O (2 x 10 min) to remove salts, then 96 % ethanol (10 min), 100% ethanol (2 x 10 min), this was followed by washes in a 1:1 mixture of ethanol and toluol (10 min) and toluol (10 min). The slides were finally covered with a coverslip using Hypermount (Shendon, Germany).

Primary antibody (DAB)	Source	Dilution	Development time	Provider
Ki67	Rabbit polyclonal	1:1000	7 min	ABIN, Aachen, Germany
NeuroD	Rabbit polyclonal	1:400	25 min	Santa Cruz, Heidelberg, Germany
Sox2	Rabbit polyclonal	1:5000	3 min	Chemicon, Darmstadt, Germany
BLBP	Rabbit polyclonal	1:10000	5 min	Chemicon, Darmstadt, Germany
DCX	Goat polyclonal	1:500	2 min	Santa Cruz, Heidelberg, Germany
GFAP	Rabbit polyclonal	1:3000	2 min	Dako, Hamburg, Germany
Iba1	Mouse monoclonal	1:1000	3 min	Wako, Japan

Primary antibody (Fluorescence)	Source	Dilution	Provider
6E10	Mouse monoclonal	1:1000	Covance, Princeton, New Jersey, United States
GFAP	Rabbit polyclonal	1:2000	Dako, Hamburg, Germany

Table 7: Primary antibodies used in immunohistochemistry of brain sections.

2.4.3. Quantification of immunolabeled cells in hippocampal sections

Quantification of immunostained nuclei or cells was performed in a series of sections (spaced 400 µm) of the dentate gyrus of the dorsal hippocampus. For Ki67 and NeuroD labelling, immunostained nuclei were counted under the microscope using the 20x objective. For Sox2, BLBP and DCX, immunostained profiles were determined and counted in a two-cell-thick region from the inner margin of the dentate granule cell layer with the help of ImageJ software (Müller et al., 2009). In addition, for each section, the length of the subgranular zone was measured using the ImageJ. Cell counts are expressed as number of immunostained cells per mm of subgranular zone.
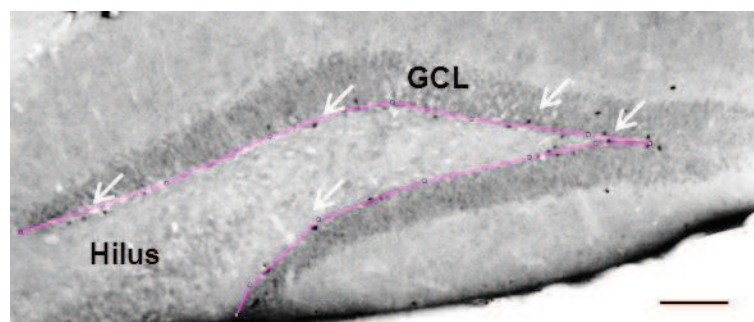
Figure 26: Photograph of a coronal section stained for Neuronal differentiation marker NeuroD. Nuclear staining (black) illustrates the presence of immunopositive cells (arrows) for NeuroD in the dentate gyrus. The length of SGZ of the dentate gyrus is measured using ImageJ. Scale bar = 100 µm. (GCL: Granular cell layer).

2.5. Determination of Aβ40 and Aβ42 concentration by ELISA

The ELISA technique (Enzyme Linked Immuno Sorbent Assay) was used for the quantitative determination of human Aβ40 and Aβ42 (Hu Aβ40 and Hu Aβ42) in the frontal cortex of Tg2576 animals. The assay recognizes both natural and synthetic forms of Hu Aβ40 and Hu Aβ42. The anti-human Aβ40 and Aβ42 antibodies used in both kits are capable of selectively detecting Aβ40 and Aβ42, respectively.

2.5.1. Sample preparation

Frontal cortex samples were obtained by cutting frozen brain hemispheres coronally at the level of the anterior commissure. Samples were placed into a small volume dounce homogenizer and weighed. Then a volume corresponding to 8x the weight of the tissue of cold solution containing guanidine-HCl (5 M) in 50 mM Tris HCl, pH 8

was added in 80 µl aliquots and the tissue was homogenized thoroughly with a hand-held pestle after each addition. The homogenates were further incubated by overhead mixing at RT for 4 hours and stored frozen at -80°C until further processing.

2.5.2. Human Aβ40 and Aβ42 ELISA

The quantification of Aβ40 and Aβ42 by ELISA was performed using the commercial kits (Invitrogen, Human Aβ40 ELISA kit Cat#KHB3481 and Human Aβ42 ELISA kit Cat#KHB3544) according to the manufacturer's instructions.

Samples prepared in guanidine-buffer (see above) were first diluted 30x with solution B (2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8.1 mM Na2HPO$_4$, 5 % BSA, 0.03 % Tween-20) supplemented with a protease inhibitor cocktail (AEBSF at 1.04 mM, aprotinin at 0.80 µM, bestatin at 40 µM, E-64 at 14 µM, leupeptin at 20 µM and pepstatin A at 15 µM) before they were centrifuged at 16000 g for 20 min at 4°C. The supernatant was diluted again 5x and 10x with Standard diluent buffer containing protease inhibitors as above.

Human Aβ40 standard was reconstituted to 100 ng/ml and Human Aβ42 standard was reconstituted to 1 µg/ml with Standard Reconstitution Buffer (55 mM bicarbonate, pH 9). This was used to prepare a standard curve in Standard diluent buffer with protease inhibitors (0, 7.81, 15.63, 31.25, 62.5, 125, 250, 500 pg/ml of Hu Aβ40 standard and 0, 10, 50, 100, 200 and 400 pg/ml of Hu Aβ42 standard).

50 µl of Aβ peptide standards, controls and diluted samples were added to duplicate wells of a 96-well plate coated with a monoclonal antibody specific for the NH2-terminus of Hu Aβ40 for quantification of human Aβ40 and with a monoclonal antibody specific for the NH2-terminus of Hu Aβ42 for quantification of human Aβ42 followed by 50 µl of Hu Aβ40 detection antibody (Rabbit anti-Hu Aβ40) or Aβ42 detection antibody (Rabbit anti-Hu Aβ42), respectively. The plate was incubated overnight at 4°C.

Bound detection antibody was detected with a horseradish peroxidase-labeled anti-rabbit antibody (anti-rabbit IgG HRP). After washing the plate 4 times with Working Wash Buffer, 100 µl of stabilized chromogen (tetramethylbenzidine TMB) was added to each well and incubated for 30 min at RT in the dark. The reaction was stopped by addition 100 µl of stop solution.

Absorbance of each well was read at 450 nm having blanked the plate reader against a chromogen blank composed of 100 µl each of Stabilized Chromogen and Stop Solution. Hu Aβ40 and Aβ42 concentrations for unknown samples and controls were determined using values from their respective standard curve.

Aliquots of the brain lysates were used in parallel for determining protein concentration by a BCA-protein assay using BSA as standard (Pierce BCA Protein Assay Kit).

2.6. Statistical analysis

All statistical analyses were performed using GraphPad Prism (version 6) using one-way ANOVA Kruskal-Wallis test, followed by Dunnet´s multiple comparison test for cohorts with 5 groups of animals. Significance is indicated as follows: * $p < 0.05$, ** $p < 0.01$.

RESULTS

Allopregnanolone (AP) promotes proliferation in rodent hippocampal neural progenitor cells and human neural stem cells from the cerebral cortex (Wang et al., 2005) and stimulates neurogenesis in the 3xTgAD Alzheimer´s disease mouse model (Wang et al., 2010). aNSCs play an important role in adult neurogenesis and have been considered instrumental for therapeutic approaches in neurodegenerative diseases. Therefore, we aimed to investigate the effects of AP analogs on proliferation in aNSC cultures and their capacity in enhancing neuronal differentiation.

1. Proliferation-stimulating effects of AP analogs in aNSCs

1.1. Growth of aNSC neurospheres in the presence of neurosteroids

To measure potential proliferation-promoting properties of allopregnanolone (AP) and the two analogs O-allyl-epiAP and O-allyl-AP in aNSC neurosphere cultures, we used the MTT assay, a well-established colorimetric assay for assessing the number of viable cells. It relies on the spectrophotometric estimation of cell numbers as a function of mitochondrial activity in living cells. Freshly dissociated neurospheres cells were grown for 5 days under standard growth factor conditions, i.e. in the presence of EGF and bFGF, with varying concentrations of the neurosteroids (ranging from 0 to 10000 nM) and then the cell number was analyzed.

None of the three tested compounds showed a stimulatory effect on neurosphere growth (Fig. 27). The number of viable cells as assessed by the MTT assay was not significantly different from controls at all concentrations tested. Only at the highest concentration (10000 nM) O-allyl-epiAP and O-allyl-AP resulted in a decrease in cell numbers (Fig. 27B,C), which was interpreted as a toxic effect.
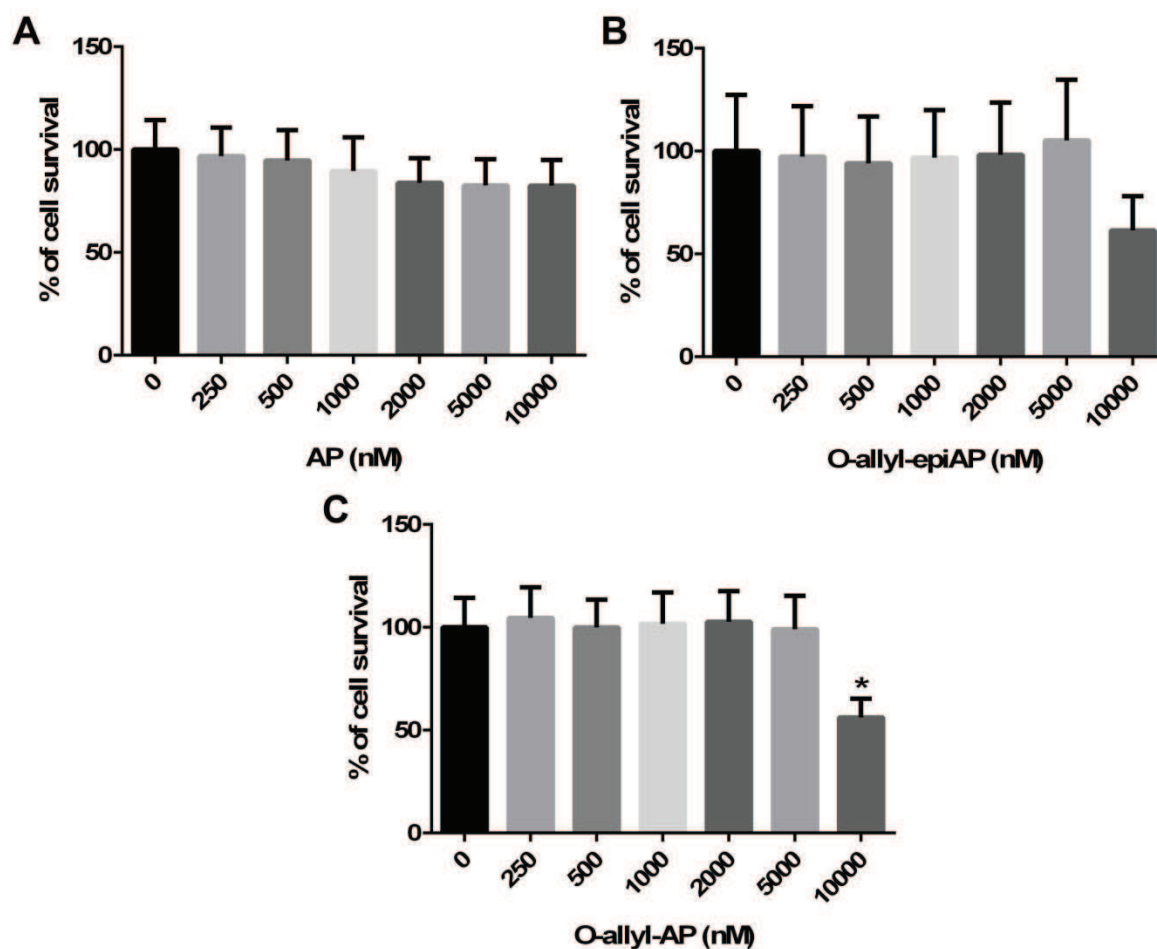
Figure 27: Growth of neurospheres cultures in the presence of neurosteroids: AP (A), O-allyl-epiAP (B) and O-allyl-AP (C). The MTT assay was used to determine relative cell numbers after culturing dissociated neurospheres cultures for 5 days in the presence of different concentrations of the compounds. No stimulatory effect of AP, O-allyl-epiAP or O-allyl-AP could be detected. Values shown represent the mean $\pm$ SEM of 4-5 independent experiments and are expressed relative to values obtained from control (vehicle-treated) cultures, which were set to 100 %. *$p < 0.05$.

Previous studies analyzing the dose-dependence of AP on the growth of SH-SY5Y neuroblastoma cells had shown a stimulatory effect on cell viability, which was maximal at 500 nM (Wendt, 2014). Moreover, AP had been reported to stimulate proliferation (determined by measuring BrdU incorporation) in a dose-dependent manner with a maximal effect at 500 nM in rat embryonic hippocampal neural progenitor cells and a maximal effect between 100 and 250 nM in human neural stem cells (Wang et al., 2005). The observation that neither of the compounds tested had a positive effect of proliferation could have a number of explications:

- As the experiments were performed in the presence of high concentrations of the mitogens EGF and bFGF, additional stimulatory effects of the compounds could have been masked, as the proliferative capacity of the cells was already maximal; Wang and colleagues (2005) performed their studies in the absence of mitogens.

- The MTT-assay as employed in this study is not sensitive enough to resolve small effects on proliferation; in fact AP in the above mentioned study stimulated proliferation only by 27 % (Wang et al., 2005).

- As nothing is known about the biological half-life or stability of the compounds, adding the compounds only once at the beginning of a five day incubation period, may not have led to a sufficiently high concentration to exert an effect.

We therefore chose to use the BrdU or EdU incorporation and reduced mitogen concentrations to determine the proliferative activity of AP and its analogs in all subsequent experiments.

1.2. Effects of AP analogs on aNSCs proliferation: EdU incorporation

1.2.1. 12 oxo-AP and O-allyl-AP stimulate proliferation of aNSCs

Based on the arguments above the experimental paradigm was modified as follows:

- EdU incorporation in combination with microscopic evaluation was used in order to increase sensitivity and enable phenotypic characterization by immunocytochemistry.

- The concentration of mitogens was reduced, in pilot experiments it was determined that reducing EGF to 1 ng/ml and bFGF to 0.5 ng/ml maintained normal proliferation for at least 3 days.

- Treatment with the compounds was only for the last 24 hours, before EdU was added and cells were fixed.

Dissociated neurosphere cells were grown on poly-D-Lysine-coated coverslips in "low growth factor conditions" for 3 days. All compounds (AP, 12 oxo-epiAP, O-allyl-epiAP, 12 oxo-AP and O-allyl-AP) were added at 500 nM for 24 hours, as none of them has shown a toxic effect at this concentration. Cells were exposed to 1 µM of

EdU for 2 h before fixation. This labeling paradigm is estimated to label approximately 20 % of the dividing population of cells based on a cell cycle length of about 10 hours (Chakrapani, 2011). Cell counting showed that 15.2 ± 1.7 % of all cells incorporated the S-phase marker under control conditions. The percentage of proliferating cells in untreated condition was arbitrarily set to 100 %. Treatment with the two analogs 12 oxo-epiAP and O-allyl-epiAP did not influence proliferation, whereas 12 oxo-AP and O-allyl-AP induced a significant increase in the number of EdU-positive cells by 15.4 % and 21 %, respectively (Fig. 28). Different from what has been described for hippocampal neuronal progenitors (Wang et al., 2005), the natural neurosteroid AP showed no proliferation-promoting effect in this culture system (but see below for experiments with hippocampal cultures). It is particularly interesting that the two 3α analogs (12 oxo-AP and O-allyl-AP) were active and obviously more efficient than AP which has the same conformation at the carbon 3-position.
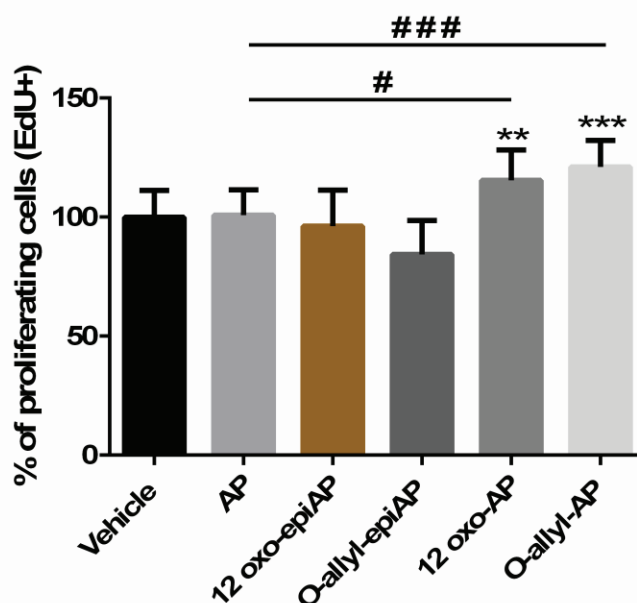
Figure 28: Proliferation-promoting effects of neurosteroids on aNSCs. The incorporation of the S-phase marker, EdU, was used to determine the percentage of proliferating cells. AP, 12 oxo-epiAP and O-allyl-epiAP did not affect proliferation, whereas 12 oxo-AP and O-allyl-AP increased the number of EdU+ cells. The percentage of proliferating cells in control cultures was arbitrarily set to 100 %. Each value represents the mean ± SEM of six independent experiments for AP, 12 oxo-AP and O-allyl-AP and three independent experiments for 12 oxo-AP and O-allyl-AP. [#]$p<0.05$, **$p<0.01$ and *** [###]$p<0.001$.

1.2.2. Phenotype of proliferating cells

Under growth factor conditions, the majority of cells in aNSC cultures are progenitor cells (> 90 %) but neuronal precursors/immature neurons and astrocytes can also be shown to be present. Therefore, it was of interest to identify the phenotype of the cells responding to the neurosteroids.

For this, cultures were analyzed after double labeling for EdU-incorporation and immunoreactivity against specific cell markers by using an antibody against the intermediate filament protein nestin which is expressed by neural progenitor cells (Doetsch et al., 1997; Romero-Ramos et al., 2002), an antibody against glial fibrillary acidic protein (GFAP) which is a marker for astrocytes and an antibody against the class III beta-tubulin protein (Tuj-1) which is expressed in immature post-mitotic neurons. Cells expressing nestin were determined to represent 97 ± 0.5 % of all cells. Quantification of the cells double labeled for EdU and nestin showed that 14.2 % of all cells were proliferating cells (EdU+, Nestin+) (Fig. 29A). Comparison of this value with the population of all EdU-positive cells (15.2 %) reveals that the great majority of the proliferating cells represented nestin-positive progenitors. Accordingly, the effect of the neurosteroids on this cell type reflected changes seen for the total population of EdU-incorporating cells. Treatment with 12 oxo-AP and O-allyl-AP increased the number of these cells by 10.3 % and 25.5 %, respectively, with a significant increase only for O-allyl-AP (Fig. 29A). Very few cells were double labeled for EdU and the astrocyte marker GFAP, 1.21 ± 0.34 %, under the same experimental conditions. Treatment with 12 oxo-AP and O-allyl-AP increased the number of these cells by 21.7 % and 12.5 %, respectively, but the increase was not statistically significant (Fig. 29B). Similarly, few cells were double labeled for EdU and the immature neuronal marker Tuj-1, 4.74 ± 1.39 %. The percentage of these cells had risen by 14.9 % and 16.7 % in the presence of 12 oxo-AP and O-allyl-AP respectively (not statistically significant; Fig. 29C). Although the effects of the analogs on GFAP- and Tuj-1-positive cells were not statistically significant, the results indicated that the neurosteroids did not act specifically on one of the cell types analyzed, but had a general proliferation-promoting activity. This is confirmed when the phenotypic composition of the population of EdU-incorporating cells is compared under the different culture conditions (Fig. 29D). There is no change in the fraction of the different cell types. This observation suggests that the AP analogs did not influence the cell fate of the aNSCs.
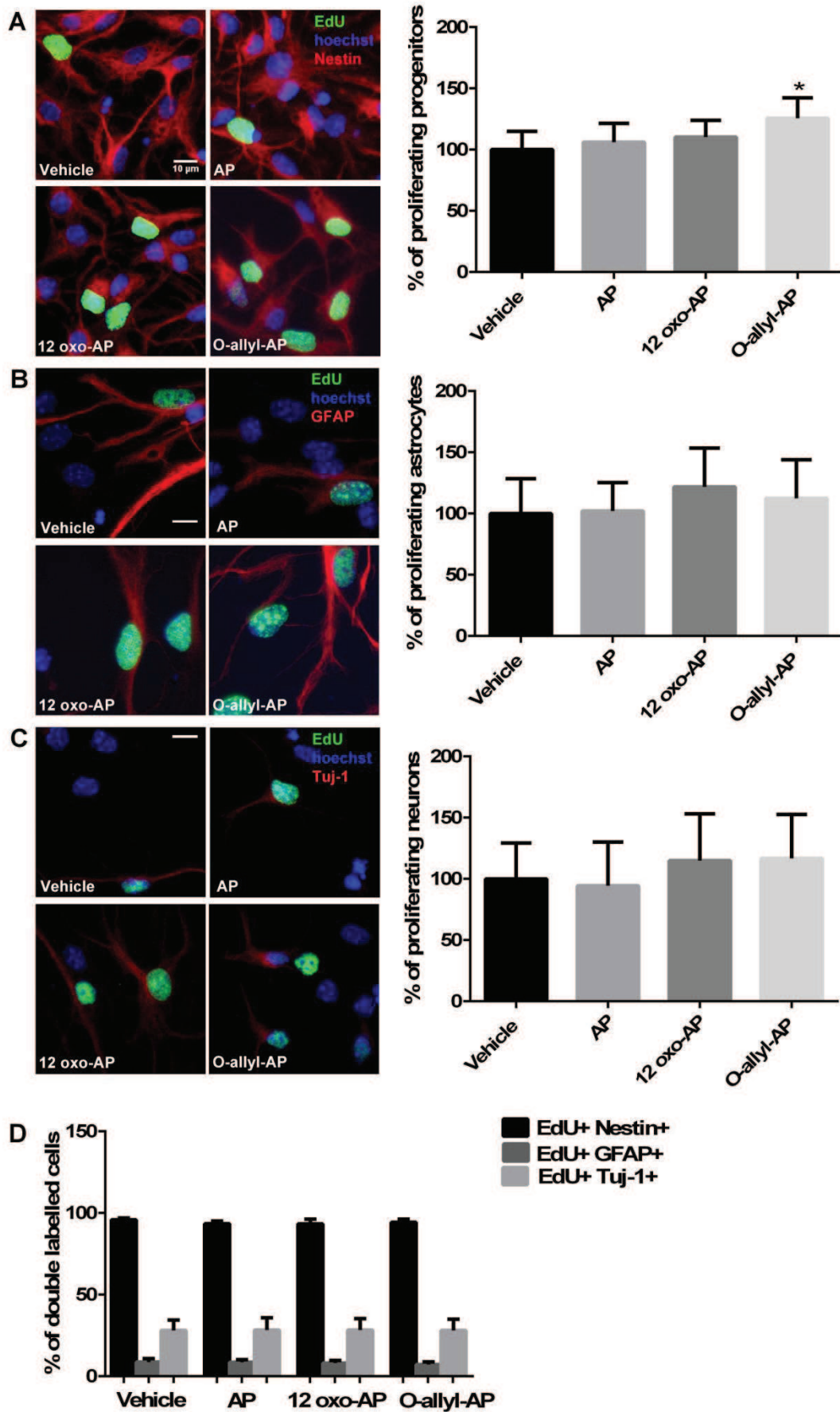
Figure 29: Phenotype of proliferating cells following treatment with AP analogs.
Proliferating cells (EdU+; green) were double-labeled with lineage markers: progenitor cell marker nestin (A), astrocyte marker GFAP (B) and immature neuronal marker Tuj-1 (C) (all in red). Hoechst nuclear staining is shown in blue; scale bar = 10 µm. (A-C) The percentage of double labeled cells in controls was arbitrarily set to 100 %. (D) The phenotypic composition of EdU-incorporating (proliferating) cells is not changed by treatment with neurosteroids. Each value represents the mean ± SEM of six independent experiments. *$p<0.05$.

1.2.3. Proliferation-promoting effects of AP analogs are mediated via L-type calcium channels

Stimulation of cell proliferation in hippocampal progenitor cells has been demonstrated to involve the activation of voltage-dependent L-type calcium channels (Wang et al., 2005; Wang and Brinton, 2008; Brinton, 2013). To investigate if the effects of the analogs on aNSCs also involve this type of calcium channels, neurosteroid treatment was performed in the presence of the inhibitor nifedipine (5 µM). Nifedipine is a dihydropyridine calcium channel blocker that primarily blocks L-type calcium channels. Application of nifedipine to control cultures did not influence their proliferation (Fig. 30).

