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Identification of functional peroxisome proliferator-activated receptor gamma (PPARγ) in mouse brain and its implication in Alzheimer's disease

Dissertation

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This work was supervised by Prof. Osborne Almeida at the Max Planck of Psychiatry and funded by the EU FP7 Consortium SWITCHBOX.

“Stupid is as stupid does.”

- *Forrest Gump*

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ABSTRACT

The risk of developing Alzheimer's disease (AD) depends on a combination of genetic and environmental factors. Disorders of metabolism (e.g. obesity, insulin resistance) are strongly linked with propensity to develop AD. The nuclear receptor, peroxisome proliferator-activated receptor (gamma subtype; PPARγ) is a key regulator of adipocyte differentiation and glucose metabolism and stimulation of PPARγ with thiazolidinediones (TZD) have been reported to improve cognition in mouse models of AD as well as in AD patients. However, there is sparse information regarding the sites of mechanisms of TZD in the brain, leaving the possibility that their effects on brain function may be secondary to improvements in peripheral fat and glucose metabolism. This work aimed to address this issue as an important step in consideration of targeting PPARγ in the delay or treatment of AD.

Here, a comprehensive mapping of PPARγ protein expression in the juvenile and adult mouse brain revealed that PPARγ are present in brain structures affected by AD pathology (frontal cortex, hippocampus) as well as areas implicated in diverse behaviours (e.g. mood, anxiety, addiction, feeding behaviour). Further, analysis of mouse primary frontocortical and hippocampal cultures revealed PPARγ expression in neurons, astrocytes but not in oligodendrocytes (microglia were not detectable in our cultures). Also reported is that functional PPARγ receptors are expressed in primary cultures of the mouse frontal cortex and hippocampus as well as in a human neural cell line; upon stimulation with pioglitazone (Pio, a potent TZD), these cells respond with an upregulation of known PPARγ target genes (*ABCB1* and *PGC-1α*); specificity of these effects was confirmed using a specific PPARγ antagonist (GW9662).

Investigations into the impact of PPARγ agonism on AD pathology focused on amyloid precursor protein (APP) misprocessing into amyloid β (Aβ) and the abnormal hyperphosphorylation of tau protein. Results showed that Pio attenuates Aβ-induced synaptic degradation and neurotoxicity, both are associated with increased misprocessing of APP and aberrant hyperphosphorylation of tau. Further, Pio treatment reduced APP levels and downregulated the expression of β- and γ-secretase (responsible for cleaving APP into Aβ) in Aβ-treated human neural cells (SH-SY5Y). Furthermore, Pio-pretreatment alleviate the generation of tau species that are phosphorylated at certain epitopes known to be involved

in the formation of insoluble tau aggregates that give rise to the tau tangle pathology that typifies the AD brain. In addition, it was demonstrated that Pio likely abrogates synaptotoxicity by interfering with accumulation of phosphorylated tau at the synapse, a phenomenon that activates the src-kinase Fyn which, in turn, leads to the degradation of the post-synapse via postsynaptic receptors (Glutamate [NMDA] receptor subunit epsilon-2 and postsynaptic density protein 95).

The results reported here support the view that PPARγ agonism may be helpful in the clinical management of AD; accordingly, the thesis discusses further steps towards translation of the present findings.

LIST OF ABBREVIATION:

15d-PGJ2	15-Deoxy-Delta-12, 14-prostaglandin J2
Aβ	Amyloid β
ABCA1	ATP-binding cassette transporter A1
ABCB1	ATP-binding cassette transporter B1
AcbC	Accumbens nucleus, core
AcbS	Accumbens nucleus, shell
AD	Alzheimer's disease
ADAM:	A disintegrin and metalloprotease 10
AF2	Activation function helix
AICD	Amyloid precursor protein intracellular domain
AKT	Protein kinase B
AMP	Adenosine monophosphate
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AMPK	AMP-activated protein kinase
aP2	Adipocyte Protein 2
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
AR	Androgen receptor
Arc	Arcuate hypothalamus
BACE1	Beta-secretase
BAT	Brown adipose tissue
BBB	Blood brain barrier
BLA	Basolateral amygdala
BMA	Basomedial amygdala
BSA	Bovine serum albumin
C99	99-residue C-terminal fragment
CA	Cornu ammonis
Cb	Cerebellum
CD36	Cluster of differentiation 36
CDK5	Cyclin-dependent kinase 5
Cg	Cingulate cortex
cDNA	Complementary DNA

CNS	Central nervous system
COX-2	Cyclooxygenase-2
CREB	cAMP responsive element binding protein
CTF	C-terminal fragment
DAB	Diaminobenzidine
DBD	DNA-binding domain
ddH_2O	Double-distilled water
DIV	Days *in vitro*
DM	Dorsomedial hypothalamus
DMDM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxid
DNA	Deoxyribonucleic acid
DPX	Distyrene Plasticizer Xylene
EC50	Half maximal effective concentration
ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked Immunosorbent Assay
ER	Estrogen receptor
ERK	Extracellular-signal Regulated Kinase
FA	Fatty acid
FC	Frontal cortex
FCS	Fetal calf serum
GC	Glucocorticoids
GFAP	Glial fibrillary acidic protein
GluN2B	Glutamate 2B receptors
Glut4	Glucose transporter type 4
GSK-3β	Glycogen synthase kinase 3β
gr	Granular layer
GR	Glucocorticoid receptor
GrDG	Granular dentate gyrus
GluN2B	Subunit 2-containing NMDAR
HPA	Hypothalamic-pituitary-adrenal
HRE	Hormone response element
HRP	Horseradish peroxidase
ICC	Immunocytochemistry

IDE	Insulin degrading enzyme
IHC	Immunohistochemistry
IL	Interleukin
ir	Immunoreactive
iNOS	Inducible nitric oxid synthase
IRS	Insulin receptor substrate
LA	Lateral amygdala
LBD	Ligand binding domain
LBP	Ligand binding pocket
LDL	Low-density lipoproteins
LH	Lateral hypothalamus
LOAD	Late-onset AD
LPL	Lipoproteinlipase
LPS	Lipopolysaccharide
LRP1	Lipoprotein receptor-related protein 1
LXR	Liver X receptor
MAP2	Microtubule-associated protein 2
MARK	MAP2 Kinase
MAPT	Microtubule-associated protein tau
MEM	Minimum Essential Medium
mo	Molecular layer
MoDG	Molecular dentate gyrus
MR	Mineralocorticoid receptor
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic acid
N-CoR	Nuclear receptor co-repressor
NFκB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NFT	Neurofibrillary tangles
NGF	Nerve growth factor
NGS	Normal goat serum
NMDAR	N-methyl-D-aspartate receptor
NR	Nuclear receptor
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction

Pe	Periventricular hypothalamus
PEPCK	Phosphoenolpyruvate carboxykinase
PET	Positron-emission tomography
PFA	Paraformaldehyde
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PHF	Paired helical filaments
PI3K	Phosphoinositid-3-Kinasen
Pio	Pioglitazone
PND	Postnatal day
PoDG	Polymorphic dentate gyrus
PPRE	Peroxisome proliferator-activated receptor element
PPAR	Peroxisome proliferator-activated receptors
PR	Progesterone receptor
PSD-95	Postsynaptic density protein 95
PV	Paraventricular thalamic nucleus
PVH	Paraventricular hypothalamus
qRT-PCR	Quantitative real time polymerase chain reaction assays
RAR	Retinoid Acid receptor
Re	Reuniens thalamic nucleus
RNAi	Ribonucleic acid interference
RSG	Retrosplenial cortex
RT	Room temperature
RXR	Retinoid X receptor
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
siRNA	Small interfering Ribonucleic acid
Sirt1	Sirtuin 1
SMRT	Silencing mediator of retinoid and thyroid hormone receptors
SN	Substantia nigra
SNP	Single-nucleotide polymorphism
SRC-1	Steroid receptor coactivator-1
st	Stria terminalis
T1DM	Type 1 diabetes mellitus
T2D	Type 2 diabetes
T2DM	Type 2 diabetes mellitus

TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with triton
THR	Thyroid hormone receptor
TNF-α	Tumor necrosis factor-α
TREM2	Triggering receptor expressed on myeloid cells 2
TZD	Thiazolidinedione
UCP-1	Uncoupling protein 1
VDR	Vitamin D receptor
VLDLR	Very-low-density-lipoprotein receptor
VMH	Ventromedial hypothalamus
v.s	versus
VTA	Ventral tegmental area
WAT	White adipose tissue

Chapter 1

General Introduction

1.1. Alzheimer's disease

Dementia is defined as a progressive loss of memory and deficits in other cognitive and behavioural functions, such as attention, language skills, aggression, as well as mood; these changes results from neural degradation in the brain. Dementia is a broad term for a range of neurodegenerative diseases (vascular dementia, dementia with Lewy bodies, fronto-temporal dementia, Creuzfeldt Jakob disease and Alzheimer's disease). Alzheimer's disease (AD) is the most prominent type of dementia in aging humans (www.alzheimers.org.uk). Professor Alois Alzheimer first described the pathology underlying the disease named after him more than 100 years ago (Toodayan, 2015). Since then, studies on AD have rocketed in number – a search for "Alzheimer' disease" in PubMed lists more than 100,000 publications.

Late onset or sporadic AD is the most common form (> 95% of cases) of AD, mainly affecting individuals over the age of 65 years. Genetics accounts for the remaining 1-5% of AD (familial) cases in which one or more mutations are known to be causally involved. Interestingly, a mutation in the Apolipoprotein E gene (Apoε4 allele) significantly raises the risk for non-familial AD and is also associated with obesity and/or hyperlipidemia (**Chapter 1 – 1.2**).

Unfortunately, AD, and in particular sporadic AD, is still poorly understood. Diagnosis of the disease is limited to questionnaires, paper-and-pen tests and neuroimaging (magnetic resonance imaging (MRI) or Positron-emission tomography (PET)) to detect changes in brain areas known to be affected by AD (Kozlov et al, 2017; www.alzheimers.org.uk). Clinicians use these various tests and markers to categorize this progressive disease according to the following stages: early stage (word-finding difficulties), middle stage (disorientation and unusual behaviour), and late stage (limited eating and self-care abilities and loss of speech) (**Fig. 1.1**).

The accumulation of pathological forms of amyloid peptide and tau protein are considered to be the neuropathological hallmarks of both the hereditable and sporadic forms of AD. Amyloid peptide (specifically, amyloid β, Aβ) forms extracellular plaques whereas hyperphosphorylated tau forms intracellular tangles; the latter correlate better with cognitive impairments in AD than Aβ load.

1.1.1. Molecular hallmarks and pathological mechanisms

Accumulations of Aβ and tau are implicated in neurotoxicity and neurodegenerative processes that lead to cognitive dysfunction that is typical of AD. The appearance of tau pathology follows a hierarchical and progressive pattern, marked by the accumulation of the protein and formation of neurofibrillary tangles. They start in the entorhinal/perirhinal cortex, spread to the hippocampus and eventually, primary cortical regions. In contrast, Aβ deposition begins in the neocortex and then gradually spreads to the hippocampus, diencephalon and basal ganglia (**Fig. 1.1**) (Serrano-Pozo et al, 2011; Goedert et al, 2015).

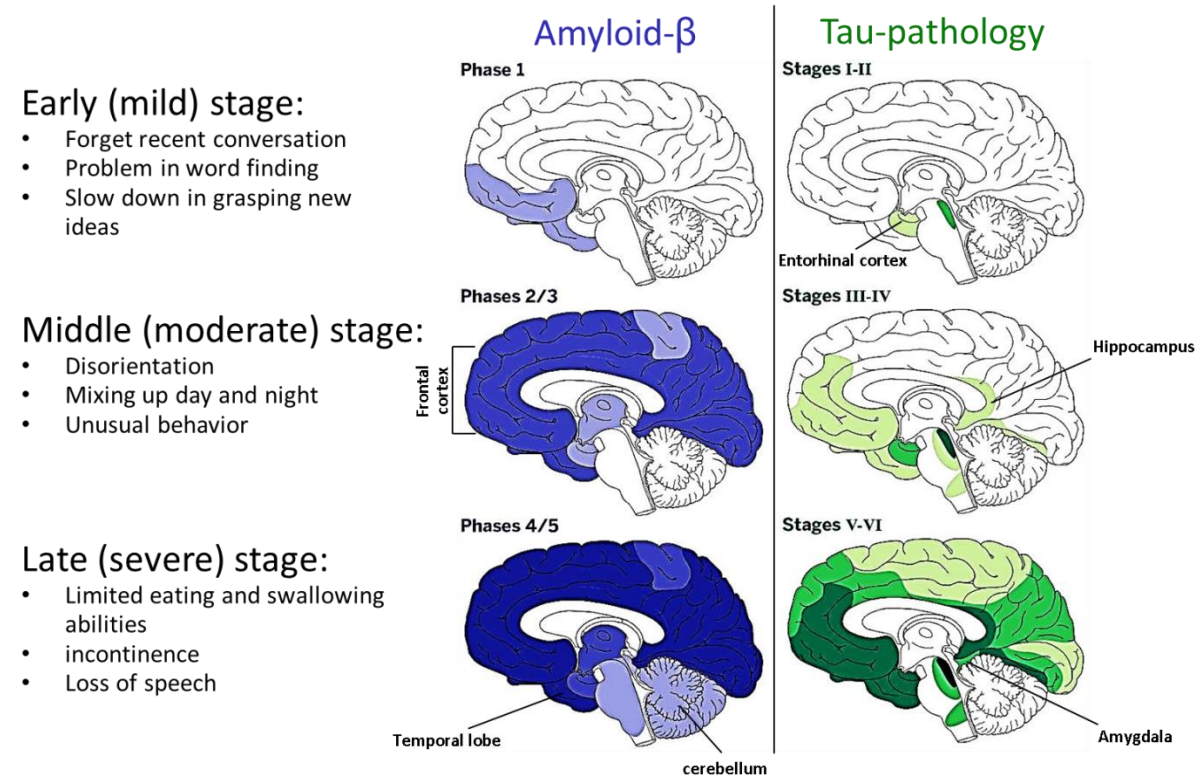

Figure 1.1. Propagation of Aβ and tau inclusions in human brain. Cognitive decline is scaled into early, middle and late stages and correlates with AD pathology (Aβ and tau accumulation) in the brain. Aβ (*shown in blue*) deposits spread from neocortex to hippocampus, diencephalon and basal ganglia. Tau-pathology (*shown in green*) begins in the entorhinal cortex from where it spreads to the hippocampus and finally, cortical areas (adapted from Goedert, 2015).

Amyloid β

Amyloid precursor protein (APP) is a single-pass transmembrane glycoprotein that can be misprocessed to Aβ by specific secretases (initially, β-secretase and then, γ-secretase).

Mutations in the *APP* gene have been shown to preferentially result in pathological breakdown of the parent protein into amyloidogenic peptides such as Aβ (principally, $A\beta_{1-40}$ and $A\beta_{1-42}$; the latter oligomers are especially prone to fibrillation and accumulation into insoluble plaques). The role of Aβ in AD, although a subject of debate, is still considered crucial in AD and has made the Aβ hypothesis attractive and prominent in the field. Briefly, this hypothesis suggests that APP misprocessing along the amyloidogenic pathway, together, with decreased Aβ degradation, lies at the core of AD pathology (Müller et al, 2017).

It is also important to note that APP may undergo non-pathological processing via α-secretase and γ-secretase into soluble APPsα (Gralle et al, 2006; Chasseigneaux & Allinquant, 2012; **Fig. 1.2**). Proteolytic cleavage of APP to generate APPsα can be induced by neuronal and synaptic activity. Although the function of APPsα is not well understood (Hoey et al, 2009; Müller et al, 2017), it is often thought to have a "protective role" in the brain; specifically, non-amyloidogenic cleavage products are implicated in synaptogenesis, and APP itself is thought to be important in synaptic adhesion (Hoe et al, 2012); on the other hand, N-terminal cleavage products are reported to serve as ligands that trigger neuroprotective cell signalling (Wang et al, 2009; Milosch et al, 2014; Hick et al, 2015).

Amyloidogenic processing of APP has been much more intensively investigated because of its implication in AD. The sequential cleavage of APP by β-secretase and γ-secretase produce a neurotoxic intracellular C-terminal fragment called C99 and Aβ (predominantly $A\beta_{1-40}$ and $A\beta_{1-42}$, mentioned previously). An increased ratio of $A\beta_{1-42}$:$A\beta_{1-40}$ in brain parenchyma partially match the degree of AD symptoms (Chang & Chen 2014; Pimplikar, 2009). Oligomers of Aβ are initially soluble peptides that polymerize into insoluble filaments which accumulate in the extracellular space to form the characteristic senile plaques of AD brains. However, whereas there is no correlation between the load of insoluble Aβ plaques and AD pathology, the amount of soluble Aβ oligomers closely corresponds with the extent of cognitive impairment (Gandy et al, 2010).

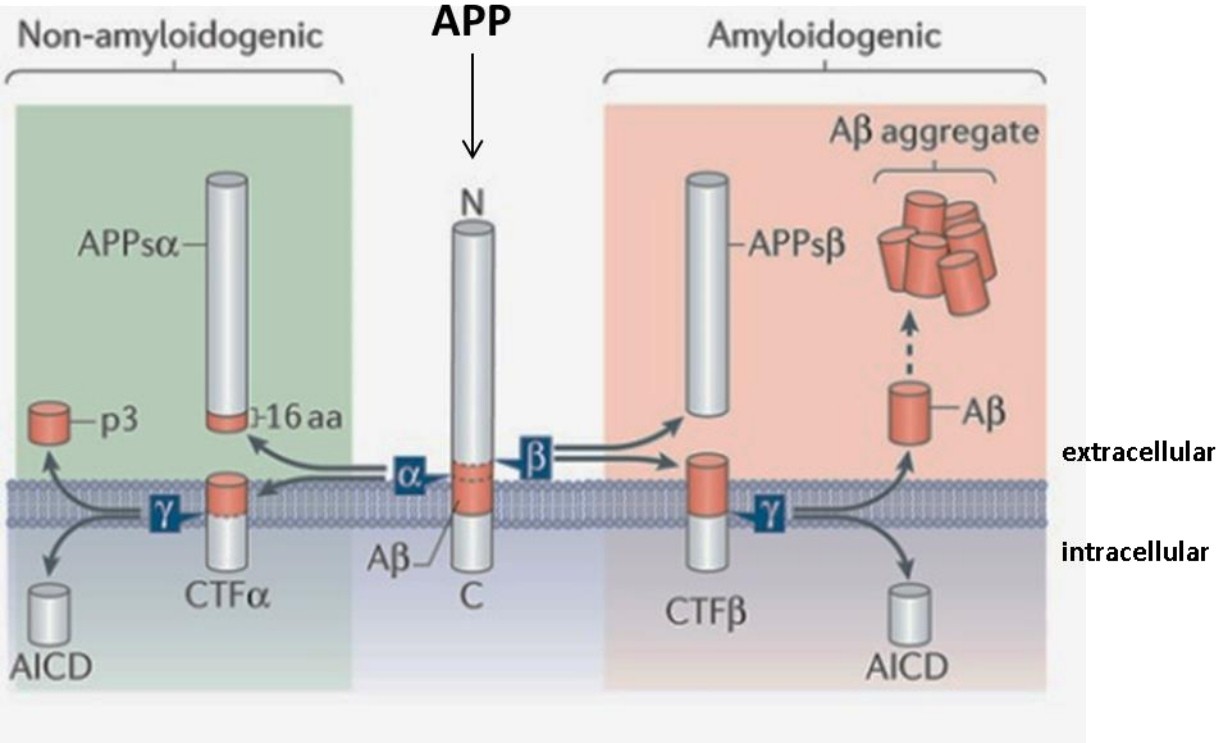

Figure 1.2. Non-amyloidogenic and amyloidogenic processing of APP. Non-amyloidogenic processing (*left, green)* shows sequential processing of APP by α-secretase to generate extracellular APPsα and an intracellular C-terminal fragment (CTFα), followed by γ-secretase-mediated cleavage into extracellular p3 and an intracellular APP domain (AICD). Amyloidogenic processing (*right, pink) shows* APP processing by β-secretase into extracellular APPsβ and intracellular CTFβ (also called C99); the latter is subsequently cleaved by γ-secretase into extracellular Aβ and intracellular AICD. The α-, β- and γ-secretases are indicated by the Greek characters α, β, γ, respectively (adapted from Müller et al, 2017).

Earlier studies from our laboratory described the ability of soluble Aβ oligomers to cause synaptic degradation (Roselli et al, 2005; Chang, et al 2016). Briefly, it appears that soluble Aβ binds to the cell surface to increase neuronal excitation mediated by glutamate, in particular via N-methyl-D-aspartate receptors (NMDAR) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), leading to synaptic loss and eventually, neuronal loss. Massive influxes of calcium ($Ca2^{+}$) into neurons are responsible for the hyperexcitation (or excitotoxicity) induced by activation of NMDAR (Roselli et al, 2005; Chang et al, 2016). Recent studies showed that intracellular Aβ oligomers can disrupt mitochondrial functionality (Kayed & Lasagna, 2008; Schaefer et al, 2016). Lastly, soluble Aβ may also compromise endogenous neuroprotective mechanisms (e.g. those mediated by various neurotrophins,

including insulin); although these mechanisms have been intensively studied, they are less-well understood, especially with regard to when exactly they are disrupted.

Tau-phosphorylation

As briefly alluded to above, Aβ does not seem to be the only or major player role in the manifestation of AD symptoms as was once thought. Rather, the current consensus is that Aβ initiates processes, especially related to the phosphorylation of tau at specific epitopes that are ultimately responsible for AD pathology (De Felice et al, 2008). Supporting this view is the fact that abnormalities in tau (e.g. hyperphosphorylation) correlate with cognitive impairments (Medina & Avila, 2014; Takashima, 2015); alternative splicing of the human tau gene, microtubule-associated protein tau (*MAPT*), can result in the production of 6 isoforms of tau. Notably, however, several tau knockout mouse strains do not show severe behavioural phenotypes (memory), raising questions about the essential role of tau in neuronal integrity and disease (Ke et al, 2012).

The physiological role of tau is to promote assembly and stabilize microtubules in the cytoskeleton. In neurons, tau is therefore involved in axonal polarity and outgrowth and generally, in the dynamic reorganization of the cytoskeleton (**Fig. 1.3 a**), but it is important to note that the protein is also expressed in other neural cells (glia), albeit at lower levels. Tau protein is a highly hydrophilic molecule, with numerous positively- and negatively-charged residues that allow interaction with other molecules or cell structures (e.g. microtubules), as well as in self-folding and -aggregation (Feinstein & Wilson, 2005; Mandelkow & Mandelkow 2012).

Abnormal hyperphosphorylation of specific epitopes in tau are known to disrupt the protein's ability to bind to microtubules with a parallel increase in free tau levels; this "free tau" undergoes folding and forms cytotoxic aggregates that give rise to the so-called tauopathies (e.g. AD, Huntington's disease, and frontotemporal dementia) (Mukrasch et al, 2009). Briefly, the hyperphosphorylation of tau, mediated by various kinases, especially MAP2 kinase (MARK), glycogen synthase kinase 3β (GSK-3β) and cyclin-dependent kinase 5 (CDK5), leads to the self-assembly of tau into paired helical filaments (PHF) and further, into neurofibrillary tangles (NFT) (Alonso et al, 2001) (**Fig. 1.3 b**).

Increasingly, research suggests that tau is transported between cells through a "seeding" process (Michel et al, 2014). Besides prion-like mechanisms which have been proposed to explain the spreading of Aβ pathology (Walker et al, 2016), other work suggests that extracellular tau released after cell death is taken up by neighbouring cells, eventually leading to increases in Ca^{2+} influx through the mediation of muscarinic receptors (Gómez-Ramos et al, 2008).

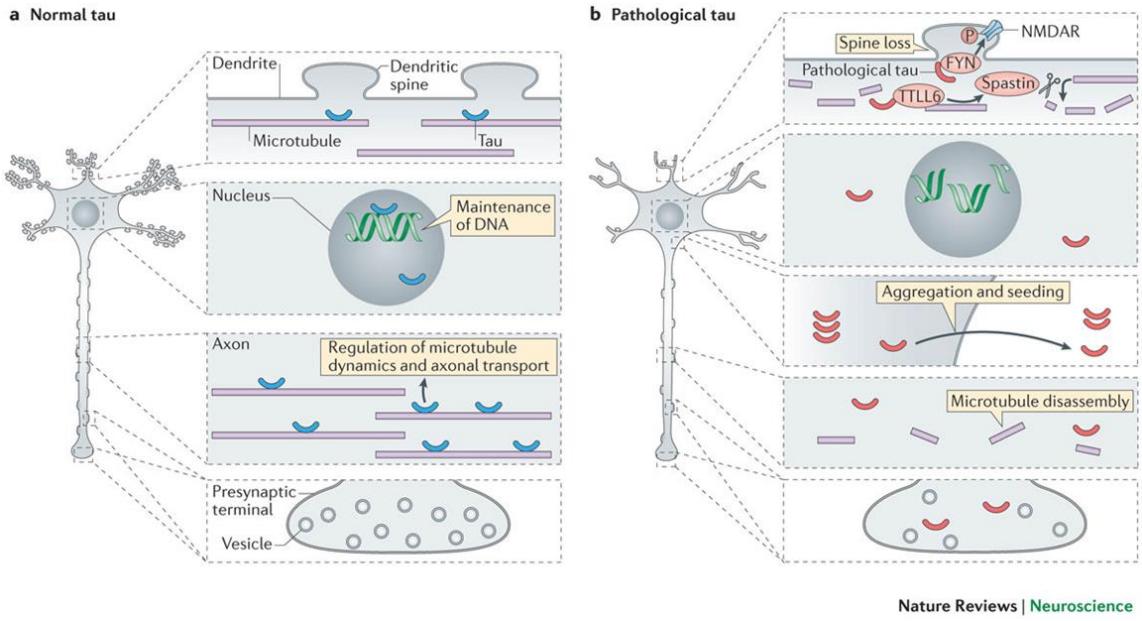

Figure 1.3. Action of normal (physiological) tau and pathological tau in neurons. a) Physiological (normal) tau is mainly found in axons where it serves to stabilize and regulate microtubular dynamics and facilitate axonal transport. The role of tau in dendrites and nucleus remains uncertain at present. **b)** Hyperphosphorylated tau generally induces pathology, causing detachment of tau from microtubules, thus resulting in disassembly of microtubules. Free hyperphosphorylated tau induces spine loss in the pre- and postsynapse. Pathological (hyperphosphorylated) tau is also able to form neurotoxic aggregates (NFT) (Wang & Mandelkow, 2016).

Recent studies have highlighted that cognitive decline correlates with the synaptotoxic effects of dendritic hyperphosphorylated tau (p-tau), before the formation of NFT (Kimura et al, 2007). Kobayashi et al (2017) recently demonstrated that tau is synthesized *de novo* in dendrites, a process triggered by supraphysiological levels of glutamate. Free p-tau in the post-synapse was shown to interact with the Src kinase Fyn to increase Ca^{2+} influx through NMDAR, causing synaptic degradation. Interestingly this mechanism was already shown to be induced by Aβ as well as by chronic stress (Ittner et al, 2010; Lopes et al, 2016).

Linking Aβ with tau in AD pathology

Both Aβ and tau accumulation are pathological hallmarks of AD. While some researchers consider Aβ to be the key cause of AD, others view tau hyperphosphorylation as the critical driver of AD. Meanwhile, it is important to note that Aβ is known to induce tau kinases such as Fyn, GSK3β and MAPK (Jin et al, 2011). Support for this has come from studies in which APP transgenic mice have been crossed with mice carrying *MAPT* mutations (see Goedert 2015 for review). Also important are studies showing that tau-deficient mice are protected against the excitotoxic effects of Aβ (Leroy et al, 2012). Hyperphosphorylation of tau at specific epitopes (e.g. pSer202, pThr231, pSer356, pSer396 and pSer404) is associated with tau-induced neurodegeneration and cognitive dysfunction; interestingly, phosphorylation of another epitope (Thr205) (Ittner et al, 2016) was suggested to confer neuroprotection (also see Schneider et al, 1999).

Hence, interactions between tau and Aβ are likely linked in a sequential and/or hierarchical fashion, with distinct individual or combined impacts on different brain areas and/or circuits (Small & Duff, 2008). All of these events (APP misprocessing and tau hyperphosphorylation) and their interactions are likely further subject to the influence of a multitude of physiological and environmental factors, some of which (e.g. stress, obesity, diabetes) pose particularly high risk for developing AD.

1.1.2. Risk factors for Alzheimer's disease

Alzheimer's disease represents 50-70% of all cases of dementia (Tarawneh & Holtzman, 2012; Winblad et al, 2016). As already mentioned, familial AD (autosomal-dominant inheritance) accounts for less than 5% of all cases, the other forms are sporadic where aging is a significant risk factor. Mutations in the genes encoding APP, α-secretase (ADAM 10) and presenilin 1 and 2 (PS-1, PS-2 are part of the γ-secretase complex) are associated with the familial early-onset cases of AD (onset before age 65 years) (Tarawneh & Holtzman, 2012; Winbald et al, 2016).

Notably, genetics also contribute to late-onset AD (LOAD); specifically, the presence of the apolipoprotein ε4 (*ApoE4*) allele, a single-nucleotide polymorphism (SNP) variant of the *ApoE* gene, being a relatively common genetic risk. Around 50 % of people with AD have at least

one apoE4 allele. Apolipoprotein E4 exerts neuropathological effects through multiple pathways, resulting in impairment of dendritic spine structure and mitochondrial function (Brodbeck et al, 2008; Verghese et al, 2011), but also disturbed lipid metabolism (Lopez et al, 2014). Thus, subjects with metabolic disorders involving hypercholesterolemia are at particularly high risk for developing AD (see **2.4.1**). *ApoE4* remains the most dominant genetic trigger of non-familial (or late onset) AD. Interestingly the risk to suffer from LOAD is higher in female *vs.* male ApoE4 carriers. This, together with sex differences in metabolism might explain why females are more affected by AD (Seshadri et al, 1997; Neu et al, 2017). The equally potent, gene variant of TREM2 (triggering receptor expressed on myeloid cells 2) occurs rarely in comparison (Guerreiro et al, 2013). Given the mechanistic link between ApoE4 and lipid metabolism, and the fact that AD takes decades to become fully manifest, suggests that lifestyle (e.g. physical inactivity, diabetes, obesity, hypertension, cognitive inactivity (see **2.4**) is an important adjunct factor, i.e. increases vulnerability to the disease. A brief overview of the main risk factors for LOAD is shown in **Table 1.1**.

Lifestyle and working habits, especially in industrialized societies, are often stressful and, stress itself determine eating and working patterns (Schiepers et al, 2018), thus potentially increasing the risk for AD. Indeed, the latter extrapolation, based on clinical epidemiological studies is supported by research in animals in which chronic stress was demonstrated to lead to memory impairments in parallel with the expression of key pathobiochemical hallmarks of AD, which induce the loss of neurons and synapses in brain areas responsible for the regulation of memory (Catania et al, 2009; Sotriropoulos et al, 2008; Sousa & Almeida 2012) (see **2.4.5**). In the overall context of this thesis, it should be mentioned that activation of a nuclear receptor called proliferator-activated receptor γ (PPARγ) is described to reverse some of the effects of an unhealthy lifestyle (e.g. overeating and stress), as dealt with in more detail in the following part. Briefly, activated PPARγ have a beneficial effect on peripheral metabolic, psychological wellbeing and anti-inflammation, and directly or indirectly on the maintenance of cognitive performance, an important aspect of AD (García-Bueno et al, 2008; Landreth et al, 2008, Ahmadian et al, 2013).

Factors that Increase Risk
Genetic (APOE ε4, other susceptibility genes)
Metabolic (cholesterol, diabetes, obesity)
Vascular (cardiovascular disease, hypertension, stroke)
Infectious/Inflammatory (chronic periodontitis, others)
Head trauma (multiple concussions)
Diet (homocysteine, Standard American Diet, deficiencies)
Lifestyle (smoking, alcohol abuse)
Sleep (poor quality, disorders)
Depression
Early menopause (natural or surgical)
Sedentary lifestyle
Certain medications
Factors that Decrease Risk
Genetic (APOE ε2)
Social (education, income, engagement)
Lifestyle (physical & mental exercise, high work complexity)
Diet (Mediterranean, polyunsaturated and fats from fish)
Vitamins (B6/B12/Folate, A, C, D, E)
Medications (NSAIDs, statins, early HRT, antihypertensives)

Table 1.1. Overview of the major factors contributing to increased risk for developing Alzheimer's disease and some factors that reduce risk for the disease. (from Fosnacht et al, 2017)

The effects of a chronic unhealthy lifestyle accumulate over time; thus, it is not surprising that aging multiplies the chances of developing late onset AD. As shown in **Fig. 1.4**, the incidence of dementia rises with increasing lifespan and demographic aging, due to improvements in healthcare, hygiene and education. A recent (2016) worldwide study reported that 46 million people currently suffer from AD; this number is expected to almost double over the next 20 years, with the greatest number of new cases arising in low- to middle income countries (www.alzheimers.org.uk). At present, there is no cure for AD and slow progress in finding means to slow or prevent the disease implies massive costs for health care for the elderly.

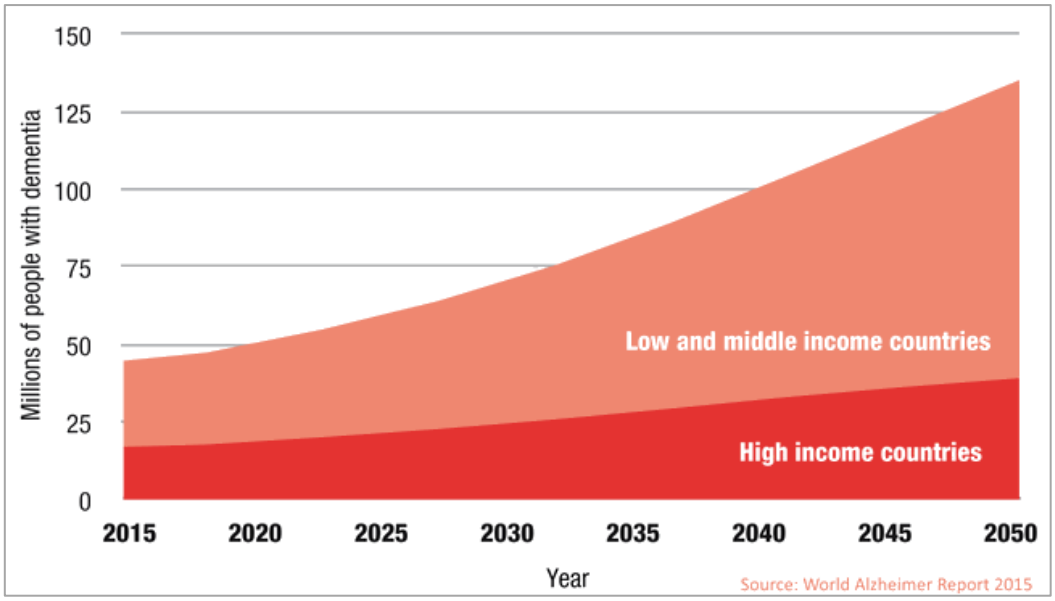

Figure 1.4. Projected cases of dementia worldwide between 2015 and 2050 (note that AD represents approximately 50-70% of all cases of dementia). The greatest increase in this age-related dementia will arise in low- to-middle income countries (www.alzheimers.org.uk).

1.2. Peroxisome proliferator-activated receptors (PPAR)

Peroxisome proliferator-activated receptors (PPAR) belong to the 48-member superfamily of nuclear receptors (NR), all of which are ligand-activated transcription factors (Laudet & Gronemeyer, 2002) (**Fig. 1.5**).

There are three isotypes of PPAR (PPARγ, PPARβ/δ and PPARγ), encoded by distinct genes. Of these, PPARα (NR1C1) was the first to be cloned and described as being activated by peroxisome proliferators (Issemann & Green, 1990). Subsequently, PPARβ/δ (NR1C2) and PPARγ (NR1C3) were characterized (Dreyer et al, 1992). Initially, PPARβ, cloned from a Xenopus oocyte library, was considered distinct from mammalian PPARδ protein (Chen et al, 1993; Schmidt et al, 1992; Zhu et al, 1993), but further work showed that PPARδ is the mammalian ortholog of amphibian PPARβ (Desvergne & Wahli, 1999; Michalik et al, 2006; Germain et al, 2006). Alternative promoter usage and splicing results in at least two sub-isoforms of PPARγ (PPARγ1 and PPARγ2) that only differ in their N-terminal residues (Zhu et al, 1993; Zhu et al, 1995; Tontonoz et al, 1994a; Elbrecht et al, 1996); since there is

considerable overlap in the tissue distribution and functions of PPARγ1 and PPARγ2, the two isoforms are usually simply referred to as PPARγ.

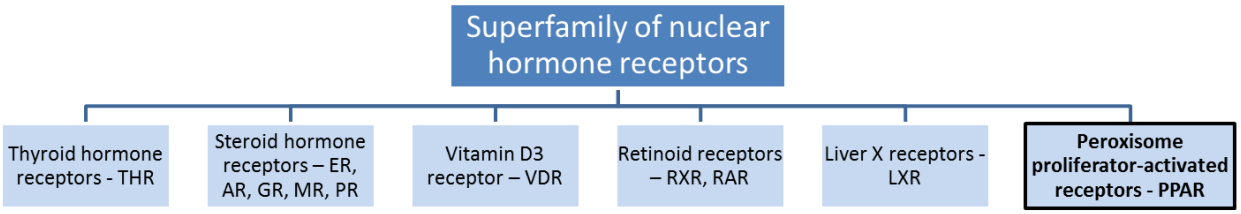

Figure 1.5. Superfamily of nuclear hormone receptors. THR: Thyroid Hormone Receptors (NR1A1, NR1A2); **ER:** Estrogen Receptors (NR3A1, NR3A2); **AR:** Androgen Receptor (NR3C4); **GR:** Glucocorticoid Receptor (NR3C1); **MR:** Mineralocorticoid Receptor (NR3C2); **PR:** Progesterone Receptor (NR3C3); **VDR:** Vitamin D Receptor (NR1I1); **RXR:** Retinoid X Receptors (NR2B1, NR2B2, NR2B3); **RAR:** Retinoid Acid Receptors (NR1B1, NR1B2, NR1B3); LXR: Liver X Receptors (NR1H3, NR1H2); **PPAR:** Peroxisome-proliferator-activated receptors (NR1C1, NR1C2, NR1C3).

1.2.1. Expression of PPAR in peripheral tissues

All PPAR play an important role in metabolic functions, including lipid metabolism and energy homeostasis, as well as in inflammation (Desvergne & Wahli, 1999; Ricote & Glass, 2007; Cho et al, 2008). Various metabolic and developmental functions of PPAR are inferred from isotype-specific expression patterns in tissues of ectodermal, mesodermal or endodermal embryonic origin (Kliewer et al, 1994), although coexpression of different PPAR (albeit with differing ratios) also occurs (Braissant et al, 1996), with potentially different tissue and spatio-temporal-specific transcriptional outcomes.