However, when nifedipine was added together with 12 oxo-AP or O-allyl-AP, the increase in proliferation was completely blocked showing that the action of the analogs on cell division involved calcium signaling via L-type calcium channels.
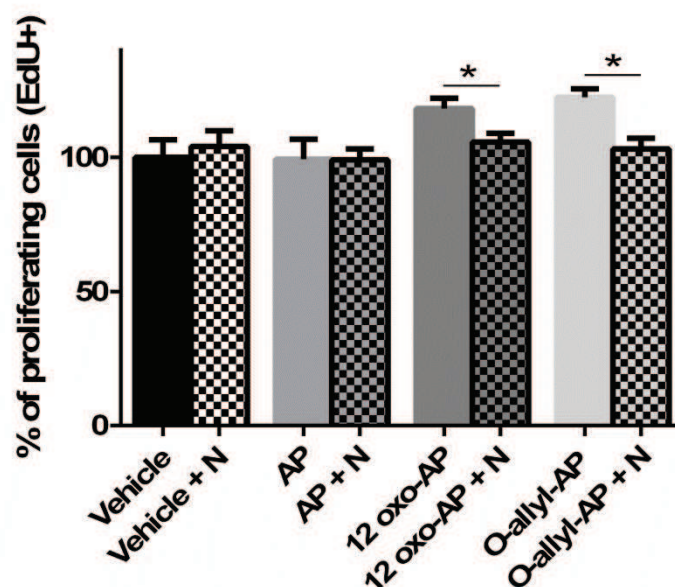
Figure 30: Proliferation promoting effects of analogs on aNSCs are mediated via L-type calcium channels. Dissociated neurospheres were treated with AP analogs in the presence or absence of Nifedipine (5 µM) for 24 h. Counting EdU+ cells shows that the proliferation promoting effect of AP analgs was completely blocked in the presence of nifedipine. The percentage of EdU+ cells in control condition was arbitrarily set to 100 %. Each value represents the mean ± SEM of three independent experiments. *$p < 0.05$.

1.2.4. Effects of AP analogs on Tuj-1 and GFAP expression in aNSCs

The immunocytochemical analysis of the phenotype of proliferating cells did not provide evidence that neurosteroid treatment of aNSC cultures under growth conditions would influence cell fate and differentiation of the cells (Fig. 29). Under these conditions, the majority of cells are progenitor cells. Few cells are positive for the neuronal marker Tuj-1 or the astroglial marker GFAP. Even if analogs would exert an effect on the neuronal or glial lineage, it would probably be difficult to detect them on the immunocytochemical level. Therefore, we analyzed the effects of neurosteroids on expression of Tuj-1 and GFAP mRNA. Expression levels of the transcripts encoding Tuj-1 and GFAP were determined by reverse transcription quantitative real-time PCR in aNSC cultures treated with AP, 12 oxo-AP and O-allyl-AP (500 nM) for 3 days. All expression levels values were normalized to that of the mRNA for the ribosomal protein S12. O-allyl-AP significantly stimulated expression of Tuj-1 by 134 % (Fig 31A), whereas the expression of the astroglial marker GFAP was unchanged (Fig. 31B). No significant changes were observed after treatment with 12

oxo-AP. These results suggest that O-allyl-AP can influence neuronal cell fate decision in progenitors and/or differentiation of neuronal precursors.
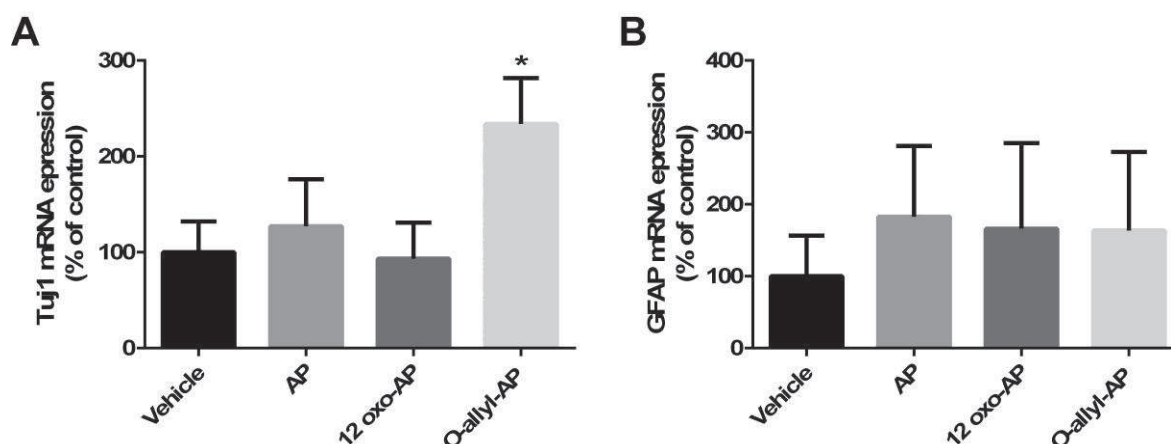
Figure 31: Neurosteroid effects on Tuj-1 (A) and GFAP (B) mRNA expression levels in aNSCs. Cells were treated with 500 nM AP, 12 oxo-AP and O-allyl-AP for 3 days before RT-qPCR analysis. Tuj-1 and GFAP mRNA expression levels were normalized to expression levels of the housekeeping gene S12 (ribosomal protein S12). mRNA expression levels under control conditions are arbitrarily set to 100%. Each value represents the mean ± SEM of five independent experiments for Tuj-1 and GFAP. *$p<0.05$.

2. Stimulatory effect of O-allyl-AP on neuronal differentiation in aNSC cultures

As already mentioned above, aNSC cultures are almost completely composed of proliferating, nestin-expressing progenitors and contain only few cells (< 5%) expressing markers for differentiating neurons and glial cells. By depriving the cultures of the mitogenic growth factors and growing them on an adhesive substrate they can be induced to stop proliferation and enter the differentiation phase. Their progeny will then differentiate into the three main cell types of the CNS: neurons, oligodendrocytes and, preferentially astrocytes (Deleyrolle and Reynolds, 2009).

To investigate if AP analogs can influence the differentiation process, freshly dissociated neurosphere cells were switched to differentiation conditions and cultured for 3 days in the presence or absence of neurosteroids (500 nM). The number of differentiating neurons was determined by counting cells stained for the neuronal marker doublecortin (DCX+ cells) (Fig. 32A). Cell counts showed that 7.3 ± 0.6 % of

all cells are DCX-positive in the control cultures. Treatment with AP or the analogs had no effect on the number of neurons in these cultures (Fig. 32B).
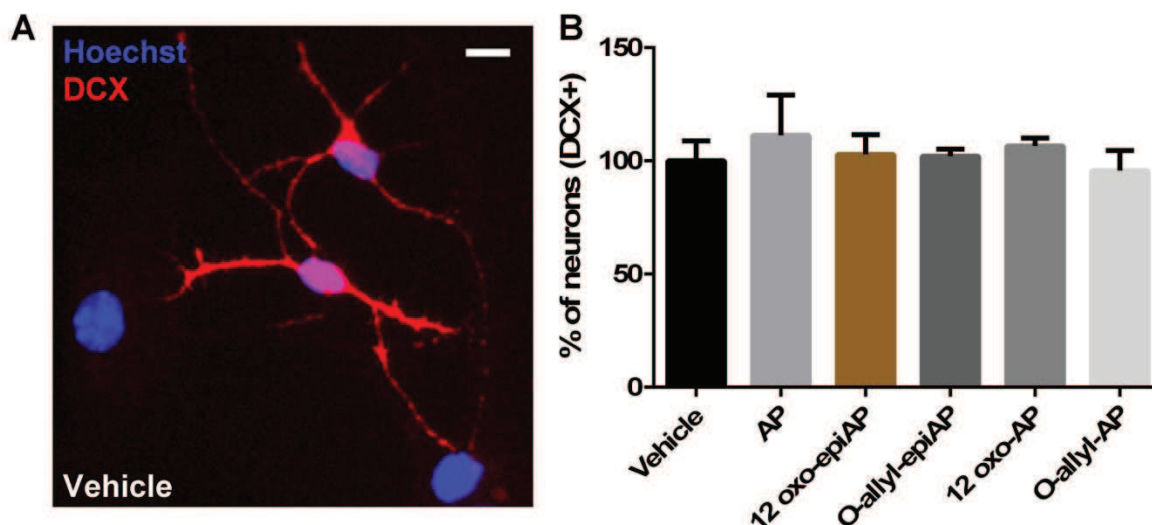
Figure 32: Effect of neurosteroids on neuronal differentiation in dissociated aNSC cultures. (A) Immunofluorescence staining of differentiated adult neural stem cell cultures immunolabeled for the neuronal marker doublecortin (DCX, red). Hoechst nuclear staining shown in blue; scale bar = 10 μm. (B) Quantification of the number of DCX-positive neurons in cultures treated with neurosteroids under differentiation conditions (i.e. without mitogenic growth factors) for 3 days. None of the neurosteroids influenced neuronal differentiation. Each value represents the mean ± SEM of three independent experiments.

The proliferation promoting effects of 12 oxo-AP and O-allyl-AP on aNSCs as well as the increase in the expression of Tuj-1 mRNA levels with O-allyl-AP treatment (Fig. 28,31A) raised the question, if the increase in progenitor cell proliferation in response to neurosteroids results in enhanced production of neurons. As the direct treatment of neurosteroids during differentiation did not influence the generation of neurons by aNSCs, we examined, if the treatment of aNSCs with the analogs during the growth phase would influence the production of DCX-positive neurons and GFAP-positive astrocytes, when the cultures were subsequently switched to differentiation conditions for 5 days (Fig. 33A).

Therefore, dissociated neurospheres were treated with 12 oxo-AP and O-allyl-AP for 5 days during the growth phase, then the neurospheres were dissociated and the cultures were subsequently switched to differentiation conditions for 5 days with or without the additional presence of the analogs. Of the two analogs that had been found to promote aNSCs proliferation (Fig. 28), only O-allyl-AP lead to an increased number of DCX-positive neurons by 37 % (Fig. 33A,B). The stimulatory effect

increased to 65 %, when the treatment was continued during the differentiation phase. The analog 12 oxo-AP had no effect independent of the treatment protocol (Fig. 33B). The number of cells differentiating into GFAP-expressing astrocytes was unaltered in the presence of the analogs (Fig. 33A,C). These results indicate that the O-allyl-AP analog can specifically promote either the choice of the neuronal cell fate or the differentiation of neurons in a population of proliferating progenitors.
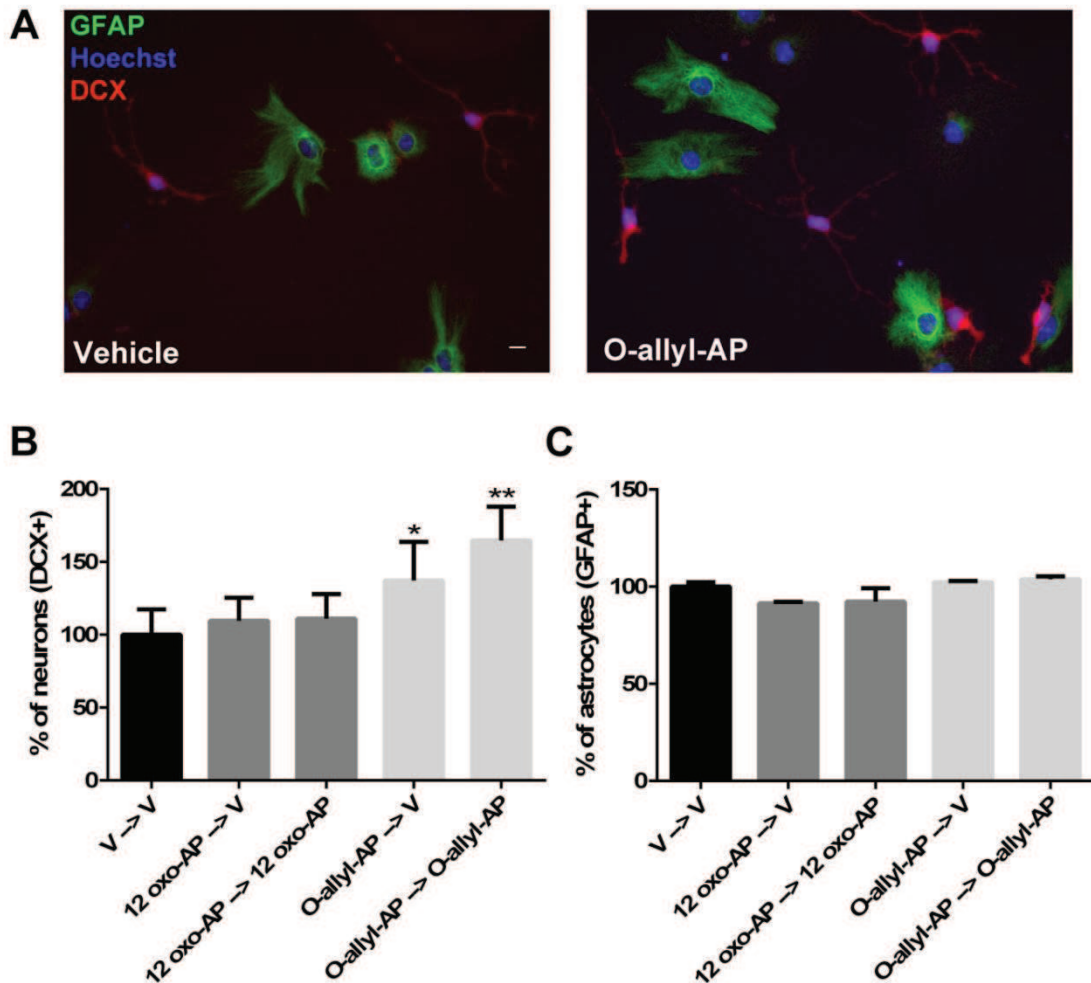
Figure 33: Stimulatory effect of O-allyl-AP on neuronal differentiation. (A) Immunofluorescence staining of differentiated aNSC cultures immunolabeled for the neuronal marker DCX (red) and the astrocyte marker GFAP (green). Hoechst nuclear staining shown in blue; scale bar = 10 µm. Quantification of the number of DCX-positive neurons (B) and GFAP-positive astrocytes (C). Cultures were first grown under proliferation conditions (with growth factors) in the presence or absence of the neurosteroids and then switched ($\rightarrow$) to differentiation conditions with or without neurosteroids. Pretreatment with O-allyl-AP promotes neuronal (B) but not glial (C) differentiation. The percentage of neurons and the percentage of astrocytes in untreated condition were arbitrarily set to 100 %. Each value represents the mean ± SEM of three independent experiments. *p<0.05 and **p<0.01.

3. Protective effects of AP analogs against beta amyloid peptide 1-42 toxicity in aNSCs

It has been shown previously, that AP not only stimulates proliferation of neuronal progenitors but also promotes their survival in models of AD (Wang et al., 2010; Singh et al., 2012). To study if analogs of AP also have neuroprotective properties, we studied their protective potential against apoptotic cell death induced by beta amyloid peptide 1-42 (Aβ42) in aNSC cultures. The production of an excess of this peptide is regarded as one of the key triggers of neuronal cell death in AD.

3.1. Effect of monomeric Aβ42 on aNSCs viability

The amyloidogenic peptide Aβ42 is a cleavage product of the membrane protein APP (amyloid precursor protein) and has been shown to exert toxic effects in a variety of cellular cultures (LaFerla et al., 1995; Li et al., 1996; Cotman, 1998; Eckert et al., 2001). In a first set of experiments, the susceptibility of aNSC cultures to the toxic effect of Aβ42 was examined. For this purpose, dissociated neurosphere cultures were incubated for 3 days with Aβ42 in different concentrations ranging from 0 to 10 µg/ml (Fig. 34). The number of viable cells was evaluated using the MTT assay. Aβ42 decreased aNSCs viability in a dose-dependent manner. The minimal concentration of Aβ42 leading to a significant reduction in cell number was found to be 1.25 µg/ml with a reduction of 35.2 % in cell survival (Fig. 34). The highest toxicity was observed at 5-10 µg/ml with a reduction of cell survival by more than 60 %. The EC50 value was around 2.5 µg/ml and this concentration was used to assess the ability of neurosteroids to protect against Aβ42-evoked toxicity.
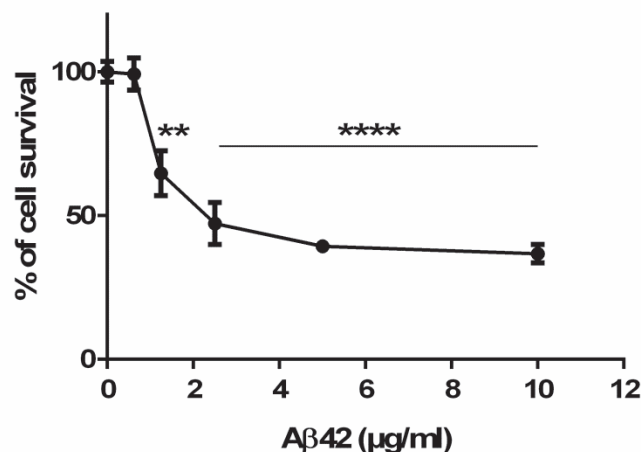
Figure 34: Dose-dependent toxic effect of Aβ42. Dissociated neurospheres were treated for 3 days with different concentrations of monomeric Aβ42 peptide. The MTT assay was used to determine cell viability. Control values (absence of Aβ42) were arbitrarily set to 100 %. Each value represents the mean ± SEM of three independent experiments. **p<0.01 and ****p<0.0001. (Shared data with Kraft S.).

3.2. Aβ42 induces apoptosis in aNSC cultures

It has been shown that Aβ42 induces cell death by activating apoptosis pathways in several cell types including neurons (Jordán et al., 1997; Troy et al., 2000; Eckert et al., 2001).

To test if reduced viability of aNSCs in the presence of Aβ42 is caused by apoptotic cell death, cells were treated with Aβ42 (2.5 µg/ml or 5 µg/ml) for 24 hours and apoptosis-related caspase-3/7 activity was determined by a fluorescence assay (Fig. 35A). Counting cells labeled for activated caspase-3/7 showed that Aβ42 treatment induced a significant increase in the number of apoptotic cells by 38 % and 50 %, respectively, with the two Aβ42 concentrations used (Fig. 35B). The toxic effect of Aβ42 was confirmed by fluorometric quantification of the fluorescence signal. Treatment with 1.25 and 2.5 µg/ml Aβ42 for 2 days resulted in a signal that was 66 % and 52 % higher, respectively, as compared to control cultures (Fig. 35C).

These results demonstrated that the aNSC cultures contain a population of cells which is highly vulnerable to acute exposure to Aβ42 and, therefore, can be used to test for the protective potential of the analogs.
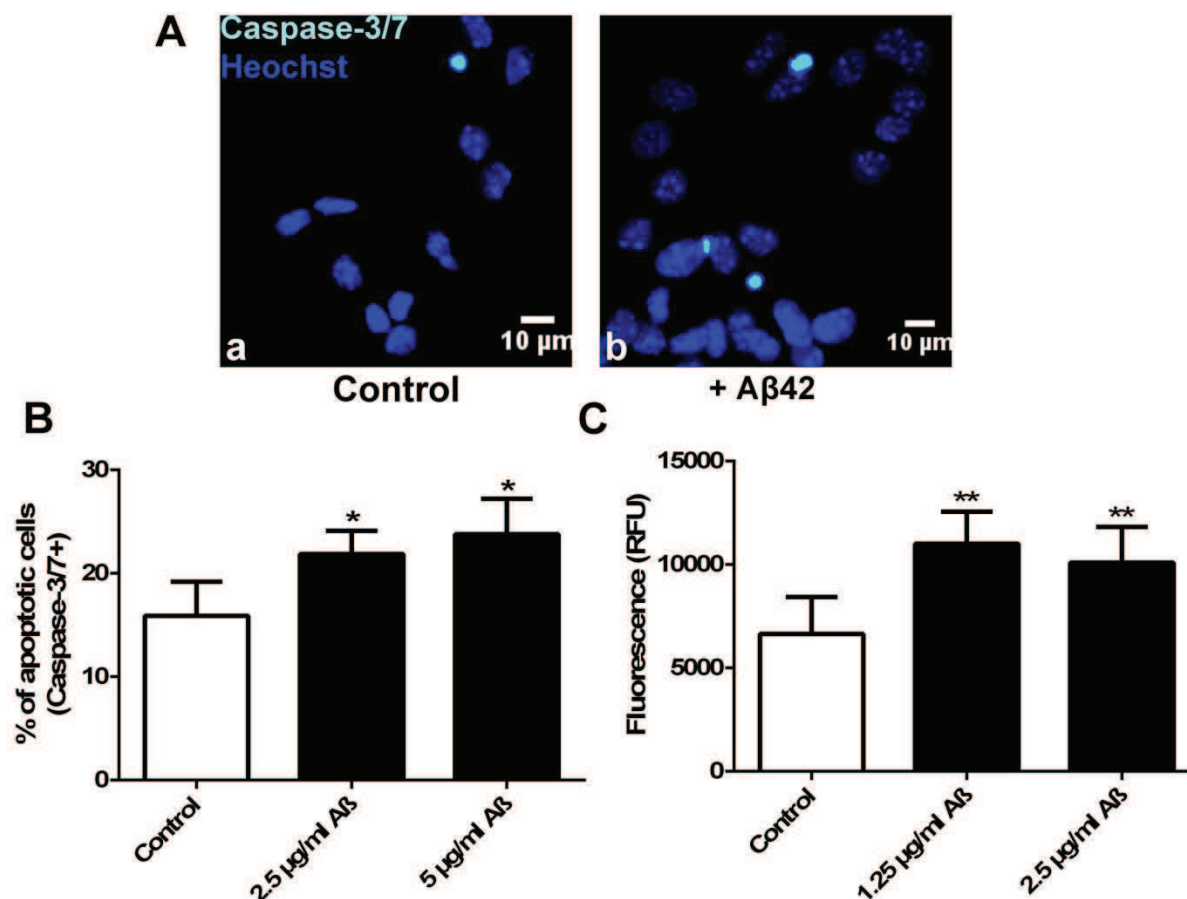
Figure 35: Induction of apoptosis by Aβ42 in aNSC cultures. (A) Photomicrographs of dissociated neurosphere cultures showing fluorescent-labeling of active caspase-3/7 (green) in control cultures (a) and in the presence of Aβ42 (b) for 24 hours. Hoechst nuclear staining shown in blue; scale bar = 10 µm. (B) Quantification of apoptotic cells (Caspase-3/7-positive cells) by cell counting. Dissociated neurospheres of aNSCs were treated with two different concentrations of amyloid beta peptide Aβ42. (C) Quantification of apoptosis by active caspase-3/7 fluorescence measurement. Each value represents the mean ± SEM of three to eight independent experiments. *p<0.05 and **p<0.01.

3.3. Protective effects of AP analogs against Aβ42-induced toxicity

The ability of the analogs to protect aNSCs against Aβ42-evoked toxicity was investigated in cultures treated with Aβ42 at a concentration of 1.25 µg/ml. Two different experimental strategies were used: a "prophylactic" strategy and a "corrective" strategy.

In the first (prophylactic) strategy, cells were pretreated with AP or its analogs (500 nM) for 1 h before addition Aβ42. Then incubation was continued for 2 days (Fig. 23A). Caspase-3/7 activity was determined by fluorometric measurement. Under

these conditions, Aβ42-induced cell death was significantly reduced (by 50 %) by AP (Fig. 23A). The two analogs O-allyl-epiAP and O-allyl-AP protected aNSCs even more efficiently against Aβ42 toxicity, reducing apoptotic activity to background levels (Fig. 23A). 12 oxo-epiAP and 12 oxo-AP were ineffective (not shown).

In the second (corrective) strategy, cells were first incubated with 1.25 μg/ml Aβ42 for 16 hours, before the application of the neurosteroids (500 nM). After two additional days of incubation, cultures were evaluated for caspase-3/7 activity (Fig. 23B). As in the prophylactic treatment, O-allyl-epiAP and O-allyl-AP completely abolished the toxic effect of Aβ42 whereas AP had no significant effect.

These results show that in both treatment regimens, the two analogs O-allyl-epiAP and O-allyl-AP were more efficient than the natural compound AP and completely prevented Aβ42-evoked apoptosis of aNSCs. With respect to the chemical structure of the compounds, it is interesting to note that different from what had been found in the proliferation studies, both 3α-derivatives (AP, O-allyl-AP) and 3β-derivatives (O-allyl-epiAP) showed protective activity. It is unclear whether the presence of the keto-group at position 12 in 12 oxo-epiAP and 12 oxo-AP leads to a loss of the neuroprotective activity or whether the etherification of the 3-OH group in AP to yield the O-allyl forms results in enhanced neuroprotective activity as compared to AP.
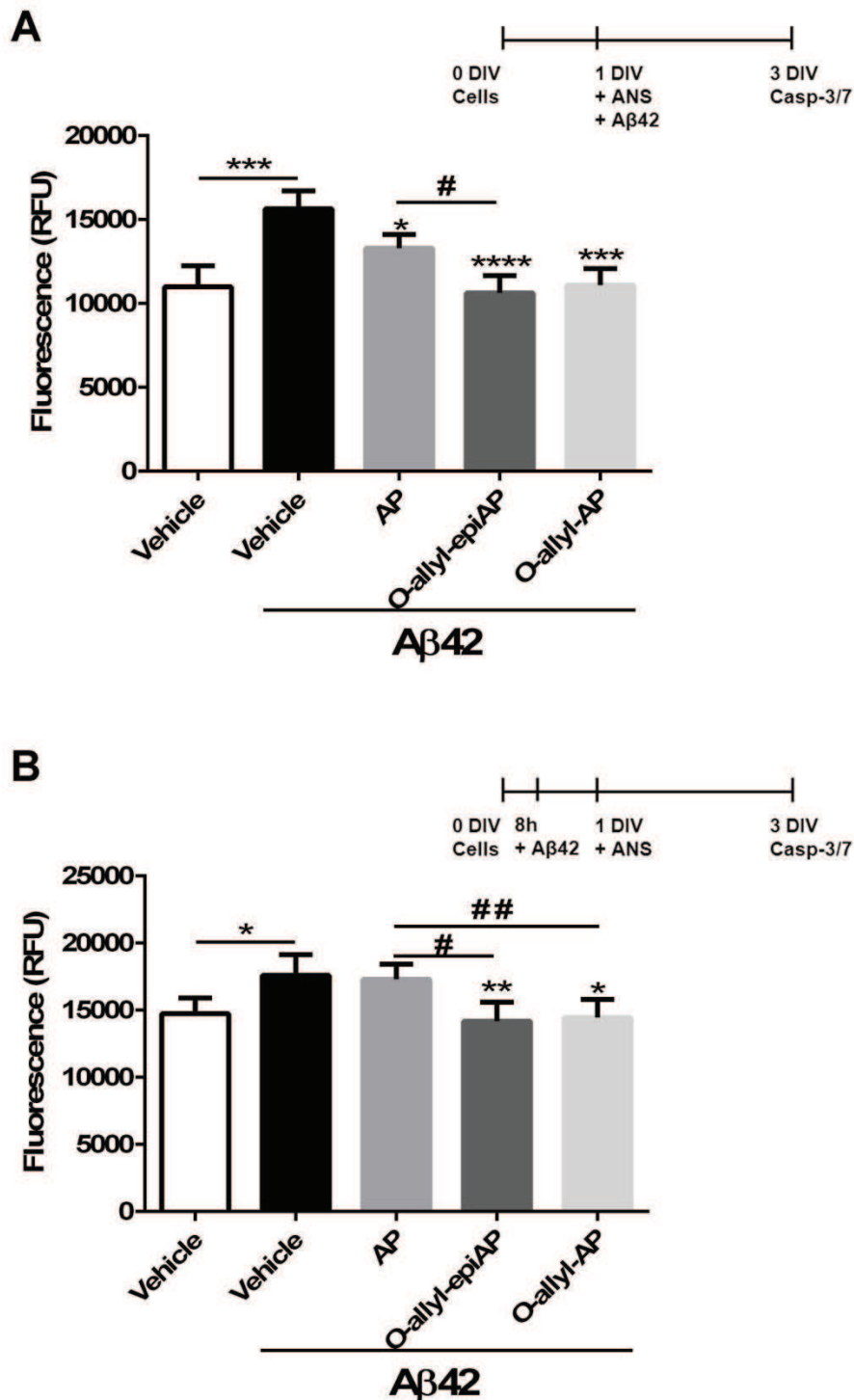
Figure 36: Anti-apoptotic effects of AP analogs in Aβ42-treated dissociated neurosphere cells. (A) Prophylactic strategy: pretreatment with 500 nM of neurosteroids 1 h before adding 1.25 µg/ml Aβ42. Afterwards cells were kept for two days in culture. Each value represents the mean ± SEM of six independent experiments. (B) Corrective strategy: neurosteroids were added 16 h after Aβ42 administration and cultures were grown for two additional days. Each value represents the mean ± SEM of five independent experiments. *[#]$p<0.05$, **[##]$p<0.01$, ***$p<0.001$ and ****$p<0.0001$.

To confirm the anti-apoptotic function of AP analogs, which was suggested by their protective effects against Aβ42 toxicity, we explored the mRNA levels of two key apoptosis modulators: Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic). The Bax/Bcl-2 ratio is regarded as an indicator of the susceptibility of a cell to apoptosis (Jung et al., 2009; Zhang et al., 2009). The experiments were performed in aNSC cultures grown for 3 days under "low growth factor conditions" in the absence or presence of neurosteroids (500 nM). Levels of mRNAs for Bax and Bcl-2 were determined by qRT-PCR and the ratio was calculated. Compared to vehicle controls, the presence of AP, O-allyl-epiAP and O-allyl-AP resulted in a decrease of the Bax/Bcl-2 ratio by 32.7 %, 53 % and 37.9 %, respectively (Fig. 37). However, only the decrease induced by O-allyl-epiAP reached statistical significance.
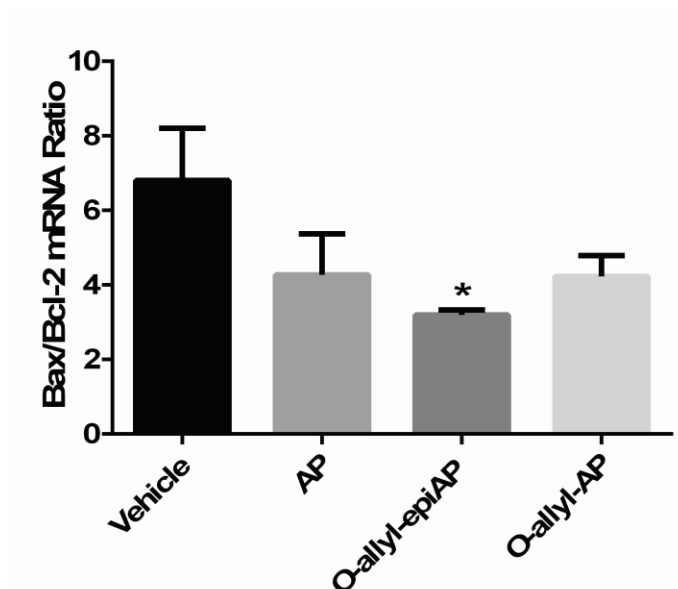
Figure 37: Neurosteroids reduce the Bax/Bcl-2 ratio in aNSC cultures. Cells were treated with AP, O-allyl-epiAP and O-allyl-AP (500 nM) for 3 days before qRT-PCR analysis. Each value represents the mean ± SEM of five independent experiments. *$p<0.05$.

3.4. Mechanisms of action of the protective effect of AP analogs against Aβ42-induced toxicity

As already mentioned in the introduction, the two neurosteroids progesterone and AP exert their neuroprotective effects using different mechanisms of action (Guennoun et al., 2015). AP acts via non genomic mechanisms as a potent allosteric modulator of $GABA_A$ receptors but does not bind to the intracellular progesterone receptor because of its hydroxyl group at C3 (Belelli and Lambert, 2005). However, AP can activate gene transcription via progesterone receptors when it is converted back to 5α-DHP by 3α-HSD. Therefore we decided to investigate a potential role of (i) $GABA_A$ receptors ($GABA_A$Rs), (ii) intracellular progesterone receptors and (iii) conversion back to 5α-DHP in AP analogs-induced neuroprotection against Aβ42 toxicity in aNSCs.

First, we examined the influence of the $GABA_A$R competitive antagonist bicuculline on the anti-apoptotic effect of AP analogs. In the presence of bicuculline (10 µM; Fig. 38A), a potent competitive antagonist at $GABA_A$Rs, the protective effect of O-allyl-epiAP and O-allyl-AP was not affected. This result suggests that $GABA_A$ receptors are not involved in mediating the protective effect of the AP analogs in aNSCs.

To test if allyl substitution at C3 would enable O-allyl-epiAP and O-allyl-AP to bind to intracellular progesterone receptors, we stimulated cells with the neurosteroids in the presence mifepristone (20 nM), an antiprogestin that acts as a competitive antagonist at intracellular progesterone receptors. The protective effect of both, O-allyl-epiAP and O-allyl-AP was maintained in the presence of mifepristone (Fig. 38B). This indicates that classical progesterone receptors do not mediate the protective effect of these analogs.

Finally, the conversion back to 5α-DHP was blocked by using medroxyprogesterone (Provera), an antagonist of 3α-hydroxysteroid oxidoreductase (3α-HSD). Provera did not inhibit the neuroprotective effect of AP analogs as shown in Fig. 38C, demonstrating that conversion to the 5α-forms of the analogs does not play a role.
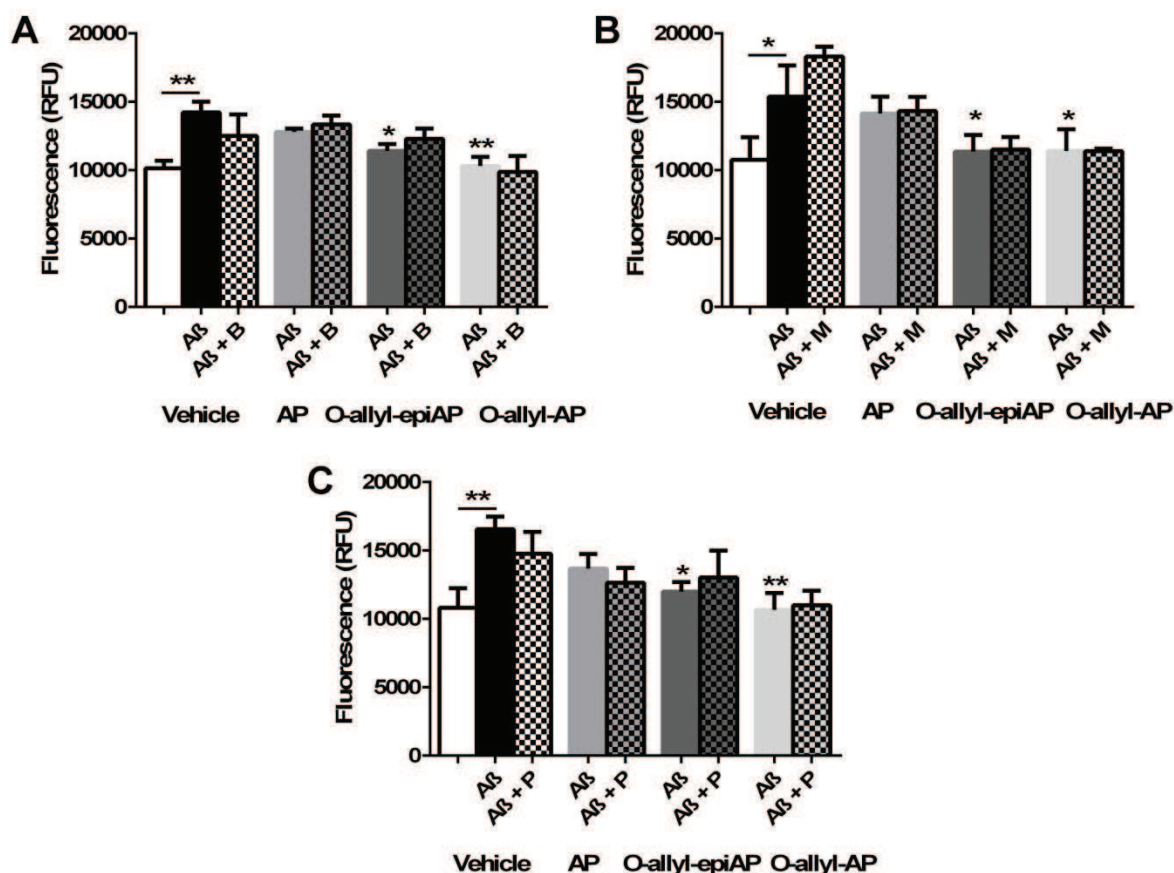
Figure 38: Neuroprotective activity of AP analogs is not mediated via GABA$_A$Rs (A), intracellular progesterone receptors (B) or conversion back to 5α-forms of the neurosteroids (C). Cells were incubated with 10 µM B (bicuculline; A), 20 nM M (mifepristone; B), 30 nM P (provera; C) for one hour, followed by addition of 500 nM of neurosteroids. One hour later 1.25 µg/ml Aβ42 was added. Caspase-3/7 activity was determined by fluorometry. Each value represents the mean ± SEM of 3-5 independent experiments. *p<0.05 and **p<0.01.

4. Proliferation-promoting effects of AP and its analogs in rat and mouse primary hippocampal cell cultures

The hippocampus plays a crucial role in regulating cognitive and memory functions which are affected in neurodegenerative diseases. It is conceivable that stimulation of hippocampal neurogenesis could have positive effects in the diseased brain. Wang and colleagues (2005) had already shown that AP stimulates progenitor cell proliferation in cultures of the embryonic (E18) hippocampus. We therefore wanted to test (i) whether the proliferation-promoting effect of AP analogs seen in our experiments with aNSCs, would extent to postnatal hippocampal cell cultures and (ii) if they are more potent than the natural neurosteroid AP.

4.1. Proliferation-promoting effect of AP analogs in postnatal hippocampal cultures

Dissociated primary cultures from postnatal day 2 rat hippocampus grown in serum-free medium were used. Cultures were treated with neurosteroids for 24 hours and then exposed to BrdU for two hours. BrdU-incorporating cells were identified immunocytochemically (Fig. 39A). In cultures treated with AP, the number of BrdU-positive cells increased significantly by 31 % as compared to vehicle-treated controls (8.1 % and 6.2 % of all cells respectively) (Fig. 39A, B). This value is virtually identical to the effect of AP observed in E18 embryonic hippocampal cultures (Wang et al., 2005). The activity of the neurosteroid was stereo-specific with the 3β-hydroxy isomer epiallopregnanolone (epiAP) having no stimulatory effect (Fig. 39B). Treatment with two of the analogs, 12 oxo-AP and O-allyl-AP, increased the number of cells incorporating BrdU by 45 % and 63 %, respectively (Fig. 39A,B). The effects were highly significant when compared to controls and in the case of O-allyl-AP significantly higher than the effect of AP. Like epiAP, the 3β-isomers of AP analogs, 12 oxo-epiAP and O-allyl-epiAP, had no proliferation-promoting activity.

We also studied the effect of the neurosteroids in primary hippocampal cell cultures from postnatal day 2 mice. The results were very similar to those obtained in rat cultures. The 3α-analogs AP, 12 oxo-AP and O-allyl-AP significantly increased the number of cells incorporating EdU by 19 %, 30 % and 45 %, respectively (Fig. 39C). As in rat hippocampal cells, the effect of the analogs was stronger than that of AP and this difference was significant in the case of O-allyl-AP. Again the 3β-analogs had no influence on proliferation (Fig. 39C).

These results confirm observations obtained with aNSC cultures. First, the stimulatory activity was restricted to the 3α-isomers and second the two novel analogs showed higher efficacy than the natural neurosteroid.
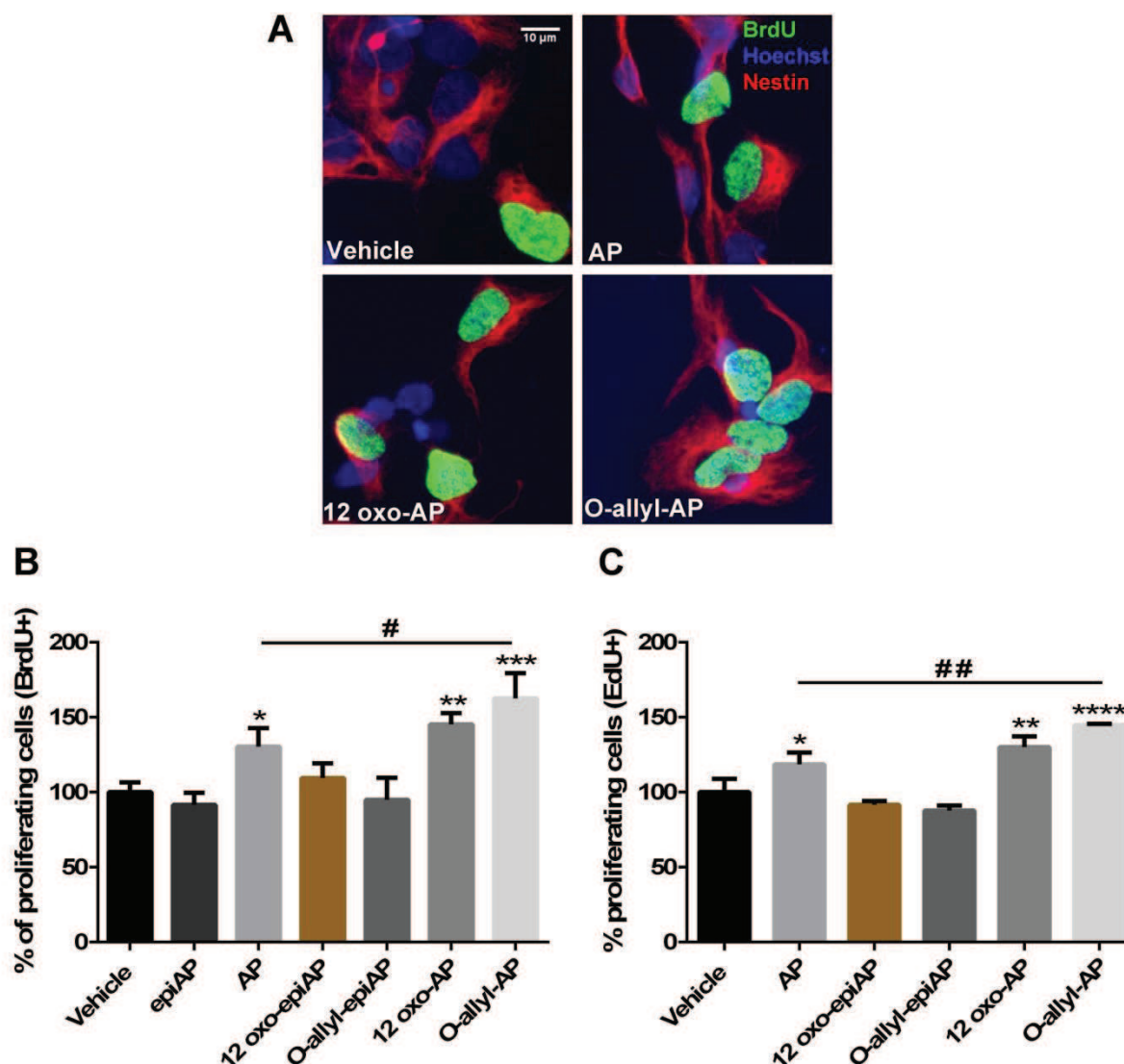
Figure 39: Proliferation promoting effect of AP analogs on rat (A,B) and mouse (C) primary hippocampal cell cultures. (A) Immunofluorescence staining for the S-phase marker BrdU (green) and the progenitor cell marker nestin (red) of rat primary hippocampal cell cultures grown for 24 h under control conditions (vehicle) or in the presence of neurosteroids as indicated. Hoechst nuclear staining shown in blue; scale bar = 10 µm. (B,C) Quantification of BrdU/EdU-incorporating cells in rat (B) and mouse (C) primary hippocampal cells grown in the absence or presence of neurosteroids for 24 h. No stimulatory effect of epiAP, 12 oxo-epiAP or O-allyl-epiAP was detected whereas AP, 12 oxo-AP and O-allyl-AP increased the number of proliferating cells. The percentage of proliferating cells in control condition was arbitrarily set to 100 %. Each value represents the mean ± SEM of three independent experiments. *[#]$p<0.05$, **[##]$p<0.01$, ***$p<0.001$ and ****$p<0.0001$.

4.2. Phenotype of proliferating cells in rat primary hippocampal cell cultures

Since the hippocampal cultures contain progenitor cells as well as differentiating neurons and astrocytes, we attempted to identify the phenotype of the cells responding to the neurosteroids. Cells labeled with antibodies against the intermediate filament protein nestin which is expressed by dividing neural progenitors were determined to represent 51 ± 7 % of all cells (Fig. 40A). We quantified the percentage of double labeled cells for BrdU and nestin. The percentage of proliferating cells in control condition was arbitrarily set to 100 %. A significant increase of 49.1 %, 70.7 % and 85 % was detected with AP, 12 oxo-AP and O-allyl-AP treatment respectively. Moreover, O-allyl-AP was more efficient than AP in stimulating proliferation of progenitor cells.

Evaluation of cells double labeled for BrdU and the neuronal marker Tuj-1 (Fig. 40B) revealed the same pattern of increase with the three neurosteroids but the results did not reach significance because of the low abundance of this cell population. In addition, no stimulatory effect was detected on cells double labeled for BrdU and the astrocytic marker GFAP (Fig. 40C).

Comparing the contribution of the three evaluated phenotypes (progenitors, astrocytes or neurons) to the total population of proliferating cells (Fig. 40D), showed that treatment with the neurosteroids did not alter cellular composition, indicating that cell fate decision is not affected.
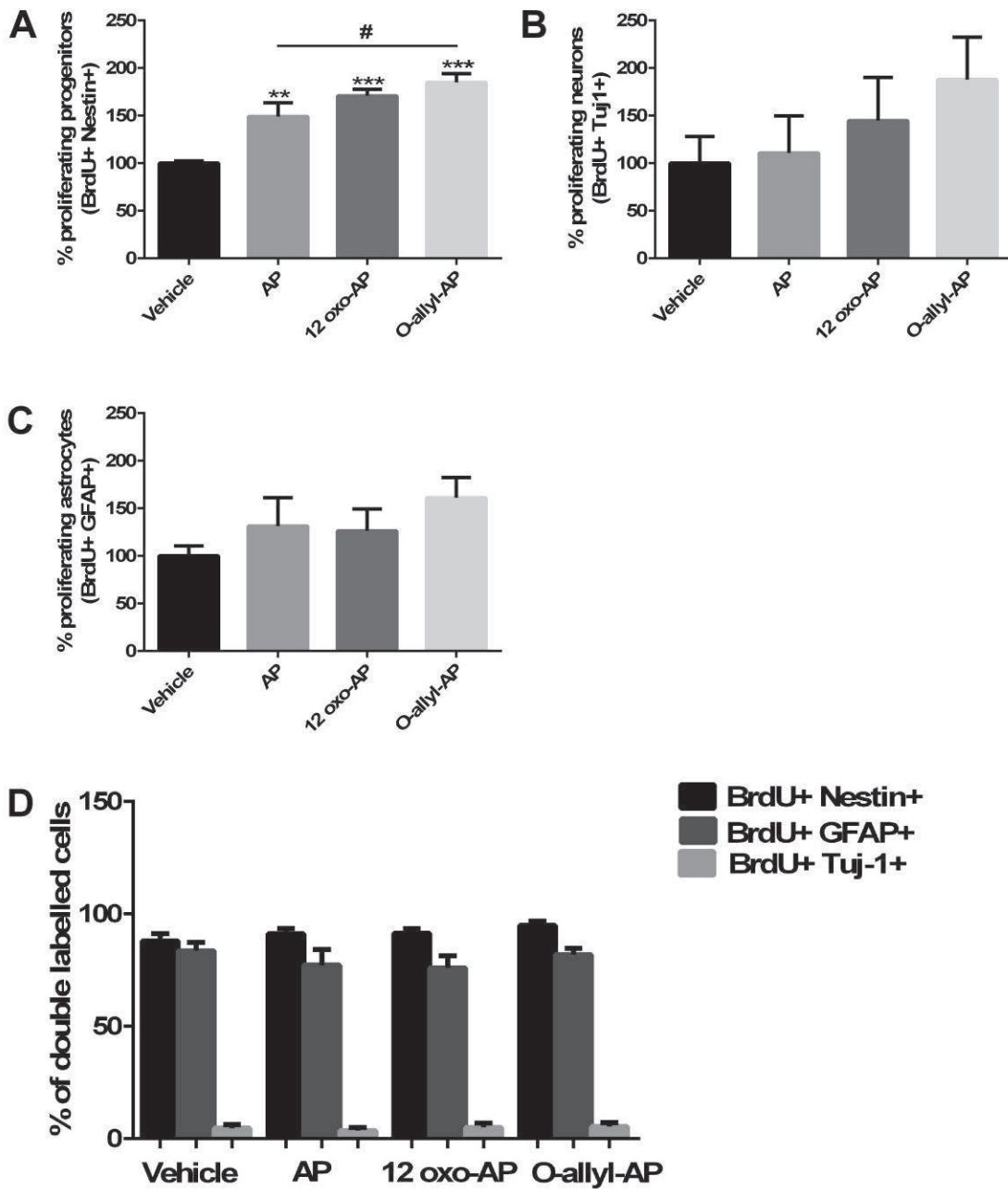
Figure 40: Phenotype of proliferating cells in AP analogs-treated hippocampal cell cultures. Proliferating cells (BrdU+) were labeled for cellular lineage markers: progenitor cell marker nestin (A), immature neuronal marker Tuj-1 (B) and astrocytic marker GFAP (C). (D) Cellular composition of the population of proliferating cells; percentage of progenitors, astrocytes or neurons of the total number of proliferating cells. Each value represents the mean ± SEM of three independent experiments. [#]$p<0.05$, **$p<0.01$ and ***$p<0.001$.

4.3. Is AP analogs-induced neural progenitor cell proliferation in mouse primary hippocampal cell cultures mediated via GABA$_A$Rs?

Evidence has been presented that the proliferation-promoting effect of AP on neuronal progenitor cells from E18 rat hippocampus is mediated via GABA$_A$Rs (Wang et al., 2005; Wang et al., 2010; Brinton, 2013). To investigate if the activity of AP analogs also involves activation of GABA$_A$Rs, hippocampal cultures were incubated with two concentrations of bicuculline, 10 µM (Fig. 41A) and 50µM (Fig. 41B), in the presence or absence of AP analogs.

Under control conditions, bicuculline had no effect on cell proliferation. The same percentage of cells entered into the S-phase and incorporated EdU (Fig. 41A,B) independent of the presence of the inhibitor.
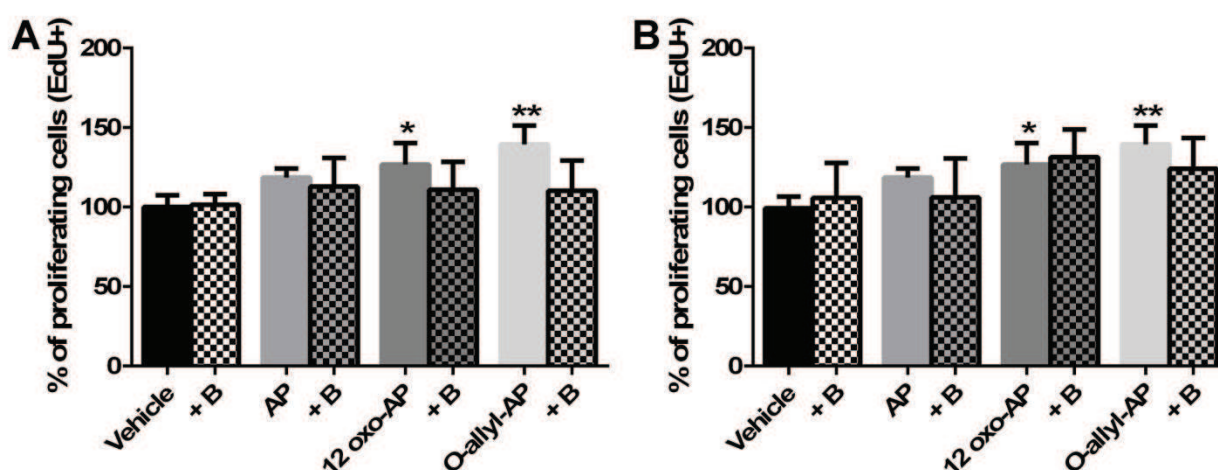
Figure 41: Effect of bicuculline on neurosteroid-induced proliferation. Mouse primary hippocampal cell cultures were treated with neurosteroids in the presence or absence of bicuculline 10 µM (A) and 50µM (B) for 24 h. The percentage of proliferating cells in control conditions (vehicle) was arbitrarily set to 100 %. Each value represents the mean ± SEM of 4-5 independent experiments. *p<0.05 and **p<0.01.