Generally, **PPARα** are expressed in cells with a catabolic function (Kliewer et al, 1994; Desvergne & Wahli, 1999). For example, they are highly expressed in cells displaying high mitochondrial and peroxisomal beta-oxidation activity, such as the liver, heart, kidney, skeletal muscle, large intestine and brown adipose tissue (BAT) (Issemann & Green, 1990; Dreyer et al, 1992; Kliewer et al, 1994; Braissant et al, 1998), as well as in macrophages where they contribute to the regulation of the inflammatory response (Hihi et al, 2002; Ricote & Glass, 2007).

PPARβ/δ are ubiquitously expressed (Dreyer et al, 1992; Kliewer, et al 1994; Braissant et al, 1996) and contribute to fatty acid catabolism and glucose homeostasis (Cho et al, 2008). Like PPARα, PPARβ/δ serve as activators of fatty acid oxidation pathways in liver, heart and skeletal muscle (Poulsen et al, 2012). Thus, they play a key role in basal metabolism (Cullingford et al 1998; Briassant & Wahli, 1998; Granneman et al, 1998). Of all the PPAR isoforms, PPARβ/δ is the, most highly expressed in the adult nervous system.

The distribution of **PPARγ** is generally more restricted as compared to PPARα and –β/δ in the mouse. In humans, PPARγ is expressed at moderate levels in kidney, heart and liver. Interestingly, PPARγ2 is highly expressed in white (WAT) and brown (BAT) adipose tissue where it regulates adipocyte development, differentiation, fatty acid storage, glucose metabolism and inflammatory responses (Elbrecht et al 1996; Dreyer et al 1992; Mukherjee et al 1997; Ricote & Glass 2007; Cho et al 2008; Tontonoz & Spiegelman 2008; Harmon et al 2011); notably, PPARγ expression is also detected in parts of the immune system (Braissant et al, 1996; Harmon et al 2011; Tyagi et al, 2011).

1.2.2. PPAR expression in the central nervous system (CNS)

Many brain areas, including the basal ganglia, hippocampus, rhomboencephalic nulclei, frontal cortex, nucleus accumbens, amygdala and ventral tegmental area in humans and mice reportedly express PPARα, -β/δ, and –γ; the relative amounts of expression of each isoform vary between areas and cell types (**Fig. 2.8**).

Expression of **PPARα** is influenced by developmental stage and is highly expressed in hippocampal granule cells and astrocytes (Braissant et al, 1996; Moreno et al, 2004); very recently, Warden et al (2016) reported more prominent expression of this isoform in neurons as compared to glia, in both mice and humans. Coexpression of PPARα with certain target genes in the basal ganglia, hippocampus, rhombencephalic nuclei have been associated with neuroprotection against oxidative stress.

PPARβ/δ is widely and prominently found throughout the adult nervous system where it is mainly localized in neurons and in oligodendrocytes. Notably, it is also the only PPAR isoform expressed in Purkinje cells; further, PPARβ/δ expression is very low in in the granular layer of the hippocampus (Granneman et al, 1998; Braissant et al, 1996; Warden et al, 2016).

Whereas most brain areas express higher levels of PPARα or β/δ as compared to **PPARγ**, the frontal cortex expresses approximately equal amounts of all three isoforms (Warden et al, 2016). The developing rat brain was reported to express PPARγ mRNA (messenger ribonucleic acid) (Braissant & Wahli 1998), and PPARγ mRNA was also detected in the brain and spinal cord of adult rats (Braissant et al 1996; Cullingford et al 1998; Moreno et al 2004; Sarruf et al 2009). In general, PPARγ expression in astrocytes tends to depend on brain region; moreover, bacterial toxins, such as lipopolysaccharide (LPS), were shown to slightly upregulate this receptor in microglia (Warden et al, 2016). However, literature reports on PPARγ (mRNA and protein) expression in different brain regions remain inconclusive, perhaps reflecting non-parallelism between the regulation of PPARγ mRNA and protein synthesis and turnover, as will be discussed further in **Chapter 2**.

Despite many attempts to characterize **PPARγ** expression in the CNS, the available reports on their cell type or brain region distribution remains poor because of the paucity of good quality antibodies and lack of use of appropriate cell type-specific markers in immunostaining analyses. Accordingly, inferring function of PPARγ in a given brain area is highly limited. While very little is known about expression of PPARγ in neurons, Moreno et al (2004) described ubiquitous PPARγ expression in astrocytes; on the other hand, the presence of **PPARα** and **β/δ** in astrocytes was seen to occur in a brain region-specific manner. Given the ontogeny of brain PPAR during development, it appears that, like in adipocytes, PPAR, including PPARγ, play a role in neural cell maturation. Interestingly, rat neural cells were shown to display increased PPARα and PPARβ/δ expression, and decreased PPARγ expression, during their maturation *in vitro* (Cimini et al, 2004). At present, there is no information about if or how the expression of the different PPAR is coordinated in a spatio-temporal manner.

Despite the unclear picture of where exactly in the brain the different PPAR are expressed, their molecular regulation and physiological functions, numerous studies have focused on the therapeutic potential of PPAR ligands in diseases of the brain. For example,

neurodegenerative diseases, ranging from Alzheimer's disease (Skerrett et al, 2014), Parkinson's disease (Chen et al, 2012; Carta, 2013), Huntington's disease (Chiang et al, 2012), and amyotrophic lateral sclerosis (Benedusi et al, 2012), have all been considered for targeting with PPARγ ligands; the anti-inflammatory properties of these ligands has provided the rationale for most of these efforts (Skerrett et al, 2014). However, given that PPARγ (and other PPAR) also regulate peripheral functions such as glucose and lipid metabolism, an important question remains open: do PPARγ ligands produce their health benefits by acting directly on the brain, or primarily by improving metabolic status in the periphery. In the context of the present thesis, it is worth illustrating this point by noting that type 2 diabetes (T2D) is strongly linked to cognitive impairments, including Alzheimer's disease, and that PPAR agonists, such as those belonging to the thiazolidinedione (TZD) family (e.g. rosiglitazone, pioglitazone) are potent sensitizers of the cellular response to insulin. This issue, with respect to PPARγ, will be discussed in greater detail in a later section of this General Introduction (see **2.3.1**).

1.2.3. Biology of PPARγ

As mentioned before, PPAR act as ligand-activated transcription factors (Laudet & Gronemeyer, 2002). However, unlike the best-studied members of the superfamily of NR that are liganded by classical hormones (estrogens, progesterone, thyroid hormone, aldosterone and cortisol/corticosterone), the primary endogenous ligands of PPAR are polyunsaturated fatty acids (Kliewer et al, 1995; Tontonoz et al, 1994; Harmon et al, 2011). While more specific details will be considered below, a schematic showing how PPAR regulate gene transcription is presented in **Fig. 1.6**; **Table 1.2** lists established PPAR target genes.

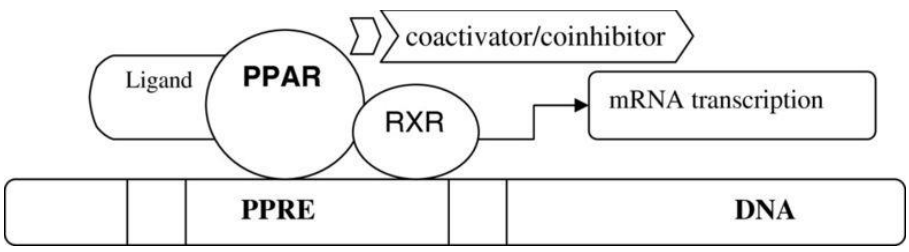

Figure 1.6. Mechanism of gene transcription by PPAR (Grygiel-Górniak, 2014)**.**

Table 1.2. Established PPAR target genes in human tissues
(from: http://www.sabioscience.com/rt_pcr_product/HTML/-PAHS-149Z.html**).**

Note some targets are shared by PPARα-β/δ and-γ; others may be unique targets of a specific PPAR isoform or shared by two isoforms. Most of these gene have been identified as PPAR in other species such as rat and mouse. However, the gene list does not imply direct targeting; neither does it consider tissue- or cell-specificity.

PPARα Targets:
Adipogenesis: ADIPOQ, LPL, NR1H3, UCP1.
Fatty Acid Metabolism: ACADL, ACADM, ACOX1, ACOX3, ACSL1, ACSL3, ACSL4, ACSL5, CPT1A, CPT1B, CPT2, CYP27A1, CYP4A11, CYP7A1, EHHADH, FADS2, HMGCS2, PLTP, SCD.
Lipid Transport: ADIPOQ, ANGPTL4, APOA1, APOA5, APOC3, LPL, NR1H3, OLR1.
Insulin Signaling: CPT1A, SORBS1.

PPARβ/δ Targets:
Adipogenesis: ADIPOQ, ECH1, FABP4, LPL, UCP1.
Fatty Acid Metabolism: ACAA2, ACADL, ACADM, ACOX1, ACOX3, ACSL1, ACSL3, ACSL4, ACSL5, CPT1A, CPT1B, CPT2, CYP4A11, CYP27A1, CYP7A1, EHHADH, ETFDH, FABP1, FABP3, FABP4, FADS2, GK, HMGCS2, MLYCD, PLTP, SCD, SLC27A1, SLC27A4.
Lipid Transport: ADIPOQ, ANGPTL4, LPL, OLR1.
Cell Proliferation: HIF1A, ILK, KLF10, PTEN.
Insulin Signaling: CPT1A, PDPK1, SORBS1.
Others: PDPK1.

PPARγ Targets:
Adipogenesis: ADIPOQ, LPL, NR1H3, UCP1.
Fatty Acid Metabolism: ACADL, ACADM, ACOX1, ACOX3, ACSL1, ACSL3, ACSL4, ACSL5, CPT1A, CPT1B, CPT2, CYP27A1, CYP4A11, CYP7A1, EHHADH, FADS2, GK, SCD.
Lipid Transport: ADIPOQ, ANGPTL4, APOE, DGAT1, LPL, NR1H3, OLR1.
Cell Proliferation: CLU, ELN, TXNIP.
Insulin Signaling: CPT1A, DGAT1, PCK1, PCK2, SORBS1.
Others: MMP9, PCK1, PCK2.

PPARγ ligands

Some known PPAR natural and synthetic ligands are shown in **Fig. 1.7**. It is worth noting that while oxidized low-density lipoproteins (LDL) display high affinities for PPARγ (Krey et al, 1997; Nagy et al, 1998), their source in the brain is presently unknown but are derived from blood circulation or by *de novo* synthesis (Mitchell & Hatch, 2011). Interestingly, some studies suggest that serotonin metabolites from the brain and/or gastrointestinal tract also serve as PPARγ ligands (Waku et al, 2010; Czimmerer et al, 2012). These authors propose that such ligands contribute to the regulation of appetite, mood and cognition, as well as central glucose and lipid sensing. In general, it is very likely that distinct ligands activate PPARγ in individual cell types (e.g. in neurons, liver, fat etc.) to induce specific gene transcription patterns.

In the context of the studies carried out in this thesis, the PPARγ agonist **pioglitazone** (Pio) that belongs to the class of drugs known as thiazolidinediones (TZD), deserves special

mention. Currently, this compound, whose structure is shown in **Fig. 1.8**, is the most specific and potent synthetic PPARγ agonist. The TZD are PPARγ-selective agonists, which show much higher affinities for PPARγ than any of the putative endogenous ligands identified thus far (Lehmann et al, 1995; Willson et al, 2000; Seimandi et al, 2005). Similar to the endogenous ligand prostanoid 15-deoxy-δ-12, 14-prostaglandin J2, Pio has a half-maximal activity in the lower micromolar range and is a potent inducer of adipogenesis (Forman et al, 1995).

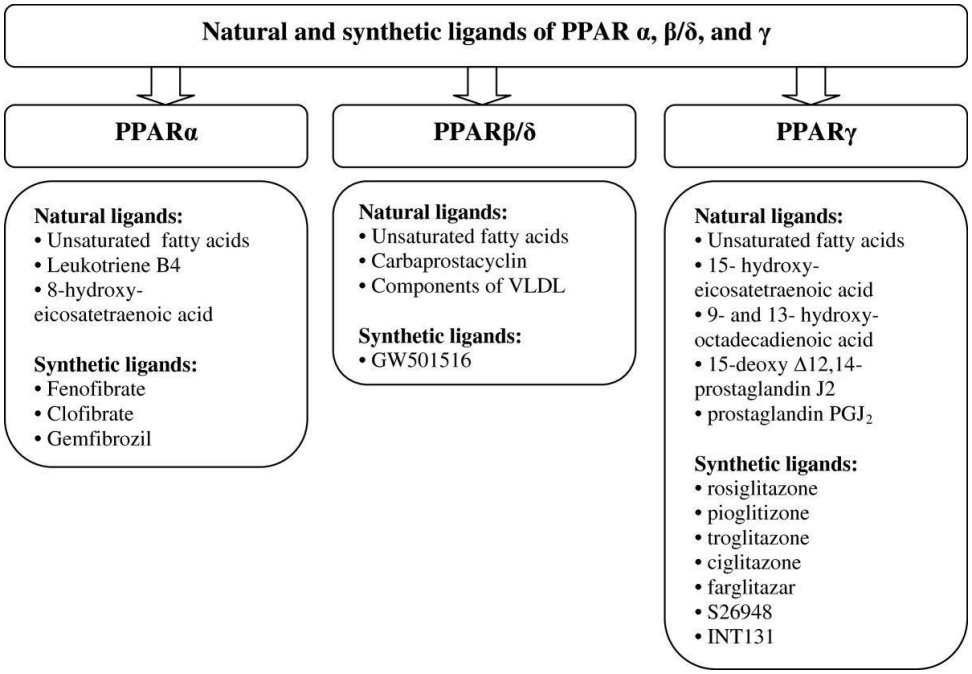

Figure 1.7. Natural and synthetic PPARγ ligands (Grygiel-Górniak B, 2014)**.**

With an EC50 in the 10^{-7}M range, Pio has proven to be a highly specific tool to activate PPARγ1 and -2 (Lehmann et al, 1995; Willson et al, 2000; Seimandi et al, 2005). A property that makes this drug attractive to work with, in the context of Alzheimer's disease and other disorders of the brain, is that it can enter the brain after oral consumption (Grommes et al, 2013); interestingly, as compared to racemic pioglitazone, (+)pioglitazone was a poorer substrate of P-glycoprotein, a protein that serves to hinder xenobiotic penetration of the blood-brain-barrier (Chang et al, 2015).

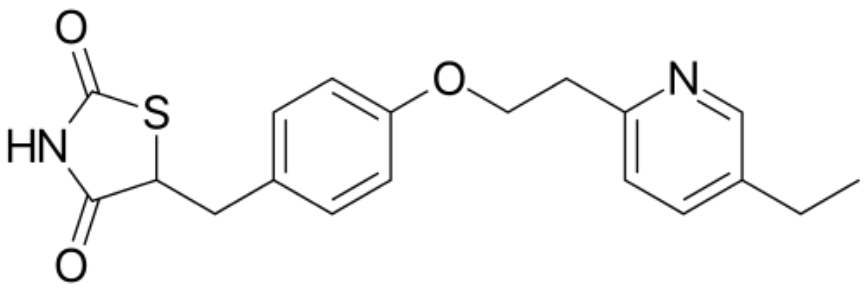

Figure 1.8. Chemical structure of pioglitazone (Pio).

Molecular aspects

In common with other NR, PPAR consist of 4 functional protein domains (**Fig. 1.9**): the NR isoform-specific **N-terminal region (A/B domain)**, which harbours a poorly-conserved cell- and promoter-specific transcriptional activation function (AF-1), can operate in a ligand independent way; a **DNA-binding region (C domain, DBD)**; a **hinge region (D domain)** which contains a nucleus-localization signal and which is thought to allow conformational changes of the receptor; and the **ligand-binding domain (E domain, LBD)** which harbours a ligand-dependent transcriptional activation function (AF-2) (Laudet & Groenemeyer, 2002; Germain et al, 2006b).

The LBD of individual NR are evolutionarily highly conserved. In general, the LBD consists of a i) dimerization surface which can form homo- or heterodimers (with nuclear receptors), ii) a ligand-binding pocket (LBP), iii) a coregulatory-binding surface that interacts with proteins that can promote or repress transcriptional activity and, iiii) a weaker activation function helix (AF2) which mediates ligand-dependent transactivation. Ligand binding results in a rigid conformation of the NR, promoting coactivator protein binding, as will be described later in this section. Molecules that bind to the AF2 pocket contain a core moiety that include an indole ring linked to a carboxyl group that can be selectively recruited by different agonists and antagonists (Waku et al, 2010).

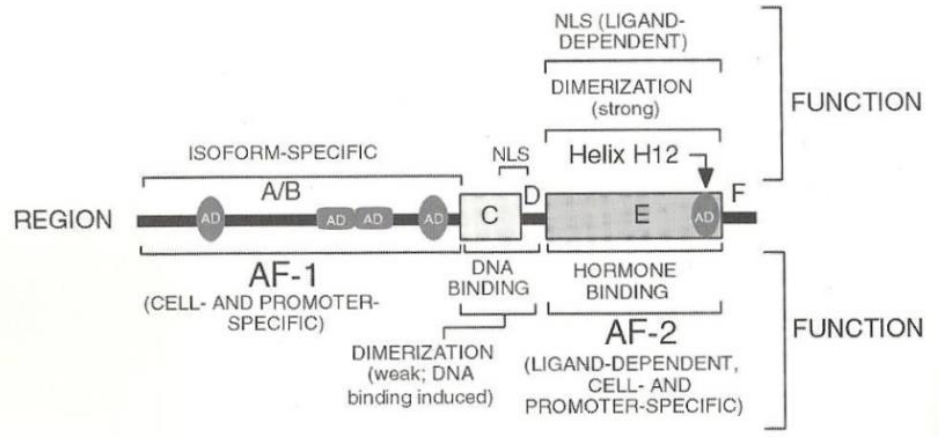

Figure 1.9. Functional domains common to all NR (Laudet V & Gronemeyer H, 2002).

The LBD of PPAR (and other NR) consists of 12 α-helices assembled into a three-layer antiparallel α- helical sandwich that forms a Y-shaped hydrophobic pocket into which cognate ligands fit. This ligand-binding cavity is larger in PPAR than in other NR, possibly accounting for why PPAR can accommodate a wide range of synthetic and natural lipophilic compounds with an acidic head-group. The specific binding of albeit diverse ligands to PPARγ can be explained by the fact that the LBD of PPARγ is larger than that of PPARβ, and less lipophilic than that of PPARα (Nolte et al, 1998; Xu et al, 2001; Nagy & Schwabe, 2004; Michalik et al, 2006; Germain et al, 2006b). Binding of fatty acids, endogenous ligands of PPARγ, trigger NR activation by stabilization of helix 12 (Itoh et al, 2008). Nanomolar concentrations of high affinity ligands (e.g. hydrophobic molecules containing an α/β-unsaturated ketone and oxidized eicosanoids) are sufficient to activate PPARγ via covalent binding to a cysteine residue in the LBD (Shiraki et al, 2005; Schopfer et al, 2005).

Like the LBD, the DBD of each NR is also strongly conserved across species. This domain includes two cysteine-rich zinc finger motifs through which specific DNA sequences, so-called hormone response elements (HRE) are recognized. The ligand binding domain (LBD) forms a Y-shaped hydrophobic pocket, which accommodates endogenous and pharmacological ligands (Michalik et al, 2006; Germain et al, 2006b); occupation of this binding site (ligand-dependent) is followed by hetero-dimerization and eventually, gene transactivation.

In contrast to steroid hormone receptors, which mostly become active after homodimerization, PPAR form heterodimers with other NR, namely, the retinoid X receptor (RXR) or liver X receptor (LXR). RXR is considered to be the main heterodimeric partner for PPARγ (Kliewer et al, 1992; Laudet & Groenemeyer, 2002; Mullican et al, 2013). Of the three RXR isoforms, RXRβ (NR2B2) is the predominant isoform in the CNS. RXR dimerize with and strengthen the DNA-binding and transcriptional activity of a variety of NR (TR, RARs, vitamin D receptor [VDR], LXRs, farnesoid X receptor [FXR], pregnane-X-receptor [PXR], constitutive androstan receptor [CAR], nerve growth factor IB [NGFIB] and nuclear receptor related 1 protein [NURR1]), including PPAR, in a "non-permissive" manner. It is important to note that RXR-RXR homodimers have transcriptional activity of their own, e.g. when liganded by 9-cis-retinoic acid, a metabolite of vitamin A (Lefebvre et al, 2010a; Mullican et al, 2013). Interestingly, different heterodimers recruit different co-regulators to produce distinct transcriptional effects, e.g. RXRα/PPARγ heterodimers are less sensitive to PPARγ activation than RXRβ/PPARγ heterodimers (Lefebvre et al, 2010b).

As already noted, PPARγ can also heterodimerize with LXR; these interactions occur in response to different ligands and at different affinities as compared with PPAR/RXR dimerization (Yue et al, 2005). LXR are cholesterol-sensing NR that are activated by oxysterols (oxidized derivatives of cholesterol like 24(S), 25-epoxycholesterol). Like PPARγ (Chawla et al, 2001; Mandrekar-Colucci et al, 2012), activation of LXR lead to transcription of ApoE and ABCA1 (both proteins are involved in cholesterol efflux transport), which are implicated in AD (see **2.4.3**). LXR are expressed as two isoforms, LXRα (NR1H3) and LXRβ (NR1H2). Whereas LXRα is mainly found in macrophages and visceral tissues (liver, kidney, intestines, adipose tissue) where its role is to catabolize cholesterol, LXRβ is ubiquitously expressed and is important for initiating cholesterol transport from blood to liver (Moore et al 2006; Zhao & Dahlman-Wright, 2010). In the brain, LXRs have been localized in astrocytes and microglia, contributing to the regulation of lipid homeostasis and neuroinflammation (Kang & Rivest, 2012).

"Permissive heterodimers" of PPARγ/RXR or PPARγ/LXR can be formed after binding of ligands of any one receptor (Kliewer et al, 1992; Willy et al, 1995; Leblanc & Stunnenberg, 1995; Varga et al, 2011; Pérez et al, 2012; Evans & Mangelsdorf, 2014). This expands the

transcriptional spectrum (and potential to facilitate adaptation to physiological challenges) of activated PPARγ. Post-translational covalent modifications of PPARγ and each of its heterodimeric partners add further diversity to the receptor's portfolio of actions.

As compared to all other NR which have five, PPAR have only three amino acids between the first two cysteines of the second zinc finger in the DBD (Issemann & Green, 1990; Dreyer et al, 1993; Chandra et al, 2008). The DBD of both parts of the heterodimer bind to the HRE in a given target gene to a direct repeat of the sequence AGGTCA separated by one nucleotide (DR1), in a specific way. The PPRE (PPAR response element) reflects PPAR/RXR polarity, where PPAR is attracted by the 5' extended half-site and RXR recognizes the 3' half site. Within the PPRE, a spacer adenine residue provides strongest heterodimer binding (as compared to the other three nucleotides). This binding site structure is unique to PPARγ (Ijpenberg et al, 1997; Germain et al, 2006a; Nielsen et al, 2008; Lefterova et al, 2008; Varga et al, 2011), confirmed by crystallographic methods which also showed how other functional NR domains besides the DBD contribute to attachment to the PPRE (Chandra et al 2008; **Fig. 1.10**).

As briefly mentioned previously, the AF-2 co-regulator-binding surface is an important determinant of the transcriptional activity of NR. Ligand binding induces conformational changes in the receptor that are conducive to the recruitment of co-regulators (co-activators and co-repressors) to the AF-2 domain; the co-regulators then promote or hinder NR binding to their cognate hormone response element. Different combinations of NR-co-activator or NR-co-repressor complexes form in different cell types, thus providing cell type-specific transcriptional responses (Ricote & Glass, 2007; Puigserver & Spiegelman, 2003).

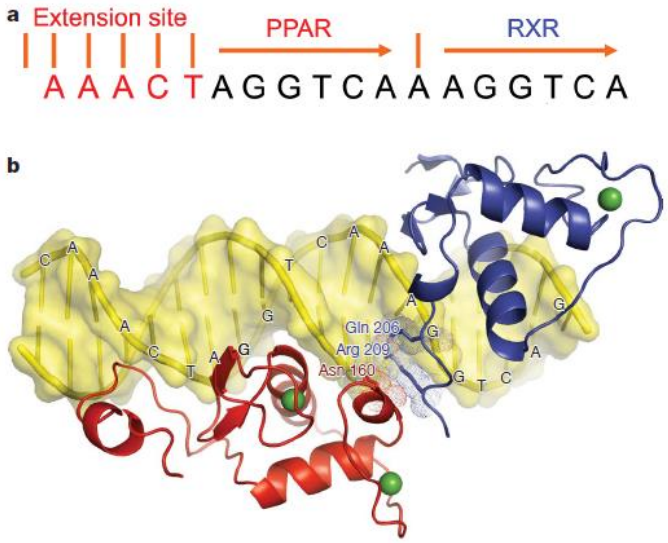

Figure 1.10. PPAR-RXR heterodimer binding to the hormone response element (PPRE) (Chandra et al, 2008)**.**

Unliganded PPARγ are held in a transcriptionally-inactive complex through to an association with **co-repressors** that bind to a LXXLL motif in the unliganded LBD; in this respect, PPAR resemble two other types of NR, thyroid hormone receptors (THR) and the retinoic acid receptors (RAR). Two well-studied corepressor molecules are silencing mediator of retinoid and thyroid hormone receptors (SMRT) and nuclear receptor co-repressor (N-CoR); these molecules repress gene transcription by recruiting histone deacetylases to chromatin (Nolte et al, 1998; Chandra et al, 2008). Ligand binding and heterodimerization of PPARγ results in recruitment of co-activators, the best-known of which are PPARγ coactivator 1-alpha (PGC-1α) and steroid receptor coactivator-1 (SRC-1) (Puigserver & Spiegleman, 2003). **Co-activators** generally bind to the N-terminal transcriptional activation domain of NR and recruit enzymes that produce chromatin modifications through acetylation, phosphorylation or methylation (Harmon et al, 2011; Chandra et al, 2008), resulting in activation of the general transcriptional machinery (see **Fig. 1.9**). Notably, PGC-1α recruits other histone acetyl transferase recruiting coactivators like SRC-1 and cAMP responsive element binding protein (CREB) to the N-terminus of PPAR (Puigserver & Spiegelman, 2003). Combinatorial usage of heterodimers, together with specific co-regulators, allow the initiation of distinct spatio-temporal transcription programmes; for instance, in adipocytes, PGC-1α/PPARγ complexes induce differentiation into brown adipocyte tissue (BAT), whereas complexing of thyroid

hormone receptor-associated protein complex 220 (TRAP220) with PPARγ induces differentiation into white adipocyte (WAT) (Puigserver & Spiegleman, 2003).

Like many other biomolecules, PPAR can undergo post-translational modifications what have important consequences for their biological activity. **Phosphorylation** is the best-known post-translational modification of PPARγ that determines the receptor's transcriptional ligand-dependent and/or independent profile. Multiple functional phosphorylation sites in PPARγ have been described to be modulated by various kinases (ERK [extracellular-signal regulated kinase] and p38-MAPK [mitogen-activated protein kinase], protein kinase A and C, AMPK [AMP-activated protein kinase] and GSK-3β [glycogen synthase kinase 3β]); in addition, PPAR are subject to phosphatase activity (Burns & Vanden Heuvel, 2007; Floyd & Stephens, 2012). It is relevant to note that CDK5-mediated phosphorylation at specific serine residues leads to conformational changes in PPARγ, resulting in inhibition of its transcriptional activity (Burns & Vanden Heuvel, 2007; Lindroos et al, 2013), and ultimately, the ability of PPAR to enhance adipogenesis and insulin sensitivity (Burns & Vanden Heuvel, 2007; Choi et al, 2010).

Repression (and trans-repression) of PPARγ activity is also observed after **sumoylation** by the protein SUMO1 of Lysine 107 in the N-terminal AF-1 domain of PPARγ. This mechanism seems to involve inhibition of corepressor N-CoR clearance (Jennewein et al, 2008; Floyd & Stephens 2012; Kang & Rivest et al, 2012). Recently, **ubiquitin,** a protein which is structurally related to SUMO-1, and which marks cells for degradation in the proteasome, was shown to play an important role in PPARγ turnover; interestingly, synthetic PPARγ agonists were found to regulate ubiquitinylation of PPARγ (Hauser et al, 2000; Floyd ZE & Stephens, 2012).

1.3. Biological and pathological roles of PPARγ

The importance of metabolic status is increasingly being recognized to influence brain health. Both, preclinical and clinical studies have demonstrated that increased body mass (obesity) impacts negatively on mood and cognition: major depression, Alzheimer's disease and stroke, three leading causes of disability and burdens on healthcare systems, are linked with abnormally high body mass. Overweight and obesity are also associated with other brain

disorders such as anxiety disorders and drug and substance abuse, as well as diseases that contribute to "metabolic syndrome", namely, hypertension, insulin resistance type 2 diabetes mellitus (T2DM) and dyslipidemia (high triglyceride and reduced high-density lipoprotein levels in blood) (Dallman et al, 2003; Barnes & Yaffe, et al 2011; Kaidanovich-Beilin et al, 2012; Hildreth et al, 2015). Overweight and obesity also interfere with the efficacy of therapeutic interventions in disorders such as major depression (Kloiber et al, 2007). Recent reports suggest that activation of the peroxisome proliferator-activated receptor γ (PPARγ) improve brain function. The PPARγ receptor is well known for its role in regulating peripheral metabolism (especially insulin sensitivity), tissue homeostasis and immune function (**Fig 1.11**), as briefly reviewed below.

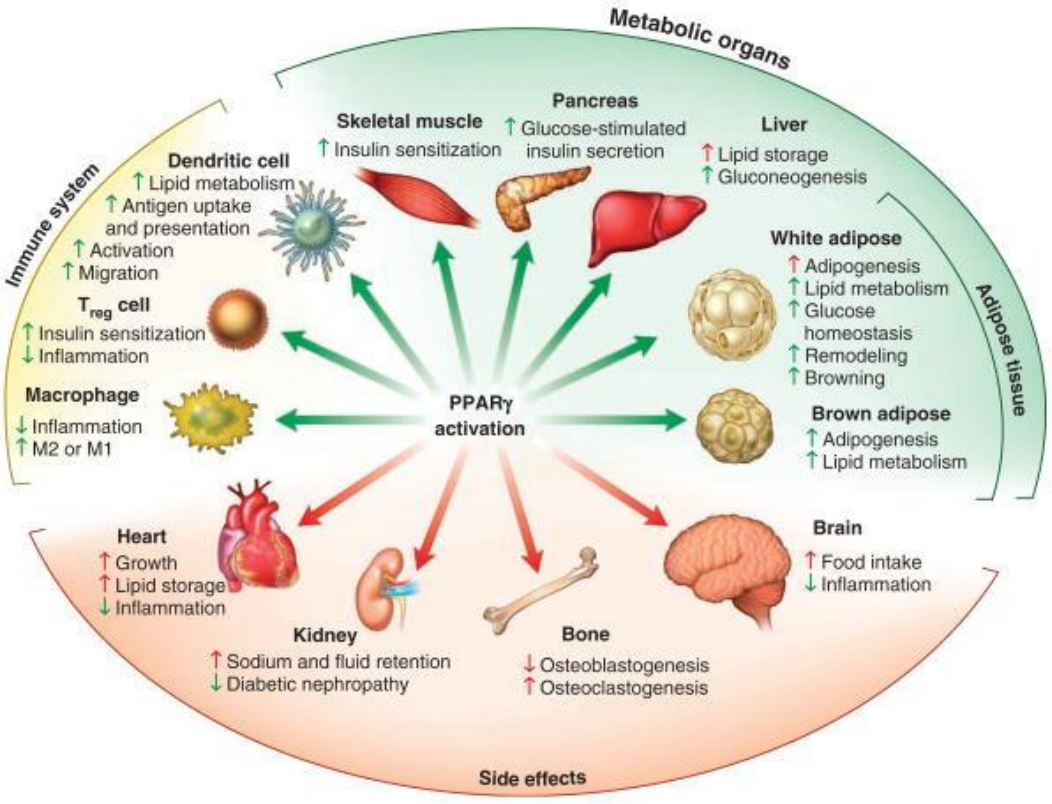

Figure 1.11. Biological and pathological actions mediated by PPARγ in various tissues (Ahmadian et al 2013).

PPARγ in adipose tissue, adiposity and some brain pathologies

Obesity is defined as a state of pathologically high adipose cell mass; it results from the ingestion of more energy than is necessary to maintain daily activities. From an evolutionary point of view, energy depots are built in order to ensure survival when conditions become inclement. Few civilizations today face such potential challenges; rather, the current rise in human obesity, especially in industrialized nations, results from foods, many of which are engineered to provide enhanced feelings of pleasure and energy in a densely-packed and readily metabolizable form (Malik et al, 2013).

The amount of food (energy) ingested is determined by intricate bi-directional communication of signals between the brain and periphery; the integrated outflow of signals from the brain occurs via the hypothalamus, whereas peripheral signals regarding satiety and overall energy status are carried to the brain primarily via the vagus nerve. Besides regulating appetite and feeding behaviour, the brain contributes to the control of blood glucose levels (Evans et al, 2004); this is perhaps not surprising given that although the brain accounts for just 2% of body mass, it uses 20% of the organism's energy supply under resting conditions (Erbsloh et al, 1958). It is important to also note that genetics, sex and age also place constraints over the behavioural and physiological regulation of energy intake and metabolism and that, the amount of energy stored in adipose tissue is ultimately determined by energy efficiency (the production of heat from fatty acids under the control of uncoupling proteins, as outlined below).

Adipose tissue is dynamic and has the potential to respond to altered nutritional status through hypertrophy and hyperplasia, and therefore to contribute to whole-body energy homeostasis (Sun et al, 2011). The current consensus is that hypertrophic white adipocytes surrounding the viscera reflect dyslipidemia (elevated cholesterol, LDL-cholesterol, triacylglycerols and apolipoprotein B). While this does not apply to subcutaneous fat tissue, both tissues are associated with defective insulin signaling and glucose metabolism in obese subjects. In contrast to hypertrophic (differentiated, increased in size) adipocytes, hyperplasia (increased number of small adipocytes) is supposed to be protective (Hoffstedt et al, 2010; Foster et al, 2012).

Adipose tissue exists in two forms, namely, white adipose (WAT) and brown adipose (BAT) tissue; the size of BAT depots is relatively small in adults but are larger in women than in men (Peirce et al, 2014). Energy is stored in the form of triglycerides in both WAT and BAT. The former releases energy in the form of free fatty acids (FA) for use by peripheral tissues (e.g. muscle, liver). Furthermore, WAT serves as an endocrine organ that regulates food intake by secreting adipokines such as leptin, adiponectin and resistin. In contrast, BAT metabolizes FA to produce thermogenic energy via activation of mitochondrial UCP-1 (uncoupling protein-1) which uncouples adenosine triphosphate (ATP) from the respiratory chain; the latter results in the dissipation of energy, by oxidation of fatty acids, as heat (Nedergaard & Cannon, 2013).

Adipocyte differentiation (Tontonoz et al, 1994 (a), Tontonoz et al, 1994(b)) and the survival of mature adipocytes (Metzger et al, 2005; Tontonoz & Spiegelman, 2008) are regulated by PPARγ. PPARγ plays a crucial role in in **FA metabolism** by regulating *acyl CoA-synthetase*, whose gene product plays a key role in **FA biosynthesis**, as well as gene products, which serve **FA transport** (adipocyte protein 2 [aP2], lipoprotein lipase [LPL], ApoE etc.) (Evans et al, 2004; Michalik et al, 2006; see **Table 1**). Further, PPARγ triggers differentiation of brown adipocytes and activates *UCP-1* (Sears et al, 1996) which, as mentioned earlier, is a critical molecule for energy dissipation by BAT through non-shivering thermogenesis. Here it is worthwhile noting that pharmacological agonists of PPARγ, such as TZDs transform unhealthy white fat into BAT like cells called beige/brite cells (Sell et al, 2004; Petrovic et al, 2010; Ohno et al, 2012; Qiang et al, 2012, Nedergaard & Cannon, 2014, Rosenwald et al, 2014). The conversion of WAT into BAT involves sirtuin 1 (Sirt1)-mediated deacetylation of PPARγ (Qiang et al, 2012).

Regulation of glucose metabolism and insulin sensitivity by PPARγ

Obesity is a risk for diabetes resulting from reduced or loss of sensitivity to insulin (type 2 diabetes; T2D) (Kahn et al, 2000). Insulin, a peptide hormone, is a key regulator of glucose uptake by metabolic tissues such as liver, fat and muscles. Loss of insulin sensitivity leads to supraphysiological levels of plasma glucose (ca. 72 to 108 mg/dL or 4.0 to 6.0 mmol/L after a fast, 140 mg/dl or 7.8 mmol/L when measured 90 min after a meal). Insulin acts after binding to the insulin receptor, induction of the phosphoinositide-3-Kinase/ protein kinase B (PI3K/AKT) pathway and synthesis of glucose transporter type 4 (Glut4), which transports glucose into the cell. Like PPARγ, insulin is also involved in the differentiation and

maintenance of adipocyte maturity. Furthermore, it regulates the transport of glucose and FA into adipocytes, while simultaneously inhibiting lipolysis.

Clinical interest in the biology of PPARγ mainly stems from its role in the control of insulin sensitivity and therefore, maintenance of blood glucose levels in the physiological range (Tondonoz & Spiegelman, 2008). Notably, polymorphisms in the ligand-binding domain of PPARγ were shown to increase risk for metabolic syndrome which consists of coexisting insulin resistance, hypertension and dyslipidemia (Barroso et al, 1999; Agarwal & Garg, 2002; Hegele et al, 2002). Specifically, a cis-regulatory variant that regulates PPARγ2 gene expression in adipocytes was identified for T2DM (Claussnitzer et al, 2014).

The importance of PPARγ in insulin sensitivity was demonstrated by knockout and fat-specific PPARγ activation experiments, manipulations that respectively, resulted in either insulin resistance (He et al, 2003) or improved insulin sensitivity (Sugii et al, 2009). Other studies revealed that PPARγ is required for the uptake of free FA by adipocytes as well as for lipolysis, resulting in decreased liver and muscle levels of free FA and triglycerides (Yamauchi et al, 2001; Tamori Y et al, 2002). In a seemingly paradoxical way, although TZDs are efficacious in increasing insulin sensitivity and lowering plasma glucose levels (effects that are accompanied by reductions in circulating free FA) (Bays et al, 2004), these drugs result in body weight gain. This may be ascribed to the fact that activated PPARγ stimulate the sequestration of FA by WAT as well as increase adipogenesis (hyperplasticity) (Tondonoz & Spiegelman, 2008; Ahmadian et al 2013); at the same time, TZD inhibit leptin, a satiety hormone which also induces lipolysis and glucose utilization in adipocytes (Kallen & Lazar, 1996; Havel, 2002).