In the presence of bicuculline, the number of EdU-positive cells was reduced (except for 12 oxo-AP at 50 µM) and no significant stimulatory effect of 12 oxo-AP and O-allyl-AP was observed in comparison with the control cultures indicating that bicuculline at least partially blocked the action of the analogs (Fig. 41). However, comparing the values obtained in the presence and absence of the inhibitor, the reduction of the neurosteroid effects was found not to be statistically significant.

Thus, although these results provided some evidence for an inhibitory action of bicuculline on neurosteroid-induced proliferation, they did not unequivocally demonstrate that the reaction of progenitor cells in hippocampal cultures to AP analogs involves the activation of GABA$_A$Rs.

5. Proliferation-promoting effects of O-allyl-AP on human neural stem cells

Our results showed a stimulatory effect of O-allyl-AP on murine cells: aNSCs and primary hippocampal cells. To determine whether our findings in murine cells are also observed in cells of human origin, we investigated the effect of the most potent analog of AP, O-allyl-AP, on proliferation of human neural stem cells (HNSC).

These analyses indicate that O-allyl-AP induced a highly significant increase in BrdU incorporation by 28.7 % at a concentration of 25 nM and 27 % at 500 nM in comparison to vehicle (Fig. 42). In contrast to its effect on murine cells, O-allyl-AP significantly stimulated proliferation already at 25 nM, whereas the minimally effective dose in murine cultures was 500 nM. The natural neurosteroid AP did not significantly stimulate proliferation in HNSC at 25 and 500 nM.
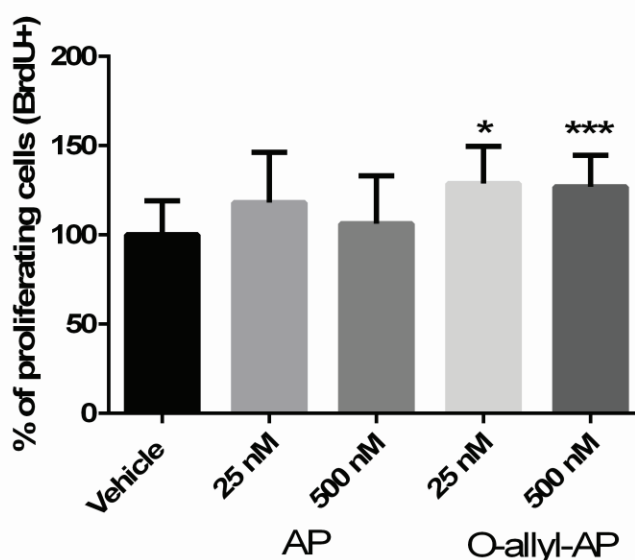
Figure 42: Effects of AP and O-allyl-AP on proliferation in HNSC. The incorporation of the S-phase marker BrdU was used to determine cell proliferation. AP did not significantly stimulate proliferation, whereas O-allyl-AP increased the number of BrdU-labeled cells. Proliferation in control cultures (vehicle) was arbitrarily set to 100 %. Each value represents the mean ± SEM of three independent experiments for AP and five independent experiments for O-allyl-AP. *p<0.05 and ***p<0.001.

The natural neurosteroid AP has already been shown to very efficiently prevent the decline in cognitive performance in aged mice and to restore deficits in learning and memory formation in an AD mouse model (Wang et al., 2010; Singh et al., 2012). Our *in vitro* results show that we have identified two synthetic steroids, O-allyl-AP and O-allyl-epiAP which not only show AP-like activity but are superior to the natural neurosteroid with respect to neuroprotective and/or neurogenesis-promoting actions in different *in vitro* model systems. It was therefore interesting to investigate if the novel analogs have beneficial effects *in vivo*. In collaboration with the lab of Dr. C. Mathis, we performed a series of behavioral tests using aged and transgenic AD mice. On the basis of toxicological pre-tests, the range of doses and the treatment regimen was chosen: the two compounds were injected intraperitoneally three times per week for 4 consecutive weeks at three different doses: 1, 2, and 4 mg/kg (O-allyl-AP) and 2, 4 and 8 mg/kg (O-allyl-epiAP). Animals were then tested for their performance in a pattern separation and an object location test. O-allyl-AP was tested in both aged and transgenic animals, whereas O-allyl-epiAP was only tested in the transgenic AD model.

After completing the behavioral tests, we used the animals for a morphological analysis of neurogenic activity in the SGZ of the dentate gyrus, although the number of animals available appeared very low, precluding in most cases a robust statistical analysis. However, we expected to get at least preliminary information on the *in vivo* actions of the novel analogs of AP.

6. Effects of O-allyl-AP on proliferation and neurogenesis in the aged adult brain

Age is considered as a principal risk factor of neurodegenerative diseases. The aging process is associated with structural, chemical, functional, neurocognitive and genetic changes which also result in a decline of neurogenesis. Adult neurogenesis, the generation of new neurons that integrate into existing neuronal networks, consists of a series of biological processes, including e.g. maintenance of the stem cell pool, proliferation of stem cells and progenitors, differentiation and controlled survival. Which of these processes are impeded by aging or in AD is not fully understood. To examine the influence of the neurosteroids on the number of stem cells and progenitors, we used a set of immunocytochemical markers for different

stages of the adult neurogenic lineage. Stem cells were identified with antibodies to Sox2 and BLBP, proliferating cells with antibodies to Ki67 and newly generated, differentiating cells were labeled with NeuroD or DCX. Aged control mice (21 months old) were compared to young mice and to aged mice treated with the AP analog over 4 weeks.

Serial coronal sections from paraformaldehyde-fixed brains were cut on a vibratome and immunostained using the peroxidase-DAB technique. Immunoreactive cells located in the SGZ of the dentate gyrus were counted and cell numbers were normalized to the length of the SGZ in individual sections.

6.1. Effect of O-allyl-AP on neural stem and progenitor cells

6.1.1. Sox2 labelling

The neural stem cell population in the hippocampus was determined using the cellular marker Sox2, which is a transcription factor essential for maintaining self-renewal or multipotency of neural stem cells. In the SGZ, the protein is expressed in stem cells and early progenitors but is also found in astrocytes throughout the brain (Bonaguidi et al., 2012). The number of immunopositive cells (Sox2+) was counted in the SGZ using ImageJ to define the region of interest (Fig 43A-C). In young 7-month-old mice, 149 Sox2+ cells were counted on average per mm of SGZ (Fig. 43D). The number was significantly decreased by 20.2 % to 119 cells/mm in aged 21-month-old mice (Fig. 43D). Treatment of aged animals with O-allyl-AP at 1, 2 and 4 mg/kg resulted in an increase of the number of Sox2+ cells to 148, 140 and 138 cells/mm, respectively, an increase of 16 to 25 % in comparison to the vehicle-treated aged animals. A dose of 1 mg/kg of O-allyl-AP proved to be most effective in increasing the number of Sox2+ cells, which reached the same density as seen in young animals.
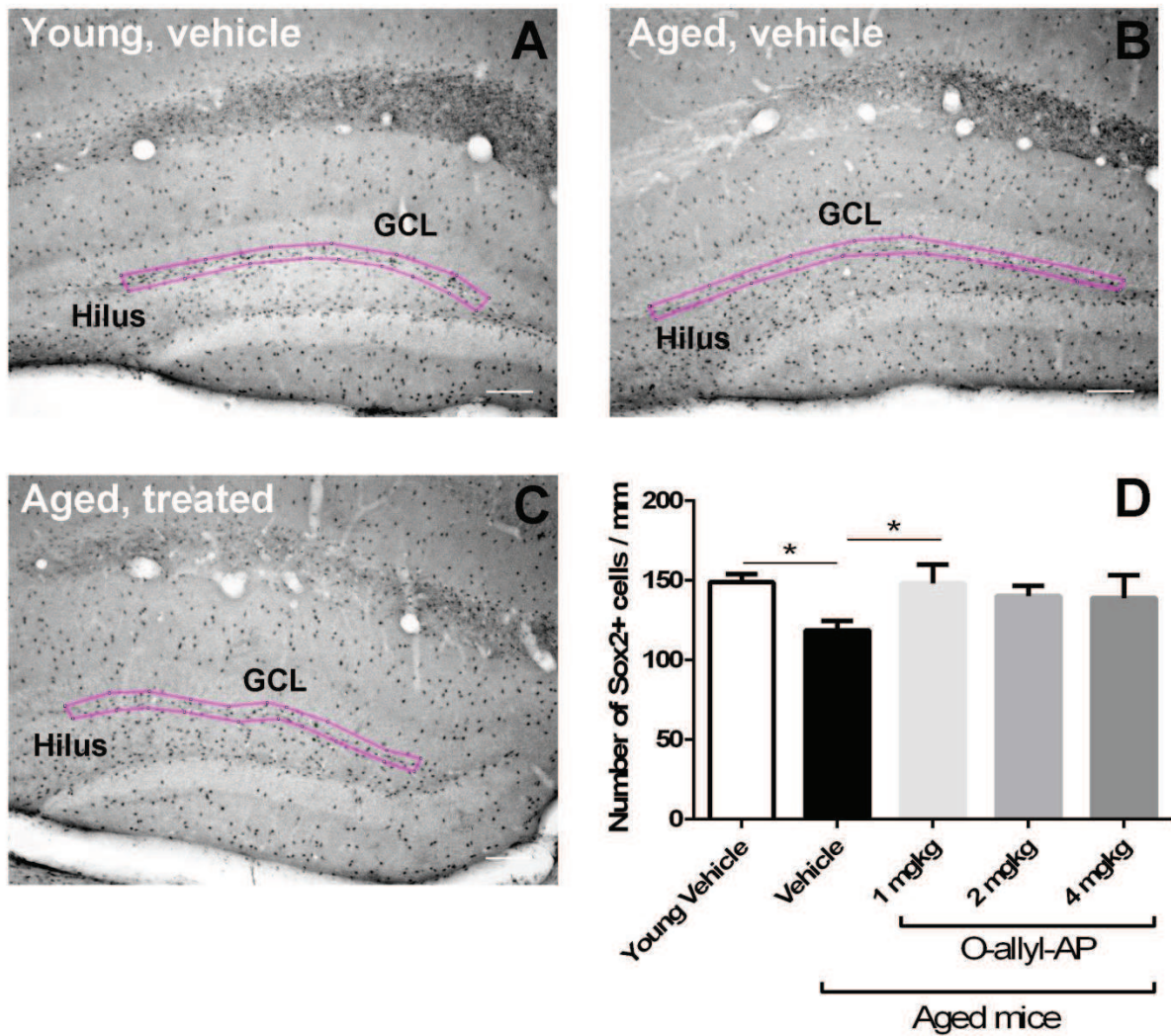
Figure 43: Effect of O-allyl-AP on the number of Sox2-expressing cells in the SGZ. (A-C) Vibratome sections of the hippocampus immunolabeled with an anti-Sox2 antibody. The purple line delineates the SGZ. Scale bar: 100µm. (D) Quantification of Sox2-immunoreactive cells in the SGZ across the experimental groups. GCL: granule cell layer. n=3, *p<0.05.

6.1.2. BLBP labelling

BLBP is a specific marker for radial glial-like neural stem cells and is implicated in neuron-radial glial cell signaling in the developing brain. Antibodies against BLBP label cell somata in the SGZ and the radial processes of these cells extending through the granule cell layer (Fig. 44A-C). The average number of BLBP+ cells per mm of SGZ decreased from 51.7 in young mice to 39.2 in aged mice, a decrease of 24.3 %. Although significance was not reached, 1 mg/kg of O-ally-AP increased the number of BLBP+ cells to a level similar to that found in young animals, while higher doses had no effect. As described above, the high inter-individual variability and the

low number of animals available for this analysis precluded a robust statistical evaluation.
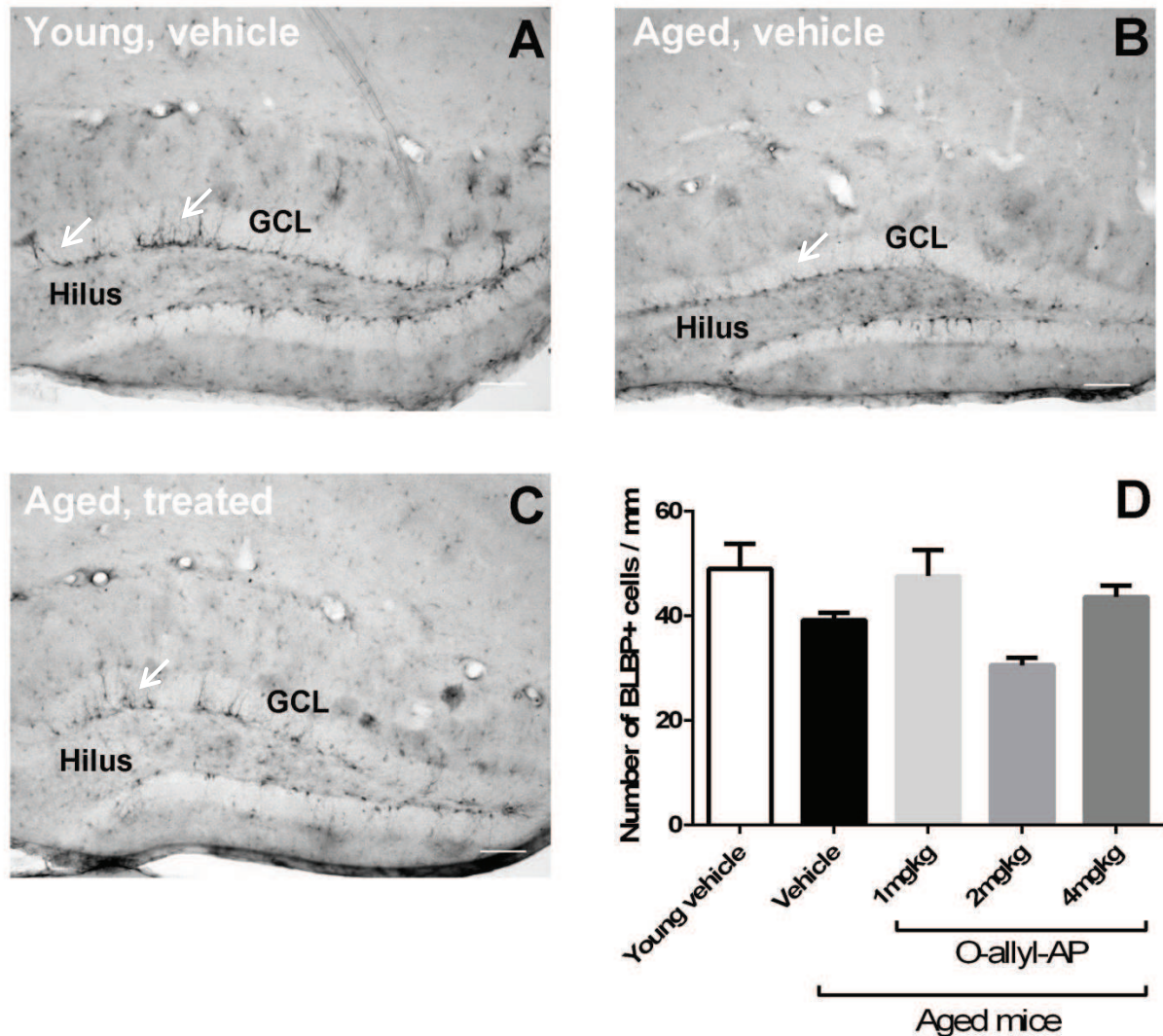
Figure 44: Effect of O-allyl-AP on the number of BLBP-immunoreactive radial glia-like stem cells. (A-C) The antibody against BLBP labels stem cell somata in the SGZ and radial processes extending through the granule cell layer (GCL). Scale bar: 100µm. (D) Quantification of BLBP-immunoreactive cells in the SGZ across the experimental groups. n=3.

6.2. O-allyl-AP effect on proliferating cells

The effect of O-allyl-AP on proliferative activity in the SGZ was analyzed by Ki67 staining which is a marker for cells that are in the cell cycle (Fig. 45A-C). The number of Ki67-immunoreactive cells was low (4 cells/mm) already in young mice (Fig. 45A,D). In the old control animals, proliferative activity was reduced by about 90 % to levels almost undetectable by Ki67 staining (Fig. 45B,D). Treatment with O-allyl-AP partially prevented this decrease (or partially restored proliferative activity; Fig.

45C,D). In animals treated with 1 mg/kg, the number of Ki67-positive cells was significantly higher (by 236 %) in comparison to the vehicle-treated mice.
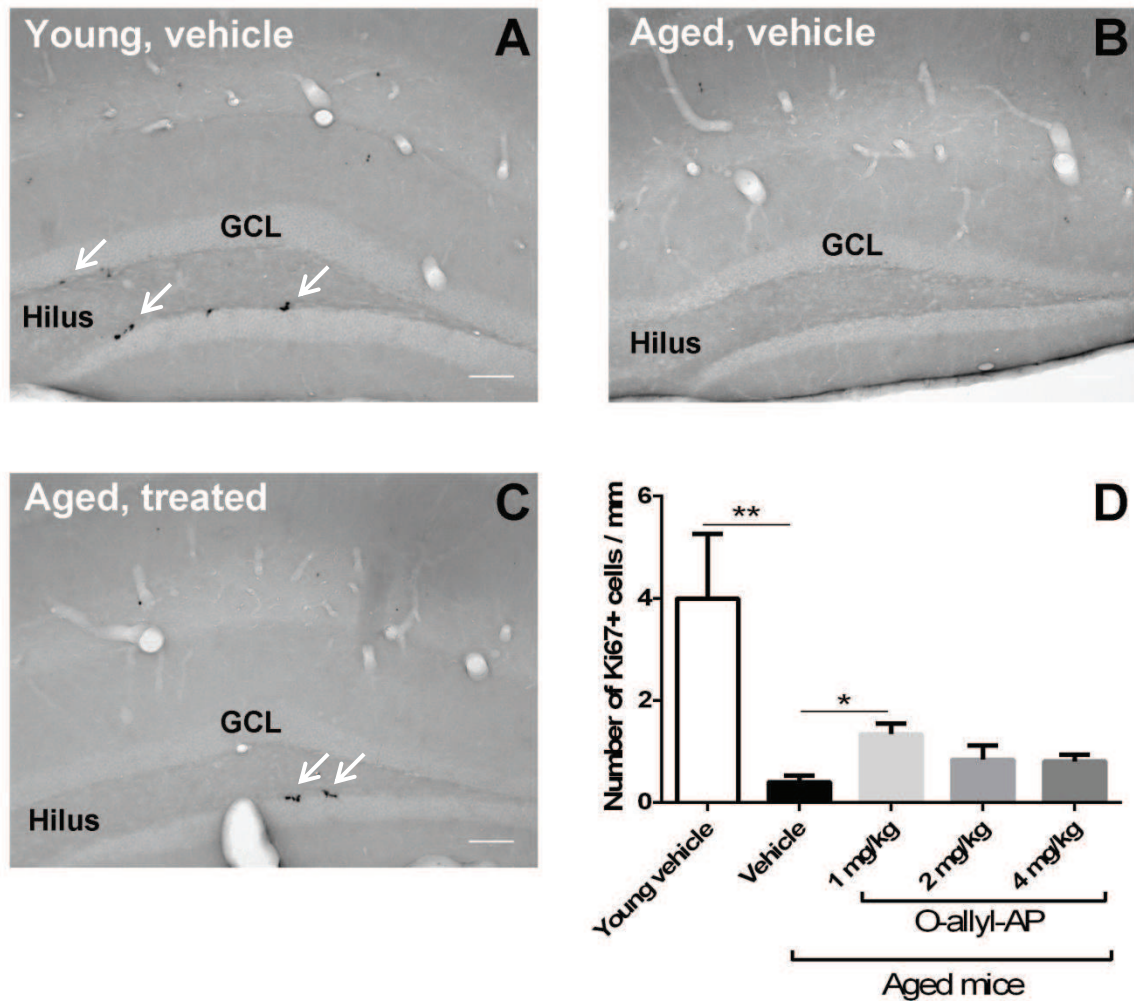
Figure 45: Effect of O-allyl-AP on proliferative activity in the SGZ of adult mice. (A-C) The antibody against the cell cycle marker Ki67 labels proliferating cells in the SGZ (arrows). Scale bar: 100µm. (D) Quantification of Ki67-immunoreactive cells in the SGZ across the experimental groups. GCL: granule cell layer. n=3, $^*p<0.05$; $^{**}p<0.01$.

6.3. O-allyl-AP effect on newborn neurons

To analyze the effects of O-allyl-AP treatment on the generation of immature neurons, hippocampal sections were immunostained with an antibody against NeuroD, a transcription factor involved in early steps of neuronal differentiation (Fig. 46A-C). As shown above for stem cells and proliferating cells, the number of newborn neurons in the SGZ of the DG is dramatically (-97.4 %) lower in aged as compared to young animals. The average number of immunopositive cells (NeuroD+) dropped

from 17.1 to 0.44 cells/mm of SGZ (Fig. 46A,B,D). The number of NeuroD+ cells was increased to 0.58, 0.59 and 0.64 cells in aged O-allyl-AP-treated animals with 1, 2 and 4 mg/kg respectively; which represent an increase of 30 to 45 % in comparison to the vehicle-treated aged animals. However, these effects were not significant.
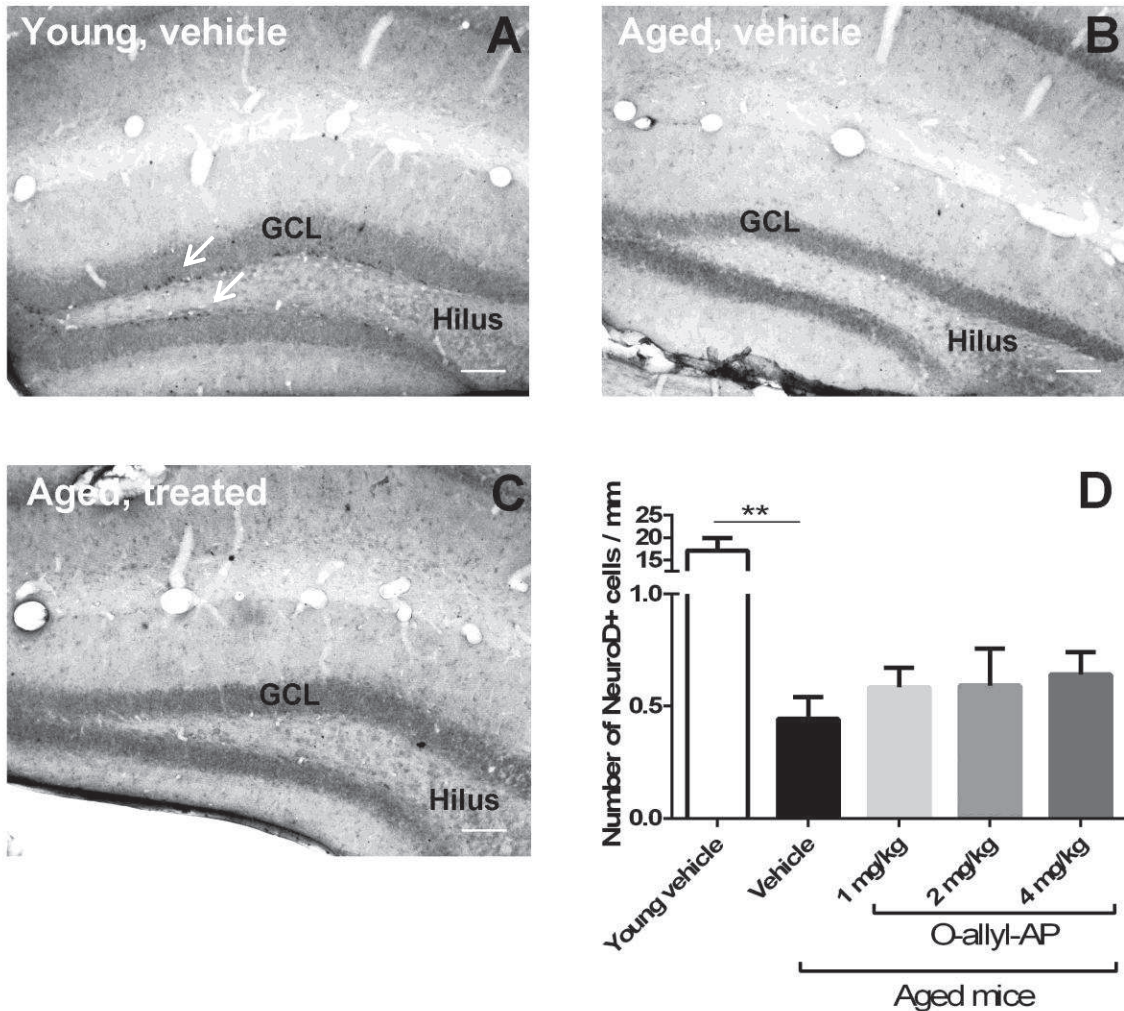
Figure 46: Effect of O-allyl-AP on generation of NeuroD-immunoreactive immature neurons. (A-C) Nuclear staining (black) illustrates the presence of immunopositive cells for NeuroD in the SGZ (arrows). Scale bar: 100µm. (D) Quantification of the number of NeuroD-immunoreactive neurons across the experimental groups. GCL: granule cell layer. n=3, [**]$p<0.01$.