The mechanisms responsible for the improved insulin sensitivity seen after activation of PPARγ by TZD are not completely understood; the issue is made further complex since TZD differentially influence insulin sensitivity in skeletal muscle, liver and adipose tissue by acting at different steps of the insulin signalling pathway, e.g. expression and/or tyrosine phosphorylation of insulin receptor substrate (IRS), PI-3-kinase/Akt (Tamori et al, 2002; Bays et al, 2004; Leonardini, et al 2009); interestingly, activation of PPARγ was found to attenuate tumour necrosis factor-α (TNF-α)-mediated block on insulin signaling (Evans et al, 2004). Further, pharmacological activation by TZD de-represses insulin-dependent GLUT4, thus

enhancing glucose transport into muscle and fat (Wu et al, 1998; Leonardini et al, 2009). It should also be noted that PPARγ directly regulates genes involved in glucose metabolism, e.g. *PEPCK* (*phosphoenolpyruvate carboxykinase*) (Tontonoz et al, 1995), whose protein product is involved in gluconeogenesis. Lastly, PPARγ also regulates adipokines (cytokines produced by adipocytes, e.g. leptin and TNF-α (direct target genes of PPARγ) as well as of adiponectin; the latter is the only adipokine whose levels are inversely related to metabolic diseases and which has significant antidiabetic, antiatherogenic and anti-inflammatory properties (Scherer et al, 2006; Matsuzawa et al, 2005). Some features of the present understanding of the mechanisms through which PPARγ facilitate metabolic homeostasis (specifically, insulin sensitivity) are shown in **Fig. 1.12**.

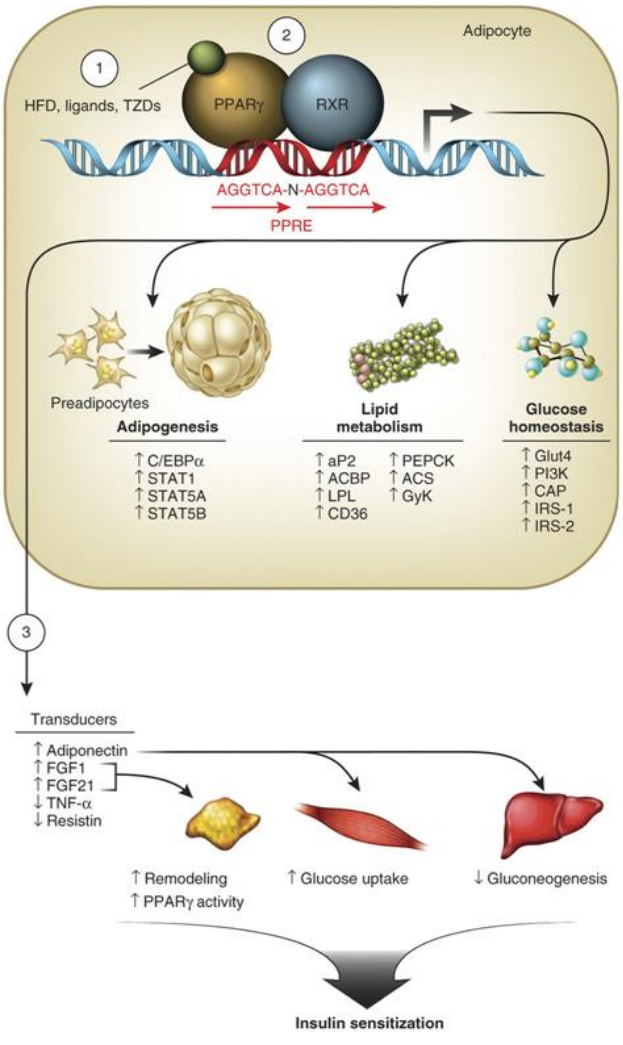

Figure 1.12. Effects of PPARγ activation on the regulation of target genes that contribute to improved insulin sensitivity in different tissues (*from* Ahmadian et al 2013).

Recent work, involving brain-specific PPARγ knockout mice and overexpression experiments showed a regulatory effect of

central PPARγ on feeding behaviour and peripheral metabolism (Lu et al, 2011; Ryan et al, 2011). Conversely, peripheral metabolic modulation of the central nervous system has been gaining attention. It is now known that insulin can cross the blood brain barrier (BBB) and that insulin receptors are expressed at high levels in the hippocampus and medial temporal cortex, two brain regions with key roles in memory (Wallum et al, 1987; Gaspar et al, 2016) and therefore of pertinence to AD.

Implication of PPARγ in Alzheimer's disease pathology

Interestingly, brain insulin signaling is reportedly reduced in AD patients (Messier et al, 2005) and insulin administration was found to improve cognition in AD patients (Craft et al, 2003); for this reason, some authors refer to AD as Type 3 Diabetes. Another interesting fact is that insulin degrading enzyme (IDE), which plays an important role in degradation of extra cellular Aβ, was shown to be reduced in AD, a phenomenon reversible by rosiglitazone (Pedersen et al, 2006). Supporting the latter observations, Du et al (2009) showed that the IDE gene promoter contains a PPRE which can be activated in primary neuron cultures. In the context of "Type 3 diabetes", it is also worth mentioning that insulin prevents Aβ-induced synaptic degradation (a key event in AD), an effect that can be enhanced with TZD treatment (Zhao et al, 2008; De Felice, 2013).

Other mechanisms thought to contribute to AD pathology via disturbed glucose homeostasis involve mitochondria; these organelles display altered morphology in AD and mitochondrial biogenesis can be improved by PPARγ activation *in vitro* and *in vivo* (Roses et al, 2007; Strum et al, 2007). A role for peroxisome proliferator-activated receptor γ co-activator 1-α (PGC1α), which itself is up-regulated by PPARγ and which acts as a coactivator of PPARγ, has also been proposed on the basis of the finding that PGC1α levels are reduced in AD brains. Interestingly PGC1α has also been linked to insulin resistance and obesity (Puigserver & Spiegelman, 2003). Like TZD, PGC1α stimulates a variety of genes involved in the mitochondrial oxidative phosphorylation pathway and mitochondrial genes such as *nuclear respiratory factor 1, nuclear respiratory factor 2* and *mitochondrial transcription factor A* (Chiang, et al 2014).

The PPARγ has been proposed as a therapeutic target for the treatment of AD in light of reports that TZDs (PPARγ agonists – see **2.3.2**) can ameliorate AD pathology. For example,

rosiglitazone treatment over 6 months was shown to improve memory, when compared to placebo-treatment in patients with mild-to-moderate AD (Watson et al, 2005); similar results were found in an independent pilot study on AD patients (Hanyu et al, 2010). Further, a large phase II clinical trial (Risner et al, 2006), including over 500 patients with mild-to-moderate AD, reported that rosiglitazone (6 months) improved attention and memory, as compared to placebo-treated patients; interestingly, treatment efficacy was restricted to subjects carrying the ApoE3 allele, whereas patients carrying the ApoE4 allele, did not benefit from the treatment (the role of ApoE in metabolism and AD is discussed later in this Introduction). In another study, which included 42 T2D-patients with mild AD, pioglitazone (also a TZD) reduced cognitive deficits; in parallel, pioglitazone prevented an increase in the plasma ratio of Aβ40/Aβ42 and improved insulin sensitivity (Sato et al, 2011); the latter is a primary, established action of TZD (see **2.4.2**). Interestingly, Sastre et al (2006) observed decreased (up to 40%) levels of PPARγ protein in the frontal cortex of AD patients.

In comparison to clinical studies, most studies on the impact of TZD in AD have been conducted in mutated human APP transgenic mice. These studies have generally been promising with respect to the potential efficacy of these drugs in improved learning and reversing cognitive impairments as well reducing Aβ42 peptide levels (but not amyloid plaque burden) in the brain (Pedersen et al, 2006; Rodriguez-Rivera et al, 2011; Denner et al, 2012; Jahrling et al, 2014). Other work reported that pioglitazone treatment of AD transgenic animals not only improved learning and memory (Mandrekar-Colucci et al, 2012; Searcy et al, 2012) but also decreased Aβ levels and -plaque pathology in the brain (Heneka et al, 2005; Sastre et al, 2006; Mandrekar-Colucci et al, 2012; Searcy et al, 2012). In a more detailed study, Brodbeck et al (2008) showed that rosiglitazone dose-dependently increased dendritic spine density in rat primary cortical neurons, while preventing dendritic spine loss in cells carrying the human ApoE4 mutation. Further, pioglitazone was also shown to prevent synaptic degradation induced by toxic Aβ treatment *in vitro* (Xu et al, 2014).

It is still unclear as to how TZD act on specific AD-related pathological pathways (APP-processing, Aβ-clearance and tau-hyperphosphorylation). Activation of PPARγ was found to reduce *BACE 1* (a direct target gene of PPARγ) mRNA and protein levels *in vitro* and *in vivo*, suggesting a potential mechanism through which PPARγ counteracts APP misprocessing (see

Fig. 1.2). This effect very likely involves the PPARγ co-activator PGC-1α (Heneka et al, 2005; Katsouri et al, 2011; Wang et al, 2013). Other studies have focussed on the role of PPARγ on Aβ-clearance, specifically by inducing the lipidation of ApoE by ABCA1 and the clearance of Aβ by lipidated ApoE. Insulin degrading enzyme (IDE), which is also involved in Aβ degradation, was also found to be increased by exposure of primary mouse neurons to a PPARγ agonist *in vitro* (Du et al, 2009). The effect of activated PPARγ on tau hyperphosphorylation and aggregation has been less-intensively investigated. In *in vitro* studies, TZD were shown to reduce tau-phosphorylation, albeit via a PPARγ-independent mechanism (Yoon et al, 2010; Cho et al, 2013). Interestingly, tau-aggregates were also decreased by activation of PPARγ in a tau-overexpressing cell model (Hamano et al, 2016). Lastly, research suggests that PPARγ help reduce AD pathology by suppressing neuroinflammation in the brain (see **2.4.4**).

Increased plasma cholesterol levels have been reported in a significant number of AD patients. Moreover, several studies have indicated that statins, drugs that inhibit cholesterol synthesis, reduce the risk of AD (Panza et al, 2006). In the brain, cholesterol is mainly carried by apolipoprotein E (ApoE). A polymorphism of the *APOE* gene that results in the production of the ApoE4 isoform which is the best-known risk factor for sporadic, late-onset AD (Verghese et al, 2011). However, the mechanisms through which ApoE contributes to amyloid β metabolism and pathology (Aβ is a key factor in AD pathology, as was described in **1.1.1**) remains unclear, although a role for ApoE in astrocytes and microglia has been suggested (Michikawa et al, 2000). Notably, pioglitazone treatment was found to enhance ApoE lipidation and transport out of the cell by ABCA1 (see below) in transgenic mouse models of AD animals and to stimulate Aβ clearance by lipidated ApoE (Mandrekar-Colucci et al, 2012; Kang & Rivest, 2012). This results in reduced Aβ levels and plaque load in the brain, and memory improvement. Importantly, blockade of PPARγ or LXR in microglia and astrocytes markedly reduce the clearance of Aβ (Mandrekar-Colucci et al, 2012; Kang & Rivest, 2012), suggesting that PPARγ or LXR act by suppressing neuroinflammatory processes (note that astrocytes and microglia express ABCA1 and ApoE) (Abuznait & Kaddoumi, 2012). Interestingly, while TZD such as rosiglitazone (a predecessor of pioglitazone) induce ABCA1 expression, they do not increase ApoE levels (Escribano et al, 2010), suggesting that besides

the level of ABCA1 and ApoE, the rate of ApoE lipidation be an additional factor that determines the effects of TZD on Aβ clearance (Jiang et al, 2008) (**Fig. 1.13**).

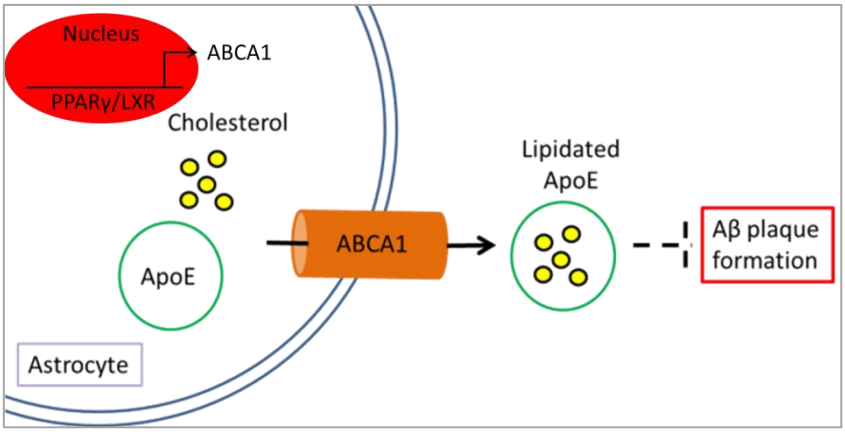

Figure 1.13. A model showing how activated PPARγ is involved in Aβ clearance (adapted from Kang & Rivest, 2012).

It is thought that following PPARγ activation, Aβ molecules are transported out of the brain into the periphery for metabolism by the liver. Transport out of the brain, across the blood-brain-barrier (BBB), is likely mediated by low-density lipoprotein receptor-related protein 1, a member of the low-density lipoprotein receptor gene family, which serves as a receptor for ApoE (Shibata et al, 2000; Liu et al, 2008). Expression of LRP1 was shown to be upregulated by nanomolar concentrations of rosiglitazone in cultured human brain microvascular endothelial cells, followed by increased Aβ uptake (Moon et al, 2012). Other work showed that LRP1-mediated transfer of ApoE-Aβ complexes across the BBB is ApoE isoform-dependent, i.e. does not operate optimally in subjects carrying an ApoE4 allele (Rebeck et al, 1993; Deane et al, 2008).

Role of PPARγ in (neuro-) inflammation

Interestingly chronic inflammation often appears together with metabolic diseases as well as neurodegenerative diseases such as Alzheimer's disease (Sun et al, 2011; Chung et al, 2008; Träger & Tabrizi, 2013). Activated PPARγ are known to repress the expression of genes encoding pro-inflammatory molecules (e.g. various cytokines and metalloproteases) by inhibiting nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NFκB) (Chen et al, 2012). As mentioned previously, low-grade inflammation is often seen in obesity, alongside

insulin resistance. Adipose tissue produces a number of pro-inflammatory peptides (e.g. Interleukin-6 and Interleukin-1β); both cytokines are implicated in the development of T1DM and/or T2DM through their actions on adipocytes, hepatocytes, myocytes, and pancreatic β cells (Kristiansen & Mandrup-Poulsen, 2005; Esterson et al, 2013). Further, macrophages are seen to invade the adipocytes of obese subjects (Weisberg et al, 2003). It is also relevant to mention that it is thought that hypoxia resulting from the limited angiogenesis in hypertrophic adipose tissue leads to the generation of free radical species (e.g. nitric oxide via iNOS (inducible nitric oxide synthase)) which have been associated with the development of insulin resistance (Esterson et al, 2013). In fact, early studies showed that activated PPARγ stimulate macrophage differentiation by promoting the expression of genes (e.g. CD36 (cluster of differentiation 36) and aP2), causing macrophages to shift to a state of oxidative metabolism, an event necessary for their phagocytotic actions (Tontonez et al, 1998; Chawla 2010).

Patients with AD often display leaky BBB, making it possible for inflammatory cytokines to enter the brain (Takeda et al, 2014). Importantly, however, oligomeric amyloid β is known to induce pro-inflammatory cytokine expression by microglia, which serve a macrophagic role in the brain (Heneka et al, 2015a; Heneka et al, 2015b). Recent studies have shown that PPARγ agonists increase microglial expression of the scavenger receptor CD36, thereby facilitating the phagocytosis of amyloid β (Yamanaka et al, 2012).

Implication of PPARγ in depression, a stress-related psychiatric disorder

Environmental stressors trigger physiological responses, in particular, the secretion of glucocorticoids (GC) under the control of the hypothalamic-pituitary-adrenal (HPA) axis. As a result, the physiological response to stress includes increased heart rate and blood pressure and mobilization of stored energy. Hypersecretion of GC can induce insulin insensitivity and hyperglycemia (diabetes), a condition associated with cognitive impairment and depression (Ulrich-Lai & Ryan, 2013; Detka et al, 2014). At the same time, chronic exposure to high GC levels is causally related to synaptic loss, neuronal atrophy (Sotiropoulos et al, 2008; Sotiropoulos et al 2011), features that are observed in association with the same impairments in brain function found in subjects experiencing extended hyperglycemia (Detka et al, 2014). In the context of this thesis, it is also interesting to note that there is growing evidence that depressive illness pre-dispose individuals to develop AD (Green et al, 2003; Kida et al, 2016).

Several studies have reported on the ability of PPARγ agonists to reverse depressive-like symptoms in both rodents and humans (Rosa et al, 2008; Salehi-Sadaghiani et al, 2012; Kemp et al, 2012). Interestingly, suppression of inflammation, one possible mediator of depression (Kemp et al, 2012; Kemp et al, 2014), has been suggested to be a prime mechanism through which activated PPARγ receptor act to reduce symptoms of this pathological behaviour. Specifically, immobilization stress (a paradigm used to induce depressive-like symptoms in rodents) was shown to increase cortical levels of both, PPARγ as well as of their endogenous ligand 15-PGJ2 (15-Deoxy-Delta-12,14-prostaglandin J2) (García-Bueno et al, 2008). Further, endogenous and pharmacological agonists of PPARγ were shown to decrease stress-induced increases in proinflammatory cytokine (e.g. COX-2 (Cyclooxygenase-2), TNFα) and iNOS levels in the rodent cortex. Besides, their anti-inflammatory effects, PPARγ agonists also seem to reverse stress-induced inhibition of cortical synaptosomal glucose uptake (García-Bueno et al, 2008; García-Bueno et al, 2014), a phenomenon that might at least partly explain their anti-depressive properties.

1.4. Specific aims of this thesis

This work addressed the following primary questions:

- Are PPARγ expressed in the mouse brain and, if so, in which structures and cell types?
- If PPARγ are expressed in the brain, are they functional, i.e. does their activation result in biologically-relevant effects?
- Are suggestions in the literature that activated PPARγ may be therapeutically relevant to Alzheimer's disease plausible?

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Chapter 2

Expression patterns and function of PPARγ in brain and neural cell lines

2.1. Abstract

Although the nuclear receptor (NR), peroxisome proliferating activating receptor γ (PPARγ) has been implicated as a potential treatment target in a number of brain disorders, to date there are few definitive reports of their expression in the human or rodent brain. The present work set out to systematically map PPARγ protein expression in the mouse brain, focusing on regions that may be targets of PPARγ ligands either in the context of Alzheimer's disease (AD) or metabolic disease (such as type 2 diabetes). Thiazolidinediones (TZD) are a well, known class of PPARγ agonists and are used for the treatment of diabetes; they have also been implicated for use in AD. Using immunocytochemistry, we identified PPARγ in nucleus accumbens, amygdala, hypothalamus, thalamus and cerebellum, as well as the frontal cortex and hippocampus; function of the two latter regions is strongly compromised in AD. Our observations that the expression of PPARγ tends to increase with age were largely confirmed by Western assay-based methods as well as by measurements of PPARγ mRNA by quantitative real time polymerase chain reaction assays (qRT-PCR). Likewise, these methodological approaches revealed that PPARγ mRNA and protein expression are also developmentally regulated in primary neural cultures from mouse frontal cortex and hippocampus. Chemotypical characterization of these cultures showed PPARγ expression in nestin$^+$ neural precursor cells, microtubule-associated protein 2$^+$ (MAP2) mature neurons and glial fibrillary acidic protein$^+$ (GFAP) astrocytes; while microglia were absent from our cultures, O4$^+$ oligodendrocytes were PPARγ$^-$. Two neural cell lines (mouse HW3-5 and human SH-SY5Y cells) were also found to express PPARγ mRNA and/or protein; specificity of the immunochemical results, were confirmed by knockdown experiments in SH-SY5Y cells. Semi-quantitative studies in frontocortical and hippocampal lysates further demonstrated sex- and site-specific differences in PPARγ expression. Importantly, we also demonstrated that the immunochemically-detectable PPARγ in the various *in vitro* neural cell preparations were transcriptionally active. Thus, Pio induced the expression of *peroxisome proliferator-activated receptor gamma coactivator 1-alpha* (*PGC-1α*) and *ATP-binding cassette transporter ABCA1* (*ABCA1*), two known PPARγ downstream target genes. Together, these observations provide robust support for earlier claims of the presence of PPARγ in the brain, thus justifying more detailed studies to investigate the mechanisms through which TZD may act to reverse the neuropathology associated with AD and other neuropsychiatric diseases.

2.2. Introduction

Peroxisome proliferator-activated receptors (PPAR), like other members of the superfamily of nuclear receptors are ligand-activated transcription factors (Issemann & Green, 1999). Their role in the periphery has been intensively studied, in particular with respect to the regulation of lipid metabolism, energy homeostasis and inflammation (Desvergne & Wahli, 1999; Cho et al, 2008). Of the three PPAR isoforms (α, β/δ and γ), PPARγ (or NR1C3) plays a fundamental role in the control of adipocyte activation. Fatty acids are the best-known PPARγ agonists and activated PPARγ become transcriptionally active after heterodimerization with either the retinoid X receptor (RXR) or liver X receptor (LXR), both of which are also nuclear receptors (Desvergne & Wahli, 1999; Laudet & Gronemeyer, 2002). The thiazolidinedione (TZD) class of drugs, in particular pioglitazone (Pio), are potent pharmacological agonists of PPARγ that have been implicated for use patients with Type 2 diabetes because of their powerful insulin-sensitizing actions (Tontonoz & Spiegelman, 2008; Hauner, 2002).

Given that diabetes is a known risk factor for Alzheimer's disease (AD) (Tontonoz & Spiegelman, 2008; Ahmadian et al, 2013), an interest has developed in the potential use of TZD to prevent, delay or treat this devastating disease, characterized by gradual loss of memory and higher brain functions. While there are a number of reports that support the view that TZD can improve cognition in both patients (Watson et al, 2005; Risner et al, 2006; Hanyu et al, 2010; Chawla, 2010; Heneka et al, 2015) and mouse models of AD (Mandrekar-Colucci et al, 2012; Searcy et al, 2012) there remains a paucity of evidence for the presence of functional PPARγ in the brain; indeed, the available data obtained in rats or mice (Gofflot et al, 2007; Moreno et al, 2004; Sarruf et al, 2009; Warden et al, 2016) are often inconsistent and generally of relatively poor resolution – limitations of sensitivity and/or specificity partly explains difficulty in interpreting or relying upon studies in which PPARγ antibodies were applied.

Considering that definitive demonstration of PPARγ in relevant brain areas would bolster the view that TZD may be useful therapeutic agents for the management of AD and would also provide clarity as to whether these drugs act indirectly (by resetting peripheral metabolic disturbances) or directly upon the neural substrate, this work aimed to explore if, and where,

PPARγ are expressed in the central nervous system (CNS). Our analysis included mRNA expression assays on micro-dissected brain regions of interest and immunochemical assays on mouse brain sections, primary cultures from the mouse hippocampus and frontal cortex, and neural cell lines; in the latter cells, PPARγ knockdown experiments were performed. Emphasis was placed on assuring specificity of the detection reagents, as well as spatial expression (specific brain areas from wildtype and PPARγ knockout mice) and localization of PPARγ in specific cell types (e.g. neurons, astrocytes, oligodendrocytes, microglia) and intracellular compartments (nucleus *vs.* cytoplasm). In addition, we examined the possibility of developmentally-regulated patterns of PPARγ expression in neural cells *in situ* and *in vitro.*

2.3. Materials and Methods

Animals: All studies were performed on mouse tissues, in compliance with European Union Council Directive (2010/63/EU) and local regulations. Mice belonging to the CD1 and C57/Bl6 strains were used, as described in Results for individual experiments. Unless otherwise stated, animals were bred and maintained in the local animal facility (Max Planck Institute of Psychiatry; stock animals obtained from Charles River, Sulzfeld, Germany). Animals were sacrificed by decapitation after rapid cervical dislocation. Their brains were then excised and areas of interest were dissected. Primary neural cell cultures were prepared from male and female mice aged 5 days (PND 5), as described below. Protein and mRNA assays were performed on mice aged 5, 14 or 90 PND (see later in this section) and from mice with a conditional knockout of PPARγ in the brain (PPARγ$^{-/-}$); the latter and appropriate controls were kindly provided by Professor Michael Heneka (Deutsches Zentrum für neurodegenerative Erkrankungen, University of Bonn; unpublished). Tissues from transgenic J20 mice overexpressing amyloid precursor protein (APP, from Dr. L. Mucke, University of San Francisco – Harris et al, 2010) and mice with a deletion of β-secretase 1 (BACE$^{-/-}$, Jackson labs (B6.129-Bace1tm1Pcw/J mice)) were kindly provided by Dr. I. Sotiropoulos (University of Minho, Braga, Portugal – Dioli et al, 2017). An overview of animals (strains, ages, sexes, numbers) is given in **Supplementary Information (2.7 – Fig. S.2.1)**.

Cell culture: All cells were maintained in an incubator with 5% CO2, at 37°C and 95% relative humidity. All reagents were from Life Technologies (Eggenstein, Germany), unless indicated otherwise.

Cell lines: Three neural cell lines were used in these studies:

1. **HW3-5:** A murine, hippocampal cell line (Kuwahara et al, 1999; Baj et al, 2005; courtesy of Dr. Dietmar Spengler, Max Planck Institute of Psychiatry) was cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum. When cultures had reached 95% confluence, cells were split using 2.5% trypsin and replated at a density of approximately 0.3 x 10^4/well in 6-well plates.
2. **SH-SY5Y:** A human neuroblastoma cell line (American Tissue Culture Collection [ATCC®], CRL-2266™, Germany) was initially seeded in Minimum Essential Medium with glutamax®, supplemented with 10% fetal calf serum (FCS), 1% penicillin-

streptomycin, and 2 mM L-glutamine. When confluency was reached (95%), cells were split with 2.5% trypsin. Differentiation was induced by reducing the serum content of the growing medium from 10% to 1% FCS and the addition of 0.05 mM retinoic acid (Sigma Adrich, Taufkirchen, Germany) for 5 days, followed by 20 ng/ml nerve growth factor (NGF – Bio-Techne, Wiesbaden, Germany) for a further 5 days (**Supplementary Informaton - Fig. S2.1**).

3. HT-22 a mouse hippocampal cell line was employed for overexpression experiments of mouse-PPARγ. Experiments were performed by Kathrin Hafner and Nils Gassen according to Gassen et al (2014). Following vector (ordered by Addgene) were used: pSV Sport PPAR gamma 1 was a gift from Bruce Spiegelman (Addgene plasmid # 8886) and pSV Sport PPAR gamma 2 was a gift from Bruce Spiegelman (Addgene plasmid # 8862) (Tontonoz et al, 1994).

Primary neural cell cultures: Cells were cultivated in either 6- or 12-well plates that had been coated with 0.08% gelatin (Sigma Aldrich) in 0.024 M borate buffer (**Supplementary Information - Table S2.2**) for 2 h, at room temperature (RT), 0.03 mg/ml poly-D-lysine (Sigma Aldrich) in borate buffer (2 h, RT), followed by 3 washes with ddH_2O before drying under an airstream. Sterile conditions were maintained throughout, and coated plates were stored in a humidified incubator until use. For immunocytochemical analysis, pre-cleaned (ethanol:sulphuric acid) glass coverslips were similarly coated and placed in 12-well dishes.

Primary hippocampal and frontocortical cultures were prepared from mouse pups on PND5. After decapitation, brains were quickly removed and placed in NeurobasalA medium, containing 2% 50xB27 and 1% kanamycin. Brain meninges and blood vessels were removed under a dissecting microscope and the isolated hippocampi and frontal cortex were sliced (250 µm) along the horizontal and vertical axes, using a McIlwain tissue chopper (Camden Instruments, Leicester, UK). Tissue slices were then incubated in 2 ml NeurobasalA-B27 (37°C, 5 min) before centrifugation (70 *g*, RT, 30 s) after which the pellet was resuspended and digested in 5 ml 0.05% trypsin (**Supplementary Information - Table S2.2**) (for 37°C, 10 min, in shaking water bath). Tissue digestion was stopped by adding 5 ml trypsin blocking solution (**Supplementary Information** - **Table S2.2**) and centrifugation (70 *g*, RT, 30 s). The supernatant was filtered through a 30 µm nylon mesh (VWR International, Schwalbach, Germany) and the

pellet was resuspended in 5 ml trypsin blocking solution. The last step was repeated until the pellet was fully dissolved. Dead cells and other debris were discarded by carefully transferring the suspension to the top of a 5 ml separation gradient consisting of 3 ml 7.5% BSA and 2 ml NeurobasalA before centrifugation (6 min, 70 *g*). After dissolving the pellet in 2 ml growing medium (**Supplementary Information - Table S2.2**), a sample of cells was counted in a Neubauer hemocytometer (Neubauer improved/DHC-N01). Subsequently, cells were seeded at a density of 400-500 cells/mm^2 in cultured growth medium. One half-volume of the medium was replaced after 3 days and every 2-3 days thereafter.

Drugs: Primary cell cultures were treated with pioglitazone (Sigma Aldrich) at doses between 0.1 and 10 µM or with the PPARγ-specific antagonist GW9662 (Sigma Aldrich). Both drugs were dissolved in 0.1% (final concentration) dimethylsulfoxid (Roth, Karlsruhe, Germany).

Immunocytochemistry (ICC): Cells, grown on glass coverslips, were prepared for immunofluorescent staining using a protocol described by (Roselli et al, 2005). Throughout, the standard buffer (also for washing steps) used was 0.01 M phosphate-buffered saline (PBS). Briefly, cells were fixed in ice-cold 4% paraformaldehyde for 15 min, washed in PBS (3x, 5 min) before permeabilization with 0.1% Triton X-100 (30 min), and blocking in 10% FCS (30 min, RT). Primary and secondary antibody solutions were prepared in 0.01 M PBS containing 0.1% Triton X-100 and 10% FCS. Cells were incubated in primary antibodies (**Table 2.1**) for 16 h at 4°C, after which they were washed (3x, 30 min) and incubated with appropriate secondary antibody (**Table 2.1**) for 1 h, at RT. Samples were then rinsed (3x, 30 min) and, their nuclei stained with Hoechst dye 33341 (1:50 000; 10 min, RT). After 3 washes (5 min each), specimens mounted on SuperfrostPlus™ glass slides. Optical section images and stacks of images from fluorescence-labelled cells were obtained using a laser scanning confocal microscope (Olympus Fluoview 1000, Hamburg, Germany) outfitted with plan apo-chromat 40x and 63x (oil) lenses. Semi-quantitative image analysis (counting of cells displaying single or double staining of antigens of interest) was performed on 100 cells in 5 separate fields on each coverslip; 3-6 coverslips from each treatment were analysed.

Table 2.1. Details of primary and secondary antisera used for immunocytochemical analysis of primary neural cell cultures.

	Antibodies	Supplier	Cat.No	Dilution/ Concentration	Conjugate
Primary	CD11b, mouse monoclonal IgG	abcam	ab86860	5 - 50 µg/ml	
	CD68, mouse monoclonal IgG1	abcam	ab31630	1:100 - 1:25	
	GFAP, mouse monoclonal IgG1	Sigma	G3893	1:1500	
	Iba-1, rabbit monoclonal IgG	Wako Chem	#019-19741	2 - 20 µg/ml	
	MAP2, chicken polyclonal IgY	AVES	#MAP	1:1500	
	Nestin, mouse monoclonal IgG1	Chemicon	MAB353	1:1500	
	O4, mouse monoclonal IgM	Millipore	MAB345	1:25	
	PPARγ, rabbit polyclonal IgG	abcam	ab19481	1:100	
	PPARγ, rabbit monoclonal IgG	Cell Signaling	#2435	1:250	
Secondary	Goat-anti chicken, polyclonal IgY	Jackson Immuno Research	#103-505-155	1:1000	Dye light 594
	Goat-anti mouse, polyclonal IgG	Invitrogen	Inv#A110029	1:1000	Alexa Fluor 594
	Goat-anti rabbit, polyclonal IgG	Invitrogen	Inv# A110374	1:1000	Alexa Fluor 488

Immunohistochemistry (IHC): Throughout, the standard buffer (also for washing steps) used was 0.01 M phosphate-buffered saline (PBS); in some cases, tris-buffered saline (TBS), with or without 0.3% triton X-100 (TBS-T) was used. Mice (CD1 strain) aged PND 7 or 90 were perfused with 4% paraformaldehyde (PFA; Sigma Aldrich). Upon excision, brains were stored in 4% PFA over night followed by 10% sucrose at 4°C until used. Coronal cryosections (30 µm thick) were cut and mounted on SuperfrostPlus™ glass slides and stored at -20° C until further processing. Sections were washed (5x, 5 min each) before permeabilization in 0.5% Triton X-100 (Sigma-Aldrich) for 20 min at RT, washed (2x, 5 min) and treated with 0.5% of hydrogen peroxide (Sigma-Aldrich) for 10 min (RT). Following 2 washes (5 min each), an antigen retrieval was performed by incubating sections in 0.01 citrate buffer (**Supplementary Information - Table S2.3**) for 20 min (RT) and then 30 min at 80°C. After cooling, sections were blocked with 1% normal goat serum (NGS; Sigma Aldrich)/ TBS-T for 30 min (RT) before incubation with anti-PPARγ (Cat. #2435; Cell Signaling, Danvers, MA, USA) at a dilution of 1:250 in 1% NGS/0.3%TBS-T (40 h; 4°C) and after washing (3x, 10 min in PBS), subsequently incubated with biotinylated secondary goat-anti-rabbit antibody (Vector Laboratories, Burlinghame, CA, USA; 1:1000 in 1% NGS/0.3%TBS-T) for 2 h, at RT. After 3 washes (10 min each), sections were labelled with ABC solution (Vectastain® Elite® ABC Kit; Vector Labs), according to manufacturer's instructions. Immunolabeled signal was then visualized with diaminobenzidine (DAB) staining (DAB Substrate Kit for peroxidase; Vector) and cell nuclei were subsequently stained with methyl green (Lee & McKinnon, 2009). Sections were then washed in running tap water, dehydrated in graded ethanols, cleared with xylene, and mounted with DPX mounting solution (Fluka Chemie, Buchs, Switzerland). Sections were

examined under a Zeiss (Jena, Germany) microscope, using AxioVision Rel. 4.7 software (Carl Zeiss, Göttingen, Germany).

PPARγ knockdown *in vitro*: SH-SY5Y cells were seeded on gelatin-coated 6 well plates at a density of 200 000 cells/well in Opit-MEM (Life technologies). Transfection was achieved using *Stealth™/siRNA technology* (ThermoFisher, Dreieich, Germany); for this, a mix of 3 primer pairs (Invitrogen PPARGHSS108295 (3_RNAI), PPARGHSS108296 (3_RNAI) and PPARGHSS108296 (3_RNAI)) was used (**Table 2.2**). Briefly, cells were transfected (at a 30-50% confluency) with 100 pM RNAi mix, using 5 µl/well of Lipofectamine^{TM}2000 (Life technologies) to transfer RNAi into cells; the RNAi mix was incubated separately from the lipofectamine for 5 min (RT) each. Cells were incubated with both solutions for 8 h in the incubator, after which the transfection solution was replaced with DMEM containing 1% FCS. For analysis (60 h later), cells were lysed with homogenization buffer (Active Motif, La Hulpe, Belgium).

Table 2.2. Primers used for RNAi knockdown of PPARγ in SH-SY5Y cells.

Primer name	Sequence	Millimolar Extinction Coeff (OD/µmol)
PPARGHSS10295(3_RNAI)	5' - UCAGCUCCGUGGAUCUCUCCGUAAU - 3'	249.0
PPARGHSS10295(3_RNAI)	5' - AUUACGGAGAGAUCCACGGAGCUGA - 3'	288.6
PPARGHSS10296(3_RNAI)	5' - GCCUGCAUcUcCACCUUAUUAUUCU - 3'	238.4
PPARGHSS10296(3_RNAI)	5' - AGAAUAAUAAGGUGGAGAUGCAGGC - 3'	308.8
PPARGHSS10297(3_RNAI)	5' - GGCUGUGCAGGAGAUCACAGAGUAU - 3'	286.4
PPARGHSS10297(3_RNAI)	5' - AUACUCUGUGAUCUCCUGCACAGCC - 3'	251.2

Immunoblotting: Two methods – classical Western blotting and the highly-sensitive Protein Simple Wes assay **–** were used to measure PPARγ in cell and/or brain tissue extracts.

Protein extraction: Cytosolic and nuclear fractions from cultured cells and brain tissue (both stored at -80°C until time of analysis) were lysed and processed using a commercial nuclear extraction kit (Cat40410; Active Motif), following the manufacturer's suggested protocols. When biomaterial was to be used for mRNA plus protein measurements, tissues were processed according to instructions provided by the manufacturer of the PARIS kit (Thermo Fisher). The protein content of the nuclear and cytosolic lysates, were measured in triplicate using Lowry's method (Lowry et al,

1951); absorbance (750 mm) was measured on a Synergy-HT plate reader (BioTek Instruments, Winooski, VT, USA).

Conventional Western blotting: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate defined amounts of protein lysates (30 µg) that had been denatured by heating at 95^{o} C for 10 min. Briefly, samples and a pre-stained molecular weight marker (Thermo Fisher) were mixed with 16% Laemmli buffer, loaded onto a 0.1% SDS-PAGE gel topped with a 5% stacking gel (**Supplementary-Information - Table S.4**) which was then placed in an electrophoresis chamber (Biorad System, Hercules, CA, USA) filled with running buffer (0.1% SDS in 0.25 M Tris Base and 1.92 M Glycine; **Supplementary Information – Table S.4**). Electrophoresis was performed using a 2.5 A power supply (stacking gel: 90 V; SDS-PAGE gel: 120 V) for up to 1.5 h. Separated proteins were semi-dry transferred onto 0.2 µm nitrocellulose membranes (BioRad) using the Turbo Transfer System (BioRad) (2.5 A, 25 V, 7.5 min). Transfer quality was assessed after visualizing protein bands with Ponceau-S Solution (Sigma-Aldrich). After washing and blocking for 1 h (RT) in 5% non-fat milk powder (Roth) or with 5% BSA (Sigma Aldrich), both dissolved in TBS-T, when primary antisera from Cell Signaling were used, (**Supplementary Information - Table S.2.4**), membranes were incubated with primary antibodies (**Table 2.3**) for 16 h at 4°C. Following 3 washes with TBS-T, membranes were incubated with a corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (**Table 2.3**) for 1 h (RT). Thereafter, membranes were washed (3x) before incubation with Clarity™ Western enhanced chemiluminescence (ECL) Blotting Substrate to visualize proteins in a chemiluminescence reader (ChemiDoc MP Imaging System, BioRad). Blots were semi-quantitatively assessed using ImageLab 5.1 Software (BioRad).

ProteinSimple Wes: Immunoblotting of tissue samples (frontal cortex and hippocampus) was performed employing the 2-40 kDa Wes Separation Module (SM-Ws012; ProteinSimple, San Jose, CA, USA) and anti-rabbit detection module for Wes (DM-001), following the manufacturer's instructions. Samples were homogenized and processed using a commercial nuclear extraction kit (Cat 40410; Active Motif), following the manufacturer's suggested protocols. Samples containing 5 µg protein (measured by Lowry assay) each were loaded into individual slots of the ProteinSimple instrument and assayed for PPARγ by semi-automatic capillary electrophoresis, using anti-PPARγ (rabbit, monoclonal; Cell Signaling #2435; diluted

1:250) and, anti-beta-Actin (rabbit, polyclonal; Cell Signaling #4967, 1:200) to assist normalization; corresponding labelled secondary antisera, provided in the kit, were used for signal detection.

Table 2.3. Primary and secondary antibodies used in immunoblotting assays

	Antibodies	Supplier	Cat.No	Dilution	Conjugate
Primary	Actin, mouse monoclonal IgG2bκ	Chemicon	MAB1501R	1:2500	
	PPARγ, mouse monoclonal IgG1	Santa Cruz	sc-7273	1:500	
	PPARγ, rabbit monoclonal IgG	Cell Signaling	#2435	1:500	
	PPARγ, rabbit polyclonal IgG	abcam	ab19481	1:400	
Secondary	Goat anti mouse, polyclonal IgG	BioRad	170-6516	1:2000	(H+L) HRP-conjugated
	Goat anti-rabbit, polyclonal IgG	Thermo	#31460	1:1000	(H+L) HRP-conjugated

Polymerase Chain reaction assay

RNA Isolation: Total RNA was isolated from cell lysates using the NucleoSpin (RNA) kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions. RNA concentrations were determined with a NanoPhotometer (SmartSpec ™ Plus, Biorad).

Reverse transcription: Rewriting of 1 µg RNA into complementary DNA (cDNA), was done by using RevertAid RT Reverse Transcription kit (Thermo Scientific) with an oligo deoxythymidine (dT) primer, according to the manufacturer's instructions.

PCR: Polymerase chain reaction (PCR) was performed using the Taq DNA Polymerase Kit (Fermentas/ThermoFisher) in a total volume of 25 µl. The primers used are listed in **Table 2.4**. After loading of PCR products onto a 2% agarose gel, containing 0.005% ethidium bromide samples were electrophoresed at 100 V for ca. 15 min.

Digestion of PCR-products with corresponding restriction enzymes: Digestion of PCR-products was performed according to the manufacturer's (Fermentas) recommendations. Briefly, 5 µl of PCR product was digested over night at 37o C (unless specified otherwise). The reaction was performed in a 10 µl reaction volume using 0.5 µl restriction enzyme (Table 2.5) in an appropriate buffer. The digested PCR product and an undigested control were analysed on agarose gels.

Quantitative real time polymerase chain reaction (qRT-PCR): cDNA was used for quantification of mRNA levels using the SYBER Green MasterMix (FastStart Essential DNA Green Master kit - Roche, Mannheim, Germany) and the Roche LightCycler 96,

following the manufacturer's instructions. Primers used are shown in Table 2.6. Calculation of relative expression levels of target genes were done according to Pfaffl (2001).

Table 2.4. Primers used in qRT-PCR assays

Primer	Sequence	Tm oC	Position	Length of PCR product
LXRα fwd	5' - GATGCTTCTGGAGACATCG - 3'	61.0	exon 6	
LXRα rev	5' - GAAGATGCTGATGGCTATGAG- 3'	61.4	exon 7	205 bp
LXRβ fwd	5' - CACTTCACTGAGCTAGCCATC - 3'	61.6	exon 7	
LXRβ rev	5' - GGAAGTCGTCCTTGCTGTAG - 3'	61.5	exon 5	214 bp
PPARγ fwd	5' - CAGCTACAGCAACATTCCTCACG - 3'	67.9	exon 6	
PPARγ rev	5' - CTCTGCATGGCTTCCTGTCTTC - 3'	67.3	exon 7	223 bp
RXRα fwd	5' - CTTCAAGAGGACAGTACGC - 3'	57.5	exon 4	
RXRα rev	5' - CTTCTCTACAGGCATGTCCT - 3'	58.5	exon 5	229 bp
RXRβ fwd	5' - GTGAAGCCACCAGTCTTAG - 3'	57.4	exon 3	
RXRβ rev	5' - GTTATCTCGGCATGAGTAGG - 3'	58.8	exon 4	196 bp
RXRγ fwd	5' - CTTCAAGAGGACAGTACGC - 3'	59.8	exon 4	
RXRγ rev	5' - GAGCTCTCCACACTCATGTC - 3'	60.1	exon 5	211 bp

Table 2.5. Restriction enzymes used to examine specificity of PCR products

PCR product	Restriction enzyme
RXRα	NcoI (Fermentas)
RXRβ	PpuMI (Fermentas)
RXRγ	XbaI (Fermentas)
LXRα	PstI (Fermentas)
LXRβ	PstI (Fermentas) (1 h)
PPARγ	BstNI (Fermentas) (55 C)

Table 2.6. Primers used in RT-qPCR assays

Primer	Sequence	Tm oC	Position	Length of PCR product	Genebank ID
ABCA1 fwd	5' - CAGCATTAAGGACATGCAC - 3'	59.3	5. exon	214 bp	
ABCA1 rev	5' - CTGGAGACCAACATTCGC - 3'	62.0	6. exon		
GAPDH fwd	5' - TGGAGAAACCTGCCAAGTATG - 3'	63.9	5. - 6. exon	116bp	Bockmühl I
GAPDH rev	5' - GTTGAAGTCGCAGGAGACAAC - 3'	63.8	6. exon		
PGC-1α fwd	5' - CGTGTCGAGACTCAGTGTC - 3'	59.6	9. exon	154 bp	
PGC-1α fwd	5' - GTGTCTGTAGTGGCTTGATTC -3'	58.8	10. exon		
Pgk1 fwd	5' - CAATGAGATGATCATTGGTG - 3'	58.9	7. exon	164 bp	
Pgk1 rev	5' - CAGTCTTGGCATTCTCATC - 3'	47.6	8. exon		
PPARγ fwd	5' - GAAGAACCATCCGATTGAAGC - 3'	64.7	4. exon	201 bp	
PPARγ rev	5' - GGTCGATATCACTGGAGATCTC- 3'	61.5	5. exon		
PPARγ1 fwd	5' - TTCTGACAGGACTGTGTGACAG - 3'	63.1		354 bp	U01841
PPARγ1 rev	5' - ATAAGGTGGAGATGCAGGTTC - 3'	61.9			
PPARγ2 fwd	5' - GCTGTTATGGGTGAAACTCTG -3'	61.2		350 bp	NM011146
PPARγ2 rev	5' - ATAAGGTGGAGATGCAGTTC -3'	61.9			

Statistical analysis: Prism 6 software (GraphPad, San Diego, CA) was used to evaluate statistical differences between groups. Data were first tested for normality, followed by Student's *t*-test or 2-factor analysis of variance (2-way ANOVA), with Tukey's *post hoc* test. All data passed the normality test (Shapiro-Wilk-Test), except for that shown in **Fig.2.4; accordingly, those data were tested** with the Kruskal-Wallis test, corrected for multiple (pair-wise) comparisons by Dunn's test. The level of significance was set at $p \leq 0.05$; numerical data are presented as ± standard error of the mean (SEM). Statistical advice was kindly provided by Dr. Darina Czamara (Max Planck Institute of Psychiatry, Munich).

2.4. Results

2.4.1. Immunohistochemical mapping of PPARγ in the neonatal and adult mouse brain

The relative paucity of consistent information regarding the presence of PPARγ in the mouse brain prompted a systematic mapping study of the expression of this protein in the mouse brain. For this, an antibody (CST #2435) shown to specifically recognize the nuclear receptor (see **Fig. 2.5**) was used. In particular, specificity of signal was tested by negative control staining (see **Fig. S2.2**). Since many proteins show differential expression during development, we here performed immunohistochemical analysis on coronal brain sections obtained from male mice of the CD1 strain, aged 7 PND (neonates) and 90 PND (adults). Focus was especially placed on examining brain regions implicated in cognition, mood-emotion, endocrine-metabolic regulation and motivation-reward mechanisms, all of which are directly or indirectly relevant to Alzheimer's disease. Representative results are shown in **Fig. 2.1.** (neonates) and **Fig. 2.2.** (adults) and their qualitative/comparative evaluation is depicted in **Table 2.7.**

Immunoreactive (ir)-PPARγ was detected at low-to-moderate levels and high levels, respectively, in two sub-regions of the neonatal and adult mouse **frontal cortex** (**Fig. 2.1 a-c, e** and **Fig. 2.2 a, b, g, h**), respectively. The cingulate cortex (*Cg*) and retrosplenial cortex (*RSG*) were prominently labeled in this respect. While the *Cg,* which interconnects with the neocortex, thalamus and entorhinal cortex (main input to the hippocampus), is involved in the processing, learning and memory of emotional information, the *RSG* which shares input/outputs with the visual cortex, thalamus and hippocampal formation, plays a role in episodic memory, spatial navigation and predictive functions. As can be gleaned from **Table 2.7**. PPARγ-ir followed rostrocaudal gradient in the neonatal frontal cortex (higher in caudal parts), whereas PPARγ expression peaked in the middle part of the adult frontal cortex (Rudebeck, 2007; Zhang et al, 2017; Epstein et al, 2017).

The **hippocampus**, another important structure with a major role in learning and memory formation was also seen to express low-to-moderate levels of PPARγ protein in both neonates (**Fig. 2.1 d-f, h, i**) and adults (**Fig. 2.2 f, g, i**). In both age groups, PPARγ-ir was relatively high in the CA1-3 layers (cornu ammonis), while lower levels of expression were observed in the

polymorphic layer (hilus) and very low levels in the molecular layer (neonates only), as indicated in **Table 2.7**. Expression of PPARγ was higher in mid-portions of the neonatal hippocampus but was more uniform (weak rostro-caudal gradient) in the adult hippocampus, as can be seen by inspection of **Table 2.7**.

The **nucleus accumbens** (**Acb**) is implicated in motivation and reward and aversion. Consistent with these roles, that have cognitive components, it also contributes to reinforcement learning. Comprised of an outer shell (AcbS) and inner core (AcbC), it forms part of the ventral striatum. Among others, the Acb receives glutamatergic inputs from the frontal cortex, amygdala, thalamic nuclei, hippocampus and ventral tegmental area (VTA); in addition, is innervated by dopaminergic neurons originating in the VTA. In turn, axonal projections from the Acb project to the basal ganglia (and via the globus pallidum and thalamus, innervates the frontal cortex and striatum), the VTA, substantia nigra (SN) and brainstem. The pattern of connections underlies the ability of the Acb to coordinate motor responses to stimuli related to reward and reinforcement. In this study, ir- PPARγ was observed in the Acb (shell and core) of the neonatal (**Fig. 2.1 a**) and adult (**Fig. 2.2 a**) mouse brain (also see **Table 2.7**) (Gipson et al, 2014; Xia et al, 2017). However, in all cases, staining was weak and caution is needed in interpreting the obtained results.

The **amygdala**, like the hippocampus and Acb, is an important part of the limbic system. It is important in memory processing, decision-making and generating emotional reactions. While the amygdala proper is strongly implicated in fear, the stria terminalis (st, part of the so-called extended amygdala) plays a role in anxiety. The amygdala fulfils these functions that are critical to the survival of the organism through neuronal inputs from sensory systems (such as the olfactory system) and its projects (among others) to the hypothalamus, VTA and brainstem nuclei (e.g. the locus coeruleus in which all noradrenergic neurons originate). Further details of the complex functional neuroanatomical subdivision of the amygdala may be found in Ventura-Silva et al (2012). The results of our immunohistochemical analysis of the amygdala are summarized in **Table 2.7**. Specifically, as shown in **Fig. 2.1 c, d, h**, low levels of PPARγ protein expression was observed in the basolateral (BLA) and basomedial (BMA) amygdala of the neonatal brain, whereas low levels of the nuclear receptor were found in the

lateral (LA) and basomedial (BMA) amygdala (but not the stria terminalis, st) of the adult mouse (**Fig. 2.2 e-h**).

The **thalamus** is a forebrain structure located above the midbrain and close to the lateral walls of the third ventricle. This area relays information between subcortical (e.g. hippocampus, hypothalamus) and various cortical areas. Briefly, it multiple connections convey sensory signals that make a critical contribution to cortical control of alertness, consciousness and sleep, all of which are crucial for healthy cognition; more recently, the paraventricular thalamic nucleus (PV) and the reuniens thalamic nucleus (Re) have been strongly implicate in the regulation of the neuroendocrine and behavioural responses to stress (Hsu et al 2014; Kafetzopoulos et al 2017). Whereas only weak PPARγ-ir signal was detected in the neonatal thalamus (**Fig. 2.1 c, e; Table 2.7**), moderate levels of PPARγ expression were observed in more rostral parts of the adult mouse thalamus (low in Re, moderate in PVA), as depicted in **Fig. 2.2 c, e, f; Table 2.7**.

The **hypothalamus**, located in the mediobasal part of the brain, contributes crucially to the regulation of neuroendocrine functions as well as to the control of energy balance. It is comprised of a number of nuclei, including the arcuate (Arc, which plays a key role in energy balance, reproductive function and stress responses), dorsomedial (DM) and lateral (LH) hypothalamic areas (among others, regulation of appetite, feeding and sleep, often together with the ventromedial nucleus), periventricular nucleus (Pe, implicated in analgesia, but also growth and metabolism) and paraventricular nucleus (PVH, important for regulating thyroid and adrenocortical activity, energy metabolism). These important homeostatic roles of the hypothalamus reflect its neural inputs from a variety of forebrain, midbrain and hindbrain which allow it to integrate signals regarding the internal and external environments, and to make appropriate responses to them. Importantly, it communicates with peripheral tissues through both, neural (via projections to posterior pituitary gland or neurohypophysis from where its hormonal secretions enter the general circulation to act on organs such as the mammary glands, uterus and kidney) and non-neural pathways; in the latter case, hypothalamic neurons directly secrete neuropeptidergic modulators into the portal vessels in the median eminence from where they activate endocrine cells in the anterior pituitary gland) (Long et al, 2014; Garretson et al, 2015).

Figure 2.1. *(a-k, continued on following pages).* **Representative images of immuno-histochemical staining of PPARγ in the neonatal (PND 7) mouse (CD1) brain, focusing on brain regions implicated in cognition, mood-emotion, endocrine-metabolic regulation and motivation-reward processes.** Immunopositive cells are visualized as brown-black staining (nuclear and cytoplasmic staining marked by black and white arrowhead, respectively). Arrows indicate neuritic (axon/dendrite) staining. Coronal sections (30 µm); nuclei counterstained with methyl green. Nomenclature and coordinates based on Paxinos et al (2007) (rostro-caudal, a-k). All images at the same magnification (scale bars: 50 µm). A qualitative assessment of staining intensity and number of immunopositive cells (relative to staining in adult mice) provided in **Table 2.7.**

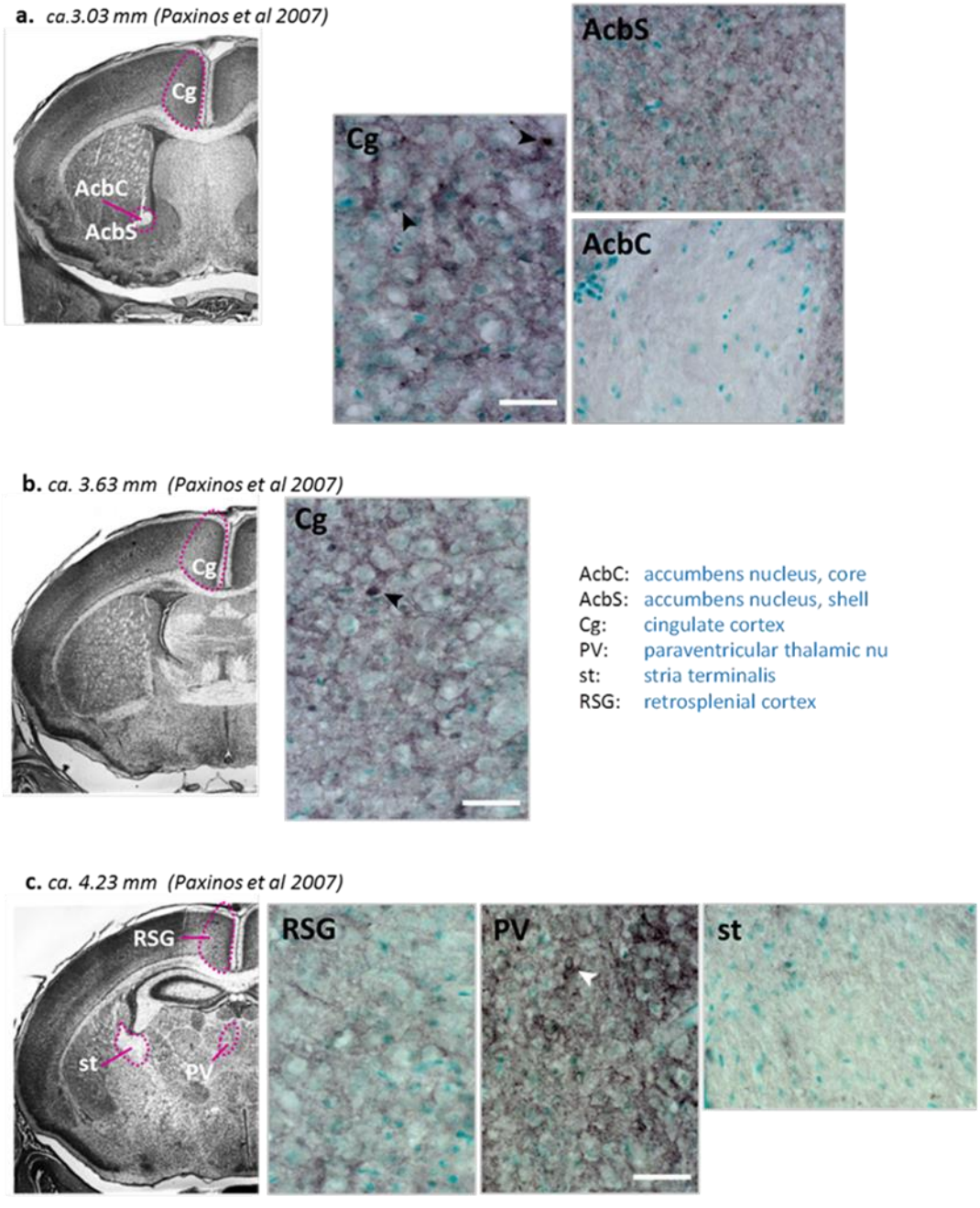
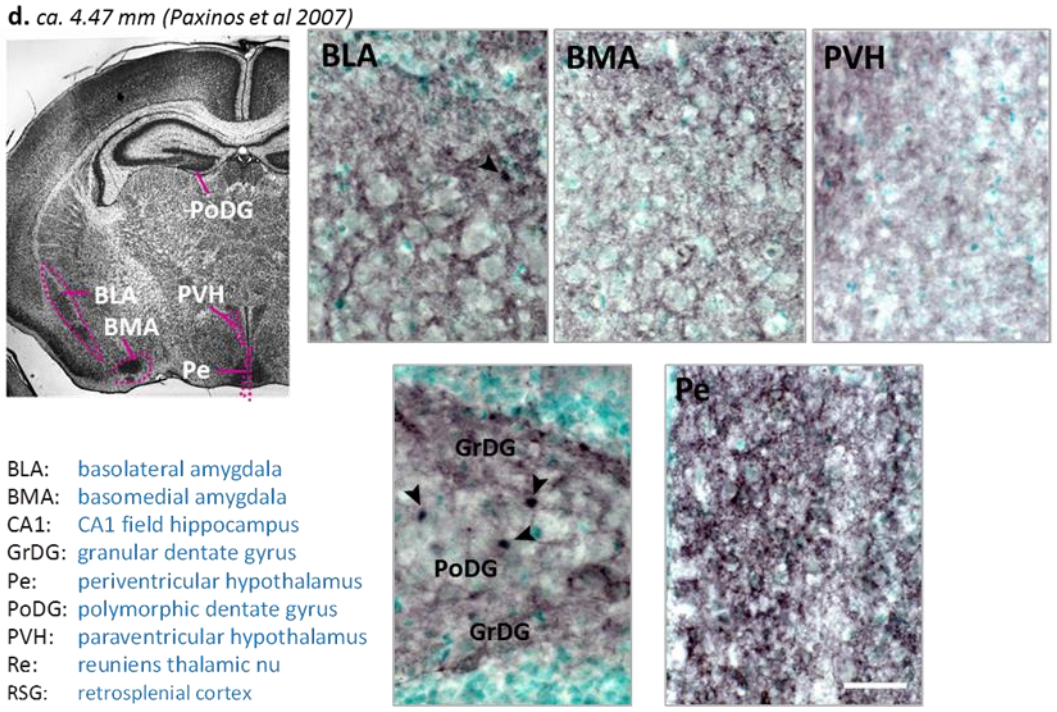


Figure 2.1. (continued)



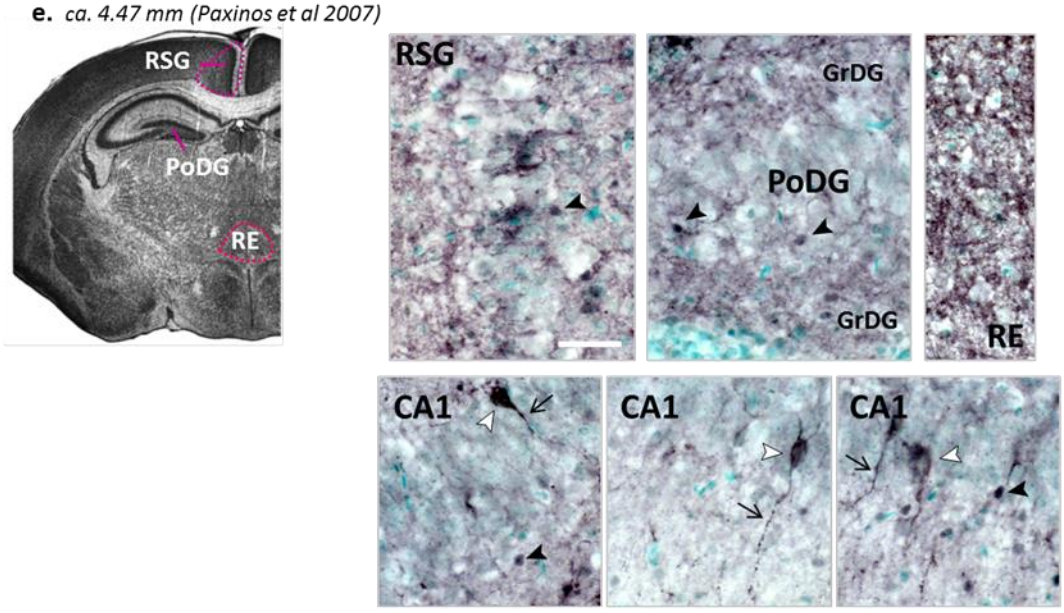


Figure 2.1. (continued)

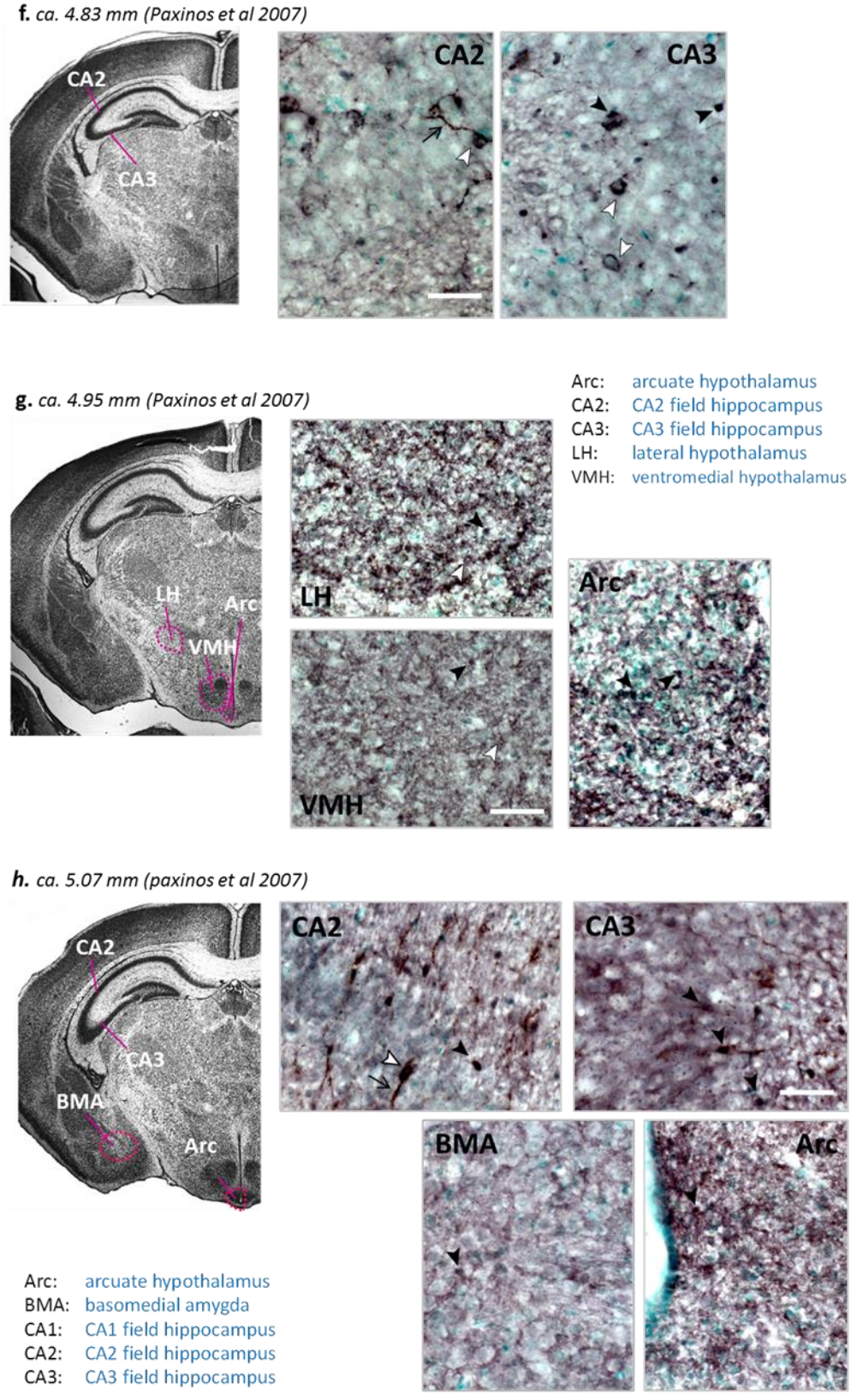
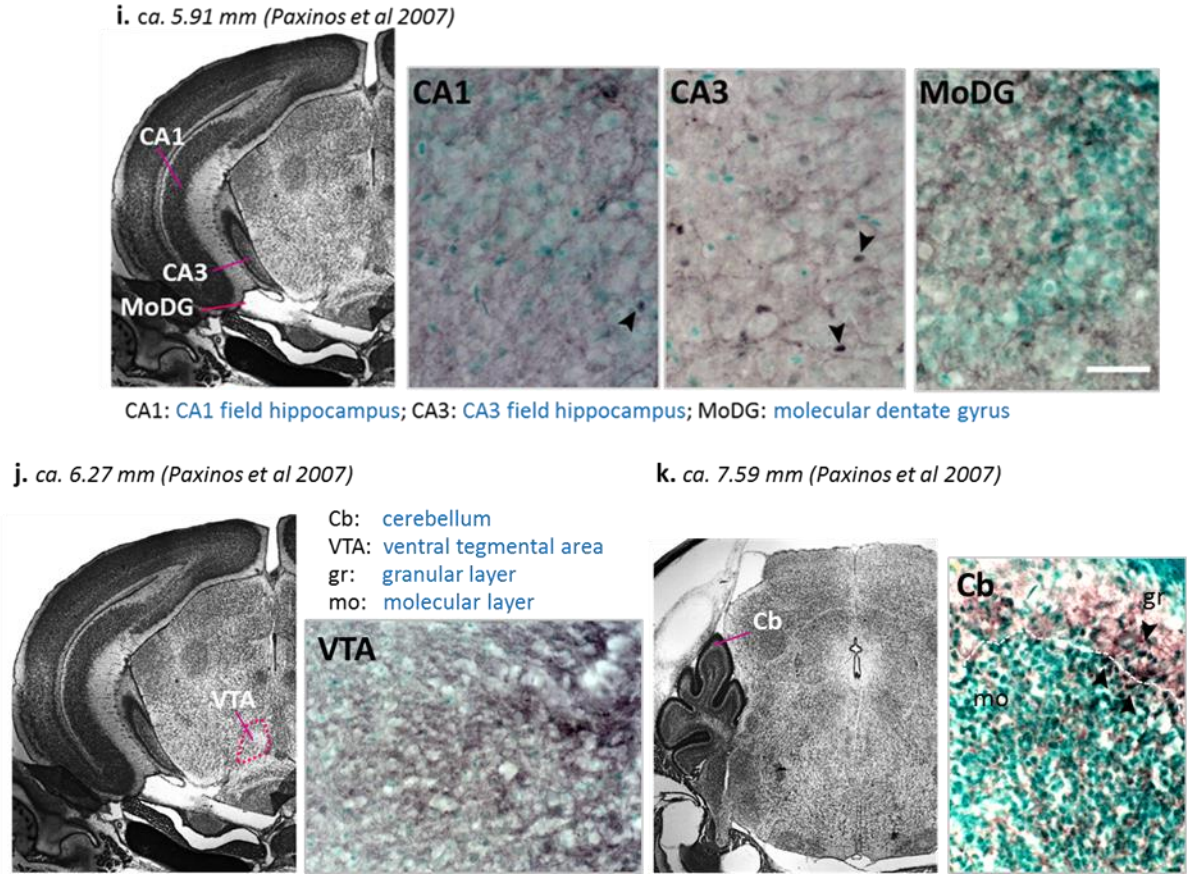


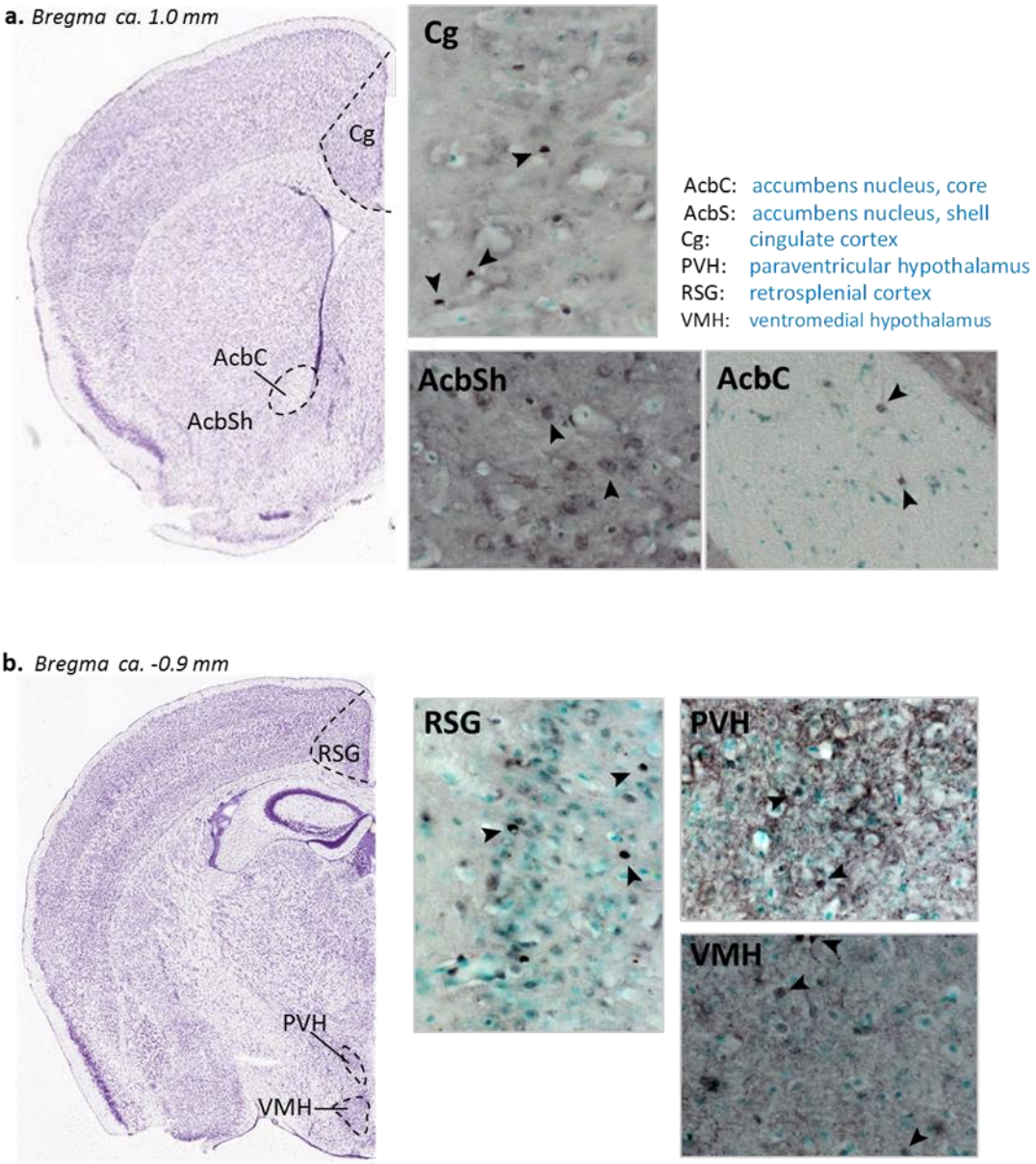
Figure 2.1. (continued)



The present immunohistochemical analysis showed low-to-moderate PPARγ staining in most of the aforementioned hypothalamic nuclei of neonatal (**Fig. 2.1 d, g, h**) and adult (**Fig. 2.2 b, d-g**) mice; generally, signal intensity and/or number was greater in adults than in neonates (see **Table 2.7**).

The **ventral tegmental area** (VTA), a midbrain structure, is the site of origin of dopaminergic neurons that are part of the cortico-mesolimbic neurocircuitry involved in the control of cognition, emotions and motivation. Among others, the VTA sends projections to the brainstem (Gipson et al, 2014). In this study, low-to-moderate PPARγ-ir signals were seen in the caudal parts of the VTA in both neonatal (**Fig. 2.1 j**) and adult (**Fig. 2.2 i**) mice (also see **Table 2.7**).

Figure 2.2 *(a-j, continued on following pages).* **Representative images of immuno-histochemical staining of PPARγ in the adult (PND 90) mouse (CD1) brain, focusing on brain regions implicated in cognition, mood-emotion, endocrine-metabolic regulation and motivation-reward processes.** Immunopositive cells are visualized as brown-black staining (nuclear and cytoplasmic staining marked by black and white arrowhead, respectively). Arrows indicate neuritic (axon/dendrite) staining. Methyl green was used as a nuclear counterstain. The nomenclature and coordinates used to indicate levels at which coronal sections were obtained (30 µm) are based on Paxinos et al (2008) (rostro-caudal, *a-j*). All images at the same magnification (scale bars: 50 µm). A qualitative assessment of staining intensity and number of immunopositive cells (in relation to that in adult mice) is provided in **Table 2.7.**



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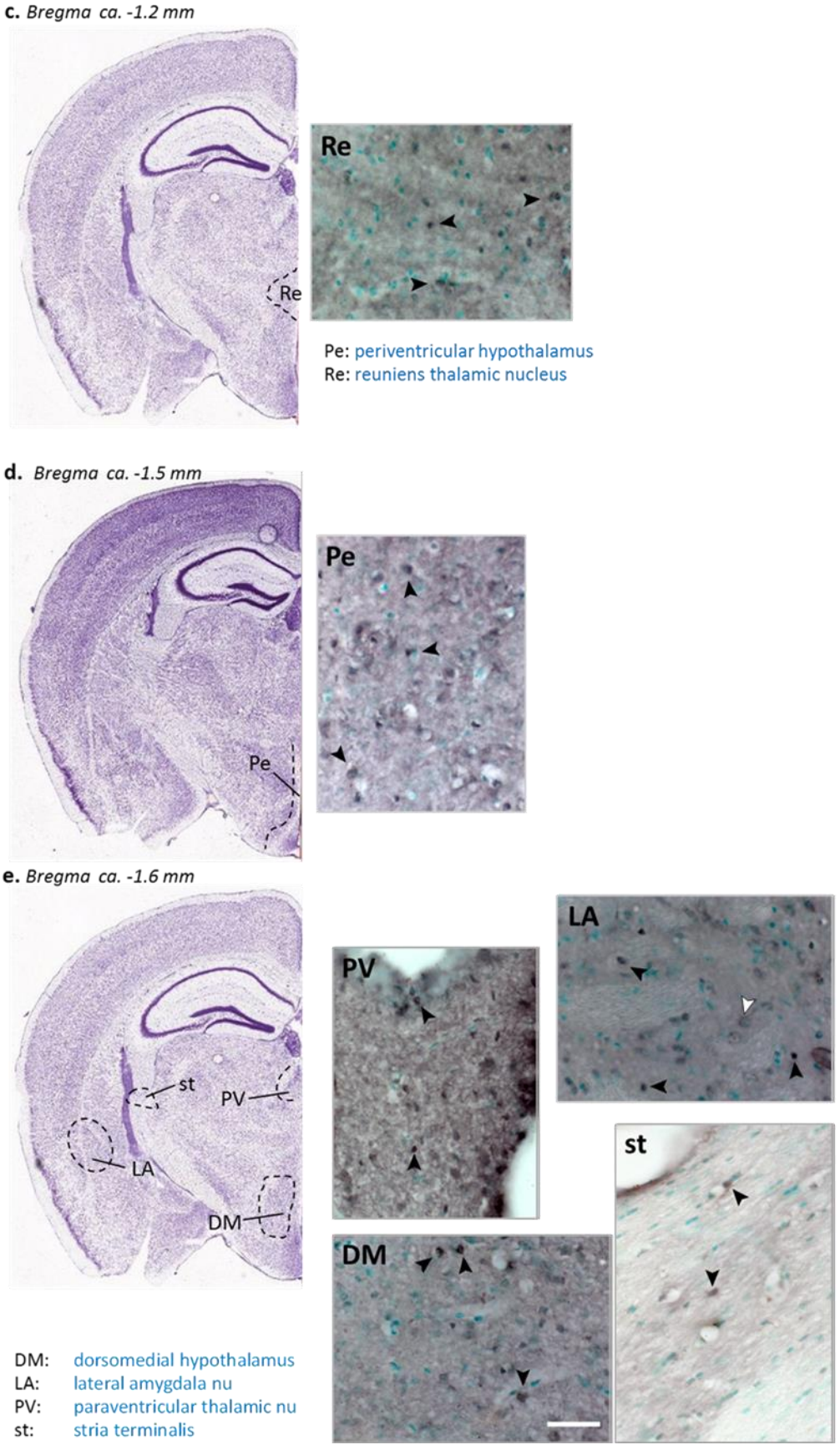

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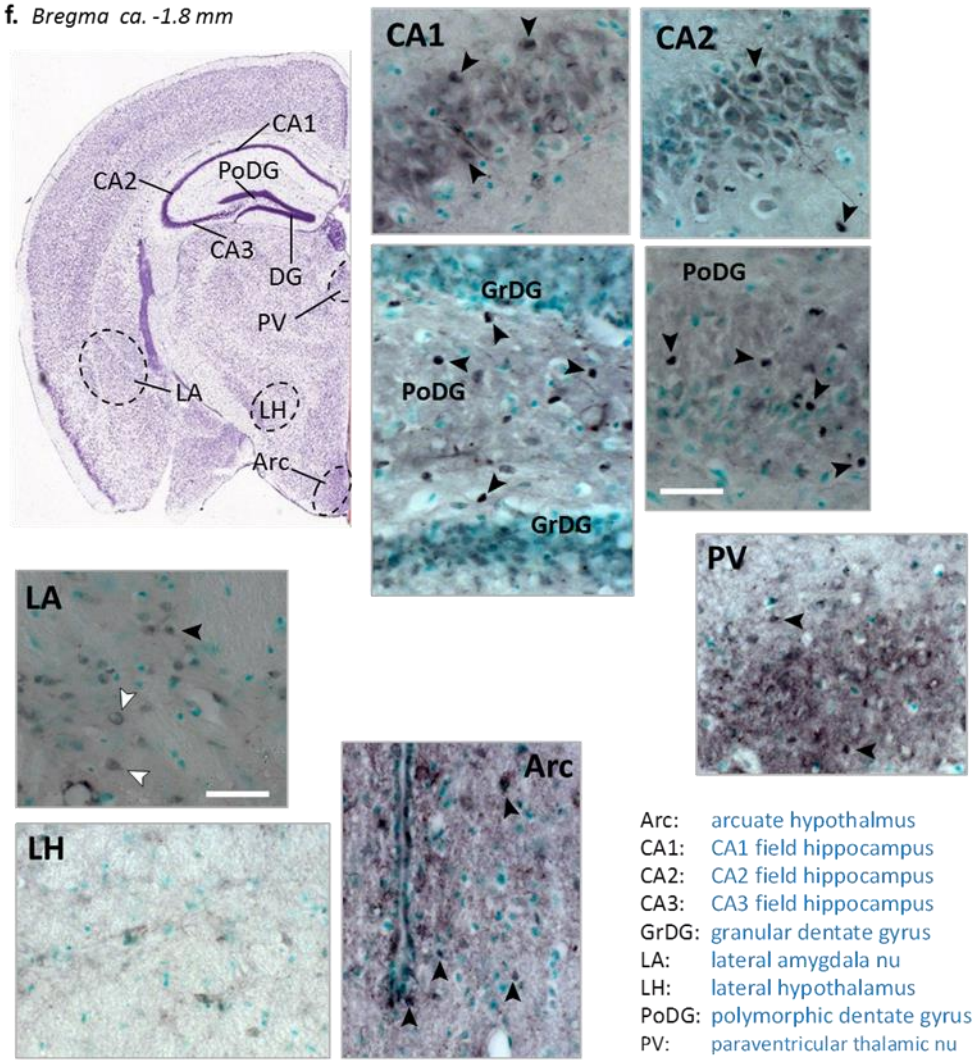


The **cerebellum** (Cb) is an important hindbrain structure that is crucial for the coordination of movement-related functions. It is also implicated in a diverse set of other cerebral functions such as cognition (attention, language, fear, hedonia). Underlying these roles are its inputs from sensory systems, including the spinal cord. It is characterized by folded layers of cortex (mainly small granule neurons) and a ventricle; Cb white matter lies beneath the cortical folds. In addition, the whole Cb is organized into several smaller lobules (Jörntell, 2017). The present analysis revealed PPARγ staining in granule cells of the neonatal (**Fig. 2.1 k**) and adult (**Fig. 2.2 j**) Cb, with relatively higher staining intensity in the neonatal *vs.* adult Cb (**Table 2.7**).