Taken together, these results indicate a moderate decrease in the size of the stem cell pool in aged mice and a pronounced reduction in proliferative activity of progenitors as well as in the production of new neurons. Treatment of aged animals for four weeks with O-allyl-AP tended to enhance neurogenic activity (progenitor cell

number, proliferation, generation of neurons) but the experimental setting did not allow to consistently demonstrate a neurogenesis-promoting action of the AP analog.

7. Effects of AP analogs in an AD mouse model (Tg2576)

7.1. Effects on proliferation

7.1.1. O-allyl-AP treatment

Encouraged by the proliferation-promoting and neuronal differentiation-stimulating effect of O-allyl-AP in our *in vitro* studies, as well as its protective action, we examined its effects in an APP transgenic AD mouse model (Tg2576). Experimental protocols were identical to those described above for experiments with aged animals. Controversial data has been reported regarding the proliferative activity in neurogenic areas in the Tg2576 AD mouse model. In one study, Tg2576 and aged-matched control non-transgenic (NTg) mice were described to have similar proliferative activities in the DG from 3 to 12 months of age (Krezymon et al., 2013). Another study reported that proliferation was decreased in the DG of 3-, 6-, and 9 month-old transgenic mice as compared to aged-matched control NTg (Dong et al., 2004).

Results of our experiments supported the findings of Krezymon and colleagues. Counting Ki67-immunoreactive cells to measure proliferative activity showed no difference between Tg2576 and NTg vehicle-treated animals (Fig. 47A). Treatment with 1 mg/kg of O-allyl-AP increased the number of proliferating cells by 95 %; from 4.7 to 9.1 Ki67+ cells/mm of SGZ (Fig. 47A).

To evaluate the generation of new neurons, sections were immunolabeled with antibodies against NeuroD (Fig. 47B) and doublecortin DCX, a microtubule-associated protein used as a marker for immature migrating neurons (Fig. 47C-F). A difference in the number of newly generated neurons was observed with both markers between NTg and Tg2576 mice, cell number being lower in the mutants by 36.1 % for DCX+ cells and 55.6 % for NeuroD+ cells (Fig. 47B,C). However, the differences were not significant. Following treatment of the Tg2576 mice with 1 mg/kg O-allyl-AP, we observed, with both markers, an increase in the number of newborn neurons in the SGZ, 34.8 % for DCX+ cells and 41.8 % for NeuroD+ cells (Fig. 47B,C). Again these changes were statistically not significant and therefore have to be interpreted carefully.
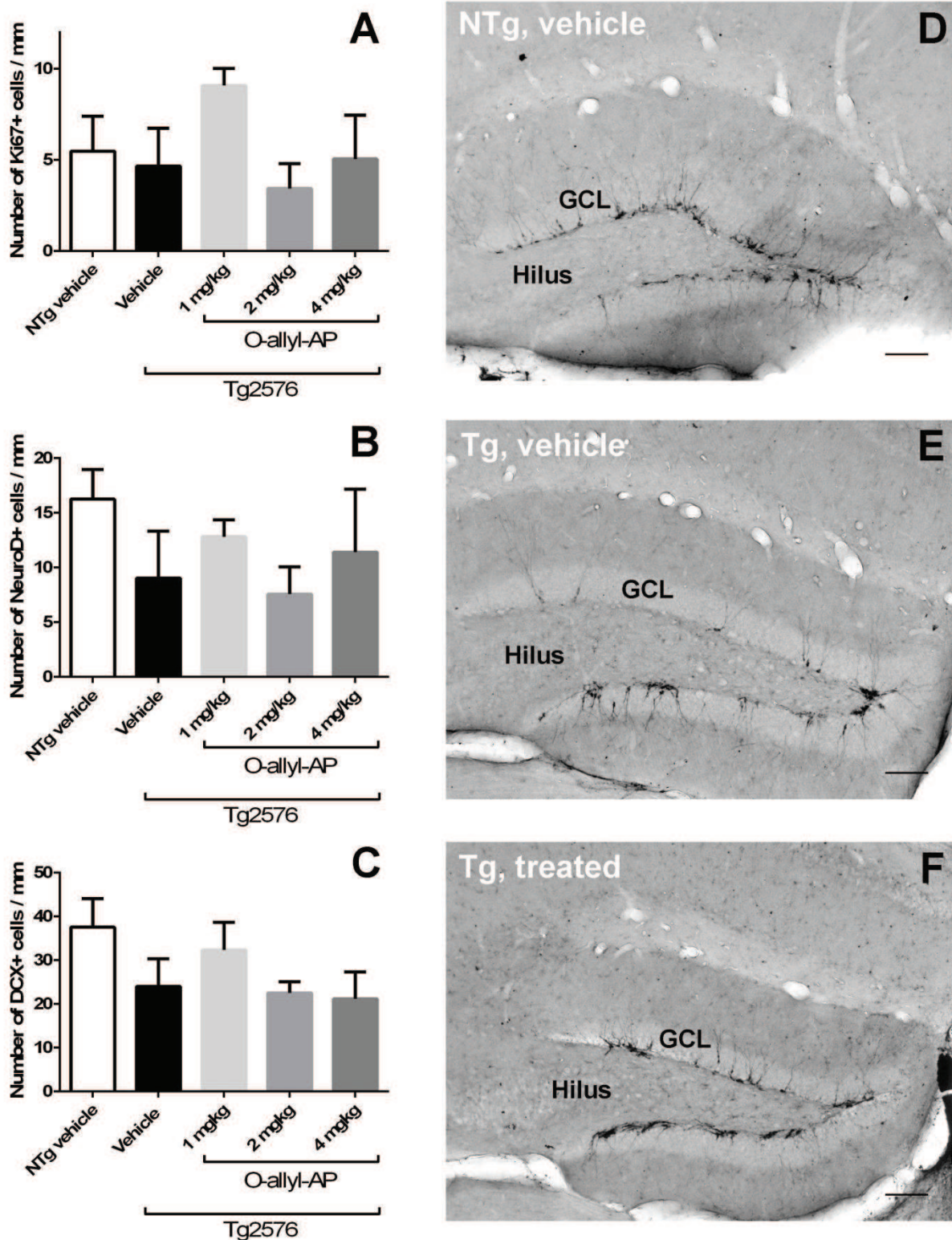
Figure 47: O-allyl-AP effects on proliferating cells and newborn neurons in Tg2576 mice. (A-C) Quantification of the number of immunoreactive cells for the cell division marker Ki67 (A), neuronal differentiation factor NeuroD (B) and immature neuronal marker DCX (C). (D-F) Vibratome sections of the hippocampus immunolabeled with an anti-DCX antibody. Nuclear and dendritic staining (black) illustrates the presence of immunopositive cells for DCX in the SGZ of the DG. Scale bar: 100µm. GCL: granule cell layer. n=3.

7.1.2. O-allyl-epiAP treatment

The compound O-allyl-epiAP has been shown to exert a strong neuroprotective action without any effect on proliferation of neural stem/progenitor cells in our *in vitro* studies. We examined its action in the Tg2576 mouse model of AD to determine if the protective effect against Aβ toxicity may result in beneficial effects in the pathological *in vivo* situation. As above, cohorts of animals were injected with three different doses of the compound (2, 4 and 8 mg/kg).

The results from this set of experiments confirmed that there was no significant difference in the number of proliferating cells between transgenic and normal mice and a reduction in the number of differentiating (DCX+, NeuroD+) neurons (Fig 48A-C). Treatment of Tg2576 mice with O-allyl-epiAP did not result in any detectable change in the number of cells expressing the marker proteins.

Since the neural stem cell cultures used to demonstrate the neuroprotective effects of O-allyl-epiAP *in vitro* were prepared from the SVZ neurogenic area, we wanted to examine whether the compound may show *in vivo* activity in this particular area. For this purpose, mRNA extracts from the frontal cortex including the SVZ were analyzed by qRT-PCR for the expression of DCX and of the proliferation marker PCNA (proliferating cell nuclear antigen). In agreement with the morphological data from the SGZ, the expression data indicated only a trend to a reduced expression in the Tg2576 mice suggesting lower neurogenic activity (Fig. 48D,E). Treatment of the transgenic animals with O-allyl-epiAP did not significantly influence mRNA levels of DCX and PCNA.

In summary, we did not find evidence for a protective effect of O-allyl-epiAP in the AD model.
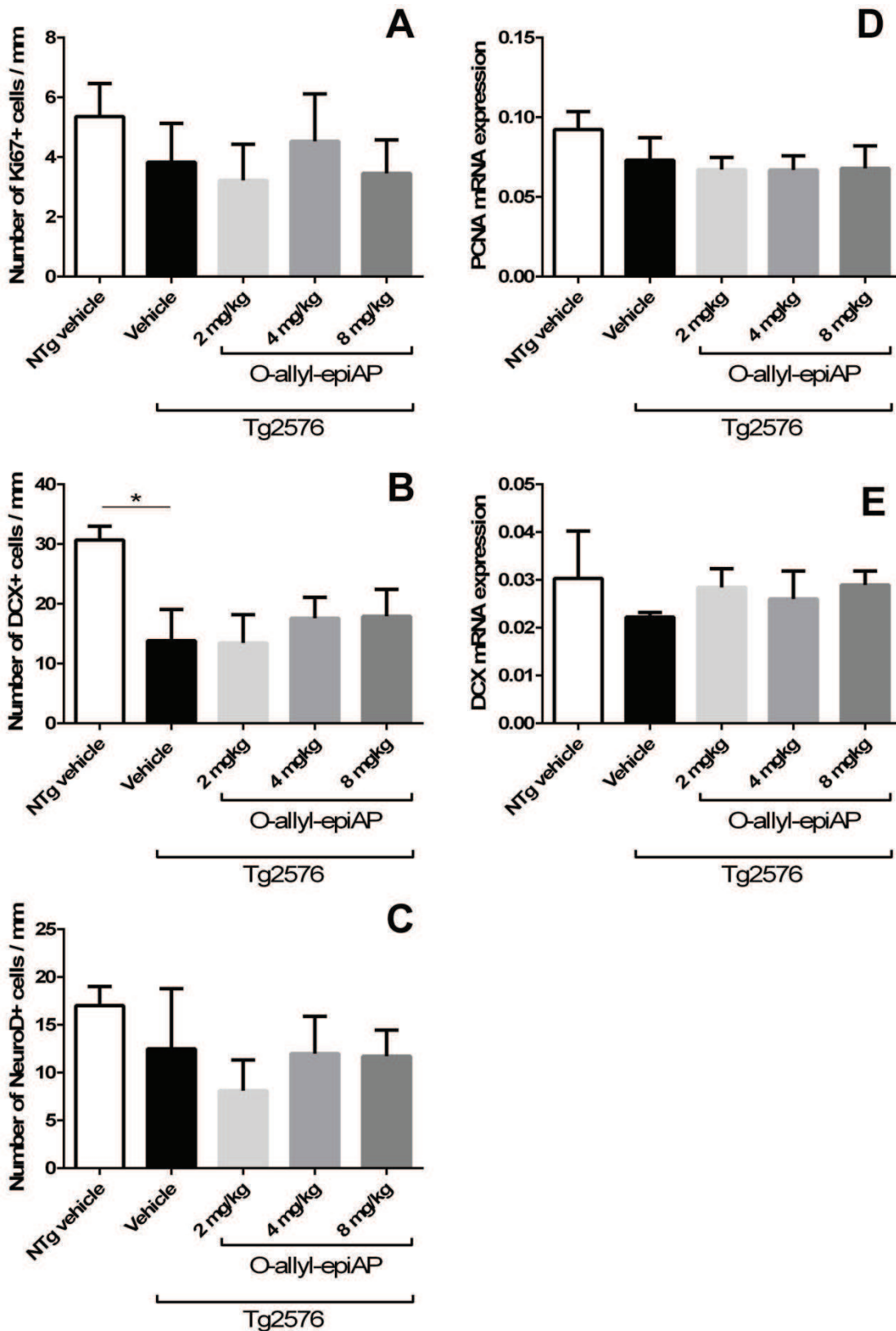
Figure 48: Effects of O-allyl-epiAP on the expression of neurogenesis markers in Tg2576 mice. (A-C) Quantification of cells immunoreactivity for Ki67 (A), DCX (B) and NeuroD (C) in the SGZ of non-transgenic (NTg) mice and in Tg2576 mouse mutants treated with different doses of O-allyl-epiAP. (D,E) mRNA-expression levels of PCNA (D) and DCX (E) in the frontal cortex of NTg and mutant mice analyzed by qRT-PCR. n=3, *p<0.05.

7.2. Effects of AP analogs on Aβ burden in Tg2576 mutant mice

It has been reported that the positive influence of AP on neurogenesis, neuronal survival and cognitive performance in a mouse model of AD is paralleled by a reduction of brain Aβ oligomer levels (Chen et al., 2011). The amyloid precursor protein is cleaved by β- and γ-secretase releasing soluble Aβ peptides. Two of those, Aβ40 and Aβ42 are assumed to represent the most important ones with regard to AD pathology (Lesné et al., 2006; Shankar et al., 2008). The aggregation of Aβ peptides generates different forms of toxic oligomers which then aggregate further to form amyloid plaques (Shankar et al., 2009).

In a previous study of Singh and colleagues (2012), animals had been treated with AP over a total period of 6 months spanning the phase from onset to amyloid plaque formation. In our experiments, animals were treated with AP analogs for 4 weeks and we wanted to examine if such a short treatment regimen would already affect levels of amyloid peptides and Aβ plaques.

Immunostaining for amyloid plaques, using a beta amyloid-specific antibody (6E10), confirmed the absence of beta-amyloid plaques in the non-transgenic control animals and its presence in the transgenic Tg2576 mice (Fig. 49). Plaques were more abundant in the cerebral cortex as compared to hippocampus. Double labeling with an antibody against GFAP demonstrated that amyloid plaques were surrounded by activated astrocytes (Fig. 49). As plaque formation in this mouse model only starts at the age the animals were used in our studies, only a small number were detected per animal, precluding a quantitative analysis.
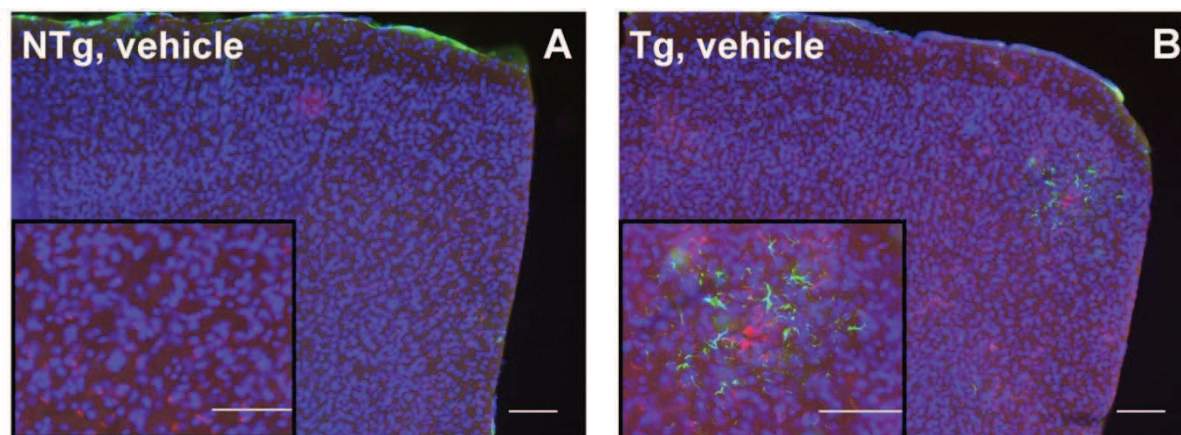
Figure 49: Amyloid plaques are surrounded by activated astrocytes in Tg2576 mutant mice. Representative photomicrographs of immunofluorescence stained sections from non-transgenic control animals (A) and Tg2576 mutant mice (B) are shown, green: staining for GFAP, red: plaques stained with amyloid beta antibody 6E10. Hoechst nuclear staining shown in blue; Scale bar = 100 μm.

Finally, we examined whether treatment with analogs had an effect on the concentration of amyloid beta peptide Aβ42 and Aβ40 in the brain of Tg2576 mice. Aβ levels were determined in extracts of the frontal cortex of vehicle or analog-treated Tg2576 mice by ELISA (Fig. 50).

Very similar results were obtained for the two peptides (Fig. 50). First, the levels of both Aβ peptides in vehicle-treated control animals differed between the two cohorts of animals. Vehicle-treated animals of the O-allyl-epiAP cohort had lower levels of both peptides as compared to the vehicle-treated O-allyl-AP cohort. This difference is probably due to the fact that Tg2576 animals in the O-allyl-AP cohort were one month older (11 vs. 10 months) at the time of analysis. It has been shown that Aβ-levels increase most strongly between the age of 6 and 12 months (Hsiao et al., 1996; Kawarabayashi et al., 2001; Arendash and King, 2002; King and Arendash, 2002; Westerman et al., 2002; Lesné et al., 2006). O-allyl-epiAP treatment at a dose of 4 mg/kg lead to a pronounced reduction of Aβ42 levels, had no effect at other doses tested and did not affect Aβ40 at all (Fig. 50).

Treatment with O-allyl-AP resulted in a dose dependent decrease of Aβ42 and Aβ40 levels. However, these effects did not reach significant because of a high inter-individual variation.
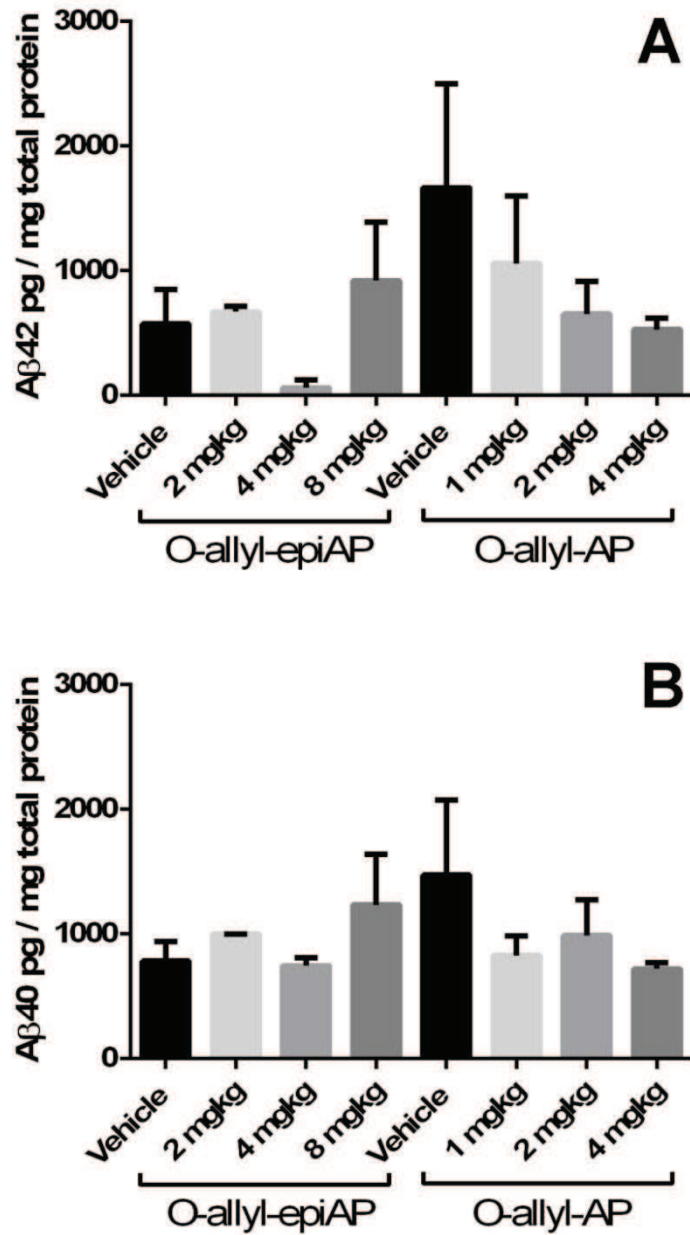
Figure 50: Effects of AP analogs on amyloid beta peptide levels in the brain of Tg2576 mutants. The amount of Aβ42 (A) and Aβ40 (B) was measured by ELISA. Each value represents the mean ± SEM of three animals (n=3).

DISCUSSION

In this study, we investigated the ability of different structural analogs of the endogenous neurosteroid allopregnanolone to promote proliferation of neural progenitor cells and adult neurogenic activity. We also tested the ability of these compounds to protect against cell death induced by Aβ42 peptide, a key factor involved in AD physiopathology.

Previous studies have shown that AP has a broad spectrum of biological activities, including neuroprotective effects in various animal models for neurodegenerative disorders (Chen et al., 2011; Brinton, 2013; Melcangi and Panzica, 2014; Patte-Mensah et al., 2014) and anxiolytic, analgesic, anesthetic, antidepressants and sedative properties in psychiatric disorders (Uzunova et al., 1998; Rasmusson et al., 2006; Marx et al., 2009; Rupprecht et al., 2009; Frye et al., 2011; Marx et al., 2011; Nelson and Pinna, 2011). On a cellular level, AP has been demonstrated to promote survival, proliferation, neuronal differentiation and integration of newly generated neurons *in vitro* and in animal models (Wang et al., 2005; Wang and Brinton, 2008; Singh et al., 2012). Although this neurosteroid is already tested in clinical trials, the diversity of its biological effects may actually impede its therapeutical use by causing undesired side effects. Therefore, in search for neuroactive steroids with higher efficacy and better selectivity with respect to their biological actions, we synthesized four novel structural analogs of AP and examined the effects of these compounds on proliferation, differentiation and neuroprotection.

The major results of this study are as follows: First, analogs of AP (12 oxo-AP, O-allyl-AP) were identified which were superior to the natural neurosteroid in stimulating progenitor cell proliferation and in promoting the generation of neurons in aNSC cultures. Findings were confirmed in primary hippocampal cultures and in a human neural stem cell line. Second, two AP analogs (O-allyl-epiAP, O-allyl-AP) were demonstrated to have enhanced neuroprotective activity against Aβ-induced cell death in aNSC cultures. Third, the stereoisomeric analogs O-allyl-AP and O-ally-epiAP were found to have distinct activity patterns with respect to promotion of neurogenesis and neuroprotection. Fourth, *in vivo* analysis in aged mice and a transgenic AD mouse model suggested that the analog O-allyl-AP with enhanced *in*

vitro activity can also attenuate the age-dependent and AD-related decline of neurogenic activity *in vivo*. These morphological observations could be correlated with the results of cognitive tests obtained by our collaborators.

1. Effect of AP analogs on proliferation and differentiation

Cultures of aNSCs from the SVZ of adult mouse, having the capacity for self-renewal and multilineage differentiation, were used to examine proliferation-promoting effects of AP analogs. Proliferative activity determined by counting the number of cells incorporating the thymidine analog EdU was increased in cultures grown in the presence of the 3α-analogs 12 oxo-AP and O-allyl-AP while the 3β-analogs did not affect proliferation. The effects were observed only at reduced concentrations of growth factors (EGF, bFGF) in the medium. At saturating standard concentration, effects of neurosteroids were masked by the mitogenic effect of the growth factors indicating that analogs may exert a rescue effect on cells deprived of optimal growth conditions. Unexpectedly, no stimulatory effect was observed with AP treatment.

Several studies have identified AP as a proliferative factor in the nervous system. AP stimulated proliferation of rat embryonic hippocampal cultures, human neural progenitor cells and oligodendrocyte progenitors in the CNS (Wang et al., 2005; Wang et al., 2010; Schumacher et al., 2012). AP also increased proliferation of immature granule cells in postnatal rat cerebellar cultures (Keller et al., 2004). One may suppose that a small effect of AP in the aNSC cultures might have escaped detection due to the presence of growth factors. This assumption was supported by results obtained in rat and mouse primary hippocampal cell cultures. The same experimental protocol as with aNSCs was applied in these culture systems except that cells were cultured without growth factors. Under these conditions, the number of BrdU- or EdU-incorporating cells increased both in rat and mouse cultures treated with AP in comparison to vehicle-treated controls. The extent of stimulation was virtually identical to the effect of AP observed in embryonic rat hippocampal cultures (Wang et al., 2005). Obviously, the stimulatory effect of AP on hippocampal precursors is more pronounced as compared to that on aNSCs. As in the aNSCs, treatment with the analogs, 12 oxo-AP and O-allyl-AP, induced a significant increase in the number of proliferating cells in both rat and mouse hippocampal cell cultures, whereas the two 3β-isomers, 12 oxo-epiAP and O-allyl-epiAP, were ineffective.

Correspondingly, the 3β-isomer of AP, epiallopregnanolone, was also inactive in agreement with results obtained previously in rat embryonic hippocampal cultures where the proliferative effect was also α-isomer-specific (Wang et al., 2005).

Together, these results demonstrate that appropriate derivatization of AP, especially its etherification at the 3 hydroxyl group, results in enhanced proliferation-promoting activity. This conclusion is supported by additional preliminary experiments indicating that replacement of the allyl-group by other functional groups can further increase the efficacy of AP analogs.

At least partially based on its proliferative effect, AP is regarded as a candidate compound for the treatment of injuries and diseases associated with neurodegeneration. Clinical trials are already underway: a phase 2 trial for the treatment of traumatic brain injury (*NCT01673828*, *University of California, Davis*) and a phase 1 trial for mild cognitive impairment due to AD (*NCT02221622*, *Roberta Brinton, University of Southern California*). With these potential applications in mind, we examined the activity of O-allyl-AP on human cells. In a human stem cell line, the efficacy of the analog in stimulating proliferation was very similar to that observed in rodent cells. The potency seemed to be even higher in the human cells, since effects were seen at concentrations as low as 25 nM. Again, AP was less effective and induced no significant changes in the number of proliferating human neural stem cells showing no significant effect. Our results indicate that certain AP analogs have a general proliferation-promoting activity at least with respect to neural stem cells and/or progenitors. It would be interesting to investigate if this also applies to other proliferatively active cell populations.

The observation of an enhanced proliferation in the presence of two analogs, 12 oxo-AP and O-allyl-AP, raised the question if this change leads to an increase in the production of neurons which would be the aim of the therapeutic use of the compounds in the context of neurodegeneration. Actually, results of an *in vivo* study in the 3xTgAD mouse model indicated that AP treatment leads to enhanced production of neurons in the SGZ (Wang et al., 2010).

In vivo, the proliferatively active cell population of the SVZ contains stem cells and mainly intermediate progenitors and immature neurons (Doetsch, 2003b). In aNSC cultures under growth conditions, the great majority of all cells were nestin-positive progenitors. Very few cells of a neuronal and astrocytic phenotype could be detected.

Analysis of the phenotype of neurosteroid-responsive cells by immunocytochemical double labeling provided no evidence that the analogs preferentially stimulated cell division in neuronal progenitors. However, analysis of the expression of astroglial and neuronal markers by qRT-PCR revealed that O-allyl-AP increased expression of Tuj-1 mRNA by more than 2-fold suggesting that O-allyl-AP might support neuronal cell fate decision in progenitors and/or differentiation of neuronal precursors. Immunocytochemical analysis did not show a corresponding increase in the number of Tuj-1-positive immature neurons in aNSC cultures. Therefore, the increase in the Tuj-1 mRNA levels seen with O-allyl-AP treatment may indicate an increase in the expression of this marker in neuronal cells and not an induction of a neuronal phenotype in additional progenitor cells. Alternatively, the stimulation period of 24 hours was too short to permit the detection of Tuj-1 protein expression by immunocytochemistry. Thus, it remains to be shown if AP analogs can directly induce neuronal differentiation under growth conditions.

Experiments making use of the fact that differentiation can be induced in aNSC cultures by depriving them of growth factors provided evidence that O-allyl-AP, in parallel with its effect on proliferation, can influence cell fate decision and/or early neuronal differentiation. When cultures were treated with the analogs for 3 or 5 days in differentiation conditions, the number of cells differentiating into DCX-positive neurons was unchanged in comparison to vehicle control. This indicated that the analogs did not directly influence the differentiation process in cells already specified to become neurons or astrocytes.

The above results described which had shown that neural progenitor cells responded to O-allyl-AP with enhanced cell division and with increased expression of Tuj-1 mRNA during growth phase raised the question, if this response to neurosteroid resulted in an enhanced production of neurons. Our results showed that treating aNSCs with O-allyl-AP during the growth phase resulted in the generation of more neurons during a subsequent differentiation phase while the number of astrocytes was unaffected. This demonstrated that the stimulatory effect of O-allyl-AP initiated during the proliferation phase has consequences for the future fate of the progenitors. As neuronal progenitors in aNSC cultures are multipotent, this can be interpreted to show that either a neuronal phenotype was induced or that the survival of a subpopulation of specified neural progenitors was increased. Continuous treatment with the neurosteroids during the differentiation phase further increased the

number of differentiating neurons. O-ally-AP is also a potent neuroprotective agent; it is therefore possible that the observed effect on the number of differentiating neurons may result from a neuroprotective rather than from a differentiation-inducing effect. Effects of neurosteroids on differentiation have been reported previously in the literature. Cell culture experiments suggest that neurosteroids may increase the survival and differentiation of both neurons and glial cells (Schumacher et al., 1996; Marx et al., 2000). Suzuki and colleagues (2004) have shown that treatment of human neural stem cells with dehydroepiandrosterone (DHEA) but not with several of its metabolites, increased the number of GFAP-positive astrocytes and also stimulated production of neurons. These effects were not mediated by GABA receptors but involved NMDA and sigma 1 receptors. Moreover, DHEA can induce differentiation of neuronal cells from mesenchymal stem cells and increase their cell proliferation *in vitro* (Shiri et al., 2009). Studies by Wang and colleagues (2010) indicated that newly formed cells in hippocampal dentate gyrus, generated following AP treatment in 3xTgAD mice, expressed a neuronal phenotype suggesting that AP may specifically foster the generation of neurons *in vivo*. *In vitro* results presented here also support the conclusion that the AP analog O-allyl-AP can promote the generation of neurons from neural precursors in neurogenic areas of the adult brain.

2. Protective effects of AP analogs against Aβ42-induced toxicity on aNSCs

AP has been shown to have great potential as a neuroprotective agent, for example, in prophylactic and corrective strategies with AP treatment of neuropathic pain caused by antineoplastic treatment of cancer patients (Patte-Mensah et al., 2014), and also in protecting the nervous system from the deleterious effects of amyloid deposition in AD (Wang et al., 2010; Chen et al., 2011; Singh et al., 2012).

The amyloid peptide Aβ42, a pathophysiological hallmark of AD, has been shown to exert toxic effects in a variety of cellular cultures (LaFerla et al., 1995; Li et al., 1996; Cotman, 1998; Eckert et al., 2001). However, controversial effects of Aβ42 in aNSC cultures were described in the literature. Some studies showed that monomeric and fibrillar Aβ42 peptide forms were toxic for aNSCs, while low concentrations of the oligomeric Aβ42 peptide stimulated their proliferation and neuronal differentiation and enhanced their ability to migrate (Heo et al., 2007). Others observed that soluble and

aggregated Aβ42 increased the growth of neurospheres prepared from the adult SVZ (Itokazu et al., 2013). Therefore, we first explored the effect of monomeric Aβ42 in our aNSC cultures. When Aβ was applied to these cultures, it rapidly induced apoptosis of neural stem/progenitor cells. This observation of Aβ42-induced apoptosis is in agreement with results of previous studies (Jordán et al., 1997; Troy et al., 2000; Eckert et al., 2001). The mechanism by which Aβ42 exerts its detrimental effect is not known. It is unclear, for instance, if Aβ needs to enter the cell or is acting via membrane receptors or pore formation. Several intracellular pathways which may be involved in mediating the toxic effect of Aβ are discussed (see part 2.2.5, pages 16-18) (Small et al., 2001; Larson and Lesné, 2012). Most of them lead to activation of apoptotic pathways and involve either ER-stress or lead to mitochondrial or neuronal dysfunction (Li et al., 1996; Heinitz et al., 2006; Eckert et al., 2008; Götz et al., 2011).

Two of the novel analogs, O-allyl-epiAP and O-allyl-AP, were able to completely prevent the pro-apoptotic effect of Aβ42 on the aNSCs. Importantly, this protective effect was also observed when the neurosteroids were added one day after initiation of Aβ treatment in the corrective protocol as we call it. This observation is of relevance when considering a possible therapeutic use of neurosteroids in neurodegenerative diseases, where a prophylactic application does not seem to be realistic. *In vivo* experiments in an AD mouse model have shown that application of AP could restore progenitor survival in the SGZ as well as memory performance in animals which already showed intraneuronal accumulation of Aβ, but were ineffective at later stages when extracellular plaques had developed (Singh et al., 2012). In our *in vitro* experiments, the delay between initiation of toxic treatment and the addition of the analogs was rather short. Therefore, it would be interesting to examine in more detail the time course of the beneficial action of the analogs and to try to correlate it with markers of cellular integrity.

As in the experiments measuring proliferative activity, the analogs were clearly more efficient than AP also in the neuroprotection paradigm. Interestingly, different from the proliferation-stimulating effect, the neuroprotective activity was not isomer-specific as the two 3 O-allyl compounds exhibit similar efficiency.

3. The differential pattern of biological activities efficacy of AP analogs

The four structural analogs of AP showed different pattern of activity and efficacy regarding their effect on proliferation and protection.

Two analogs, 12 oxo-AP and O-allyl-AP, promoted both proliferation of progenitor cells in rodent aNSCs and primary hippocampal cell cultures more efficiently than AP. Interestingly, these both analogs are 3α isomers and have a proliferation stimulatory effect irrespective of the presence of a keto group at position 12 for 12 oxo-AP or substitution with an allyl group at position 3 for O-allyl-AP. This substitution has been chosen to prevent enzymatic conversion of the compound by 3α-HSD as is possible for AP (Patte-Mensah et al., 2014). Therefore, the observed stimulatory effect of the O-allyl-form on proliferation is likely attributable to the unmetabolized form of the compound. These results indicate that the proliferation-promoting effect is stereoisomer specific and it is crucial to have a α-isomer at position 3. In contrast, the 3β-isomers of these compounds, 12 oxo-epiAP and O-allyl-epiAP, are completely inactive in stimulating proliferation. These findings are in line with a previous study by Wang and colleagues (2005), in which they showed that in embryonic hippocampal neurons, the proliferation-promoting effect of AP was stereospecific as AP stereoisomers, epiallopregnanolone and 5 α-pregnan-3β-ol, were without effect.

O-allyl-AP exerts stronger effects on proliferation than 12 oxo-AP and in addition O-allyl-AP stimulates progenitor cells differentiation into neurons whereas 12 oxo-AP was ineffective. This could be explained by the presence of the O-allyl group which may increase the bioavailability which is known to be low for AP due to rapid inactivation by glucuronidation and sulphate conjugation at the 3α-hydroxyl. The allyl ether also prevents the oxidation at the 3α-hydroxyl group which can occur in AP and 12 oxo-AP.

Using an Aβ42 toxicity assay in our aNSC culture system, we demonstrated that only the O-allyl substituted compounds, O-allyl-epiAP and O-allyl-AP, prevent apoptosis induced by addition of Aβ42. The protective effect was not isomer-specific as both the α- and the β-form of O-allyl-substituted compounds were able to fully protect cells against the toxic effect of Aβ42. Interestingly, we could show that both compounds were protective in prophylactic and corrective strategies. In the prophylactic

paradigm, both analogs were more potent than AP in preventing the Aβ42-induced apoptosis. Whereas in the corrective paradigm, in which analogs were added one day after inducing apoptosis by Aβ42 treatment, AP was ineffective in protecting cells against the toxic effect of Aβ42. These findings suggest that the etherification of the hydroxyl group at position C3 by an allyl group prevents its metabolism and oxidation and thus enhances the bioavailability of the analogs without altering their neuroprotective action.

4. Mechanisms of action of AP analogs

The GABA$_A$ receptor (GABA$_A$R) is regarded as the major target for most neurosteroids and for AP in particular (Majewska et al., 1986; Majewska, 1992; Reddy, 2003). AP has been shown to be a potent positive allosteric modulator of GABA$_A$Rs (Majewska et al., 1986). Also, the proliferation-promoting and neuroprotective effects of AP have been reported to involve GABA$_A$Rs (Brinton, 2013). Here, the potential mechanisms of action of AP analogs in regard to their proliferative and protective effects will be discussed separately.

The proliferation-promoting effect of AP was reported to be mediated via GABA$_A$Rs in neural stem/progenitor cells (Keller et al., 2004; Wang et al., 2005; Wang and Brinton, 2008; Wang et al., 2010; Brinton, 2013). In early postnatal rodent hippocampal cells as well as in neuronal progenitors and in migrating neuroblasts of the adult SVZ, GABA exhibits excitatory properties (Wang et al., 2003; Valeeva et al., 2013). This is due to a reversed intracellular/extracellular chloride concentration gradient. Thus activation of GABA$_A$Rs leads to an efflux of chloride, depolarization of the membrane potential and activation of voltage-dependent L-type calcium channels (VDLCC).

It was not one of the major objectives of this study to elucidate the mechanisms of action of the AP analogs. Nevertheless we performed a set of pharmacological experiments to examine if the novel compounds are mechanistically similar to AP or not. To investigate if analogs of AP mediate their proliferative effect via the GABA$_A$R, primary hippocampal cell cultures were treated with analogs in the presence of the GABA$_A$R antagonist bicuculline. The results of these experiments were ambiguous. In the presence of the antagonist, the effects of the two analogs, 12 oxo-AP and O-allyl-AP, were consistently reduced and were no longer statistically significant. This

could be taken to indicate that bicuculline partially blocks the action of the analogs. However, comparing the values obtained for each compound in the presence and absence of the inhibitor, the reduction of the neurosteroid effects was found not to be statistically significant. Thus, the results provided some evidence for an inhibitory action of bicuculline on neurosteroid-induced proliferation but do not allow to finally conclude that actions of AP analogs on proliferation are mediated via GABA$_A$R activation. Interestingly, although it is frequently stated that AP actions on neurogenesis involve GABA$_A$Rs, this has never been directly demonstrated (Wang et al., 2005; Wang et al., 2010; Irwin et al., 2011; Brinton, 2013; Irwin and Brinton, 2014). Rather it has been shown in an electrophysiological *in vitro* study that bicuculline can block the AP-induced rise in intracellular calcium (see below) in embryonic hippocampal neurons (Wang et al., 2008). Thus, different from the anxiolytic, sedative and anticonvulsant actions of AP which have convincingly been shown to be based on positive modulation of GABA$_A$R inhibitory transmission (Reddy, 2010), the involvement of GABA$_A$R activation in proliferative and neuroprotective effects of neurosteroids remains to be proven.

It has been reported that the proliferative effect of AP in embryonic hippocampal progenitor cell cultures requires activation of VDLCC (Wang et al., 2005) in response to GABA$_A$R-mediated membrane depolarization; therefore, we examined the effect of the VDLCC inhibitor nifedipine on proliferation in our aNSC culture system. Presence of nifedipine in the cultures indeed completely abolished the effects of 12 oxo-AP and O-allyl-AP on cell proliferation indicating that L-type calcium channels are involved in the proliferation-promoting effect of analogs in aNSCs. AP had been shown to have a selective effect on the neuronal VDLCC α1 subtypes Cav1.2 and Cav1.3 along with β3 and α2δ-1 subunits by using the whole-cell voltage-clamp technique (Earl and Tietz, 2011).

Thus, our findings are in agreement with previous studies which came to the conclusion that neurosteroid-enhanced activation of the GABA$_A$R resulting in depolarizing responses in progenitor cells increased influx of calcium through L-type channels and activation of intracellular signaling (Wang et al., 2005; Irwin et al., 2011; Brinton, 2013). However, our results do not exclude the possibility that the analogs can directly influence VDLCC. Such an interaction has been demonstrated for AP and T-type calcium channels in the peripheral nervous system (Pathirathna et al., 2005). Therefore, we are currently examining the role of GABA$_A$Rs in mediating the

proliferative effect of analogs in aNSCs by using the $GABA_AR$ antagonist bicuculline to determine if the observed effect of AP analogs on VDLCC is a consequence of $GABA_AR$ activation.

Several studies aimed to identify the structure-activity relationship of neurosteroid actions on the $GABA_AR$ (Laubach et al., 1955; Gyermek et al., 1968; Gyermek and Soyka, 1975; Sear, 1997). Studies testing the biological activity of structural analogs of AP identified functionally important structural characteristics: the geometry between rings A/B, a hydrogen-bond donor in C3 position, a hydrogen-bond acceptor in C20 position and/or flexible bond at C17 position (Purdy et al., 1990; Zorumski et al., 2000; Ragagnin et al., 2007). In particular, the 3α-hydroxyl group was described to be an essential element required for binding of the ligand to the receptor (Upasani et al., 1997). Replacing the hydrogen of the hydroxyl with methyl, thus eliminating the ability of the neurosteroid to act as a hydrogen donor in hydrogen bond formation, resulted in 250-fold reduction in potency (Upasani et al., 1997). Intriguingly, the two AP derivatives O-allyl-epiAP and O-allyl-AP which were most efficient in our experiments in stimulating proliferation and/or protecting neurons from Aβ-induced apoptosis do not fulfil these criteria. First, they do not have a free 3-OH group and would not be able to function as hydrogen donors at this position. Instead, the oxygen of the O-allyl could be a hydrogen acceptor in hydrogen bond formation. Second, in the case of O-allyl-epiAP, the O-allyl group is in the β-configuration. This was unexpected, since the β-isomer of AP has been described to be inactive at the $GABA_AR$ or even to inhibit the action of AP (Wang et al., 2000b; Wang et al., 2005). There are different possibilities to interpret these observations. Either the structural features postulated to be essential for interaction with the $GABA_AR$ must be modified or different from what was suggested by our pharmacological experiments, the analogs do not act via modulation of the $GABA_AR$. In view of the significant structural differences between AP and the analogs tested here, it is conceivable that they use different mechanisms of action. It is also imaginable that the different biological effects (proliferation, neuroprotection) involve different cellular mechanisms.

Actually, studies from many laboratories have provided evidence for additional cellular mechanisms of AP action. Our pharmacological analysis did not indicate that the neuroprotective effect of the two O-allyl analogs requires activation of the

GABA$_A$R. It also excluded that the classical progesterone receptors were involved, not unexpectedly, since there is no known enzymatic pathway to convert these compounds back to 5α-DHP. A number of other mechanisms have been described to form the basis of AP actions in different experimental paradigms and may also be considered in the context of our results.

Pregnane-X-receptors (PXR) could be candidates because of their implication in AP neuroprotective effects in reducing Aβ pathology in 3xTgAD (Chen et al., 2011) and in delaying the onset of neurological symptoms in a mouse model of Nieman-Pick type C disease (Langmade et al., 2006). Moreover, Chen and colleagues (2011) showed that in the 3xTgAD mouse model, AP treatment was only effective in reducing Aβ and AD-associated parameters when treatment was started already during the pre-pathology stage, prior to overt extraneuronal Aβ plaque formation. They found that AP induced upregulation of protein levels for PXR and also of LXR and HMG-CoA reductase, all of which are known to be involved in regulating cholesterol homeostasis by increasing cholesterol efflux. This, in turn, is thought to lead to a reduction of γ-secretase activity and thus to lower Aβ levels. Another action of AP, a peripheral analgesic effect was proposed to be mediated by T-type calcium channels and glycine membrane receptors (Pathirathna et al., 2005; Mitchell et al., 2007).

The Bax/Bcl-2 ratio is used as a key index to evaluate the apoptotic status of cells (Jung et al., 2009; Zhang et al., 2009; Zárate et al., 2010). Recent studies demonstrated APs efficiency in reducing glucose-induced oxidative stress and apoptosis by down-regulating the Bax/Bcl-2 ratio and caspase-3 activation (Afrazi and Esmaeili-Mahani, 2014; Afrazi et al., 2014). It has also been shown that AP is efficient in reducing cellular oxidative stress and subsequent apoptosis (Zampieri et al., 2009), in reducing cellular ROS levels, lipid peroxidation and activation of the NF-kB pathway. To get some insights into the mechanisms of action of the protective effect of AP analogs in aNSCs, we measured the effects of O-allyl-epiAP and O-allyl-AP on the Bax/Bcl-2 mRNA expression ratio by quantitative real-time PCR in aNSCs. Both analogs and AP decreased the Bax/Bcl-2 mRNA ratio in aNSCs indicating an anti-apoptotic effect of the neurosteroids even in the absence of an apoptosis-inducing agent. Thus, the promotion and/or the repression of Bcl-2 and Bax gene

expression appears to contribute to the protection of progenitors by AP and its analogs in the aNSC cultures.

There have been reports that AP can also influence the phosphorylation of Akt (protein kinase B) through either stimulation of $GABA_A$ receptors or inhibition of NMDA receptors (Xilouri and Papazafiri, 2006). Treatment of P19 neurons with AP reduced pro-apoptotic pathways by maintaining Akt kinase activation, and by interfering with the mitochondrial apoptotic pathway, preserving cytochrome c levels in the mitochondria and Bax levels in the cytoplasm. Similar effects of AP were also found in other models of brain injury (Djebaili et al., 2005). Other influences of AP have been observed in primary cortical neurons. There, AP was shown to regulate ATP levels and basal respiration (Grimm et al., 2014). Data from our collaborators (lab of Dr. A. Eckert, Basel) showed that O-allyl-epiAP and O-allyl-AP are able to increase ATP production and to improve mitochondrial respiration in normal and APP transfected SH-SY5Y cells (Lejri et al., in preparation). Moreover, O-allyl-epiAP protects the mitochondrial respiratory chain against oxidative stress in APP transfected SH-SY5Y cells (Lejri et al., in preparation). SH-SY5Y cells do not express the AP-binding $GABA_A$R subunits α1 and β2 (Hosie et al., 2006; Grimm et al., 2014), indicating that $GABA_A$Rs do not play a role in the effects of AP analogs on mitochondria in this neuroblastoma cell line.

It is obvious that more information is required to allow conclusions about the mechanisms involved in mediating the effects of the novel AP analogs described here. It will be interesting to perform additional pharmacological and electrophysiological experiments in the different experimental models, as these may also help to better understand the different patterns of activity of the AP analogs and their improved efficiency in comparison with the natural neurosteroid.

5. Effects of O-allyl-AP on neurogenesis in the aged brain

Since O-allyl-AP has been shown to efficiently stimulate proliferation, differentiation and to have potent neuroprotective properties *in vitro*, we initially focused our efforts on this compound and investigated whether it would counteract the age-dependent decline of neurogenic activity *in vivo*. In parallel our collaborators performed behavioral tests.

Three doses were chosen based on data from acute and chronic toxicology studies: 1, 2 and 4 mg/kg. Morphological analysis of cellular markers for stem cells, progenitor cells and neurons in the SGZ of the dentate gyrus of the hippocampus of adult 7-month-old and aged, 21-month-old mice confirmed the decrease in adult neurogenic activity due to aging.

O-allyl-AP-treated aged animals had a higher number of stem (Sox2+) and proliferating cells (Ki67+) in the dentate gyrus, with a dose of 1 mg/kg having the strongest stimulatory effect. Higher doses had no positive effect. This may indicate that at these doses toxic side effects become apparent. In fact, the initial toxicology study had shown significant anxiogenic effects with chronic administration (three injections per week for 4 weeks) of O-allyl-AP at 8 mg/kg. In general, an increased dose at the same time increases efficacy and toxicity. Therefore, it is important to carefully identify the minimum effective dose and try to optimize the treatment regimen in future experiments to reach maximal efficacy and minimal toxicity. For AP the importance of developing an optimal treatment regimen has been emphasized by Irwin and colleagues (2014). A reduced efficacy at higher doses could also be explained by development of tolerance, leading to reduced efficacy of the compound due to chronic administration. Currently there is no information about any of the pharmacologically relevant parameters of O-allyl-AP. While positive, stimulatory effects were observed for the number of stem and proliferating cells, no increase of the number of NeuroD+ cells could be demonstrated. NeuroD is a bHLH transcription factor which is transiently expressed in neural progenitors in the SGZ (von Bohlen und Halbach, 2007). The number of NeuroD+-cells is dramatically decreased in the aged brain. O-allyl-AP treatment only led to a small, non-significant increase in their number. An increase in the number of proliferating (stem) cells without a concomitant increase in the number of their progeny, suggests that the survival and differentiation of the latter is compromised by age-related factors, the effects of which are not efficiently counteracted by neurosteroid treatment, at least not with the treatment regimen employed.

Using the pattern separation test, the spatial episodic memory of mice was assessed. In rodents and humans, performance in this task is reduced with normal aging and correlated with an atrophy of the dentate gyrus and the CA3 subregion of the hippocampus (Holden and Gilbert, 2012). Newborn granule neurons have been

shown to play a critical role in mediating pattern separation (Holden et al., 2012). They become integrated into the neuronal network of the dentate gyrus 3-4 weeks after their generation and are thought to enable discrimination of similar inputs (Lacar et al., 2014). Therefore, drug treatment in our experiments lasted for 4 weeks and the behavioral experiments were performed 1 week after the last injection. Behavioral analysis showed an improvement in pattern separation with all three doses of O-allyl-AP. The discrepancy between the positive effect of O-allyl-AP with all three doses in behavioral analysis and only an effect with 1 mg/kg of O-allyl-AP in morphological analysis could be explained by the fact that mice were sacrificed 3 to 5 weeks after completion of the pattern separation test. At this time newborn cells could have already been eliminated again due to the lack of survival promoting factors missing in the aged brain. Optimization of the treatment and analysis regimen in future experiments could help to resolve this issue. Work by Chen and colleagues (2011) e.g. has shown for AP, that injecting animals once per week for 6 months was superior in promoting neurogenesis and reducing AD pathology as compared to injection every other day for 3 months or once per month.

6. Effects of O-allyl-AP and O-allyl-epiAP in Tg2576

The Tg2576 AD mouse model expresses a double mutated form of the amyloid precursor protein under the control of a promoter of the hamster prion protein (Hsiao et al., 1996). This model develops a progressive amyloid pathology. Starting at 6 months of age, Tg2576 mice have subtle learning and memory defects and show an increase in levels of soluble forms of the amyloid peptide (Jacobsen et al., 2006). At the age of 10 to 11 months, Tg2576 mice suffer from an advanced stage of amyloid pathology, with presence of amyloid plaques in the hippocampus and neocortex, and significant memory deficits (Hsiao et al., 1996; Kawarabayashi et al., 2001; Arendash and King, 2002; King and Arendash, 2002; Westerman et al., 2002; Lesné et al., 2006). Previous studies have shown for another AD mouse model (3xTgAD) that AP administration was most effective in a pre-Aβ pathology stage: AP promoted neurogenesis in the SGZ of the hippocampus, reduced Aβ pathology and restored learning and memory deficits (Wang et al., 2010; Chen et al., 2011; Singh et al., 2012). In our experiments, treatment of Tg2576 mice with the two compounds O-allyl-epiAP and O-allyl-AP was therefore initiated at 7 and 8 months of age respectively,

before the appearance of a large number of Aβ plaques. Based on initially performed acute and chronic toxicological studies O-allyl-epiAP was used at 2, 4 and 8 mg/kg and O-allyl-AP at 1, 2 and 4 mg/kg.