Figure 2.2. (continued)

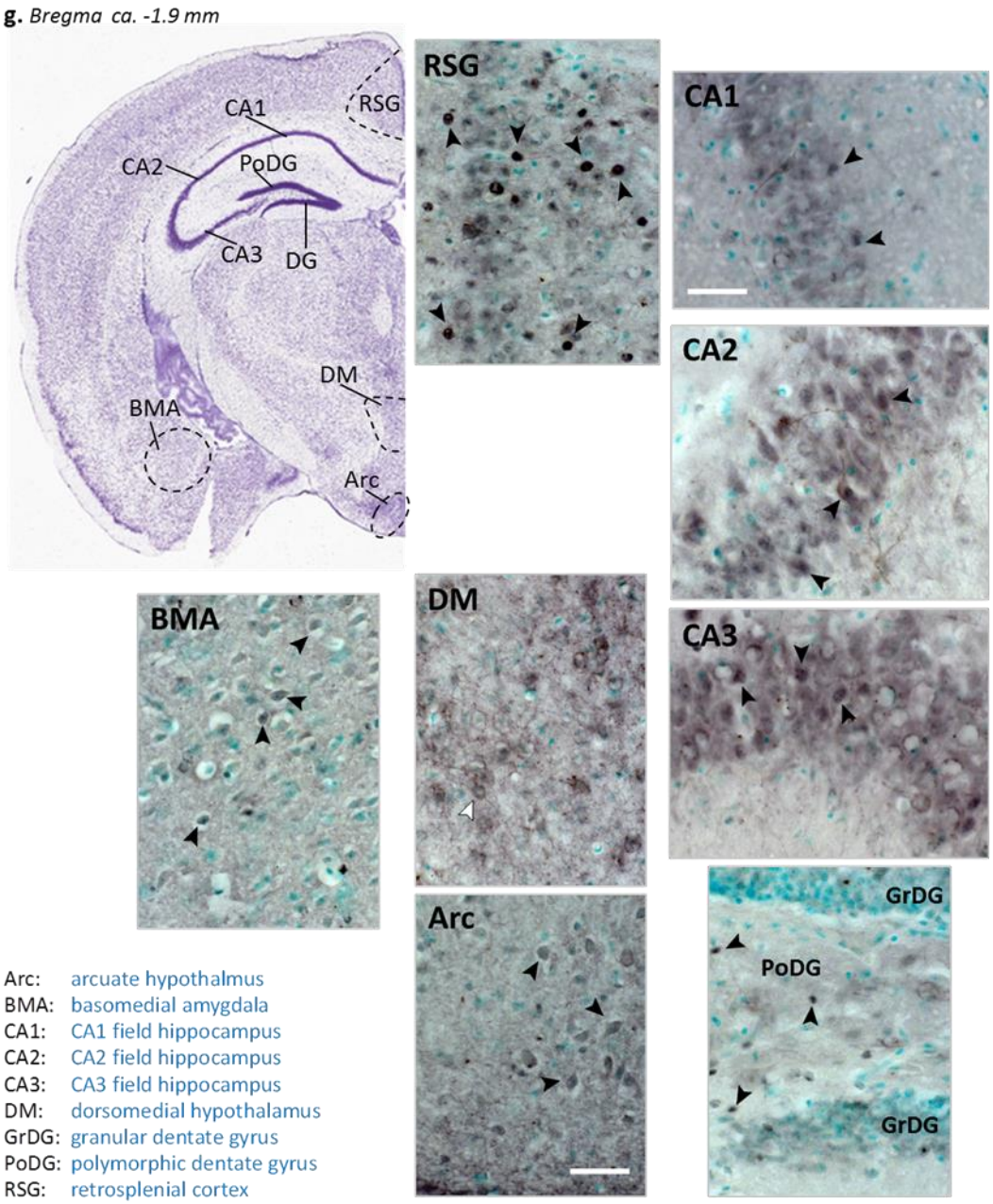


Figure 2.2. (continued)

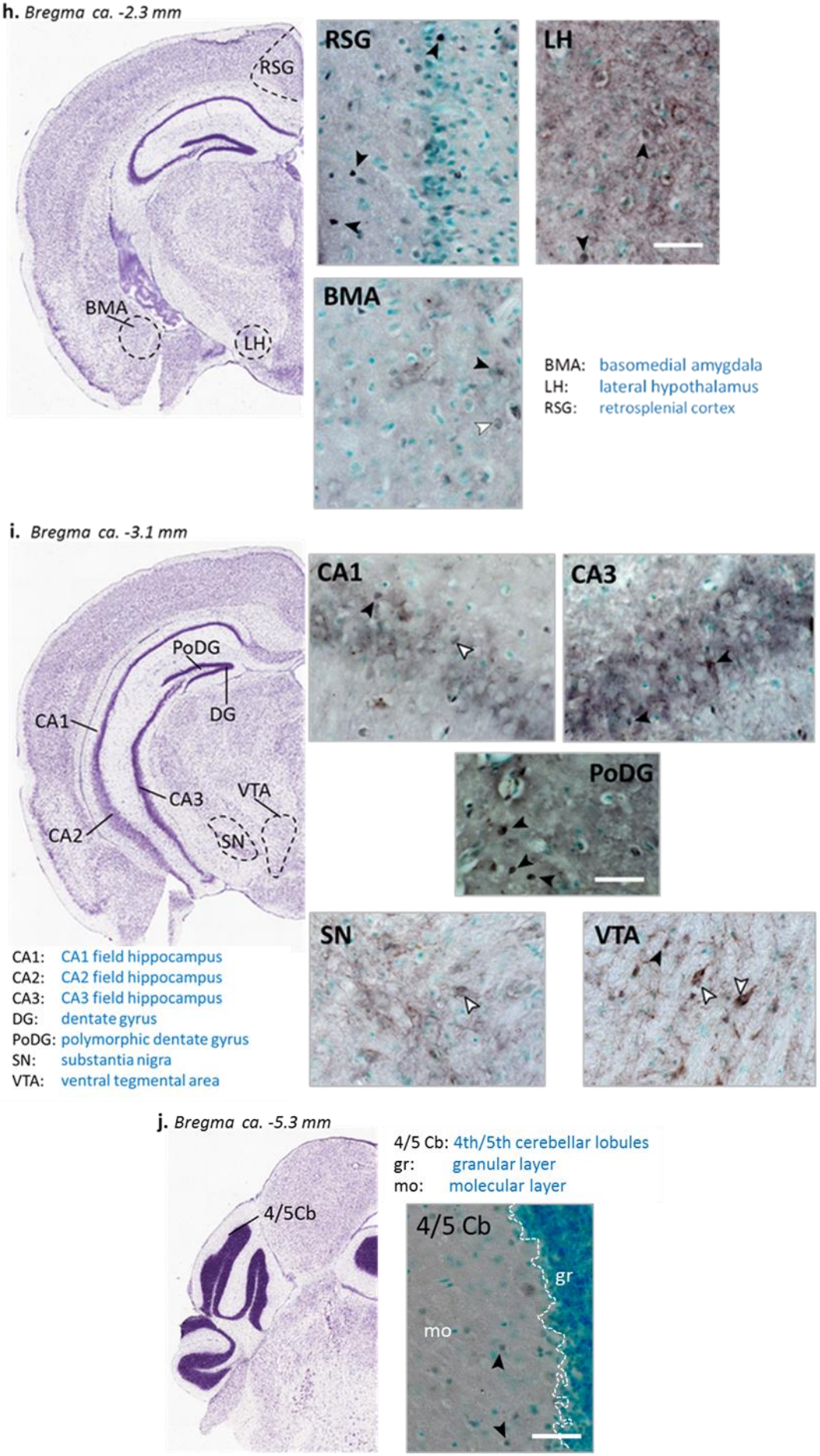


Table 2.7. Subjective rating of staining intensity and number of cells detected by immunohistochemistry for PPARγ in neonatal (PND 7) and adult (PND 90) mouse brains (described in **Figs. 2.1** and **2.2**, respectively). For convenience, gross anatomical (sub)regions are tabulated; for information regarding structure-function relations, please see main text referring to **Figs. 2.1** and **2.2**. **+/-** indicates areas in which very low (or poorly resolved) staining was found; **+, ++** and **+++** indicate low, moderate and strong staining, respectively. ? Indicates questionable staining due to high background.

		PND 7 (coordinates based on Paxinos,2007)					PND 90 (coordinaes based on Paxinos,2008)					
		rostral ←				→ caudal	rostral ←			→ caudal		
	Sub-region	3.03	4.23-4,47	4.71-4.95	5.07	5.91-7.59	-1.0-1.6	-1.8	-1.9	-2,3-5,4	Sub-region	
	Cg	+	+				+				Cg	
Frontal cortex	RSG		+/-	++			++		+++	++	RSG	Frontal cortex
	AcbC	+/-					++				AcbC	
Nucleus accumbens	AcbS	+/-					+				AcbS	Nucleus accumbens
	LA						+	+/-			LA	
	BLA		+								BLA	
	BMA		+		+/-				+	+/-	BMA	
Amygdala	st		-				+/-				st	Amygdala
	CA1			+/++		+/-		+	+/-	+/-	CA1	
	CA2			++	++			++	+ /++		CA2	
	CA3			++	++	+			+ /++	++	CA3	
	MoDG					+/-					MoDG	
	GrDG		+	+				+/-	+		GrDG	
Hippocampus	PoDG		+	+				++		+	PoDG	Hippocampus
	Arc			+/++	+			+/++	+		Arc	
	DM						+		+/-		DM	
	LH			+/-				+/-		+/-	LH	
	Pe		?				+ /++				Pe	
	PVH		+/-				+ /++				PVH	
	Re			+/-			+				Re	
Hypothalamus	VMH			+/-			+				VMH	Hypothalamus
	Re						+				Re	
Thalamus	PV		?				+	+ /++			PV	Thalamus
	SN									+/-	SN	
Midbrain	VTA					+				++	VTA	Midbrain
Cerebellum						++				+/-		Cerebellum

Although PPARγ is described as a nuclear receptor, since it acts in the nucleus to regulate gene transcription, it should be noted that in this study ir-PPARγ appeared to have a diffuse intracellular location, partly cytoplasmic (**Fig. 2.1 c, e-h** and **2.2 a, d-i**) and sometimes neuritic staining (**Fig. 2.1 e, f, h**).

On the other hand, it should be noted that the quality of the brain sections used here, as well as the resolution of the images does not allow a definitive conclusion regarding the intracellular localization of PPARγ protein in the mouse brain. This tissue is further considered later in the section in which immunocytochemical staining of neural cells *in vitro* is described.

2.4.2. Expression of PPARγ (and heterodimerization partners) in neural cells

Following the *in situ* mapping of PPARγ protein expression in neonatal and adult mouse brains (**Figs. 2.1** and **2.2; Table 2.1**), and given the described limitations of the immunohistochemical approach, we next sought to examine the expression of PPARγ in primary neural cultures derived from the frontal cortex and hippocampus of CD1 mice (and Wistar rats in one specific case) aged PND 5. The analysis was extended to include mouse and human neural cell lines.

Our initial analysis (reported in Moosecker, MSc Thesis, TUM, 2013) examined the expression of the mRNA encoding *PPARγ* and its heterodimerization partners (isoforms of the retinoid X receptor [RXR] and liver X receptor [LXR]). We observed that primary rat hippocampal cultures express not only *PPARγ* mRNA, but also *RXRα* (*NR2B1*), -*β* (*NR2B1*) and -*γ* (*NR2B3*) and *LXRα* (*NR1H3*) and *β* (*NR1H2*) mRNA, as shown in a representative, real time polymerase chain reaction (RT-PCR) experiment in **Fig. 2.3 a**; restriction enzyme assays subsequently validated these findings. The expression of mRNAs for *PPARγ* in parallel with its dimerization partners gives credence to the view that PPARγ in neural cells has the potential to be transcriptionally active.

In light of earlier studies suggesting that the expression of PPARγ is developmentally regulated (Michalik et al, 2002), quantitative real time PCR (qRT-PCR) analysis was used to examine *in vitro* age/maturation-dependent alterations in the levels of *PPARγ* in primary frontocortical and hippocampal cultures (**Fig. 2.3 b**); cultures were monitored after 6, 9 and 14 days *in vitro* (DIV). Interestingly, this approach revealed that whereas levels of the mRNA coding for *PPARγ1* (the dominant isoform previously reported in neural tissue (Michalik et al, 2006) in

frontocortical cultures does not vary with age, those in the hippocampus show a transient, but significant ($p < 0.05$), peak on DIV 9 (**Fig. 2.3 b**, *right-hand panel*). Interestingly, PPARγ (total) mRNA levels did not differ over time in frontocortical and hippocampal cultures (**Fig. 2.3 b**, *left-hand panel*).

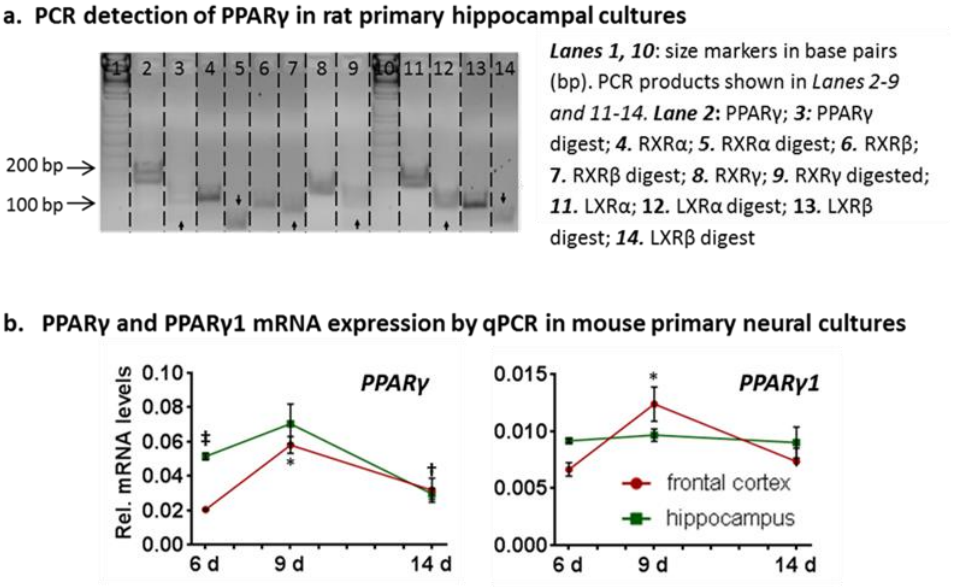


Figure 2.3. Rat and mouse primary frontocortical- and hippocampus-derived neural cultures express *PPAR* and *PPARγ1* mRNA. (a) PCR assays were performed on cell extracts from rat hippocampal cultures after 14 DIV; black arrows indicate digested mRNA (also see Moosecker, MSc Thesis, TUM, 2013). **(b)** Shows *in vitro* maturation-dependent changes in *PPARγ and PPARγ1* mRNA levels in mouse frontocortical and hippocampal cultures after 6, 9 and 14 DIV; mRNA levels assayed by qPCR. All numerical data (n = 6) are presented as means ± S.E.M. Symbols. * indicate differences compared to 6 DIV, † indicate differences compared to 9 DIV, and ‡ indicate differences between frontocortical and hippocampal cultures.

2.4.3. Parallel expression of total *PPARγ* mRNA and PPARγ1 protein in the male and female mouse frontal cortex and hippocampus *in situ*

Complementing the data in the previous section (**Fig. 2.3**), expression of total *PPARγ* mRNA and PPARγ1 protein was measured in 5- and 14-day old mouse (CD1) brains *in situ*. The lysates from the same tissue were analysed by qPCR and Western blotting analyses. As shown in **Fig. 2.4 a**, higher levels of *PPARγ* mRNA expression were found in the frontal cortex and hippocampus of 5-day old females *vs.* males ($p = 0.57$). Immunoblotting (**Fig. 2.4 b**) revealed higher levels of PPARγ1 protein in the frontal cortex of 5-d old females *vs.* similarly-aged males

(p = 0.10); there were no age- or sex-related differences in PPARγ protein levels in the hippocampus although these measures showed a certain degree of parallelism with PPARγ mRNA expression in this brain area.

2.4.4. Specificity of PPARγ measurements obtained by immunoblotting

In light of concerns regarding the limited specificity of commercially-available antisera against PPARγ, we next sought to verify the results obtained by Western blotting in the previous section; this work was also intended to verify the specificity of the immunostaining results reported in **Figs. 2.1 and 2.2** above, and **Figs. 2.5** (below). To do this, we initially performed an *in vitro PPARγ* knock-down experiment, using a human neuroblastoma cell line (SH-SY5Y) and compared immunoblotting results obtained by the application of two commonly-used antisera (ab19481 and sc7273). As shown in **Fig. 2.5 a** (*right-hand panels*), both antisera significantly diminished PPARγ1 levels in cells in which *PPARγ* was effectively silenced using a RNA interference (RNAi) approach ($p \leq 0.05$; **Fig. 2.5 a**, *left-hand panels*). Although both antisera gave comparable results, we subsequently chose to use ab19481 for a large portion of the immunocytochemistry experiments (Note: Antibody ab19481 was withdrawn from the market shortly after completion of these studies).

Next, we used a highly sensitive quantitative assay (Protein Simple™) to measure PPARγ1 protein levels in the brains of floxed/floxed mice and mice in which the *PPARγ* gene had been conditionally deleted in the forebrain deleted ($PPAR\gamma^{-/-}$). The results shown in the *leftward-most panel* of **Fig. 2.5 b**, shows that antibody CST #2435 specifically detects PPARγ protein (only in brains from $PPAR\gamma^{fl/fl}$ mice but not from $PPAR\gamma^{-/-}$ mice). These data were supported by using a positive control that consisted of a mouse cell line (HT22) overexpressing *PPARγ1* and *-2*. Further support for specificity of antibody ab19481 was provided by data showing sex-specific differences in the expression of PPARγ1 in the mouse frontal cortex and hippocampus (**Fig. 2.5 b,** *middle and right-hand panels*). Moreover, whereas PPARγ1 protein levels showed age-related increases in the frontal cortex of both sexes, such changes were only observed in the male hippocampus. Indirect support for the specificity of the CST #2435 antibody is provided by the observation that the levels of both, PPARg1 and PPARγ2 were

altered in genetically-manipulated mice, namely in J20 (*APP*-overexpressing) mice and in BACE1-/- mice that are deficient in Aβ production. (**Fig 2.5 c**).

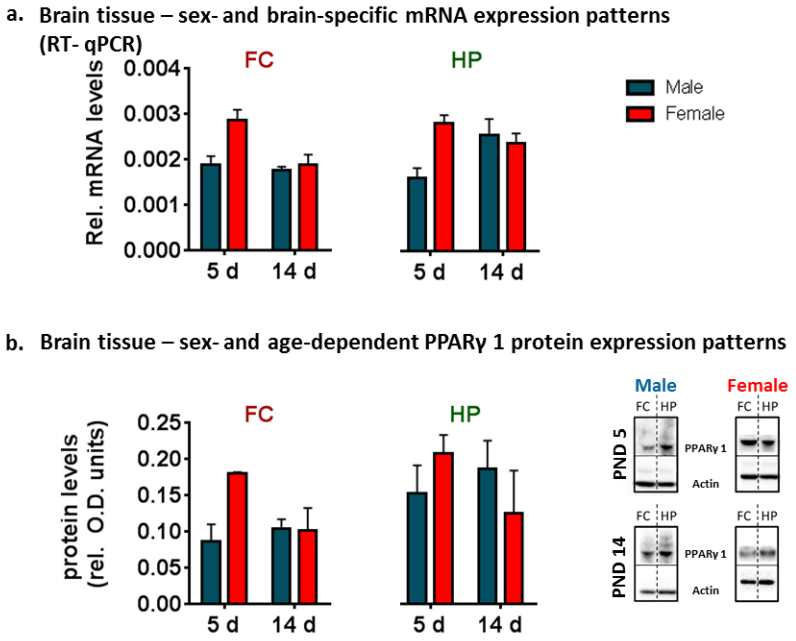


Figure 2.4. Parallel expression patterns of *PPARγ* mRNA and PPARγ1 protein mouse frontal cortex and hippocampus *in situ*. Relative expression of **(a)** mRNA levels of total *PPARγ* (measured by qRT-PCR, normalized to *Pgk1*) and **(b)** protein levels of PPARγ1 (measured by Western blotting (CST #2435), normalized to actin) in frontocortical (FC) and hippocampal (HP) tissue extracts from male (blue bars) and female (red bars) mice aged 5 and 14 days. All numerical data (n = 4) are presented as means ± S.E.M. * indicate significant difference between sexes, † indicate significant age differences within one sex.

2.4.5. Primary neural cell cultures express PPARγ, detectable by immunocytochemistry

As mentioned above, antibody ab19481 was withdrawn by its suppliers during the course of this work, necessitating the validation of an alternative product (CST #2435) for use in above-reported immuno-histochemical and following -cytochemical analyses.

To do this, we compared residual stocks of ab19481 with CST #2435 to stain primary cultures prepared from the frontal cortex and hippocampus of PND 5 mice (CD1), co-labelling cells with anti-glial fibrillary acidic protein (GFAP), anti-microtubule-associated protein2 (MAP2) or anti-nestin, which recognize astroglia, mature neurons and neuroprogenitors. A semi-quantitative analysis (immunopositive cell counts) of CST #2435 generally gave similar results

to those obtained with ab19481 (**Fig. 2.6 a**). Immunoreactive PPARγ was expressed in a large proportion of neural progenitors (nestin-positive), astroglia (GFAP-positive) and mature neurons (MAP2-positive) in hippocampal and frontocortical cultures. Notably, PPARγ+ neuroprogenitors were more abundant in the hippocampus than in the frontal cortex; *vice versa,* PPARγ immunoreactivity was more prominent in forntocortical than hippocampal astrocytes (**Fig. 2.6 a**).

In order to gain further insight into the expression of PPARγ by other cell phenotypes seen in our primary cultures, we subsequently performed double immunostaining for PPARγ (CST #2435-reactive) with either nestin, GFAP, MAP2 and O4 (the last recognizes oligodendrocytes) and followed this by a triple stain for cell nuclei (Hoechst 33341). As shown in **Fig. 2.6 b**, the results recapitulate those shown in **Fig. 2.6 b** in the case of astrocytes, mature neurons and neuroprogenitors; none of the O4-positive oligodendrocytes expressed PPARγ and our cultures did not contain any microglia (antibody Iba-1a, CD68 and CD11b, data not shown).

As previously noted, PPARγ is a nuclear receptor. As can be seen in immunostaining experiments on primary mouse frontocortical cells, using CST #2435, ir-PPARγ was not expressed in the nucleus exclusively; several cells also showed cytoplasmic labelling (**Fig. 2.6 c**), probably reflecting the fact that the receptor is synthesized in this cell compartment before translocation to the nucleus. On the other hand, PPARγ was found exclusively in the nucleus of differentiated human neuroblastoma cells (SH-SY5Y) and an undifferentiated murine neural cell line (HW3-5) by immunocytochemistry as well as immunoblotting (**Fig. 2.6 d**).

2.4.6. PPARγ expressed by primary mouse neurons are transcriptionally active

Having shown that primary neural cultures derived from the mouse prefrontal cortex and hippocampus express PPARγ mRNA and protein, we next aimed to investigate their transcriptional activity. Experiments were performed on primary neural cultures as well as the differentiated human neuroblastoma cell line (SH-SY5Y). At a dose of 10^{-5} M, pioglitazone (Pio), a potent PPARγ agonist, induced the mRNA expression of two known PPARγ target genes, namely *ATP-binding cassette transporter 1* (*ABCA1*) *and peroxisome proliferator-activated receptor gamma coactivator 1-alpha* (*PGC-1α*) in SH-SY5Y cells (**Fig. 2.7 a**); induction

was observed after 24 hours of exposure to Pio. To demonstrate that this result reflected specific engagement of PPARγ, cells were also co-treated with Pio (10^{-5} M) and GW9662 (10^{-6} M), a specific PPARγ antagonist. As shown in (**Fig. 2.7 a**), the effects of Pio were significantly blocked by GW9662 ($p \leq 0.05$).

Experiments in primary frontocortical and hippocampal cultures showed that *ABCA1* mRNA is more highly expressed in hippocampal *vs.* frontocortical cells, whereas *PGC-1α* mRNA show an opposite pattern (*insets*, **Fig. 2.7 b**). Pio dose-dependently induced *ABCA1* mRNA expression in hippocampal, but not frontocortical cells (**Fig. 2.7 b**, left-hand panel). On the other hand, *PGC-1α* mRNA levels were only significantly upregulated in frontocortical (but not hippocampal) cells when Pio was added at a dose of 1.0 µM (**Fig. 2.7 b**, right-hand panel). Further, the effects of Pio on *ABCA1* gene expression were seen to partly depend on duration of exposure of both frontocortical and hippocampal cells to the drug (**Fig. 2.7 c**, left-hand panel); in contrast, *PGC-1α* mRNA levels in both cultures were less clear (no significant change in frontocortical cells and a significant decrease in hippocampal cells) (**Fig. 2.7 c**, right-hand panel).

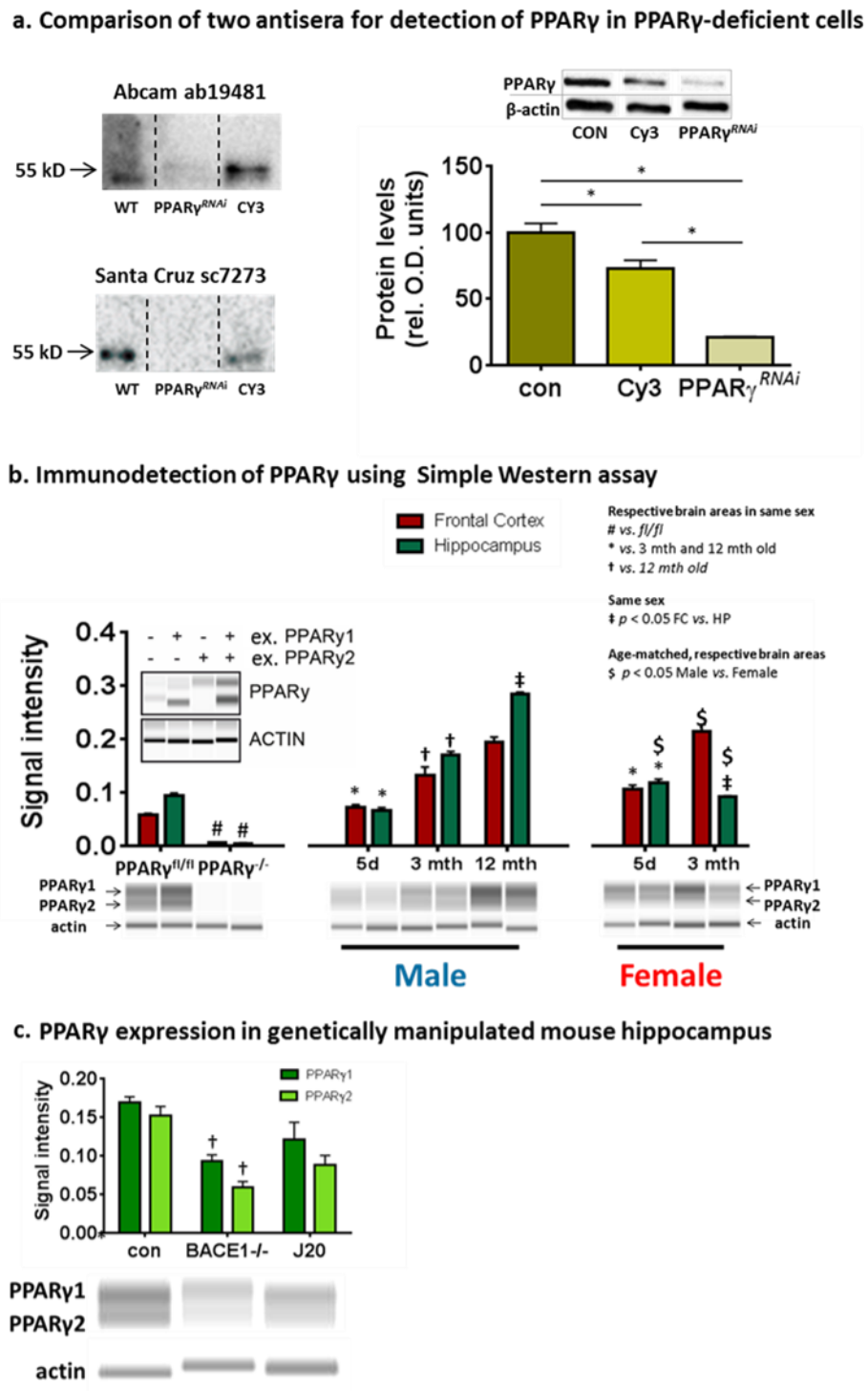


Figure 2.5. Specificity of PPARγ antisera verified by RNAi-transduced knockdown of PPARγ. (a) Undifferentiated SH-SY5Y cells were transfected with either PPARγ RNAi or Cy3 (control). *Left-hand panel* compares two antisera against PPARγ (ab19481 and sc#7273) by Western blotting; RNAi-mediated knockdown of *PPARγ* resulted in low or absent PPARγ protein in RNAi-treated cells *vs. WT* or *Cy3*-transfected cells. *Right-hand panel* depict semi-quantitative results obtained using ab19481 antibody. **(b)** Protein levels of PPARγ1 in tissue extracts from the frontal cortex (red bars) and hippocampus (green bars) from male and female mice aged between 5 d and 12 months. Tissue extracts from *PPARγ*$^{-/-}$ (knockout) mice were used as a negative control. Extracts from the neural cell line HT22 in which exogenous *PPARγ1* and *PPARγ2* were expressed served as positive controls (inset). **(c)** Tissue extracts from *BACE1* knockout and *APP*-overexpressing mice. Data for control mice is same as that shown in *panel b* (3 month old male). Protein levels were measured using the ProteinSimple Wes assay. All numerical data (2-3) are shown as means ± S.E.M; significant differences are $p \leq 0.05$.

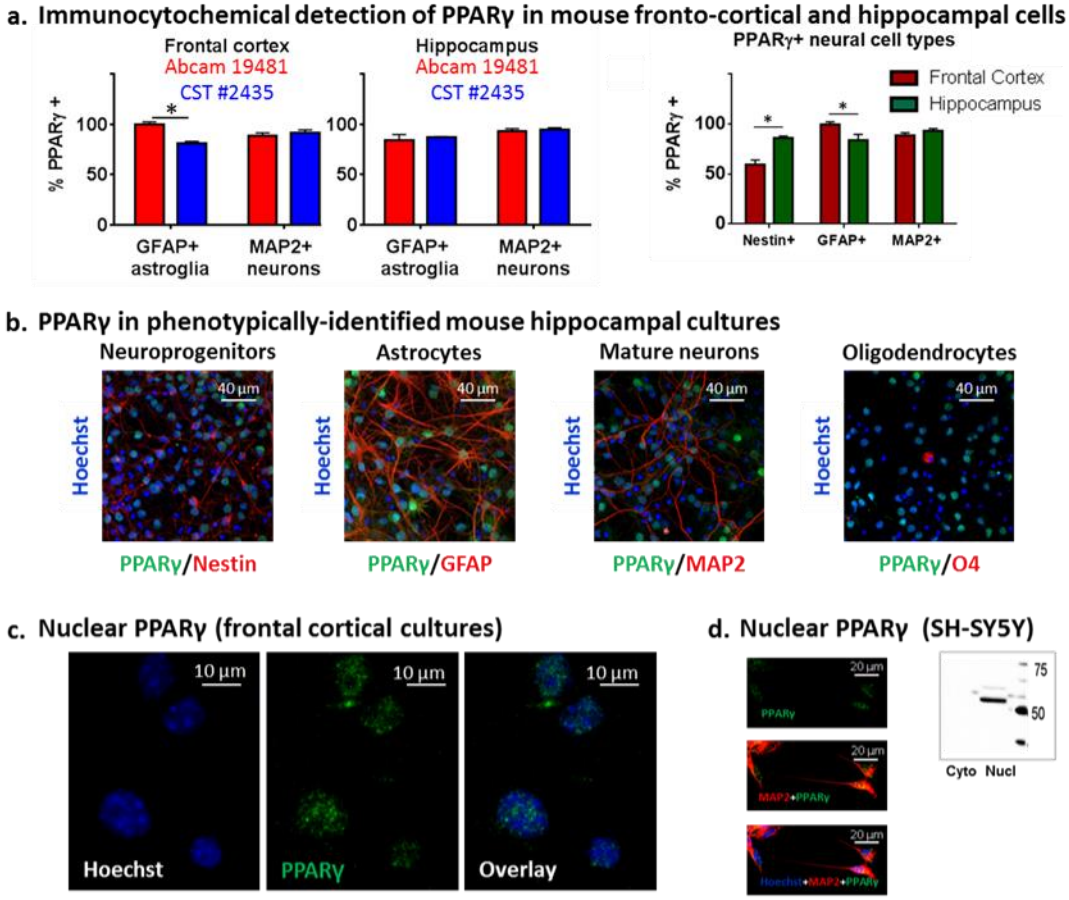
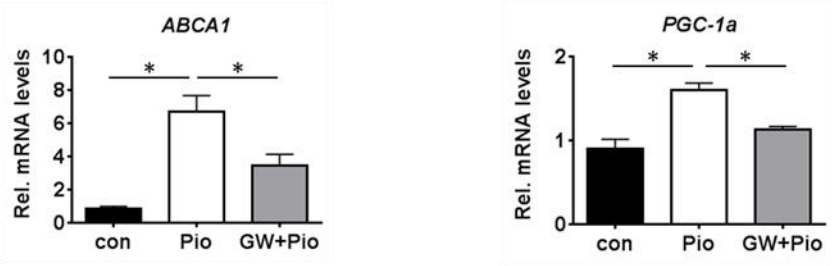
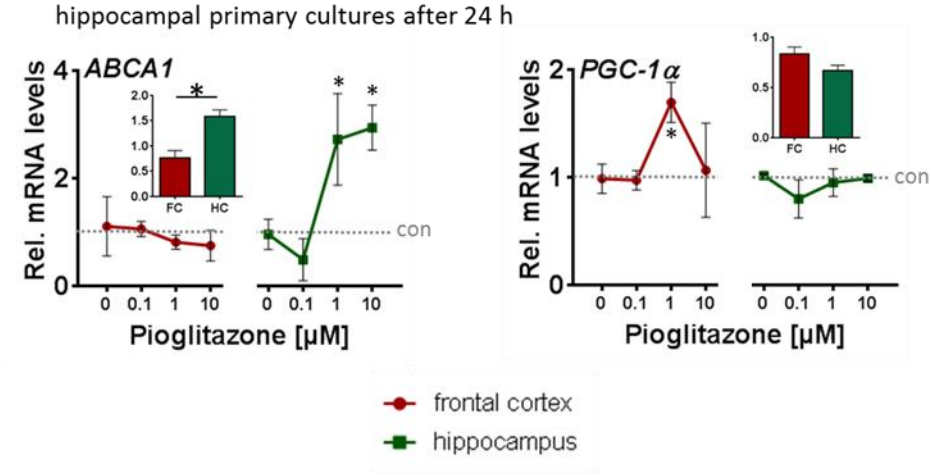


Figure 2.6. Neural cell cultures express PPARγ, detectable by immunocytochemistry. (a) Immunocytochemical detection of PPARγ in mouse fronto-cortical and hippocampal cells (primary cultures, DIV 14); shown are the co-localization of PPARγ (detected antibody ab19481 *[red bars]* or antibody CST#2435 *[blue bars]* with GFAP (astroglia), MAP2 (mature neurons) and nestin (neural precursors). Numerical data is based on counting of 1500 cells of each cell type on a total of 3 independent coverslips. Note that the relative number of cells co-stained for PPARγ and different chemophenotypic markers (nestin, GFAP and MAP2) was similar with ab19481 and CST#2435 antisera (*far-right panel*). **(b)** shows examples of microscopic fields (40x) from hippocampal cultures using antibody CST#2435; PPARγ-stained cells appear green and those stained for nestin, GFAP and MAP2 appear red. In addition, cells stained with antibody O4 that labels oligodendrocytes are shown (red); note the latter cell type were few and did not show obvious colocalization of PPARγ. In all cases, nuclei are labelled with Hoechst dye 33341 (blue). **(c)** Demonstrates nuclear PPARγ immunoreactivity (using antibody CST #2435) in primary mouse frontocortical cultures (60x); nuclear staining appears blue and PPPARγ as green. **(d)** Shows nuclear localization of immunoreactive PPARγ in SH-SY5Y cells, a human neuroblastoma cell line (staining performed the same as in (c) plus MAP2 appearing in red). Also shown are the results of a single experiment which demonstrates PPARγ immunoreactivity (by Western blotting) in the nuclear, but not cytosolic, fraction of HW3-5, a mouse hippocampal cell line.

a. Induction of target genes (*ABCA1* and *PGC-1α*) by pioglitazone in human SH-SY5Y cells is sensitive to the PPARγ antagonist GW9662



b. Dose-related transcriptional effects of pioglitazone in frontocortical and hippocampal primary cultures after 24 h



c. Time-dependent induction of PPARγ target genes in frontocortical and hippocampal primary cultures by pioglitazone [10 µM]

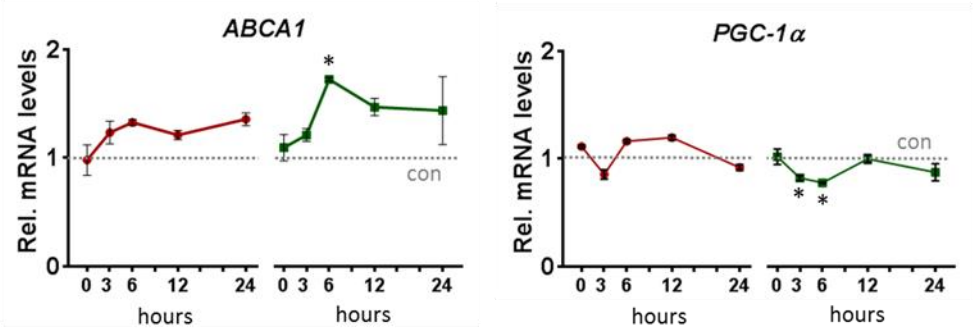


Figure 2.7 Transcriptional effects of activated PPARγ in mouse neural cells. Relative expression of target genes (*ABCA1* and *PGC-1α*) in response to a PPARγ agonist (pioglitazone). **(a)** Differentiated human cell line SH-SY5Y treated with 1 µM GW9663 for 30 min before, 10 µM pioglitazone (PIO) for 24 h. Relative mRNA expression of *ABCA1* and *PGC-1α* normalized to *GAPDH*. **(b)** Relative expression of mRNA levels of *ABCA1* and PGC-1α normalized to *GAPDH* in primary frontocortical (insets: *dark red bars*) and hippocampal (insets: *dark green bars*) cell cultures from CD1 mice (PND5). The line graphs show dose-response curves where Pio was added to DIV 14 primary hippocampal and frontocortical cultures at 0, 0.1, 1 and 10 µM for 24 h. **(c)** Show time course of responses by DIV 14 primary hippocampal and frontocortical cultures to Pio treatment (10 µM for 0, 3, 6 or 12 h). All numerical data (n = 6) are represented as means ± S.E.M, * $p \leq 0.05$.

2.5. Discussion

The tissue distribution of peripheral PPARγ and their function in the regulation of fat and glucose metabolism is well documented (Evans et al, 2004; Rosen & Spiegelman, 2006). On the other hand, much less is known regarding the expression and role of this receptor in the CNS despite reports that PPARγ agonists can ameliorate cognitive impairments in AD patients and animal models of the disease (Watson et al, 2005; Risner et al, 2006; Hanyu et al, 2010; Chawla, 2010; Heneka et al, 2015; Mandrekar-Colucci et al, 2012; Searcy et al, 2012). Since PPARγ agonists such as pioglitazone can potently improve insulin sensitivity in type II diabetes (Filipova et al, 2017) and because metabolic dysfunction is strongly linked with AD (Rojas-Gutierrez et al, 2017), a critical question is whether the cognitive-improving effects of PPARγ activation result from direct effects on neural substrates in the brain or are secondary events resulting from improved metabolic control. To begin to resolve this question, the present study was designed to localize PPARγ in specific mouse brain regions *in situ* as well as in primary neural cultures from the neonatal mouse brain and human- and mouse-derived neural cell lines; further, studies were undertaken to demonstrate the functionality of PPARγ in neural cells.

Several earlier studies attempted to map PPARγ expression (mRNA and protein) in the rodent brain. The summary of representative studies shown in **Table 2.8** shows consistencies, but also discrepancies, in the distribution of PPARγ, either with respect to presence or intensity of signal in different brain regions and/or mRNA *vs.* protein; when differences occurred, these could be attributed to either differences in the methods used to measure gene (using RNAse protection assays, PCR, *in situ* hybridization) and protein (using immunohistochemistry) expression, specificity and sensitivity of the reagents used and/ or species, age and sex of the animals investigated. Inspection of **Table 2.8** also demonstrates that existing maps are somewhat fragmented, probably reflecting the particular research interest (e.g. neuroendocrine *vs.* cognitive, emotional or motivational) of the various authors; therefore, gaps in the summary do not necessarily represent "absence of PPARγ expression". The first part of the work presented in this chapter is based on immunohistochemical analysis of the neonatal (**Fig. 2.1**) and adult (**Fig. 2.2**) mouse brain in which we focussed on regions (primarily)

Table 2.8. Overview of rodent PPARγ brain mapping (mRNA and protein level) in literature: For comparability with the present work, same anatomical (sub)regions as in **table 2.7** are tabulated; for information regarding structure-function relations, please see main text referring to **Figs. 2.1** and **2.2. +/-** indicates areas in which very low (or poorly resolved) staining was found and **-** indicates regions, which were included in analysis, but not positiv for PPARγ; mRNA level is indicated as light (low expression) to dark green (moderate) and protein leve as light (low signal), darker (moderate signal) and very dark (high signal) green. Cs (Cingulate) and RSG (retrosplenial) are subregions of the frontal cortex.

		Cullingford et al, 1998	Braissant et al, 1996	Moreno et al, 2004	Sarruf et al, 2009	Liu et al, 2015	Gofflot et al, 2007	Lu et al, 2011	Warden et al, 2016	Lu et al, 2011	Warden et al, 2016	Pissioti, MSc 2012	Present study, 2018
Species		Rat				Mouse							Mouse
Age		neonatal		adult		juvenile	adult			adult			neonatal, adult
mRNA/Protein		mRNA	mRNA	Antibody 2	Antibodies 3-7	mRNA				Antibody 8	Antibody 4	Antibody 8	Antibody 8
Brain region, in situ	**Subregions**												
frontal cortex		-/+	-									+/-	Cg and RSG
nucleus accumbens	AcbC												
	AcbS												
amygdala	LA												
	BLA												
	BMA												
	st												+/-
hippocampus	DG		+/-				-						
	CA1		-										
	CA2												
	CA3		-										
hypothalamus	Arc		-			+/-	-						
	DM						-						
	LH						-						+/-
	Pe						-						
	PVH					+/-	-						
thalamus	Re				-								
	PV												
midbrain	VTA	-+		+/-								+/-	-
cerebellum		-+	+/-										
Forebrain, in vitro	**Cell type**												
	neuron												
	astrocyte												
	oligodendrocyte			-									-
	microglia										-		

Antibody 1	Affinity Bioreagents
Antibody 2	Chemicon (07-466)
Antibody 3	Abcam (19481)
Antibody 4	Genetex (GTX19481)
Antibody 5	Santa Cruz (sc7273)
Antibody 6	Cell Signaling (#2435)
Antibody 7	Cell Signaling (?)

mRNA			
Protein			
Signal intensity	Low	Moderate	High

involved in the regulation of cognition (frontal cortex, hippocampus, cerebellum), reward processing (nucleus accumbens, ventral tegmental area), emotion (amygdala), sensory gating (thalamus), neuroendocrine functions and energy metabolism (hypothalamus) and motor functions (cerebellum).

The present immunohistochemical analysis was completed using a polyclonal antiserum supplied by Cell Signaling (#2435) after comparing its performance (specificity) against another commercial reagent (abcam 19481) (see **Figs 2.6 a-b**), including demonstration that antibody #2435 from Cell Signaling did not detect PPARγ immunoreactivity in brain extracts from PPARγ$^{-/-}$ mice (**Fig. 2.5 b**). The results obtained largely confirmed previous results from our lab (Pissioti, 2012), as shown in **Table 2.8**.

Moderate-to-high PPARγ immunoreactivity was observed in the frontal cortex and hippocampus, two areas of particular relevance to AD because of their susceptibility to develop Aβ plaques and tau tangles, events that are accompanied by gradual memory deficits. To our knowledge, this report represents the first to describe PPARγ expression in the cingulate (Cg) and granular retrosplenial cortex (RSG), two sub-divisions of the frontal cortex. The Cg serves as a hub for the reception of sensory information from the thalamus and neocortex which is then transmitted to the entorhinal cortex, an area where AD pathology is initiated before it spreads to the hippocampus and frontal cortex; besides contributing to contextual memory (Zhang et al, 2017), this network is crucial for the processing, learning and memory of emotions (Rudebeck et al, 2007). Through its connections to the thalamus and hippocampus, the RSG is involved in spatial episodic memory, spatial navigation and predictive functions, all of which are compromised even at relatively early stages of AD (Epstein et al, 2017).

Our finding of PPARγ expression in the hippocampus confirms several previous reports (Briassant et al, 1996, Moreno et al, 2004; Gofflot et al, 2007, Lu et al, 2011, Liu et al, 2015; Warden et al, 2016). The hippocampus, with its reciprocal connections to cortical, subcortical and brainstem structures (Mufson et al, 2015) plays a crucial role in memory formation, especially that of episodic and spatial memory (anterior hippocampus) as well as in the processing of emotional information (posterior hippocampus) (Bubb et al, 2017). A unique

observation in the present investigation was that there is a rostro-caudal gradient (high to low) in PPARγ signal intensity in the hippocampus (**Table 2.7**), suggesting a greater contribution of PPARγ to the regulation of cognitive *vs.* emotional behaviour.

As previously mentioned (**see Chapter 1**), major depression, a stress-related disorder, may be a risk factor for developing AD (Catania et al, 2005; Sotiropoulos et al, 2007), and clinical studies report mood dysfunction in many AD patients (Sampath et al, 2017). It is also relevant to mention the majority of depressed patients also show increased anxiety and fear, behaviors in which the extended amygdala plays a central role (Ventura-Silva et al, 2012). Since anhedonia (impaired ability to perceive pleasure) is a characteristic of major depression, and because (over)eating is pleasure/reward-driven that places individuals at risk for developing metabolic disorders (de Guglielmo et al, 2015, Quaresma et al, 2016; Pissioti 2016), our current investigation not only included analysis of PPARγ expression in the amygdala but also in two key areas of the reward circuitry, namely the nucleus accumbens (Acb) and ventral tegmental area (VTA); all of which are reciprocally connected with the frontal cortex, hippocampus, thalamus and hypothalamus (Gipson et al, 2014). Whereas some previous studies reported PPARγ mRNA and protein expression in the amygdala (Liu et al, 2015; Warden et al, 2016), the present study is the only one in which semi-quantitative differences in receptor expression in specific amygdaloid subnuclei, the LA, BLA and BMA were found; this allows for detailed structure-functional analyses in the future. Similarly, with respect to the Acb, the present study could resolve differences in PPARγ in the core and shell subdivisions, unlike previous studies which only provided global descriptions of PPARγ expression in the Acb (Moreno et al, 2004; Warden et al, 2016). The question of whether the VTA, a midbrain structure that sends (mainly) dopaminergic afferents to the Acb, amygdala and frontal cortex, expresses PPARγ unfortunately remains poorly-defined at present: whereas two earlier studies reported PPARγ in the VTA (Sarruf et al, 2009; Warden et al, 2016), like Moreno et al, (2004) (in rat) and Pissioti 2012 (in mouse), we here could not convincingly detect PPARγ immunoreactive signal in this structure.

Our analysis of the thalamus supports previous reports of PPARγ protein expression in the thalamus (Moreno et al, 2004; Liu et al, 2015), a structure that is intricately linked to cortical and subcortical areas and which helps “gate” information to those areas to allow stimulus

perception. The thalamus comprises several sub-nuclei that have distinct afferent-efferent loops with distinct functions. Here, we focused on two thalamic nuclei – the paraventricular (PV) and reuniens (Re) thalamic nucleus; the former connects with amygdala and contributes to the regulation of motivation and mood (Hsu et al, 2014) and the latter relays stress-related information from the cortex to hippocampus and is thus implicated in depressive behaviour (Kafetzopoulos et al, 2017). Whereas PPARγ mRNA and protein were previously described in the PV (Moreno et al, 2004; Liu et al, 2015), the present study is the first to demonstrate PPARγ expression in the Re.

Relatively high PPARγ signal intensities were also found in various hypothalamic nuclei implicated in the control of appetite and energy metabolism (Arc, DM, LH and PVH) and the endocrine response to stress (PVH). Whereas the former are interesting because of their ultimate contribution to energy homeostasis (or dysbalance, including type 2 diabetes) (Long et al, 2014; Garretson et al, 2015), the latter is interesting because exaggerated stress hormone (glucocorticoid) secretion was previously shown to result in AD-like pathology, specifically Aβ overproduction (Catania et al, 2005) and abnormal hyperphosphorylation of tau protein (Sotiropoulos et al, 2007; Lopes et al, 2017). To a large extent, the present findings add a quantitative element to previous reports (Moreno et al, 2004; Sarruf et al, 2009; Long et al, 2014 and Garretson et al, 2015) and generally confirm the potential importance of PPARγ in the control of endocrine functions and energy balance.

The important role of age in determining PPARγ expression in the brain is particularly evident when immunohistochemical data from the neonatal and adult mice cerebellum are compared. We found that PPARγ expression is markedly diminished during ageing, although it should be mentioned that at least three other studies reported relatively high levels of PPARγ mRNA (Gofflot et al, 2007, Lu et al, 2011) and protein (Moreno et al, 2004) expression in the cerebella of adult rodents. In contrast to our observations of rage-dependent decreases in PPARγ expression in the cerebellum, our results show a striking increase in PPARγ protein levels in the frontal cortex and hypothalamus, and to some extent in the hippocampus, during aging of the mouse (see summary **Table 2.7**). These brain area-specific age-related changes in expression not only suggests lifetime alterations in the physiological significance of PPARγ but also changes in tissue sensitivity to PPARγ agonists across the lifespan.

To summarise this discussion of the present immunohistochemical results: although our analysis involved very limited material (1 neonatal and 1 adult mouse brain per anatomical structure), the results are generally in agreement with previous observations and in several cases, extend the earlier work in providing additional information regarding age differences, brain areas not previously studied (or not with the same degree of resolution, e.g. definition of sub-nuclei within a given structure). Our findings show that PPARγ are expressed in brain regions concerned with the regulation of cognition and energy balance as well as with the control of emotional and neuroendocrine functions, all of which together interact to maintain cognitive health or dysfunction. While these findings give credence to the view that activation of central PPARγ can have consequences for a variety of brain functions, they represent only a first step towards dissecting the site and mechanisms of PPARγ agonist (and relative importance of peripheral *vs.* central actions) in the context of the reported therapies for disorders of the brain, such as AD (Kummer et al, 2015; Ulrich-Lai & Ryan, 2013).

Other approaches were used to complement and confirm the immunohistochemical results. Using a highly sensitive immunoblotting method (ProteinSimple Wes) to analyse brains from various wildtype and PPARγ knockout mice, we confirmed the specificity of the antiserum (CST #2435) used in the *in situ* morphological studies. As reported here, this antiserum gave similar results to those obtained with antibody (ab19481) in immunocytochemical analysis of primary neural cultures; the latter antibody, as well as another antiserum (sc2723) was shown to be specific in another set of experiments in which RNAi was used to knockdown *PPARγ*. Moreover, we confirmed that the expression of PPARγ protein (especially in the hippocampus) increases with age and showed (by conventional Western blotting) that the levels of PPARγ protein in the mouse brain differ in a region- and sex-specific manner.

To address the important question of which cell type expresses PPARγ in the mouse frontal cortex and hippocampus, we performed immunocytochemical assays (using antibody ab19481 [also used by Sarruf et al, 2009 and Warden et al, 2016] from Abcam which was subsequently withdrawn by the producer) on primary cultures from frontal cortices and hippocampi of postnatal (PND 4) mice. The cultures were grown in a chemically-defined medium (Neurobasal A/B27 medium including the likely PPARγ agonists linoleic and linolenic acids, and retinoic acid) and stained after 14 days *in vitro* (DIV). Co-immunolabeling revealed

that neurons (MAP2+) and astrocytes (GFAP+), but not oligodendrocytes (O4+), express PPARγ protein, albeit to varying extents (see **Fig. 2.6 b**); interestingly, our cultures did not contain microglia.

Whereas the cultured neurons and astrocytes displayed PPARγ protein that had high proportion of nuclear localization (**Fig. 2.6 c**), our immunohistochemical analysis (brain tissue sections) showed that PPARγ may be present in the nucleus and cytoplasm (**Fig. 2.1 c, e-h** and **2.2 a, d-i**); in particular, PPARγ staining was seen in hippocampal (CA) neurites *in situ* (**Fig. 2.1 e** and **f**). At least one previous report (Isaac et al, 2006) described PPARγ signal in both, cytosolic and nuclear fractions derived from neural cell lines. Indeed, cytoplasm-located PPARγ is not entirely unexpected since translation of the receptor occurs in that compartment. On the other hand, it should be mentioned that the restricted nuclear localization of PPARγ in cultured cells may reflect the presence of agonist molecules (linoleic and linolenic acid in the B27 used for primary neural cells, or other fatty acids in fetal calf serum used to culture cell lines) in culture media.

In this work, primary neural cultures were used to ascertain the functionality of PPARγ detected by immunochemistry. First, we demonstrated by qPCR assays that primary rat neural cultures express the mRNA encoding *PPARγ* as well as its heterodimerizaton partners, *RXR* (*α, β* and *γ* isoforms) and *LXR* (*α* and *β* isoforms) (**Fig. 2.3 a**); previous work showed that at least one of these partners is necessary for the transcriptional activity of PPARγ (Laudet & Gronemeyer, 2002; Gofflot et al, 2007). Next, we showed that the levels of two well-known PPARγ target genes, *ABCA1* (Mandrekar-Colucci et al, 2012) and *PGC-1α* (Puigserver & Spiegelman 2003), are upregulated in a dose-dependent manner when neural cultures exposed to pioglitazone, a potent pharmacological PPARγ agonist. Interestingly, one of these downstream targets were differentially regulated in frontocortical *vs.* hippocampal cultures: whereas pioglitazone only induced *ABCA1* expression in hippocampal cells, *PGC-1α* levels were only upregulated in frontocortical cultures. These observations suggest brain area-specific responses to PPARγ ligands. Lastly, experiments involving co-treatment of a human neuroblastoma cell line (SH-SY5Y) with pioglitazone and the PPARγ antagonist GW9662 abrogated the ability of the PPARγ agonist to induce *ABCA1* and *PGC-1α* transcription, thus lending support to the specificity of the qPCR measurements.

In summary, this work provides convincing evidence for the presence and functional activity of PPARγ in the mouse (with preliminary confirmatory data for rat) brain. Especially within the context of AD, the results demonstrate that PPARγ are expressed in the hippocampus and frontal cortex; in addition, they show that other areas involved in the regulation of motivation (primarily, nucleus accumbens, midbrain), emotion (amygdala), sensory gating (thalamus), endocrine/metabolic regulation (hypothalamus) and movement (cerebellum) also express PPARγ. Together, these findings add clarity to the sometimes contradictory reports in the literature and are particularly important because they give credence to the view that the reported ability of PPARγ agonists to improve cognition and other related behaviours may result, at least partially, from ligand binding to neural substrates (neurons, astrocytes) in the brain. However, it is important that future investigations focus more closely on the question of whether microglia express PPARγ. Since most studies implicating a role of these receptors in AD suggest that the ameliorative effects of PPARγ agonists might be related to their anti-neuroinflammatory properties (García-Bueno et al, 2014; Heneka et al, 2013). The results of this study also demonstrate, for the first time, that PPARγ expression in the brain is regulated by sex and age; these two parameters are relevant in the context of brain diseases such as AD: women are more prone to AD and aging is the greatest risk factor for developing AD (Prince et al, 2015).

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2.7. Supplementary Information

Table S2.1. Overview of animals used for the different types of analysis

	Analysis	Animals	Age	Sex	n
Primary cell culture	RT-PCR	Wistar rats	PND 5	mixed	12 pups/culutre
	qRT-PCR	CD1	PND 5	mixed	10 - 20 pups/culture
	ICC	CD1	PND 5	mixed	10 - 20 pups/culture
Hole brain analysis	IHC	CD1	PND 5	male	1
			3 months	male	1
Tissue (FC and HP)	qRT-PCR and WesternBlot	CD1	PND 5	male	4
			PND 5	female	4
			PND 14	male	4
			PND 14	female	4
	protein simple WES	PPARγ [fl/fl]	3 months	male	2
		PPARγ NexCRE[+/-]	3 months	male	3
		J20	3 months	male	3
		BACE[-/-]	3 months	male	2
		C57/BL6	PND 5	male	3
				female	3
			3 months	male	3
				female	3
			12 months	male	3

Figure S2.1. Solutions used for primary cultures

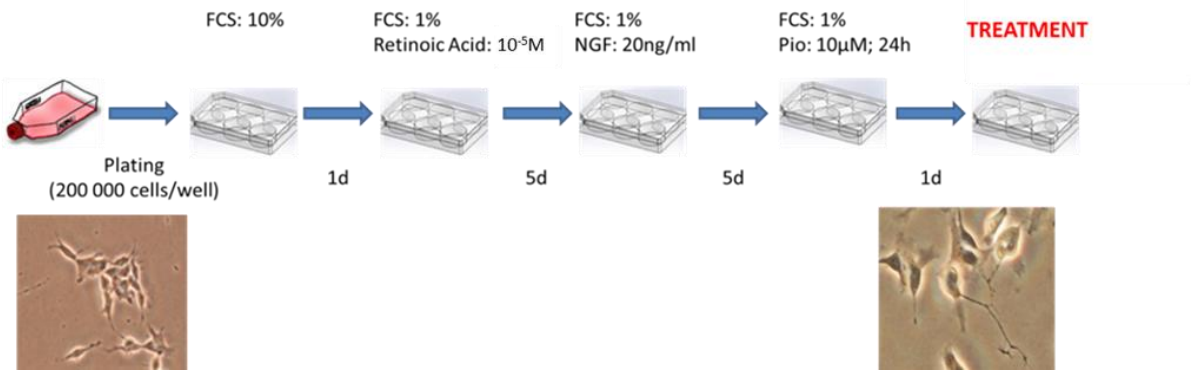


Table S2.2. Solutions used for primary cell culture

	Ingredients	Quantity	Supplier
Borate Buffer	Sodium borate	0.9 g	Sigma Aldrich
	-> 100 ml ddH2O adjusted to pH 8.3		
Trypsin solution	trypsin (10x)	0.025%	Gibco
	BSA 7,5%	3.0 mg/ml	life technologies
	Dnase	50 U/ml	Worthington
	-> in EBSS		life technologies
Trypsin blocking solution	Soy bean trypsin inhibitor	0.4 mg/ml	Sigma Aldrich
	BSA 7,5%	3.0 mg/ml	life technologies
	FCS	2.0%	life technologies
	50x B27	1.0 mM	life technologies
	-> in Neurobasal A		
Growing medium	50x B27	1.0 mM	life technologies
	Kanamycin	1.0%	life technologies
	Glutamax 100X	200 mM	life technologies
	bFGF	10.0 ng/ml	life technologies
	-> in Neurobasal A		

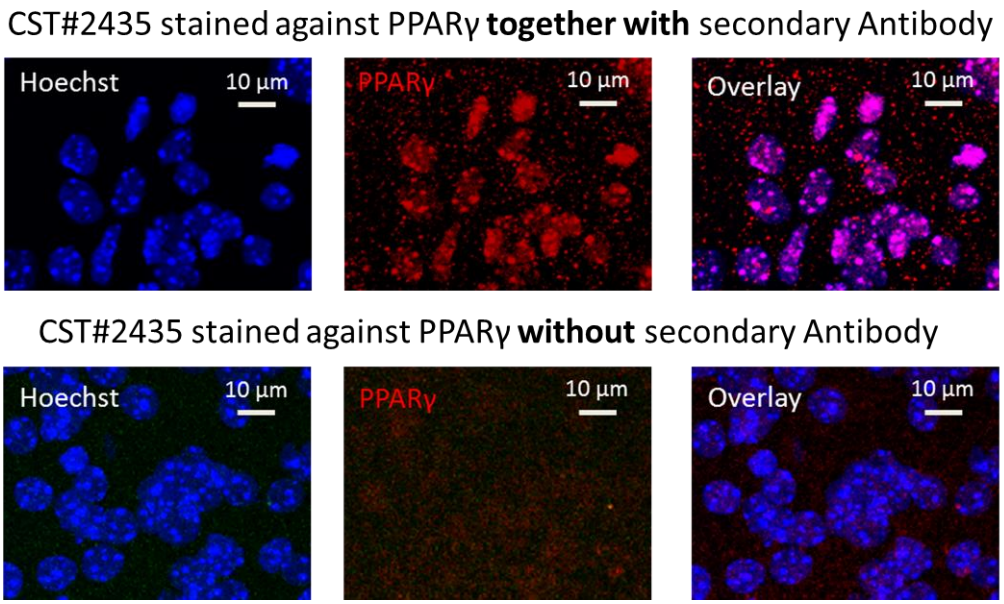
Table S2.3. Solutions used for immunohistological analysis

	Ingredients	Quantity	Supplier
0.1 M PBS	NaCl	80.0 g	Roth
	Na_2HPO_4	14.4 g	Merk
	KH_2PO_4	2.4 g	Merk
	KCl	2.0 g	Merk
	-> in 800 ml ddH_2O adjusted to pH 7.4		
Sodium citrate buffer	0.1 M solutio Citric Acid		
	0.1 M solution Sodium citrate		
	-> up to 500 ml ddH_2O adjusted to pH 6.0	9.0 ml	
		41.0 ml	

Table S2.4. Solutions used for WesternBlot analysis

Western blot	Ingredients	Quantity	Supplier
6x Laemmli Buffer	0,5 M Tris pH 6,8	6.8 ml	
	Glicerol	3.2 ml	Sigma-Aldrich
	SDS (20%)	1.6 ml	Sigma-Aldrich
	β-mercaptoethanol	0.8 g	Sigma-Aldrich
	1% bromophenol blue	1.6 mg	Sigma-Aldrich
	-> 5 ml dH_2O storage 20 C		
10 % SDS-PAGE gel	Acrylamide (40%)	12.5 ml	Roth
	1,5 M Tris (pH 8,8)	12.5 ml	
	10% SDS	0.5 ml	
	10% APS	0.5 ml	
	TEMED	0.04 ml	Roth
	-> up to 50 ml H_2O		
Stacking gel	Acrylamide (40%)	1.3 ml	Roth
	1,0 M Tris (pH 6,8)	1.25 ml	Sigma Aldrich
	10% SDS	0.1 ml	Sigma Aldrich
	10% APS	0.1 ml	Sigma Aldrich
	TEMED	0.01 ml	Roth
	-> up to 10 ml H_2O		
1 % Running Buffer	Tris Base	30.3 g	Sigma Aldrich
	Glycine	144 g	Roth
	SDS	10 g	Sigma Aldrich
	-> up to 1000 ml ddH_2O		
1 % TBS-T	Trizma HCl	24.2 g	Sigma Aldrich
	NaCl	80.0 g	Sigma Aldrich
	-> up to 1000 ml ddH2O adjusted to pH = 7.6		

Fig. S2.2. Negative control for PPARγ immunohistochemistry. Representative images of immunohistochemical staining of neonatal (PND 7) mouse (CD1) brain (frontal cortex). Nuclei were stained with Hoechst 33341 (blue). Cells expressing PPARγ were labelled with antibody CST#2435 (red), detected with secondary antibody (goat anti-rabbit conjugated to Alexa Fluor 594) (*upper panel*); the *lower panel* shows a section incubated with primary, but no secondary, antiserum.



Chapter 3

Activated PPARγ interrupt pathways underlying Alzeimer's disease pathology

3.1. Abstract

Metabolic disorders such as obesity and type II diabetes have been associated with risk for developing dementia, including Alzheimer's disease (AD). Pioglitazone (Pio), a pharmacological agonist of the nuclear receptor peroxisome proliferated-activated receptor γ (PPARγ), which effectively improves insulin sensitivity, has therefore been considered as a potential agent in the amelioration of AD symptoms. However, to date, there is sparse information regarding the sites and mechanisms of Pio actions. The present *in vitro* studies were based on the fact that amyloidβ (Aβ) is a primary trigger of the neurodegeneration and cognitive deficits found in AD. Results obtained in a human neural cell line (SH-SY5Y) and primary cultures from mouse frontal cortex and hippocampus, show that Pio reduces the pathological processing of amyloid precursor protein (APP) into Aβ, improves clearance of toxic Aβ, and inhibits Aβ-induced hyperphosphorylation of tau protein; the latter event is considered critical for the characteristic loss of synapses and neurons in AD. Importantly, we show that Pio interferes with the dendritic localization of tau; we postulate (and are currently testing) that the latter results from the phosphorylation of tau by glycogen synthase kinase 3β (GSK3β) and the subsequent Fyn kinase-mediated linking of hyperphosphorylated tau to glutamate 2B receptor (GluN2B)-dependent synaptotoxicity.

Together, these findings provide mechanistic support for the view that PPARγ may be drug target worthy of further exploration in the quest for anti-AD treatments.

3.2 Introduction

Extended lifespan in the modern era is resulting in increased incidence of Alzheimer's disease (AD), a life-threatening and cost-intensive disease that is still incurable (www.alzheimers.org.uk). Besides age, modern lifestyles increase the risk for developing obesity and Type II diabetes which are causally implicated in AD (Fosnacht et al, 2017). Transcriptional activity of the peroxisome proliferated-activated receptor γ (PPARγ) contributes to the regulation of adipocyte differentiation and has also been found to improve insulin sensitivity (**Chatper 1 - 2.3.1**). Treatment of AD patients and mouse models of the disease, with PPARγ agonists has been shown to improve cognition (Risner et al, 2006; Hanyu et al, 2010; Mandrekar-Colucci et al, 2012; Searcy et al, 2012; Ahmadian et al, 2013), albeit through mechanisms that have not been fully elucidated. Our previous demonstration of PPARγ in the mouse brain (**Chapter 2**), especially in areas affected by AD pathology (frontal cortex and hippocampus), make it plausible that central PPARγ are at least partly responsible for the previous observations on cognitive improvement, i.e. the improvements are not necessarily secondary to improvements in peripheral metabolism.

This study focussed on two proteins, whose aggregation is causally associated with AD symptomology: amyloid β (Aβ) (that eventually forms senile plaques) and tau (hyperphosphorylation of which gives rise to neurofibrillary tangles) (Wang & Mandelkow, 2016). The 40- and/or 42 amino acid peptide Aβ is generated from the misprocesing of amyloid precursor protein (APP), a membrane protein, following cleavage by β-secretase (BACE1) and γ-secretase; while oligomeric/soluble Aβ is highly toxic to synapses ($A\beta_{42} > A\beta_{40}$) (Roselli et al, 2005) it remains unclear as to whether (and how) aggregated Aβ, that forms extracellular plaques results in neurodegeneration (Müller et al, 2017; Iqbal et al, 2014). While cytoskeletal tau, especially that in axons, has received much attention in research on neurodegeneration (hyperphosphorylated tau detaches from microtubules and results in instability of the cytoskeleton), tau-regulated synaptic toxicity has recently become a central focus of AD research. Studies have shown the *de novo* synthesis and hyperphosphorylation of tau in dendrites which, through the mediation of Fyn kinase, leads to the interaction of glutamatergic receptors (GluN2B) and PSD-95 (Postsynaptic density protein 95) inducing spine loss and dendritic atrophy (Kimura et al, 2007; Ittner et al, 2010; Lopes et al, 2016;

Kobayashi et al, 2017). Whereas abnormal tau is now broadly accepted as the primary trigger of AD-like pathology, it is important to note that Aβ is a key inducer of the tau kinases cyclin dependent kinase 5 (CDK5) and glycogen synthase kinase 3β (GSK 3β) (Dolan & Johnson, 2010).

Previous work showed that the PPARγ agonist Pio can prevent Aβ-induced neuronal death and synaptotoxicity *in vitro* (Xu et al, 2014). The present experiments, using a human neural cell line (SH-SY5Y) and primary cultures from the mouse frontal cortex and hippocampus, were intended to confirm and extend the observations by Xu et al (2014). Briefly, we show that Pio-activated PPARγ abrogate Aβ and tau pathology. Specifically, pre-treatment with Pio reduced Aβ-induced misprocessing of APP to Aβ and promoted Aβ clearance. The PPARγ agonist also reduced Aβ-induced hyperphosphorylation of tau protein at a number of epitopes that are implicated in AD pathology (pSer202, pThr205, pThr231, pSer356 and PHF1 [pSer396 and pSer404]); notably, the accumulation of hyperphosphorylated tau in dendrites was markedly inhibited by Pio.

3.3. Materials and Methods

Animals and tissues: All animals were used in compliance with the European Union Council's Directive (2010/63/EU) and local regulations. Animals were bred and kept at the animal facility of the MPI of Psychiatry. For primary neural cell cultures, male and female CD1 mice (10-20), were used on postnatal day 5 (PND 5). Pups were born to mothers that were housed under standard laboratory conditions [temperature 22°C; relative humidity 50 ± 10%; 12h light/dark cycle (lights on at 7:00)], with *ad libitum* access to food (#1324 laboratory diet; Altromin Spezialfutter GmbH & Co. KG, Lage, Germany) and water. Brains were excised after cervical dislocation and decapitation of the pups.

Drugs and treatments: Primary cell cultures were treated with 1 µM GW9662 (Sigma Aldrich, dissolved in dimethyl sulfoxide (DMSO), final concentration 0.01%), 10 µM of pioglitazone (Sigma Aldrich; Pio, dissolved in DMSO) and/or 1 µM Aβ 1-42 (American Peptide #62-0-80). The latter was prepared according to Stine et al (2011).

Cell culture: All cells were maintained in an incubator (95% air, 5% CO_2; 37°C, 95% relative humidity).

Primary neural cell cultures: Frontocortical and hippocampal primary cultures were generated from PND 5 mice (CD1 strain), as described in **Chapter 2**. Cells were used after 14 days *in vitro* (14 DIV) by which time they had developed neurites that were microtubule-associated protein 2 (MAP2) positive, indicative of their differentiated state (**Fig. 2.6**).

Cell lines: The human neuroblastoma cell line (SH-SY5Y) and the murine hippocampal cell line (HW3-5; (Kuwahara et al, 1999; Baj et al, 2005; courtesy of Dr. Dietmar Spengler, Max Planck Institute of Psychiatry) were cultured and differentiated (SH-SY5Y) as described in **Chapter 2**.

Cell viability assay: A MTS assay kit (CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS - 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); Promega) was used to assess cell viability of differentiated SH-SY5Y cultures

(grown in 24-well plates), following the manufacturer's suggested protocol. Briefly, after exchanging the growth/differentiation medium for pure DMEM, cells were incubated (3 h, in dark) with MTS solution after which the optical density (490 nm) of the supernatant was measured.

Immunofluorescent cytochemistry (ICC) and image analysis:

Cultures were stained using immunofluorescence, as described in **Chapter 2**. Details of primary and secondary antisera used are summarized in **Table 3.1**. The number of stained puncta, on a defined dendritic length, were quantified using SynPAnal, a software for rapid quantification of synaptic density (Danielson & Lee, 2014; PLOS ONE Staff, 2015).

Table 3.1. Summary details of antisera used for immunoctyochemical staining

	Antibodies	Supplier	Cat.No	Dilution	Conjugate
Primary	PSD-95, mouse monoclonal IgG2a	Neuromab	#75-028	1:1000	
	Synapsin 1,2, rabbit polyclonal IgG	SYSY	#160002	1:1500	
	anti-Tau (phospho S396), rabbit monoclonal IgG	Abcam	ab109390	1:1000	
Secondary	Goat-anti rabbit polyclonal IgG	Alexa Fuor	Inv# A110374	1:1000	Alexa Fluor 488
	Goat-anti mouse polyclonal IgG	Alexa Fluor	Inv#A110029	1:1000	Alexa Fluor 594

Immunoblotting

Protein extraction: Treated cells were washed once with 0.01 M PBS and lysed in homogenization buffer that included phosphatase inhibitors II and III (SIGMA P5726-5ML and P0044-5ML) (**Supplementary Information - Table S.3.1**). Lysates were sonicated on ice and centrifuged (14.000 *g*, 15 min, 4°C); the supernatants were stored at -80°C until further analysis (Western blotting or Enzyme-linked Immunosorbent Assay (ELISA), see below).

Western blotting: The protein contents of thawed supernatants were assayed using the Lowry method (Lowry et al, 1951), as described in **Chapter 2** after which Western blotting was performed on samples as previously described (**Chapter 2**), using primary and secondary antibodies listed in **Table 3.2**.

Enzyme-linked Immunosorbent Assay (ELISA): Insulin-degrading enzyme levels were determined in cell lysates (50 µg protein) using ELISA kits (Cloud-Clone Corp, Katy, TX; Cat. SEB897Mu), according to the manufacturer's instructions.

Table 3.2. Characteristics of antisera used for Western blot analysis

	Antibodies	Supplier	Cat.No	Dilution	Conjugate
Primary	PPARγ, rabbit monoclonal IgG	Cell Signaling	#2435	1:500	
	anti-APP A4, Clone 22C11, mouse monoclonal IgG	Millipore	#MAB348	1:500	
	anti-BACE (D10E5), rabbit monoclonal IgG	Cell Signaling	#5606	1:1000	
	anti-nicastrin, rabbit polyclonal IgG	Sigma	#N16660	1:1000	
	anti-Tau (phospho S202), rabbit monoclonal IgG	Abcam	ab108387	1:1500	
	anti-Tau (phospho T205), rabbit monoclonal IgG	Abcam	ab4841	1:1500	
	anti-Tau (phospho T231), rabbit monoclonal IgG	Abcam	ab151559	1:1500	
	anti-Tau (phospho S356), rabbit monoclonal IgG	Abcam	ab92682	1:1500	
	PHF1 (p396/404-Tau), mouse	kind gift from Dr. Ioannis Sotiropoulos		1:1000	
	anti-Tau (TAU-5), mouse monoclonal IgG	Abcam	ab80579	1:1500	
	Actin, mouse monoclonal IgG	Chemicon	#MAB1501R	1:2500	
Secondary	Goat anti-rabbit polyclonal IgG	Thermo	#31460	1:1000	(H+L) HRP-conjugated
	Goat anti mouse polyclonal IgG	BioRad	170-6516	1:2000	(H+L) HRP-conjugated

Polymerase Chain Reaction (PCR) assay: Quantitative real time PCR (qRT-PCR) was performed on cDNA transcripts from RNA extracts of lysed cells (**Chapter 2**). **Table 3.3** lists the primers used in this study.