In the Tg2576 AD mouse model, controversial data have been reported with respect to proliferation and neurogenesis. Some studies observed a reduced proliferative activity in Tg2576 animals in comparison to their non-transgenic littermates (Dong et al., 2004), while others showed that Tg2576 mice even at an advanced age, do not exhibit reduced proliferation or neuronal loss (Irizarry et al., 1997; Krezymon et al., 2013). Dong and colleagues (2007) found a reduced synaptic density in the outer molecular layer of the dentate gyrus of the hippocampus in the vicinity of Aβ deposits.

In our experiments, with both animal cohorts (O-allyl-epiAP and O-ally-AP), we observed only a small decrease in the number of proliferation cells in vehicle treated animals, which is in accordance with results of Irizarry (1997) and Krezymon (2013). Reduction of the number of their progeny (NeuroD, DCX) was consistently larger but did not reach statistical significance. It is conceivable that even a slight reduction in proliferative activity could result in a more pronounced loss of neurons over time, so that the choice of the time point of examination is critical. Examination of animals at a more advanced stage of disease may reveal such a difference.

For O-allyl-epiAP, we could confirm our *in vitro* results by showing that it had no effect on proliferation. In contrast to the *in vitro* situation, where O-allyl-epiAP protected cells against acute Aβ42 toxicity, it was ineffective in ameliorating cognitive deficits, nor did it affect levels of Aβ42 and Aβ40 peptides. The chemical structure of the compound would not per se preclude crossing of the blood brain barrier. However, no information is available to date about its pharmacokinetic and pharmacodynamic properties.

In O-allyl-AP-treated animals, we observed a tendency of the compound to increase the number of proliferating cells (Ki67+) and neurons (DCX+ and NeuroD+) in mice treated with 1 mg/kg of O-allyl-AP. Amyloid load as measured by the concentration of Aβ42 and Aβ40 decreased in a dose-dependent manner. In the object location test O-allyl-AP-treated animals showed a small improvement at the dose of 1 mg/kg, but the results again did not reach significance. In summary, treatment with O-allyl-AP seems to have the capacity to stimulate proliferation and neurogenesis, reduce Aβ

pathology and improve cognitive deficits. While improved behavioral performance can be correlated to increased neurogenesis, as this particular test has been shown to depend on newborn neurons in the DG, it is not clear, how this AP-analog would cause a reduction of Aβ-levels. Chen and colleagues (2011) also observed a reduction of certain oligomeric Aβ species in AP-treated 3xTgAD animals. As there was no indication that APP processing was affected by AP-treatment, the underlying mechanism remained unclear.

Our results identified O-allyl-AP as a proliferative, neurogenic and neuroprotective compound, whereas O-allyl-epiAP, although being neuroprotective *in vitro*, did not show any beneficial effects *in vivo*. Unless this is related to e.g. poor bioavailability, this could suggest that a neuroprotective effect alone is not enough to ameliorate deficits associated with normal aging or neurodegenerative diseases such as AD. A combination of stimulation of neurogenesis and neuroprotection would therefore be necessary for an effective therapy.

In order to further develop AP-analogs into therapeutic tools, several points have to be taken into consideration. First of all, the treatment regimen appears to be important (reviewed by Brinton et al., 2013). Studies by Chen and colleagues (2011) have shown that an intermittent, once per week AP treatment most efficiently stimulated neurogenesis and had the most pronounced anti-amyloidogenic effects, whereas injecting animals three times per week for three months was significantly less efficient. AP had significant effects on survival of BrdU+ cells and on learning and memory in the state of intraneuronal Aβ accumulation (Wang et al., 2010). The same group, however, has shown that starting the treatment at 3 or 6 months (one injection per week for 6 months) and analyzing the animals at the end of treatment, AP still had a positive, although reduced, effect in the 6-month-group which exhibited intraneuronal Aβ at the beginning of the treatment (Chen et al., 2011). This would further emphasize the importance of an optimized treatment regimen.

Second, establishing the optimal treatment dose is critical. AP itself, e.g., exerts biphasic effects with an inverted U-shaped dose response profile (Wang et al., 2005; Wang and Brinton, 2008). Similar observations have been made in another study, which found that low (nM) levels of AP stimulate proliferation of neural progenitors, while higher (µM) concentrations are inhibitory due to conversion to allopregnanediol, which involved reduction of the keto group at C20 (Wiebe and Lewis, 2003). It is

conceivable that such a chemical conversion contributes to the lack of a positive effect of the analogs at higher concentrations. Recent data furthermore have shown that chronically elevating AP-levels within the physiological range lead to increased Aβ accumulation and impairment of learning and memory (Türkmen et al., 2006; Bengtsson et al., 2012; Bengtsson et al., 2013), further underlining the necessity to establish an optimal treatment regimen. As the maximal effect of O-allyl-AP was already detected with 1 mg/kg, lower doses should be tested in the future.

Its low molecular weight and low number of hydrogen bond donors and acceptors would make it possible in principal to administer AP by an oral route. However, the water solubility of AP and the analogs required to render them able to cross the blood-brain-barrier is low (logP-value > 5; Luchetti et al., 2010). Furthermore, their bioavailability may be limited due to first-pass metabolism in the digestive tract and liver. For that reason, formulations of AP in β-cyclodextrins were developed to enable parenteral administration (intramuscular, subcutaneous, intravenous or intranasal) (Irwin and Brinton, 2014; Irwin et al., 2014). While extensive studies have been performed for AP, no such data are available yet for the analogs used in this study.

Finally, clinical human trials with AP have revealed sex differences between male and females (van Broekhoven et al., 2007; Kask et al., 2008; Kask et al., 2009). Whether this is the case for O-allyl-AP is not known and would have to be addressed in future studies.

In conclusion, dosing regimen, formulation and route of administration are crucial parameters to be determined for O-allyl-AP to test its potential as a therapeutic candidate for AD.

CONCLUSIONS AND PERSPECTIVES

The PhD presented here shows results of experiments to test biological activities of novel synthetic analogs of the endogenous neurosteroid AP. Using cellular, biochemical and pharmacological approaches we identified analogs of AP which exhibit increased efficacy and differential patterns of biological activities and can, therefore, be considered to have improved therapeutic potential in comparison with the natural neurosteroid.

Two of the analogs, 12 oxo-AP and O-allyl-AP, were found to have the ability to promote proliferation of neural stem/progenitor cells. Both of them exhibited stronger proliferative effects than AP in mouse aNSCs, as well as in mouse and rat primary hippocampal cell cultures. They stimulated primarily the proliferation of progenitor cells. Our results revealed a relationship between the function and the chemical structure of the analogs. 3α isomers of AP analogs, including AP itself, had a proliferation promoting effect, irrespective of the substitution at C3, while the corresponding 3β isomers were ineffective. This demonstrates the importance of the α-configuration at C3 for this particular biological activity. O-allyl-AP was more potent than 12 oxo-AP in promoting proliferation in both system models. In addition, O-allyl-AP exerted a proliferative effect on human neural stem cells. Contrary to 12-oxo-AP, O-allyl-AP had the additional ability to stimulate differentiation of proliferating progenitors into neurons. These data suggest that etherification of the hydroxyl group at C3 with an allyl group resulted in enhanced effects on proliferation and an additional action on neuronal differentiation, most likely by protecting the neurosteroid from re-oxidation of the OH-group and from enzymatic degradation.

The molecular mechanisms mediating the proliferation-promoting effect of AP analogs are not clear at the moment. Our results showed that activation of L-type calcium channels is one of the signaling steps but they did not provide conclusive evidence for the involvement of $GABA_A$Rs. Thus, it remains to be clarified if analogs of AP act by potentiating $GABA_A$R responses thereby leading to enhanced chloride efflux and membrane depolarization and, as a consequence, to calcium influx through voltage-gated L-type calcium channels, a mechanism that has been postulated for AP actions on neural progenitor cells. For this, additional pharmacological experiments should be performed using $GABA_A$R agonists and

antagonists. Moreover, electrophysiological studies will allow us to directly compare characteristics of the effects of the analogs on $GABA_A$Rs to that of AP also on a quantitative level.

In addition, gene expression analysis will be performed to explore the influence of 12 oxo-AP and O-allyl-AP on expression of cell cycle activators (cyclin E, cyclin B, CDK1, PCNA) and inhibitors (DK4, CDK6, p16, p18) in aNSCs. CREB phosphorylation will be also examined using western blot. Again, this will not only provide information on signaling mechanisms but also on potential differences between the analogs and the natural neurosteroid.

The effect of O-allyl-AP on neuronal differentiation is another aspect of our results which should be investigated in more detail. To demonstrate if the analog promote neuronal fate in proliferating progenitors, primary hippocampal cell cultures will be pulse-labeled with BrdU and the phenotypes of the BrdU-incorporating cells will be analyzed after extended culture periods of up to 10 days required for the neurons to fully mature. Immunochemical analysis for different neuronal markers will reveal the cellular fate of dividing cells in response to O-allyl-AP.

Our study revealed a second activity of the AP analogs with potential clinical relevance. The O-allyl-substituted AP analogs efficiently protected aNSCs against cell death induced by amyloid beta peptide 1-42, a major etiological factor of AD. The neuroprotective potential of AP was previously shown in various experimental models. Here, we showed that analogs of AP were more potent than AP and, in particular, that they were protective in a curative strategy in which cells were treated with analogs several hours after inducing apoptosis, whereas AP was ineffective. Whether this reflects quantitative differences in efficacy or different mechanisms of action of AP and the analogs, remains to be demonstrated. The structure-activity relationship revealed that the protective effect was not isomer-specific as both the α- and the β-confirmation of O-allyl analogs of AP completely protected cells from Aβ-induced apoptosis. The substitution of the hydroxyl group by an O-allyl group at position 3 prevents both the re-oxidation of the hydroxyl into a ketone and the degradation of the neurosteroid following e.g. glucuronidation. It is likely that the resulting increase in bioavailability contributes to the higher efficacy of the analogs when compared to the endogenous neurosteroid.

Pharmacological analysis indicated that neuroprotective effects of AP analogs are not mediated via $GABA_A$Rs or by classical intracellular progesterone receptors. In view of the differential pattern of activity of AP and the stereoisomeric analogs, it would be of particular interest to clarify the mechanism involved in the respective activities. Therefore, additional pharmacological experiments should be performed to investigate the involvement of L-type calcium channels and membrane progesterone receptors in mediating the neuroprotective action of AP analogs. Furthermore, effects of AP analogs on the expression of PXR, LXR and proteins involved in cholesterol homeostasis (e.g. HMG-CoA-R, ApoE) should be examined by combining molecular and pharmacological methods.

Results of our collaborators showed that neuroprotective AP analogs exert a positive influence on mitochondrial functions. We also found that AP analogs influenced mitochondria-associated pathways by reducing caspase-3/7 signaling and decreasing the Bax/Bcl-2 ratio. These results indicate that the protective action of AP analogs against cell death involves anti-apoptotic mechanisms. Further investigations should explore the anti-apoptotic pathways activated by AP analogs.

In conclusion, the *in vitro* part of our study identified O-allyl-derivatives of AP with proliferative and/or neuroprotective effects, as promising candidates for the treatment of neurodegenerative diseases such as AD. As we observe differential effects on proliferation and protection, in the same system (aNSCs), we suggest that both effects may involve different mechanisms. Future studies should aim at gaining more insight into the molecular mechanisms underlying the activities of the AP analogs.

With the *in vitro* experiments we were able to identify AP analogs with enhanced activities in neurogenic and neuroprotective paradigms. Behavioral studies performed in parallel by our collaborators in aged mice and in an AD mouse model confirmed, at least for O-allyl-AP, that AP analogs may have therapeutical potential in the context of age-dependent and AD-related neuronal cell death and neurogenic deficits. With the aim to directly correlate behavioral observations with cellular alterations, we performed a morphological analysis of the SGZ neurogenic lineage in mice which had undergone behavioral testing. Since the treatment regimen had been designed solely on the basis of toxicological pre-tests and the number of animals was limited, the validity of the results was necessarily restricted. Nevertheless, the results we

obtained showed an interesting correlation with behavioral observations and the *in vitro* data.

In agreement with an improvement in the pattern separation test seen in aged mice treated with O-allyl-AP (1 mg/kg), these animals showed increased numbers of stem cells and proliferating progenitors as compared to age-matched controls. As already mentioned in the discussion, additional experiments need to be performed with larger numbers of animals per group and with an optimized experimental protocol: (i) lower doses of O-allyl-AP will be included to exclude toxic effects; (ii) the treatment regimen will be changed, less injection per week but for a longer period of time; (iii) mice will be injected with BrdU to trace the fate of dividing cells and to identify their phenotype in order to confirm the neurogenic effect of O-allyl-AP; (iv) mice will be sacrificed directly after behavioral studies. Neurogenic areas will be analyzed morphologically and biochemically with respect to gene expression, particularly those regulating the cell cycle, and the CREB signaling. To get information on the pharmacokinetics of O-allyl-AP, concentrations in blood samples and in the brain will be measured.

In the transgenic Tg2576 mice which reproduce the symptoms of AD, O-allyl-epiAP was tested for its ability to reduce physiopathological hallmarks of AD because of its strong neuroprotective effect observed in our *in vitro* studies. O-allyl-AP was tested for its ability to attenuate the decrease of neurogenic activity.

Morphological analysis did not show any effect of O-allyl-epiAP on the cellular composition of the SGZ. This was in line with the lack of behavioral improvements with this neurosteroid possibly indicating that neuroprotective activity is not sufficient to prevent neurogenic and cognitive deficits in the AD mice.

In O-allyl-AP-treated animals, results were consistent though statistically not significant in indicating an increase in the number of proliferating cells as well as of newborn neurons and a simultaneous reduction of Aβ42 and Aβ40 levels. To confirm these results, experiments need to be repeated with larger groups of mice, including younger animals, and with modifications in the experimental protocol as described above. With potential molecular mechanisms in mind, we will also analyze the effect of O-allyl-AP on the expression of PXR, LXR and proteins involved in cholesterol homeostasis (HMG-CoA-R, ApoE).

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DESCRIPTIF SYNTHÉTIQUE EN FRANÇAIS DES TRAVAUX DE LA THÈSE

Les maladies neurodégénératives sont des affections fréquentes du système nerveux qui touchent plus de 45 millions de personnes dans le monde et génèrent une souffrance importante chez les patients ainsi qu´un coût médico-social de plusieurs milliards d'euros par an. Les troubles neurologiques provoqués par les maladies neurodégénératives peuvent, selon la région du système nerveux concernée, affecter la mémoire, la cognition, le langage, la perception et la sensibilité à la douleur et la locomotion. Ces maladies présentent toutefois une caractéristique commune qui est la dérégulation des processus contrôlant la protection et la survie des cellules nerveuses, ce qui conduit à la dégénérescence d´une partie du système nerveux central ou périphérique et à la mort neuronale. Les mécanismes cellulaires et moléculaires impliqués dans les pathologies neurodégénératives sont encore mal connus ce qui explique l´absence de traitement efficace malgré le nombre élevé de personnes atteintes. Au cours des recherches de traitements efficaces contre les maladies neurodégénératives, il est important de prendre en considération deux concepts importants: (i) le concept de la neuroprotection qui consiste à élaborer des stratégies pour protéger les neurones contre la mort cellulaire et ainsi préserver leurs fonctions essentielles; (ii) le concept de la neurogenèse à visée thérapeutique qui repose sur la capacité intrinsèque du système nerveux à générer des nouveaux neurones pour compenser les pertes induites par les lésions, les traumatismes, les substances neurotoxiques, le stress oxydant, la sénescence ou le vieillissement cérébral. En conséquence, l'exploitation de ces deux concepts dans le cerveau adulte constitue des pistes très prometteuses pour la recherche visant à trouver de nouvelles approches thérapeutiques contre les maladies neurodégénératives.

Le neurostéroïde naturel allopregnanolone, exerce de nombreuses actions bénéfiques, comme des effets neurotrophiques et neuroprotecteurs, sur le système nerveux sans provoquer d'effets secondaires majeurs (Ciriza et al., 2004; Griffin et al., 2004; Ciriza et al., 2006; Patte-Mensah et al., 2006; Meyer et al., 2008; Wang et al., 2010; Brinton, 2013). L´allopregnanolone réduit le volume d'infarctus cérébral, améliore l'intégrité de la barrière hémato-encéphalique ainsi que la mémoire et l'apprentissage dans des modèles animaux d'AVC ischémique aigu (Sayeed et al.,

2006; Ishrat et al., 2010; Moralí et al., 2012). L´allopregnanolone atténue aussi la perte neuronale après axotomie des neurones moteurs et favorise la remyélinisation dans le système nerveux central ou périphérique (Garay et al., 2007; Garay et al., 2009; Kipp and Beyer, 2009). L´administration d'allopregnanolone diminue l'expression des protéines pro-apoptotiques (caspase-3 et Bax), la fragmentation de l'ADN apoptotique et la réponse astrocytaire au niveau du site de lésion, ce qui permet une amélioration des performances cognitives (Djebaili et al., 2004; Djebaili et al., 2005; Sayeed et al., 2009). Par ailleurs, plusieurs études de notre laboratoire ont mis en évidence l´efficacité de l´allopregnanolone contre la douleur neuropathique dans des modèles expérimentaux (Mensah-Nyagan et al., 2008; Meyer et al., 2008; Mensah-Nyagan et al., 2009; Patte-Mensah et al., 2010; Patte-Mensah et al., 2014). En effet, l´administration *in vivo* de l´allopregnanolone supprime les symptômes neuropathiques (allodynie/hyperalgésie) évoqués par les médicaments antinéoplasiques, tels que la vincristine (Meyer et al., 2010) et l'oxaliplatine (Meyer et al., 2011). De plus, l´allopregnanolone est également capable d'atténuer la douleur neuropathique provoquée chez le rat par constriction ou ligature lâche du nerf sciatique (Mensah-Nyagan et al., 2008; Meyer et al., 2008; Mensah-Nyagan et al., 2009; Patte-Mensah et al., 2010). Des travaux d'autres équipes montrent que l´allopregnanolone stimule la neurogenèse et restaure l'apprentissage et la mémoire dans un modèle de souris triple transgénique (3xTgAD) de la maladie d'Alzheimer (Wang et al., 2010; Irwin et al., 2011; Brinton, 2013). Dans ce modèle animal, l´allopregnanolone réduit la neuro-inflammation et la charge en bêta-amyloïde, tout en augmentant les marqueurs de production de la substance blanche et l'homéostasie du cholestérol (Chen et al., 2011; Irwin et al., 2011; Singh et al., 2012). L'ensemble des données évoquées ci-dessus suggèrent que les neurostéroïdes, en particulier, l'allopregnanolone, peuvent constituer des pistes intéressantes à exploiter pour le développement de stratégies efficaces contre la maladie d´Alzheimer et les pathologies neurodégénératives (Melcangi et al., 2011b; Brinton, 2013; Irwin and Brinton, 2014; Melcangi and Panzica, 2014; Patte-Mensah et al., 2014). La variété des effets de l'allopregnanolone s'explique par sa capacité à moduler divers récepteurs canaux comme les récepteurs GABA$_A$, glycine, et les canaux calciques de type T (Belelli and Lambert, 2005; Pathirathna et al., 2005; Jiang et al., 2006). En conséquence, en fonction de la cible modulée, l'allopregnanolone peut exercer des effets anti-apoptotiques pour contrecarrer les

mécanismes neurodégénératifs ou l'allopregnanolone peut promouvoir la prolifération des cellules souches neurales. Alors que l'effet proliférateur de l'allopregnanolone pourrait servir à stimuler la neurogenèse et compenser les pertes neuronales dans le cerveau des patients Alzheimer, la prolifération cellulaire évoquée par l'allopregnanolone n'est pas adaptée pour la prévention ou le traitement des symptômes neuropathiques causés par la chimiothérapie du cancer. En effet, les stratégies neuroprotectrices pour lutter contre les neuropathies périphériques chimio-induites nécessitent idéalement l'utilisation de composés neuroprotecteurs capables d'enrayer la neurotoxicité sans stimuler la prolifération cellulaire qui est contre-indiquée chez les patients cancéreux. Nous avons alors émis l'hypothèse que l'optimisation de la structure de l'allopregnanolone pourrait permettre de caractériser deux catégories différentes d'analogues structuraux (ANS): (i) une série d'ANS stimulant la neuroprolifération (à développer pour des stratégies contre les symptômes de la maladie d´Alzheimer) et (ii) une deuxième série d'ANS (à utiliser dans les stratégies contre les neuropathies périphériques chimio-induites) qui seraient capables de réduire sélectivement la neurotoxicité sans induire d'effets proliférateurs indésirables. Pour vérifier notre hypothèse, nous avons travaillé en collaboration avec des chimistes (Equipe du Dr M. Miesch, UMR 7177) qui ont synthétisé 4 ANS de l'allopregnanolone à savoir le Pregnane-12,20 dione 3-hydroxy (3α, 5α) ou 12 oxo-AP, le Pregnane-12,20 dione 3-hydroxy (3β, 5α) ou 12 oxo-epiAP, le Pregnan-20 one 3-(2-propen-1-yloxy) (3α, 5α) ou O-allyl-AP et le Pregnan-20 one 3-(2-propen-1-yloxy) (3β, 5α) ou l'O-allyl-epiAP.

Ces analogues structuraux de l´allopregnanolone ont fait l'objet d'une étude multidisciplinaire dans le cadre de notre travail de thèse dont les principaux objectifs sont les suivants:

1- Déterminer les effets des ANS sur la neurogenèse adulte dans le cerveau des rongeurs, plus spécifiquement, examiner l´action des ANS sur la prolifération et la différenciation des cellules souches neurales adultes de la zone périventriculaire du cerveau de souris.

2- Evaluer la capacité des ANS à protéger les cellules souches neurales adultes contre l'apoptose induite par le peptide beta amyloïde 1-42 (Aβ42) impliqué dans l´étiologie de la maladie d´Alzheimer.

3- Vérifier si les effets des ANS sur les cellules souches neurales adultes sont reproductibles sur des cultures primaires des cellules de l'hippocampe qui joue un rôle crucial dans la régulation des fonctions cognitives et mnésiques altérées par les pathologies neurodégénératives.

4- Evaluer l´action des ANS contre le déclin de la neurogenèse et le déficit cognitif liés au vieillissement normal du cerveau.

5- Tester la capacité des ANS à améliorer les performances cognitives des souris transgéniques Tg2576 qui reproduisent les symptômes de la maladie d´Alzheimer.

Pour étudier l'effet proliférateur des ANS, les cellules souches neurales adultes de souris ont été prétraitées avec les ANS pendant 24 heures avant d´être incubées avec du BrdU/EdU (analogues de la thymidine), pendant deux heures et d´être fixées pour le marquage des cellules en phase S du cycle cellulaire. Les cellules incorporant le BrdU/EdU ont été identifiées par immunocytochimie. Nos résultats montrent que seuls deux des 4 ANS, à savoir le 12 oxo-AP et l´O-allyl-AP, stimulent significativement la prolifération des cellules souches neurales adultes avec un effet proliférateur plus important que celui exercé par l´allopregnanolone. Ces résultats suggèrent que la position isomérique 3α dans la structure des ANS est déterminante pour la potentialisation de l´effet proliférateur exercé sur les cellules souches neurales adultes. Des expériences de double marquage immunocytochimique ont permis d´identifier le phénotype des cellules qui dérivent de la prolifération des cellules souches neurales adultes évoquée par les ANS. Les cellules ayant incorporé le BrdU ont donc été marquées simultanément soit (i) avec un anticorps dirigé contre la Nestine, un marqueur des cellules progénitrices, (ii) avec un anti-GFAP, un marqueur des astrocytes ou (iii) avec un anticorps contre la protéine βIII-tubuline ou Tuj-1 présente dans les neurones immatures post-mitotiques. Les résultats ont révélé que la grande majorité des cellules en prolifération sont des cellules progénitrices nestine-positives. Il était alors important de savoir si ces cellules progénitrices

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(nestine-immunoréactives) issues de la prolifération des cellules souches neurales adultes induite par les ANS sont capables de se différencier en neurones. Pour résoudre cette question, nous avons réalisé des expériences supplémentaires avec un anticorps dirigé contre la doublecortine (DCX), une protéine associée aux microtubules qui est fortement exprimée dans le soma et les axones des neurones en différenciation. Nos résultats montrent que l'administration de l'O-allyl-AP aux cellules souches neurales adultes pendant la phase de croissance augmente le nombre de neurones DCX-immunoréactifs sans modifier le nombre d'astrocytes GFAP-positifs. Cet effet est amplifié lorsque l'O-allyl-AP est maintenu dans le milieu de cultures pendant la phase de différenciation des cellules, ce qui confirme la capacité de l'O-allyl-AP à stimuler fortement la différenciation des cellules progénitrices (nestine-positives) en neurones (DCX-immunoréactifs).

Le deuxième objectif important de notre travail de thèse était d'évaluer l'effet protecteur des ANS contre la cytotoxicité provoquée par le peptide Aβ42 dont l'accumulation dans le cerveau constitue l'un des principaux facteurs étiologiques de la maladie d´Alzheimer. Pour ce faire, nous avons exposé les cellules souches neurales adultes au peptide Aβ42 pendant 24 heures et mesuré ensuite grâce à une approche fluorimétrique l´activité des caspases-3/7 cytoplasmiques témoignant de l'apparition d'une apoptose cellulaire. Deux stratégies différentes ont été utilisées pour évaluer la capacité des ANS à exercer un effet protecteur contre l'apoptose induite par le peptide Aβ42. Une stratégie "prophylactique" consistant à prétraiter les cellules souches neurales adultes avec les ANS avant de les exposer au peptide Aβ42 et une stratégie "corrective" dans laquelle l´application des ANS intervient après l'exposition des cellules souches neurales adultes au peptide Aβ42. Les mesures fluorimétriques réalisées montrent que, quelle que soit la stratégie d'administration des ANS utilisée, les deux analogues O-allyl (O-allyl-AP et O-allyl-epiAP) sont les composés neuroprotecteurs les plus puissants qui exercent une forte protection des cellules souches neurales adultes contre l'apoptose Aβ42-dépendante et cette protection est plus importante que celle évoquée par l'allopregnanolone. En effet, le traitement des cellules souches neurales adultes avec chacun des deux ANS O-allyl permet de maintenir ou de restaurer dans les cellules souches neurales adultes un niveau basal d'activité caspases-3/7 similaire au niveau basal observé dans les cellules témoins ou non-apoptotiques qui n'ont pas été exposées au peptide

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Aβ42. Ces données suggèrent que l'éthérification du groupe 3-OH dans la structure de l´allopregnanolone entraîne une amélioration ou une amplification de la capacité neuroprotectrice de ce neurostéroïde.