Table 3.3. Primers used in PCR assays

Primer	Sequence	Tm oC	Position	Length of PCR product	Genebank ID
ABCA1 fwd	5'GACATCCTGAAGCCAATCC -3'	62.1	30. exon	212 bp	
ABCA1 rev	5' - GTAGTTGTTGTCCTCATACC - 3'	53.3	33. exon		
ApoE fwd	5' - GCACGGCTGTCCAAGGAG - 3'	67.3	3. exon	190 bp	Hixon and Vermier, 1990
ApoE rev	5' - CGGCCTGGCTAGTCCAAGGAG - 3'	56.8	3. exon		
GAPDH fwd	5' - CCATCACCATCTTCCAGG - 3'	61.6	3. exon	165bp	
GAPDH rev	5' - GTTGAAGTCGCAGGAGACAAC - 3'	52.4	5. exon		

Statistical analysis

Numerical data are presented as mean $\pm$ SEM. Data were subjected to outlier analysis (Iglewicz & Hoagin's robust test for multiple outliers, 2-sided with modified *z* score ≥ 1) before identification of significant differences ($p \leq 0.05$) using Student's *t*-test or 2-factor analysis of variance (2-way ANOVA), with Tukey's *post hoc* test (Prism 6 software (GraphPad, San Diego, CA). All data passed the normality test (Shapiro-Wilk-Test), except for that shown in **Fig. 3.1c(ii)** and **Fig. 3.2; accordingly, those data were tested** with the Kruskal-Wallis test, corrected for multiple (pair-wise) comparisons by Dunn's test. Dr. Darina Czamara (Max Planck Institute of Psychiatry, Munich) kindly advised on the statistical analysis.

3.4. Results

3.4.1. Counteraction of Aβ-induced cytotoxicity by pioglitazone (Pio)

Neuronal death in AD is thought to result from the triggering of toxic mechanisms (e.g. generation of reactive oxygen species) by Aβ under variably reported conditions (Müller et al, 2016; Behl & Moosmann, 2002). As shown in **Fig. 3.1 a**, treatment of differentiated SH-SY5Y cells with 1 µM of soluble $A\beta_{1\text{-}42}$ for 24 h, but not 3 h, led to a significant loss of cell viability ($p \leq 0.05$), as measured by MTS assays; importantly, pre-treatment with Pio significantly abrogated Aβ-induced loss of cell viability ($p \leq 0.05$), indicating that activated PPARγ have potentially neuroprotective actions against Aβ-induced neural cell death.

3.4.2. Upregulation of APP misprocessing by Aβ is blocked by pioglitazone (Pio)

Using SH-SY5Y cells, we found that Aβ upregulates the production and misprocessing of APP to Aβ, with the contemporaneous upregulation of the two key enzymes responsible for the amyloidogenic cleavage of APP, β-secretase (BACE1) and γ-secretase (the latter measured as nicastrin, a component of γ-secretase) (**Fig. 3.1 b**); these results confirm previous *in vivo* findings in our laboratory (Catania et al, 2009). Further, **Fig. 3.1 b** shows that pre-treatment of SH-SY5Y cells with Pio prevents Aβ-induced increases in the generation of APP and expression of BACE1 and nicastrin. These observations thus demonstrate that PPARγ activation can interfere with the generation of Aβ.

3.4.3. Pio upregulates mechanisms potentially contributing to reduction of Aβ levels

Based on the postulation that Aβ-induced pathology should be mitigated by increased clearance of Aβ (Baranello et al, 2015) we next examined whether PPARγ activation by Pio induces the expression of target genes (in SH-SY5Y cells) whose protein translational products are implicated in Aβ clearance. Specifically, we used qRT-PCR to measure the mRNA levels of the *ATP-binding cassette transporter A1* (*ABCA1*) and of *apolipoprotein E* (*ApoE*). The clearance of Aβ by ABCA1 depends on its ability to lipidate ApoE; the latter then binds Aβ, and exports it out of the brain, through the mediation of the *very-low-density-lipoprotein receptor* (*VLDLR*) in the BBB (note: a point mutation in ApoE, resulting in the overexpression

of ApoE4, is a known genetic risk factor for AD in humans (Jiang et al, 2008). The results of this experiment, shown in **Fig. 3.1 c (i)**, are not easy to interpret since Aβ, like Pio, upregulated ABCA1 and ApoE mRNA expression; moreover, co-treatment of cells with Pio and Aβ reversed the effects produced by the individual compounds. It should be noted that the transcriptional effects of Pio on *ABCA1* and *ApoE* mRNA were shown to be mediated by PPARγ insofar that the Pio effects were significantly reduced in the presence of the PPARγ antagonist GW9662; however, GW9662 alone upregulated the levels of *ABCA1* and *ApoE* mRNA, raising questions about whether the drug has mixed agonist-antagonist properties.

We subsequently investigated another potential mechanism through which Pio might reduce Aβ accumulation, namely via degradation by extracellular (secreted) insulin-degrading enzyme (IDE). This set of experiments was performed on primary mouse frontocortical and hippocampal cultures. As can be seen from **Fig. 3.1 c (ii)**, Pio ($p = 0.1094$) and Aβ ($p= 0.1094$) alone increased levels of IDE in frontocortical cultures. However, the only significant difference detectable was between Aβ and Pio + Aβ co-treatment ($p = 0.0486$) in primary hippocampal cultures.

In summary, this set of results unfortunately fails to provide a clear answer regarding the importance of Aβ-clearing mechanisms that were initially hypothesized to underlie the ability of Pio to counteract Aβ-induced cytotoxicity. We found that Pio upregulates at least two relevant molecules (ABCA1, IDE) but acted in an unpredicted fashion when added in the presence of exogenous Aβ, at least under the experimental conditions used in this work.

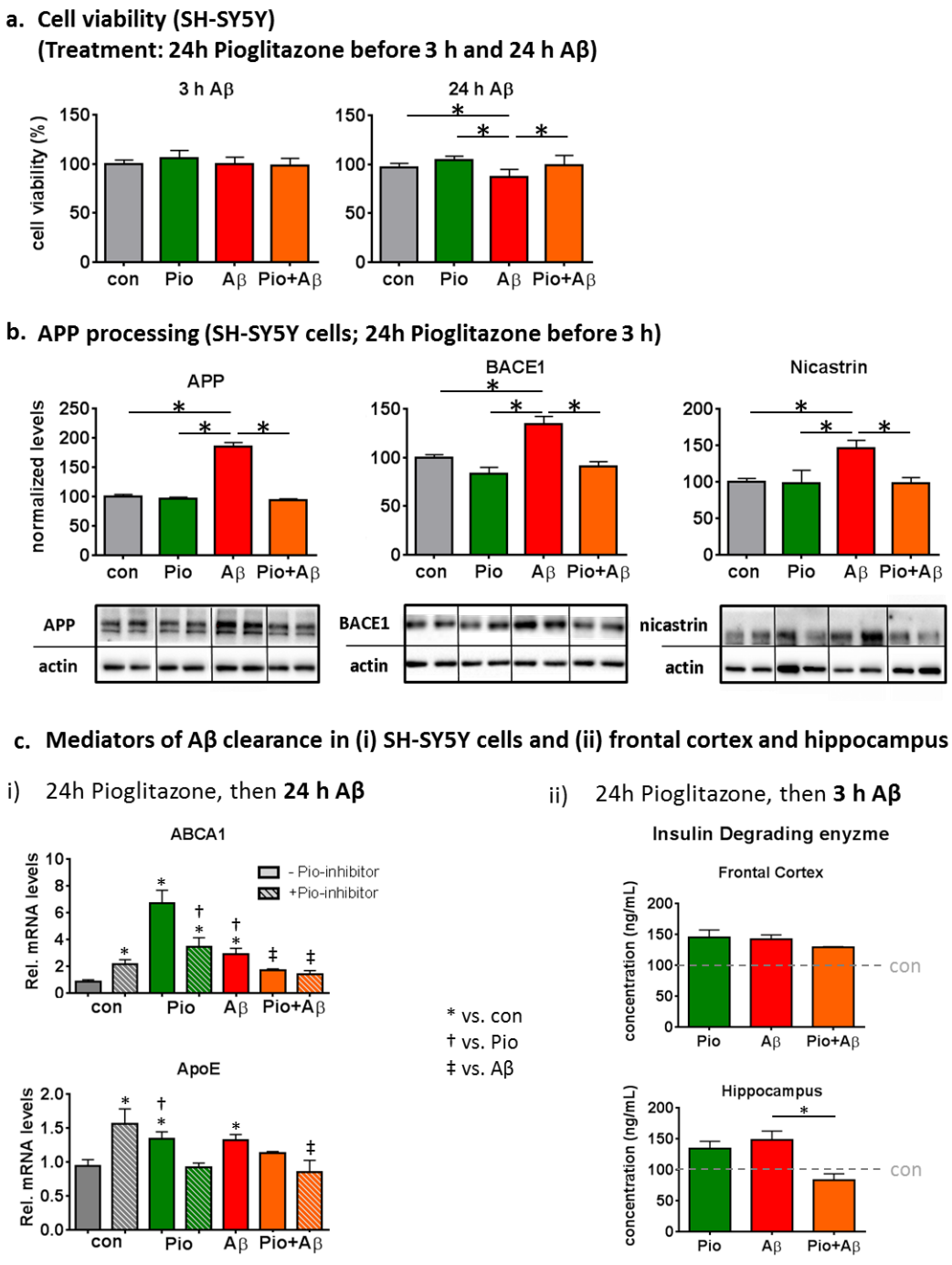


Figure 3.1. Pioglitazone prevents cytotoxic and Aβ effects *in vitro*. The human neural cell line SH-SY5Y (**a-d(i)**) and primary neural cultures from mouse frontal cortex and hippocampus (**d (ii)**) are treated with 1 µM GW9662 (PPARγ antagonist) for 30 min (**d (i)**), 10 µM Pio (PPARγ agonist) for 24 h (**a-d**) alone or followed by 1 µM soluble Aβ 1-42 for 3 h (**a** left graph), **b** and **d (ii)**) or 24 h (**a** right graph and **d(i)**. A MTT assay is employed to measure cell viability (**a**). Westernblot experiments show protein levels normalized to actin of APP, BACE1 and nicastrin as blots below the graphs and as percentages normalized to control (100%) as graphs (**b**). qPCR measurements show mRNA levels of *ABCA1* and *ApoE* (**c (i)**) normalized to GAPDH. ELISA assay results show percentage of protein concentration of IDE in culture medium compared to control levels (100%) (**c (ii)**). All numerical data (n = 3-6) are represented as means ± S.E.M; significant differences are $p \leq 0.05$.

3.4.4. PPARγ activation influences tau biochemistry

Hyperphosphorylation of tau protein at specific epitopes plays a significant role in triggering neuropathological events (e.g. synaptic dysfunction) and behavioural impairments that are characteristic of AD (Wang & Mandelkow, 2016). While several studies, including those reported above have focused on PPARγ-mediated modulation of APP synthesis and processing and Aβ pathology, there is very little information regarding the involvement of PPARγ in tau pathology (Escribano et al, 2010; Yoon et al, 2010; Cho et al, 2013; Yu et al, 2015). In a pilot study we examined whether activation of PPARγ by Pio modulates tau biochemistry. As shown in **Supplementary Information Fig. S.1**, exposure of murine neural cells (HW3-5 cell line) resulted in a significant decrease ($p \leq 0.05$) in the expression of total tau protein as well as of levels of phosphorylated tau at Ser396; phosphorylated Ser396 is a component of extracellular tangles (Metaxas & Kempf, 2016).

Prompted by the above observation, we next exploited the known ability of $A\beta_{1-42}$ to induce the aberrant phosphorylation of tau (De Felice et al, 2008) to examine whether Pio can prevent post-translational modifications of tau that are potentially toxic. The investigations were performed on primary frontocortical and hippocampal cultures that were pre-treated with 10 μM Pio for 24 h before the addition of $A\beta_{1-42}$ for 24 h. As shown in **Fig. 3.2 a** and **3.2 b**, although $A\beta_{1-42}$ did not alter total tau levels, treatment with Aβ was associated with an increase in the relative amounts of five pTau isoforms (pSer202, pThr205, pThr231 (only in frontocortical cultures), pSer356 (only in frontalcortical cultures) and pSer396/Ser404 [PHF-1]) ($p < 0.05$), all of which have been implicated in AD pathology (Metaxas & Kempf, 2016); these changes were more pronounced in frontocortical *vs.* hippocampal cells. The effect was significantly ($p < 0.05$) reversed by co-treatment with Pio for pSer202 (only in hippocampal cultures), pThr205 and pThr231, whereas this difference was only a trend for pSer396/Ser404 [PHF-1]) ($p \leq 0.1$).

In summary, these results demonstrate that Pio-activated PPARγ can counter the ability of Aβ to induce aberrant hyperphosphorylation of tau at epitopes relevant to AD.

3.4.5. Pioglitazone reduces expression of pSer396-tau in synapses via PPARγ

Previous studies indicated that the neurodegenerative effects of tau may be secondary to Aβ-induced tau-hyperphosphorylation (Dolan & Johnson, 2010; Stancu et al, 2014). More recently, Aβ was shown to induce hyperphosphorylation of synthesized tau in dendrites where it leads to the activation of Fyn kinase and synaptic degradation (Ittner et al, 2010; Kobayashi et al, 2017).

Given the results in the previous section showing that PPARγ activation antagonizes Aβ-induced tau hyperphosphorylation, we next investigated whether 24 h pretreatment with Pio (10 µM) abrogates the effects of $A\beta_{1-42}$ (1 µM, 24 h) on the localization and phosphorylation status of dendritic tau and on the preservation/loss of synapses. Our analysis was done by laser scanning confocal microscopy for which postsynaptic densities were labelled with anti-PSD-95 (green) and quantified (green puncta) using SynPAnal software, and immunolabeling with an anti-pSer396-tau (red) that detects a tau phospho-epitope known to be expressed in AD brains.

Consistent with literature reports (Roselli et al, 2005; Liu et al, 2010) Aβ treatment of frontocortical primary cultures led to significantly ($p \leq 0.05$) decreased PSD-95-positive puncta and concomitantly increased pSer396-tau immunoreactive signal ($p \leq 0.05$), as shown in **Fig. 3.3**. Pre-treatment with Pio over-rode the ability of Aβ to reduce PSD-95 expression and to upregulate pSer396-tau. Moreover, Pio pre-treatment reduced the colocalization of pSer396-tau in PSD-95-immunoreactive elements, represented relative to the total number of PSD95-positive puncta ("colocalization index"). Similar results were obtained when the above experimental design was applied to primary hippocampal cultures, as shown, qualitatively (upper panel) and quantitatively (lower panel), in Fig. 3.4 a. Briefly, pre-treatment with Pio counteracted the Aβ-induced decrease (3 h) in PSD-95 expression, increase in pSer396-tau levels and colocalization index (ratio between elements that were co-labelled with antisera against pTau-Ser396 and PSD-95 to the total number of PSD-95-immunopositive puncta).

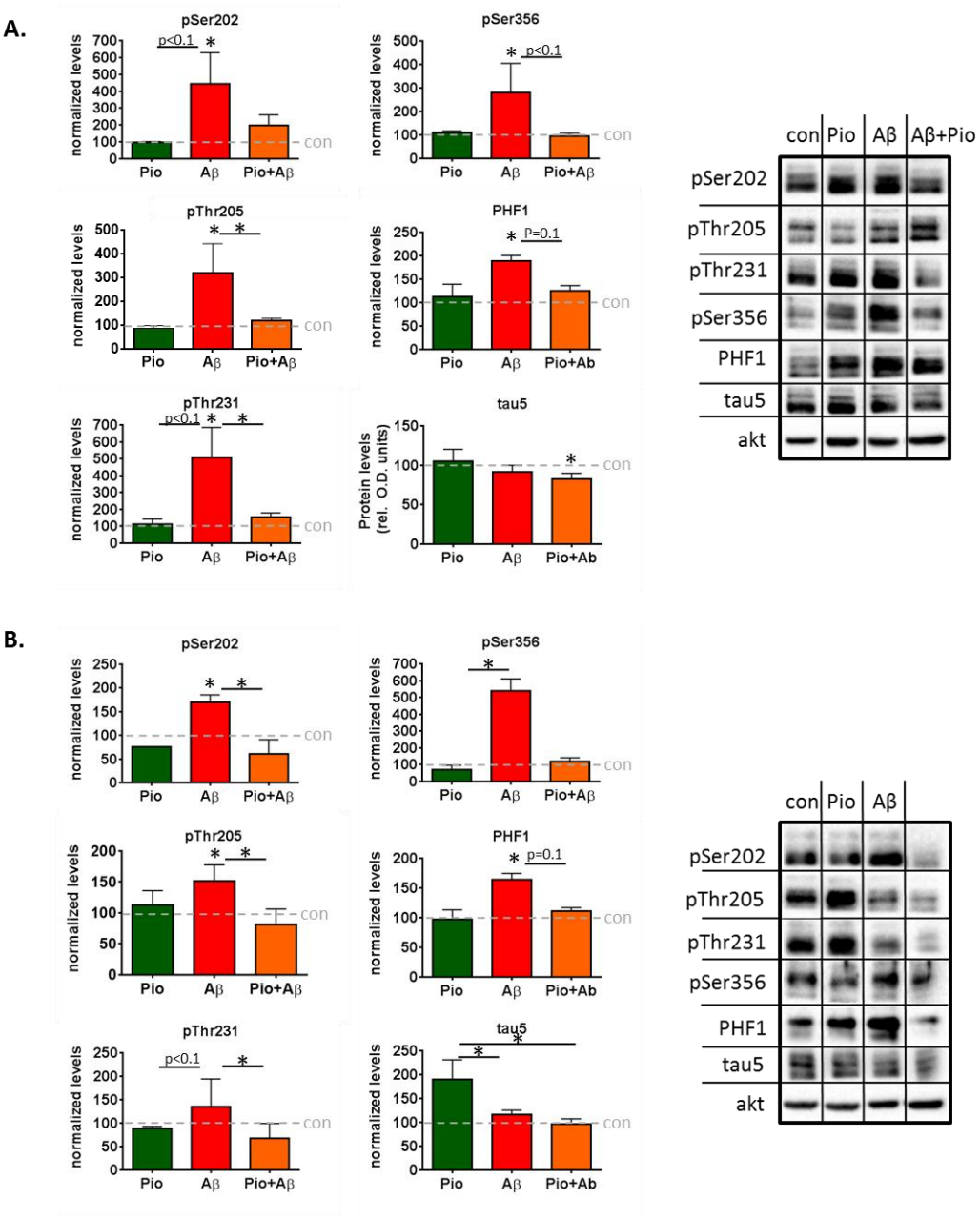


Figure 3.2. Pioglitazone prevents tau-hyperphosphorylation at specific tau-epitopes. Primary neural cultures from mouse frontal cortex (**a**) and hippocampus (**b**) are treated with 10 µM Pio (PPARγ agonist) for 24 h alone or followed by 1 µM soluble $A\beta_{1-42}$ for 24 h. Westernblot experiments show protein levels. Total tau is normalized to actin and tau-epitopes: pSer202, pThr205, pThr231, pSer356 and PHF1 (pSer394/Ser404) are normalized to total tau. Blots are shown right handed to the graphs and results are shown as percentages normalized to control (100%) as graphs left handed. All numerical data (n = 3-6) are represented as means ± S.E.M; significant differences (Mann-Whitney test) are $p \leq 0.05$.

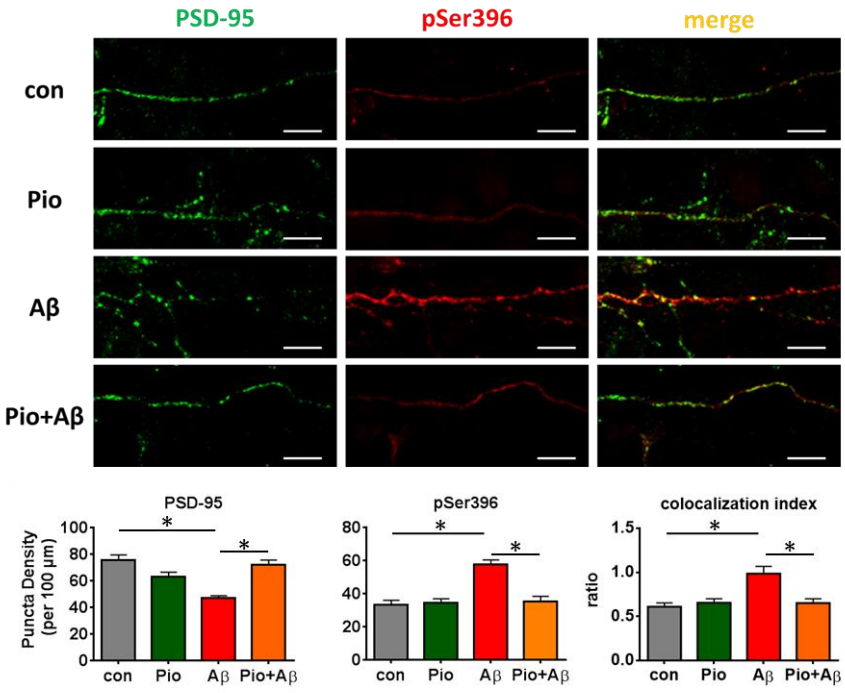


Figure 3.3. Pioglitazone prevents Aβ-induced downregulation of PSD-95 and postsynaptic localization of hyperphosphorylated tau (pSer396) in mouse frontocortical cultures. Cells were pre-treated with Pio (10 µM, 24 h) and then exposed to 1 µM $A\beta_{1-42}$ for 24 h. Fixed cells were stained for PSD-95 (green fluorescence) and pSer396 tau epitope (red fluorescence), as shown in *upper panels*. *Lower panels* show analysis of dendrites (each 100 µm apart) by confocal laser scanning microscope (bars: 10 µm); SynPAnal software was used to analyse synaptic puncta. The "colocalization index" was computed as the ratio between elements that were co-labelled with antisera against pTau-Ser396 and PSD-95 to the total number of PSD-95-immunopositive puncta. All numerical data (n = 10-22) are represented as means ± S.E.M; significant differences are $p \leq 0.05$.

This set of studies also demonstrated that Pio countered the actions of Aβ through the mediation of PPARγ since an antagonist of the receptor, GW9662 (1 µM, administered for 30 min before Pio), blocked the ability of Pio to prevent Aβ-induced changes in PSD-95 and pSer396-tau expression (and co-localization of the two proteins), as shown qualitatively (upper panel) and quantitatively (lower panel) in Fig. 3.4 b. Interestingly, but inexplicably, GW9662, together with Pio and Aβ caused a significant increase in pSer396-tau levels as well as in the synaptic localization of this tau epitope ($p \leq 0.05$) (**Fig. 3.4 b**).

Next, we focused on the question of whether Pio can serve to reduce synaptic degradation following *in vitro* exposure of frontocortical and hippocampal neurons to Aβ for either 3 h or 24 h. For this, we monitored synaptic integrity by evaluating puncta labelled with antibodies against the pre- and post-synaptic proteins, synapsin 1,2 (red fluorescence) and PSD-95 tau;

the latter has previously been shown to trigger synaptic loss through the mediation of the src-secretase Fyn (Ittner et al, 2010; Lopes et al, 2016).

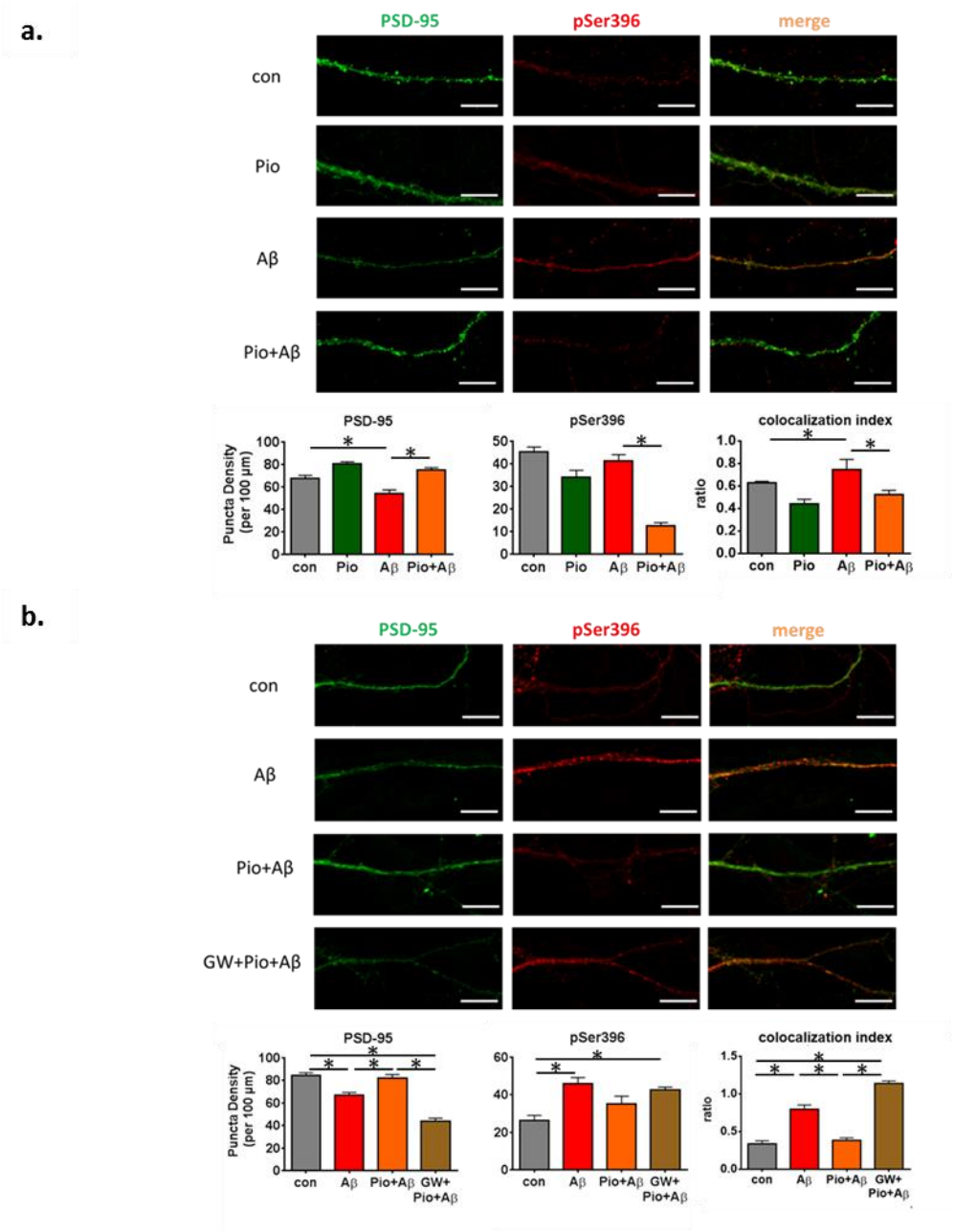


Figure 3.4. Pioglitazone prevents Aβ-induced downregulation of PSD-95 and postsynaptic localization of hyperphosphorylated tau (pSer396) in mouse hippocampal cultures. Cells were treated with (**b**) and without (**a**) GW9662 before treatment with Pio (10 µM, 24 h) and then exposed to 1 µM Aβ $_{1\text{-}42}$ for 3 h. Fixed cells were stained for PSD-95 (green fluorescence) and pSer396 tau epitope (red fluorescence), as shown in *upper panels*. *Lower panels* show analysis of dendrites (each 100 µm apart) by confocal laser scanning microscope (bars: 10 µm); SynPAnal software was used to analyse synaptic puncta. The "colocalization index" was computed as the ratio between elements that were co-labelled with antisera against pTau-Ser396 and PSD-95 to the total number of PSD95-immunopositive puncta. All numerical data (n = 8-22) are represented as means ± S.E.M; significant differences are $p \leq 0.05$.

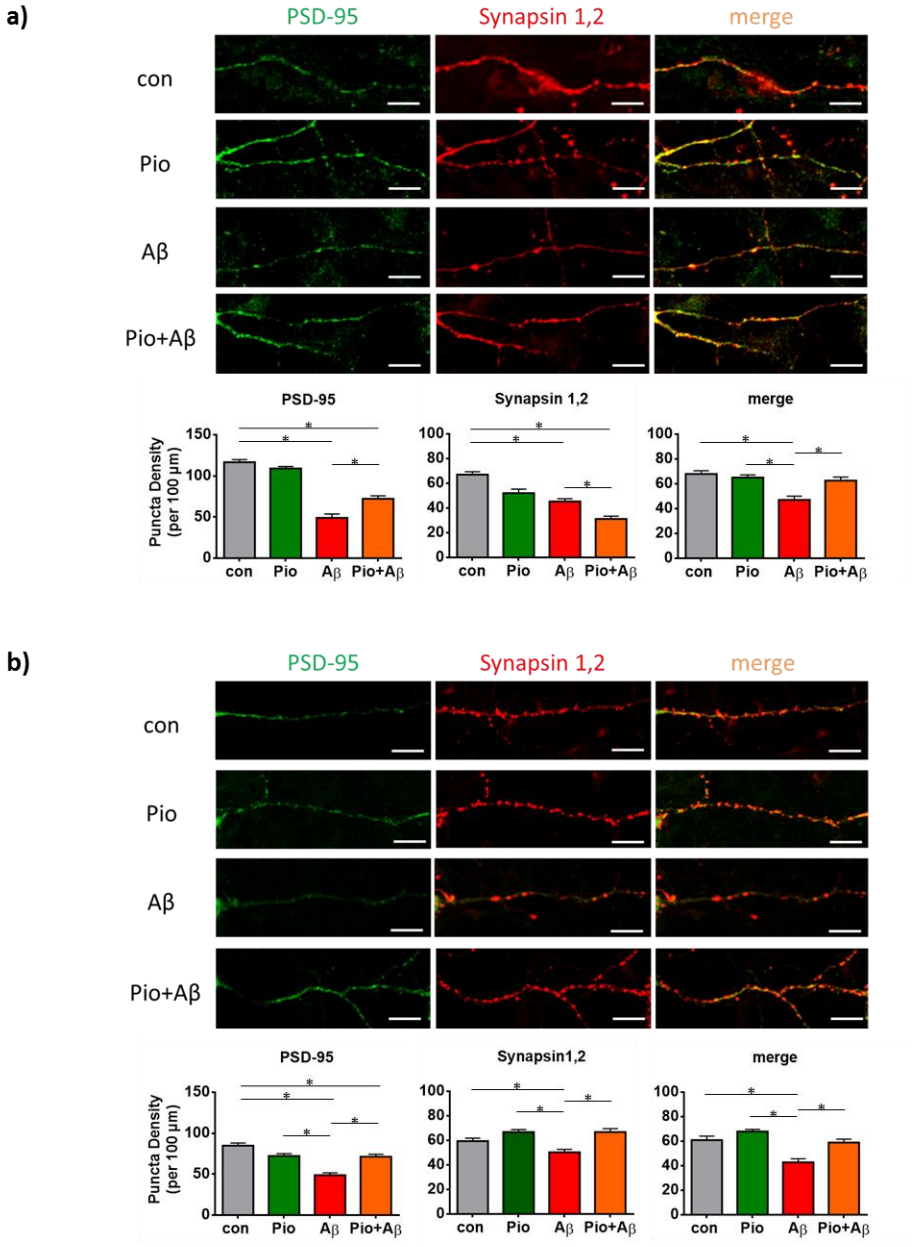


Figure 3.5. Pioglitazone prevents synaptic degradation in frontocortical (a) and hippocampal (b) neurons exposed to $A\beta_{1-42}$ for 3 h (*in vitro*). Primary neural cultures were tested after 14 DIV. The PPARγ agonist, pioglitazone (Pio) was added to cells at 10 µM 24 h prior to treatment with 1 µM $A\beta_{1-42}$ for 3 h. Results for DIV14 fronto cortical (**a**) and hippocampal (**b**) cultures are shown. Immunoreactive PSD-95 and synapsin were identified (laser scanning confocal microscopy) as green and red puncta, respectively, with orange puncta representing intact synapses (apposition of synapsin and PSD-95 immunoreactivity). Puncta analysis was performed on dendrites that were 100 µm using SynPAnal software. Scale bars represent 10 µm. All numerical data (n = 16-22) are represented as means ± S.E.M; significant differences are $p \leq 0.05$.

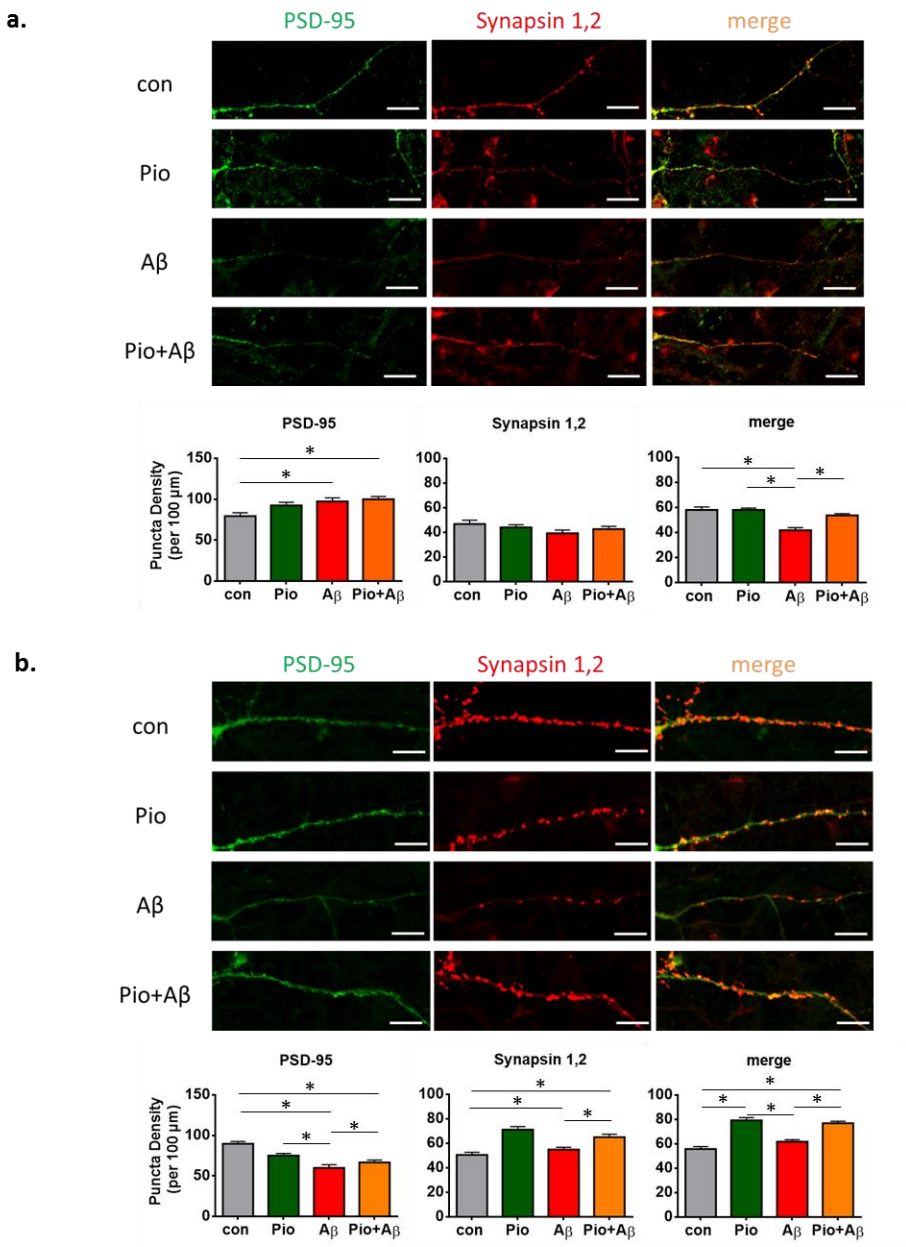


Figure 3.6. Pioglitazone prevents synaptic degradation in frontocortical (a) and hippocampal (b) neurons exposed to $A\beta_{1-42}$ for 24 h (*in vitro*). Primary neural cultures were tested after 14 DIV. The PPARγ agonist, pioglitazone (Pio) was added to cells at 10 µM 24 h prior to treatment with 1 µM $A\beta_{1-42}$ for 24 h. Results for DIV14 fronto cortical (**a**) and hippocampal (**b**) cultures are shown. Immunoreactive PSD-95 and synapsin were identified (laser scanning confocal microscopy) as green and red puncta, respectively, with orange puncta representing intact synapses (apposition of synapsin and PSD-95 immunoreactivity). Puncta analysis was performed on dendrites that were 100 µm) using SynPAnal software. Scale bars represent 10 µm). All numerical data (n = 16-22) are represented as means ± S.E.M; significant differences are $p \leq 0.05$.

Taken together, this set of data demonstrate that Pio, a PPARγ agonist, can counter synaptic degradation caused by Aβ. Further, these results suggest that these actions of Pio at least partly result from the drug's ability to block Aβ-induced hyperphosphorylation of dendritic

(green fluorescence), respectively; apposed immunoreactive puncta (orange fluorescence) were considered representative of intact synapses. As shown in **Figs. 3.5 a** and **b**, treatment with $A\beta_{1-42}$ (1 µM) for 3 h caused a significant reduction in PSD-95 and synapsin immunoreactivity ($p \leq 0.05$) in frontocortical and hippocampal cultures, as well as in the merging of signals for the two synaptic marker proteins. The same treatment, albeit over 24 h, resulted in a loss of signals representing PSD-95 and synapsin immunoreactivity signal in hippocampal, but not frontocortical, cells (**Figs. 3.6 a** and **b**). All of these effects were markedly attenuated when both types of cultured cells were pre-treated with Pio (10 µM, 24 h before application of 1 µM $A\beta_{1-42}$).

3.5. Discussion

Pioglitazone (Pio), a pharmacological antagonist for the nuclear receptor PPARγ, has potential therapeutic value in the management of diabetes type II, which is a risk factor for Alzheimer's disease (AD) (Ahmadian et al, 2013). The latter view is supported by recent publications that report that Pio results in cognitive improvement in AD patients and in mouse models of AD. Investigations by our group (**Chapter 2** and Pissioti, 2016) demonstrated the expression of PPARγ (mRNA and protein level) in the mouse brain, with strong expression in the frontal cortex and hippocampus, two brain regions involved in cognitive processes that are affected by AD pathology. While our results largely confirm previous findings (**Chapter 2**), their added value lies in the fact that we used carefully characterized antisera and also examined tissues originating from animals with differing physiological backgrounds. Moreover, we demonstrated the transcriptional activity of the centrally-expressed PPARγ. Extending those findings, the work described in this chapter shows that PPARγ activity influences neural viability and synaptic integrity after exposure of neurons to amyloid β (Aβ), a protein that serves as a pathological hallmark of AD. Specifically, they show that the neuro- and synaptotoxic actions of Aβ can be abrogated by Pio *in vitro* (SH-SY5Y human neuroblastoma cells and primary frontocortical and hippocampal neurons from postnatal mice) (Inestrosa et al, 2005; Xu et al, 2014). Further, this work shows that Pio can alter at least two cellular mechanisms that are crucial in the development of AD pathology: Amyloid precursor protein (APP) processing and tau-hyperphosphorylaton. Previous complementary studies reported that thiazolidinediones (TZD), such as Pio, can reduce Aβ generation and deposition in

transgenic mouse models of AD (Escribano et al, 2010; Mandrekar-Colucci et al, 2012; Skerrett et al, 2015) as well as in primary neural cultures (neurons, astrocytes and microglia) (Mandrekar-Colucci et al, 2012; Skerrett et al, 2015) and in neural cell lines overexpressing APP (with/out PPARγ overexpression) (Camacho et al, 2004). Only a few earlier authors examined the influence of TZD on tau pathology; two notable ones, carried out in primary cell cultures and cell lines suggested that PPARγ activation counteracts the aberrant hyperphosphorylation of tau and expression of tau kinases such as GSK-3β (Yoon et al, 2010; Cho et al, 2013).