Pour atteindre le troisième objectif de notre thèse, nous avons utilisé des cultures primaires d'hippocampe de rats et de souris nouveaux-nés pour réaliser les mêmes séries de travaux effectuées avec les cellules souches neurales adultes. De façon intéressante, nos résultats montrent que sur les cultures primaires de cellules d'hippocampe, les ANS reproduisent les mêmes effets proliférateurs comme observés sur les cellules souches neurales adultes. En effet, les deux ANS, 12 oxo-AP et O-allyl-AP, demeurent toujours plus efficaces que l'allopregnanolone dans la stimulation de la prolifération des cultures primaires des cellules de l'hippocampe et plus spécifiquement des cellules progénitrices BrdU/nestine-positives.

Il est bien connu que l'âge est le principal facteur de risque des maladies neurodégénératives. La baisse de la neurogenèse adulte est aussi identifiée comme étant un des principaux facteurs qui contribuent à la genèse des déficits cognitifs associés aux maladies neurodégénératives (Ming and Song, 2011; Walter et al., 2011; Braun and Jessberger, 2014; Winner and Winkler, 2015). Encouragés par les résultats de nos études *in vitro* montrant les effets proliférateurs, stimulateurs de la différenciation neuronale des cellules progénitrices, anti-apoptotiques et neuroprotecteurs de certains ANS, nous avons décidé de tester la capacité de ces composés à contrecarrer *in vivo* le déclin de la neurogenèse lié à l'âge et à améliorer ainsi les performances cognitives des animaux âgés. Etant donné que le composé O-allyl-AP s'est révélé très efficace *in vitro* à la fois sur la prolifération, la différenciation neuronale et la neuroprotection des cellules, nous avons dans un premier temps focalisé nos efforts sur ce composé. Des souris âgées de 21 mois ont été traitées avec trois doses différentes (1, 2 et 4 mg/kg) du composé O-allyl-AP pendant un mois à raison de trois injections par semaine. La mémoire spatiale des souris a été évaluée grâce au test comportemental du *Pattern Separation* qui est connu comme étant sensible à l'activité neurogénique dans le gyrus denté de l'hippocampe adulte (Sahay et al., 2011a; Sahay et al., 2011b; Holden and Gilbert, 2012; Holden et al., 2012; Nakashiba et al., 2012; Hunsaker and Kesner, 2013). Cette étude

comportementale a été complétée par des analyses histologiques effectuées sur des prélèvements de cerveaux des souris préalablement testées dans le *Pattern Separation test*. En particulier, nous avons utilisé des marqueurs cellulaires spécifiques pour évaluer le nombre de cellules souches, de cellules en prolifération et de cellules en différenciation dans la zone sous-granulaire du gyrus denté de l'hippocampe. Nos résultats montrent que l'O-allyl-AP (1 mg/kg) permet une amélioration des performances des souris âgées dans le *Pattern Separation test* qui est positivement corrélée à une augmentation du nombre de cellules souches neurales adultes et de cellules progénitrices dans le gyrus denté de l'hippocampe.

Pour atteindre notre 5ème objectif consistant à vérifier l'éventualité d'une action bénéfique des ANS chez les souris transgéniques Tg2576 reproduisant les symptômes de la maladie d´Alzheimer (Hsiao et al., 1996), nous avons utilisé un protocole similaire à celui décrit ci-dessus mais le test comportemental réalisé est celui du déplacement d'objets qui est bien validé pour l'évaluation des déficits cognitifs dans le modèle Tg2576 (Yassine et al., 2013). Par ailleurs, en plus des études histopathologiques pour l'évaluation du nombre de cellules prolifératrices et de neurones nouvellement générés, nous avons réalisé des analyses ELISA pour déterminer l'effet des ANS sur la concentration cérébrale des peptides $A\beta42$ et $A\beta440$ chez les souris Tg2576. Les animaux ont été traités avec l'O-allyl-AP (1, 2 ou 4 mg/kg) ou l'O-allyl-epiAP (2, 4 ou 8 mg/kg). Nos résultats préliminaires montrent une tendance de l'O-allyl-AP à la dose de 1 mg/kg à améliorer la performance des souris Tg2576 dans le test de déplacement d'objets. Cette observation est corrélée d'une part, à une augmentation du nombre de cellules prolifératrices et du niveau de neurogenèse, et d'autre part à une diminution de la concentration cérébrale des peptides $A\beta42$ et $A\beta40$. En revanche, l'O-allyl-epiAP semble être inefficace aussi bien sur la performance cognitive des souris Tg2576 que sur l'amélioration des paramètres cellulaires et biochimiques évalués. Néanmoins, il convient de signaler que toutes nos données préliminaires sur les souris Tg2576 nécessitent d'être vérifiées davantage avec des effectifs d'animaux plus importants pour valider les analyses statistiques avant de faire des déductions appropriées.

En conclusion, notre travail de thèse a permis de caractériser avec succès des analogues structuraux de l´allopregnanolone présentant pour certains d'entre eux des effets bénéfiques et des avantages par rapport à la molécule de référence. En particulier, nous avons identifié un ANS, notamment l'O-allyl-AP, qui est un composé très prometteur. En effet, l'O-allyl-AP, qui stimule *in vitro* la prolifération des cellules nerveuses, la différenciation neuronale et protège les cellules souches neurales adultes contre l'apoptose ou la cytotoxicité induite par le peptide Aβ42, est aussi efficace *in vivo* pour contrecarrer le déclin de la neurogenèse lié à l'âge et améliorer les performances cognitives au cours du vieillissement. L´ensemble de nos résultats ouvre des perspectives intéressantes pour l´utilisation des ANS dans le développement de stratégies thérapeutiques contre la maladie d´Alzheimer et les maladies neurodégénératives.

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ZUSAMMENFASSENDE BESCHREIBUNG DER ARBEIT AUF DEUTSCH

Neurodegenerative Erkrankungen sind häufige Erkrankungen des Nervensystems, welche mehr als 45 Millionen Menschen weltweit betreffen und erhebliche Leiden für die Patienten, sowie medizinische und soziale Kosten von mehreren Milliarden Euro pro Jahr verursachen. Neurodegenerative Erkrankungen können abhängig von der betroffenen Gehirnregion, Gedächtnis, Kognition, Sprache, Sensibilität, Schmerzwahrnehmung und die Motorik betreffen. Allen gemeinsam ist jedoch eine Störung von Prozessen, die Schutz und Überleben von Nervenzellen regulieren, was letztlich zur Degeneration von Teilen des zentralen oder peripheren Nervensystems und zum Absterben von Neuronen führt. Die zellulären und molekularen Mechanismen, die für die Entstehung von neurodegenerativen Erkrankungen verantwortlich sind, sind noch weitgehend unverstanden, was das Fehlen von wirksamen Therapien erklärt, trotz der hohen Zahl betroffener Menschen. Im Zusammenhang mit der Entwicklung wirksamer Ansätze zur Therapie neurodegenerativer Erkrankungen sind zwei wichtige Gesichtspunkte zu berücksichtigen: (i) Neuroprotektion, d.h. der Schutz von Nervenzellen vor dem Zelltod und die Erhaltung ihrer Funktionen; (ii) die Steigerung der intrinsischen Fähigkeit des Nervensystems neue Nervenzellen zu bilden (Neurogenese), um den Zellverlust durch Verletzungen, Traumata, neurotoxische Substanzen, oxidativen Stress oder Alterung zu kompensieren.

Das endogene Neurosteroid Allopregnanolon hat ein breites Spektrum positiver neurotropher und neuroprotektiver Wirkungen im Nervensystem, ohne gravierende Nebenwirkungen aufzuweisen (Ciriza et al., 2004; Griffin et al., 2004; Ciriza et al., 2006; Patte-Mensah et al., 2006; Meyer et al., 2008; Wang et al., 2010; Brinton, 2013). Allopregnanolon reduziert z.B. das infolge eines Infarkts geschädigte Volumen, hat positive Wirkungen auf die Integrität der Blut-Hirn-Schranke sowie auf Lernen und Gedächtnis in Tiermodellen für akuten ischämischen Schlaganfall (Sayeed et al., 2006; Ishrat et al., 2010; Morali et al., 2012). Es reduziert das Absterben von Motorneuronen nach Axotomie und fördert die Remyelinisierung im zentralen oder peripheren Nervensystem (Garay et al., 2007; Garay et al., 2009; Kipp and Beyer, 2009). Die Behandlung mit Allopregnanolon reduziert weiterhin die

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Expression der pro-apoptotischen Proteine Bax und Caspase-3, die DNA Fragmentierung und die Astrozytenaktivierung an der Läsionsstelle und verbessert die kognitive Leistung (Djebaili et al., 2004; Djebaili et al., 2005; Sayeed et al., 2009). Untersuchungen unserer Arbeitsgruppe haben darüber hinaus gezeigt, dass Allopregnanolon die Entstehung neuropathischer Schmerzen verhindert (Mensah-Nyagan et al., 2008; Meyer et al., 2008; Mensah-Nyagan et al., 2009; Patte-Mensah et al., 2010; Patte-Mensah et al., 2014). So verhindert eine *in vivo* Behandlung mit Allopregnanolon neuropathischen Schmerz (Allodynie/Hyperalgesie), der sich häufig in Folge einer Zytostatika Behandlung mit Vincristin (Meyer et al., 2010) oder Oxaliplatin (Meyer et al., 2011) entwickelt. Auch neuropathischer Schmerz nach Kompression peripherer Nerven wird im Modell der Ligation des N. ischiadicus der Ratte reduziert (Mensah-Nyagan et al., 2008; Meyer et al., 2008; Mensah-Nyagan et al., 2009; Patte-Mensah et al., 2010). Arbeiten anderer Arbeitsgruppen haben gezeigt, dass Allopregnanolon die Neurogenese stimuliert und die Lern- und Gedächtnisleistung in einem Mausmodell der Alzheimer-Krankheit (3xTgAD) erhöht (Wang et al., 2010; Irwin et al., 2011; Brinton, 2013). In diesem Tiermodell reduziert Allopregnanolon auch neuroinflammatorische Parameter und die Beta-Amyloid-Level, steigert die Expression von Markern der weißen Substanz und verbessert die Cholesterin-Homöostase (Chen et al., 2011; Irwin et al., 2011; Singh et al., 2012). Die oben aufgeführten Daten zeigen, dass Neurosteroide, insbesondere Allopregnanolon, interessante Kandidaten für die Entwicklung wirksamer Therapiestrategien bei Alzheimer und neurodegenerative Erkrankungen sind (Melcangi et al., 2011; Brinton, 2013, Irwin and Brinton, 2014; Melcangi and Panzica, 2014; Patte-Mensah et al., 2014). Die Vielzahl der Wirkungen von Allopregnanolon beruhen auf einer Vielzahl von Zielmolekülen und Mechanismen, die beeinflusst werden können: GABA$_A$-Rezeptoren, Glyzinrezeptoren, T-Typ-Calciumkanäle (Belelli and Lambert, 2005; Pathirathna et al., 2005; Jiang et al., 2006). Dadurch kann Allopregnanolon anti-apoptotische Wirkungen entfalten und Neurodegeneration verhindern sowie die Proliferation von neuralen Stammzellen fördern. Allerdings wäre die proliferationsfördernde Wirkung von Allopregnanolon, die im Zusammenhang mit der Kompensation von Zellverlust bei neurodegenerativen Erkrankungen wünschenswert ist, bei der Behandlung des neuropathischen Schmerzes infolge einer Chemotherapie-Behandlung von Tumoren kontraindiziert, da dadurch das Wachstum nicht eliminierter Tumorzellen stimuliert werden könnte. Die vorliegende

Arbeit basiert auf der Hypothese, nach der Strukturanaloga von Allopregnanolon (ANS) identifiziert werden können, die (i) die Proliferation von neuralen Stamm-/Vorläuferzellen stimulieren können und gleichzeitig neuroprotektiv sind, z.B. für die Entwicklung einer Therapieoption für Morbus Alzheimer und die (ii) selektiv neuroprotektiv wirken, ohne gleichzeitig die Proliferation zu stimulieren (zur Anwendung bei chemisch induzierter peripherer Neuropathie). Dazu wurden von unseren Kooperationspartnern (Dr. M. Miesch, UMR 7177, Strasbourg) 4 Allopregnanolon-Analoge synthetisiert: Pregnane-12,20 dione 3-hydroxy- (3α, 5α) oder 12 oxo-AP; Pregnan-20 one 3-(2-propen-1-yloxy) (3β, 5α) oder 12 oxo-epiAP; Pregnane-12,20 dione 3-hydroxy- (3β, 5α) oder O-allyl-AP; Pregnan-20 one 3-(2-propen-1-yloxy) (3α, 5α) oder O-allyl-epiAP.

Die Charakterisierung dieser Strukturanaloga von Allopregnanolon stand im Zentrum einer multidisziplinären Studie mit den folgenden Hauptzielen:

1- Untersuchung ihrer Wirkung auf die Neurogenese im Gehirn von Nagetieren, insbesondere auf die Proliferation und Differenzierung von adulten neuralen Stammzellen der Subventikular Zone (SVZ).

2- Evaluation, ob Apoptose neuraler Stamm-/Progenitorzellen, induziert durch Beta-Amyloid 1-42 (Aβ42), das in der Ätiologie von Morbus Alzheimer eine zentrale Rolle spielt, durch die Analoga verhindert werden kann.

3- Da der Hippokampus, dem bei kognitiven und Gedächtnisfunktionen eine besondere Rolle zukommt, bei neurodegenerativen Erkrankungen eine zentrale Rolle spielt, soll untersucht werden, inwieweit Effekte auf neurale Stammzellen auch auf Zellen des Hippokampus übertragen werden können.

4- Untersuchung der Wirkung der ANS auf die Reduktion der Neurogeneseaktivität im Verlauf der physiologischen Alterung des Gehirns.

5- Überprüfung, ob ANS positive Wirkungen auf Neurogenese und kognitive Leistung in einem Alzheimer Mausmodell (Tg2576) haben.

Um die proliferationsfördernde Wirkung von ANS zu untersuchen, wurden adulte neurale Stammzellen der Maus für 24 Stunden damit behandelt. Anschließend wurden die Zellen für zwei Stunden mit BrdU bzw. EdU (Thymidin-Analoga) inkubiert, um die Zahl der Zellen bestimmen zu können, die sich in der S-Phase des Zellzyklus befinden. Dies erfolgte durch immunzytochemische Färbung und Zellzählung. Unsere Ergebnisse zeigen, dass zwei der 4 Analoga, nämlich 12-Oxo- und O-allyl-AP, die Proliferation von adulten neuralen Stammzellen signifikant stimulieren, wenn sie an der Position C-3 die α-Konfiguration aufweisen. Die 3β-Formen dieser Analoga haben keine proliferationsfördernde Wirkung. Immunzytochemische Doppelmarkierungen wurden dazu eingesetzt den Phänotyp der proliferierenden Zellen zu bestimmen. BrdU/EdU-inkorporierende Zellen wurden dazu zusätzlich mit folgenden Antikörpern markiert (i) gegen Nestin, einem Marker für Vorläuferzellen (ii) gegen GFAP, einem Marker für Astrozyten oder (iii) gegen βIII-Tubulin (Tuj-1) für post-mitotische unreife Neurone. Dabei zeigte sich, dass die Mehrzahl der proliferierenden Zellen Nestin-positive Vorläuferzellen sind. Im nächsten Schritt wurde untersucht, ob ANS auch die Differenzierung der Nachkommen der Vorläuferzellen beeinflussen. Dafür wurden die Zellen nach Differenzierung mit Antikörpern gegen Doublecortin (DCX), einem Mikrotubuli-assoziierten Protein, das vor allem im Soma und Axon differenzierender Neurone exprimiert wird, gefärbt. Die Behandlung adulter neuraler Stammzellen mit dem O-allyl-Analogon während der Wachstumsphase erhöht die Zahl neuronal differenzierender Zellen. Die Zahl glial differenzierender Zellen bleibt unverändert. Dieser Effekt ist noch ausgeprägter, wenn O-allyl-AP auch während der Differenzierung anwesend ist. Zusammengefasst zeigen diese Ergebnisse, dass das O-allyl-Analogon in der Lage ist, sowohl die Proliferation von Vorläuferzellen, als auch deren neuronale Differenzierung zu stimulieren.

Das zweite wichtige Ziel dieser Arbeit war es, die protektive Wirkung der Analoge gegenüber der zytotoxischen Wirkung von Aβ42, das eine zentrale Rolle für die Ätiologie von Morbus Alzheimer spielt, zu untersuchen. Dafür wurden Kulturen neuraler Stammzellen für 24 Stunden mit dem Aβ42 Peptid behandelt. Anschließend wurde die Apoptose durch eine fluorimetrische Bestimmung der Aktivität von Caspase 3/7 quantifiziert. Zwei Behandlungsstrategien wurden untersucht: eine "prophylaktische" Behandlung, bei der ANS vor Aβ42 gegeben wurden und eine

"therapeutische" Behandlung, bei der ANS erst nach der Applikation von Aβ42 zugegeben wurden. Die Ergebnisse zeigen, dass unabhängig von der Art der Applikation, die beiden O-allyl-Analoga (O-allyl-AP und O-allyl-epiAP) am wirksamsten neuroprotektiv wirken, und adulte neurale Stammzellen vollständig vor der Zelltod-induzierenden Wirkung von Aβ42 schützen können. In allen Behandlungsschemata entspricht die Apoptoseaktivität der von Kontrollzellen, bzw. liegt sogar etwas darunter, was darauf hindeutet, dass diese Analoge sogar eine generell protektive Wirkung besitzt. Die neuroprotektive Wirkung ist dabei unabhängig von der Konformation der Äthergruppe an C-3 des Moleküls.

Um das dritte Ziel dieser Arbeit zu erreichen, wurden dissoziierte Primärkulturen des Ratten-bzw. Maus Hippokampus verwendet. Interessanterweise zeigen unsere Ergebnisse, dass ANS, die in Kulturen neuraler Stammzellen proliferationsfördernd wirken, dies auch in Kulturen hippokampaler Zellen tun. Auch in diesem Kultursystem ist die Wirkung von 12 oxo-AP und AP-O-Allyl größer als die von Allopregnanolon.

Es ist bekannt, dass Alter der Hauptrisikofaktor für neurodegenerative Erkrankungen ist. Neben dem Untergang von Neuronen spielt auch der Rückgang der Neurogeneseaktivität für das Entstehung kognitiver Defizite eine Rolle (Ming and Song, 2011; Walter et al., 2011; Jessberger and Braun, 2014; Sieger and Winkler, 2015). Die Ergebnisse unserer *in vitro* Experimente hatten gezeigt, dass ANS die Proliferation und Differenzierung neuraler Stamm-/Vorläuferzellen stimulieren und zusätzlich neuroprotektive Wirkung haben. Um nun zu überprüfen, ob dies auch für die *in vivo* Situation gilt, haben wir zunächst untersucht, ob sie auch in der Lage sind, dem altersbedingten Rückgang von Neurogenese und kognitiver Leistung entgegen zu wirken. Da sich in den *in vitro* Untersuchungen das O-allyl-Analoge als sehr wirksam erwiesen hat, konzentrierten wir unsere Bemühungen zunächst darauf. 21 Monate alten Mäusen wurden dazu für einen Monat (drei Injektionen pro Woche) mit drei verschiedenen Dosen (1, 2 und 4 mg/kg) O-allyl-AP behandelt. In Verhaltenstests (pattern separation) wurde dann zunächst das räumliche Gedächtnis der Tiere untersucht. Die Leistung in diesem Verhaltenstest korreliert mit der Neurogeneseaktivität im Hippokampus (Sahay et al., 2011a; Sahay et al., 2011b; Holden and Gilbert, 2012; Holden et al., 2012; Nakashiba et al., 2012; Hunsaker and

Kesner, 2013). Danach wurde das Gewebe für morphologische Untersuchungen aufgearbeitet und mit zellspezifischen Markern die Zahl von Stammzellen, proliferierenden Zellen und differenzierenden Zellen bestimmt. Unsere Ergebnisse zeigen, dass O-allyl-AP (1 mg/kg) die Leistung im Verhaltenstest steigert. Dies korreliert mit einer Zunahme der Anzahl von adulten neuralen Stammzellen und Vorläuferzellen im Gyrus dentatus des Hippokampus.

Schließlich wurde getestet, ob Behandlung mit ANS einen positiven Effekt in einem Mausmodell der Alzheimer-Krankheit hat (Tg2576 transgene Mäuse) (Hsiao et al., 1996). Auch hierfür wurden Verhaltenstest (Yassine et al., 2013) und morphologische Analysen kombiniert. Zusätzlich zu den histopathologischen Untersuchungen zur Quantifizierung der Anzahl proliferierender Zellen und neu entstandener Neurone, führten wir ELISA-Assays durch, um die Wirkung von ANS auf die Konzentration der toxischen Peptide Aβ42 und Aβ40 zu bestimmen. Die Tiere wurden mit O-allyl-AP (1, 2 bzw. 4 mg/kg) oder O-Allyl-epiAP (2, 4 oder 8 mg/kg) behandelt. Unsere vorläufigen Ergebnisse deuten darauf hin, dass O-Allyl-AP in einer Dosis von 1 mg/kg die kognitive Leistung von Tg2576 Mäuse im Verhaltenstest verbessert. Dies korreliert einerseits mit einer höheren Zahl proliferierender Zellen, andererseits mit einer erniedrigten Konzentration von Aβ42 und Aβ40. Anders als *in vitro* hatte O-Allyl-epiAP keine Wirkung auf die untersuchten Parameter. An dieser Stelle sei darauf hingewiesen, dass unsere Daten bislang auf einer relativ geringen Zahl von Tieren beruhen und dass aufgrund großer interindividueller Unterschiede größere Kohorten von Tieren untersucht werden müssten, um diese Ergebnisse zu validieren.

Zusammenfassend lässt sich sagen, dass es gelungen ist Strukturanaloga von Allopregnanolon zu synthetisieren, die einerseits besser wirksam sind als das Referenzmolekül und darüber hinaus ein differentielles Wirkungsspektrum aufweisen. Vor allem das O-allyl-Analogon erwies sich dabei als vielversprechend: es stimuliert *in vitro* die Proliferation von Nervenzellen, die neuronale Differenzierung und schützt adulte neuralen Stammzellen vor der zytotoxischen Wirkung von β-Amloid. *In vivo* wirkt es dem altersbedingten Rückgang der Neurogenese entgegen, verbessert die kognitiven Leistungen und reduziert die Konzentration von Aβ. Insgesamt ergeben sich aus diesen Ergebnissen interessante Perspektiven für die Entwicklung von

Strategien für die Behandlung von Alzheimer und anderen neurodegenerativen Erkrankungen.

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Mona Karout

Multidisciplinary analysis of biological effects of novel analogs of the neurosteroid allopregnanolone: evidence for a proliferative, neurogenic and neuroprotective action

RESUME:

Ce travail de thèse a permis de caractériser avec succès des analogues structuraux de l´allopregnanolone présentant pour certains d'entre eux des effets bénéfiques et des avantages par rapport à la molécule de référence. En particulier, l'analogue O-allyl-AP, qui stimule *in vitro* la prolifération des cellules progénitrices, la différenciation neuronale et protège les cellules souches neurales adultes contre l'apoptose induite par le peptide Aβ42, est aussi efficace *in vivo* pour contrecarrer le déclin de la neurogenèse lié à l'âge et améliorer les performances cognitives au cours du vieillissement. De façon intéressante, les effets proliférateur et neuroprotecteur de l´O-allyl-AP semblent impliquer différents mécanismes d'action. Des expériences supplémentaires sont nécessaires pour conclure sur la capacité de l´O-allyl-AP à stabiliser le déclin de l'activité neurologique et à réduire les caractéristiques physiopathologiques de la Maladie d'Alzheimer (MA) chez les souris Tg2576. Nos résultats ouvrent des perspectives intéressantes pour l'application de l´O-allyl-AP dans le développement de stratégies thérapeutiques contre la MA et les maladies neurodégénératives.

Mots clés: Allopregnanolone, Neurogenèse, neuroprotection, cellules souches neurales adultes, hippocampe, beta amyloïde.

ABSTRACT:

This PhD work allowed us to successfully characterize structural analogs of allopregnanolone. Some of these analogs showed beneficial effects and advantages with respect to the molecule of reference. In particular, the analog O-allyl-AP stimulates proliferation of progenitor cells in different neural *in vitro* models, neuronal differentiation and protects adult neural stem cells against Aβ-induced apoptosis. In addition, O-allyl-AP is effective in counteracting the decline in neurogenesis related to age and in improving cognitive performance during aging. Interestingly, proliferative and neuroprotective effects seem to involve different mechanisms of action. Additional experiments are needed to confirm our preliminary data about the ability of O-allyl-AP to attenuate the decrease of neurogenic activity and to reduce pathophysiological hallmarks of Alzheimer disease (AD) in Tg2576 mice. Our findings provide interesting perspectives for using O-allyl-AP in the development of therapeutic strategies against AD and other neurodegenerative diseases.

Keywords: Allopregnanolone, neurogenesis, neuroprotection, adult neural stem cells, hippocampus, beta amyloid.