Given the paucity of reports in which the influence of TZD was examined on Aβ and tau pathology in parallel (for an exception, see Escribano et al, 2010), the present work was designed with hindsight of the fact that amyloid pathology is detectable before tau abnormalities and current consensus holds that the overproduction of Aβ is an essential trigger of abnormally hyperphosphorylated tau (De Felice et al, 2008). In our experiments, cultured cells were pretreated with Pio at a dose (10 μM) and for a duration (24 h) shown to induce transcription after PPARγ activation (**Chapter 2**); cells were subsequently exposed to soluble $A\beta_{1-42}$ peptide (3 or 24 h) in an attempt to mimic one aspect of AD although it should be mentioned that multiple species of Aβ are found in the brains of AD patients (Roher et al, 2017). We chose to use soluble Aβ because the cytotoxic and memory-impairing actions of Aβ correlate better with soluble, rather than with the insoluble forms of the peptide of which the classical senile plaques seen in advanced stages of AD are composed (Klein et al, 2001). It is known that $A\beta_{1-42}$ rapidly self-aggregates into an insoluble form; to overcome this, we here used an established protocol (Stine et al, 2011) to ensure that our preparations consisted of soluble oligomers. Importantly, the results reported here show that our treatment protocol could replicate previous findings that soluble Aβ is neurotoxic (e.g. Stine et al, 2011), induces synaptic degradation (e.g. Roselli et al, 2005), increases APP misprocessing (e.g. Catania et al, 2009) and trigger the hyperphosphorylation of tau (e.g. De Felice et al, 2008).

Treatment of differentiated SH-SY5Y neuroblastoma cells with Aβ reduced cell viability after 24 h, an effect that was attenuated by prior activation of PPARγ with Pio. In keeping with previous work (Roselli et al, 2005, Liu et al, 2010), we also observed that effects of Aβ on synaptic degradation (reduction of synapsin and PSD-95 apposed elements). These effects

were detectable within 3 h of exposure to amyloid peptide but proved more difficult to detect after 24 h; the latter observation is consistent with the view that when cells are exposed to Aβ, synaptic toxicity precedes neuronal cell death (Bredesen et al, 2006). Interestingly, detectable, synaptotoxicity (and its attenuation by Pio pretreatment) was more marked in primary hippocampal than frontocortical cultures; this finding suggests that the hippocampal cells are more sensitive (respond earlier) to the cytotoxic effects of Aβ.

Our findings that Pio counteracts the deleterious effects of Aβ on neural cell (SH-SY5Y cell line) survival as well as synaptic integrity (primary frontocortical and hippocampal neurons) support previous observations by Xu et al (2014) who obtained similar results in primary hippocampal cells[1]. Together, these observations add validity to the hypothesis that activation of PPARγ may be a therapeutic approach to delay or reverse AD neuropathology.

Having shown that Pio can effectively reverse AD neuropathology simulated *in vitro* by addition of Aβ to neural cells, and in light of previous studies that showed that with Aβ can exacerbate APP misprocessing (Catania et al, 2009), we also investigated whether Pio has the potential to attenuate the latter. Semiquantitative immunoblotting (Western blotting) analysis of differentiated SH-SY5Y cells revealed that Pio protects against Aβ-induced APP misprocessing and that the effects of Pio include a reduction in the expression of the two key amyloidogenic proteolytic molecules, β-secretase (BACE1) and nicastrin; the latter is an essential component of γ-secretase, and interestingly, previous authors failed to detect any effect of PPARγ agonists on γ-secretase protein levels and/or activity in either *in vivo* or *in vitro* setups (Camacho et al, 2004; Escribano et al, 2010; Kummer et al, 2015). Further, it should be noted that Pio was previously reported to influence APP metabolism in divergent ways (d'Abramo et al, 2005; Escribano et al, 2010; Liu et al, 2013). On the other hand, a growing consensus in the literature is that PPARγ activation suppresses BACE1 activity (Rossner et al, 2006; Liu et al, 2013; Wang et al, 2017) although authors who examined this in TZD-treated cell lines overexpressing PPARγ failed to observe such effects (Camacho et al, 2004; d'Abramo et al, 2005). Further experiments are needed to explain the disparate results;

[1] The reason for using a neural cell line (for cell viability studies) and primary neuronal cultures (for synaptic analysis) in the present work is that, in contrast to cell lines, the latter are difficult and expensive to prepare and the yield of viable neurons in culture is limited; on the other hand, even differentiated neural cell lines do not develop mature synapses, limiting their use for the questions asked here.

in particular, in the context of the present work, it will be important to establish whether the effects measured in differentiated SH-SY5Y neural cells, commonly used in AD research, are in fact artefactual.

The brain is supposedly equipped with mechanisms that normally act to reduce the accumulation of Aβ; besides insulin-degrading factor (see below), these include ABCA1 (Mandrekar-Colucci et al, 2012; Kang & Rivest, 2012), whose transcription is upregulated by PPARγ activation. The latter protein plays an important role in Aβ clearance since it carries lipidated ApoE to the extracellular space which can then bind Aβ and exported out of the brain via low density lipoprotein receptor-related protein 1 (Shinohara et al, 2017). Here, it is worth mentioning that the significance of this mechanism is reflected by the fact that a short nucleotide polymorphism in the *ApoE* gene, resulting in the production of ApoE4 which cannot be lipidated, represents the main genetic risk factor for late-onset AD (Jiang et al, 2008). Whereas modulation of the ABCA1-ApoE pathway by TZD has thus far only been described in glial cultures (Jiang et al, 2008; Escribano et al, 2010; Mandrekar-Colucci et al, 2011; Skerrett et al, 2015), the present work shows PPARγ-dependent induction of *ABCA1* and *ApoE* in differentiated SH-SY5Y neural cells. We also show that exogenous Aβ upregulates the expression of these genes, possibly reflecting a compensatory response, and that Pio cannot reverse the effects of Aβ. Unexpectedly, we also observed an upregulation of *ABCA1* and *ApoE* mRNA expression after exposing SH-SY5Y cells to GW9662, a specific PPARγ antagonist for 24 h; finding cannot be easily explained but it may be due to the extended (24 h) treatment of the cells with Aβ which could confound the detection of mRNA species in degenerating cells. Nevertheless, in view of the potentially exciting implication of TZD for boosting Aβ clearance, these experiments deserve repetition using different dose-time conditions.

As mentioned above, IDE is involved in reducing Aβ load; the enzyme is known to be involved in the proteolytic degradation of extracellular Aβ (Kurochkin & Goto, 1994). Here, we used ELISA to measure IDE protein in extracts from primary frontocortical and hippocampal neurons that had been previously exposed to Aβ, with or without pre-treatment with Pio. Increased levels of IDE were observed after treatment with either Pio or Aβ alone; while the latter may be explained as a compensatory effect, our finding that IDE levels were reduced

after co-treatment with Pio and Aβ remains puzzling. Notably, while some authors reported that activated PPARγ regulate IDE as well as that of neprilysin which can also degrade Aβ (Du et al, 2009; Kalinin et al, 2009; Li et al, 2015; Zhang et al, 2015), others failed to see such effects under either *in vivo* or *in vitro* conditions (Escribano et al, 2010; Mandrekar-Colucci et al, 2012; Kummer et al, 2015). Again, variables such as neural cell types (e.g. neurons *vs.* astrocytes or microglia) may contribute to the non-uniformity in the results obtained in different studies.

In this study we also investigated the role of PPARγ in the regulation of tau since abnormal hyperphosphorylation of this cytoskeletal protein results in its detachment from microtubules and neuritic dystrophy. Further, tau undergoes several post-translational modifications such as phosphorylation. Tau that is hyperphosphorylated at certain epitopes self- aggregates and gives rise to the neurofibrillary tangles (NFT) characteristic of the AD brain (Wang & Mandelkow, 2016). In addition, recent research has shown that tau is also localized in dendrites; its hyperphosphorylation at this site triggers synaptic dysfunction (Kimura et al, 2007; Ittner et al 2010; Lopes et al 2016; Kobayashi et al 2017).

Hyperphosphorylation of tau is induced by Aβ, with CDK5 and GSK-3β being the most commonly implicated kinases (De Felice et al, 2008; Noh et al, 2009; Dolan & Johnson, 2010). Here, we not only confirmed that exposure of frontocortical and hippocampal neurons to $A\beta_{1-42}$ oligomers for 24 h induces hyperphosphorylation of specific tau epitopes, but also demonstrated that this phenomenon can be blocked by pretreatment with Pio. The following phosphoepitopes were analysed in the present study: pThr231 (mainly present at the pre-NFT stage), pSer356 (found in mature NFT), and pSer202, pThr205, pSer396 and pSer404 (mark extracellular tangles) (Metaxas & Kempf, 2016). Recently, pThr205 has triggered considerable discussion because, although it is among the pTau epitopes recognized by the AT8 antibody which is commonly used to detect tau-pathology, Ittner et al (2016) suggested that pThr205 may actually confer protection in a mouse model of AD.

Using primary mouse frontocortical and hippocampal neurons, we failed to see any effect of either Aβ on the expression levels of total tau protein; these findings agree with those reported previously in a study of the SH-SY5Y neural cell line (Yoon et al, 2010). However,

upregulation of pThr205 was observed after treatment of primary neuronal cultures with Aβ. However, this was not accompanied by signs of neuroprotection (reduced cytotoxicity and synaptotoxicity), possibly because the effect was not sufficiently strong under the experimental conditions used. Our analysis also revealed, that as reported by other authors, studying individual or pairs of pTau epitopes only using conditions quite different from those used in the present work (D'Abramo et al, 2005; Yoon et al, 2010; Cho et al, 2013), Pio-activated PPARγ reduced the expression levels of pSer202, pThr205, pThr231 and pSer396/404 after exposure of primary neuron cultures to Aβ. Although the general pattern of tau epitope regulation by Pio, Aβ and Pio+Aβ was similar in frontocortical and hippocampal cultures, statistical differences were not easily detectable; the latter may be due to the low sample (power) size (n = 3-6).

Interestingly, Aβ-treated frontocortical neurons display higher levels of pThr231 as compared to hippocampal neurons; since this pTau epitope is usually found in pre-tangles, this result suggests that tau pathology may start earlier in the frontal cortex than in limbic areas of the brain.

As previously mentioned, pTau has been recently implicated in synaptic dysfunction and atrophy. The molecular mechanism responsible for the latter is suggested to involve the induction of src-kinase Fyn activity by pTau, resulting in the association between glutamate receptors (GluN2B receptors) to PSD-95 and ultimately, synaptic degradation; such a mechanism has been shown to be triggered by Aβ (Ittner et al, 2010) and, more recently, by chronic stress (Lopes et al, 2016). To investigate the effects of PPARγ activation on synaptic integrity, we here focussed on pSer396 since phosphorylation of this epitope is sensitive to Pio (see above and Yoon et al, 2010). Our investigations showed that pre-treatment with Pio abrogates Aβ-induced hyperphosphorylation of tau protein at Ser396 by Aβ; this effect was shown to be PPARγ dependent since it could be blocked by the PPARγ antagonist GW9662.

Conclusion: The experiments reported in this chapter support previous suggestions that activated PPARγ can protect against Aβ-induced neurotoxiciy and synaptic degradation. Specifically, the results, obtained in either primary forntocortical and hippocampal neural cultures or in a human neuroblastoma cell line (SH-SY5Y), show that activation of PPARγ with Pio attenuates the misprocessing of APP (and therefore into Aβ) by reducing BACE1 and

nicastrin levels, and also reduces the amount of aberrantly hyperphosphorylated tau and localization of such species in the post-synapse. Thus, Pio was found to reduce both, Aβ-induced cytotoxicity as well as synaptotoxicity.

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3.7. Supplementary Information

Table S.3.1 Homogenization buffer for protein extraction

Ingredients	Final Concentration	Supplier
NaCl	250 mM	Roth
EDTA	1 mM	Gibco
MgCl	2.5 mM	Gibco
Glyerol	10%	Roth
NP-40	1%	Nonidet, Pierce
-> 100 mM Tris-Cl adrusted to pH 8		
just before use add: - each 1 % phosphatase inhibitor cocktail II & III (Sigma) - 2 % protease inhibitor cocktail (Sigma)		

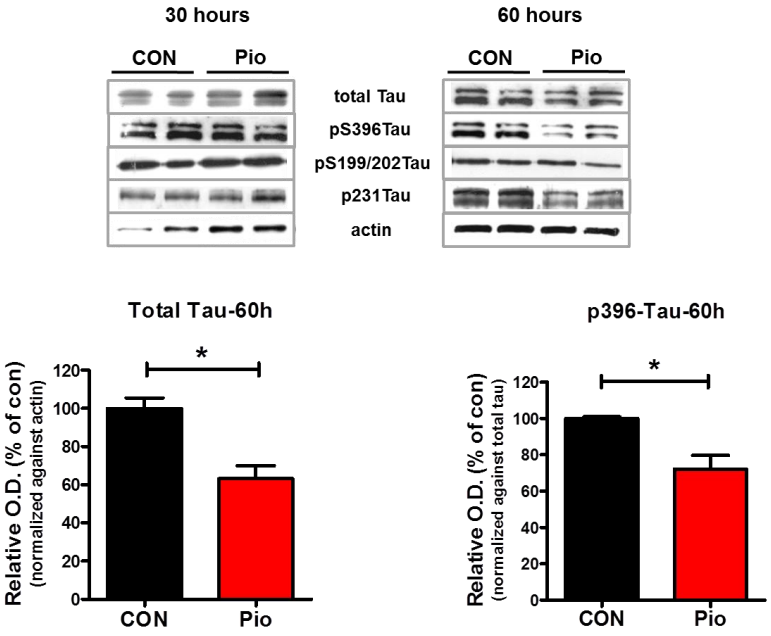

Figure S.3.1 Pio treatment reduces total tau and pS396. HW3-5 cells were treated with Pio for 30 h or 60 h in order to measure protein levels of total tau normalized to actin and several phosphorylated tau epitopes (Serin396, Serin199 and 202, Threonine 231) normalized to total tau. Westernblot measurements are shown as blots (upper picture) and as percentages as graphs. All numerical data (n = 3-6) are represented as means ± S.E.M; significant differences are $p \leq 0.05$. Experiment conducted in collaboration with Sarah Pinheiro and Dr. Inoannis Sotiropoulos (ICVS, Braga/PT).

Chapter 4

Outlook: Integration of Results and Considerations Regarding the Development of PPARγ-targeted Neurotherapeutics

4.1. Summary of aims and main findings

The rationale behind this work was:

- PPARγ agonists suggested for AD and other brain disease therapy, but without robust evidence for sites or mechanisms of action
- Above suggestions based on role of PPARγ in clinical management of imbalances in glucose and fat metabolism, conditions causally linked with diabetes and obesity, which themselves are thought to contribute to AD, PD and disorders of mood and emotion

Results presented in **Chapter 2** provide the first comprehensive descriptions of distribution of PPARγ in brain tissue and neural cells; for this, methods for detecting both, *PPARγ* mRNA transcripts and PPARγ protein were applied to mouse brain tissue, frontocortical and hippocampal primary cultures from mouse brain, and neural cell lines derived from humans. An important question underlying the work in this thesis was to examine whether the reported positive effects of PPARγ agonist ligands can be attributed to direct actions on brain tissue or whether they may be secondary to improvements in metabolism. For this reason, all biological material used for analysis in this work originated from healthy, lean animals; in addition, a human (SH-SY5Y) and a mouse (HW3-5) neural cell line was used for some experiments.

Focus on primary cultures from frontal cortex and hippocampal cultures was because these two brain areas are strongly affected by AD pathology. However, because such cultures are difficult to prepare and use for functional studies, that work was complemented by the neural cell line cultures which are more amenable to transfection and analysis. With regard to the analysis of brain tissue: several previous studies examined the rodent brain, but those works were usually restricted to a limited number of brain areas and/or their results are difficult to interpret due to questions regarding the specificity of the reagents (antisera) used. On the other hand, the only similar attempt at comprehensive investigation of PPARγ distribution in the rodent brain is that provided in the Allen Mouse Brain Atlas (http://mouse.brain-map.org/); however, descriptions therein are based on high-throughput *in situ* hybridization

histochemistry, an approach that cannot accommodate the problem of assay sensitivity and which does not necessarily reflect functional protein levels.

As reported in **Chapter 2** of this thesis, immunohistochemical (and immunoblotting) analyses performed on juvenile and adult mouse brains, demonstrated PPARγ protein expression in a variety of brain areas, including the frontal cortex and hippocampus, but also in regions concerned with the regulation of neuroendocrine function, feeding and metabolism (hypothalamus), motivation and reward (nucleus accumbens, ventral tegmental area) and fear/anxiety (amygdala). Besides observing age-related differences in the distribution pattern of PPARγ protein expression, this analysis confirmed the presence of PPARγ in the frontal cortex and hippocampus, two brain regions related to cognition and memory and affected early during the onset of AD pathology (**Fig. 4.1(1)**). The specificity of the immune-labelling was verified by using brains from mice in which PPARγ was conditionally deleted as a negative control; cells in which PPARγ was overexpressed and brains derived from obese mice (see Pissioti, 2016) and mice overexpressing APP served as positive controls. Furthermore, labelling was performed with antisera from three different commercial sources.

The above protein expression studies were taken a step further by analysing PPARγ expression in primary frontocortical and hippocampal cultures (from mice aged 5 days; cultures from older ages remains technically challenging) which revealed presence of PPARγ mRNA and protein in specific cell types - neurons and astrocytes (**Fig. 4.1(2)**). Complementing these findings, we also detected PPARγ (mRNA and protein) in rodent and human neural cell lines. Protein measurements were validated using knockdown experiments (RNAi against PPARγ) in a human neural cell line (SHSY-5Y). In parallel, primary cultures and cell lines were exploited to demonstrate the functional activity of the detected PPARγ protein (**Fig. 4.1(3a)**). Specifically, activation of PPARγ with the agonist pioglitazone (Pio) led to an upregulation of the mRNA encoding the PPARγ-target genes (*ABCA1* and *PGC-1α*) this effect was blocked in the presence of the PPARγ antagonist (GW9662), proving PPARγ mediation of the Pio actions.

Together, these results provide convincing support for the presence of functional PPARγ in the mouse brain, and in particular, that the two areas of greatest significance to AD, the frontal cortex and hippocampus which play critical roles in cognitive function, express PPARγ.

These findings therefore validate the use of mice as a model for examining the therapeutic potential of PPARγ agonists for the delay/prevention/treatment of diseases of the brain in humans.

Although Alzheimer's disease is ultimately characterized by massive neurodegenerative changes (loss of neurons and neural tracts), it is important to note that the disease is increasingly viewed as a disorder that results from the synaptic dysfunction that precedes gross anatomical changes. The pathology involves two key molecules: Aβ- and tau which induce synaptic and neural damage, followed by loss of memory and cognitive decline. Toxic soluble $A\beta_{1-40}$ and $A\beta_{1-42}$ are formed by successive processing of APP by β- and γ-secretase. Furthermore, this misprocessing induces tauhyperphosphorylation (p-tau) at epitopes that ultimately results in cytoskeletal (microtubule) destabilization and, as more recently shown, translocation of p-tau to dendrites and activation of a molecular cascade (including fyn-kinase, NR2B and PSD-95) that culminates in synaptic degradation (Ittner et al, 2010; Lopes et al, 2016).

In order to investigate the role of PPARγ in AD pathology, primary frontocortical and hippocampal cultures from mouse and a neural human cell line were pre-treated with Pio before the addition of soluble $A\beta_{1-42}$. Treatment with $A\beta_{1-42}$ alone induced neurotoxicity and synaptic degradation, effects that was attenuated by Pio pre-treatment. Furthermore, we demonstrated that pre-activation of PPARγ with Pio reduced the protein levels of APP as well as its cleavage by β- and γ-secretase to generate Aβ (**Fig. 4.1(3b)**). Pre-treatment with PPARγ agonist Pio also alleviated Aβ-induced generation of p-Tau, including at epitopes previously known to be implicated in AD, and results showed that Pio also prevented synaptotoxicity associated with p-Tau. Specifically, the latter findings are consistent with reports that p-Tau is present in synapses (Kimura et al 2007; Ittner et al 2010; Kobayashi et al 2017) where it promotes src kinase-mediated interaction of PSD-95 and NR2B; importantly, in the present experiments, the actions of Pio were blocked by GW9662, a specific PPARγ antagonist (**Fig. 4.1 (3c)**).

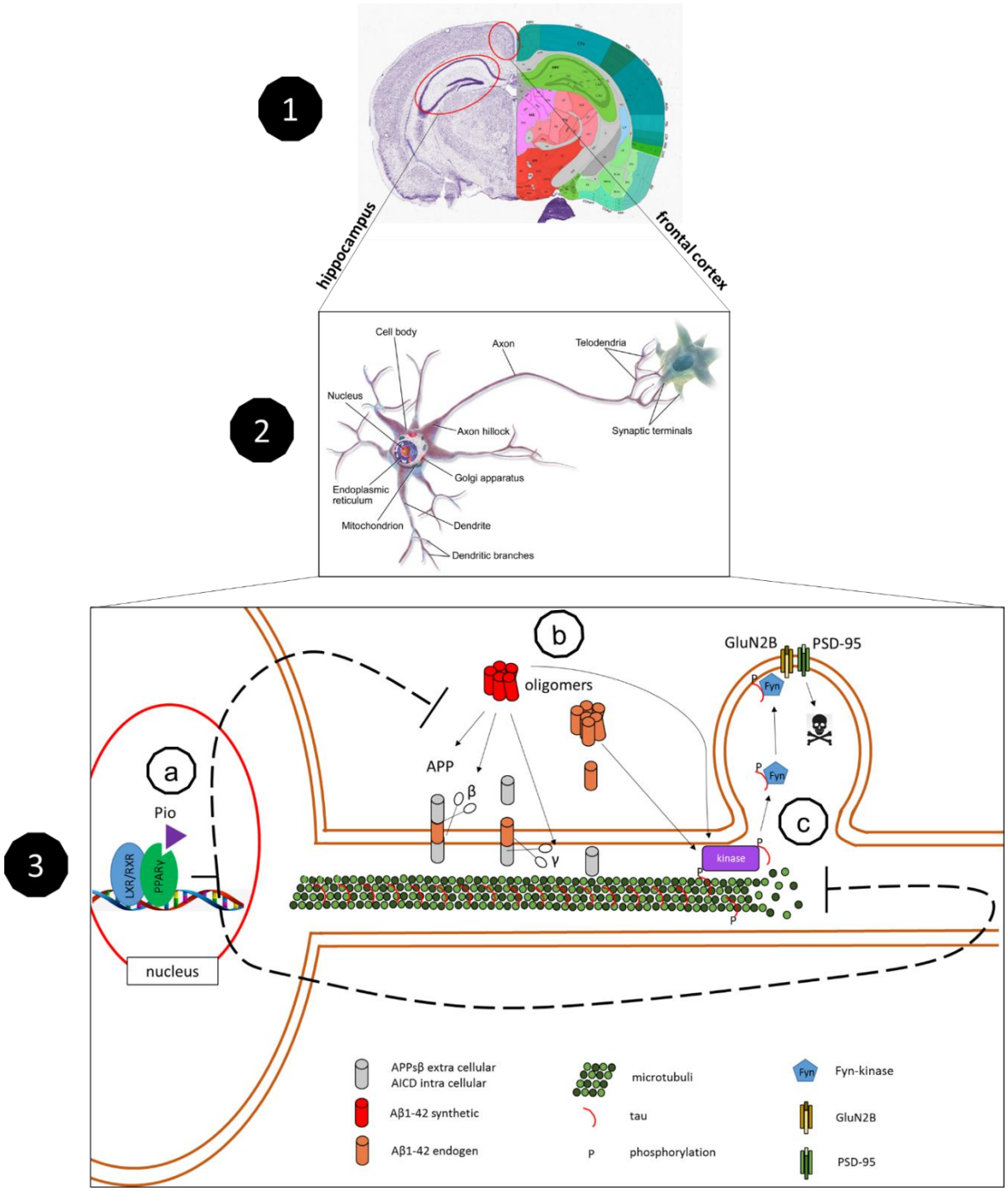

Figure 4.1. Pioglitazone attenuates Aβ- and tau-pathology. Expression of PPARγ was detected in several areas of the mouse brain, with the frontal cortex and hippocampus being two regions that displayed high levels (**1**); PPARγ protein was detected both, *in vitro* and *in vivo*, including in frontocortical and hippocampal neurons and astrocytes (**2**). Activation of PPARγ with Pioglitazone (Pio) leads to heterodimerization with LXR or RXR and induction of target genes (**3 a**). Soluble oligomeric Aβ induces APP expression and its misprocessing by β-secretase (β) and γ-secretase (γ), resulting in neurotoxic Aβ oligomer production. This process is inhibited by Pio (**3 b**). Soluble oligomeric Aβ promotes amyloidogenic processing of amyloid precursor protein (APP) and induces tau hyperphosphorylation via specific kinases such as GSK-3β and CDK5; both are inhibited by Pio. Hyperphosphorylated tau in the dendrite (induced by Aβ, for example) activates the src -kinase Fyn, promoting association of glutamate NR2B receptors (GluN2B) with PSD-95, resulting in synaptic degradation and loss. The present research showed, that Pio can disrupt this mechanism in a PPARγ-dependent manner (**3 c**).

4.2. Perspectives

Given the results obtained in this work, and in light of the implication of PPARγ as a therapeutic target (Galimberti & Scarpini, 2016; Lee et al, 2018), the rest of this discussion considers factors that need to be kept in mind in any future development of PPARγ-targeting drugs for brain disorders such as AD.

Pharmacology, absorption, metabolism: Pioglitazone (Pio), the thiazolidinedione used in the present work, binds to PPARγ with a relatively high affinity (Kd ~ 40 nM) with high specificity (Lehmann et al, 1995), but at higher molecular levels (mM range) which means less affinity, also activates PPARα (Sakamoto et al, 2000). Oral pioglitazone is easily, absorbed with 83% of the drug entering the blood circulation (Galimberti & Scarpini, 2016). In humans, the drug is metabolized by the hepatic cytochrome P450 (CYP) enzyme system, mainly by CYP2C8 and, to a lesser extent by CYP3A4.

Bioavailability to brain structures: Oral Pio at a dose of 20.4 mg/kg produced low nanomolar range concentrations of the drug in the mouse brain after 4 days of administration (Grommes et al, 2013). However, the situation in human might be more complicated. As already mentioned Pio is partly metabolized CYP3A4. Notably substrates for P-glycoprotein (P-gp) a protein located at the blood-brain-barrier (BBB) which extrudes xenobiotics, are mainly metabolized by this CYP and further substrates of CYP3A4 are very likely a substrate of P-gp (Lüllmann et al, 2016; Galimberti & Scarpini, 2016). Accordingly, access of Pio to brain tissue may be limited or subject to modulation by gene variants of the ABCB1 gene which encoded P-glycoprotein (Uhr et al, 2008; Breitenstein et al, 2014; 2015; 2016). Data which are supporting this hypothesis come from animal studies employing a chemical selective P-gp inhibitor showing improved brain penetration of Pio (Chang et al, 2015).

Clinical use and adverse effects of TZD: In light of its insulin-sensitizing actions, Pio was approved by the US Food and Drug Administration (FDA) as an adjunct to diet to treat diabetes mellitus in 1999. However, like other TZD, Pio has undesired side-effects, namely weight gain and fluid retention. The former reflects the fact that TZD increase fatty acid uptake and stimulation of adipogenesis, probably due to PPARγ-independent, non-specific effects

(Ahmadian et al, 2013). Previously, the use of Pio in treating type 2 diabetes has been suspended in many countries because of the associated (small) risk of bladder cancer (Pio) (Lewis et al, 2011; Nissen et al, 2010). Meanwhile, the FDA lifted its restriction on the use of rosiglitazone as an antidiabetic in 2013 in response to a review by the Duke Clinical Research Institute that reported that this TZD did not increase the risk of heart attacks more than other medications for type 2 diabetes.

Prospects for TZD and other PPARγ agonists in AD therapeutics: A TZD that shows the potential to effectively delay or arrest the progression of AD would not be likely to undergo the same scrutiny for side effects and safety afforded to other drugs for chronic use, given the fact that most AD patients are aged and would likely be suffering from other health- and life-compromising conditions. Although rosiglitazone failed a final phase (Harrington et al, 2011), its sister TZD, Pio, is being evaluated in a Phase III trial (NCT0228406) in patients with mild cognitive impairment due to AD; the trial will close in 2021.

Although this thesis is focussed on PPARγ as a target in AD, the other PPAR isotypes (PPAR-α, -β) also deserve consideration. As mentioned in **Chapter 1**, PPARα and -β are expressed in (mouse) frontocortical and hippocampal neurons from frontal cortex and hippocampus in mouse, and recent publications have suggested PPARα as a promising therapeutic target in AD since their activation shifted APP processing towards the non-amyloidogenic pathway by inducing ADAM metallopeptidase domain 10 (**Chapter 1 - Fig. 1.2**) (Corbett et al, 2015; D'Orio et al, 2018). Given this, it is plausible that dual or pan-agonism could increase more effective anti-AD pathology actions; however, ligands that activate multiple PPAR with high potency and which are free from adverse effects, are lacking at present. Nevertheless, two compounds, lobeglitazone and saroglitazone, which activate PPARα/γ were recently licensed for the treatment of type 2 diabetes in Korea and India, respectively (Tan et al, 2016). No other known dual PPARα/γ agonist (e.g. LT175, TZD18, MHY908, DSP-8658) has found clinical use or registered on the Clinical.Trial.gov homepage.

Experimental studies using mouse models of AD have examined the use of the so-called PPAR pan-agonists (compounds that can simultaneously activate all 3 PPAR isotypes PPAR-α, -β and -γ). One such study showed that GFT1803 reduced Aβ deposition and had cognition-

improving effects (Kummer et al, 2015). While only one weak pan-PPAR agonist (bezafibrate) has to date been approved for treatment of hyperlipidemia, insulin resistance and myocardial infarction in patients with metabolic syndrome, the results of clinical trials with other pan agonists (IVA337, tetradecylthioacetic acid and chiglitazar) that were associated with low side-effects are awaited (Tan et al, 2016); if successful, testing of such agonists for their utility in AD therapeutics can be expected.

Future investigations to target PPARγ for the clinical management of AD will likely include consideration of liver X receptor (LXR) or retinoid X receptor (RXR) agonists due to non-permissive activation with PPARγ (as well as PPARα and/or PPARβ); LXR and RXR agonists are both heterodimer partners of PPARγ that are essential for the transcriptional activity if activated PPARγ (**Chapter 1**). Both PPARγ heterodimerization partners have been reported to have beneficial effects in animal models of AD (Moutinho & Landreth, 2017), suggesting the eventual development of combinatorial nuclear receptor-based drug therapy. The latter would require more a detailed knowledge of cellular signalling pathways related to AD so as to increase therapeutic potency while, at the same time, minimizing undesired side-effects (Moutinho & Landreth, 2017). In this context, it is relevant to mention that activation of PPARγ, like that of LXR promotes Aβ clearance by ABCA1 mediated lipidation of ApoE (**Chapter 1** and **3**). Moreover, activated PPARγ have the potential to promote Aβ clearance by upregulating the expression of insulin-degrading enzyme (IDE) (Jiang et al, 2008; Du et al, 2009; Mandrekar–Colucci et al, 2012); these observations also support the potential efficacy of combinatorial agonist approaches to reduce Aβ pathology. Along the same, multi target lines discussed above, the RXR agonist bexarotene (which heterodimerizing with, both LXR and PPAR) was shown to have an additive effect on Aβ uptake by microglia when combined with a PPARγ agonist (Pioglitazone) in animals (Yamanaka et al, 2012) and interestingly, a clinical Phase II trial with bexarotene reported a reduction of brain amyloid burden in patients carrying the ApoE4 allele (Cummings et al, 2016).

Recent work in mice has shown that two phytochemicals (magnolol and honokiol) can co-activate PPARγ and RXR and stimulate glucose uptake with few side-effects as compared to Pio (Tan et al, 2016). It will be interesting to examine how such molecules impact on the development and course of AD pathology. Another plant-derived molecule that activate

PPARγ include amorfrutin B, extracted from *Amorpha fruticose*, apparently specifically targets PPARγ; it reportedly has beneficial effects on insulin sensitivity and blood lipid profiles and lacks the most common side-effects associated with TZD (e.g. weight gain, fluid retention) (Weidner et al, 2013; Tan et al, 2017); at present there is no information on whether the pharmacological activity of this compound may directly, or indirectly, have a use in the management of AD.

Strikingly, almost all research on nuclear receptor (NR) target modulation for treating AD have focussed on Aβ pathology; this includes studies on neuroinflammation, a phenomenon known to be exacerbated in Aβ-induced pathology (McManus & Heneka, 2017). The other major player in AD pathology, has been generally neglected, a gap partially filled by the studies reported in this thesis (**Chapter 3**). On the other hand, work by our laboratory, and continued elsewhere, has examined the role of stress and glucocorticoids, the actions of which are mediated by another NR class, glucocorticoid receptors (GR). Specifically, our group has previously reported that exposure to chronic stress or exogenous glucocorticoids results in APP misprocessing, amyloid deposition and tau phosphorylation, while disrupting memory in rats (Sotiropoulos et al, 2008; Catania et al, 2009; Sotiropoulos et al, 2011). The effect of glucocorticoids and stress on p-tau could be extended to a mechanism where p-tau induces a molecular cascade ending up in synaptotoxicity (Lopes et al, 2016).

Biomarkers and drug development: The importance of biomarkers to diagnose and stratify patients with a given disease as well as to monitor efficacy of experimental drugs is now well-recognized. Until very recently, biomarkers to detect and predict AD were limited to monitoring Aβ in the brain using brain imaging tools (functional magnetic resonance imaging [fMRI], positron emission tomography [PET]) or measurements of Aβ levels in the cerebrospinal fluid (CSF). Although Aβ is a useful predictive biomarker, because APP misprocessing is an early step in AD pathology (**Chapter 1** - **Fig. 1.1**), both neuroimaging and CSF detection methods require qualified staff and adequate monitory resources. For example, brain imaging is an expensive investigative tool and its availability is limited; it is estimated that a single PET scan costs to US $ 5,000 (https://www.ahcmedia.com/articles/-139835-how-useful-is-amyloid-pet-imaging-in-the-diagnosis-of-dementia, Caselli & Woodruff, 2016), a sum that is exorbitant when one considers that monitoring of disease progress or treatment

efficacy requires scanning at multiple intervals. Moreover, patients may be reluctant to such diagnostic tools because of fears associated with exposure to magnetic or isotopic radiation (Boccardi et al, 2016; Caselli & Woodruff, 2016). Similarly, patients may be anxious about providing CSF samples by lumbar puncture, an invasive procedure that is, nevertheless safe when performed by a skilled neurologist. Given these concerns, the trend has been to detect Aβ in blood, and this approach is commonly used to monitor AD pathology in clinical trials.

One of the challenges associated with using Aβ as a biomarker is that Aβ a "sticky" peptide that complexes with other blood proteins, easy and accurate detection remains a challenge (Janelidze et al, 2016). However, progress in the development of highly sensitive methods to detect Aβ in blood is being made and Fandos et al (2017) recently reported use of an enzyme-linked immunosorbent assay (ELISA) to distinguish between AD patients from healthy subjects; by measuring blood plasma amyloid β 42/40 ratios the ELISA could distinguish between AD patients and healthy controls with 81% certainty and the results were confirmed by PET imaging. Similar levels of sensitivity were recently reported by two other independent groups; those assays were based on a combination of immunoprecipitation and mass spectrometry (IP-MS) in order to separate and to quantify Aβ-species (Ovod et al, 2017; Nakamura et al, 2018).

While a lot of focus has been placed on Aβ as a biomarker in AD, recent presentations at the 1st Advances in Alzheimer's and Parkinson's Therapies Focus Meeting (Bowman Rogers, 2018 – article on ALZFORUM) provided convincing evidence that tau phosphorylated at threonine 181 (p-tau181) in the blood might be a useful diagnostic tool. One group used magnetic spin technology to quantify very low blood levels of p-tau (Thr181) levels (Yang et al, 2018) whereas another used an ELISA method to detect this p-tau and was able to discriminate cohorts of AD patients and healthy subjects (Mielke et al, 2018). In addition, Tatebe et al (2017) used an ultrasensitive immunoassay for p-tau181 to identify AD-patients. Together, these reports promise that measurements of specific phospho-epitopes of tau may soon complement the neuroimaging and Aβ biomarker portfolio.

4.3. Epilogue

The identification of transcriptionally active PPAR in the mouse brain (in this work, PPARγ specifically, but other studies have shown or suggested expression of other PPAR isoforms in the brain) indicates that, notwithstanding likely bidirectional interactions between peripheral and central regulatory mechanisms, PPAR agonists can directly influence brain functions. The latter include the potential amelioration of Alzheimer disease (AD)-related pathology, specifically, the generation, neurotoxic capacity and clearance of amyloid β (Aβ) and the aberrant hyperphosphorylation of tau and its associated synaptotoxic actions. Despite these findings, further work is needed to elucidate the mechanisms that underlie the ability of PPARγ ligands such as pioglitazone (Pio) to interrupt the development and progression of AD pathology. To date, only one recent study has reported PPAR expression in the human brain (Warden et al, 2016); given this, certain caution is needed when applying results obtained in mice to the development of treatments for humans. Secondly, while studies in animal models of AD contribute to understanding the neurobiology of disease, their translatability to humans may be limited; notably, genetic predisposition seems to account for a minor (< 5%) of AD, suggesting that, besides advanced age, gene X environment interactions play a crucial role in the etiology of the disease. Obesity and insulin resistance, two conditions that can be managed by PPARγ agonist-based therapeutics, rank highly among the environmental factors causally linked to AD. In addition, other variables such as sex and glucocorticoid responses to stressful stimuli (chronic exposure to glucocorticoids can induce insulin resistance, women display higher hormonal responses to stress and have a greater propensity to develop AD) need to be considered. Lastly, advances in the prevention and/or treatment of AD by activating central PPARγ will depend on the design of compounds that have optimal pharmacological and pharmacokinetic properties, that can access brain tissues and cells, lack toxic or other adverse effects, and most importantly, that lead to measurable delays or reduction in AD symptomatology. The latter will depend critically on the availability of sensitive, accurate and affordable biomarkers; reliability of pharmaceutical claims would be markedly improved by a combination of biomarkers (proteomic, neuroimaging and neuropsychological) – inter-disciplinary collaboration would make this feasible in the near future.

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